De-*O*-acylated lipooligosaccharide of *E. coli* B reduces the number of metastatic foci via downregulation of myeloid cell activity

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Abstract. Lipopolysaccharides are the main surface antigens and virulence factors of gram-negative bacteria. Removal of four ester-bound fatty acid residues from hexaacyl lipid A of *Escherichia coli* lipooligosaccharide (LOS) resulted in the de-*O*-acylated derivative *E. coli* LOS-OH (LOS-OH). This procedure caused a significant reduction in the toxicity of this compound compared to the native molecule. We investigated the effect of such a structural LOS modification on its biological activity using *in vitro* assays with monocytic cells of the RAW264.7 line, dendritic cells of the JAWS II line, bone marrow-derived dendritic cells (BM-DCs), and spleen cells. Furthermore, in *in vivo* experiments with a melanoma B16 metastasis model, the anti-metastatic activity of the compounds and spleen cell reactivity mediated by them representing a systemic response were analyzed. The results revealed that LOS-OH demonstrated weaker ability than LOS to stimulate and polarize an immune response both *in vitro* and *in vivo*. It induced lower cytokine production by cells of myeloid lines. Multiple applications of LOS-OH into mice injected intravenously with B16 cells significantly ($P<0.05$; $P<0.01$) reduced the number of metastatic foci in the lungs, presumably via silencing of myeloid cell reactivity as well as the inability to stimulate lymphoid cells both directly and indirectly. These findings suggest that LOS-OH maintained in the body of metastasis-bearing mice appears to modulate or downregulate the innate response, leading to the inability of blood myeloid cells to support the migration of melanoma cells to lung tissue.

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Introduction

Lipopolysaccharides (LPSs, endotoxin) represent the main surface antigens and virulence factors of gram-negative bacteria. LPSs when isolated from smooth strains of bacteria are composed of three distinct regions: lipid A, a core oligosaccharide, and the O-specific polysaccharide (O-antigen). The lipid A of *E. coli* type is built of a GlcN-containing disaccharide substituted with six acyl residues. This hexaacylated form critically affects the biological activity of endotoxins by mediating the interaction of LPS with pattern recognition receptors presented on a wide range of cells, mainly on monocytes/macrophages, neutrophils and endothelial cells (1). LPSs elicit both systemic and local reactions by induction of a massive proinflammatory response which involves the release of cytokines participating also in anticancer activity (2). Therefore, for several decades, it has been postulated that LPSs or synthetic lipid A molecules can be used as anticancer agents. Unfortunately, both of them demonstrated only a slight therapeutic effect and overall toxicity (3). The relationship between chronic inflammation and carcinogenesis can therefore be considered in the context of the delivery of various LPS doses into the host environment (4,5) which can affect tumor growth or delay tumor progression. A better understanding of the mode of multifaceted action generated by LPSs or their derivatives in induction of antitumor immune activity is still of interest.

In contrast to the smooth type of LPSs, the rough LPS synthesized by strains such as *E. coli* B are devoid of the O-specific chain (lipooligosaccharide, LOS). It may be related to a partial reduction in virulence and increase in susceptibility to phagocytosis and serum bactericidal reactions (6,7), but it is still able to be an immune stimulator (8).

It has been reported that changes in the proinflammatory activity of LPS derivatives could be induced by the modified structure of lipid A associated with a different number of fatty acid residues (9). Recently, we identified de-*O*-acylated LOS *E. coli* B (LOS-OH) as a major constituent of bacteriophage T4 lysate with reduced biological activity.

In the present study, an LOS derivative with four ester-bound fatty acid residues removed from the lipid A was prepared by

de-*O*-acylation using anhydrous hydrazine. There are a few reports concerning such derivatives differing in the presence of fatty acid residues which may modulate LOS activity. However, a variety of de-*O*-acylated analogues characterized by lower toxicity have been proposed as anticancer agents or adjuvants for anticancer vaccines (3).

To investigate the effect of structural modifications of *E. coli* B LOS-OH (LOS-OH) on its activity, *in vitro* experiments using myeloid and lymphoid cells were performed. Moreover, to examine this compound's effect on lung colonization by B16 cells, a B16 melanoma metastasis *in vivo* model was employed.

Our findings revealed that LOS-OH in *in vitro* experiments induced lower stimulation of myeloid cells, resulting in low cell surface expression, low cytokine production and limited capacity of activation of the BM-DCs to trigger splenocyte priming. Moreover, multiple administration of this compound led to a statistically significant ($P<0.05$; $P<0.01$) reduction in lung metastatic foci formation. It was accompanied by substantial (but not statistically significant) changes in the ratio of granulocytic to monocytic cells in blood and prolonged, low-level activation of myeloid but not lymphoid spleen cells. Thus, LOS-OH demonstrated weaker ability than LOS to stimulate and polarize an innate immune response both *in vitro* and *in vivo*.

Materials and methods

LOS and LOS-OH preparation. Bacteria were grown for 24 h and harvested as previously reported (9). *E. coli* B LOS was extracted from bacterial cells by the PCP method (10) and purified by ultracentrifugation (105,000 x g, 4 h, 4˚C). The yield of LOS was 4% of the bacterial cells.

LOS (50 mg) was de-*O*-acylated by treatment with anhydrous hydrazine as previously described (11) (3 ml, 30 min, 37˚C), followed by precipitation with cooled acetone (-20˚C) and centrifugation (27,000 x g, 30 min, -10˚C). The sediment was washed with acetone, then dissolved in water, and freeze-dried (yield of de-*O*-acylated LOS *E. coli* B: 34 mg, 68% of LOS) (12). Both LOS and LOS-OH compounds were diluted in PBS for *in vitro* and *in vivo* tests. Applied doses did not affect cell viability in *in vitro* and *ex vivo* cultures.

Mice and ethics statement. Female 6-9 week-old C57BL/6 (B6) mice were maintained in the Animal Facility of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland) under specific pathogen-free (SPF) conditions. Mice were supplied from the Center for Experimental Medicine of the Medical University of Bialystok (Bialystok, Poland). All experiments were performed according to the EU Directive 2010/63/EU for animal experiments and were approved by the 1st Local Ethics Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (number 48/2008).

Cell lines and in vitro stimulation. All cells were cultured in standard conditions at 37˚C in a humid atmosphere (5% CO_2). All culture media were supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin and 0.5% sodium pyruvate (all from Sigma-Aldrich Chemie GmbH; Merck KGaA).

B16 cells of a mouse melanoma line (ATCC; Rockville, Maryland, USA) were maintained *in vitro* in RPMI-1640 GlutaMAX and Opti MEM GlutaMAX (1:1) (both from Gibco; Thermo Fisher Scientific, Inc.,) additionally supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Chemie GmbH; Merck KGaA).

RAW264.7 cells of a monocyte line (ATCC) were maintained in DMEM culture medium (ATCC) supplemented with 10% FBS. For tests, cells were stimulated with LOS or LOS-OH (both *E. coli* B) at concentrations of 50, 10, 1 or 0.1 μg/ml for 24 h in 24-well plates ($5x10^5$ /ml/well).

