Anti-Inflammatory Modulation of Microglia via CD163-Targeted Glucocorticoids Protects Dopaminergic Neurons in the 6-OHDA Parkinson’s Disease Model

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Increasing evidence supports a decisive role for inflammation in the neurodegenerative process of Parkinson’s disease (PD). The immune response in PD seems to involve, not only microglia, but also other immune cells infiltrated into the brain. Indeed, we observed here the infiltration of macrophages, specifically CD163+ macrophages, into the area of neurodegeneration in the 6-hydroxydopamine (6-OHDA) PD model. Therefore, we investigated the therapeutic potential of the infiltrated CD163+ macrophages to modulate local microglia in the brain to achieve neuroprotection. To do so, we designed liposomes targeted for the CD163 receptor to deliver dexamethasone (Dexa) into the CD163+ macrophages in the 6-OHDA PD model. Our data show that a fraction of the CD163-targeted liposomes were carried into the brain after peripheral intravenous injection. The 6-OHDA-lesioned rats that received repeated intravenous CD163-targeted liposomes with Dexa for 3 weeks exhibited better motor performance than the control groups and had minimal glucocorticoid-driven side effects. Furthermore, these animals showed better survival of dopaminergic neurons in substantia nigra and an increased number of microglia expressing major histocompatibility complex II. Therefore, rats receiving CD163-targeted liposomes with Dexa were partially protected against 6-OHDA-induced dopaminergic neurodegeneration, which correlated with a distinctive microglia response. Altogether, our data support the use of macrophages for the modulation of brain neurodegeneration and specifically highlight the potential of CD163-targeted liposomes as a therapeutic tool in PD.

Key words: CD163; dexamethasone; dopamine; macrophages; microglia; Parkinson’s disease

Introduction

Parkinson’s disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) and the presence of intraneuronal aggregated α-synuclein in the Lewy bodies. It has been proposed that the immune system plays an active part in the symptoms and progression of PD (Doorn et al.,...
2012; Blandini, 2013). Supporting this, epidemiological studies show that the use of nonaspirin NSAIDs decreases the risk of developing PD (Rees et al., 2011) and genetic studies described a significant association between polymorphisms in the HLA gene loci and the risk for late-onset PD (Hamza et al., 2010; International Parkinson Disease Genomics Consortium, 2011).

Microgliosis in PD brain has been shown repeatedly in patients and has also been observed in animal models of PD (Cerbrián et al., 2015). PET brain imaging and postmortem studies in PD shows early but sustained microglial activation that was not only limited to areas with significant neuronal death, but was also correlated with α-synuclein pathology (Hunot et al., 1996; Knott et al., 2000; Imamura et al., 2003; Croisier et al., 2005; Ouchi et al., 2005; Gerhard et al., 2006). Importantly, α-synuclein released from neurons acts as a proinflammagen on microglia, further contributing to the microgliosis (Sanchez-Guajardo et al., 2013a). The microglia response is complex, as shown by the variety of proteins upregulated, such as MHC-II, CD68 (Banati et al., 1998; Croisier et al., 2005), COX2, and iNOS (Hunot et al., 1996; Knott et al., 2000). The immune response is also noticed in periphery and involves both pro-inflammatory signals such as TNF and IL-1β, but also anti-inflammatory molecules such as IL-4 and IL-10, all of which are increased in the serum and/or CSF of PD patients (Mogi et al., 1994; Garcia-Esparcia et al., 2014). This widespread and chronic inflammatory reaction in PD patients suggests that microglia fail to solve the insult that triggers their activation and such maintained chronic activation might lead to neuronal death (Herrera et al., 2005; Joers et al., 2016).

The immune response in PD patients and in animal PD models seems to involve, not only microglia, but also infiltrated peripher al macrophages (Zhao et al., 2011) and lymphocytes (Lossinsky and Shivers, 2004; Brochard et al., 2009). It has been proposed that CD4+ T cells may play a key role in the dopaminergic cell death in the MPTP and the α-synuclein PD models (Brochard et al., 2009; Sanchez-Guajardo et al., 2013b). Further supporting a role of peripheral cells, unrelated inflammatory peripheral events increase neurodegeneration in PD models (Vil- laran et al., 2010; Gao et al., 2011).

Synthetic glucocorticoids are the most potent known drugs used to relieve inflammation. Among them, dexamethasone (Dexa) was shown previously to be neuroprotective in the MPTP mouse PD model (Kurkowska-Jastrzebska et al., 2004) and in an LPS rat PD model (Castano et al., 2002). However, chronic treatment with Dexa and glucocorticoids are well known to induce serious side effects, so we used a low-dose Dexa-targeted approach. We have shown previously that both in vitro and in vivo, anti-CD163-Dexa-conjugate attenuated the LPS-induced inflammatory response in rats (Graversen et al., 2012). CD163 is a hemoglobin scavenger receptor (Kristiansen et al., 2001) expressed in monocytes/macrophages that has been involved in different inflammatory diseases (Etzerodt and Moestrup, 2013; Dultz et al., 2016; Liu et al., 2015). In the brain, CD163 is expressed in perivascular macrophages (PVMs) but not in microglia, so it constitutes a potential marker for infiltrated peripheral immune cells in the brain parenchyma (Pollifet et al., 2006).

Microgliosis and increased pro-inflammatory mediators in brain are also found in the 6-hydroxydopamine (6-OHDA) PD model (Cicchetti et al., 2002; Nagatsu and Sawada, 2005; McCoy et al., 2006; Na et al., 2010). In the present study, we investigated macrophage infiltration in the brains of 6-OHDA-lesioned rats and tested whether anti-inflammatory modulation of infiltrating macrophages protects against neurodegeneration.

Materials and Methods

Animals. Adult female Sprague Dawley rats (n = 120; Taconic) weighing 225–250 g at the time of the surgery were housed two per cage with ad libitum access to food and water in a climate-controlled facility under 12:12 h light/dark cycle. The animal experiments were conducted under humane conditions with ethical approval from the Danish Animal Inspectorate that adheres to the European Rules.

