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# Targeted delivery of glucocorticoids to macrophages in a mouse model of multiple sclerosis using inorganic-organic hybrid nanoparticles

Elena Montes-Cobos [a,b], Sarah Ring [a,1], Henrike J. Fischer [a,b], Joachim Heck [c], Judith Strauß [b], Markus Schwaninger [d], Sybille D. Reichardt [a], Claus Feldmann [c], Fred Lühder [b,2], Holger M. Reichardt [a,*,2]

[a] Institute for Cellular and Molecular Immunology, University Medical Center Göttingen, 37073 Göttingen, Germany
[b] Institute for Multiple Sclerosis Research and Neuroimmunology, University Medical Center Göttingen, 37073 Göttingen, Germany
[c] Institute of Inorganic Chemistry, Karlsruhe Institute of Technology, 76131 Karlsruhe, Germany
[d] Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, 23562 Lübeck, Germany

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ABSTRACT

Glucocorticoids (GC) are widely used to treat acute relapses in multiple sclerosis (MS) patients, but their application is accompanied by side effects due to their broad spectrum of action. Here, we report on the therapeutic option to apply GC *via* inorganic-organic hybrid nanoparticles (IOH-NP) with the composition $[ZrO]^{2+}[(BMP)_{0.9}(FMN)_{0.1}]^{2-}$ (designated BMP-NP with BMP: betamethasone phosphate; FMN: flavinmononucleotide). We found that these BMP-NP have an increased cell type-specificity compared to free GC while retaining full therapeutic efficacy in a mouse model of MS. BMP-NP were preferentially taken up by phagocytic cells and modulated macrophages *in vivo* more efficiently than T cells. When GC were applied in the form of BMP-NP, treatment of neuroinflammatory disease in mice exclusively depended on the control of macrophage function whereas effects on T cells and brain endothelial cells were dispensable for therapeutic efficacy. Importantly, BMP-NP were not only active in mice but also showed strong activity towards monocytes isolated from healthy human volunteers. We conclude that application of GC *via* IOH-NP has the potential to improve MS therapy in the future.



## 1. Introduction

Chronic inflammatory diseases are a group of highly variable pathogenic conditions that may affect any organ or tissue. Their total prevalence in western countries is currently around 5% and still rising [1]. A common denominator of these diseases is their immune-mediated pathomechanism. Loss of self-tolerance or the response to harmless foreign antigens lead to leukocyte infiltration into affected organs, and causes a misbalance of circulating cytokines and the release of cytotoxic mediators. Despite the availability of highly effective biologicals [2], application of synthetic GC remains the mainstay in the treatment of many of such inflammatory diseases. They possess strong immunosuppressive activity and potently ameliorate clinical symptoms by inhibiting innate and acquired immune responses [3]. GC achieve these effects by employing a variety of mechanisms such as inhibiting cytokines, redirecting leukocyte migration, inducing T cell apoptosis, and altering macrophage polarization [4]. While the therapeutic efficacy of GC is unsurpassed, their use is accompanied by various complications [5]. Some patients are resistant to GC treatment while others experience side effects such as opportunistic infections, muscle wasting, and diabetes. Hence, it would be desirable to improve currently available GC-based therapeutic regimens to overcome these problems.

MS is a chronic neuroinflammatory disease of autoimmune origin that affects more than two million people worldwide [6,7]. It is initiated by self-reactive T cells that recognize antigens present in the central nervous system (CNS), subsequently leading to the recruitment of other cells of the innate and adaptive immune system resulting in destruction of neuronal cells. The most common form is relapsing-remitting MS, which is characterized by acute disease bouts and intervening periods that are largely free of symptoms. The mononuclear cell infiltrate found in the CNS of affected patients is mainly composed of T cells and macrophages, which closely cooperate in the pathogenesis of MS. The main role of pathogenic T cells is disease initiation and exacerbation by recognition of antigen, production of cytokines and induction of oligodendrocyte apoptosis, processes with all of which GC are able to interfere [8,9]. Macrophages can commit to different phenotypes and thereby fulfill distinct functions in MS depending on their polarization

* Corresponding author: Institute for Cellular and Molecular Immunology, University Medical Center Göttingen, Humboldtallee 34, 37073 Göttingen, Germany.
*E-mail address:* hreichardt@med.uni-goettingen.de (H.M. Reichardt).
[1] Present address: Priority Area Infections, Research Center Borstel, 23845 Borstel, Germany.
[2] These authors contributed equally to the work.

