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(54) Title: NOVEL DISEASE TREATMENTS

(57) Abstract: Aberrant infection of B cells by EBV leads to autoimmune disease, especially multiple sclerosis, myasthenia gravis and Hashimoto's thyroiditis, and can be treated or immunised against.



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NOVEL DISEASE TREATMENTS

The present invention relates to treatments for chronic inflammatory diseases characterised by autoimmune features.

The cause(s) of multiple sclerosis (MS), the most common chronic inflammatory disease of the central nervous system (CNS), remain unknown but likely involve genetic and environmental factors^{1,2}. Among the latter, Epstein-Barr virus (EBV) is the virus most consistently associated with MS, as indicated by a higher prevalence and titre of anti-EBV antibodies³⁻⁷ and enhanced EBV-specific cellular immune responses^{8,9} in MS patients compared to controls, and by molecular mimicry between certain EBV and CNS components¹⁰. However, the mechanisms linking EBV infection, which is latent and harmless in most individuals, to MS-associated immune dysfunction remain unknown. EBV latently infects B cells, promotes their proliferation and activation, and, in doing so, provides constant antigenic challenge to the immune system¹¹. A variety of abnormalities in humoral immunity are associated with MS, including: intrathecal synthesis of immunoglobulins (predominantly oligoclonal IgG)¹²; presence of clonally related B cells^{13,14} and unusual B-cell subsets (germinal centre centroblasts and centrocytes)¹⁵ in demyelinated lesions and cerebrospinal fluid (CSF); and, in a subset of early-onset MS patients, formation of intrameningeal lymphoid-like structures resembling B-cell follicles^{16,17}.

Surprisingly, it has now been found that aberrant infection by Epstein-Barr virus (EBV) of B cells present in the brain is a primary cause of autoimmune disease, particularly multiple sclerosis, in humans.

Thus, in a first aspect, the present invention provides the use of at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent, in the manufacture of a medicament for the treatment of an autoimmune disease.

Preferably, the use includes only an anti-EBV substance. Alternatively, it is also preferred that the use includes only a B cell cidal agent. However, most preferably the use includes both an anti-EBV substance and a B cell cidal agent.

Autoimmune diseases may include, *inter alia*, systemic lupus erythematosus, Hashimoto's thyroiditis, Grave's disease, Sjögren's syndrome, multiple sclerosis, rheumatoid arthritis, myasthenia gravis and inflammatory myopathies (dermatomyositis, inclusion body myositis, and polymyositis). In all, at a recent count, there were more than eighty types of autoimmune disease.

Hashimoto's thyroiditis is a disease that commonly causes the condition known as hypothyroidism. Thus, the present invention is also useful in treating said condition.

In a further aspect, the invention also provides at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent for the treatment of an autoimmune disease. Also provided is a pharmaceutical composition comprising at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent, for the treatment of an autoimmune disease. The invention also provides a method for treating an autoimmune disease, comprising administering, to a patient in need thereof, a therapeutically effective amount of at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent.

It is apparent that EBV establishes a low level, persistent, infected state in B cells. In this state, little or no active virus production is seen, and B cells are actively encouraged to replicate, with concomitant suppression of normal apoptotic regulation. This type of infection is referred to herein as latent infection. Infected plasma cells, which arise from infected B cells, are the cell type in which viral reactivation occurs and may optionally be further targeted by the medicaments of the present invention.

In susceptible individuals, levels of latent infection may be high, or may alternate on an occasional basis with lytic infection, where whole virus is expressed by infected cells.

The latent infection of B cells stimulates a cytotoxic T cell response directed at the infected B cells. It is apparent that brain damage is caused predominantly by the antiviral immune response. In multiple sclerosis, the B cells form ectopic follicles in the central nervous system (CNS), especially in the meninges, and these lymphoid-like structures represent the main intracerebral foci of viral reactivation. Without being bound by theory, it appears that the meningeal localisation of B cells leads to extended cortical lesions by causing the clustering of cytotoxic T cells. The effect may be enhanced by the apparent dysregulation, as described above, of EBV infection in susceptible individuals. Around 90% of the population is EBV-seropositive, but the virus is generally efficiently controlled. Any deviation from this delicate host-virus balance is considered to be 'dysregulation', whether it is caused by EBV, by other viruses or by genetic factors.

Where the autoimmune disease is associated with regions of the body not restricted to the CNS, then it is preferred, in one embodiment, not to employ a B cell cidal agent in a systemic medicament, and to just use one or more substances (a). Thus, for the treatment of systemic lupus erythematosus or rheumatoid arthritis, for example, it is preferred to use one or more anti-EBV substances, and no B cell cidal agents.

However, in another embodiment, it is preferred to use a B cell cidal agent, for instance a monoclonal antibody, such as rituximab, for treating both lupus and rheumatoid arthritis. This has been shown to work well. Indeed, in 2008, the results of a phase II trial of rituximab in multiple sclerosis were published (Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, Langer-Gould A, Smith CH; HERMES Trial Group. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis *N Engl J Med*. 2008 Feb 14;358(7):676-88)), again showing a good response to the drug, with a rapid decrease in disease activity and a long-lasting effect.

Without being bound by theory, it is believed that B cell cidal agents may help reduce the pool of infected B cells in the body and lower the EBV-specific immune response which mediates brain damage. Plasma cell cidal agents may also be employed in addition to or separately from B cell cidal agents, although it is generally preferred to target B cells, as these are the progenitors of the plasma cells. Cidal agents as defined herein may be thought of as being capable of reducing or leading to a reduction in the number of B-cells, including those infected with EBV, or rendering said cells inactive.

