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The vaccine adjuvant MPLA activates glycolytic metabolism in mouse mDC by a JNK-dependent activation of mTOR-signaling

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ABSTRACT

Introduction: The detoxified TLR4-ligand MPLA is a successfully used adjuvant in clinically approved vaccines. However, its capacity to activate glycolytic metabolism in mDC and the influence of MPLA-induced metabolic changes on cytokine secretion are unknown.

Aim: To analyze the capacity of MPLA to activate mDC metabolism and the mechanisms contributing to MPLA-induced metabolism activation and cytokine secretion.

Methods: C57BL/6 bone-marrow-derived myeloid dendritic cells (mDCs) were stimulated with LPS or MPLA and analyzed for intracellular signaling, cytokine secretion, and metabolic state. mDC were pre-treated with rapamycin (mTOR-inhibitor), U0126, SP600125, SB202190 (MAPK kinase inhibitors), as well as dexamethasone (MAPK- and NFκB-inhibitor) and analyzed for MPLA-induced cytokine secretion and cell metabolic state.

Results: Stimulation of mDCs with either LPS or MPLA resulted in a pronounced, mTOR-dependent activation of glucose metabolism characterized by induction of the Warburg Effect, increased glucose consumption from the culture medium, as well as release of LDH. Compared to LPS, MPLA induced significantly lower cytokine secretion. The activation of mDC metabolism was comparable between LPS- and MPLA-stimulated mDCs. The MPLA-induced cytokine secretion could be partially inhibited using mTOR-, MAP kinase-, and NFκB-inhibitors, whereas the activation of glucose metabolism was shown to depend on both mTOR- and JNK-signaling.

Summary: The MPLA-induced activation of glycolytic metabolism in mouse mDC was shown to depend on a JNK-mediated activation of mTOR-signaling, while both MAPK- and NFB-signaling contributed to pro-inflammatory cytokine secretion. Understanding the mechanisms by which MPLA activates dendritic cells will both improve our understanding of its adjuvant properties and contribute to the future development and safe application of this promising adjuvant.

1. Introduction

Adjuvants are used to boost immune responses against otherwise poorly immunogenic antigens. Currently licensed adjuvant preparations include oil emulsions, liposomes, aluminum salt derivatives (alum), “Toll”-like receptor (TLR)-activating innate immune agonists such as monophosphoryl lipid A (MPLA), or complex combinations of these (Alving et al., 2012; Fox et al., 2011).

Among those adjuvants, TLR-ligands like MPLA are of special interest because they harbor an intrinsic capacity to activate innate immune cells, resulting in the efficient induction of antigen-specific adaptive immunity.

The detoxified LPS-derivative and TLR4-ligand MPLA, was derived from the lipopolysaccharide (LPS) of *Salmonella minnesota* R595 by a series of organic extractions followed by mild acid and alkaline treatments (Ulrich and Myers, 1995), resulting in three distinct modifications compared to its parent molecule: (1) The removal of the core polysaccharide containing the O-antigen, (2) the removal of one phosphate group, and (3) the removal of one fatty acid chain (Ulrich and Myers, 1995).

MPLA has been evaluated in human clinical trials where it has been safely administered to more than 300,000 human subjects (GlaxoSmithKline Vaccine HPV-007 Study Group et al., 2009) and several vaccines containing MPLA have been licensed or are in phase III trials including Fendrix® (hepatitis B), Cervarix® (human papillomavirus-16 and -18), Pollinex® Quattro (pollen allergies), and RTS,S® (malaria) (Agnandji et al., 2011; GlaxoSmithKline Vaccine HPV-007 Study Group et al., 2009; Kundi, 2007; Patel and Salapatek, 2006).

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Therefore, MPLA is a successfully used adjuvant in both prophylactic and therapeutic vaccination settings.

Immunologically, MPLA which is usually applied as part of adjuvant systems (AS) has been shown to predominantly induce Th1-biased immune responses in mice (Casella and Mitchell, 2008) and to efficiently induce non-specific resistance to infection for up to 2 weeks post-exposure (Fensterheim et al., 2018).

We previously showed that, in direct comparison to LPS, MPLA-stimulation induced similar, but attenuated immune responses in several important immune cell types such as mouse epithelial cells, myeloid dendritic cells (mDCs), B and T cells, as well as human *ex-vivo* isolated monocytes, while being unable to activate either human or mouse mast cells (Schülke et al., 2015).

To improve both the efficacy and safety of existing and novel adjuvants, we need to understand the complex mechanisms by which these substances activate immune cells. Recent results have suggested a newly arising paradigm in which the activation of immune cells is not only mediated by classical immunological signaling pathways but also by distinct changes in immune cell metabolism (O'Neill et al., 2016). In macrophages TLR stimulation was reported to induce a shift towards a glycolytic phenotype (without changing oxygen consumption), thereby strongly reducing oxidative phosphorylation while favoring the generation of lactate from glucose in a process termed the Warburg Effect (Galván-Peña and O'Neill, 2014; Jha et al., 2015; Rodríguez-Prados et al., 2010). In immune cells, this increased glucose consumption sustains essential immune-related functions such as cytokine production, phagocytosis, reactive oxygen species (ROS) generation, and cell proliferation (Linke et al., 2017). Moreover, the increased rate of glycolysis increases cellular adenosine tri-phosphate (ATP) production, while also allowing for the production of biosynthetic intermediates needed by the activated immune cells (O'Neill, 2011). Therefore, immune cell function and consequently adjuvant activity is critically linked to the metabolism of the corresponding cell. LPS, the parent molecule of MPLA, also induces a metabolic shift towards aerobic glycolysis characterized by increased glucose consumption and lactate release and decreased oxygen consumption rate (Warburg Effect) in both monocytes (Lachmandas et al., 2016; Raulien et al., 2017) and macrophages (Fensterheim et al., 2018; O'Neill, 2011; Zhu et al., 2015).

While the influence of LPS on macrophage and monocyte metabolism is well studied (Lachmandas et al., 2016; O'Neill, 2011; Raulien et al., 2017; Zhu et al., 2015), up to now very few data on MPLAs ability to activate macrophage metabolism (Fensterheim et al., 2018) and no data on MPLAs impact on glycolytic metabolism in DCs are available. Therefore, the aim of this study was to analyze the influence of the vaccine adjuvant MPLA on glycolytic metabolism in myeloid dendritic cells (mDCs) and the mechanisms connecting MPLA-induced cytokine secretion to a possible activation of mDC metabolism.

2. Material and methods

2.1. Endotoxins

MPLA was purchased from Invivogen (Toulouse, France) and LPS (L5886) from Sigma-Aldrich (Steinheim, Germany). Both endotoxins were reconstituted in DMSO at a concentration of 1 mg/ml.

2.2. Mice

C57BL/6 mice were bred at the animal facility of the Paul-Ehrlich-Institut under specific pathogen-free conditions. All animal experiments were performed in compliance with the German animal protection law.

2.3. In vitro generation and stimulation of mouse bone marrow-derived dendritic cells

Mouse myeloid dendritic cells (mDCs) were generated as described previously (Schülke et al., 2010). On day 8 mDCs were seeded at 3.2×10^5 cells/ml in 24-well plates (Thermo Scientific, Dreieich, Germany) and stimulated with the indicated concentrations of either LPS or MPLA for 24–72 h. Supernatants were analyzed for cytokine secretion by ELISA using either the CCL2 Ready-Set-GO ELISA Set (eBiosciences, Frankfurt, Germany), matched antibody pairs (eBiosciences, IL-1β: purified anti mouse IL-1β (clone B122) plus polyclonal biotin anti mouse IL-1β (#13-7112-81), TNF-α: purified anti mouse TNF-α (clone 1F3F3D4) plus biotin anti mouse TNF-α cocktail (clones MP6-XT3 & MP6-XT22), IL-12p70: purified anti mouse IL-12p70 (clone C18.2) plus biotin anti mouse IL-12p70 (clone C17.8), or the IL-10 mouse ELISA development kit (Peprotech, Hamburg, Germany) according to the manufacturers recommendations.