Dopaminergic lesion. Rats were anesthetized with medetomidine hydrochloride (1 mg/ml) diluted 1/15 in fentanyl (50 μg/ml) (1.6–1.8 ml injection, i.p.) and placed in a stereotaxic apparatus (Stoeling). The skull was exposed by an incision in the midline and three 2 mm holes were drilled to allow 2 μl injections of 6-OHDA (7 μg in saline and 0.02% ascorbic acid) per site or vehicle for sham injections. The solution was injected over 2.5 min using a 5 μl Hamilton syringe attached to a glass capillary (outer diameter of 60–80 μm) at the following coordinates in mm: AP +1.0; 0.0; −1.0 and ML ±3.0; ±3.4; ±4.2 from bregma and −4.9 ventral from dura (nose bar at −3.3) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). After the injection, the cannula was left in place for 5 min before slow retraction. Animals were sutured using metal clips and injected with buprenorphine (0.36 mg/kg) for pain relief and awakened with a single injection of antipamol hydrochloride (0.7 mg/kg). Fully awakened animals were placed back in their cages.

The 6-OHDA (Sigma-Aldrich) was dissolved at 4.2 mg/ml (the 6-OHDA used contains 82% free base) with 0.02% ascorbic acid in sterile saline, kept on ice for no more than 2 h, and protected from light throughout the entire procedure. For sham injections, 0.02% ascorbic acid in sterile saline was used.

Liposome preparation. Liposomes were prepared and modified for CD163 targeting as described previously (Etzerodt et al., 2012). In short, liposome formulations were formed using the ethanol injection method from a mixture of hematopoietic stem/progenitor cells Hydrogenated soy L-α-phosphatidylcholine, cholesterol, and mPEG2000-PE (molar ratio of 55:40:5; Lipoid and Sigma-Aldrich). Lipids were dissolved in 100 μl of EtOH at 65°C for 10 min, followed by hydration for 1 h at 65°C in 900 μl of aqueous buffer. Liposomes were sized by extrusion 25 times through a 0.1 μm filter using a mini-extruder kit (Avanti Polar Lipids) and dialyzed against 150 mM NaCl (0.9% NaCl) overnight at 4°C. Lipid content, drug content, and encapsulation efficiency were estimated from high-pressure size-exclusion chromatography (UV absorbance 210 nm) using a Dionex UltiMate 3000 HPLC system (Thermo Scientific) equipped with an Ascentis C18 column (Sigma-Aldrich). Liposome sizes were estimated using dynamic light scattering and the DynaPro NanoStar system (Wyatt Technology). Modification of liposomes for CD163 targeting was done as described previously by the postinsertion method of lipidated antibody using the CD163-antibody clone E2D (AbD Serotec) (Torchilin et al., 2001; Etzerodt et al., 2012).

For liposomes encapsulating calcine, lipids were rehydrated in 250 mM calcein disodium (Sigma Aldrich) and 150 mM NaCl and dialysis was repeated five times to remove excess calcine. Liposomes encapsulating Dexa-21-hemisuccinate lipids were rehydrated in 200 mM CaAcO3 and remote loading of Dexa was done using the remote loading technique for amphiphatic weak acids (Clerc and Barenholz, 1995) with a Dexa to lipid ratio of 1:10 (mol/mol). Formulation of liposomes encapsulating 125I-labeled Bolton–Hunter reagent was done by rehydrating lipids in a 80 mM HBS buffer (80 mM HEPES, 150 mM NaCl), pH 8.6, supplemented with 80 mM 1-arginine (Mougin-Degraef et al., 2006). On the day of injection, 1-arginine-containing liposomes were radioactively labeled by incubating liposomes for 30 min at room temperature with 125I-Bolton–Hunter reagent (0.48 MBq/μmol lipid) in 5 mM CBS (5 mM citrate, pH 5.0, 150 mM NaCl). Excess 125I-Bolton–Hunter reagent was subsequently removed using a Zeba Spin desalting column (7 K MWCO; Thermo Fisher Scientific).

Biodistribution of radioactive CD163 liposomes. Three weeks after striatal injections, 6-OHDA or sham (n = 4 per group) rats were anesthetized with 2.5% isoflurane (IsoVet 1000 mg/g inhalation vapor, liquid; Channelle) in 0.4 L/min O2 and 1 L/min N2O that was maintained during the intravenous injections. A single intravenous, 0.5 ml injection of 125I-
anti-CD163-liposomes (0.125 mM <span>liposome solution, 1.5 x 10^6 cpm</span>/animal, n = 8) was administered to each animal. Blood samples were collected from the sublingual vein at several time points after liposome injection (2, 20, 40, and 60 min). Rats were killed after 2 h by cervical dislocation under anesthesia, at which time a final intracardiac blood sample was taken and selected organs were rapidly dissected (heart, two distant pieces of the spleen, liver, and both kidneys), weighed, and analyzed for 125I radioactivity using an alpha-counter (COBRA 5002; PerkinElmer). The radioactivity results were adjusted to the mass of tissue and are presented as a percentage of the total injected dose for each organ. Time course of the blood clearance of 125I was determined in 0.5 ml of blood and is presented as relative radioactive emission compared with emission 2 min after 125I-anti-CD163 injection.

**Tracking of peripherally injected liposomes.** One and 3 weeks after surgery (6-ODHA; n = 4 and sham; n = 2), lesioned and naive (n = 2) rats received a single intravenous injection of CD163-targeted calcein-loaded liposomes (ED2-LCL-Calcein; dose lipid = 1.25 mmol and IgG = 0.075 mg/ml; injection volume = 2 ml/kg) following the protocol from the Qtracker565 cell-labeling kit provided by the manufacturer (Thermo Fisher Scientific). Rats were briefly kept in 4% ice-cold paraformaldehyde (PFA; in 0.1 M NaPB, pH 7.4). When relevant and before PFA perfusion, blood was collected and the heart, thymus, liver, and/or spleen were dissected and frozen in dry ice for storage at −80°C. PFA-perfused brains were postfixed in the same PFA solution for 2 h, transferred to 25% sucrose solution (in 0.02 M NaPB), sliced into 40-μm-thick coronal sections (Microm HM 450; Brock and Michelsen), separated in serial sections (series of 8 for the striatum and 6 for the SN), stored at −20°C. For selected experiments, animals were deeply anesthetized with iso-flurane and killed by cervical dislocation. Thereafter, selected organs were dissected, frozen in dry ice, and kept at −80°C until use.