Anti-EBV substances include drugs, antibodies and siRNA, for example. Suitable anti-EBV drugs include nucleotide and nucleoside analogues such as, *inter alia*, acyclovir, valacyclovir, and ganciclovir, and Type-1 interferons, such as IFN- α and IFN- β . Of these, it is generally preferred to use at least valacyclovir, and it may be advantageous to use valacyclovir in combination with at least one another anti-EBV substance, preferably at least one of those mentioned above. Other anti-EBV substances will be apparent to the skilled person.

Antibodies against EBV may be against any suitable target. Clinical trials have been performed on vaccines comprising the gp350/220 surface glycoprotein, as well as the HLA B8 restricted epitope from EBNA-3A. When used in treatment, as opposed to prophylaxis, of an autoimmune disease, then antibodies specific for latent markers may be employed, these markers including Epstein Barr Nuclear Antigen 2 and latent membrane protein 1 (LMP1). Where a vaccine against EBV is to be administered to prevent EBV infection and subsequent autoimmune disease, then it is generally preferable to target antigens associated with active infection, such as BFRF1 and gp220/350.

siRNA (short interfering RNA) may be employed that targets any part of the EBV genome necessary for EBV replication, such as the EBNA1 and EBNA2 genes. Particularly preferred targets are EBNA1 sequences such as 5'-ggaggttccaacccgaaat-3' (SEQ ID NO: 23) and 5'-

ggactaccgacgaaggaac-3' (SEQ ID NO: 24), as disclosed in Virology 2006; 346:385, and those derived from LMP1, such as 5'-ggaatttgacggacaggc-3' (SEQ ID NO: 25), as disclosed in Cancer Letters 2006; 232:189. Other suitable targets will be readily apparent to those skilled in the art. Based on these target, SEQ ID NOs: 26-28 (the RNAi sequences directed at SEQ ID NOs: 23-25 respectively), or variants or fragments capable of hybridising to SEQ ID NOs: 23-25 under highly stringent conditions such as 6 x SSC, are useful for siRNA.

The anti-EBV substances may be administered systemically, or directly to the CNS, either by injection into the ventricles or intrathecally. Administration direct to the CNS is referred to herein as intrathecal administration, although intracerebroventricular administration is also contemplated by the present invention. Administration to the CNS is particularly preferred for treatment of MS. For treatment of other conditions, such as thyroiditis or arthritis, then administration may be to the area affected, for instance the thyroid or neck, or the affected joint. Delivery may be intravenous or by transdermal, transmucosal by oral means, although suitable encapsulation or formulation may be required in respect of oral delivery.

In conditions where intrathecal administration is indicated, such as multiple sclerosis, for example, then it is desirable to employ at least one B cell cidal agent in the medicament. Any physiologically acceptable B cell cidal agent may be used, as appropriate. B cell cidal agents are apparent to those skilled in the art, and include monoclonal antibodies targeting B-cell surface proteins, such as the chimaeric monoclonal antibody known as Rituximab developed by IDEC Pharmaceuticals, to activate antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity and or/apoptosis. As Rituximab does not target plasma cells, other plasma cell cidal agents, such as a depleting plasma cell-specific antibody or the anti-CD138 plasma-cell-specific immunotoxin (IT, B-B4-SO6), may also be used to specifically eliminate EBV-infected plasma cells.

It is preferred that both the anti-EBV substance or the B cell cidal agent are administered, preferably contemporaneously. However, it is also preferred that only one of these two is administered.

Either the anti-EBV substance or the B cell cidal agent alone may be effective in controlling, suppressing, and even eliminating B cell infections, but it is particularly preferable to employ at least one of each where possible, especially intrathecally. A particularly preferred medicament employs both Rituximab and valacyclovir. Another preferred combination is Rituximab and acyclovir.

Where the medicament comprises more than one active ingredient, these may be administered together or separately. Where a disease has both CNS and systemic components, then, where a B cell cidal agent is employed, this is advantageously restricted to intrathecal administration, while an anti-EBV substance may be administered both systemically and intrathecally, if desired.

Medicaments of the present invention may also comprise, additionally to, or alternatively to, one of the active ingredients, a T cell inhibitory, blocking, suppressing, or cidal substance when administered intrathecally. Such a substance is preferably targeted at CD8+ T cells. The substance is preferably a specific antibody. Type II topoisomerase inhibitors are effective as CD8+ T cell cidal agents, and include etoposide, which induces apoptosis of EBV-infected lymphocytes.

It is particularly preferred that the medicament of the present invention be directed to multiple sclerosis, and it is preferred that any such medicament be suitable for intrathecal administration.

The components of the disease in human brain are; the latent EBV infection, the infected B cells, the periodic reactivation of EBV, and the bystander tissue damage caused by cytotoxic T cells in response to the latently and lytically infected B cells. Any one of these aspects is targetable by the medicaments of the present invention. Acyclovir and valacyclovir are particularly preferred, especially when it is desired to only target the lytic infection and not the latent infection.

As mentioned above, the present invention further extends to a vaccine for the prevention of multiple sclerosis, or any other autoimmune disease, comprising at least one epitope characteristic of EBV. Suitable vaccines are well known to those skilled in the art, and it is preferred to administer such a vaccine in early childhood (or during the first year after birth), before the individual becomes EBV-seropositive.