2.4. Inhibitors

For inhibitor experiments mDCs were pre-incubated with the indicated amounts of rapamycin (mTOR inhibitor), SB-202190 (p38α/β MAPK inhibitor), SP600125 (JNK MAPK inhibitor), dexamethasone (MAPK- and NFκB-inhibitor) (all Invivogen, Toulouse, France), or U0126 (MEK1/2 MAPK inhibitor, Cell Signaling Technologies, Leiden, The Netherlands) for 90 min (ELISA and metabolic state) to 3 h (Western Blot) and subsequently stimulated with either 1 µg LPS or MPLA for either 30 min (Western Blot) or 24–72 h (ELISA and analysis of cell metabolic state). Toxicity of the used inhibitors was determined by flow cytometry using an LSR II flow cytometer (BD Bioscience, Heidelberg, Germany) and the fixable viability dye eFlour 780 (Thermo Fisher Scientific, Dreieich, Germany). Data were analyzed using FlowJo V.7 (Treestar Inc., Ashland, OR, USA). Here, compared to unstimulated mDCs, none of the used inhibitor concentrations showed toxic effects (Repository Fig. E1).

2.5. SDS-PAGE and Western Blot

SDS-PAGE was performed according to the method described by Laemmli (Laemmli, 1970) (cross linker c = 5%, total bis/acrylamid 15%) under reducing conditions. For Western Blot experiments mDCs were cultured in RPMI1640 + 10% FCS (Sigma-Aldrich) with or without the indicated inhibitor concentrations and cultured for 3 h at 37 °C, 5% CO_2 in FACS tubes (BD Bioscience). Subsequently, 1×10^6 mDCs were stimulated with the indicated proteins for 30 min in 200 µl RPMI1640 without additions. 30 min post stimulation, cells were washed with ice cold PBS and subsequently lysed with 200 µl lysis buffer (62.5 mM Tris-HCl (pH = 6.8 at 25 °C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) for 10 min on ice. Target proteins in lysates were detected by Western Blot using the iBind System (Thermo Fisher Scientific) and antibodies from Cell Signaling Technologies: NFκB pathway sampler kit, MAPK family antibody sampler kit, phospho-PI3 kinase p85, mTOR substrates antibody sampler kit, or loading control antibody sampler kit (HRP Conjugate). Detection was performed using ACE Glow substrate (VWR, Darmstadt, Germany) and a Fusion-Fx7 Spectra (Vilber Lourmat, Eberhardzell, Germany).

Band intensities were quantified using ImageJ software (version 1.51, NIH, Bethesda, USA), obtained RLU values were normalized to unstimulated samples and plotted using GraphPad Prism for Mac (version 8).

2.6. Analysis of cell metabolic state

The Warburg Effect in stimulated mDC cultures was documented by taking photos of the culture plates and determined photometrically 24 and 72 h post stimulation by quantifying the optical density (OD) at 570 nm and calculating the Warburg effect as 1/OD570 normalized to unstimulated controls. Glucose concentrations in culture supernatants were determined 72 h post-stimulation using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically

from the measured glucose concentrations by calculating the glucose consumption in % in relation to medium without mDCs (glucose conc. in RPMI1640 = 2 mg/ml). LDH activity in supernatants of mDCs was determined 72 h post-stimulation using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6 to v8 for Mac or Windows (GraphPad Software, La Jolla, USA) using 2-way ANOVA tests with confidence intervals adjusted for multiple comparisons according to either Bonferroni or Turkey. For statistically significant results the following convention was used: * - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001.

3. Results

3.1. LPS and MPLA activate glucose metabolism in stimulated mDCs

To analyze the impact of MPLA on glucose metabolism in bone marrow-derived mDCs we stimulated mDCs with either MPLA or LPS for 72 h and analyzed the induced Warburg Effect, cell metabolic state, and cytokine secretion (Fig. 1A). Here, both MPLA and LPS induced a clearly detectable Warburg Effect (Fig. 1B&C) which was not significantly different between LPS and MPLA-stimulated mDCs for a stimulation concentration of 1 µg/ml (Fig. 1C), but was less pronounced in MPLA-stimulated mDCs in comparison to the corresponding amount of LPS for other concentrations (for dose-dependent responses see Repository Fig. E2). Moreover, glucose consumption from the culture medium, increased metabolic rates, and the increase in LDH-activity in culture supernatants after stimulation were not significantly different between MPLA- and LPS-stimulated mDCs when stimulated with 1 µg/ml of either endotoxin, whereas for other concentrations significant differences were observed (Fig. 1C, for dose-dependent responses see Repository Fig. E2).

When analyzing mDC-derived cytokine secretion, MPLA induced lower levels of all investigated cytokines (approximately 2.5-fold lower IL-1β, 6.1-fold lower IL-10, 2.5-fold lower TNF-α, and 3.4-fold lower IL-12 production when comparing a stimulation dose of 1 µg/ml of both endotoxins, Fig. 1D) with the exception of CCL2. Here, CCL2 production was not statistically different when stimulating with high dosages of MPLA and LPS (1–10 µg/ml endotoxin, Fig. 1D & Repository Fig. E2) while in a stimulation dose of 0.01 µg/ml MPLA even induced significantly higher CCL2 production than observed for LPS (Repository Fig. E2).

Therefore, interestingly, compared to the corresponding effects of LPS, the MPLA-induced activation of mDC metabolism was comparable to the effects induced by LPS (Fig. 1B&C) whereas the induced cytokine secretion was much lower in MPLA-stimulated mDCs (Fig. 1D).

3.2. MPLA-stimulation results in activation of MAPK-, NFκB, and mTOR-signaling in mDCs

We next checked whether we could detect differences in the signaling pathways activated in mDCs upon MPLA- or LPS-stimulation (Fig. 2). There were no differences in MPLA- and LPS-induced NFκB signaling or p42/44 phosphorylation between MPLA- and LPS-stimulated mDC and only slightly lower p38 and stress-activated protein kinase/c-Jun N-terminal kinase (SAP/JNK) phosphorylation in MPLA-stimulated mDC. Interestingly, in accordance with the strong activation of glucose metabolism by both MPLA and LPS shown in Fig. 1, we observed phosphorylation of both phosphatidylinositol 3 kinase (PI3K) and the down-stream mTOR-target protein p70 S6K in both MPLA- and LPS-stimulated mDCs (Fig. 2, for quantification of WB results see Repository Fig. E3).

3.3. MPLA-induced activation of glycolytic metabolism in mDCs has a strong dependency on mTOR-activation

The observed PI3K and p70 S6K phosphorylation upon stimulation of mDCs with MPLA (Fig. 2, Repository Fig. E4) suggested that, the observed MPLA-induced mDC activation might depend on signaling events located downstream of PI3K activation such as for example activation of mammalian target of rapamycin (mTOR). Therefore, we determined the dependency of MPLA-induced glucose metabolism and cytokine secretion on mTOR-activation by pre-treating either MPLA- or LPS-stimulated mDCs for 90 min with the specific mTOR-inhibitor rapamycin (Fig. 3A).

