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dPGS Regulates the Phenotype of Macrophages via Metabolic Switching

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The synthetic compound dendritic polyglycerol sulfate (dPGS) is a pleiotropic acting molecule but shows a high binding affinity to immunological active molecules as L-/P-selectin or complement proteins leading to well described anti-inflammatory properties in various mouse models. In order to make a comprehensive evaluation of the direct effect on the innate immune system, macrophage polarization is analyzed in the presence of dPGS on a phenotypic but also metabolic level. dPGS administered macrophages show a significant increase of MCP1 production paralleled by a reduction of IL-10 secretion. Metabolic analysis reveals that dPGS could potently enhance the glycolysis and mitochondrial respiration in M0 macrophages as well as decrease the mitochondrial respiration of M2 macrophages. In summary the data indicate that dPGS polarizes macrophages into a pro-inflammatory phenotype in a metabolic pathway-dependent manner.

Monocytes play a vital role in the initiation and progression of inflammation.[1] By the aid of adhesion molecules, for instance selectins, monocytes are recruited to the site of injury or infection.[2] The successive infiltration of macrophages leads to the tissue damage in chronic inflammation.[3] Thus, preventing the extravasation of monocytes has been considered as a strategy to reduce the innate immune response.

Migrated monocytes polarize into mature macrophages in the damaged tissue, which in turn facilitates the clearance of pathogens as well as the regeneration. Macrophages are divided into two major subsets: Classically activated macrophages promote inflammation while alternatively activated macrophages exhibit an anti-inflammatory face.[4] Recent studies reported that these two populations utilize distinct metabolic pathway during the activation.[5] Pro-inflammatory macrophages rely on glycolysis to gain energy rapidly while anti-inflammatory macrophages prefer mitochondrial respiration. On the other hand, modulating metabolism in macrophages could also switch their phenotype.[6,7] High glycolytic rate pro-inflammatory macrophages clear the intracellular bacteria via metabolites. In contrast, anti-inflammatory macrophages permit the persistence of bacteria, which leads to a non-effective antibiotic treatment.[8,9] Thus it is desirable to develop novel drugs targeting the macrophage metabolic phenotype to control bacterial infection.

Multivalent macromolecules such as dendritic polyglycerol sulfates (dPGS) were designed to interrupt the interaction between leukocytes and endothelial cells. Our previous data showed that dPGS exhibit a high binding affinity for L- and P-selectin and target complement proteins in vitro.[10–12] dPGS treatment could also effectively prevent the extravasation of immune cells in a dermatitis and polymyositis model in vivo.[10,13]

In this paper we focus on a modulatory effect of dPGS on the polarization of macrophages. Our data demonstrate that administration of dPGS elevates the expression of *MCP1* in both, mRNA and protein level. In contrast, anti-inflammatory signature gene *Mgl2* as well as the anti-inflammatory cytokine IL-10 were decreased. Metabolic analysis demonstrated that dPGS treatment reinforced glycolysis as well as mitochondrial respiration in macrophages. Therefore, our research provides more mechanistic insights how dPGS modulates inflammation by targeting myeloid cells.

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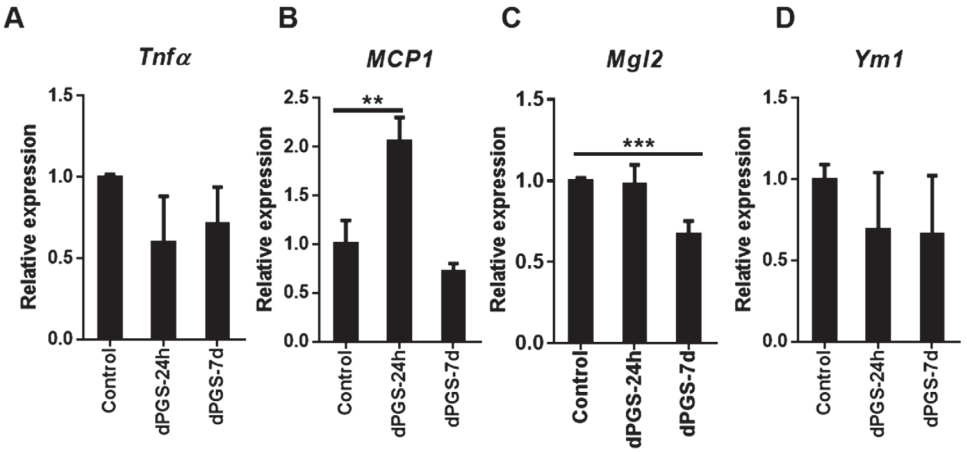