**Immunohistochemistry.** Immunohistochemical stainings were performed on free-floating brain sections as described previously (Febbraro et al., 2013) using the following mouse primary antibodies: tyrosine hydroxylase (TH; 1:3000; Millipore), CD11b (Mac1; 1:500; AbD Serotec), CD163 (ED2; 1:1000; AbD Serotec), CD68 (ED1; 1:200; AbD Serotec), and MHCIId (OX-6; 1:250; AbD Serotec). The sections stained with CD11b antibody were counterstained with cresyl violet (0.5% solution) and MCBB. Bright-field images were acquired with a Leica DMi6000B microscope.

For immunofluorescence, CD11b (Mac1; 1:500; AbD Serotec) primary antibody incubation was followed by several washes and incubated with species-specific fluorescein-conjugated antibodies (Alexa-Fluor 647 and Alexa-Fluor 568; Invitrogen) in 0.25% Triton X-100 in KPBS with 2.5% horse serum at room temperature for 2 h and DAPI (1:2000; Sigma-Aldrich) was added for the last 10 min for nuclear staining. After final washes, sections were mounted and confocal images were obtained using an LSM 710 Meta confocal microscope (Zeiss) with a 63x lens.

**Microscopic analysis and stereological analysis.** The unbiased stereological estimation of the total number of either TH+ or CD11b+ cells in the SN or CD163+ cells in the striatum were made by an observer blinded to the identity of the animals using the optical fractionator principle (Westermeyer, 1991). This sampling technique is not affected by tissue volume changes and does not require reference volume determinations. Sample points were performed using the NewCast module in VIS software (Visiopharm). A 1.25x low-power objective on at least a single CD163+ cell was used to delineate the borders of the SN and striatum based 

entire striatum (typically 10–12 sections in a series per animal), from the rostral tip of the striatum to the level of −1.80 relative to bregma, were used. The actual counting was performed with a 40× objective (numerical aperture 0.75). The counting frame (56.89 μm × 42.66 μm) was placed randomly by the VIS module and moved systematically until the entire delineated region was sampled. The sampling frequency was chosen by adjusting the X-Y step length between 190 and 240 μm for CD163 cell counting, between 230 and 280 for CD11b+ cell counting, and between 140 and 280 for TH+ cell counting such that between 100 and 200 cells were counted on each side of the brain for every animal. The estimated total number of positive cells was calculated according to the optical fractionator formula and a coefficient of error of <0.10 was accepted.

For MHCI+ cell counting, one section of the SN from each animal located between −5.60 mm and −6.04 mm from bregma according to the mouse brain atlas (Paxinos and Watson, 1998) was selected and the quantification was made using a bright-field Leica DMI600B microscope at a magnification of 10×.

**Striatal fiber density measurement.** The optical density of TH+ fibers in the striatum was measured at 6 different rostro-caudal levels according to the rat brain atlas (Paxinos and Watson, 1998): AP: +1.60; +1.00; 0.20; −0.30; −0.92; −1.40 mm relative to bregma. The different brain sections were scanned using a densitometer (Bio-Rad GS-710) and the digital images obtained were analyzed by Image J software using grayscale. The optical density for each section was corrected for nonspecific background measured from corpus callosum. The data are presented as a percentage of the lesioned side to the intact control side.

**Activity of cortisol levels.** Sera were obtained from the collected blood samples by centrifugation 400 × g for 15 min at 4°C. The cortisol assay was performed using 1:20 diluted samples. The endogenous cortisol concentrations were measured using the Cortisol Parameter Assay Kit (R&D Systems). Sample dilutions were performed by adding 20 μl of serum sample into 380 μl of calorimeter diluent from the kit. The samples were read using a Cobas 6000 analyzer (Roche Diagnostics) with 450 nm wavelength according to the manufacturer’s instructions.

**High-performance liquid chromatography.** At the day of the analysis frozen SN or striata were briefly sonicated (Branson Sonifer 250) on ice in using GraphPad Prism 6.0 for Mac using one-way ANOVA test followed by Tukey’s multiple-comparisons test. When appropriate, paired t tests were used. Values of p < 0.05 were considered to be significant.

**Results**

6-OHDA induced degeneration results in the increase of CD163+ cells in striatal parenchyma

The expression of the scavenger receptor CD163 in the brain is limited to meningeal macrophages and the PVM (Polliet et al., 2006). Therefore, the presence of CD163+ cells in the brain parenchyma may suggest an infiltration of peripheral, meningeal, or PVM cells or an abnormal upregulation of the CD163 expression by the local microglia. To determine whether the CD163+ population changed during the neurodegenerative process occurring in the 6-OHDA rat PD model, we performed stereological quantification of the CD163+ cells in the striatum at 1 and 3 weeks after 6-OHDA surgery. To account for the possible infiltration of CD163+ cells into the striatal parenchyma due to the physical damage induced by the surgery, we performed a sham surgery in the contralateral side. CD163 immunostaining revealed two types of cells: rod cells, which were elongated and typically associated with blood vessels and thus resemble the previously described PVMs (Fig. 1A, B, C), and polygonal cells that were not associated with blood vessels but located in the parenchyma and consequently might correspond to infiltrating macrophages (Fig. 1B, B’). One-week after surgery, no significant changes in the total number of CD163+ cells were observed between the ipsilateral and contralateral striatum. In the ipsilateral sham striatum, >95% of the CD163 + cells were found mainly as rod cells (PVMs) that were clearly associated with blood vessels (Fig. 1B, C). Similar results were obtained from the contralateral striata, although with lower numbers of polygonal cells, suggesting a small but non-significant increase of CD163+ polygonal cells in the ipsilateral striatum. However, 3 weeks after surgery and 6-OHDA injection, the total number of CD163+ cells was significantly increased in the 6-OHDA lesion striatum (Fig. 1C). This increase was due to an elevated number of CD163+ polygonal cells located in the striatal parenchyma compared with the sham contralateral side (22% of total CD163+ cells), suggesting a recruitment of peripheral immune cells to the side of neurodegeneration.

