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Early modulation of pro-inflammatory microglia by minocycline loaded nanoparticles confers long lasting protection after spinal cord injury

Simonetta Papa [a], Ilaria Caron [a], Eugenio Erba [b], Nicolò Panini [b], Massimiliano De Paola [c], Alessandro Mariani [c], Claudio Colombo [d], Raffaele Ferrari [e], Diego Pozzer [a], Elisa R. Zanier [a], Francesca Pischiutta [a], Jacopo Lucchetti [f], Andrea Bassi [g], Gianluca Valentini [g], Giulio Simonutti [g], Filippo Rossi [e], Davide Moscatelli [e], Gianluigi Forloni [a], Pietro Veglianese [a, *]

[a] Dipartimento di Neuroscienze, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy
[b] Dipartimento di Oncologia, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy
[c] Dipartimento di Ambiente e Salute, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy
[d] Institute for Chemical and Bioengineering, ETH Zurich, Vladimir Perlog Weg 1, 8093 Zurich, Switzerland
[e] Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta", Politecnico di Milano, via Mancinelli 7, 20131 Milan, Italy
[f] Dipartimento di Biochimica e Farmacologia Molecolare, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", via La Masa 19, 20156 Milan, Italy
[g] Dipartimento di Fisica, Politecnico di Milano, P.zza Leonardo da Vinci 32, 20133 Milan, Italy

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ABSTRACT

Many efforts have been performed in order to understand the role of recruited macrophages in the progression of spinal cord injury (SCI). Different studies revealed a pleiotropic effect played by these cells associated to distinct phenotypes (M1 and M2), showing a predictable spatial and temporal distribution in the injured site after SCI. Differently, the role of activated microglia in injury progression has been poorly investigated, mainly because of the challenges to target and selectively modulate them *in situ*. A delivery nanovector tool (poly-ε-caprolactone-based nanoparticles) able to selectively treat/target microglia has been developed and used here to clarify the temporal and spatial involvement of the pro-inflammatory response associated to microglial cells in SCI. We show that a treatment with nanoparticles loaded with minocycline, the latter a well-known anti-inflammatory drug, when administered acutely in a SCI mouse model is able to efficiently modulate the resident microglial cells reducing the pro-inflammatory response, maintaining a pro-regenerative milieu and ameliorating the behavioral outcome up to 63 days post injury. Furthermore, by using this selective delivery tool we demonstrate a mechanistic link between early microglia activation and M1 macrophages recruitment to the injured site via CCL2 chemokine, revealing a detrimental contribution of pro-inflammatory macrophages to injury progression after SCI.

1. Introduction

Mounting evidence indicates that acute inflammation is a central component of the spinal cord injury (SCI) contributing to the spreading, amplification and chronicity of tissue injury [1–5]. Microglia and peripheral macrophages are consistently detected in the peri-contusional tissue after SCI and represent the main cellular contributors to post-SCI inflammation [6–8]. In particular, microglial cells have been reported to exist in a dynamic equilibrium between a classical pro-inflammatory and an alternative beneficial activated state [7]. The microglial response appears to be finely tuned by the local microenvironment and immediately after SCI acquire a pro-inflammatory-phenotype, promoting an early self-propelling local inflammation [7] that can lead to the recruitment of both destructive (M1) and beneficial (M2) macrophages [4,6–10]. However, despite the recent growing effort to

* Corresponding author.
E-mail address: pietro.veglianese@marionegri.it (P. Veglianese).

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characterize the role associated to the innate immunity response, the specific role played by activated resident microglia during the inflammatory phase in the injury progression remains elusive. Various reasons could explain the difficulties encountered to understand the involvement of activated microglia in this neuropathology: i) microglial cells are extremely complex to target experimentally; ii) their beneficial or detrimental effects are spatially related and temporally defined [4,6,7,10]; iii) several subsets of microglia are involved [4,6,7,10]. Given these critical issues, new strategies to understand the distinct role played by microglia in injury progression are necessary. In recent years, nanomedicine has provided many innovations and it has been increasingly applied in drug development, in particular with the use of nanoparticles (NPs) for biomedical applications. Polymeric NPs show relevant potential advantages in pharmacological delivery by enhancing drug targeting and concentration in the injury site, increasing the pharmacological activity and decreasing potential drug-related side effects [11–16]. An interesting application of this innovative cell-targeted delivery regards the pharmacological modulation of microglia/macrophages. Indeed, new *in vitro* and *in vivo* evidence suggests that NPs can be selectively internalized by a specific endocytic/phagocytic activity of the macrophagic cells after different insults, allowing to exploit them as Trojan horses [14,15,17,18]. It is well known that microglia/macrophages assume phagocytic activity after traumatic stimuli [2,14,15,19,20] and this makes NPs a potential tool for drug targeting in SCI. Thus, in order to deeply understand the potential role of the inflammatory response related to activated microglia during the progression of SCI, we propose here a cell-targeted *in situ* modulation by using a selective delivery tool loaded with a promising and well known anti-inflammatory drug (minocycline) [21–26]. In particular, we describe the detailed spatial and temporal changes of the pro-inflammatory response that are associated to microglia and their implication in injury progression. We demonstrate that minocycline-loaded NPs acutely administered in mice after SCI are able to modulate the inflammatory milieu specifically targeting activated microglia and leading to a significant and persistent amelioration of the behavioral outcome. Furthermore, we provide a mechanistic link between early microglia activation and M1 macrophages recruitment via CCL2 that can be mitigated by acute delivery of minocycline-loaded NPs following SCI. We therefore show that an early modulation of the pro-inflammatory microglia by minocycline-loaded NPs is able to skew toward a reparative environment the injured tissue, ameliorating outcome after SCI.

