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Inflammatory response of endothelial cells to a human endogenous retrovirus associated with multiple sclerosis is mediated by TLR4

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Abstract

The MSR (multiple sclerosis-associated retrovirus) belongs to the human endogenous retrovirus HERV-W family. The envelope protein originating from the MSR has been found in most patients with multiple sclerosis (MS). This protein (Env-ms) has pro-inflammatory properties for several types of immune cells and could therefore play a role in MS pathogenesis by promoting the leukocyte diapedesis observed in the central nervous system of patients. Our study aims to analyze the effects of Env-ms on the blood–brain barrier (BBB) at a molecular and functional level. We demonstrate that the recombinant MSR envelope is able to stimulate several inflammatory parameters in a human BBB in vitro model, the HCMEC/D3 brain endothelial cell line. Indeed, Env-ms induces over-expression of ICAM-1, a major mediator of leukocyte adhesion to endothelial cells, in a dose-dependent manner as well as a strong dose-dependent production of the pro-inflammatory cytokines IL-6 and IL-8. Furthermore, using a silencing approach with siRNAs, we show that Env-ms is recognized via the Toll-like receptor 4 receptor, a pattern recognition receptor of innate immunity present on endothelial cells. We also show, using functional assays, that treatment of brain endothelial cells with Env-ms significantly stimulated the adhesion and the transmigration of activated immune cells through a monolayer of endothelial cells. These findings support the hypothesis that MSR could be involved in the pathogenesis of MS disease or at least in maintenance of inflammatory conditions, thus fueling the auto-immune disorder. MSR could also play a role in other chronic inflammatory diseases.

Keywords: auto-immune disease, blood–brain barrier, leukocyte adhesion, retroviral envelope

Introduction

Multiple sclerosis-associated retrovirus (MSRV) is an enveloped retrovirus initially isolated from cell cultures from patients with multiple sclerosis (MS) (1). It is the first identified member of the human endogenous retrovirus W (HERV-W) family (2) which has infected the germline and then has been transmitted to the offspring of their hosts in the course of primate evolution (3). Most of the integrated HERV-W display deletions or mutations in their open reading frames encoding functional proteins (4), thus preventing a complete expression. However, genetic elements of endogenous HERV are involved in genetic rearrangements (5) that can contribute to their genetic diversification and their occasional expression (3).
The recombinant Env-ms and Env-syn proteins were obtained as previously described (8). Monoclonal antibodies GN-mAb01 and GN-mAb03, detecting both Env-ms and Env-syn proteins and GN-mAb12, detecting gag protein, were obtained from GeNeuro (Geneva, Switzerland).

**Cells and culture conditions**

HCMEC/D3 cells were obtained from P. O. Couraud (Institut Cochin, Paris, France) and cultured as previously described (19). Briefly, cells were seeded onto collagen type 1 (Sigma-Aldrich, St Louis, MO, USA) coated flasks in supplemented EBM-2 medium (Lonza ‘bullet kit’, Basel, Switzerland) containing 2.5% fetal bovine serum (FBS) and growth factors, bFGF, VEGF, IGF, EGF at a final concentration 4x lower than recommended by the furnisher. Cells were obtained at passage 26 and cultured up to passage 36.

Primary HUVECs were cultured in M199 medium containing 20% heat-inactivated FBS, ECGS (Endothelial Cells Growth Supplement, 50 µg ml⁻¹), heparin (100 µg ml⁻¹) and antibiotics. Cells were cultured up to passage 5 onto collagen type 1 (Sigma) coated flasks.

HL-60 cells, a human promyelocytic leukemia cell line, was cultured in RPMI 1640 containing 10% FBS.

**Cell stimulation**

HCMEC/D3 or HUVECs were seeded onto collagen type 1 coated 24-well plates until reaching confluency. Media were then replaced with media containing recombinant proteins or stimulating cytokines for 16h. All conditions were tested in triplicate. After stimulation, cells were trypsinized and analyzed by flow cytometry for the expression of ICAM-1 and lyzed by flow cytometry for the production of cytokines (IL-6, IL-8 and TNF-α) detection kits purchased from PromoKine/Promcell, Heidelberg, Germany).