**Peripherally transferred CD163+ cells infiltrate the striatal parenchyma in the 6-OHDA PD model**

The stereological data suggest that the CD163+ polygonal cell population is recruited to the brain, specifically to the striatum, upon induction of neurodegeneration using 6-OHDA. To confirm that peripheral CD163+ macrophages could be indeed recruited to the brain parenchyma, CD11b+/CD163+ peritoneal cells were isolated from a group of naïve rats (Fig. 2B, C) labeled with fluorescent Qdot 565 nm (565 nm) for in vivo tracking and injected intravenously in 6-OHDA-lesioned animals 3 weeks after brain surgery. Immunohistochemistry of brain parenchyma for CD11b (expressed by microglia and macrophages) from the rats killed 18 h after intravenous cell transfusion showed Qdot565 nm-positive CD11b+ cells of polygonal body shape in the striatum (Fig. 2D), confirming the infiltration of peripheral CD163+ cells to the brain.

**CD163-targeted liposomes target the brain efficiently, especially in the 6-OHDA model**

The infiltration of peripheral CD163+ cells into striatum enabled us to use the CD163+ cells as mediators and/or tools to modify the immune environment in the brain of 6-OHDA-injected animals. To do so, we designed long-circulating liposomes (LCLS), which were specifically targeted toward the CD163 receptor, by coating them with an anti-CD163 antibody (ED2...
clone). To assess the potential of the ED2-LCLs as drug carriers and to evaluate biodistribution in the 6-OHDA model, we loaded the ED2-LCLs with $^{125}$I radiolabel and injected a single dose intravenously ($0.03$ MBq) in 6-OHDA-lesioned animals and sham rats 3 weeks after brain surgery. We followed the clearance of the radioactive liposomes from the blood circulation, taking regular blood samples for 2 h; thereafter, all animals were killed.

We collected brain, spleen, kidneys, heart, and a portion of the striatum. $^{125}$I-labeled liposomes were detected in the brain, spleen, and kidneys, with the highest concentration in the spleen. The radioactivity was highest in the contralateral striatum, indicating preferential accumulation in the lesioned side. The radioactivity decreased with time, indicating gradual clearance from the circulation and accumulation in the spleen and kidneys.

Stereological quantification of the CD163+ cells in the 6-OHDA-injected striatum 3 weeks after surgery shows a significant increase of total CD163+ cells, which was mainly driven by the increase of polygonal cells in the parenchyma. Values are mean ± SEM ($n = 5$). Paired t test analysis: **$p < 0.001$; ###$p < 0.01$.

**Figure 1.** CD163+ cells in striatum. A, B, Representative photos of striatum immunostained against CD163 3 weeks after surgery; the contralateral striatum received sham vehicle injection and the ipsilateral side received $3 \times 7 \, \mu g$ of 6-OHDA (see Materials and Methods). CD163+ cells in the contralateral striatum appeared mostly as rod-shaped cells associated with blood vessels (A', B'), whereas the 6-OHDA-lesioned side also exhibited numerous cells of polygonal shape in the parenchyma (B'', B'''). C, Bar graph showing the average number of two types of CD163+ cells in the striatum at 1 and 3 weeks after surgery. Stereological quantification of the CD163+ cells in the 6-OHDA-injected striatum 3 weeks after surgery shows a significant increase of total CD163+ cells, which was mainly driven by the increase of polygonal cells in the parenchyma. Values are mean ± SEM ($n = 5$). Paired t test analysis: **$p < 0.001$; ###$p < 0.01$.

**Figure 2.** Tracking of ex vivo fluorescence-labeled CD163+ macrophages in the 6-OHDA model. A, Schematic representation of the experimental design: peritoneal macrophages were isolated from a naive rat and CD11b+/CD163+ cells were FACS sorted. Thereafter, the cells were ex vivo loaded with Qdot565 and transfused intravenously to rats 3 weeks after 6-OHDA lesion. The animals were killed 24 h later and the brain processed for immunofluorescence (for details, see Materials and Methods). B, Representative FACS dot plot showing our selection strategy of CD11b+/CD163+ cell population: selection of live cells with FSC and SSC plot, doublets exclusion based on FCS-A/FCS-H and SSC-A/SSC-H, and a final gate based on CD11b and CD163 expression and on isotype controls. C, Sort quality control of CD163+/CD11b+ cells. D, Confocal microscopic analysis showing CD11b+ containing Qdot565 in the brain parenchyma suggesting infiltration of the ex vivo-loaded cells. Scale bar, 6 μm applies to both.
The 125I radiolabel was easily detectable in circulation soon after intravenous injection, but decreased rapidly, with only 55% of the original dose detected in the blood of 6-OHDA-injected rats after 20 min. Thereafter, the levels of radioactivity remained almost stable in the blood until 2 h after injection (Fig. 3A). Interestingly, the 6-OHDA-injected animals had always the lowest radioactive readouts in circulation at all time points tested, although these were not significantly different from sham (Fig. 3A). When we measured the levels of radioactivity accumulated in tissue after 2 h in selected tissues, we could detect signal in all tissue analyzed, but the highest levels were found in the liver, spleen, and kidneys (Fig. 3B). The high radioactivity signal from the kidneys suggests an increased filtration of liposome-free 125I, which correlates with the suggested increased blood clearance in the 6-OHDA animals. Moreover, compared with sham animals, increased radioactivity levels were also detected in the brains of animals injected with 6-OHDA (Fig. 3C), thus confirming the putative potential of these liposomes as therapeutic tools for brain neurodegeneration. The uptake of radioactive liposomes in the brain may be direct; that is, circulating 125I-radiolabeled ED2-LCLs are taken up by CD163-expressing cells in the brain parenchyma (likely a PVM); or indirect, 125I-radiolabeled ED2-LCLs are taken up by peripheral CD163+ macrophages, which are then recruited to the brain.