Here, the MPLA-induced Warburg Effect, glucose consumption from

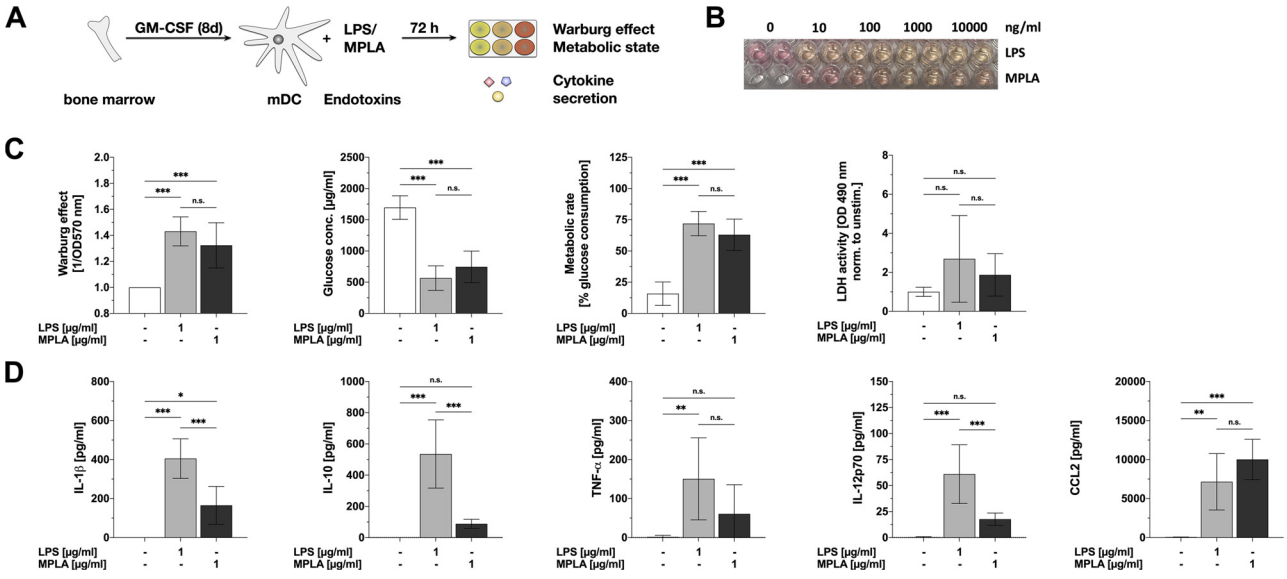

Fig. 1. LPS and MPLA both activate glucose metabolism in myeloid dendritic cells. 0.32 × 10^6 C57BL/6 mDCs were stimulated with 1 µg/ml of either LPS or MPLA for 72 h and analyzed for their metabolic state and cytokine secretion (**A**). The induced Warburg Effect is depicted in (**B**). The metabolic state of the stimulated mDCs in terms of Warburg Effect, glucose consumption, metabolic rate, and release of LDH into the culture medium was determined 72 h post-stimulation (**C**) and cytokine secretion into cell supernatants was determined 72 h post-stimulation by ELISA (**D**). Data are mean results of four independent experiments ± SD.

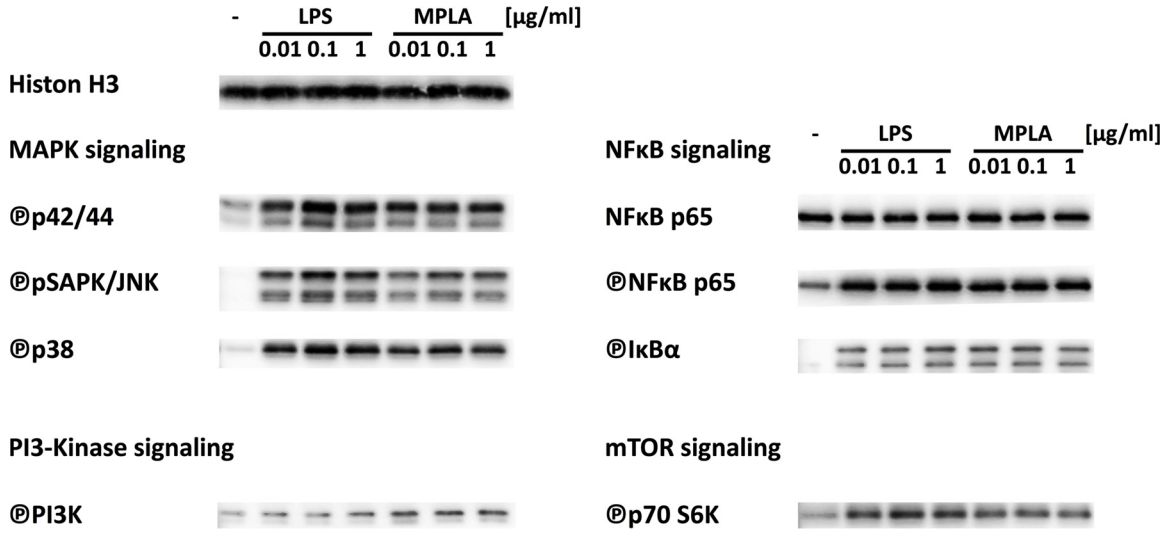

Fig. 2. LPS and MPLA induce comparable MAP kinase, NfκB-, and mTOR-activation. 1×10^6 C57BL/6 mDCs were stimulated for 30 min with the indicated amounts of either MPLA or LPS (0.01–1 µg/ml). Target proteins in the lysates were detected by Western Blot. Data are representative results of three independent experiments.

the culture medium, metabolic rate, and increase in LDH-activity were inhibited by 100%, whereas the LPS-induced Warburg Effect, glucose consumption, as well as metabolic rate were only inhibited up to approx. 50% (Fig. 3B&C, for dose-dependent responses see Repository Fig. E4).

When analyzing the influence of rapamycin-mediated mTOR-inhibition on mDC-derived cytokine secretion, MPLA-induced IL-1β secretion was increased, MPLA-induced IL-10 secretion was reduced, whereas MPLA-induced TNF-α and IL-12 production were not influenced by rapamycin pre-treatment (Fig. 3D, for dose-dependent responses see Repository Fig. E4). Here, LPS-induced IL-1β and IL-12 secretion were increased by rapamycin pre-treatment while IL-10 and TNF-α production were dose-dependently suppressed (Fig. 3D, Repository Fig. E4). Additionally, CCL2 secretion induced by both endotoxins was dose-dependently and significantly inhibited by rapamycin pre-treatment (Fig. 3D, Repository Fig. E4).

24 h post stimulation, the Warburg Effect was not yet detectable and activation of mDC metabolism was very low and completely inhibited for both endotoxins by rapamycin pre-treatment (Repository Fig. E5). In contrast to this, MPLA- and LPS-induced cytokine secretion was already clearly detectable (Repository Fig. E5).

Taken together, these results suggest that indeed MPLA-induced glycolysis and levels of some of the secreted cytokines (IL-10, CCL2, IL-1β) strongly depend on mTOR-activation.