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[10]. When exposed to GC, they assume an anti-inflammatory phenotype, which is characterized by reduced pro-inflammatory cytokine secretion, an upregulation of scavenger receptors, intensified phagocytosis, and diminished antigen presentation [11]. These changes eventually enable macrophages to reduce inflammation, limit the adaptive immune response, initiate repair mechanisms, and contribute to the resolution of disease symptoms.

Although there are a number of new drugs on the market that slow down disease progression of MS [12], GC administration has persisted as the gold standard with which to interfere with acute relapses and optic neuritis for the last 30 years [13]. Nevertheless, GC have also been tested as an add-on to disease-modifying drugs in relapsing-remitting MS, and in the treatment of primary and secondary progressive forms of MS [14–16]. Besides clinical trials, studies in mice have made an important contribution towards our current understanding of this therapeutic regimen, in particular the analysis of experimental autoimmune encephalomyelitis (EAE), an animal model that mimics many pathophysiological features of MS [17]. These studies revealed that T cells are the major target of conventional GC therapy, and respond by reduced cytokine production, apoptosis induction and altered migration [18]. In contrast, encapsulation of GC in PEGylated liposomes causes their redirection to the myeloid cell compartment, which induces polarization of macrophages and monocytes to an anti-inflammatory phenotype [19]. Modifying the delivery method of GC thus makes it possible to alter their mechanism of action and accordingly, application of liposomal GC in animal models of autoimmune diseases provided favorable results [19–21]. However, liposomes are also known to cause adverse effects, including the activation of the complement system, the latter which may lead to life-threatening hypersensitivity reactions [22]. Since the outcome of human trials to treat MS with liposomal GC has not been reported in literature, the clinical success of this approach is difficult to estimate. Meanwhile, phospholipid and polymer-based nanostructured carrier systems have been developed as alternative delivery vehicles for GC, which show potent anti-inflammatory activity and reduced side effects in mouse models [23–25]. Whether the new GC formulations will be suitable to treat neuroinflammatory disorders, however, remains to be determined.

Recently, we reported on novel inorganic-organic hybrid nanoparticles (IOH-NP) that could be used for the treatment of inflammatory diseases [26]. Precipitation of inorganic cations such as $[ZrO]^{2+}$ with the negatively charged organic molecules betamethasone (BMZ) phosphate $[BMP]^{2-}$ and flavinmononucleotide $[FMN]^{2-}$ in aqueous solution results in the formation of nanoparticles with the composition $[ZrO]^{2+}[(BMP)_{0.9}(FMN)_{0.1}]^{2-}$ with a hydrodynamic diameter of 30–40 nm. These BMP-NP reduce the production of pro-inflammatory cytokines and thus are able to act as immunosuppressive drugs [26]. In addition, they can be tracked due to the fluorescent dye contained therein. It is against this background that we investigated the mechanism of GC delivered in the form of BMP-NP in the modulation of the two major cell types involved in MS, and to characterize the features of BMP-NP *in vivo* in the treatment of this disease by using EAE, a relevant mouse model of MS. Our results indicate that BMP-NP strongly increase the target cell-specificity of GC for macrophages without compromising therapeutic efficiency. Furthermore, they are capable of modulating functions of human monocytes, suggesting that they will also be biologically active in patients. These findings feed the hope that it might become possible to improve GC therapy using this novel drug formulation in the future.