The present invention will now be further illustrated with regard to the accompanying, non-limiting Examples section. All references cited herein are hereby incorporated by reference, unless otherwise apparent.

EXAMPLE 1

Using *in situ* hybridisation, we examined human *post-mortem* brain tissue for the presence of EBV-encoded small nuclear mRNAs (EBERs) that are expressed during the latent phase of EBV infection. Nuclear EBER signals were detected in the meninges and immunologically active white matter lesions, mostly in perivascular positions, in 19 of 20 MS cases analysed, but not in controls and patients with other neuroinflammatory diseases (**Table 1**). The highest frequency of EBER⁺ cells was observed in a subset of 8 MS patients (hereafter termed EBV-high) who died during

secondary progressive disease. These cases had been previously extensively characterised for the presence of abundant intralesional and intrameningeal mononuclear cell infiltrates and ectopic B-cell follicles displaying germinal centre-like features^{16,17} (**Fig.1a-f**). In this subset, all ectopic follicles (n=15) and the acute (n=4) and chronic active (n=16) white matter lesions analysed were enriched in EBER⁺ cells (**Fig.1g-l**).

Among the MS cases with less CNS inflammation and a low frequency of EBV-infected cells (EBV-low cases) (**Fig.1m-o** and **Fig. 7**), only one had two small EBER⁺ ectopic follicles in the meninges despite negligible parenchymal inflammation. Occasional perivascular EBER⁺ cells were also found in areas of normal-appearing white matter that contained clusters of activated, MHC class II⁺ microglia (pre-active lesions) (**Fig.1p-r**). No EBER signals were detected in inactive lesions, indicating a relationship between inflammatory activity and viral gene expression. By combining *in situ* hybridisation for EBER with immunohistochemistry for the pan B-cell marker CD20, 70-90% of EBER⁺ cells were identified as B-cells, whereas 40-90% of B-cells were EBER⁺, depending on the MS case analysed (**Fig.1i,k**). The highest percentage of infected B-cells (about 90%) was detected in the meninges and ectopic follicles of EBV-high MS cases. A substantial proportion of CD138⁺ plasma cells (50-80%) was also EBER⁺ (**Fig.1i**, inset), while no EBER⁺ cells with the morphological features of neurons, glial, meningeal or endothelial cells were detected. The identification of EBV-infected cells as predominantly B-cells was confirmed by the existence of a positive correlation between the number of EBER⁺ and CD20⁺ cells in the brain of all MS cases analysed (**Fig.1s**).

We further analysed the status of EBV infection in MS brains using immunohistochemical techniques with antibodies against latent and lytic viral proteins. As markers of viral latency, we studied Epstein-Barr nuclear antigen 2 (EBNA2) and latent membrane protein 1 (LMP1)¹⁸. EBNA2 is the first viral protein to be expressed in EBV-infected B-cells *in vitro* and acts as a transactivator of viral and cellular genes inducing B-cell proliferation (growth program). LMP1 is an integral membrane protein that promotes B-cell proliferation and survival and is expressed during the growth program and the more restricted viral transcription program, named default program¹⁸. LMP1⁺ and EBNA2⁺ cells were detected in the immune infiltrates of 15 and 14 of the 20 MS cases analysed, respectively (**Table 1**), the highest frequency being observed in EBV-high MS cases (**Fig.2a-f**). Ectopic follicles contained numerous LMP1⁺ (**Fig.2d**), but no EBNA2⁺ cells (not shown), reminiscent of what has been observed in tonsillar germinal centre and memory B-cells¹¹. Expression of EBV latent proteins provides a mechanism for B-cell expansion and activation in the MS brain and is consistent with the presence of B-cells expressing the proliferation marker Ki67

and the anti-apoptotic molecule bcl2 in intrameningeal follicles (**Fig.1b,f**) and acute lesions (**Fig.3c**). LMP1 may also induce expression of the enzyme activation-induced cytidine deaminase¹⁹ (**Fig.1d**), which is normally restricted to germinal centre B-cells. These findings raise the possibility that germinal centre-like features in the MS meninges might be induced by EBV, independently of antigenic stimulation and T-cell help. In turn, according to a current model of EBV infection in healthy carriers¹¹, EBV could exploit the B-cell maturation process occurring in ectopic follicles to establish a persistent infection in the MS brain.

As markers of the lytic program, we studied BFRF1 which, along with BFLF2, is expressed during early viral replication²⁰, and gp350/220, the most abundant glycoprotein of the viral envelope¹⁸. BFRF1⁺ cells were present inside and around all intrameningeal follicles and acute lesions analysed in EBV-high MS cases (**Fig.2g-m**). A few BFRF1⁺ cells were also detected in the small ectopic follicles identified in one EBV-low MS case (not shown). Double immunostainings showed that BFRF1 immunoreactivity was present in a substantial proportion of B-cells and plasma cells (30-55%) but was much stronger in plasma cells than in B-cells (**Fig.2 h-m**), in agreement with findings in healthy carriers where terminal differentiation into plasma cells initiates the viral replicative cycle²¹. Rare gp350/220⁺ cells were found only in the parenchymal lesions of one EBV-high MS case (**Fig.2o**), indicating occasional production of infectious virus, probably due to abortive replication or efficient immune surveillance.