3.4. Inhibition of MAP kinase signaling partially inhibits MPLA-induced cytokine secretion but only JNK inhibition suppresses activation of mDC metabolism

To further analyze the pathways contributing to the MPLA-mediated mDC activation in more detail we focused the remaining experiments on MPLA-stimulated mDCs. In Fig. 2 we observed, that MPLA-stimulation of mDCs resulted in significant activation of MAP kinase (MAPK) signaling (phosphorylation of p42/44, SAPK/JNK, as well as p38). It has been reported, that MAPK-signaling can cross-activate PI3K-mTOR-signaling (Mendoza et al., 2011). Therefore, to further analyze the contribution of the observed MPLA-induced MAPK activation to the activation of mDC cytokine secretion and cell metabolism mDCs were pre-treated with 3 different inhibitors of MAP kinase-activation (U0126 a MEK1/2 and p42/44 MAPK inhibitor, SB202190 an p38α/β MAPK

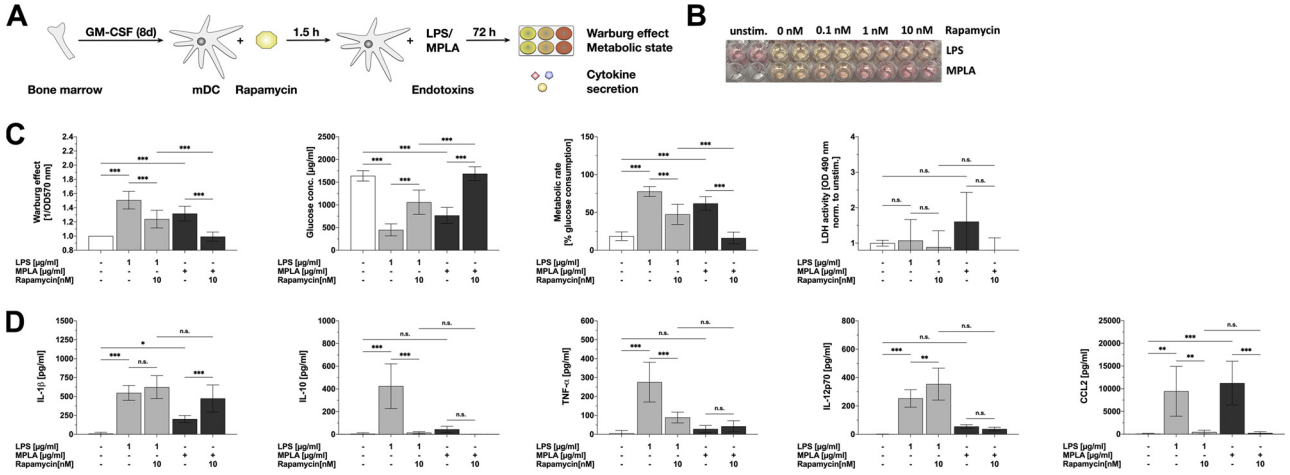

Fig. 3. The MPLA-induced Warburg effect can be more efficiently inhibited by pre-treatment with rapamycin. 0.32×10^6 C57BL/6 mDCs were pre-treated with 10 nM of rapamycin (mTOR-inhibitor) for 90 min and subsequently stimulated with either 1 µg MPLA or 1 µg of LPS for another 72 h (**A**). The induced Warburg Effect is depicted in (**B**). The metabolic state of the stimulated mDCs in terms of Warburg Effect, glucose consumption, metabolic rate, and release of LDH into the culture medium was determined 72 h post-stimulation (**C**) and cytokine secretion into cell supernatants was determined 72 h post-stimulation by ELISA (**B**). Data are mean results of four independent experiments ± SD.

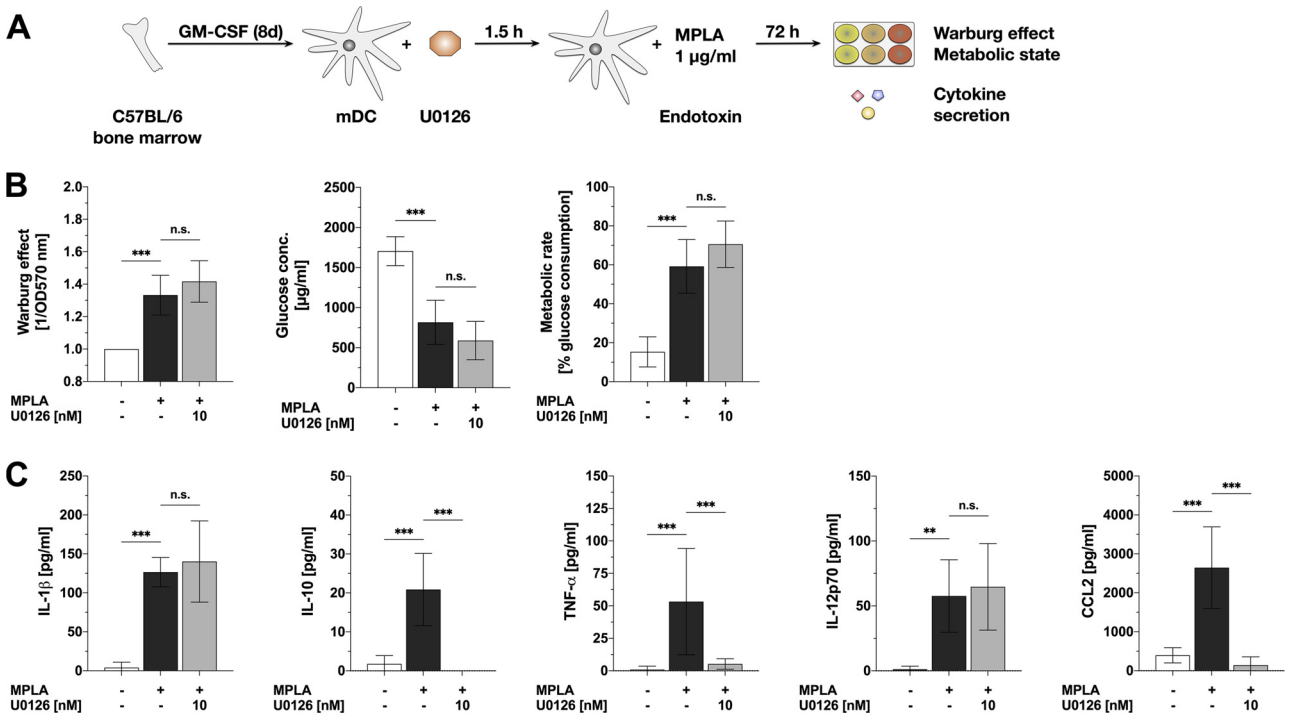

Fig. 4. Inhibition of p42/44 MAP kinase-signaling by U0126 partially inhibits MPLA-induced cytokine secretion but has no influence on activation of mDC metabolism. 0.32×10^6 C57BL/6 mDCs were pre-treated with 10 nM of U0126 (MEK1/2 and p42/44 MAPK-inhibitor (**A**)) for 90 min and subsequently stimulated with 1 µg MPLA for another 72 h. mDCs were analyzed for metabolic state (**B**) and cytokine secretion into cell supernatants 72 h post-stimulation by ELISA (**C**). Data are mean results of four independent experiments ± SD.