JAWS II cells of a dendritic cell line (ATCC) were cultured in RPMI-1640 GlutaMAX and αMEM (both from Gibco; Thermo Fisher Scientific, Inc.) in a ratio of 1:1 supplemented with 10% FBS and 5 ng/ml rmGM-CSF (Immunotools, Germany). Cells were stimulated with LOS or LOS-OH at concentrations of 10, 1, 0.1 μg/ml for 24 h in 12-well plates ($5x10^5$ /ml/well).

The supernatants from these myeloid cultures were collected and analyzed using commercially available ELISA sets.

Cell culture and ex vivo stimulation. Bone marrow-derived dendritic cells (BM-DCs) obtained from six-day culture in medium RPMI-1640 GlutaMAX supplemented with 10% FBS, GM-CSF (40 ng/ml) and IL-4 (10 ng/ml) (both from Immunotools, Germany) [as described in (13,14)] were applied into 12-well plates ($1x10^6$ /ml/well). The next day (7th day), cells were stimulated with LOS or LOS-OH at concentrations of 10, 1, 0.1 μg/ml for 24 h in 12-well plates. Concentrations of cytokines in the collected supernatants were measured using ELISA. Phenotypic characteristics of stimulated BM-DCs were analyzed using flow cytometry.

Co-culture of spleen cells with preincubated BM-DCs. Spleen cells were obtained from healthy C57BL/6 mice, wiped through a sterile nylon filter into a RPMI-1640 GlutaMAX culture medium with 3% FBS, washed, resuspended and stored in liquid nitrogen. After thawing, splenocytes were primed with BM-DCs which were preincubated with LOS and LOS-OH at concentrations of 10, 1, 0.1 μg/ml for 24 h in 12-well plates ($5x10^5$ /ml/well) prior to mixed culture. The co-culture was carried out for 5 days (10:1 ratio) in RPMI-1640 GlutaMAX culture medium with 10% FBS and 200 U/ml IL-2. Afterwards the cells and supernatants were harvested. The supernatants were analyzed using commercially available ELISA sets. Phenotypic characteristics of spleen cells were analyzed using flow cytometry. Splenocytes were separated in FACS analysis based on SSC-FSC parameters.

Phenotypic characterization of the BM-DCs. For phenotypic characterization of eight-day BM-DCs the following fluorophore-labeled anti-mouse monoclonal antibodies (mAbs) were used: mouse anti-mouse FITC I-A^b (BD Pharmingen; clone 25-9-17), rat anti-mouse RPE-CD40 (BD Pharmingen; clone 3/23), hamster anti-mouse APC-CD80 (BD Pharmingen; clone 16-10A1) and rat anti-mouse PE-Cy7-CD86 (BD Pharmingen; clone GL1), and the appropriate isotype controls: FITC-labeled mouse IgG2a (BD Pharmingen; clone G155-178), R-phycoerythrin (RPE)-labeled IgG2a

(BD Pharmingen; clone R35-95), APC-labeled Hamster IgG2k (BD Pharmingen; clone B83-3), PE-Cy7-labeled Rat IgG2a (BD Pharmingen; clone R35-95). The cells were stained for 45 min at 4˚C. Expression of the cell-surface molecules was analyzed by a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson; BD Biosciences).

Evaluation of cytokine production. Supernatants from all cultures were analyzed using commercially available ELISA kits (IL-6, IL-10 from Becton Dickinson (BD Biosciences) and TNF-α, IL-12, IL-17A, IFN-γ from eBioscience) in accordance with the manufacturer's instructions.

In vivo experiments and determination of antimetastatic activity of LOS-OH. B16 cells of a melanoma cell line were harvested from *in vitro* cultures. A single-cell suspension in Hank's buffered salt solution ($2x10^5$ cells/200 ml/mouse) with cell viability over 90% was inoculated intravenously (i.v.) into the lateral tail vein. LOS and LOS-OH were administered intraperitoneally (i.p.) at doses of 250 and/or 2,500 μg/kg body weight (b.w.)/dose, 4 times (1 h before the B16 cell inoculation and on the 1st, 7th and 14th day following it). During the experiments, mouse body weight and temperature were monitored, but no changes were observed. Experiments were terminated on the 14th and/or 21st day. Mice were sacrificed by cervical dislocation. For visualization of lung metastases, organs (from 8-10 mice per group) were fixed in formalin overnight, which allowed metastatic foci to be distinguished from lung tissue (15). Lung colonies were counted under a dissecting stereomicroscope (x10 magnification). The blood morphology was evaluated in each blood sample using the Mythic 18 analyzers (C2 Diagnostics, Montpellier).

Analysis of spleen cells. Spleens obtained from tumor-bearing mice were prepared as described above. Then, parts of the cells ($2x10^6$ cells/ml) were stimulated with ConA (0.5 μg/ml) or LOS (1 μg/ml) for 48 h and afterwards the supernatants were collected. Concentration of cytokines in collected supernatants were measured using ELISA sets. For lymphoid cell analysis, splenocytes were stained with the following fluorophore-labeled anti-mouse monoclonal antibodies (mAb): anti-CD4-APC (BD Pharmingen; clone RM4-5), anti-CD8-PE-Cy7 (BD Pharmingen; clone 53-6.7), anti-CD49b-PE (BD Pharmingen; clone DX5) and anti-CD19-FITC (BD Pharmingen; clone 1D3). For determination of myeloid splenocytes, anti-B220-APC (BD Pharmingen; clone RA3-6B2), anti-CD11b-PerCP-Cy5.5 (BD Pharmingen; M1/70), anti-Ly6C-PE (BD Pharmingen; clone AL-21), anti-Ly6G-APC-Cy7 (BD Pharmingen; clone 1A8), anti-MHC class II-FITC (BD Pharmingen; clone 25-9-17) and anti-CD86-PE-Cy7 (BD Pharmingen; clone GL1) antibodies were used. To determine their viability DAPI was used. Phenotypic analysis was carried out using an LSRFortessa cell analyzer with Diva software (Becton Dickinson; BD Biosciences).

Statistical analysis. In all remaining analyses, the statistical differences were calculated using the nonparametric Kruskal-Wallis test for multiple independent groups followed by Dunn's multiple comparison post hoc test or two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Analyses were performed using the GraphPad Prism 7.02 software (GraphPad, San Diego, CA, USA). A P-value <0.05 was considered to be indicative of statistical significance. Asterik(s) at the top of a histogram bar describe statistically significant differences of the experimental group when compared to the control group ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$), while hash symbol(s) placed above brackets indicate statistically significant differences between the two indicated groups ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$).

Results

LOS-OH demonstrates weaker ability than LOS to stimulate myeloid cells for production of IL-6 and TNF-α and phenotypic maturity. We evaluated the biological effect of LOS-OH on the RAW 264.7 macrophage-like cell line (Fig. 1A) and JAWS II dendritic cell line (Fig. 1B) in comparison to LOS activity. For this purpose, RAW 264.7 cells were exposed to both compounds at concentrations ranging from 50 to 0.1 μg/ml while JAWS II cells were exposed to concentrations ranging from 10 to 0.1 μg/ml. The unstimulated cells were treated as a control. Observed differences in the cytokine production depended on the concentration and nature of the stimulators. RAW264.7 cells treated with LOS-OH produced IL-6 (P<0.05) and TNF-α (P<0.01, P<0.0001) at extremely low levels, showing a statistically significant decrease in relation to the effect of LOS (Fig. 1A). JAWS II cells were also able to respond to compounds in a concentration-dependent manner. However, in contrast to former cells, differences in the level of cytokine production between LOS and LOS-OH were less expressed. Compounds induced a statistically significant difference in TNF-α production only at a concentration of 0.1 μg/ml (P<0.05) (Fig. 1B) Hence, the concentration range within which LOS-OH appeared to be able to stimulate cytokine production is extremely narrow.