Figure 1. dPGS increases the expression of MCP1 in bone marrow derived macrophages. 5×10^5 bone marrow cells were treated with 1 μM dPGS for 24 h or 7 days in the presence of 20 ng mL^{-1} M-CSF. The expression level of *Tnfα*, *MCP1*, *Mgl2*, and *Ym1* were detected by real-time PCR. Shown is the mean ± SD from two to four independent experiments and analyzed by one-way ANOVA with Tukey's post hoc test. ***$p \leq 0.001$

Dendritic polyglycerol sulfates (dPGS) were designed to block L- and P- selectin mediated leukocyte adhesion to activated endothelia. However, the directly regulatory role of dPGS on immune cells, for instance myeloid cells, is still unclear. Here, bone marrow derived macrophages were treated with dPGS for either 24 h or 7 days in the presence of M-CSF. *MCP1* and *Tnfα* were used as pro-inflammatory signature genes and *Mgl2* plus *Ym1* for the anti-inflammatory phenotype. M1 macrophages prefer to produce pro-inflammatory cytokines, for instance TNFα, CCL2(MCP-1), IL6, IL-12, and IL23 et al. Mgl2 is the abbreviation of macrophage galactose *N*-acetyl-galactosamine specific lectin 2, which could be translated into the lectin protein CD301b. CD301b positive macrophages play a role in would healing and widely used as a M2 macrophage marker both in vitro and in vivo.[14] Ym1 is a secreted protein which is highly expressed in IL-4 conditions. In type II immune response (Helminth infection or allergy), Ym1 is dramatically expanded in macrophages to against fungal or other pathogens containing chitin. Although the exact role of Ym1 in type II immune response is not clear yet, it could be used to identify M2 macrophage in vitro and in vivo.[15] Our data demonstrated that 24 h administration of dPGS could dramatically increase the expression of *MCP1*. In long-term treatment group, *Mgl2* was significantly downregulated by dPGS and no difference was detected for *Tnfα* and *Ym1* (**Figure 1**). These data indicate a tendency of pro-inflammatory macrophage polarization induced by dPGS.

Different macrophage phenotypes secrete distinct cytokines at inflammation sites. Lipopolysaccharide (LPS)- and IFNγ–polarized macrophages produce typical pro-inflammatory cytokines, for instance TNFα and MCP1. Th2 cytokines for instance IL-10, are provided by anti-inflammatory macrophages to suppress further immune responses.[16,17] Supernatants collected from M-CSF differentiated macrophages with or without dPGS were used here to measure the cytokine profile. Consistent with real-time PCR results, the protein level of MCP1 was also increased after dPGS treatment (**Figure 2**A). Our data