Because cytotoxic CD8⁺ T-cells have a key role in controlling latent EBV infection and in preventing viral replication²², we predicted that the presence of EBV-infected cells in the MS brain would associate with CD8⁺ T-cell infiltration. Indeed, intracerebral accumulation of CD8⁺ T-cells was much more prominent in EBV-high than in EBV-low MS cases (**Fig. 7**). Participation of CD8⁺ T-cells in the clearance of EBV-infected cells was supported by the findings that these cells did infiltrate all sites where infected B-cells/plasma cells were located and that the frequency of CD8⁺ T-cells in the meninges and white matter strikingly correlated with that of EBER⁺ cells (**Fig.3a,b,k**). Notably, CD8⁺ T-cells expressing the proliferation marker Ki-67 were detected in some ectopic follicles and all acute lesions, consistent with recent antigen stimulation likely related to viral reactivation (**Fig.3c**). At these sites, many CD8⁺ T-cells established close anatomical contacts with B-cells, plasma cells and BFRF1⁺ cells, and about 3% of them expressed perforin, a key component of lytic granules, indicating recognition and attack of EBV-infected B-cells/plasma cells (**Fig.3d-g**). Even in the EBV-low MS cases, some of the scattered CD8⁺ T-cells were found in direct contact with B-cells (**Fig.3h**). Prominent macrophage activation was also associated with intracerebral EBER⁺ and CD8⁺ T-cell accumulation (not shown). The presence of a large number

of EBV-infected cells and cytotoxic T-cells in the brain of the MS cases with ectopic follicles is compatible with the severe clinical and neuropathological features of this patient subset¹⁷. Consistent with the idea that an antiviral immune response occurs in the MS brain, blood dendritic cell antigen 2 (BDCA2)⁺ plasmacytoid dendritic cells, which are the main source of type-I interferon and have a key role in antiviral immunity, were detected in many MS immune infiltrates, predominantly in meningeal follicles and acute lesions of EBV-high MS cases (**Fig. 3 i,j**).

We also investigated whether frequency of EBV-infected cells and status of EBV infection in MS brains correlated with viral load and oligoclonal IgG bands (OCBs) in the corresponding post-mortem CSF. EBV DNA was generally undetectable, being found at low copy number in the CSF of only 2 of 16 MS cases (2 primary progressive MS with 1310 and 4900 copies/ml, respectively). None of the other viruses investigated [herpes simplex viruses type 1 and 2 (HSV-1/2), varicella-zoster virus (VZV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6) and JC virus (JCV)] were detected in the 16 CSF samples analysed.

Oligoclonal bands (OCBs), which are generally CSF-restricted and relatively constant throughout the disease¹², were found in the CSF of 15 of 16 MS cases. Interestingly, the number of CSF OCBs was significantly higher in EBV-high MS cases (**Fig.4a**), further indicating a relationship between EBV infection and intracerebral B-cell activation. Consistent with previous findings^{8,23}, EBV-specific OCBs were detected in 7 of 16 MS cases (44%), but showed no preferential distribution in the EBV-high and EBV-low MS groups (**Fig.4b**). Except for one case, EBV-specific OCBs, when present, were few (median number = 2; range 1-5) and generally faint (**Fig.4c**), indicating that persistent EBV infection in the MS brain does not yield a robust, virus-specific oligoclonal humoral response which is typical of CNS infections associated with viral replication²⁴.

Although the prevailing hypothesis is that MS is caused by an autoimmune response toward CNS antigens¹, conclusive evidence supporting this idea is lacking. Without being bound by theory, we have demonstrated that EBV infection is dysregulated in the MS brain, thereby providing an explanation for altered humoral and cellular immune responses to EBV in MS patients³⁻⁹. Our findings implicate EBV in intrathecal Ig synthesis, the MS hallmark, through infection and activation of CNS-infiltrating B-cells, and in recurrent CNS inflammation through induction of an antiviral immune response. Perturbed EBV infection also provides an explanation for the dominance and persistence of CD8⁺ T-cell clones in the MS brain^{25,26}, and implies that bystander tissue damage mediated by cytotoxic T-cells is a major determinant of MS lesions,

similar to what happens in other EBV-associated diseases²². Stimulation of autoreactive B-cell clones by EBV is not excluded and may explain the variable pattern of antibody reactivities toward CNS-restricted and ubiquitous self-antigens observed in MS patients²⁷. This also extends to other chronic diseases with autoimmune features, such as systemic lupus erythematosus and rheumatoid arthritis, in which an association with EBV is indicated by epidemiological, serological and DNA studies²⁸.

METHODS

Post-mortem brain tissue specimens and CSF

This study was performed on post-mortem brain tissue obtained from 20 MS patients with different disease courses, 6 patients affected by other inflammatory neurological diseases, 2 control patients without neurological disease and 1 patient with Alzheimer's disease. Patient details are given in **Table 1**. Brain tissue specimens were provided by the UK Multiple Sclerosis Tissue Bank at Imperial College London (confirmation of MS diagnosis provided by Drs F. Roncaroli and R. Nicholas), the Department of Neurosciences, Ophthalmology and Genetics, University of Genova, Italy, and the Institute of Pathological Anatomy, U.C.S.C. Policlinico A. Gemelli, Rome, Italy. The *post-mortem* delay ranged between 7 and 27 h (median time, 18.5 h). The MS cases analysed had not received immunosuppressive therapies, except for MS114 (treated with ACTH for 12 months), MS80 (treated with ACTH for 11 years), MS5 (treated with mitoxantrone for 3 years), and MS153 (treated with ACTH for 30 months). Control tissue specimens, lymphoma and normal human lymph node were provided by the Institute of Pathological Anatomy, A. Gemelli Hospital, and the Pathology Department of S. Andrea Hospital, Rome.