BM-DCs were exposed to LOS-OH for 24 h. We found that their response was very similar to that observed for JAWS II cells. Initially high IL-6 production diminished along with the decrease in concentrations of both compounds. However, cytokine production induced by LOS-OH at 10 μg/ml corresponded to that obtained for LOS at 0.1 μg/ml and was subsequently drastically decreased. A similar tendency of TNF-α and IL-12 production was observed (Fig. 2A). BM-DCs responded to a high LOS concentration with strong IL-10 production but reduction of the compound's concentration, and the use of corresponding LOS-OH concentrations markedly reduced cytokine production. Thus, LOS-OH was less potent in the stimulation of BM-DCs compared to LOS, regardless of the type of cytokine produced.

The effect of both stimulators on the changes in expression of BM-DC surface markers (MHC class II, CD40, CD80, CD86) was evaluated (Fig. 2B). Cell stimulation with LOS caused statistically significant increases in surface marker expression over the control at the concentration of 10 μg/ml [MHCII, CD40 (P<0.001); CD80, CD86 (P<0.0001)] and 1 μg/ml [MHCII (P<0.05); CD80, CD86, CD40 (P<0.01)]. After LOS-OH stimulation, we observed a slight increase in the expression of surface markers compared to untreated cells; however, these changes were not statistically significant. The

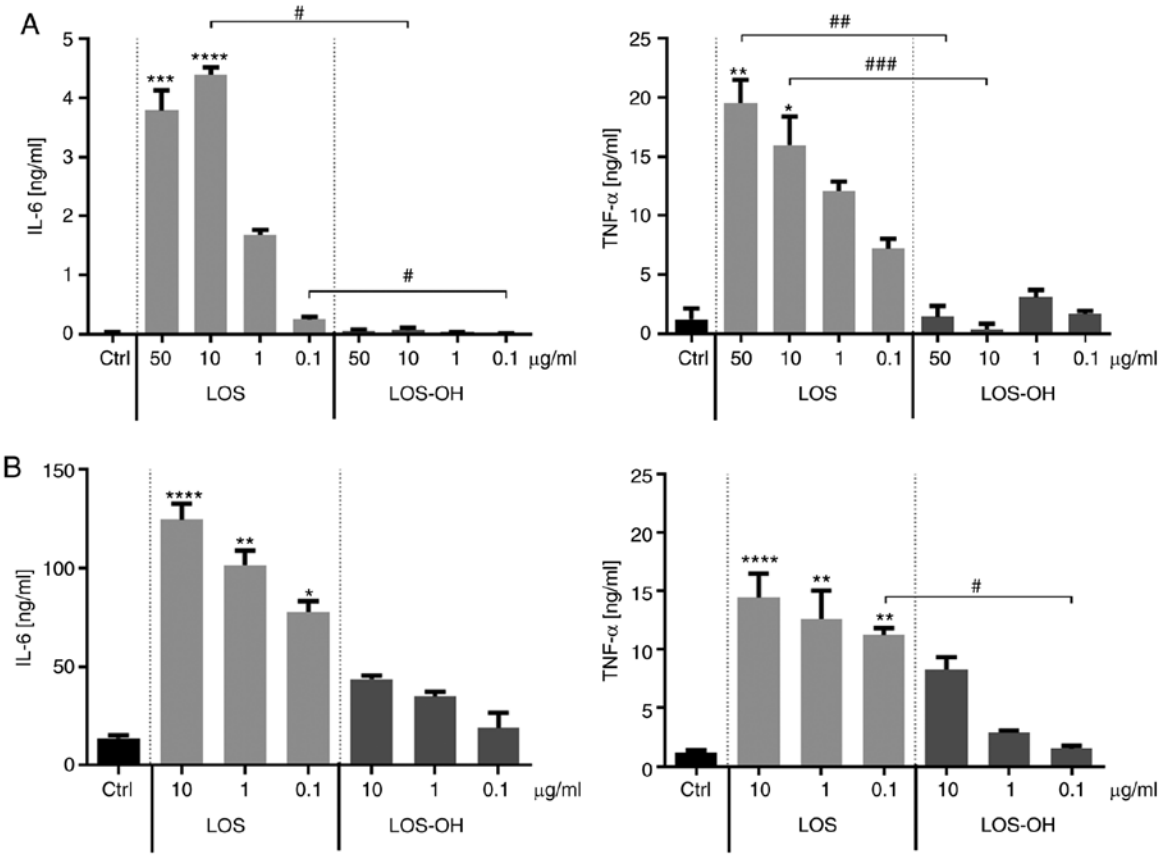

Figure 1. Effects of LOS and LOS-OH on the capacity of RAW 264.7 and JAWS II cells for cytokine production. Concentrations of IL-6 and TNF-α in supernatants collected after 24 h of culture in (A) RAW264.7 and (B) JAWS II cells with LOS or LOS-OH as measured using ELISA. Unstimulated cells were used as a control (Ctrl). The results are shown as the mean ± SD (n=6). The differences between groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*P<0.05, **P<0.01, ***P<0.001, ***P<0.0001 vs. Ctrl; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 between the two indicated groups). LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; Ctrl, control; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α.

obtained data confirmed that LOS-OH exhibited substantially lower efficacy in the activation of BM-DCs than LOS but still in a concentration-dependent manner.

In order to analyze the indirect effect of both compounds on lymphoid cells, we used a mixed cell culture of splenocytes and BM-DCs preincubated for 24 h with LOS (BM-DC/LOS) or LOS-OH (BM-DC/LOS-OH) prior to the test and compared it to untreated cells (Fig. 3A). Preincubated BM-DCs were used in a role of antigenic stimulators. No statistically significant changes in the percentage of $CD4^+$ T helper cells and $CD19^+$ B cells were observed. Although there were some statistically significant differences between BM-DC/LOS-OH groups, changes in the percentage of $CD8^+CD49b^-$ T cytotoxic (P<0.05) or $CD8^-CD49b^+$ natural killer (NK) cells were slight compared to the BM-DC/LOS groups. We found only an increase in the $CD8^+49b^+$ natural killer T (NKT) cell percentage. Thus, the treatment with BM-DC/LOS-OH did not cause any substantial changes in the percentages of spleen-derived lymphoid cell subpopulations. Even if changes were observed, they were comparable with those induced by BM-DC/LOS at 0.1 μg/ml.