**Adhesion and transmigration assays**

For adhesion assays, HL-60 cells were cultured at a concentration of 2.10^5 cells ml⁻¹ and activated with vitamin D3 (18.7 µg ml⁻¹) and indomethacin (10⁻⁷ M; Sigma-Aldrich) for 72h. HL-60 cells were detached with a scraper and labeled with calcine AM (Invitrogen Molecular Probes, Carlsbad, CA, USA) just before the experiment. Then 1.10^4 cells were left to adhere for 35min onto a confluent HCMEC/D3 monolayer representing a surface of ~3.8 cm². After three washing steps with PBS, the remaining adherent cells were lysed with 1% SDS. For each condition, cell lysate is plotted in triplicate on a Costar, Cambridge, MA, USA) for 3–4 days at 37°C and stained with TNF-α (100 U ml⁻¹) or 2 µg ml⁻¹ of Env-ms for 16h prior to the assay. For inhibition experiments, Env-ms (2 µg ml⁻¹) was pre-incubated with monoclonal antibodies specific for Env-ms or gag protein (30 µg ml⁻¹, 30min 4°C). Activated HL-60 cells (10⁵ cells per well) were added to the upper compartment. A concentration of 2 x 10⁻⁶ M fMLP (Calbiochem-Novabiochem) was added in the lower compartment, to create a chemotactic gradient for peripheral mono nuclear cells. After 1h at 37°C, migrated HL-60 cells were recovered from the
bottom well, centrifuged and quantified using a CyQUANT assay kit (Molecular Probes, Eugene, OR, USA).

**Immunostaining for flow cytometry**

After trypsinization, living cells were incubated for 1 h at 4°C with anti-ICAM-1 primary antibody (clone 2D5 produced locally as previously described) (20). After centrifugation, pellets were re-suspended in 50 µl of RPMI 1640 containing the secondary antibody coupled with FITC (goat anti-mouse-FITC purchased from Jackson ImmunoResearch, Philadelphia, PA, USA) and left to incubate for 30 min at 4°C. After centrifugation, cells were re-suspended in 500 µl of RPMI 1640 + 10% FBS and fluorescence was measured with a FACSCAN flow cytometer (Becton Dickinson, Le Pont-De-Claix, France). Results were analyzed using WinMDI software.

**Immunostaining for fluorescence microscopy**

Cells were seeded onto LabTek chambers (Nunc, Rochester, NY, USA) until confluence and were then stimulated for 16 h in the chambers. Cells were then washed twice with pre-warmed (37°C) PBS containing Ca ++ and Mg ++ and then fixed for 30 min at room temperature with PBS Ca-Mg containing 2% PFA and 2% sucrose. After fixation, we performed two quenching steps with PBS containing glycine 0.1 M and NaCl 0.01% for 10 min and a permeabilization step using PBS containing 0.2% saponin, 2% BSA, for 20 min at room temperature. Staining was performed using the anti-ICAM-1 primary antibody clone 2D5 with a secondary goat anti-mouse antibody coupled to TRITC (Jackson ImmunoResearch); anti-TLR4 (Santa Cruz, Santa Cruz, CA, USA) with a secondary goat anti-rabbit antibody coupled to Alexa 488; or the anti-golgin 97 (Invitrogen) with a secondary goat anti-mouse antibody coupled with Alexa 555. Nuclei were labeled with Hoechst 33342. Cells were observed with an epi-fluorescence microscope (Carl Zeiss AxioImager) with the pseudo-confocal module APOTOME. Images were analyzed with ImageJ software (ImageJ, NIH, USA). For co-localization experiments, the Pearson’s correlation coefficient ($R_P$) and the Mander’s overlap coefficient ($R_M$) were calculated using the ImageJ co-localization plugin after background subtraction.

**Western blotting**

After reaching confluence, HCMEC/D3 cells were trypsinized and lysed for 30 min on ice with a Tris–HCl 50 mM, pH 7.5, NaCl 150 mM buffer containing 1% NP40 or 0.1% SDS (as mentioned in each case) and a protease inhibitor cocktail (Sigma-Aldrich). Whole cell extracts were submitted to SDS–PAGE (10% acrylamide gels), blotted onto polyvinylidene difluoride membranes and stained with a rabbit polyclonal primary antibody raised against TLR4 (Santa Cruz) or actin (Sigma-Aldrich) and a secondary goat anti-rabbit antibody coupled with peroxidase (Invitrogen). Bands were quantified with BioRad Image Lab software.