To further confirm the potential of CD163-targeted-LCLs to reach the brain, we injected ED2-LCLs loaded with calcein intravenously in 6-OHDA-lesioned rats 1 and 3 weeks after brain surgery. Subsequent analysis of the brains by confocal microscopy confirmed the uptake of ED2-LCLs in the brain tissue because calcein was observed in CD11b+ cells located in the brain parenchyma (Fig. 4), further supporting the potential of CD163-targeted LCLs as tools to target cells that reach the brain parenchyma. However, we cannot discard at this point that the CD163-targeted LCLs loaded with calcein were taken up by CD163+ PVM because some of the calcein-loaded cells appeared in close proximity to blood vessels (Fig. 4A–D).

Experimental design: repeated injections of Dexa encapsulated in liposomes targeted for the CD163 in the PD model

Our data showed that the CD163+ peripheral cells are recruited to the brain, where they infiltrate the striatum of rats in the 6-OHDA model, and that we could access the brain parenchyma using this CD163+ population by specific targeting via ED2-LCLs. Therefore, we designed an experimental approach to use CD163+ cells to modify the neuroinflammatory process in striatum and limit the pro-inflammatory environment in the neurodegenerative area. To do so, we loaded the CD163-targeted LCLs with Dexa with the aim of modifying CD163+ cells toward an anti-inflammatory profile. We hypothesized that these modified infiltrated CD163+ cells would modulate the inflammatory process associated with the 6-OHDA lesion in the striatal parenchyma, resulting in a neuroprotective effect in the nigrostriatal dopaminergic system. Treatment was initiated 1 d before the intracerebral 6-OHDA lesion, followed by treatment 3 times per week for 3 weeks with Dexa-loaded-ED2-LCLs (Dexa 0.02 mg/kg, 0.125 mM liposome solution; ED2-LCLs-Dexa). The following were used as controls: (1) free phosphorylated-Dexa (Dexa 1 mg/kg; free Dexa), (2) Dexa loaded in nontargeted liposomes (Dexa 0.02 mg/kg, 0.125 mM liposome solution; LCL-Dexa), and (3) PBS-loaded liposomes (0.125 mM liposome solution, PBS, 0.15 M NaCl in 10 mM phosphate buffer, pH 7.4; PBS-LCL) in three parallel groups. In a pilot experiment, we confirmed that...
the 6-OHDA lesion was similar in PBS-loaded CD163-targeted LCLs or PBS-untargeted LCLs (data not shown), therefore, we used the last one as a control. The Dexa doses were chosen based on our own previous study in rats, where we showed that 0.02 mg of CD163-targeted Dexa/kg was as efficient as 1.0 mg of free Dexa/kg at decreasing the LPS-induced TNFα release (Graversen et al., 2012).

Glucocorticoid side effect alleviation using liposome encapsulation of Dexa

One of the major advantages of our approach is that, by targeting specifically the CD163 population, the dose needed is substantially lower than the normal Dexa doses used otherwise for anti-inflammatory purposes; doses reported in the literature range from 0.1 to 10 mg/kg (Quan et al., 2014). First, the barrier of polyethylene glycol has been used to ensure that the LCLs are not bound by opsonins and do not inhibit unspecific uptake in phagocytic cells (Allen and Hansen, 1991). Second, we have shown before that coating with polyethylene-glycol-linked anti-CD163 antibodies or drug targeting via the ED2-anti-CD163 antibody ensures fast uptake in CD163+ cells (Etzerodt et al., 2012; Graversen et al., 2012). All of these reduce the dose and therefore the undesired side effects of a chronic treatment. To analyze the side effects commonly observed with prolonged corticoid treatments, we measured the endogenous serum cortisol level and monitored body weight, which decreases in rodents after chronic glucocorticoid treatment (Liu et al., 2011). In addition, we measured thymus size, a commonly used parameter in rodent studies because it reflects the corticosteroid-mediated apoptosis of lymphocytes, dominating cells type of the thymus (Ben Rhouma and Sakly, 1994; Cole et al., 2000). As expected, animals receiving free Dexa showed a significant reduction in all three markers compared with PBS-LCL animals, which did not received any Dexa (p < 0.001; Fig. 5). Animals receiving Dexa in targeted or untar-
ggeted liposomes were not different from PBS-LCL animals, con-
fiming that the reduction of the dose achieved by the targeting decreased the undesired side effects of the drug.

CD13-targeted Dexa alleviates motor defects

During the third week of treatment and after surgery, the animals were tested for motor performance using the cylinder test. As a result of the dopaminergic unilateral 6-OHDA neurodegeneration, all animals showed a biased use of the ipsilateral paw versus the lesioned ipsilateral one. However, animals that received ED2-
LCL-Dexa showed significantly better use of the contralateral paw compared with the LCL-PBS control animals. Free Dexa treatment or nontargeted Dexa-loaded liposomes did not result in a better motor performance (Fig. 6). This supports a protective ability of the treatment with CD163-targeted liposomes.

**CD163-targeted glucocorticoid treatment protects dopaminergic nigral neurons**

To analyze the status of the dopaminergic nigrostriatal system, we measured the striatal TH+ fiber innervation by densitometric analysis. All groups showed a decrease of the dopaminergic innervation in the ipsilateral striatum 3 weeks after surgery. Although there was a slightly higher density of TH+ fibers in the ED2-LCL-Dexa, no significant difference was found upon statistical analysis (one-way ANOVA, $F_{(3,20)} = 1.288, p = 0.30$; Fig. 7).