For the MS cases, 32 tissue blocks (2 x 2 cm) from the cerebral hemispheres were analysed and classified by histopathological methods, i.e. hematoxylin/eosin staining, and the combined Luxol fast blue-periodic acid-Schiff reaction, or by immunohistochemistry using anti-myelin oligodendrocyte glycoprotein antibody, to identify areas of inflammation and demyelination^{15,16}. Immunohistochemical stainings for T and B lymphocytes, macrophages, and MHC class II molecule expression (**Table 2**) were performed to evaluate the degree of lesion activity (pre-active, acute, chronic active, and chronic inactive), as previously described^{15,16}.

Post-mortem CSF samples were also available from some of the MS cases (n = 16) obtained from the UK MS Tissue Bank and were used for OCB determination and viral DNA analysis. The set of CSF samples used in this study was rather homogeneous (mean albumin concentration = 31.7

mg/dL, with a standard error of 2.1 and a 95% CI of 4.9; mean post-mortem delay of CSF collection = 17.1 hours, with a standard error of 1.2 and a 95% CI of 2.6).

EBER – *In situ* hybridisation

In situ hybridisation experiments were carried out using the Epstein-Barr virus (EBER) PNA Probe/Fluorescein and the PNA ISH detection kit (both from Dako), according to the manufacturer's instructions. The kit also included negative and positive control fluorescein-conjugated PNA probes. Treatment with proteinase-k was performed on frozen, PFA fixed-frozen and paraffin-embedded sections at the following dilutions in TBS: 1:500 for 10 min, 1:100 and 1:10 for 20 min, respectively. An EBV-associated B-cell lymphoma (paraffin-embedded sections) was used as positive control tissue for EBER signals. To perform double stainings for EBER and CD20 or CD138, after the *in situ* hybridisation procedure sections were subjected to antigen retrieval in citrate buffer pH6.0 and then incubated overnight with anti-CD20 or anti-CD138 monoclonal antibody (mAb), extensively washed, incubated with a biotinylated rabbit anti-mouse secondary Ab (Jackson Laboratories) for 1 h, washed and treated with avidin-biotin alkaline phosphatase complex (Standard ABC-AP, Vector Laboratories) for 45 min. The reaction was visualised with fast red (Sigma, St. Louis, MO, USA). Sections were sealed in aqueous medium (Ultramount, Dako) and viewed and photographed with an Axiophot Zeiss microscope equipped with an Axiocam digital camera, using the Axiovision 4 AC software.

Immunohistochemistry

Deparaffinised 5- μ m-thick and air dried, acetone fixed 10- μ m-thick cryosections were rehydrated with PBS and immunostained with the Abs listed in **Table 2**. For single immunostainings, post-fixed sections were subjected to the antigen retrieval procedure and incubated for 20 min with 0.1% H₂O₂ in PBS to eliminate endogenous peroxidase activity, then for 1 h with 10% of normal sera (all from Jackson Immunoresearch Laboratories, Cambridgeshire, UK), and overnight at 4°C with the primary Abs diluted in PBS containing 1% BSA. For EBNA2 and cleaved-caspase 3 immunostainings, 0,05 % Triton X 100 and 0,1% Tween 20 were added to the medium, respectively. The binding of biotinylated secondary Abs (**Table 2**) was visualised with avidin-biotin horseradish peroxidase complex technique (ABC Vectastain Elite kit; Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (DAB) (Sigma) containing 0.01% H₂O₂ as substrate. All sections were counterstained with hematoxylin, sealed with Canadian Balsam viewed and photographed as described above. Negative controls included the use of IgG isotype controls or preimmune serum, or omission of the primary antibody.

Indirect immunofluorescence and confocal analysis

After an initial blockade with 10% normal goat or donkey serum in PBS, sections were incubated overnight at 4°C with the primary Abs indicated in **Table 2**, either alone or in combination, as indicated in the legend. Sections were then incubated for 1 hour at room temperature with fluorochrome-conjugated second Abs (**Table 2**) and sealed in Vectashield (Vector Laboratories). For negative controls, primary Abs were replaced with preimmune serum and IgG isotype controls. Images were analysed and acquired with a laser scanning confocal microscope (LSM 5 Pascal, Carl Zeiss).

Quantitative analysis

The number of EBER⁺, CD20⁺ and CD8⁺ cells was counted in 6 adjacent sections per tissue block (two 2 x 2 cm-sections were analysed for each immunohistochemical staining) at 20x magnification by one investigator blinded to the case number (B.S.). One-two blocks per MS case were analysed. Values are expressed as the mean number of positive cells per mm² in the white matter and per mm in the meninges.

IEF immunoblot

The pattern of OCBs in autopsy CSF was investigated with agarose isoelectric focusing (IEF) (pH 3.0-10.0, Cambrex, Rockland, ME, USA) and affinity immunoblotting using a standard protocol²⁹. Although the lack of paired serum samples, which were not available at autopsy, inherently affected the CSF analysis, in MS OCBs are typically restricted to the CSF, without corresponding serum bands¹². IgG and albumin were determined with turbidimetry (Cobas Integra, Roche, Milan, Italy). EBV-specific OCBs were determined using a previously published IEF/affinity-mediated immunoblotting protocol²⁹, using nitrocellulose membranes that had been previously coated with highly pure EBV Antigen, strain P3HR1, and a mouse monoclonal anti-EBV EA-D IgG as a positive control (both from Fitzgerald Industries International, Concorde, MA, USA). The serum of a patient with a monoclonal gammopathy tested on EBV-precoated papers and EBV-specific OCB-positive CSF samples tested on lanes coated with a Blocking Solution containing casein (WesternBreeze, Invitrogen, Carlsbad, CA, USA) only were used for assessing non-specific binding. For each sample, 0.3 µg of IgG were used.