**Treatment with peptide N-glycosidase**

Before analysis by western blotting, some extracts were treated by peptide N-glycosidase (PNGase) using a deglycosylation kit (Sigma, GlycoProfile II, Enzymatic In-solution N-Deglycosylation kit). Extracts were treated according to the manufacturer’s recommendations but omitting the denaturation step before adding the PNGase to avoid precipitation of the proteins. Briefly, 50 µg of extracts were treated with 2.5 units of PNGase or water as a negative control for 2 h at 37°C, then immediately analyzed by western blotting.

**siRNA transfections**

siRNA targeting TLR4 were transfected according to the manufacturer’s recommendations (Invitrogen). Briefly, we transfected the cells with a set of three siRNAs targeting TLR4 or with a negative control at a final concentration of 20 nM (Stealth RNAi all provided by Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) when cells reached 30% confluency. Cells were re-transfected 48 h later under the same conditions. Forty-eight hours later, the transfected and control cells were treated with Env-ms or LPS and then analyzed.

**Statistics**

All results are expressed as mean ± SE. Data were analyzed using R statistical software; two-sided Student $t$-test was used. Differences between conditions were considered significant at $P < 0.05$.

**Results**

**Effects of Env-ms on HCMEC/D3 brain endothelial cells**

First, we analyzed the effect of Env-ms on confluent monolayers of HCMEC/D3 cells. We observed by fluorescence microscopy an increase in expression of ICAM-1 adhesion molecules (Fig. 1A). In order to quantify ICAM-1 expression, cells were analyzed by flow cytometry for surface expression of ICAM-1 after incubation with TNF-α, which is known to activate endothelial cells, or with Env-ms; both treatments induced a strong ICAM-1 expression (Fig. 1B). The induction of ICAM-1 expression was specific for Env-ms, since treatment with Env-syn had no significant effect and was dose dependent on the Env-ms concentration (Fig. 1C). To further confirm the specificity of Env-ms pro-inflammatory properties, we pre-incubated Env-ms with monocular antibodies recognizing either Env-ms, or the gag protein of HERV as a negative control, before treating HCMEC/D3 cells. After overnight incubation with the treated Env-ms, ICAM-1 surface expression by HCME/D3 cells was measured by flow cytometry as detailed above. Anti-Env-ms antibodies blocked ~40% of Env-ms-induced ICAM-1 expression as compared to the irrelevant anti-gag antibodies (Fig. 1D). We performed a complementary control to ensure that there is no LPS contamination in the recombinant envelope protein. For that purpose, we used polymyxin B (PB) antibiotic that can neutralize LPS activity. LPS or Env-ms were pretreated or not with PB and then incubated overnight with HCMEC/D3 cells. The pre-treatment with PB almost completely abolished the LPS-induced ICAM-1 increased expression but had no effect on Env-ms-induced ICAM-1 expression, indicating that Env-ms pro-inflammatory activity was not caused by LPS contamination (Fig. 1E).
We investigated the effects of Env-ms at a functional level by measuring the adhesion between activated immune cells and a monolayer of HCMEC/D3 cells. For that purpose, we used activated calcein-labeled HL-60 cells adhering to the HCMEC/D3 monolayer. After several washing steps and lysis of all remaining adherent cells, we quantitated adherence of HL-60 cells by fluorescence readings. We observed an increase of adherent HL-60 cells when HCMEC/D3 cells had previously been treated with Env-ms (2 µg ml⁻¹) (Fig. 1F). This increase was even stronger than the positive control with LPS treatment, but this was not statistically significant. These results are consistent with our observations (Fig. 1A and B) of increased expression of ICAM-1 following Env-ms treatment, since ICAM-1 is a major mediator of the adhesion between activated immune cells and endothelial cells [(20, 21), see (22) for review].

Adhesion of leukocytes to the endothelium can be followed by leukocyte transendothelial migration. As shown in Fig. 1 (G), Env-ms was able to stimulate transendothelial migration of HL-60 cells; this increased transmigration was inhibited (by 80%) when Env-ms was pre-incubated with a specific monoclonal antibody (GN-mAb03), while an antibody specific for Gag protein was devoid of any inhibitory effect.