## 2. Materials and methods

### 2.1. Nanoparticle preparation, characterization and application

Synthesis of $[ZrO]^{2+}[(BMP)_{0.9}(FMN)_{0.1}]^{2-}$ IOH-NP was performed as previously described [26]. The BMP-NP were composed of 90 mol-% BMP and 10 mol-% FMN. Synthesis was carried out by admixing a solution of $ZrOCl_2 \times 8H_2O$ (5 mg) in $H_2O$ (2.5 ml) to a solution of sodium betamethasone 21-phosphate ($Na_2$(BMP), 50 mg) and sodium riboflavin-5′-mono-phosphate dihydrate (Na(HFMN), 5.1 mg) in $H_2O$ (50 ml). After nucleation, the IOH-NP were purified by repeated centrifugation and redispersion from/in water. Finally, they were redispersed (2.8 mg/ml) into HEPES buffer (30 mM) at pH 7.4 with an effective concentration of 4.4 μM of the pharmacologically active drug. The stoichiometry of the nanoparticles was confirmed by energy-dispersive X-ray spectroscopy and elemental analysis as described previously [26]. Dynamic light scattering (DLS) data and Zeta potentials of $[ZrO]^{2+}[(BMP)_{0.9}(FMN)_{0.1}]^{2-}$ IOH-NP freshly dispersed in PBS are depicted in Supplementary Fig. S1A,B. Empty nanoparticles not containing any BMP (designated EP-NP) were obtained through replacing $[BMP]^{2-}$ against $[HPO_4]^{2-}$ by using $Na_2(HPO_4)$ as a starting material instead of $Na_2$(BMP) and used as a reference in most experiments.

The chemical stability of the BMP-NP in PBS was determined based on the carbon content of the nanoparticles after 12 and 24 h stirring at 37 °C. Elemental analysis was performed after centrifugation and purification. Accordingly, the as-prepared BMP-NP exhibited an initial carbon content of 42 ± 2 wt-%, which matches very well the expectation (44 wt-%). After 12 and 24 h, the carbon contents were 40 ± 2 and 38 ± 2 wt-%, respectively. These data point towards a slow release of BMZ and FMN *via* hydrolytic cleavage of the phosphate ester bond [26]. It is noteworthy that the colloidal stability did not show any relevant changes over time. In terms of the significance of the experiment, the particle size and size distribution of BMP-NP freshly dispersed in PBS (Supplementary Fig. S1) was similar compared to BMP-NP stirred for 24 h in PBS (data not shown).

*In vitro*, BMP-NP were added to the cell culture such that the BMZ contained in the nanoparticles corresponded to a concentration of $10^{-7}$ or $10^{-6}$ M. Equal volumes of EP-NP were used for each concentration of BMP-NP to ensure that the amount of ZrO in control cultures was identical. *In vivo*, the volume of BMP-NP was calculated such that the dosage of the BMZ contained in the nanoparticles corresponded to 10 mg of active drug per kg of body weight. Equal volumes of EP-NP were injected as controls to ensure that the amount of ZrO applied to each mouse was the same. All calculations were based on the mass content of the BMP-NP as mentioned above.

### 2.2. Macrophage isolation and culture

Bone marrow-derived macrophages (BMDM) were obtained by culturing single-cell suspensions of bone marrow obtained from femur and tibiae of C57BL/6 wildtype mice for seven days in the presence L929-conditioned medium (LCCM) as described previously [27]. After completion of macrophage differentiation, BMDM were harvested, resuspended in DMEM medium with 10% FCS, and incubated for 24 h with BMP-NP or EP-NP as a control. Alternatively, BMDM were treated with DEX or its corresponding vehicle PBS. BMDM were analyzed by FACS, bright field microscopy, or quantitative RT-PCR.

Peritoneal macrophages (PMΦ) were elicited by injecting 1 ml 4% thioglycolate solution i.p. four days prior to isolation. For *in vivo* analysis of nanoparticle effects, mice were injected on three consecutive days with BMP-NP, EP-NP, DEX, or PBS. One day after the last treatment a peritoneal lavage was performed using PBS with 0.1% BSA. The cells were seeded in 10 cm plates and after 1 h of incubation at 37 °C, adherent macrophages were collected in PBS with 2 mM EDTA and analyzed by FACS or bright field microscopy. Alternatively, the cells were cultured at a density of $2 \times 10^5$ cells/ml in DMEM medium with 10% FCS for another 48 h with 20 ng/ml LPS and 50 ng/ml IFNγ followed by the analysis of TNFα levels in the supernatant by ELISA. In case not only macrophages but also T and B cells contained in the peritoneal lavage should be analyzed, the adherence step was omitted.