To analyze whether, despite the lack of protection of dopaminergic terminals, we had a change in neuronal survival, we did stereological quantification of the number of TH+ neurons in SN 3 weeks after surgery. The animals receiving ED2-LCL-Dexa had indeed a significant higher number of surviving TH+ neurons compared with those receiving free Dexa or control LCL-PBS (Fig. 8A–L). Therefore, Dexa, when targeted to macrophages expressing CD163, could protect dopaminergic neurons, whereas treatment with free Dexa or nontargeted LCL-Dexa did not achieve similar neuroprotection.

**Biochemical analysis of striatal DA metabolism**

To analyze the effect of the treatment on DA metabolism, we performed biochemical analysis of DA and metabolites in striatum and SN 3 weeks after surgery by HPLC. We observed a similar profile to that observed upon densitometric analysis of dopaminergic terminals mentioned above, with the highest striatal DA content in the animals receiving ED2-LCL-Dexa, even though it did not reach significant difference (one-way ANOVA, $F_{(3,26)} = 2.420, p = 0.089$; Fig. 9A). The levels of HVA and DOPAC and the turnover ratios indicated an increase in DA turnover in the 6-OHDA-injected side compared with the contralateral side in all groups except in the ED2-LCL-Dexa animals (Fig. 9G–J). No change was observed in the DA, DOPAC, or HVA content in SN and the turnover did not show any signs of significant changes (one-way ANOVA, $F_{(3,22)} = 1.851, p = 0.168$; Fig. 9D–F, J–L).

**CD163-targeted liposomes loaded with low doses of Dexa induced MHCII expression in nigral microglia**

As expected, we observed a significant increase in the number of microglia CD11b+ cells in the ipsilateral side in all groups (Fig. 10A). However, no difference of the ipsilateral microgliosis was noticed between the CD163 liposomes loaded with Dexa and the control treatments (Fig. 10B). Therefore, our treatment did not avoid the proliferation (or recruitment) of microglia induced by 6-OHDA. We analyzed the expression of the CD68 phagocytic marker in our samples. As described previously, we observed upregulation of the protein in the ipsilateral SN, but no obvious difference was found among groups (data not shown).

We also studied the phenotypic changes in activated microglia by quantifying the number of MHCII-expressing microglia in a single representative SN section. As expected, MHCII+ cells were only seen occasionally in the contralateral side of the 6-OHDA lesion at the level of the SN regardless of any treatment. However, MHCII+ cells were densely stained with an activated hyperramified morphology on the ipsilateral side of the 6-OHDA lesion (Fig. 11A–D). Animals that received ED2-LCL-Dexa treatment had a significantly higher number of MHCII+ cells compared with the free Dexa group, although animals receiving LCL-Dexa also had elevated MHCII+ cells in SN (Fig. 11E). Interestingly, when we plotted and analyzed neuronal dopaminergic survival versus MHCII+ microglia in the SN, we observed a clear trend of positive correlation ($p = 0.06$). Indeed, animals showing an elevated number of surviving TH+ cells also exhibited higher numbers of MHCII+ microglia in SN, but only in the ED2-LCL-Dexa group, not in any of the other groups (Fig. 11F).

At the striatal level, all animals showed a robust upregulation of MHCII expression in microglia. When we analyzed the area covered by MHCII+ cells in striatal sections, we observed that animals receiving ED2-LCL-Dexa and LCL-Dexa showed a more widespread MHCII+ cell distribution (Fig. 12B, C) versus the dense, delimited area covered by MHCII+ cells in the control groups (Fig. 12A, D).

**Discussion**

We report here that the CD163+ macrophage population significantly infiltrates the neurodegenerating striatal parenchyma in the 6-OHDA PD model. We also show that neuroprotection can be achieved by targeting the infiltrating CD163+ cells using the anti-inflammatory drug Dexa. To do so, we specially designed liposomes targeted for the CD163 and loaded with glucocorticoids. We show that the CD163-targeted-liposomes reached the brain parenchyma, which may occur directly (CD163+PVM) or indirectly via CD163+-recruited cells. Repeated intravenous injections of these liposomes in the 6-OHDA rats resulted in better motor performance and higher dopaminergic survival. This neuroprotection was observed mainly at the SN, where we also found a significant microgliosis. However, the microglia response appeared different, with a significantly higher number of MHCII+ cells that correlated positively with the neuronal survival. Our
data suggest that modifying the CD163 population by peripheral injections of ED2-LCL loaded with Dexa induces a specific immune response, which results in a distinctive microglial response in the brain correlating with decreased dopaminergic cell death and better motor performance.

We observed a distinguishable increase in the number of CD163/H11001 cells in the lesioned striatum 3 weeks after 6-OHDA. This was not just a consequence of blood–brain barrier damage during surgery because sham surgery did not result in similar increases. Previously, CD163 was defined as the scavenger receptor in monocytes/macrophages responsible for the hemoglobin–haptoglobin complex uptake (Kristiansen et al., 2001). CD163 changes during inflammation and seems related to wound healing (Kowal et al., 2011; Liu et al., 2015). As described, we found that CD163 expression was normally confined to meningeal macrophages (data not shown) and PVM (Polfliet et al., 2006). In healthy rat brain, only occasional CD163+ cells appear in the parenchyma; however, this was significantly increased in the 6-OHDA-lesioned striatum. CD163+ microglia-like cells were reported in certain brain lesion models, although it is unclear whether these cells were of peripheral origin (Zhang et al., 2012). To address whether peripheral CD163+ cells could be recruited to the brain parenchyma, we performed adoptive transfer of CD163+ peritoneal macrophages in 6-OHDA rats. Ex vivo labeling of the CD163+ macrophages allowed us to follow them in vivo and confirmed their infiltration from the periphery to the brain parenchyma. This is consistent with previous reports documenting infiltration of macrophages, but also T cells, in different neurodegenerative models including PD (Kurkowska-Jastrzebska et al., 1999; Kokovay and Cunningham, 2005; Simard et al., 2006; Rodriguez et al., 2007; Depboylu et al., 2012; Theodore and Maragos, 2015). These infiltrated macrophages may acquire later microglia characteristics (Simard and Rivest, 2004). This recruitment into the area of neurodegeneration seems to happen before cell death, but persists after this occurs (Rodriguez et al., 2007). We saw a significant