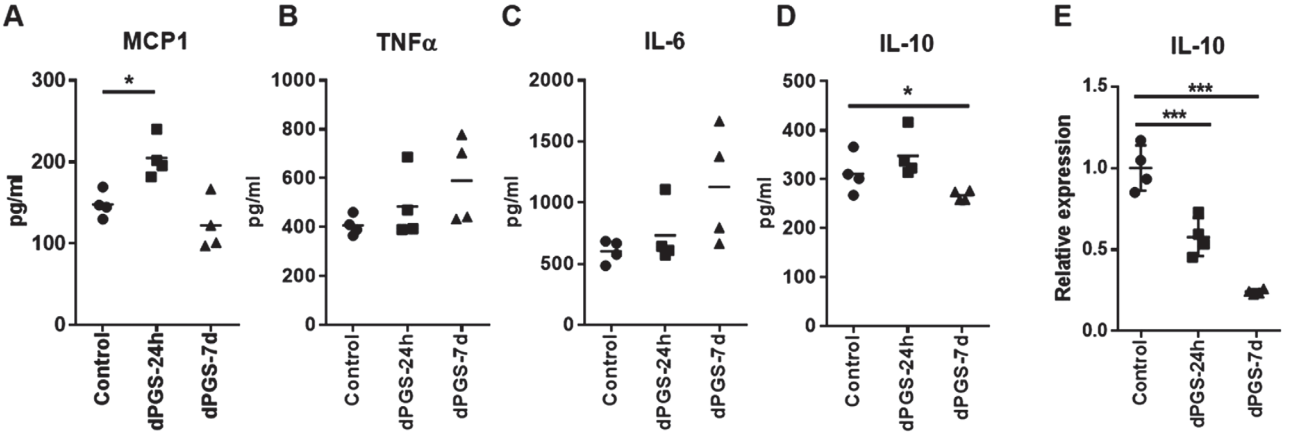

Figure 2. dPGS increases the protein level of MCP1 in bone marrow derived macrophages. 5×10^5 bone marrow cells were treated with 1 μM dPGS for 24 h or 7 days in the presence of 20 ng mL^{-1} M-CSF. On day 6, myeloid cells were stimulated by 1 μg mL^{-1} LPS. The supernatant was collected for cytokine detection. The cells were lysed to detect the expression of IL-10 by real-time PCR. Shown is the mean ± SD from two to four independent experiments, statistics were calculated by one-way ANOVA with Tukey's post hoc test. ***$p \leq 0.001$

