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Therapeutic depletion of monocyte-derived cells protects from long-term axonal loss in experimental autoimmune encephalomyelitis☆

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ABSTRACT

Studies in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (EAE) suggest that peripheral monocyte-derived cells (MDCs) are instrumental for disease initiation. MDCs, however, are plastic, and may exert various functions once in the central nervous system (CNS) for prolonged periods. Furthermore, the long-term effect of MDC depletion on continuing axon loss is not known. We show that long-lasting depletion of MDCs, after onset of EAE clinical deficits, is accompanied by decreased CNS infiltration by pathogenic T lymphocytes. Although this treatment does not reverse clinical disease, it prevents worsening of neurological deficits and long-term axonal loss.

1. Introduction

In the central nervous system (CNS), inflammatory disorders such as multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), lesions are often populated mainly by myeloid cells (Huang et al., 2001; Huitinga et al., 1990; Jiang et al., 2014; Lee et al., 2012; Lucchinetti et al., 2000; Mildner et al., 2009). These cells include infiltrating monocyte-derived cells (MDCs), resident microglia, and in some cases neutrophils. Interestingly, it was previously shown that both monocytes and neutrophils expand in the blood preclinically in EAE (Rumble et al., 2015) and neutrophil-related chemokines and monocyte subsets are increased in the blood of MS patients (Chuluundorj et al., 2014; Rumble et al., 2015).

MDCs enter the CNS in an immature state and mature locally acquiring macrophage- or dendritic cell-like properties. The effects of depleting peripheral MDCs before or shortly after the onset of clinical EAE have been well studied (Brosnan et al., 1981; Fife et al., 2000; Huang et al., 2001; Huitinga et al., 1990; Izikson et al., 2000; Mildner et al., 2009; Polfliet et al., 2002; Tran et al., 1998). Furthermore, MDC depletion in a toxin-induced demyelination model was shown to affect the generation of growth factors, thereby affecting the efficiency of remyelination (Kotter et al., 2005). In a model of axonal injury, depletion of peripheral macrophages had no effect on the percentage of infiltrating T cells (Fux et al., 2008). However, it is unknown whether the CNS is infiltrated by peripheral immune cells when peripheral MDCs are deleted in EAE, and, if so, what are the effector functions of these cells. Furthermore, the degree of axonal damage and loss during the chronic phase of the disease and long after the completion of clodronate treatment has not yet been examined. This information is important in order to assess whether there is a long term therapeutic potential, and if so, what the effective treatment window is.

Although largely regarded as pathogenic in CNS inflammation, MDCs may also exert reparative functions (Murray and Wynn, 2011). In a spinal cord transection animal model, early macrophage deletion protects from severe deficits, but MDCs depletion in later stages worsened the clinical outcome (Shechter et al., 2013). Similarly, the protective functions of MDCs populations are observed in EAE (Boven et al., 2006; Vogel et al., 2013). There is now a vast literature on the two antipodes of macrophage polarization M1 and M2, with M2 being further subdivided to M2a, b and c subsets (Lacey et al., 2012; Mantovani et al., 2004; Martinez and Gordon, 2014; Martinez et al., 2006). Although these characteristics are commonly attributed to macrophages, most MDCs are able to polarize in a similar manner (Kapsenberg, 2003; Martinez and Gordon, 2014; Mazzoni and Segal, 2004; Scotton et al., 2005; Sironi et al., 2006). This M1/M2 classification is widely accepted, but bona fide M1 and M2 polarized cells are usually generated in vitro, when naïve MDCs are stimulated with a single or a mixture of

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specific cytokines. In an in vivo setting, such as EAE, many competing stimuli are released concomitantly (Soulika et al., 2009), and may relay opposing signals. Hence, MDCs may transiently and dynamically express both M1 and M2 markers in response to microenvironmental stimuli.

Furthermore, the role of microglia in the progression of the disease is unclear. Microglia are a self-renewed population that originates from precursors arising in the extra-embryonic yolk sac (Ajami et al., 2007; Bruttger et al., 2015; Ginhoux et al., 2010). However, readily available immunohistochemical techniques are not sufficient to differentiate MDCs from activated microglia in wild type mice. Although genetically modified mice may allow discrimination of these populations, the relative contributions of microglia and infiltrating MDCs during the course of EAE are still under debate (Goldmann et al., 2013; Parkhurst et al., 2013; Yamasaki et al., 2014). Activation of microglia occurs immediate after injury (Davalos et al., 2005, 2012); but whether microglia acquire monocyte-like functions during inflammation, or whether they exert neuroprotective or anti-inflammatory effects, is still not clarified (Goldmann et al., 2013; Heppner et al., 2005; Howell et al., 2010; Lewis et al., 2014; Yamasaki et al., 2014).

Our previous study showed that accumulation of MDCs within the CNS is reduced when astroglial CCL2 (previously known as monocyte chemoattractant protein 1) is deleted. Specifically, astroglial derived CCL2 deletion inhibited the accumulation of pathogenic M1 MDCs and reduced clinical severity and axon loss in mice (Moreno et al., 2014). The total numbers of infiltrating $CD4^+$ T cells were not affected by deleting astroglial CCL2. However, unexpectedly, we found that the frequency of Th17 cell subsets was significantly increased in the absence of astroglial derived CCL2 compared to controls with normal levels of astroglial CCL2. These data suggest that, in EAE, clinical disease and axonal damage are largely mediated through an astroglial CCL2-facilitated infiltration of MDCs and/or activation of local microglia. However, it is also plausible that CCL2 may have MDC independent effects (Lee et al., 2013), influencing the course of EAE.