2. Materials and methods

2.1. Nanoparticles

PEGylated poly-ε-caprolactone-based NPs (PCL) were synthesized and loaded with minocycline as previously described [15]. Briefly, HEMA-CL_3 macromonomer was synthesized through ring opening polymerization of caprolactone initiated by 2-hydroxyethyl methacrylate (HEMA) without solvent and using $Sn(Oct)_2$ as catalyst [27]. Then NPs were prepared using free radical emulsion polymerization performed in a monomer starved semi-batch emulsion polymerization (MSSEP) by loading in the reactor 0.05 g of potassium persulfate as initiators, 0.25 g of Tween80 as surfactant, 0.5 g of polyethylene glycol methyl ether methacrylate (HEMA-PEG_{19}), while 2 g of HEMA-CL_3 were added in starved condition together with 25 mg of HEMA-RhB (the dye Rhodamine B has been linked to HEMA molecule, to have a polymerizable dye and thus covalently linked to the polymer backbone). Reaction was performed in a three necked flask, temperature was controlled with an external oil bath set to 80 ± 1 °C and the reactor was kept inert through vacuum–nitrogen cycles. The NPs dimension was checked with dynamic light scattering and transmission electron microscopy. Minocycline (Sigma; St Louis, MO, USA) free-base, as previously reported [15] was entrapped in the partially swollen preformed NPs as follows: a PTFE cylinder of 1 cm of diameter and 1 cm of length with an axial perforation of 1 mm diameter and a radial one of 500 μm was used as a mixing device for the drug loading procedure. The NP latex (300 mg in 6 mL of deionized water, concentration of 50 mg NPs/mL water) and the dissolved drug (2 mg of minocycline in 1 mL of DMSO) were loaded in syringe pumps and injected radially in the device at a flow rate of 30 mL/min and 5 mL/min, respectively. Four-hours dialysis was employed to remove free minocycline. The amount of minocycline present in PLC-based NPs at the end of the dialysis period was determined by Micromass Quattro Micro triple quadrupole mass spectrometer (Waters Corp) as previously reported [15]. The *in vitro* release kinetics of minocycline from NPs was studied at 37 °C and presented In Figure S5.

2.2. Animals care

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS Istituto di Ricerche Farmacologiche Mario Negri (Quality management System Certificate UNI EN ISO 9001:2008 – Reg. N.8576-A), in compliance with national (D.lgs. 26/2014; Authorization n. 19/2008-A issued March 6, 2008 by Ministry of Health) and international laws and policies (EEC Council Directive 2010/63/UE; US NIH Guide for care and use of laboratory animals, 2011).

2.3. Surgery

C57BL/6J mice (Charles River Laboratories International, Inc.) or B6.129P-Cx3cr1tm1Litt/J mice (The Jackson Laboratory) were used for *in vivo* studies. Animals received an antibiotic and analgesic treatment before surgery, respectively with a subcutaneous injection of ampicillin (50 mg/kg) and buprenorphine (0.15 mg/kg). The surgical procedure was carried out in deep anesthesia by intraperitoneal injections of ketamine hydrochloride (Imalgene, 100 mg/kg) and medetomidine hydrochloride (Domitor, 1 mg/kg).

To induce SCI, an incision was made in the skin, T11 and T12 vertebrae were identified and exposed by separation of dorsal and intervertebral muscles. Animals were then placed on a Cunningham Spinal Cord Adaptor (Stoelting) mounted on a stereotaxic frame and laminectomy of T13 vertebra was done to uncover the lumbar spinal cord. Mechanical trauma of the spinal cord at T12 vertebral level was achieved using an aneurysm clip with a closing force of 60 g (left in place for 1 min and then removed). After spinal cord compression, dorsal muscles were juxtaposed using absorbable sutures and the skin sutured. Manual compression of the bladder was also performed twice daily for two days after SCI induction.

At several time points mice were treated with intraparenchymal injections of minocycline loaded NPs (PCL-mino, 0.25 mg of minocycline within 0.25 gr of polymer/mL), empty NPs (PCL, NPs 0.25 gr polymer), free delivered minocycline (mino free, 0.25 mg in addition to PCL) or phosphate buffered saline, PBS (Saline). Using a glass capillary (40 ± 2 μm diameter) the solutions (0.250 μL/site) were injected in the injured area of spinal cord with a flow rate of 0.2 μL/min. Six injections were performed in order to cover the whole injured area (Fig. 1a). The capillary was positioned at +0.5 mm aside from the midline, then it was deepened into the parenchyma to 0.6 mm below the pia mater. After treatment, dorsal muscles were juxtaposed using absorbable sutures and the skin sutured and disinfected. During the experiment, mice were housed