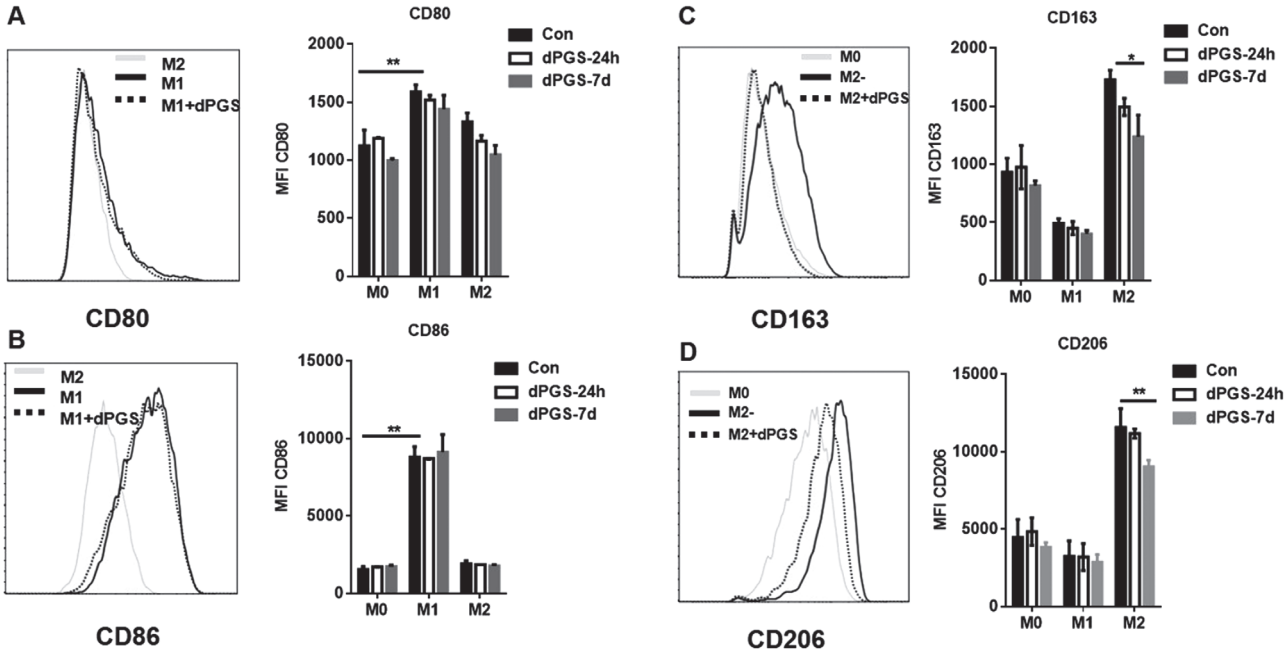

Figure 3. dPGS cannot alter the expression of pro- or anti-inflammatory markers in bone marrow derived macrophages. 5×10^5 bone marrow cells were polarized into M0, M1, or M2 macrophages and then treated with 1 μM dPGS for 24 h (dPGS-24h) or 7 days (dPGS-7d) in the presence of 20 ng mL^{-1} M-CSF. The expression level of CD80, CD86, CD163, and CD206 were measured via flow cytometry. Shown is the mean ± SD from two to four independent experiments, statistics were calculated by one-way ANOVA with Tukey's post hoc test. ***$p \leq 0.001$

showed a trend for an increased expression of both, TNFα and IL-6 in the 7 days-dPGS treated group. (Figure 2B,C). In contrast to pro-inflammatory cytokines, the secretion of IL-10 was slightly reduced (Figure 2D). To further confirm the reduction of IL-10 after dPGS treatment, we measured the expression level of IL-10 via qPCR. Our data showed dPGS significantly reduces the expression of IL-10 (Figure 2E).

Matured macrophages maintain distinct surface markers. Classically activated macrophages express co-stimulatory molecules CD80 and CD86 to present antigen in inflammation. Scavenger receptor CD163 and mannose receptor CD206 are classical markers to identify M2 macrophages.[18,19] The expression of these markers on polarized macrophages were monitored by flow cytometry. Compared with LPS-stimulated pro-inflammatory macrophages, unpolarized macrophages expressed only modest levels of CD80 and CD86, which could not be altered by dPGS administration (**Figure 3**A,B). Similarly, both the expression of CD163 and CD206 remained stable (Figure 3C,D). However, both the expression of CD163 and CD206 in M2 conditions were impaired by the treatment of dPGS, which indicates a regulatory role of dPGS in M2 polarization.

Recently, a metabolic assay was described to distinguish functional macrophage phenotypes.[5] Aerobic glycolysis in pro-inflammatory macrophages supports the abundant consumption of ATP, whereas anti-inflammatory macrophages exhibit an enlarged mitochondrial respiration. Extracellular acidification rate (ECAR), proton efflux rate (PER), and oxygen consumption rates (OCR) were measured in the presence of variant metabolic inhibitors in order to detail the metabolic process in polarized macrophages. Our results showed that the ECAR, PER, and OCR values were increased in dPGS administrated macrophages (**Figure 4**A–C). Technically, the PER value consists of both lactate acid from glycolysis and mitochondrial respiration derived CO_2. After discounting the contribution of mitochondria/CO_2, glycoPER was utilized here to clarify the exact glycolysis rate in macrophages. Our data confirmed the increase of glycolysis via the glycoPER value after dPGS treatment in macrophages (Figure 4D). When analyzing the detail of glycolysis, we found that basal glycolysis, post 2-Deoxy-D-glucose (2-DG) acidification, basal proton efflux rate as well as compensatory glycolysis were coincidently elevated in the dPGS administered group (Figure 4E–H).