We also evaluated the ability of splenocytes co-cultured with BM-DC/LOS or BM-DC/LOS-OH to produce cytokines (Fig. 3B). It was observed that stimulation with BM-DC/LOS-OH resulted in IFN-γ production equal (at 10 μg/ml of LOS-OH) or lower (at subsequent doses of LOS-OH) compared to splenocytes stimulated with untreated BM-DCs. Moreover, we demonstrated a statistically significant reduction in the IFN-γ production by splenocytes co-cultured with BM-DC/LOS-OH at 1 μg/ml in relation to the production of this cytokine by splenocytes stimulated with BM-DC/LOS at 1 μg/ml (P <0.05). We did not observe significant differences in the production of IL-17A by splenocytes stimulated with BM-DC/LOS, BM-DC/LOS-OH or untreated BM-DCs. Splenocyte activation for IL-10 production by BM-DCs preincubated with both compounds was altered in a concentration-dependent manner and the difference between LOS-OH and LOS at 1 μg/ml was statistically significant (P<0.05). Regardless of this fact, considerable differences between the levels of IFN-γ and IL-10 production were found, and the IFN-γ/IL-10 ratio showed that the effect of activated BM-DCs on splenocyte priming was found only when LOS was used at 10 μg/ml (Fig. 3B). Also, BM-DC/LOS-OH were not able to activate a lymphocyte-dependent immune response. In our previous study, spleen cells obtained from healthy mice were able to produce IL-10 after stimulation with LOS and LOS-OH (10, 1, 0.1 μg/ml). The concentration of IL-10 in the supernatants was in the range 0.5-2.0 ng/ml after LOS use and after LOS-OH stimulation was in the range 0-0.5 ng/ml and was dependent on the compound dose (data not shown). In

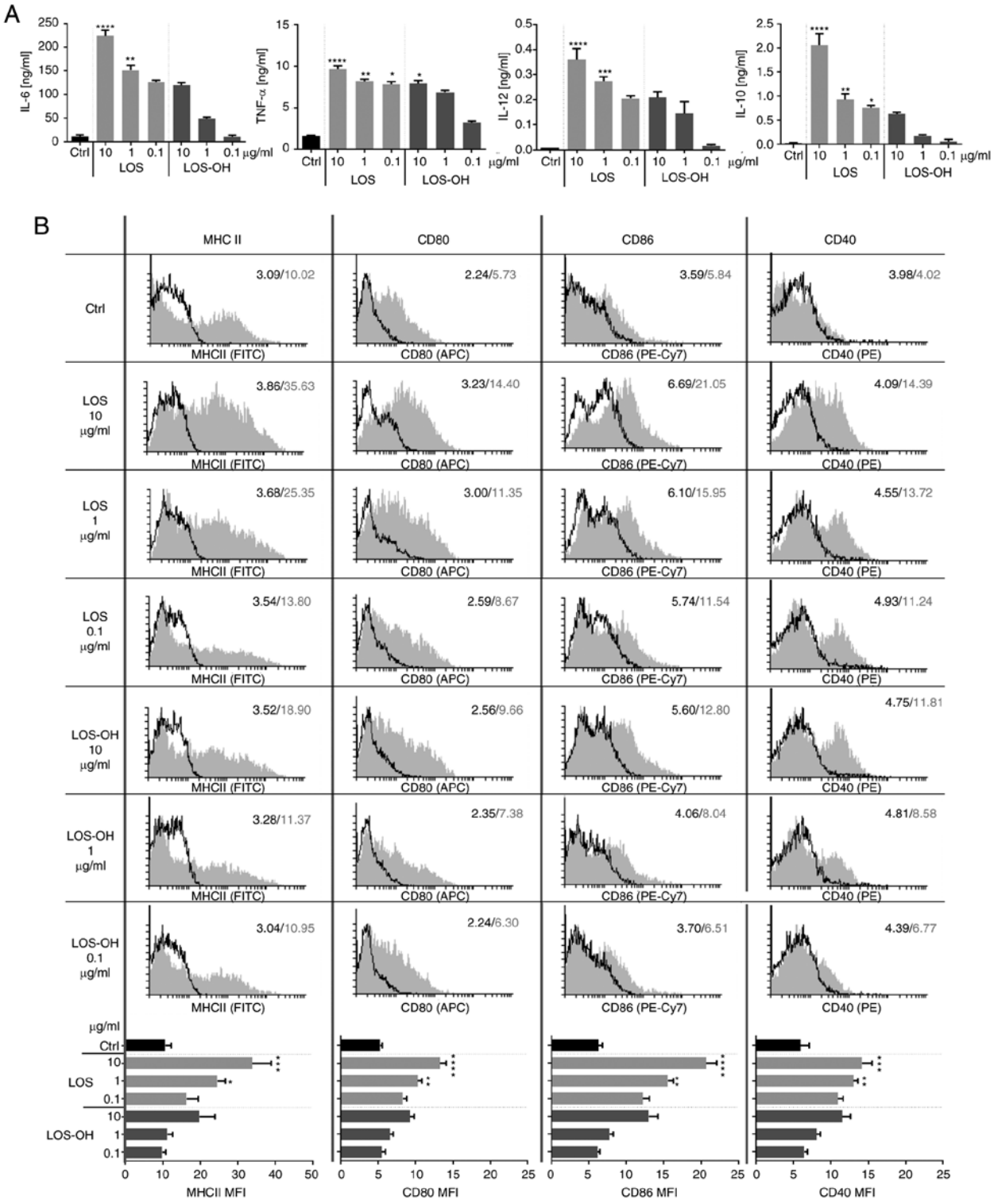

Figure 2. Influence of LOS and LOS-OH on the activity of BM-DCs. (A) Cytokine production by dendritic cells after LOS and LOS-OH stimulation *in vitro*. Unstimulated cells were used as a control (Ctrl). Concentration of cytokines in collected supernatants was measured using ELISA. (B) Phenotypic characteristics of dendritic cells after LOS and LOS-OH stimulation *in vitro*. The histograms present results from one representative experiment. The numbers correspond to MFI (mean fluorescence intensity) for the isotype control (black) vs. examined surface antigen (gray). The results are shown as the mean ± SD (n=6). The differences between groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. Ctrl). LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; Ctrl, control; IL, interleukin; TNF-α, tumor necrosis factor-α; BM-DCs, bone marrow-derived dendritic cells.

contrast to the effects obtained from mixed cultures, splenocytes directly exposed to compounds did not reveal capacity for IL-10 production. Moreover, they did not produce IFN-γ or IL-17A.