Viral DNA detection

Using TaqMan® technology, CSF samples were tested by real-time PCR for DNA of the following viruses: EBV, HSV-1, HSV-2, VZV, CMV, HHV6-A, HHV6-B, and JCV. PCR

protocols are listed in **Table 3**. In all virus assays, an internal control was used to control for PCR inhibitors in the samples.

Statistical analysis

The Mann Whitney *U*-test was used to compare the median values of EBER⁺, CD20⁺ and CD8⁺ cells counted in adjacent MS brain sections and of CSF OCBs between EBV-low and EBV-high groups of MS cases. Spearman's rank correlation was calculated to analyse the relationship between number of EBER⁺ cells and number of CD20⁺ or CD8⁺ T cells in MS brain sections. A *p* value lower than 0.05 was considered as significant.

Table 1 Demographic and clinical data of MS and control cases and summary of inflammatory degree and EBV marker expression in their brain tissue

Patient/type of MS ^a	Sex/ Age at death (years)	Age at onset	Disease duration	Cause of death	Degree of CNS inflammation ^b / number of ectopic follicles analysed	Frequency of EBER ⁺ cells ^c : meninges/ white matter	Expression of latent EBV antigens ^d	EBNA2 ^e	BFRF1	Gp350/220
MS2/RR	F/28	18	10	Disseminated intravascular coagulopathy	±/0	0 ^f /low	n.p. ^g	n.p.	n.p.	n.p.
MS274 /RR	M/56	24	32	Gut carcinoma	±/0	Low/low	+	+	n.p.	n.p.
MS107/PR	M/38	21	17	Bronchopneumonia	+/0	Low/moderate	+	+	-	-
MS98/PR	M/57	44	13	MS	+/0	Low/moderate	+	+	-	-
MS83/PP	M/53	36	17	Bronchopneumonia	±/0	Low/moderate	+	+	-	-
MS102/PP	M/73	21	52	Bronchopneumonia	+/0	Low/moderate	+	+	-	-
MSG1/PP	F/43	33	10	Cerebrovascular disease	±/0	Low/moderate	-	+	n.p.	-
MS114 /SP	F/52	37	15	Bronchopneumonia	±/0	Low/low	+	+	-	-
MS104/SP	M/53	42	11	Urinary tract infection	±/0	Low/low	-	-	-	-
MS80/SP	F/71	36	35	Heart failure	±/0	0/0 ^h	-	-	-	-
MS5/SP	M/66	unknown	unknown	Bronchopneumonia	±/0	Low/0	+	-	-	-
MS109/SP ⁱ	F/60	35	25	Myocardial infarct	±/2	Moderate/low	-	+	+	-
MS154/SP	F/35	23	12	Bronchopneumonia	++/1	High/high	+	+	+	-
MS92/SP	F/37	20	17	MS	++/5	High/high	+	+	+	-
MS180/SP	F/44	26	18	MS	++/2	High/high	+	+	+	-
MS121/SP	F/49	35	14	Bronchopneumonia	++/2	High/high	+	+	+	-
MS79/SP	F/49	25	24	Bronchopneumonia	++/2	High/high	+	+	+	+

MS153/SP	F/50	18	32	MS	++ / 1	High/ moderate	+	+	+	-
MS85/SP	F/59	24	35	Cerebrovascular disease	++ / 1	High/ moderate	+	+	+	-
MS160/SP	F/44	26	16	Bronchopneumonia	++ / 1	High/ moderate	+	-	+	-
Other inflammatory neurological diseases (OIND)										
OIND1	M/60			Primary cerebral vasculitis	++/0	Absent				
OIND2	F/55			Primary cerebral vasculitis	++/0	Absent				
OIND4	M/60			Viral encephalitis (arbovirus)	+++/0	Absent				
OIND6	F/68			Mycotic meningitis	+++/0	Absent				
OIND7	F/ unknown			Mycotic meningitis	++/0	Absent				
OIND8	M/23			Lymphoblastic leukemia with leukemic blasts in the CNS; CMV IgM ⁺	+++/0	Absent				
Controls										
C1	M/35			Crohn's disease	-/0	Absent				
C16	M/92			Cardiac failure	-/0	Absent				
Alzheimer disease/ NIND2	F/ unknown			Pneumonia <i>ab ingestis</i>	-/0	Absent				

^a RR = relapsing remitting; PR = progressive relapsing; SP = secondary progressive; PP = primary progressive.

^b The degree of CNS inflammation represents an average estimate of the degree of immune cell infiltration in MS meninges and white matter: - = negative; [±] = scarce; ⁺ = moderate; ⁺⁺ = abundant; ⁺⁺⁺ = massive.

^c The frequency of EBER⁺ cells was calculated in the meninges and parenchyma (white matter). Based on the cell counts shown in Fig. 7, the following scores were used: for the cerebral meninges, 0 = no EBER⁺ cells; low = 0,1-5 EBER⁺ cells/mm; moderate = 5-20 EBER⁺ cells/mm high = 20-100 EBER⁺ cells/mm; for the white matter, 0 = no EBER⁺ cells; low = 0,02-0,2 EBER⁺ cells/mm²; moderate = 0,2-1,5 EBER⁺ cells/mm², high = 1,5-5 EBER⁺ cells/mm². In the white matter, EBER⁺ cells were detected in pre-active, acute and chronic active, but not inactive, lesions.