From the glycolytic rate assay, we confirmed a pro-glycolytic capacity of dPGS on bone marrow derived macrophages, which indicates a M1 macrophage phenotype. However, if dPGS regulates the metabolism of M2 macrophages is still unclear. The phenotyping data showed a reduction of *Mgl2* expression on mRNA level as well as the expression of CD163, CD206, and IL-10 in dPGS-treated macrophages on protein level, implying a potential modulatory role of dPGS on the M2-polarization of macrophages. M2 macrophages prefer mitochondrial respiration, thus we performed mitochondrial stress assays in untreated macrophages (M0-Ctr), M2 macrophages (M2-Ctr) as well as dPGS treated macrophages (M2-dPGS-24h; M2-dPGS-7d). As described in other studies, anti-inflammatory macrophages showed a significantly reinforced mitochondrial respiration (as indicated by OCR) (**Figure 5**A).[20] In contrast, dPGS treatment comprehensively decreased the mitochondrial function within the macrophages. Especially the spare respiratory capacity and maximal respiration were significantly decreased in the dPGS-treated group compared to the M2 control macrophages (Figure 5B–G). Our phenotyping data suggested that

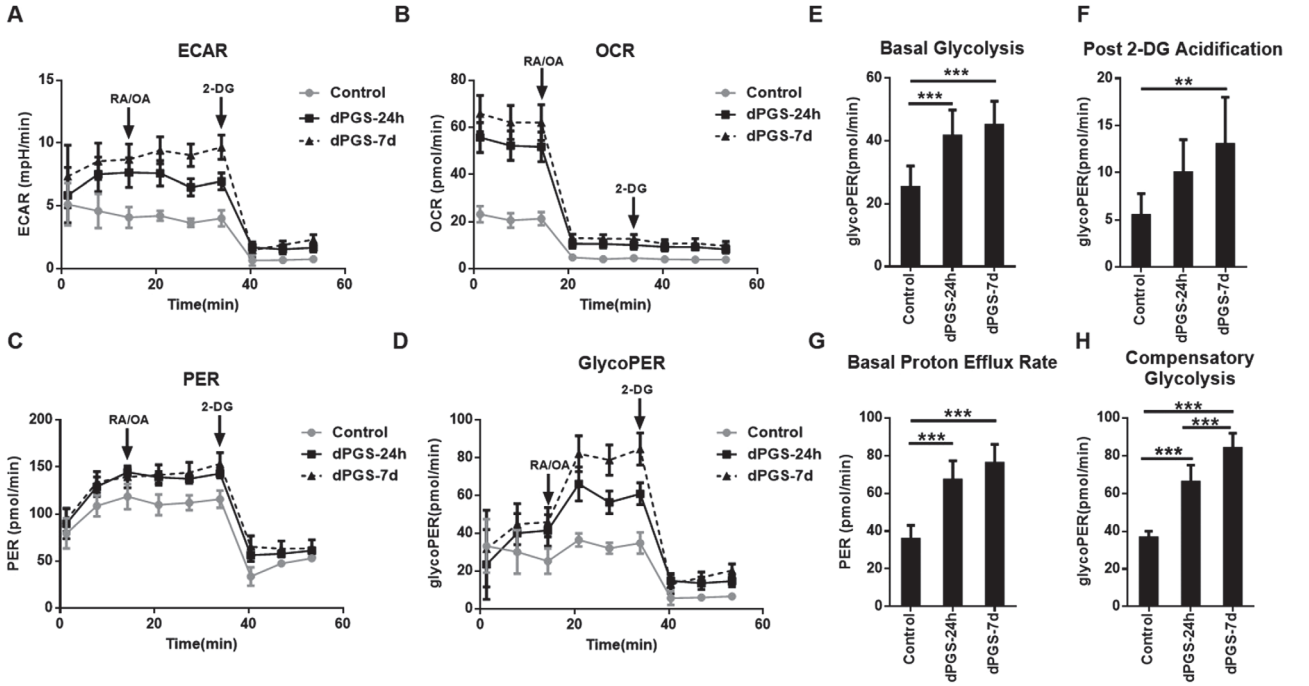

Figure 4. dPGS enhances the glycolytic rate of bone marrow derived macrophages. 4×10^6 bone marrow cells were treated with 1 μM dPGS for 24 h or 7 days in the presence of 20 ng mL^{-1} M-CSF. The extracellular acidification rate (ECAR), oxygen consumption rate (OCR), proton efflux rate (PER), and glycolytic proton efflux rate (glycoPER) of 1×10^5 differentiated myeloid cells were monitored after the addition of the electron transport inhibitor rotenone and antimycin A (R/AA) (0.5 μM) and 2-deoxy-D-glucose (2-DG, 50 mM) in indicated time points. The basal proton efflux rate, post 2-DG acidification, basal glycolysis, and compensatory glycolysis were qualified. Shown is the mean ± SD from two to four independent experiments, statistics were calculated by one-way ANOVA with Tukey's post hoc test. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