LOS-OH exhibits an anti-metastatic effect on pulmonary metastasis of B16 cells but did not affect lymphoid cell activity. Since LOS-OH appeared to be non-toxic at a wide range of doses (data not shown), for evaluation of its

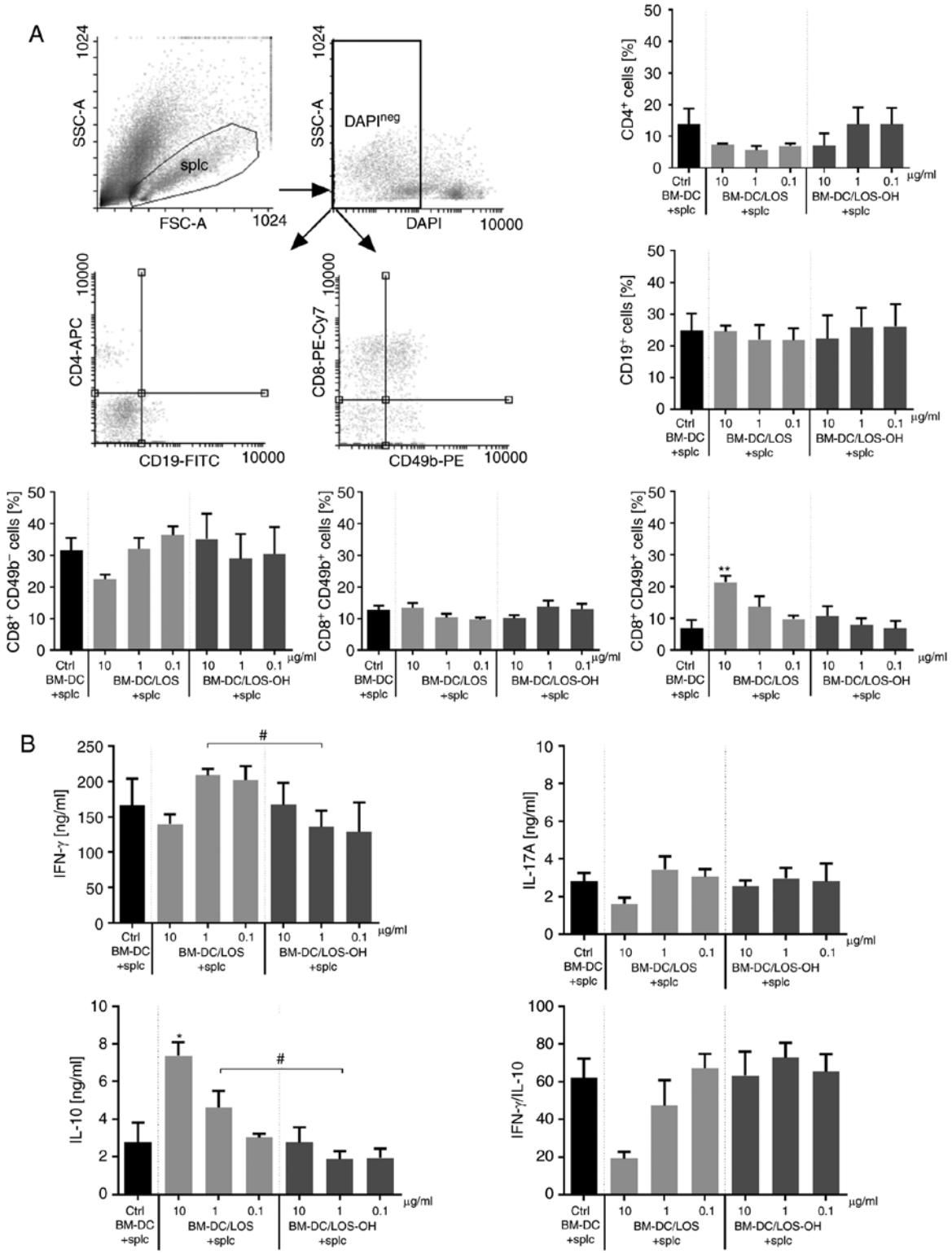

Figure 3. Phenotypic characteristics of splenocytes (splc) primed with BM-DCs previously stimulated with LOS or LOS-OH. (A) Expression of the main lymphocyte surface antigens after 5-day mixed culture of splenocytes and BM-DCs preincubated with LOS or LOS-OH for 24 h. (B) Cytokine concentrations in supernatants from mixed culture of spleen cells and preincubated BM-DCs were evaluated by ELISA. The results are shown as the mean ± SD (n=6). The differences between groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*P<0.05, **P<0.01 vs. Ctrl; $^{\#}$P<0.05 between the two indicated groups). Ctrl, control; BM-DCs, bone marrow-derived dendritic cells; LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; IFN-γ, interferon γ; IL, interleukin.

anti-metastatic activity (vs. LOS) two widely different doses of the compounds (250 or 2,500 μg/kg b.w.) were used (Fig. 4A). The lowest numbers of B16 pulmonary metastatic foci were observed in the LOS-OH treated groups, regardless of the dose administered. The administration of LOS at 2,500 μg/kg/dose caused a statistically significant (P<0.05) increase in the number of pulmonary metastases compared to untreated mice, whereas the use of LOS-OH at the same dose resulted in a