Recombinant Env-ms induced a strong and dose-dependent production of IL-6 (Fig. 2A) and IL-8 (Fig. 2B) but did not induce significant production of TNF-α (data not shown). The induction of these pro-inflammatory cytokines is specific of Env-ms since Env-syn had no effect.

Taken together, these results indicate that Env-ms exerts a specific pro-inflammatory activity on brain endothelial cells.

**Effects of Env-ms on primary endothelial cells**

The effects of Env-ms were further studied on primary HUVEC. Recombinant Env-ms induced an increased expression of ICAM-1 (Fig. 3A) similar to that observed previously with HCMEC/D3 cells. Furthermore, HUVECs responded to Env-ms by a strong dose-dependent production of IL-8 (Fig. 3C). In contrast, no significant production of IL-6 was obtained after incubation of HUVECs with either Env-ms or TNF-α (Fig. 3B). However, primary HUVECs are able to...
produce IL-6 in response to LPS stimulation (Fig. 3B). Taken together, these results indicate that Env-ms also induces a pro-inflammatory response of HUVECs but with a slightly different cytokine profile than that of HCMEC/D3 cells.

Implication of TLR4 in Env-ms recognition by endothelial cells

TLR4 is expressed by HCMEC/D3. It has been previously shown that Env-ms interacts with the receptor of innate immunity TLR4/CD14, on monocytes and dendritic cells (8); therefore, we investigated whether this pathway may be used by endothelial cells to respond to Env-ms. TLR4 expression was analyzed in permeabilized HCMEC/D3 cells by fluorescent microscopy which showed that TLR4 mostly co-localized with the Golgi apparatus (Fig. 4A) as has been previously described in other cell types (23, 24). Analysis by western blotting demonstrated that in both HCMEC/D3 and HUVECs, TLR4 was expressed as a 150kDa protein, with a minor band at 120kDa (Fig. 4B). It has been reported in the literature that the heavy form is the fully processed, glycosylated and active form which can be expressed at the cell membrane, while the light form remains intracellular (25). These observations suggest that a fully glycosylated TLR4 form could be expressed by the HCMEC/D3 cells. Treatment of the whole cell extracts with PNGase resulted in the disappearance of the heavy form in favor of the light form (Fig. 4C), showing that the heavy form is glycosylated.

TLR4 knock down abolishes HCMEC/D3 response to Env-ms. Subsequently, in order to verify that TLR4 is the receptor implicated in Env-ms recognition, we transfected HCMEC/D3 cells with TLR4-targeting siRNAs and assessed the efficiency of TLR4 knock down by western blotting (Fig. 4D). After quantification with ImageJ, we measured a 44% decrease of TLR4 expression after two rounds of transfection with TLR4-targeting siRNA when compared to control siRNA-transfected cells and a 59% decrease compared to untransfected cells.

We then analyzed the effect of reduced TLR4 expression on the induction of ICAM-1 expression and on the production of pro-inflammatory cytokines by LPS, a well-known
TLR4 ligand, or by Env-ms (Fig. 5). The results showed that ICAM-1 expression, induced by either LPS or Env-ms, was significantly reduced (by at least half) in knocked down cells compared to the cells transfected with a control siRNA (Fig. 5A). The specificity of TLR4 silencing by these siRNAs is shown in Fig. 5(B), which shows that TNF-α is still able to induce the expression of ICAM-1 after transfection with these siRNAs. Furthermore, the production of IL-6 and IL-8 cytokines induced by either LPS or Env-ms was dramatically decreased (by ~90%) after TLR4 knock down as compared to control transfected cells (Fig. 5C and D). Taken together, these results support the hypothesis that TLR4 is implicated in Env-ms recognition by HCMEC/D3 cells.

Discussion

MS is a chronic neurological disease, whose etiology remains poorly defined. However, it is well established that inflammatory process in the brain yields to the destruction of oligodendrocytes which build myelin sheaths around axons in the brain and spinal cord (26). Under inflammatory conditions, vascular endothelial cells respond by the secretion of factors and the increase of adhesion molecules that lead to an enhancement of leukocyte adhesiveness and to their migration into tissues (27). The recruitment of activated leukocytes across BBB endothelial cells is a critical step in triggering inflammation and CNS tissue injury in the course of MS (18). Therefore, the initiation of the inflammatory process, along with its outcome on the neuroimmunological context, remains a central point of interest in the understanding of MS.

