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Toll-like receptor 3 differently modulates inflammation in progressive or benign multiple sclerosis

Marina Saresella[a], Andrea Gatti[a], Paola Tortorella[a], Ivana Marventano[a], Federica Piancone[a], Francesca La Rosa[a], Domenico Caputo[a], Marco Rovaris[a], Mara Biasin[b], Mario Clerici[a,c,*]

[a] Don C. Gnocchi Foundation, Pzza Morandi, 3, 20121 Milano, Italy
[b] Department of Clinical Sciences, University of Milano, 20100 Milano, Italy
[c] Department of Physiopathology and Transplants, University of Milano, 20100 Milano, Italy

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Abstract TLR-dependent signal transduction pathways were analyzed in patients with a diagnosis of either relapsing–remitting (RRMS), secondary progressive (PMS) or benign (BMS) MS and healthy controls (HC). Prototypical TLR molecules expressed either on the cell surface (TLR4) or intracellularly (TLR3) were stimulated with specific antigens (LPS and poly I:C, respectively). Expression of factors involved in TLR signaling cascades, production of downstream immune mediators and TLR expression were evaluated. Results showed that, whereas LPS-stimulation of TLR4 had a marginal effect on cell activation, poly I:C-stimulated TLR3 expression on immune cells was significantly increased in PMS and BMS compared to HC. This was associated with a higher responsiveness to poly I:C that resulted in the activation of the TLR3-mediated pathway and the production of inflammatory cytokines in PMS and, in contrast, in the up-regulation of a peculiar mosaic of inflammation-dampening genes in BMS. Results herein might explain different MS disease phenotypes.

1. Introduction

Multiple sclerosis (MS) is an inflammatory neurodegenerative disorder of the central nervous system (CNS) of unknown etiology [1–5]. The most common clinical phenotype of MS, the relapsing–remitting (RR) form, is characterized by an acute onset of neurological symptoms followed by complete or partial recovery. The long-term prognosis of RRMS is usually unfavorable since most patients enter the so-called secondary progressive (P) phase of the disease and accumulate irreversible neurological disability. A different disease pattern is benign MS (BMS). In this case, absent or minimal neurological

* Corresponding author at: Don C. Gnocchi Foundation ONLUS, Pzza Morandi, 3, 20100, Milano, Italy. Fax: +39 02 50330414.
E-mail address: mario.clerici@unimi.it (M. Clerici).

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impairment is present years after the onset manifestations. Notably, whereas RRMS appears to be largely driven by inflammatory processes, neurodegeneration is believed to play an important role in the chronic brain and spinal cord injury in patients with PMS.

Toll like receptors (TLRs) molecules are expressed on the cell surface or intracellularly on endosomes, and recognize patterns characteristic of bacteria, fungi and viruses known as pathogen-associated molecular patterns (PAMPs); the interaction between TLRs and PAMPs triggers innate and adaptive immune responses [6–10]. In addition to playing a key role in host defense against pathogens, TLRs activation has been linked to the pathogenesis of inflammatory and autoimmune diseases [11,12] possibly including MS [13–18]. In this case, data obtained in EAE, the animal model of MS, indicate that TLR4 knockout mice have greater disease severity, which is associated with increased Th17 function [19], whereas TLR3 stimulation is protective in the same model due to the induction of IFNβ [20]. These data led to the suggestion that TLRs whose activation results in the induction of IFNβ might have a protective role against EAE, whereas TLR stimulation that leads to pro-inflammatory cytokine expression adds to disease severity [21–23]. TLR agonists are nevertheless present in the adjuvant preparations used to induce EAE in mice; it is therefore difficult to extrapolate how data from these studies would relate to a possible role for the TLR/PAMP system in MS [15].

This caveat notwithstanding, it is becoming increasingly clear that TLRs are involved in the elicitation of immune responses even in the CNS. In particular, recent data showed that TLR signaling takes part in the regulation of the antigen-specific adaptive immune response during the pathogenesis of MS [24] and that TLR3 and TLR4 possibly play a role in modulating MS as well as EAE [19,14,25].