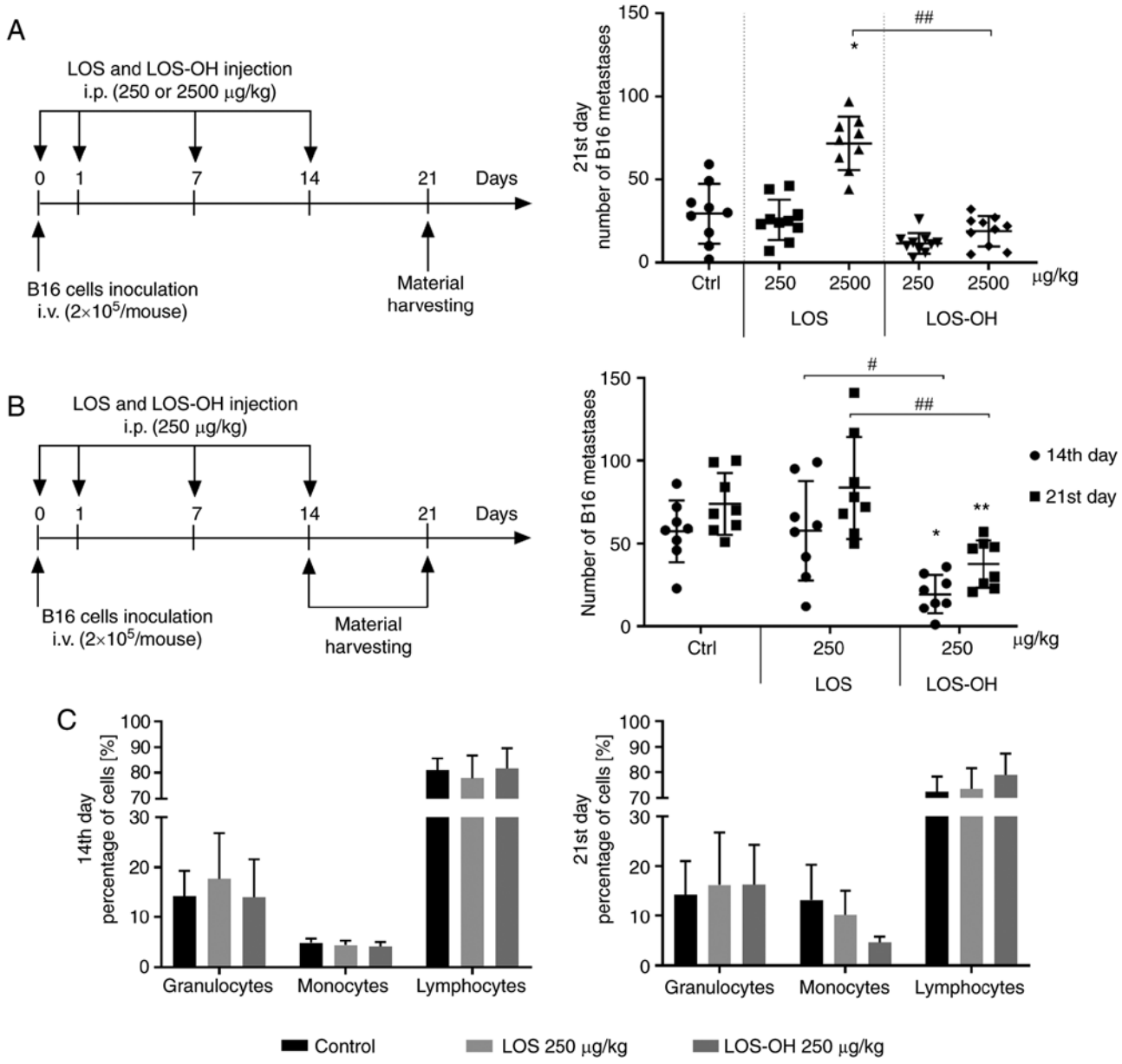

Figure 4. Estimation of the effects of LOS or LOS-OH administration to B16 metastasis-bearing-C57BL/6 mice. (A and B) Schemes of the *in vivo* experiment and average number of pulmonary metastatic foci from 9-10 mice for each mice group counted on the 14th and/or 21st day of the experiment. The results are shown as the mean ± SD for 9-10 mice per group. (C) The number of leukocytes determined in the blood collected from B16 metastasis-bearing mice on the 14th and 21st day of the experiment. The differences between groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test $P<0.05$, $^{**}P<0.01$ vs. Ctrl; $^{\#}P<0.05$; $^{\#\#}P<0.01$ between the two indicated groups). Ctrl, control; LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; i.v., intravenously; i.p., intraperitoneally.

significant reduction in metastatic foci compared to the LOS-treated mice ($P<0.01$).

In the next experiment we analyzed the time relation of the anti-metastatic effect of LOS-OH applied at 250 μg/kg/dose (Fig. 4B). The average number of pulmonary metastases in the LOS-OH-treated mice varied between 20 (the 14th day) and 38 (the 21st day), while in mice treated with LOS the number of metastases did not decrease and was estimated as 58 (the 14th day) and 84 (the 21st day). We found a time-dependent, statistically significant reduction in pulmonary metastasis number when mice were treated with LOS-OH compared to the control group ($P<0.05$, $P<0.01$ at the 14th and 21st day, respectively) or mice treated with LOS ($P<0.05$, $P<0.01$ at the 14th and 21st day, respectively).

In the course of the second experiment, blood morphological analysis was performed. The changes in cell number were related to the type of the compound and time point of observation (Fig. 4C). On the 14th day of the experiment, lymphocytes obtained from metastasis-bearing mice revealed a similar percentage regardless of the nature of stimulators. There were no significant differences in percentages of either monocytes or granulocytes at this time point. Meanwhile, inconsiderable changes in the percentage of lymphocyte and monocyte subpopulation in the blood obtained from mice on the 21st day of the experiment were observed. However, no changes in the granulocyte subpopulation were observed.

We also analyzed the influence of the administration of two doses of compounds on activation of the systemic

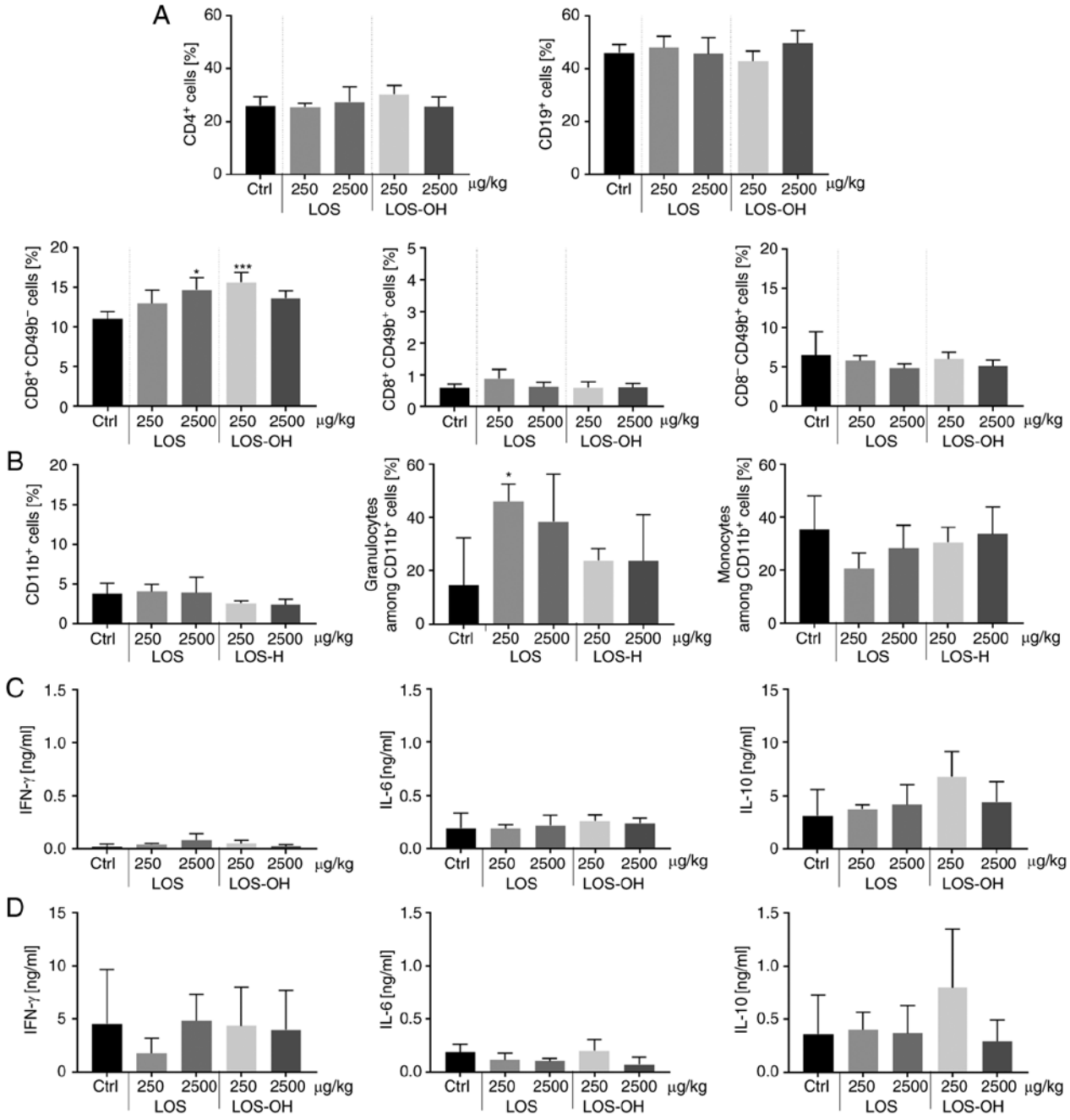

Figure 5. Activity of spleen cells obtained from tumor-bearing mice after treatment with LOS and LOS-OH at doses of 250 and 2,500 μg/kg on the 21st day of the experiment. Phenotypic characterization of (A) lymphoid and (B) myeloid cells was performed by flow cytometry. Cytokine production after 48-h *ex vivo* cell stimulation with (C) 1 μg/ml of LOS or (D) 0.5 μg/ml of concanavalin A (ConA) was analyzed by ELISA. To calculate the mean, four mice of each group were tested. The differences between the groups were estimated using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (*P<0.05, ***P<0.001 vs. Ctrl). Ctrl, control; LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; IFN-γ, interferon γ; IL, interleukin.

