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Calcitonin gene-related peptide decreases IL-1beta, IL-6 as well as Ym1, Arg1, CD163 expression in a brain tissue context-dependent manner while ameliorating experimental autoimmune encephalomyelitis

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ABSTRACT

Activation states of immune cells (among them, the so-called pro- or anti-inflammatory states) influence the pathogenesis of multiple sclerosis (MS). The neuropeptide calcitonin gene-related peptide (CGRP) can exert a pro- or anti-inflammatory role in a context-dependent manner. In mice CGRP was found to attenuate the development of experimental autoimmune encephalomyelitis (EAE, a common MS animal model). We analyzed CGRP effects on the expression of cytokines and markers of activation states, as well as its intracellular cascade, following intrathecal administration during EAE immunization. Real Time quantitative-PCR (RT-PCR) showed that IL-1beta and IL-6 (associated to a pro-inflammatory state in EAE), but also Ym1 (also known as Chil3), Arg1 and CD163 (associated to an anti-inflammatory state in EAE) were decreased in the encephalon (devoid of cerebellum). In the cerebellum itself, IL-1beta and Ym1 were decreased. TNF-alpha (associated to a pro-inflammatory state in EAE), but also IL-10 (associated to another type of anti-inflammatory state) and BDNF were unchanged in these two regions. No changes were detected in the spinal cord. Additional tendencies toward a change (as revealed by RT-PCR) were again decreases: IL-10 in the encephalon and Arg1 in the spinal cord. CGRP decreased percentage of $Ym1^{+}/CD68^{+}$ immunoreactive cells and cell density of infiltrates in the cervical spinal cord pia mater. Instead, Ym1 in the underlying parenchyma and at thoracic and lumbar levels, as well as Arg1, were unchanged. In cultured microglia the neuropeptide decreased Ym1, but not Arg1, immunoreactivity. Inducible NOS (iNOS) was unchanged in spinal cord microglia and astrocytes. The neuropeptide increased the activation of ERK1/2 in the astrocytes of the spinal cord and in culture, but did not influence the activation of ERK1/2 or p38 in the spinal cord microglia. Finally, in areas adjacent to infiltration sites CGRP-treated microglia showed a larger ramification radius.

In conclusion, CGRP-induced EAE amelioration was associated to a concomitant, context-dependent decrease in the expression of markers belonging to both pro- or anti-inflammatory activation states of immune cells. It can be hypothesized that CGRP-induced EAE attenuation is obtained through a novel mechanism that promotes down-regulation of immune cell activation that facilitates the establishment of a beneficial environment in EAE provided possibly also by other factors.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory demyelinating disease of the CNS that causes severe oligodendrocyte, axonal and general brain tissue damages, often ending in a progressive neurological disability (Lublin et al., 2014). The inflammatory CNS infiltration by immune cells induces the formation of demyelinating lesions whose location is primarily in the white matter, but it is now widely accepted that early-stage MS patients show cortical demyelination at higher prevalence than previously thought (Lucchinetti et al.,

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2011) and cortical-lesion volume is an independent predictor of the progression of disability (Calabrese et al., 2009).

MS and EAE are often referred to as T cell-mediated diseases, but myeloid cells are also very abundant in the inflammatory demyelinating foci of MS (Lucchinetti et al., 2000) and innate immune cells are critical for disease development and progression (Tran et al., 1998; Marik et al., 2007; Mildner et al., 2009). Moreover, monocytes/macrophages provide an EAE boost which is associated with the expression of markers, as iNOS, characterizing a pro-inflammatory (previously referred to as M1) macrophage activation state (Tran et al., 1998; Marik et al., 2007; Mildner et al., 2009). The balance between myeloid cell pro- and anti-inflammatory activation states is emerging as predictive of disease severity in EAE, as it can promote either persistence of inflammation with progressive neurodegeneration or initiation of remyelination and tissue regeneration (Miron et al., 2013; Yamasaki et al., 2014; Pinto et al., 2014). In EAE an imbalance toward the iNOS$^+$ macrophage population is associated to a higher risk of relapses, whereas an equilibrium between the iNOS$^+$ and the arginase 1$^+$(Arg1) macrophage populations (characterizing a reparative/protective, previously referred to as M2, activation) predicts a milder disease (Mikita et al., 2011). Disease severity is directly correlated, in particular, to the number of macrophages/microglia cells in the CNS parenchyma and the ratio of inflammatory vs reparative/immunomodulatory cells (identified by iNOS and Arg1 immunoreactivity, respectively), suggesting that an inhibition of the reparative/immunomodulatory cell profile occurs during relapses. Moreover, disease severity was associated to a constantly high iNOS expression in circulating monocytes and treatment with interleukin (IL)-10/IL-13-stimulated monocytes (isolated from healthy animals) increased Arg1 expression in CNS macrophages/microglia and suppressed EAE (Mikita et al., 2011). Finally, stimulation of the IL-4 pathway (that activates an anti-inflammatory state in microglia/macrophages; Colton, 2009) inhibits progression of ongoing relapsing-remitting EAE (Casella et al., 2016) and increases remyelination and axonal protection in lysolecithin-induced focal demyelination and EAE (Psachoulia et al., 2016), likely favoring the physiological switch from inflammatory-like to reparative/immunomodulatory-like macrophages. Furthermore, attenuation of inflammation and/or decreased disease severity is associated with switch to Arg1$^+$ macrophages (Ahn et al., 2012) and CD163 increase (Gerzanich et al., 2017; Stojić-Vukanić et al., 2018), but also IL-10 (Wang et al., 2017) and BDNF (Smith et al., 2018) increases.