To address whether observations of clinical and axonal protection in our previous studies using astroglial CCL2 deficient mice was due to MDC effects, we depleted MDCs by injecting mice with clodronate liposomes to induce apoptosis of phagocytic cells (van Rooijen and Hendrikx, 2010). Peripheral monocytes (in blood, and spleen) are effectively depleted by repeated intravenously administered clodronate liposome injections (Misharin et al., 2014; Robbins et al., 2013; Sunderkotter et al., 2004; van Rooijen et al., 1989). We initiated clodronate liposome injections after mice began to show neurological deficits, thus mimicking a clinical scenario. We found that there is a critical clinical window during which MDCs cause damage in the CNS. Our findings show that continuous axonal loss occurs as a result of continuous accumulation of MDCs over time. Furthermore, our data suggest that resident microglia are not likely to be major contributors to axonal pathology, independently of infiltrating MDCs. These findings are in agreement with our previous study (Moreno et al., 2014), showing that reduction of infiltrating macrophages into the CNS results in protection from further axonal damage and worsening of clinical disease. Although depletion of infiltrating MDCs after disease onset arrested disease progression, there was no evidence of clinical recovery.

2. Materials and methods

2.1. EAE induction

EAE was induced in 12 week-old male C57BL/6 mice, as we previously described (Lee et al., 2012; Soulika et al., 2009). Briefly, 300 μg of rodent MOG peptide (amino acids 35–55, New England Peptides) in complete Freund's adjuvant (CFA) containing 5 mg/ml killed *Mycobacterium tuberculosis* (DIFCO) on day 0, with intraperitoneal (i.p) administration of 200 ng of pertussis toxin on days 0 and 2. Wild-type CFA control mice received CFA and pertussis toxin, but no MOG peptide. Neurological deficits were graded as follows: limp tail or waddling gait = 1; limp tail and waddling gait = 2; single limb paresis and ataxia 2.5; double limb paresis = 3; single limb paralysis and paresis of second limb = 3.5; full paralysis of 2 limbs = 4; moribund = 4.5; and death = 5 (Bannerman et al., 2007; Moreno et al., 2014; Soulika et al., 2009). Only mice showing clinical deficits within a 2-day window were used for this study. Mice were randomly assigned either to the clodronate or to the control group.

Tissues were isolated at indicated time points. We used 3–6 mice for each analysis. Mice used for histology and for flow cytometry are from different experiments. All mice were maintained in a pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care approved, veterinarian-staffed vivarium, and all study protocols and procedures were approved by the University of California Davis Institutional Animal Care and Use Committee.

2.2. Clodronate injections

Clophosome® - Clodronate Liposomes (Neutral) (FormuMax, Sunnyvale, CA) (7 mg/ml) were allowed to reach room temperature, mixed thoroughly and injected intravenously (i.v.) in mice that displayed a clinical score of at least 1. The first dose administered was 1.4 mg of clodronate in a total volume of 200 μl per mouse. The following doses administered were 0.7 mg of clodronate in a total volume of 100 μl per mouse. All injections were administered every 2–3 days as shown in Fig. 1.

2.3. Isolation of mononuclear cells from the CNS

Mice euthanized by CO_2 asphyxiation were perfused with ice cold phosphate buffered saline (PBS). Brains and spinal cords were minced, digested at 37 °C for 30 min in PBS containing 0.04 units of Liberase R1 (Roche) and 10 μg of DNase I (Roche) per ml. Softened fragments were pushed through a 100 μm mesh. CNS infiltrating cells were isolated via a discontinuous 40/70% (v/v) Percoll gradient (GE Healthcare), as previously described (Lee et al., 2012; Soulika et al., 2009).

2.4. Flow cytometry

CNS mononuclear cells were immunostained after incubation at 37 °C for 3 h in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin-streptomycin, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate (Invitrogen) in the presence of brefeldin A (GolgiPlug, BD Biosciences). Immediately before immunostaining, Fc receptors were blocked for 10 min with anti-CD16/32 (BD Biosciences). MDCs were identified by brilliant violet (BV)-650 labeled anti-mouse CD11b (BioLegend) and phycoerythrin-cyanine7 (PE-Cy7) or allophycocyan (APC)-labeled anti-mouse CD45 (BD Biosciences). Classically activated M1 MDCs were identified by PE-labeled anti-mouse Ly6C (BioLegend), and APC-Cy7-labeled anti-mouse MHCII (BioLegend). Alternatively activated M2 MDCs were identified by APC-labeled anti-mouse Arginase-1 (R&D Systems), and biotinylated-labeled anti-mouse Ym1 (R&D Systems). For T helper cell subset analysis, cells were stimulated with 50 ng/ml PMA and 750 ng/ml Ionomycin in the presence of brefeldin A and stained with Pacific Blue (PB)-labeled anti-mouse CD4 (BD Biosciences), fixed, permeabilized using the Cytofix/Cytoperm Plus Kit (BD Bioscience) according to the manufacturer's protocol, and intracellularly stained with APC-labeled anti-mouse IFNγ (BD Biosciences), and PE-labeled anti-mouse IL17 (BD Biosciences) (Mills Ko et al., 2014; Moreno et al., 2014). Gating was determined based on isotype controls.

2.5. Immunohistochemistry

Mice were anesthetized by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (16 mg/kg) and intracardially perfused with