dPGS treatment enhances the expression of pro-inflammatory signature genes as well as cytokines, here we found a negative regulation of dPGS on mitochondrial respiration of M2 macrophages. Several studies already stated that metabolic reprogramming seems to be a key feature to trigger final macrophage function.[8,9,21] Regarding the correlation of metabolic pathways and the effective macrophage phenotype in bacterial infection, the immunomodulation of dPGS might pave the way to develop novel therapeutic strategies.

In this study, we evaluated the impact of dPGS on macrophage polarization via various methods. We found that the expression of MCP1 was significantly increased by dPGS at both mRNA and protein level. In M0 conditions, dPGS could also reduce the expression of Mgl2 (Figure 1) as well as IL-10 production (Figure 2) in M0 macrophages. In the metabolic assay we could clearly monitor both, the elevated glycolysis (Figure 4) in pro-inflammatory macrophages and the deficiency of mitochondrial respiration in anti-inflammatory macrophages (Figure 5). Furthermore, dPGS treatment decreased the expression of the surface markers CD163 and CD206 in M2 conditions (Figure 3), which indicates a regulatory role of dPGS in the polarization of M2 macrophages. Previously, we found that the macromolecular inhibitor dPGS reduces the leukocyte migration and decreases complement activity. Here we found that dPGS also elevates the metabolic activation in macrophages, which in turns promotes the production of the pro-inflammatory cytokine MCP1. In contrast, in M2 conditions, dPGS impaired the maturation of M2 macrophages. Therefore, our results reveal a new mechanism how dPGS affects inflammatory processes in general and macrophage activation in particular.

Experimental Section

dPGS Synthesis: The polymer was produced as recently described.[12] In brief, first the dendritic polyglycerol (dPG) as the core scaffold was synthesized via a controlled living anionic ring-opening multibranching polymerization of glycidol by slow monomer addition on partially deprotonated pentaerythritol as the starter. In a second step, sulfation of dPG was prepared with a SO_3·pyridine complex to give dPGS with a molecular weight 13 300 g mol^{-1} and a degree of sulfation of 87%, equivalent to about 60 sulfate groups.

Macrophage Polarization: To isolate bone marrow cells, the cavities of femur and tibia bones of BALB/c mice were flushed with PBS. The single cell suspensions were cultured in high glucose DMEM (4.5 g L^{-1} D-glucose) supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 20 ng mL^{-1} macrophage colony-stimulating factor (M-CSF, Peprotech, Hamburg, Germany). Non-adherent macrophage progenitor cells were isolated after 24 h incubation and cultured in the presence of 20 ng mL^{-1} M-CSF for 6 days. Medium was renewed every other day. To polarize pro-inflammatory macrophages, myeloid cells were treated with 100 ng mL^{-1} LPS plus 50 ng mL^{-1} IFN-γ (Peprotech) for 24 h. 10 ng mL^{-1} IL-4 (Peprotech) was utilized to generate anti-inflammatory macrophages.