Figure 7. Dopaminergic fiber innervation in striatum. A. Photomicrographs showing representative TH-immunostained striatal section in each group 3 weeks after 6-OHDA lesion. B. Bar graph illustrating the semiquantitative measurement of the TH+ densitometry expressed as the percentage of the contralateral side striatal; no significant change was found between groups. Values are mean ± SEM (n = 6 –9). One-way ANOVA followed by Tukey post hoc analysis.
Figure 8. Dopaminergic neuronal population in SN. A, D, G, J. Low-magnification photographs showing representative nigral sections immunostained for TH. B, C, E, F, H, I, K, L. Higher magnification of SN from the naive (contralateral) and 6-OHDA (ipsilateral) sides of each section. Notice the higher density of TH+ neurons in the ipsilateral SN in the ED2-LCL-Dexa animal compared with the LCL-PBS or free Dexa animals. L, Bar graphs illustrating the average number of surviving TH+ nigral neurons obtained by stereological quantification expressed as a percentage of the contralateral side. Animals treated with ED2-LCL-Dexa showed higher number of TH+ neurons compared with the ones treated with LCL-PBS or free Dexa. Scale bar in J, 12.5 mm and applies to A, D, G, and J; scale bar in L, 200 μm and applies to B, C, E, F, H, I, K, and L. Values are mean ± SEM (n = 12–14). One-way ANOVA followed by Tukey post hoc analysis: *p < 0.05.
increase of CD163
/H11001
after 3 weeks, when cell death had occurred, although our data may suggest that the infiltration could be initiated as soon as 1 week after surgery. The role of the infiltrated cells is unclear, but has been proposed to be deleterious based on their iNOS expression (Kokovay and Cunningham, 2005) or protective based on their GDNF expression (Rodriguez et al., 2007). In stroke, the infiltrated cells express protein characteristics of both pro-inflammatory and anti-inflammatory profiles, although their pre-

Figure 9. Analysis of DA and its metabolites and DA turnover rates. Levels of DA and its metabolites, DOPAC and HVA, were examined by HPLC in the striatum (A–C) and SN (D–F) of animals treated for 3 weeks. All animals showed an overt decrease of DA in the lesioned ipsilateral striatum; ED2-LCL-Dexa animals exhibited the highest striatal DA content, but this did not reach significant different (one-way ANOVA, \( F_{(3,26)} = 2420 \, p = 0.089 \)). The turnover of DA was calculated as the DOPAC/DA (G–I) and HVA/DA (J–L) and examined in the striatum (G–I) and SN (J–L) of animals treated for 3 weeks with ED2-LCL-Dexa and control treatments. All animals showed a significant increase in all turnover ratios in the ipsilateral striatum except the ED2-LCL-Dexa animals. No changes were observed in the SN. Values are mean ± SEM (n = 6–8). Paired t test (contralateral vs ipsilateral) and one-way ANOVA (across groups): *** \( p < 0.001 \); ** \( p < 0.01 \); * \( p < 0.05 \).
Figure 10. CD11b+ microglia population in the SN. A, Bar graph shows the average number of total cells obtained by stereological quantification of CD11b+ cells both in the contralateral and ipsilateral SN after 3 weeks of treatment. Injection of 6-OHDA in the striatum induced a significant increase of CD11b+ population in the SN regardless of the treatments. B, Bar graphs representing the CD11b+ population in the ipsilateral side expressed as a percentage of the contralateral side showing that no significant difference was found. Values are mean ± SEM (n = 8). Paired t test (in A) and one-way ANOVA followed by Tukey post hoc analysis (B).

Figure 11. MHCII+ microglia in SN. A–D, Low-magnification photos showing representative SN sections immunostained against MHCII from each group. High-power photomicrographs showing insets illustrated in the top low-magnification photos. Notice the higher density of MHCII+ cells in the ipsilateral SN from the animal treated with ED2-LCL-Dexa compared with the ones treated with LCL-PBS or free Dexa. E, Bar graph showing the mean number of MHCII+ cells in ipsilateral SN expressed as a total number of cells per nigral section. Animals treated with ED2-LCL-Dexa and untargeted LCL-Dexa showed an increased number of MHCII+ cells compared with the PBS-LCL control group. F, Positive correlation in between TH+ neurons and MHCII+ cells in SN in ED2-LCL-Dexa-treated animals. Scale bar in D, 200 μm and applies to high-power photomicrographs in A–D. Values are mean ± SEM (n = 7–11). One-way ANOVA followed by Tukey post hoc analysis: ***p < 0.001; **p < 0.01; *p < 0.05.
In humans and monkeys, CD163+ microglia-like cells appear in brain upon virus infections, trauma lesions, and immune-related neurodegeneration (Borda et al., 2008; Hollfelder et al., 2011). CD163+ cells were observed close to Aβ plaques in Alzheimer’s disease patients’ brains, suggesting a role for this population in protein aggregation-related neurodegeneration (Zhang et al., 2011; Pey et al., 2014).