response of treated mice, revealing that LOS-OH caused changes in the number of lymphoid and myeloid cells in the spleen. The percentage of $CD4^+$ T helper, $CD19^+$ B and $CD8^-CD49b^+$ NK or $CD8^+CD49b^+$ NKT cells did not show differences between groups. However, after administration of LOS-OH at 250 μg/kg/dose, a statistically significant (P<0.001) increase in the percentage of $CD8^+CD49b^-$ T cytotoxic cells was noted compared to the control group (Fig. 5A). On the other hand, treatment with the compounds did not increase the number of $CD11b^+$ cells but induced significant (P<0.05) changes within this population after LOS (250 μg/kg) stimulation, particularly in the granulocytic subpopulation (Fig. 5B).

We also evaluated the influence of both compounds on specific cytokine production. For this purpose, splenocytes from treated mice were *ex vivo* re-stimulated with LOS or stimulated with concanavalin A (Con A). Splenocytes which responded to LOS (Fig. 5C) produced a low amount of IL-6, a minor amount of IFN-γ but a high amount of IL-10 compared to the control, especially when spleen cells originated from mice treated with 250 μg/kg of LOS-OH. Splenocytes which responded to ConA (Fig. 5D) produced a similar amount

of IL-6 but lower amount of IL-10 with the same tendency corresponding with mouse treatment.

Subsequently, we analyzed the kinetics of activation of the systemic response after treatment with 250 μg/kg of LOS-OH or LOS (Fig. 6). No significant changes in the percentage of $CD4^+$ T helper, $CD8^+CD49b^{+/-}$ cells, or $CD19^+$ B splenocyte subpopulations were observed. An increase in the percentage of $CD8^-CD49b^+$ NK cells during the experiment (Fig. 6A) as well as a time-related increase in the myeloid cell number was also found (Fig. 6B). However, all observed changes were only time-dependent, and they were not associated with the applied treatment.

To conclude, the administration of LOS-OH was not able to cause differences in the size of spleen cell subpopulations compared to LOS or control groups even when some time-dependent changes could be observed (NK and myeloid splenocytes).

We also evaluated the changes in the level of cytokine production by splenocytes obtained on the 14th or the 21st day of the experiment (Fig. 6C). The data showed a time-dependent increase in cytokine production. However, differences between types of cytokines were related to the type of *ex vivo* stimulation rather than to the type of the administered compound; higher production of IL-10 was induced *ex vivo* by LOS restimulation, and a high amount of IFN-γ was produced by ConA-stimulated splenocytes.

Discussion

The variability of lipid A structures depends on the degree of acylation and the nature of the fatty acids. Removal of four ester-bound fatty acid residues from hexaacyl lipid A of *Escherichia coli* lipooligosaccharide (LOS) resulted in the de-*O*-acylated derivative (LOS-OH). Since some aspects of structure-function relationships for various lipopolysaccharides (LPS) are not fully understood (16), we investigated the influence of the structural modification of one of these on the biological activity. Toward this aim, macrophages or dendritic cells of the myeloid lines were exposed to LOS-OH for 24 h and revealed lower induction of IL-6 and TNF-α production compared to LOS possessing hexaacyl lipid A activity. Hence, a question has been raised whether LOS-OH would affect bone marrow-derived dendritic cells (BM-DCs) in a similar manner. Based on cytokine production, regardless of the type of secreted cytokines (proinflammatory IL-6, TNF-α, IL-12 or anti-inflammatory IL-10) we confirmed that LOS-OH demonstrated lower ability to stimulate BM-DCs than LOS. BM-DCs are believed to be programmed for a strong response to LPS stimulation, which resulted in upregulation of MHC class II and CD80 and CD86 expression (17-19). A similar effect was exhibited by BM-DCs stimulated with LOS (8). In contrast, LOS-OH caused only a slight increase in expression of these antigens. Similar downregulation of the immune reaction has been described both in human and mouse macrophages (20) and could be associated with low TNF-α production leading to induction of immune tolerance (3,21,22). The relationship between chronic inflammation and carcinogenesis can be considered in the context of the delivery of various doses of LPS and its derivatives into the host environment (4,5), provoking not only tumor growth. The effect associated with low LPS concentration probably results from endotoxin-induced immune tolerance (21,23,24).

LOS-OH appeared to be unable to directly trigger splenocyte reactivity *in vitro*. When these cells were co-cultured with BM-DC/LOS-OH, the cell interaction did not cause changes in surface antigen expression on splenocytes. In these mixed cultures, IFN-γ production did not exceed the control level (untreated BM-DCs). In turn, BM-DCs preincubated with LOS-OH or LOS provoked splenocytes to produce IL-10 at a higher level with a statistically significant concentration of 1 μg/ml ($P<0.05$), but significant changes in IL-10 production were observed only after the use of BM-DC/LOS at 10 μg/ml ($P<0.05$). Moreover, the calculation of IFN-γ to IL-10 ratio revealed that BM-DC/LOS-OH were not able to modulate priming of splenocytes.

The above findings suggest that LOS-OH is a weaker stimulator for *in vivo* activation and polarization of the immune response, consistent with its non-toxicity over a wide dose range in comparison to LOS. Therefore, we conducted *in vivo* experiments with a melanoma B16 metastasis model in which B16 melanoma cells were inoculated intravenously in mice and treated with LOS-OH.

LOS-OH given i.p. at a dose of 250 μg/kg b.w., caused a time-dependent, statistically significant reduction in the number of experimental metastases compared to the control group or mice treated with LOS. However, changes in the percentage of monocyte and lymphocyte cells in the blood, examined 7 days after the last compound administration in the second experiment, appeared to be inconsiderable. Admittedly, the time interval in which the immune cells retain increased activity after treatment with LPS does not exceed 3 to 4 days (25), but we observed in our previous report (8) the prolonged presence of low concentrations of IL-1β and TNF-α in blood, even 7 days after the application of LOS. However, this could be the result of a secondary action of myeloid cells rather than a direct effect of LOS administration (8). Thus, *E. coli* B LOS-OH-mediated activation of myeloid cells in blood, appeared to have low efficacy. The other issue can be the influence of multiple LPS administration on reduction of immune cell response, perhaps associated with induction of immune tolerance. However, it is unlikely that a weak stimulator such as the LOS-OH compound could cause tolerance. Alternatively, LPSs playing a role in interaction between bacteria and the cell surface especially of myeloid cells (26) trigger innate inflammatory reaction. The structure of the LOS derivative with four ester-bound fatty acid residues removed from the lipid A, such as *E. coli* B LOS-OH, led to low-level activation of myeloid cells which rather prevented proinflammatory activity. Due to increasing evidence confirming the macrophage interactions with development of metastases, we anticipated such a relationship in the case of our experiments (27). It cannot be excluded that a decrease in myeloid cell reactivity hindered B16 cell migration and anchoring in the lung. These findings suggest that a temporary anti-metastatic effect of LOS-OH might be elicited via downregulation of myeloid cell activity. Thus, it may be responsible for reduction in the local immune cell response, but not the systemic response.