Antibodies and Chemicals: If not indicated otherwise, all chemicals were obtained from Sigma-Aldrich Chemie GmbH. The following antibodies were applied for flow cytometric analysis: PE-CD80 (clone 16-10A1) and APC-Cy7-CD11b (clone M1/70) are from BD Bioscience (Heidelberg, Germany); PE-CD163 (clone ED2) was purchased from antibodies-online (Aachen, Germany); Alexa488-CD206 (clone MR5D3)

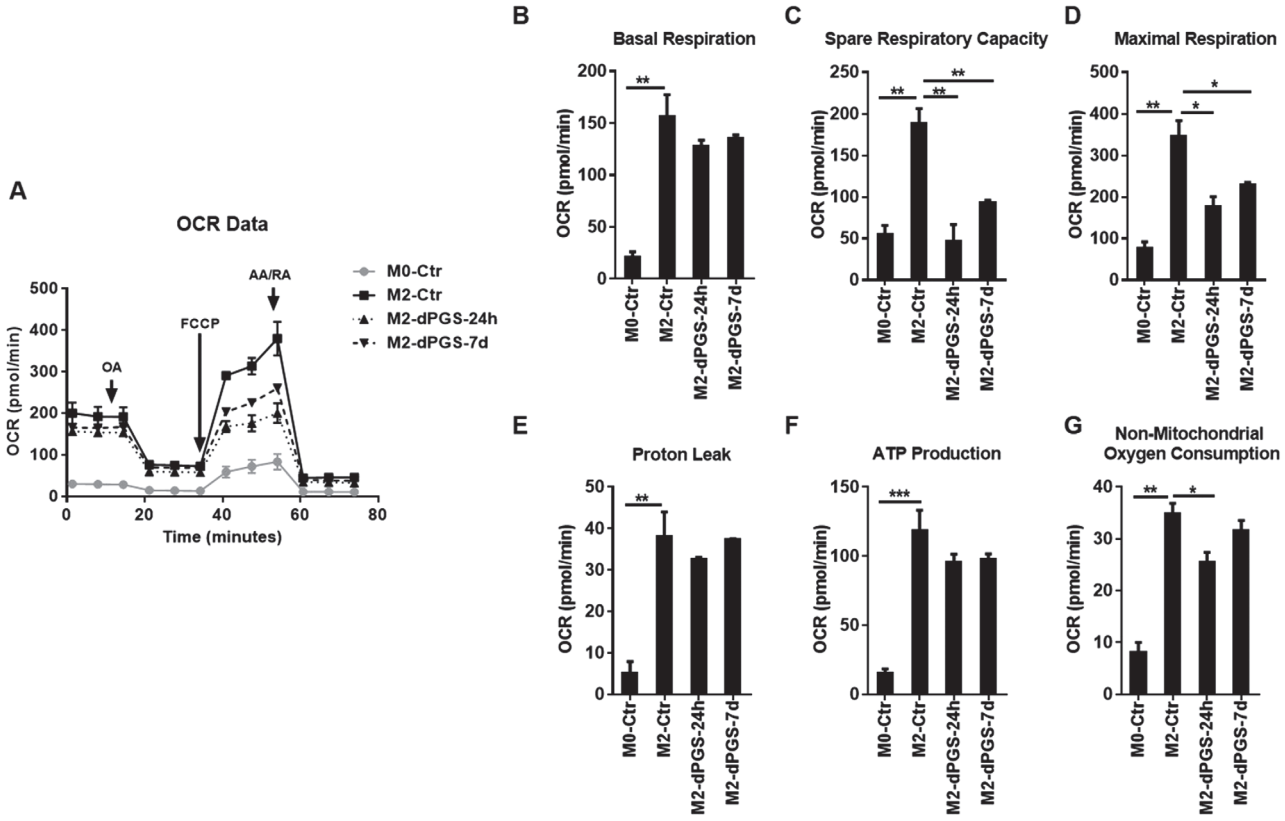

Figure 5. dPGS inhibits the mitochondrial respiration of M2 macrophages. 4×10^6 bone marrow cells were treated with 1 μM dPGS for 24 h or 7 days in the presence of 20 ng mL^{-1} M-CSF. IL-4 was used to polarize M2 macrophages on day 6. The oxygen consumption rate (OCR) of 1×10^5 polarized myeloid cells were monitored after the addition of oligomycin (OA) (1 μM), the uncoupler FCCP (1 μM), and the electron transport inhibitor rotenone and antimycin A (R/AA) (0.5 μM) in indicated time points. The basal respiration, spare respiratory capacity, maximal respiration, proton leak, ATP production, and non-mito respiration based on the OCR value were quantified. Shown is the mean ± SD from two to four independent experiments, statistics were calculated by one-way ANOVA with Tukey's post hoc test. ***$p \leq 0.001$

was purchased from Biolegend GmbH (Fell, Germany). FITC-CD86 (clone GL1) is from eBioscience (Frankfurt, Germany).