^d ⁺ = present; - : absent

^e EBNA2 immunostaining was detected in cells localised in acute and chronic active white matter lesions and in sparse meningeal infiltrates, but not in ectopic follicles.

^f Poorly preserved meninges.

^g n.p. = not performed due to lack of brain tissue.

^h The only MS case devoid of EBER⁺ cells in this series also lacked B cells/plasma cells and had largely inactive white matter lesions in the two brain tissue blocks analysed.

ⁱ MS109 is at the borderline between EBV-low and EBV-high MS cases. In this case, negligible intraparenchymal inflammation was observed in 8 brain tissue blocks analysed here and in a previous study¹⁷ whereas 2 small B-cell follicles containing a few EBER⁺ and BFRF1⁺ cells and relatively more CD8⁺ T cells were detected in the meninges. In Fig. 7 and Fig. 4 this case was classified among the EBV-low MS cases.

Table 2: List of primary antibodies used in this study

Antigen	Specificity	Type of antibody/clone	Dilution	Source
CD3	T lymphocytes	Mouse monoclonal/PS1	Pre-diluted	Immunotech, Marseille, F
CD20	B lymphocytes	Mouse monoclonal/L26	Pre-diluted	Immunotech
CD8	Tcell subset of cytotoxic/suppressor cells	Mouse monoclonal/4B11	1:30	UCS Diagnostic, Italy
CD8	Tcell subset of cytotoxic/suppressor cells	Rabbit polyclonal	1:30	ABR Affinity BioReagents, CO, USA
CD138 (Syndecan-1)	Plasma cells	Mouse monoclonal/B-B4	1:100	Serotec, Oxford, UK
Ig-A,-G, -M	Plasma cells and plasma blasts	Rabbit polyclonal	1:300	Dako, Carpinteria, CA
CD68	Macrophages	Mouse monoclonal/KP1	1:50	Dako
MHC class II antigens	Antigen presenting cells	Mouse monoclonal/CR3/43	1:50	Dako
Myelin-oligodendrocyte glycoprotein (MOG)	Myelin and oligodendrocytes	Mouse monoclonal/Z12	1:50	Kind gift of S.Piddlesden, Cardiff, UK
Ki67 nuclear antigen	Proliferating cells	Rabbit polyclonal	1:400	Novocastra Labs, Newcastle, UK
CXCL13	Follicular dendritic cells, stromal cells	Goat polyclonal	1:15	R&D Systems, Minneapolis, MN, USA
Activation-induced cytidine deaminase	Germinal centre centroblasts	Rat monoclonal/ EK2 5G9	1:5	Ascenion, Munich, Germany
Bcl2	Bcl2 oncoprotein	Mouse monoclonal/124	1:50	Dako

saved caspase-3	Apoptotic cells	Rabbit polyclonal	1:100	Cell Signaling, Boston, MA, USA
NA2	EBV latent protein	Mouse monoclonal/PE2	1:75	Dako
TP1	EBV latent protein	Mouse monoclonal/CS. 1-4	1:300	Dako
RF1	EBV early lytic protein	Rabbit polyclonal	1:800	Ref. 19
350/220	Protein of the EBV envelope	Mouse monoclonal/72A1	1:200	DSMZ, Braunschweig, Germany
Blood dendritic cell antigen 2 (BDCA2)	Plasmacytoid dendritic cells	Mouse monoclonal/AC144	1:10	Miltenyi Biotec, CA, USA
Perforin	Perforin	Mouse monoclonal/δG9	1:8	BD Biosciences, S. Diego, CA, USA

CNS tissues from 9 patients with SPMS, 2 patients with PPMS, 2 patients with PRMS and 2 control subjects were fixed in 4% paraformaldehyde (PFA) for several days, cryoprotected in sucrose, frozen by immersion in isopentane precooled on a bed of dry ice (-55°C), and stored at -80°C until use. Brain tissue blocks from 1 patient with PPMS, 2 patients with RRMS, 6 patients with OIND and one control with Alzheimer's disease were fixed in buffered formalin and embedded in paraffin wax. Brain tissue blocks from 4 patients with SPMS were snap frozen. For MS79 and MS153 cases both PFA-fixed and snap frozen tissue blocks were analysed. Antigen retrieval procedures utilised microwave of PFA-fixed and paraffin sections in citrate buffer (10 mM, pH 6.0). For CD8 (clone 4B11) immunostaining, heat treatment with EDTA solution (1 mM, pH 9.0) was performed. Sections immunostained for MOG were treated with cold methanol without antigen retrieval procedure. For single immunostainings in bright field, the biotinylated secondary Abs rabbit anti-mouse or goat anti-rabbit IgG (Jackson Laboratories) were used. For immunofluorescence stainings, rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG, and fluorescein-conjugated donkey anti-goat IgG and rhodamine-conjugated donkey anti-mouse IgG Abs diluted in PBS + 5% normal sera (all from Jackson Laboratories) were used as secondary Abs.

The primers sequences provided below are listed as SEQ ID NOs: 1-22 in the sequence listing.