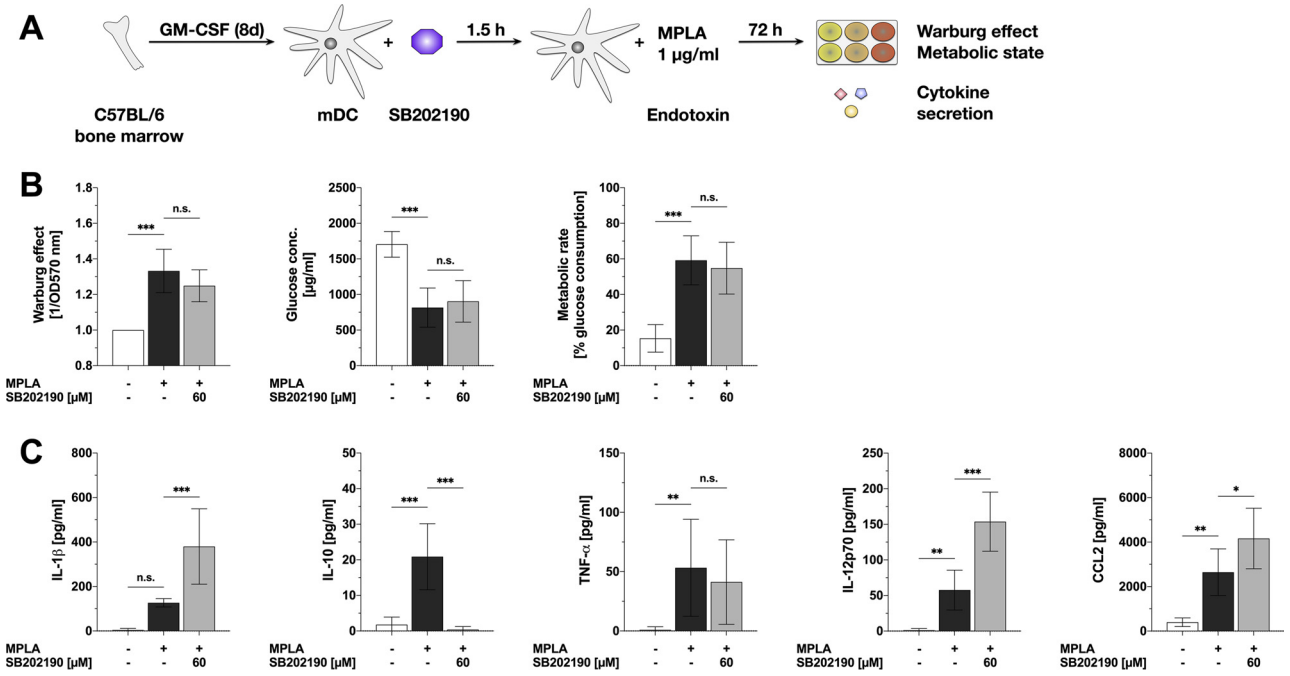

Fig. 5. Inhibition of MAP kinase-signaling by SB202190 alters MPLA-induced cytokine secretion but has no influence on activation of mDC metabolism. 0.32×10^6 C57BL/6 mDCs were pre-treated with 60 µM of SB202190 (p38α/β MAPK inhibitor (**A**)) for 90 min and subsequently stimulated with 1 µg MPLA for another 72 h. mDCs were analyzed for metabolic state (**B**) and cytokine secretion into cell supernatants 72 h post-stimulation by ELISA (**C**). Data are mean results of four independent experiments ± SD.

inhibitor, and SP600125 a JNK-inhibitor), stimulated with MPLA for 72 h, and checked for activation of mDC metabolism as well as cytokine secretion (Figs. 4–6).

Here, pre-treatment with U0126 (Fig. 4A) had no effect on MPLA-induced activation of mDC metabolism (Fig. 4B) and MPLA-induced IL-1β and IL-12 secretion but highly significantly inhibited MPLA-induced IL-10, CCL2, and TNF-α secretion (Fig. 4C, for dose-dependent responses see Repository Fig. E6). In line with these results SB202190 pre-treatment (Fig. 5A) had no effect on MPLA-induced activation of glucose metabolism (Fig. 5B) and TNF-α production (Fig. 5C) while

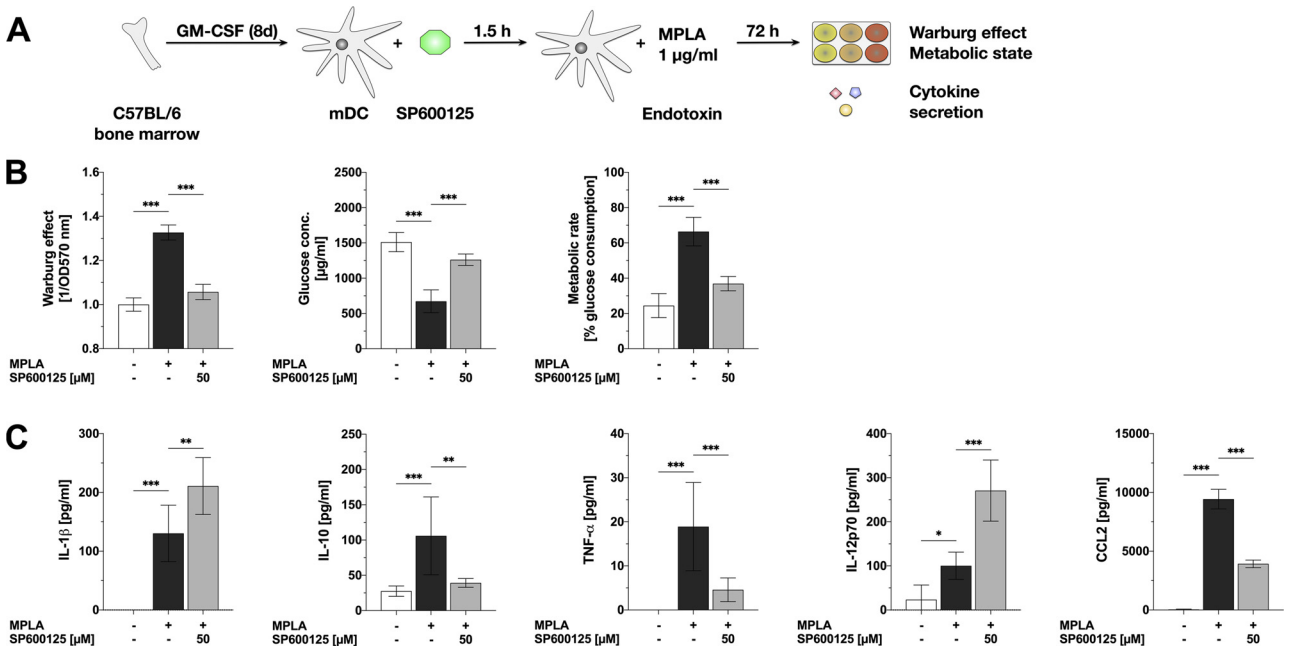

Fig. 6. Inhibition of JNK-signaling by SP600125 alters MPLA-induced cytokine secretion and inhibits activation of mDC metabolism. 0.32×10^6 C57BL/6 mDCs were pre-treated with 50 µM of SP600125 (JNK MAPK inhibitor (**A**)) for 90 min and subsequently stimulated with 1 µg MPLA for another 72 h. mDCs were analyzed for metabolic state (**B**) and cytokine secretion into cell supernatants 72 h post-stimulation by ELISA (**C**). Data are mean results of three independent experiments ± SD.

inhibiting MPLA-induced IL-10 and boosting IL-1β and IL-12, as well as CCL2 secretion (Fig. 5C, for dose-dependent responses see Repository Fig. E7).

Interestingly, pre-treatment of MPLA-stimulated mDCs with the JNK-inhibitor SP600125 (Fig. 6A) highly significantly suppressed the MPLA-induced Warburg Effect to basal levels (Fig. 6B) and partially inhibited both glucose consumption and increases in metabolic rate (Fig. 6B). Moreover, SP600125 treatment significantly suppressed MPLA-induced IL-10, TNF-α, and CCL2 production while increasing IL-12p70 as well as IL-1β secretion (Fig. 6C, for dose-dependent responses see Repository Fig. E8).

3.5. Inhibition of MAP kinase and NFκB-signaling by dexamethasone inhibits both MPLA-induced cytokine secretion and activation of mDC metabolism

Finally, the contribution of both MAPK- and NKκB-signaling to the activation of mDC metabolism and cytokine secretion by MPLA was investigated using the inhibitor of both MAPK- and NFκB signaling dexamethasone (Fig. 7A). Here, pre-treatment with dexamethasone dose-dependently and highly significantly inhibited the MPLA-induced Warburg Effect and significantly reduced glucose consumption (but not to basal levels) and metabolic rate (Fig. 7B, for dose-dependent responses see Repository Fig. E9).

Interestingly, with the exception of IL-12p70 in the highest dexamethasone concentration, dexamethasone pre-treatment significantly (and dose-dependently) suppressed the secretion of all tested cytokines (Fig. 7C & Repository Fig. E9).