Realtime PCR: Total RNA was extracted from polarized macrophages with TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using 2 μg RNA, 9 ng random primer (TaKaRa, Ohtu, Japan), and M-MLV reverse transcriptase (Promega, Heidelberg, Germany). The amounts of *Tnfα, MCP1, Mgl2*, and *Ym1* mRNA were determined using iQTM SYBR Green Supermix on a MyiQTM system (Bio-Rad Laboratories). The acidic ribosomal protein 36B4 mRNA was used as internal control. The sequence of primers were applied:

36B4:5′-AACCCTGAAGTGCTCGACATCACA-3′; 5′-ATTGATGATGGAGTGTGGCACCGA-3′;
Tnfα:5′-CCCTCACACTCAGATCATCTTCT-3′; 5′-GCTACGACGTGGGCTACAG-3′;
MCP1:5′-AGGTCCCATGTCATGCTTCTGG-3′; 5′-CTGCTGCTGGGTGATCCTCTTG-3′;
Mgl2:5′-GGCCTCCAATTCTTGAAACCT-3′; 5′-TTAGCCAATGTGCTTAGCTGG-3′;
Ym1:5′-AGAAGGGAGTTTCAAACCTGGT-3′; 5′-GTCTTGCTCATGTGTGTAAGTGA-3′.

Cytokine Measurement: Primary myeloid cells were incubated for 7 days as described above and then stimulated with 1 μg mL^{-1} LPS for 24 h. The supernatant was collected for analysis. CBA inflammation kit (BD Pharmingen) was used in accordance with the manufacturer's instructions. Samples were measured using a FACSCANTO II (BD Biosciences) device and data were analyzed via FCAP array Software (BD Biosciences).

Flow Cytometry: Flow cytometric analyses were performed using standard procedures. In brief, 10^6 cells were harvested and resuspended in 50 μL PBS/0.5% BSA buffer in the presence of the indicated antibodies for 10 min on ice. Cells were washed by PBS afterward. Data acquisition was performed using a FACSCANTO II device (BD Bioscience) and analyzed by FlowJo software (FlowJo LLC, Ashland, Oregon, USA) and the Guidelines for the use of flow cytometry and cell sorting in immunological studies.[22]

Metabolic Assays: Polarized macrophages were seeded in XF 96-well culture microplate (Agilent, Santa Clara, USA) in at 1×10^5 cells per well in 180 μL pre-warmed assay medium. For mitochondrial respiration assay, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the mitochondrial stress test procedure in XF media (non-buffered DMEM containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate). Glycolytic rate was measured following the guidance of Agilent Seahorse XFp Glycolytic Rate Assay Kit. The assay medium was then gently mixed again for 3–5 min between each rate measurement to restore normal oxygen tension and pH in the microenvironment surrounding the cells. After the baseline measurement, 20–27 μL of a testing agent prepared in assay medium was then injected into each well to reach the desired final working concentration. This was followed by mixing for 5–10 min to expedite compound exposure to cellular proteins, after which OCR and ECAR measurements were then made. Generally, two to three baseline rates and two or more response rates (after compound addition) were

measured, and the average of two baseline rates or test rates was used for data analysis.

Statistical Analysis: Statistical significance of differences between the experimental groups were determined by Student's *t*-test or factorial analysis of variance and the respective post hoc tests (Tukey's and Dunnett's multiple comparisons test) using GraphPad Prism software (GraphPad Software, La Jolla, CA).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

infection, macrophage polarization, MCP1, metabolic switch, polyglycerol sulfates

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