We also considered the interaction of blood leukocytes and auxiliary cells of vascular endothelium, which are well known to be sensitive to LPS action. The almost unaltered

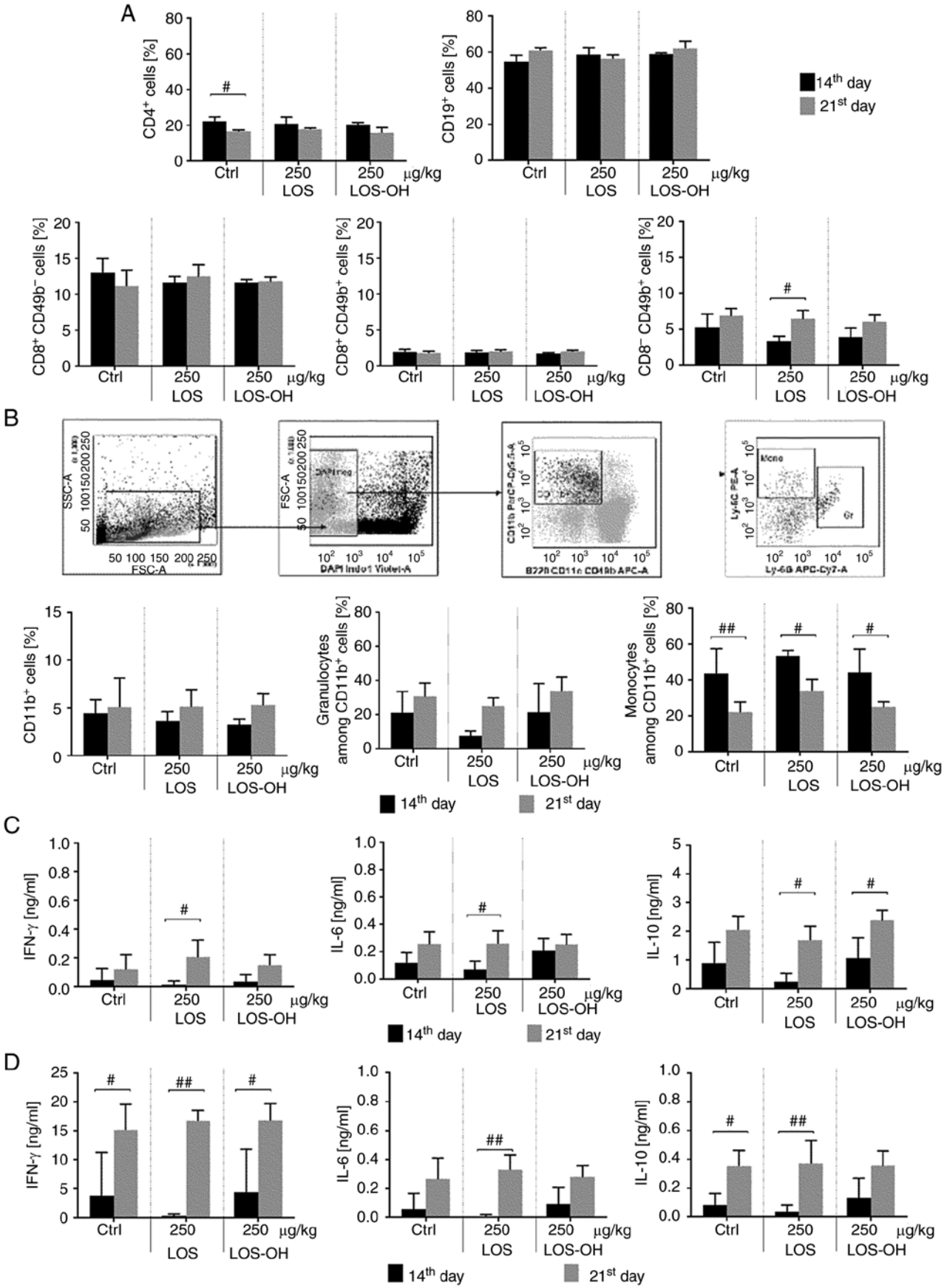

Figure 6. Phenotypic and functional characteristics of splenocytes obtained from tumor-bearing mice treated with LOS or LOS-OH in doses of 250 μg/kg or untreated controls on the 14th or 21st day of the experiment. Phenotypic analysis of (A) lymphoid and (B) myeloid cells was performed by flow cytometry. Cytokine production after 48-h *ex vivo* cell stimulation with (C) 1 μg/ml of LOS or (D) 0.5 μg/ml of concanavalin A (ConA) was analyzed by ELISA. To calculate the mean, four mice of each group were tested. The differences between the groups were estimated using two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test ($^{\#}P<0.05$, $^{\#\#}P<0.01$). Ctrl, control; LOS, *Escherichia coli* lipooligosaccharide; LOS-OH, de-*O*-acylated derivative of LOS; IFN-γ, interferon γ; IL, interleukin.

percentage of granulocytes suggested that these cells may also be responsible for inhibition of B16 cell migration to the lung, when they are considered in the context of inflammatory reactions. Granulocytes may be of great importance in the formation of a metastatic niche, but they (at least neutrophils in humans) can also reduce LPS toxicity by enzymatic activity (26).

Administration of LOS-OH to mice did not affect the diversity of the spleen cell populations, and even if the changes were visible, they were only time-dependent, and the multiple use of LOS-OH led to low-level activation of myeloid but not lymphoid spleen cells. The multiple administration of LOS as well as LOS-OH provoked splenocytes to respond to secondary stimulation (re-stimulated with LOS or stimulated with ConA), which resulted in a time- and stimuli-dependent increase in IL-10 and IFN-γ production. However, there were no differences between groups of treated mice and control suggesting that no systemic response was activated as a result of compound administration.

Altogether, multiple applications of *E. coli* B LOS-OH derivative of *E. coli* B LOS were able to elicit a statistically significant decrease in the number of metastatic foci, presumably via silencing of myeloid cell reactivity as well as the inability to stimulate lymphoid cells both directly and indirectly.

These findings suggest that LOS-OH maintained in the body of the metastasis-bearing mice seems to modulate or even downregulate the innate response, leading to the inability of blood myeloid cells to support the migration of melanoma cells to lung tissue.

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Availability of data and materials

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

Authors' contributions

JM, AS, JW, JB and EPP conceived and designed the experiments. JM, AS, NAG, JR, JJ, EPP and JW performed the experiments. JM, AS, JR, JJ, JW and EPP collected and analyzed the data. MK, AM, WJ, TN, JL and CL prepared and analyzed LOS and LOS-OH of *E. coli* B and in this manner facilitated conduction of the experiments. JM, AS, EPP, JW, JR and JL wrote the manuscript. All the authors read and approved the manuscript and provided critical feedback and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments were performed according to EU Directive 2010/63/EU for animal experiments and were approved by the 1st Local Ethics Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (number 48/2008).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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