We observed that modulation of the immune response via ED2-LCL results in significant protection of dopaminergic neurons and improved motor behavior. Our hypothesis was that the inflammatory event was actively participating in the neurodegenerative process and therefore its modulation would result in protection. We observed here a robust ipsilateral microgliosis 3 weeks after surgery, as described previously (Marinova-Mutafchieva et al., 2009; Virgone-Carlotta et al., 2013; Schlachetzki et al., 2014). CD68 expression suggests that microglial phagocytic activity precedes neuronal death that might be detrimental for dopaminergic survival (Marinova-Mutafchieva et al., 2009). Accordingly, anti-inflammatory strategies are neuroprotective in PD models, such as minocycline or Dexa treatments in the MPTP model (Wu et al., 2002; Kurkowska-Jastrzebska et al., 2004), as well as approaches decreasing pro-inflammatory mediators such as TNF (McCoy et al., 2011) and IL-1β (Koprich et al., 2008; Pott Godoy et al., 2008; Tanaka et al., 2013). However, therapeutic strategies targeting peripheral macrophages have rarely been used before in PD. Ex vivo-modified bone marrow stem cells or macrophages overexpressing GDNF showed neuroprotection in the MPTP and the 6-OHDA models (Biju et al., 2010; Zhao et al., 2014). Nano-formulated catalase administered intravenously after acute MPTP intoxication resulted in protection in SN (Brynskikh et al., 2010); in addition, intravenous transfer of ex vivo-transfected macrophages with a catalase plasmid DNA induced neuroprotection in the 6-OHDA model (Haney et al., 2013). Intranasal delivery of bone-marrow-derived stem cells has resulted in new macrophage-like cells in brain associated with neuroprotection (Danielyan et al., 2010; Danielyan et al., 2011; Danielyan et al., 2014). Therefore, targeting peripheral macrophages seems to be a promising tool for neuroprotective therapies.

In our animals, we observed neuronal protection, but no significant protection of the striatal terminals. This could be due to the lesion design and the microglia/immune response time line. Indeed, the 6-OHDA model is a retrograde neurodegenerative model, in which the degeneration is initiated in the terminals and progresses retrogradely toward the nigral cell body. Therefore, the striatal fiber loss starts as soon as 6 h after injection and is accompanied by a rapid and robust microgliosis (Walsh et al., 2011; Stott and Barker, 2014). Although microgliosis change in profile and protein expression is observed as early as 72 h in SN, the microgliosis proliferation is delayed 1 week after lesion, at the same time as neurons show the first sign of caspase-3 activation and death (Walsh et al., 2011; Stott and Barker, 2014). This may result in a better window for neuroprotection of the cell bodies using our therapeutic strategy.

Remarkably, we observed microgliosis in SN despite the significant dopaminergic survival, suggesting a modulation of the immune response toward a neuroprotective type without avoiding its proliferation or recruitment. Microglia, as macrophages, have different activation stages: pro-inflammatory, anti-

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**Figure 12.** MHCII expression in striatum. Photos from striatal section immunostained against MHCII from representative animals of each group 3 weeks after striatal 6-OHDA lesions. Insets indicate areas where the high-power photomicrographs were taken. The animals treated with ED2-LCL-Dexa and LCL-Dexa generally show a smaller area of dense MHCII+ cells at the needle track area, composed of macrophages and ramified microglia (B, C). Notice that the MHCII+ expression was not confined only to the core, but we observed MHCII+ cells spread throughout the striatum (B’, C’, insets). LCL-PBS- and free-Dexa-treated animals normally showed a bigger but less dense core covered by MHCII expression associated with the injection area (A, D), but the MHCII+ population seemed contained and did not spread through the striatal parenchyma (A’, D’, insets). Scale bar, 100 µm and applies to low-magnification photos.
inflammatory, and repairing. The dynamic response of this population will be determinant for the neuronal fate in PD (Moehle and West, 2015). Others and we have shown previously that microgliosis can occur in the absence of cell death and is associated with neuroprotection (McCoy et al., 2006; Sanchez-Guajardo et al., 2010; Sanchez-Guajardo et al., 2013b). In agreement with our previous observations, we saw increased MHCI expression in the animals in which we observed neuroprotection (Sanchez-Guajardo et al., 2010; Sanchez-Guajardo et al., 2013b). MHCI expression is elevated in microglia in PD patients and is associated with α-synuclein deposition (Croisier et al., 2005). Remarkably, genetic variances related to the MHCI complex in humans are correlated to PD, suggesting a key role for such proteins (Hamza et al., 2010; Wisemann et al., 2013). Increased MHCI has been correlated to neuroprotection upon CX3CL1 overexpression in the 6-OHDA model (Nash et al., 2015). Conversely, in an α-synuclein viral-vector-based PD model, lack of MHCI expression was protective; however, the MHCI knockout line used in the study presents a dramatic decrease of the CD4 + T-cell population, thus complicating the interpretation (Madsen et al., 1999; Harms et al., 2013). MHCI is involved in the presentation of antigens to T cells in adaptive immunity. The adaptive immune system has been also linked to the 6-OHDA PD model and, in the MPTP model, CD4 + T cells are proposed to be essential in neurodegeneration (Brochard et al., 2009; Wheeler et al., 2014; Theodore and Maragos, 2015). Interestingly, although we also observed MHCI upregulation in untargeted LCL-Dexa animals, they did not show neuronal protection, suggesting that the targeting of the LCL to the CD163 + population was key in this therapeutic event. We do not yet know whether the differences reside in changes at the peripheral level or in brain. Therefore, the observation requires further investigation to understand its significance in neuroprotection.

Our results show that peripherally injected nanoparticles targeted to the CD163 receptor can be used to modify the local CNS microglia phenotype significantly and achieve neuroprotection of dopaminergic neurons in a 6-OHDA model of PD. At this point, we cannot discard a protection accomplished through soluble mediators induced by peripheral changes. Nor can we reject the possibility that the liposomes were taken up directly by CD163 + PVM in the brain. However, our data also suggest that a CD163 macrophage population is involved in PD-like neurodegeneration, notably by infiltrating the brain parenchyma. The exact role of these cells and the mechanisms of infiltration into the brain parenchyma are still unclear. The results of our study support the use of targeted glucocorticoids for the treatment of chronic brain inflammation, but stress the importance of characterizing the time course and the associated role of the CD163 + cell population in the degenerative process.

References
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