When analyzing the influence of the used inhibitors on MAPK activation by Western Blot, pre-treatment with either rapamycin or the PI3K-inhibitor Wortmannin had no effect on MAPK activation while strongly suppressing phosphorylation of the mTOR target protein p70 S6 kinase (Repository Fig. E10A, band quantification in B). In contrast to this, U0126 pre-treatment abrogated MPLA-induced p42/44 MAPK phosphorylation and slightly reduced SAP/JNK phosphorylation without influencing p38 MAPK while increasing p70 S6 kinase phosphorylation (Repository Fig. E10 A). Here, pre-treatment with SB202190 increased p42/44 MAPK phosphorylation, but had no influence on p38 and SAP/JNK phosphorylation (Repository Fig. E10A& B).

Pre-treatment with SP600125 suppressed phosphorylation of p42/44, SAP/JNK as well as p70 S6K, but had no influence on p38 phosphorylation (Repository Fig. E10C&D), while dexamethasone suppressed only slightly suppressed phosphorylation of p38 and SAP/JNK while having no effect on phosphorylation of p42/44 and p70 S6K (Repository Fig. E10C&D).

4. Discussion

While the activation of immune cell metabolism by LPS is well described in the available literature (Lachmandas et al., 2016; O'Neill, 2011; Raulien et al., 2017), corresponding results for the commercially used vaccine adjuvant MPLA are very limited.

In fact, only one recently published study describes the effects of MPLA on macrophage metabolism (Fensterheim et al., 2018). Here, the authors showed MPLA to confer resistance to *S. aureus* and *C. albicans* infection by inducing a metabolic reprogramming of macrophages and sustained chemokine production both supporting improved pathogen clearance (Fensterheim et al., 2018). The MPLA-stimulated macrophages were characterized by a TLR4/mTOR-initiated, sustained glycolysis, mitochondrial biogenesis, increased malate shuttling, and elevated oxygen consumption (Fensterheim et al., 2018). This metabolic reprogramming allows MPLA-primed macrophages to both sustain mitochondrial ATP production and use glycolytic byproducts for antimicrobial purposes (Infantino et al., 2014; Jha et al., 2015; Michelucci et al., 2013). In this context, Fensterheim and colleagues reported the persistent macrophage antimicrobial activity, chemokine secretion, and resistance to subsequent infection induced by MPLA-application to be mTOR-dependent (Fensterheim et al., 2018).

In line with the results reported by Fensterheim, stimulation of mDCs with MPLA induced a pronounced production of the chemokine CCL2, while cytokine secretion induced upon stimulation with MPLA was significantly lower than the levels observed after stimulation with LPS. CCL2 is a potent chemoattractant that contributes to the

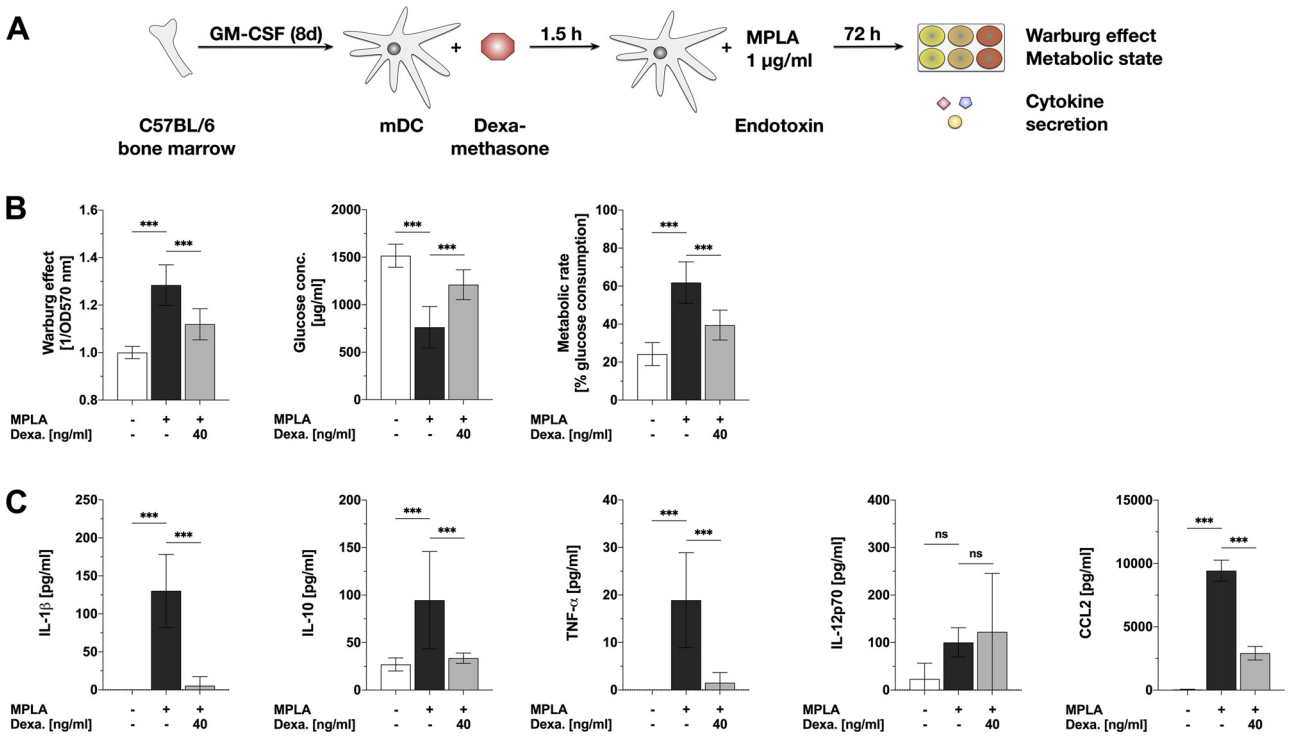

Fig. 7. Inhibition of MAP kinase and NFκB-signaling by dexamethasone inhibits both MPLA-induced cytokine secretion and activation of mDC metabolism. 0.32×10^6 C57BL/6 mDCs were pre-treated with 40 ng/ml of dexamethasone (NFκB- and MAPK-inhibitor (**A**)) for 90 min and subsequently stimulated with 1 µg MPLA for another 72 h. mDCs were analyzed for metabolic state (**B**) and cytokine secretion into cell supernatants 72 h post-stimulation by ELISA (**C**). Data are mean results of three independent experiments ± SD.

recruitment of blood monocytes to the site of inflammation (Yoshimura, 2018). Therefore, the pronounced, mTOR-dependent CCL2-secretion induced by MPLA is likely to contribute to the efficient recruitment and migration of immune cells upon application of this adjuvant.

The present study is the first report of MPLAs capacity to activate glycolytic metabolism in dendritic cells, which as professional antigen presenting cells able to prime naive T cells (Besche et al., 2010) are important cells in vaccination settings.

In line with the results published by Fensterheim et al. for macrophages (Fensterheim et al., 2018), we observed that both LPS and MPLA activated glucose metabolism in stimulated mDCs. In line with these results, García-González et al. were able to show dexamethasone plus MPLA treated human mDCs to (among other genes) strongly up-regulate genes involved in hemotactic responses, cell-to-cell signaling and interaction, fatty acid oxidation, and free radical scavenging, suggesting the activation of mDC metabolism by the combination of MPLA plus dexamethasone (García-González et al., 2017).

Interestingly, while MPLA, with the exception of the chemokine CCL2, consistently induced a much lower production of the investigated, mDC-derived cytokines than the corresponding amounts of LPS, activation of mDC metabolism by MPLA was more comparable, and for a stimulation concentration of 1 µg/ml not significantly different, to the activation of mDC metabolism induced by LPS. Here, MPLA-stimulation induced a comparable Warburg Effect as well as and similar levels of glucose consumption, metabolic rates, and identical LDH release.