Thus, a switch of macrophage activation from inflammatory-like toward reparative/immunomodulatory-like profile may attenuate the pro-inflammatory response, lead to a milder disease and promote CNS regeneration, including remyelination, and an imbalance between the pro-inflammatory and the regenerative macrophage response may contribute to the failure of remyelination in MS (Lloyd and Miron, 2016).

Also microglia are both a main source and target of cytokines and chemokines in the CNS and intervene in the complex intercellular communication occurring during MS and EAE (Muzio et al., 2007). Basal levels of cytokines and chemokines can be detected in the brain at steady state, but their expression is greatly increased during neuroinflammation. The complex signaling occurring during MS and EAE may influence involved immune cells (including microglia) in a paracrine or autocrine manner creating negative or positive feedback loops. A pro-inflammatory loop can be exerted by cytokines such as tumor necrosis factor TNF-alpha, interferon (IFN)γ, IL-1beta, IL-6 that, produced by various cell types, can activate microglia (and other immune cells) to increase production and release of these and other pro-inflammatory cytokines ultimately worsening EAE signs (Merson et al., 2010).

It is worth mentioning that activated microglia can release chemokines (e.g., CCL2/MCP-1, CCL3/MIP-1a, CCL4, CCL5, CXCL10 and CCL12) that are required for myeloid and T cells attraction under neuroinflammatory conditions (Herder et al., 2014). Among them CCL2(MCP-1)/CCR2 axis exerts a crucial role for the recruitment of inflammatory Ly6Chi monocytes (Izikson et al., 2000; Mildner et al., 2009). On the other hand, a series of different factors (e.g., IL-4, IL-10, IL-13 and transforming growth factor TGF-beta) can induce a distinct type of activated microglia that acquires anti-inflammatory, immunosuppressive, and neuroprotective properties (Jiang et al., 2014).

Microglia activation can be easily monitored by following its well characterized changes in morphological appearance, but even though the analysis of microglia morphology can be exquisitely sensitive, it must be taken into account that the activation-induced morphological changes cannot discriminate among the different types of activations (Boche et al., 2013).

Beside cytokines (and chemokines) the neuropeptide calcitonin gene-related peptide (CGRP) is among the factors which are involved in the complex signaling occurring during EAE: CGRP and CGRP receptor component protein (RCP, a cytoplasmic component of CGRP receptor) were expressed at higher levels during relapses, EAE severity was increased in CGRP-alpha knockout mice, and intrathecal CGRP delivery during immunization slowed EAE development (Sardi et al., 2014). The EAE amelioration, obtained by the intrathecal delivery of CGRP, was accompanied by the inhibition of the morphological activation of microglia (Sardi et al., 2014), and the peptide inhibited LPS-induced microglia release/production of TNF-alpha, IL-6, NO/iNOS, CCL2/MCP1, CCL3/MIP-1alpha (Consonni et al., 2011).

It is worth mentioning that CGRP receptors have been described in all sets of brain-resident cells (astrocytes, microglia, oligodendrocytes and neurons; Morara et al., 1998; Sardi et al., 2014 and references therein). In addition to microglia, CGRP was shown to modulate cultured astrocytes by increasing cAMP, calcium and cFos (see Morara et al., 2008; D'Antoni et al., 2010; and references therein) and transiently changing their morphology (D'Antoni et al., 2010). Moreover, CGRP receptors have been described in all sets of immune cell types. The peptide action on immune cells is multifaceted and can regulate both the adaptive and innate immune systems by influencing proliferation, differentiation and various aspects of the activity of T/B lymphocytes, dendritic cells, macrophages and neutrophils (Assas et al., 2014 for a review). In particular, CGRP decreases HLA-DR, CD86 in macrophages, dendritic cells and monocytes (where it increases also IL-10) (Torii et al., 1997; Carucci et al., 2000; Bracci-Laudiero et al., 2005), and induces a Th2 polarized T cell response, as it does via Langerhans cells by increasing IL-4, CCL17, CCL22 and decreasing IFN-gamma, CXCL9, CXCL10 (Ding et al., 2008), or in naive Th cells down-regulating IL-2, IFN-gamma and up-regulating IL-4 (Tokoyoda et al., 2004). However, its action is context-dependent as, for example, the peptide enhanced IFN-gamma secretion in Leishmania major susceptible splenocytes (Ahmed et al., 1999).

The present analysis was aimed at describing whether CGRP can affect EAE by modulating the expression of molecules that have been involved in microglia and other immune cell activation and how it may exert its action.

2. Materials and methods

All efforts were made to minimize animal suffering and to reduce the number of mice to be used in accordance with the European Communities Council Directive 2010/63/EU, which has been approved by the Italian Parliament (D.Lgs. 26/2014). All the present experiments were carried out following the approval of the Italian Ministry of Health. The mice were purchased from Charles River Italia (Calco, LC). Upon arrival the mice were placed in individual, sterilized cages with unlimited access to sterilized water and food (vitamin/nutrient-enriched diet: code 4RF25 purchased from Mucedola, Milano, Italy). In each cage a sterilized paper towel was inserted to allow the mice to create a nest by shredding the towel. Cages were kept in a conventional and periodically disinfected room, whose access was restricted to the research personnel involved in EAE experiments who carried hair cover, gloves, animal facility gown, surgical mask and shoe covers. If,