The discovery of the retroviral element MSRV, isolated from the cerebrospinal fluid of a patient with MS (1), has opened new issues about a putative microbial contribution in MS physiopathology, reviewed in (6). Several lines of evidence indicate that MSRV could participate to the activation of some immune cells. MSRV and its envelope protein, Env-ms, trigger T-cell receptor V\textsuperscript{β} (28) and TLR4 (8), similarly to superantigens such as those encoded by mouse mammary tumor virus (29, 30).

Since Env-ms could activate innate immunity through the TLR4/CD14 pathway, we further sought pro-inflammatory activity on endothelial cells which are known to express TLR and respond to TLR signals (27, 31). Indeed, we demonstrate that Env-ms is able to activate the vascular endothelium including the brain endothelium in a manner consistent with the fact that MS is an inflammatory disease of the CNS. Regarding the activation pathways, our results show that the inhibition of Env-ms induced pro-inflammatory effects is incomplete after TLR4 knock down. This most probably arises from the fact that TLR4 expression is not completely abolished after transfection with siRNAs as observed on our western blotting experiment (Fig. 5D). Nevertheless, we cannot exclude the implication of TLR4 co-receptor CD14 (32).

One interesting observation of our study concerns the different effects of Env-ms in endothelial cells of different origins. The envelope protein seems to exert a slightly higher pro-inflammatory activity on HCMEC/D3 cells that originate

![Fig. 4. TLR4 expression in endothelial cells. (A) TLR4 co-localizes with the Golgi apparatus. Fluorescent staining of Golgi apparatus (red), TLR4 (green) and nuclei (blue) on HCMEC/D3 cells (not confluent) after fixation with PFA 2% and permeabilization with saponin 0.2%. Negative control is obtained after staining with the secondary antibodies only. Pearson’s correlation coefficient $R = 0.67$ and Mander’s overlap coefficient $R = 0.75$. Scale bar: 20 µm. (B) TLR4 is expressed on HCMEC/D3 and on HUVECs. Cells were lysed with the Tris–HCl 50 mM, pH 7.5, NaCl 150 mM buffer containing 1% NP40. The presence of TLR4 in the whole extracts is then analyzed by western blotting. (C) TLR4 heavy form (150 kDa) leads to a light form (120 kDa) after treatment with PNGase. Whole extracts, prepared with a lysis buffer containing only NP40, were treated with PNGase, or water as a negative control, for 2h and then analyzed by western blotting. (D) TLR4 expression on HCMEC/D3 cells after double transfection with siRNAs targeting TLR4 or control siRNAs; cells were lysed with the Tris–HCl 50 mM, pH 7.5, NaCl 150 mM buffer containing 1% NP40. The presence of TLR4 in the whole extracts was then analyzed by western blotting and quantified with ImageJ software. NT: not transfected.
from the brain than on HUVECs that originate from umbilical vein. Indeed, Env-ms triggers the over-expression of ICAM-1 and the production of IL-6 and IL-8 by HCMEC/D3 while only ICAM-1 expression and IL-8 production are strongly stimulated on HUVECs. This is not due to differences in the levels of TLR4 expression between HCMEC and HUVECs since we verified by western blotting that TLR4 was expressed at a similar level in both cell types (Fig. 4B). Concerning the cytokine production, HUVECs are known to produce a much lower amount of IL-6 compared to IL-8 in response to LPS and their response to TNF-α is controversial and depends on culture conditions as already discussed by Makó et al. (35). That could explain the absence of IL-6 production after treatment with Env-ms and TNF-α. However, in our conditions, HUVECs are able to produce significant amounts of IL-6 in response to LPS but not in response to Env-ms (Fig. 3C). These variations observed between LPS and Env-ms effects on HUVECs could be explained by a lower affinity of the latter with TLR4 possibly due to structural differences. Finally, in spite of these variations, we show that Env-ms exerts its pro-inflammatory activity on both types of vascular endothelial cells.