In line with these results, MPLA-stimulation resulted in both PI3K and p70 S6K phosphorylation in mDCs, as well as comparable levels of NFκB- and MAP kinase-activation compared to LPS-stimulated mDCs. Concordantly, MPLA was shown to trigger the TLR4-TIR-domain-containing adapter-inducing interferon-β(TRIF)-PI3K-Akt in rat renal cells (Watts et al., 2017).

PI3K phosphorylation can result in mTOR-dependent activation of immune cell metabolism (Herrero-Sánchez et al., 2016). Therefore, we further analyzed the contribution of mTOR-activation to LPS- and MPLA-induced mDC activation by pre-treating the cells with rapamycin. Rapamycin pre-treatment completely and dose-dependently inhibited the MPLA-induced activation of mDC metabolism, while the LPS-induced activation of mDC metabolism was only inhibited by approximately 50%. Taken together with the observed phosphorylation of PI3K and p70 S6K by MPLA these results suggest, the MPLA-induced activation of glycolytic metabolism in mDCs to depend on mTOR-activation.

In line with results published by us for a TLR5-ligand containing fusion protein (Schülke et al., 2018), rapamycin pre-treatment abrogated MPLA-induced IL-10 production but had no influence on MPLA-induced TNF-α or IL-12 production. Therefore, we analyzed the contribution of MPLA-induced MAP kinase- and NFκB-activation to the activation of mDC metabolism and cytokine secretion using the inhibitors U0126, SB202190, SP600125, and dexamethasone: U0126 is a selective inhibitor of MAP kinase/ERK kinase-1 (MEK1) and MEK2 (Favata et al., 1998), preventing the activation of MAP kinases p42 and p44 normally triggered by the activation of different TLRs (Rabehi et al., 2001; Rhee et al., 2004), while SB202190 inhibits p38α and p38β MAPK activation (Arana-Argáez et al., 2010; Wang et al., 2014). Moreover, SP600125 specifically inhibits JNK-activation (Bennett et al., 2001; Langiewicz et al., 2018) and dexamethasone inhibits both MAP kinase- and NFκB-activation (Bhattacharyya et al., 2010).

Here, inhibition of MAP kinase signaling suppressed MPLA-induced IL-10 (U0126, SP600125, and SB202190), CCL2 (U0126 & SP60012), and TNF-α (U0126 & SP600125) secretion while increasing MPLA-induced IL-1β (SB202190 & SP60012) and IL-12p70 (SB202190 & SP60012) secretion. However, inhibition of MAP kinases, with the exception of JNK-inhibition by SP600125, was not sufficient to prevent either MPLA-induced phosphorylation of the mTOR target protein p70 S6 kinase or activation of mDC metabolism. Therefore, the obtained results suggest the activation of mDC metabolism by MPLA to be mainly mediated by activation of JNK and mTOR, while MPLA-induced

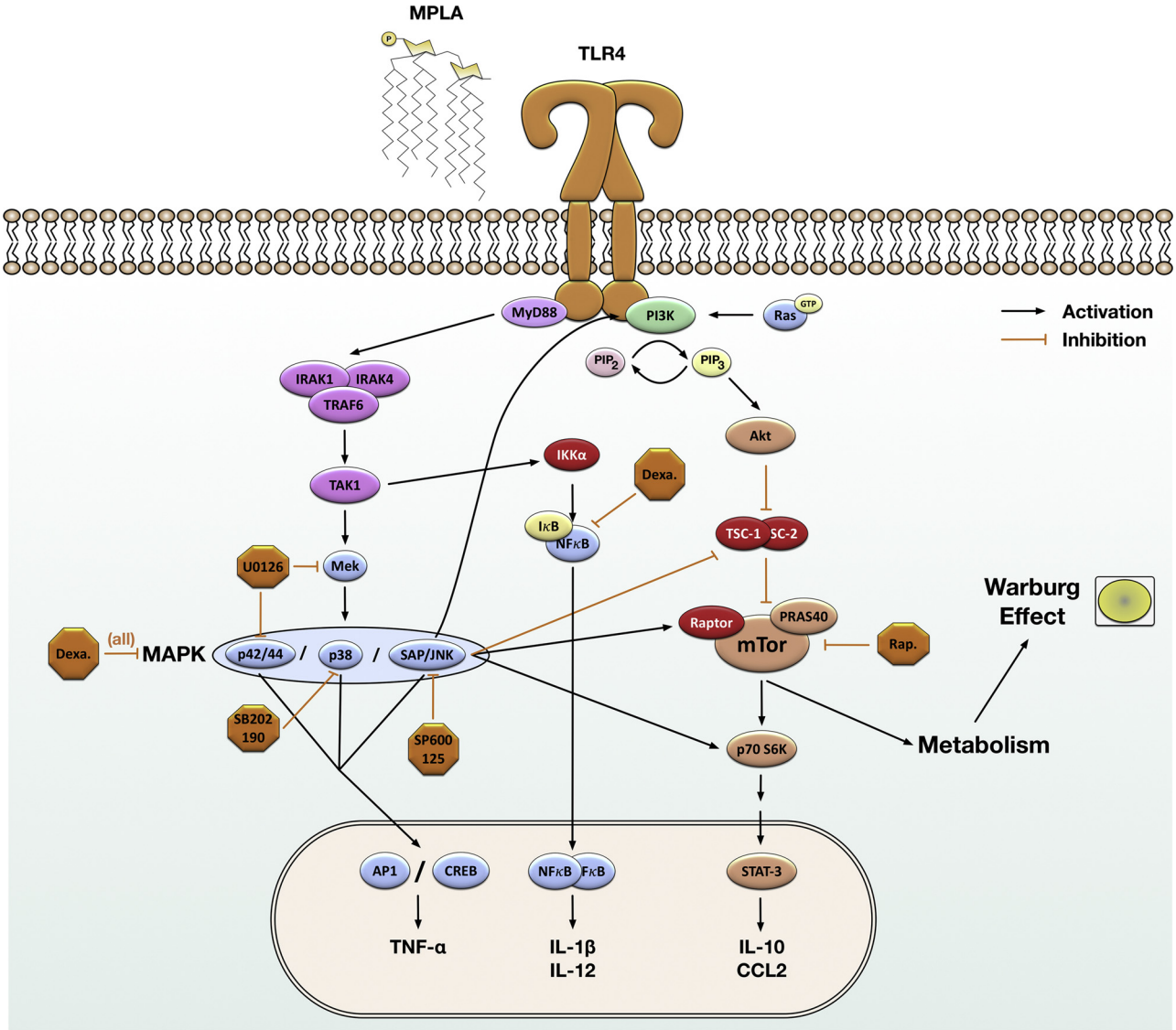

Fig. 8. Proposed mechanism of MPLA-induced mDC activation. Stimulation of mDCs with MPLA results in a mammalian target of rapamycin (complex 1) (mTORC1)-dependent activation of mDC metabolism, as well as IL-10 and CCL2 secretion, likely mediated by signal transducer/activator of transcription 3 (STAT-3)-signaling. MPLA also triggers a myeloid differentiation primary response 88 (MyD88)-dependent, interleukin-1 receptor-associated kinase 1 (IRAK1)/IRAK4/TNF receptor associated factor 6 (TRAF6)/transforming growth factor beta-activated kinase 1 (TAK1)-mediated activation of the MAP kinase signaling pathway resulting in pro-inflammatory cytokine secretion (TNF-α). Moreover, inhibition of both MAPK- and NFκB-signaling by dexamethasone additionally suppressed the secretion of MPLA-induced IL-1β and IL-12p70 production, suggesting the MPLA-induced production of IL-1β and IL-12p70 to be caused by NFκB-signaling. In addition, the activation of MAP kinase signaling can cross-activate the mTOR pathway by either inhibiting tuberous sclerosis complex 1/2 (TSC-1/TSC-2) complexes, phosphorylation of regulatory associated protein of mTOR (RAPTOR), or direct activation of mTOR downstream targets like p70 S6 kinase by ERK/ribosomal S6 kinase (RSK). The MAPK-dependent mTOR activation also contributes to IL-10 and CCL2 secretion. Among the tested inhibitors, only rapamycin, SP600125, and dexamethasone were able to suppress the MPLA-induced activation of mDC metabolism. Proteins with mainly inhibitory function on their respective downstream target molecules are shown in red. The different inhibitors used in this study are indicated in orange, orange arrows depict inhibitory signals, black arrows depict activating signals (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

cytokine and chemokine secretion can also be mediated by the other MAP kinases.