In the attempt to shed light on this issue, trying to dissect possible roles for extra- and intra-cellular TLRs in MS, we compared TLR3- and TLR4-mediated activation of immune cells and signal transduction pathways in MS patients with different disease phenotypes. Results herein indicate that TLR3 activation plays a dichotomous role in MS: supporting inflammation in patients with progressive disease, but reinforcing molecular anti-inflammatory mechanisms in those with benign disease.

2. Materials and methods

2.1. Patients and controls

Forty-eight MS patients were studied. Sixteen patients were affected by RRMS in a clinical phase of stability (RRMS) as confirmed by brain and spinal cord MRI with gadolinium (MRI-G) showing no areas of enhancement at the time of enrolment. Sixteen patients had a diagnosis of PMS on the basis of the clinical history and of MRI-G evidencing a stability of the lesion load at the time of enrolment. Finally, 16 patients were affected by BMS as confirmed by both clinical history and MRI-G showing stability or an improvement of the lesion load at the time of enrollment. No patient had received immunosuppressive drugs in the year prior to the study period; informed consent was given according to a protocol approved by the ethics committee of the Don Gnocchi Foundation. Forty-two sex- and age-matched healthy controls (HC) were also analyzed. Epidemiological and clinical characterization of patients and controls is presented in Table 1.

2.2. Cell separation and Toll-like receptors stimulation

Peripheral blood mononuclear cell (PBMC) were separated on lympholyte separation medium (Cedarlane, Hornby, Ontario, CA) and resuspended in RPMI 1640 containing 10% human serum and 2 mmol/L glutamine and penicillin/streptomycin. PBMC (1×10^6/ml) were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 6 h (mRNA analyses); 24 h (for TLR FACS analyses); or 3 days (protein analyses in supernatants by Multiplex Immunoassay or ELISA). Cells were unstimulated (medium alone), or stimulated with 10 μg/ml polyinosic: polycytidylic acid (Poly I:C), or 2 μg/ml lipopolysaccaride (LPS). Supernatants were used for cytokine detection whereas PBMC were washed in PBS and spun for 10 min at 1500 RPM. The resulting PBMC pellet was used for RNA extraction or resuspended for flow-cytometry analyses.

2.3. Flow cytometry immunofluorescent staining

PBMC were stained with anti-CD14-PC7 (clone RMO52, isotype mouse IgG_{2a}, Beckman Coulter) and anti-TLR4-PE (clone 610015, isotype mouse IgG_1, R&D Systems) mAbs. Cells were then treated with FIX and PERM Cell kit (eBioscience, San Diego, CA), and stained with TLR3 FITC (clone 11439, isotype mouse IgG_1, LifeSpan Biosciences Inc., Seattle, WA, USA) mAb. Analyses were performed using a Beckman-Coulter Cytomics FC-500 flow cytometer equipped with a single 15 mW argon ion laser operating at 488 nm and interfaced with CXP Software 2.1. Two hundred thousand cells were acquired and gated on CD14 expression and side scatter properties. Isotype control or single fluorochrome-stained preparations were used for color compensation.

2.4. Measurement of cytokines in supernatants

IFNβ, IL12 and CXCL10 concentration was determined by multiplex sandwich immunoassays (Fluorokine Multi Analyte Profiling Kit) (R&D Systems); that of IFNα and TNFα by ELISA (Quantikine Immunoassay; R&D Systems).

2.5. RNA extraction and reverse transcription

RNA was extracted and reverse transcribed into first-strand cDNA as previously described [26]. All the samples were evaluated for GAPDH expression by real-time PCR to test the quality of RNA. TLR signaling pathways were analyzed in a PCR array on 96-well plates following the procedures suggested by the manufacturer (SA Biosciences, Frederick, MD;). This approach allows the monitoring of mRNA expression of 84 genes related to the TLR pathway activation, plus five housekeeping genes. Results were expressed as ΔΔCt (where Ct is the cycle threshold) and presented as ratios between the target gene and the GAPDH housekeeping mRNA. Controls were included on each array for genomic DNA contamination,