The presence of Env-ms protein was detected in the peripheral blood of most of MS patients as a soluble protein in the serum and as a membrane-associated protein at the surface of circulating monocytes and to a lesser extend on B and NK cells (14). Histology of brain tissues of some postmortem MS patients revealed that perivascular lesions contained a large number of macrophages and microglial cells which expressed Env protein as well as HLA-DR (11). Whatever the initial origin of Env-ms, either at the periphery or in the CNS, its presence in both compartments may contribute establishing conditions for neuro-inflammatory damage. This is further strengthened by the fact that human oligodendrocyte differentiation is impaired after exposure to Env-ms leading to the reduction in expression of myelin protein (36). It is striking to note that again in this latter case, Env-ms triggers TLR4 pathway in oligodendroglial precursor cells which respond by the production of pro-inflammatory cytokines and reactive oxygen species. Interestingly, TLR4 is expressed by other cells contributing to the BBB formation. Firstly, astrocytes have TLR4 but their capacity to respond to its ligands has been a subject of debates. Recently, it has been shown that astrocyte response to LPS stimulation requires CD14 expression (37). Secondly, pericytes, which surround endothelial cells to build capillaries, possess a functional TLR4 that responds to LPS stimulation by the production of pro-inflammatory cytokines and up-regulation of ICAM-1 (38). Although the reactivity of pericytes and astrocytes to an Env-ms signal remains to be firmly established, one may consider that the convergence in the BBB of cells expressing TLR4 may favor the particular

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**Fig. 5.** Effects of Env-ms are greatly impaired after knock down of TLR4 receptor. (A) siRNA-transfected HCMEC/D3 cells were stimulated overnight with LPS or Env-ms. ICAM-1 expression was measured by flow cytometry. Cells were either not transfected with siRNA (NT) and not stimulated with LPS or Env-ms (untreated), or transfected with control siRNA (CT siRNA) or TLR4 siRNA and stimulated with Env-ms (2 µg ml⁻¹) or LPS (0.1 µg ml⁻¹). Results represent the mean ± SE of three independent experiments each performed in triplicate. (B) Same as in (A): untransfected cells (NT) or cells transfected with TLR4 siRNA were stimulated with TNFα (100 U ml⁻¹, 18 h). (C, D) HCMEC/D3 cells were transfected and then stimulated overnight with LPS or Env-ms. Production of IL-6 and IL-8 was measured by ELISA assays. Results represent the mean ± SE of two independent experiments each performed in triplicate. NT: not transfected, CT: control. *P ≤ 0.01; **P ≤ 0.001.
Endothelial cell activation by Env-ms/TLR4 pathway

**Fig. 6.** Schematic representation of the effects of Env-ms. DC = dendritic cell; EC = endothelial cell; ENV = envelope protein of HERV-W MSRV; MΦ = macrophage; ODC = oligodendrocyte cell; p38 = microglial cell; TL = T lymphocyte.

sensitivity of the CNS to the exposure of TLR4 ligands inducing synergic paracrine signals.

Altogether, these data highlight the importance of the Env-ms/TLR4 pathway in the demyelination and in the inflammation associated with MS (Fig. 6). In the blood, Env-ms can activate on the one hand circulating monocytes, dendritic cells and macrophages and on the other hand vascular endothelial cells leading to the production of pro-inflammatory cytokines, the enhancement of the adhesion of circulating cells to endothelium and then their migration through the BBB into the brain. Env-ms in the brain provokes activation of macrophages, microglial and dendritic cells which orient the T1 response of T lymphocytes. In parallel, Env-ms interferes with oligodendrocyte cells in activating their precursors to produce pro-inflammatory cytokines and in reducing their capacity to build myelin sheaths. Simultaneous inflammatory conditions together with a myelinization defect may favor the rise of myelin-specific T1 T lymphocytes leading to auto-immunity. Obviously, interfering with the Env-ms/TLR4 pathway represents a promising therapeutic approach which is currently progressing by the means of specific anti-Env-ms neutralizing antibodies (39).

In conclusion, our study brings new evidence about the pro-inflammatory properties of MSRV envelope protein, defines the mechanism by which Env-ms stimulates endothelial cells and reinforces the proposal that MSRV may play a key role in MS pathogenesis and probably in other neurodegenerative disorders, such as schizophrenia (13, 40).

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Conflict of interest statement: P.N.M. and H.P. are authors of the patent ‘Composition for Treating Pathology Associated With MSRV/HERV-W’ (WO2005080437) for the development in clinics of therapeutic antibody to treat Multiple Sclerosis: ongoing clinical trial (NCT01639300) by GeNeuro (H.P.).

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