Here, the inhibition of MPLA-induced IL-10 secretion by both mTOR- and MAPK-inhibitors, as well as the inhibition of metabolic activation by the JNK-inhibitor SP600125, suggested a connection between MPLA-induced MAP kinase-activation and the activation of the mTOR pathway.

In line with these results, it has been reported, that MAP kinase signaling can cross-activate pPI3K-mTOR-signaling (Mendoza et al., 2011) (Fig. 8). Here, rat sarcoma-guanosine tri-phosphate (Ras-GTP), generated early upon activation of MAPK-signaling, can directly bind and allosterically activate PI3K (Fig. 8) (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994; Suire et al., 2002) and the activation of p42/44/ribosomal S6 kinase (RSK)-signaling can lead to mTOR-activation by (1) phosphorylation of RAPTOR (regulatory associated protein of mTOR) (Carriere et al., 2011; Foster et al., 2010; Pearce et al., 2010) or (2) phosphorylation of tuberous sclerosis complex 2 (TSC2), which releases TSC-1/2 mediated mTOR inhibition (Roux et al., 2004; Zoncu et al., 2011) (Fig. 8). Furthermore, proteins involved in MAPK-scaffolding have been reported to have the capacity to regulate mTOR-signaling (Costanzo-Garvey et al., 2009; Dougherty et al., 2009; McKay and Morrison, 2007; Mendoza et al., 2011).

Taking these studies into account, the effects of the tested MAP kinase inhibitors on mTOR-dependent cytokine secretion and activation

of glucose metabolism may also be explained by their reported capacity to cross-inhibit the PI3K/mTOR pathway, resulting in autophagy induction (Menon et al., 2011).

Additional inhibition of MAP kinase- and NFκB-activation using dexamethasone showed that inhibition of both pathways resulted in both a reduced activation of mDC metabolism and suppressed production of all tested cytokines. Since MAPK inhibition only suppressed MPLA-induced TNF-α production (besides the mTOR-mediated IL-10 and CCL2) production, but dexamethasone treatment additionally suppressed MPLA-induced IL-1β and IL-12p70 production, these results suggest the observed IL-1β and IL-12p70 production to be likely mediated by the observed NFκB-activation (the suggested contribution of the tested signaling pathways is demonstrated in Fig. 8).

Our results suggest, that JNK MAP kinase activation by MPLA-stimulation in mDCs is an upstream event of both mTOR-mediated cytokine secretion (CCL2 and IL-10) and mTOR-dependent activation of mDC metabolism. Here, the performed inhibitor experiments using U0126 and SB202190 suggest MPLA-induced activation of the p42/44- and p38-MAP kinases to be dispensable for the activation of mDC metabolism.

We observed, that pre-treatment of the mDCs with the p38 MAPK-inhibitor SB202190 partly altered MPLA-induced cytokine secretion (see Fig. 5) but had no influence on phosphorylation levels of p38. A possible explanation for these results is provided by results from Gonzalez et al. (Gonzalez et al., 2004). They observed, that PI3K/Akt-signaling (which is also induced by MPLA-stimulation in mDCs) can cross-activate p38 MAPK signaling during myoblast differentiation via increased levels of Akt2 (Gonzalez et al., 2004).

Therefore, the observed activation of PI3K and mTOR-signaling in MPLA-stimulated mDCs may also cross-activate p38 MAPK-signaling in the investigated mDCs. Such a cross-activation would in theory at least in part negate the suppressive effects of SB202190 on p38 phosphorylation. In line with this speculation, when comparing all three tested MAPK-inhibitors, pre-treatment with SB202190 had the most moderate effect on MPLA-induced cytokine secretion.

In summary, we have shown that stimulation of mDCs with the TLR4-activating vaccine adjuvant MPLA resulted in a pronounced, mTOR-dependent activation of glucose metabolism. While MPLA-induced cytokine secretion could be partially inhibited using mTOR-, NFκB-, and MAP kinase-inhibitors, the activation of glucose metabolism was shown to be dependent of JNK- and mTOR-activation. Therefore, the obtained results suggest an activation of the JNK MAPK to promote an mTOR-dependent activation of glucose metabolism, IL-10 secretion, and CCL2 production in MPLA-activated mDCs, while both MAPK- and NFκB-activation contribute to pro-inflammatory cytokine production.

Understanding the mechanisms by which MPLA activates dendritic cells will both improve the knowledge of its adjuvant properties and contribute to the future development and safe application of this promising adjuvant.

5. Conclusions

- Stimulation of mDCs with the TLR4-activating vaccine adjuvant MPLA resulted in a pronounced, mTOR-dependent activation of glucose metabolism.
- The results suggest an MPLA-induced activation of the JNK MAPK to promote an mTOR-dependent activation of glucose metabolism, IL-10-, and CCL2-production, while both MAPK- and NFκB-activation contribute to pro-inflammatory cytokine production.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Author contributions

FB performed *in vitro* experiments, analyzed the data, interpreted the results, and revised the manuscript; AG performed *in vitro* experiments, analyzed the data, interpreted the results, and revised the manuscript; SW supported *in vitro* studies, performed and analyzed Western Blot experiments; SSÜ conceived the study, performed *in vitro* and Western Blot experiments, analyzed the data, interpreted the results, and wrote the manuscript;

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Appendix A. Supplementary data

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Glossary

APC: Antigen presenting cell
ATP: Adenosine tri-phosphate
CCL2: CC-chemokine ligand 2
C-Myc: Avian myelocytomatosis virus oncogene cellular homology
Dexa.: Dexamethasone

Erk1/2: Extracellular signal-regulated kinases 1/2
HIF-1α: Hypoxia-inducible factor 1-alpha
IκBα: Inhibitor of kappa light chain gene enhancer in B cells alpha
IL: Interleukin
LPS: Lipopolysaccharide
MAP(K): Mitogen-activated protein (kinase)
mDC: Myeloid dendritic cell
MEK1/2: MAP kinase/ERK kinase-1/2
MPLA: Monophosphoryl lipid A
mTOR(C1): Mammalian target of rapamycin (complex 1)
NFκB: Nuclear factor "kapa-light-enhancer" of activated B cells
OD: Optical density
PI3K: Phosphatidylinositol 3-kinase
RAPTOR: Regulatory associated protein of mTOR
Ras-ERK: Ras-extracelluler signal-regulated kinase
Ras-GTP: Rat sarcoma guanosine tri-phosphate
ROS: Reactive oxygen species
RSK: ERK/ribosomal S6 kinase
SAP/JNK: Stress-activated protein kinase/c-Jun N-terminal kinase
STAT(3): Signal transducer and activator of transcription (3)
TNF-α: Tumor necrosis factor alpha
Th1/2: T helper type 1/2
TLR(4): "Toll"-like receptor (4)
TRIF: TIR-domain-containing adapter-inducing interferon-β
TSC-1/2: Tuberous sclerosis complex 1/2