Table 3: Real-time PCR protocols for viral DNA detection

Virus	Genome region	Primers and probe	Primers and probe in 5'-3' orientation (and SEQ ID NO)	Amplicon length (bp)	Detection limit (c/mL)
Epstein-Barr virus (EBV) ³⁰	LMP1	LMP1 F	AAG GTC AAA GAA CAA GGC CAA G (1)	64	100
		LMP1 R	GCA TCG GAG TCG GTG GG (2)		
		LMP1 probe	6-FAM-AGG AGC GTG TCC CCG TGG AGG-TAMRA (3)		
JC virus (JCV)	VP1	VP1-F	GAG TGT TGG GAT CCT GTG TTT TC (4)	77	100
		VP1-R	GAG AAG TGG GAT GAA GAC CTG TTT (5)		
		VP1 probe	6-FAM-TCA TCA CTG GCA AAC ATT TCT TCA TGG C-TAMRA (6)		
Herpes simplex virus-1 (HSV-1)	gD	HSV-1 Q1 F	GGC CTG GCT ATC CGG AGA (7)	63	1000
		HSV-1 Q2 R	GCG CAG AGA CAT CGC GA (8)		
		HSV-1 probe	6-FAM-CAG CAC ACG ACT TGG CGT TCT GTG T-TAMRA (9)		
Herpes simplex virus-2 (HSV-2)	US4-US5	HSV-2 Q1 F	AGA TAT CCT CTT TAT CAT CAG CAC CA (10)	73	1000
		HSV-2 Q2 R	TTG TGC TGG CAA GGC GA (11)		
		HSV-2 probe	6-FAM-CAG ACA AAC GAA CGC CGC CG-TAMRA (12)		
Varicella-zoster virus (VZV)	ORF 4	Orf 4 F	ATG GCG TAC CGA GTC AAT GG (13)	86	100
		Orf 4 R	TAC GGG CCG TGC TAT TGA AG (14)		
		Orf 4 probe	6-FAM-CAC GCT GGC TCC CGC GGT-TAMRA (15)		
Human herpesvirus 6 (HHV-6) A/B	US67	HHV-6 F	ATG CTG CCA GGT ACA AAG AGC (16)	87 (A/B)	100
		HHV-6 R	AAA TGA CAA GYG CAC YGA G (17)		
		HHV-6 probe A	6-FAM-CAG CCA TAT TTC CGG TAT ATG ACC TTC GTA AGC T-TAMRA (18)		
		HHV-6 probe B	6-FAM-CAG CGA TAT TTC CGG TAT ATG ACC TTC GTA AGC T-TAMRA (19)		
Cytomegalovirus (CMV)	US17	US17 F	GCG TGC TTT TTA GCC TCT GCA (20)	151	100
		US17 R	AAA AGT TTG TGC CCC AAC GGT A (21)		
		US 17 probe	6-FAM-TGA TCG GCG TTA TCG CGT TCT TGA TC-3-TAMRA (22)		

FIGURE LEGENDS

Figure 1

Localisation, immunophenotype and frequency of EBV-infected cells in post-mortem MS brains with different degrees of CNS inflammation. (a-i) Immunohistochemical characterisation of ectopic B-cell follicles enriched in EBER⁺ B-cells in the cerebral meninges of a subset of MS patients with prominent CNS inflammation. A representative ectopic follicle located in the depth of a cerebral sulcus displays the morphological features of a germinal centre as it is composed of CD20⁺ B-cells clustered around a network of stromal/follicular dendritic cells expressing the B-cell attracting chemokine CXCL13 (a: double immunofluorescence staining for CD20 in red and CXCL13 in green). The same ectopic follicle also contains numerous proliferating B-cells (b: double labelling for CD20 in green and the proliferation marker Ki67 in red; the inset in b shows 3 double labelled CD20⁺/Ki67⁺ cells at higher magnification) and peripherally located Ig⁺ plasma cells (c). (d-f) A distinct intrameningeal ectopic follicle is shown that contains: (d) numerous cells expressing activation-induced cytidine deaminase, the key enzyme responsible for somatic hypermutation and Ig class-switch recombination in germinal centre B-cells; (e) fewer cells expressing cleaved caspase-3, which is expressed in germinal centre centroblasts undergoing apoptotic cell death; and (f) numerous cells expressing the anti-apoptotic molecule bcl-2, which is expressed in positively selected germinal centre B-cells. (g, h) *In situ* hybridisation for EBER shows enrichment of EBER⁺ cells in 2 follicles from 2 different MS cases. (i) *In situ* hybridisation for EBER (blue-black nuclei) combined with immunostaining for the pan B-cell marker CD20 (red membrane staining) shows that most EBER⁺ cells in a meningeal follicle express CD20. The inset shows a cell double labelled for the plasma cell marker CD138 (red staining) and EBER (blue-black nucleus). Enrichment of EBER⁺ B-cells and plasma cells in MS follicles suggests that these structures are generated by clonal expansion of EBV-infected B-cells. Note that EBER signals in the MS brain are confined to the cell nuclei, similar to what is observed in a control, EBV-associated B-cell lymphoma (j). (k) Presence of EBER⁺ cells in a large, B-cell rich perivascular cuff located in an acute white matter lesion of an EBV-high MS case (double staining for CD20 and EBER, as above); the inset shows a high magnification picture of a CD20⁺/EBER⁺ cell from the same infiltrate. (l)

Perivascular and scattered intraparenchymal EBER⁺ cells in a chronic active white matter lesion of an EBV-high MS case (the inset shows CD20 staining of the same blood vessel in an adjacent section). (m-o) Scattered EBER⁺ cells in the meninges (m) and around scarcely infiltrated blood vessels in chronic active white matter lesions (n, o) of EBV-low MS cases; the insets in m and o show CD20 immunostaining of the same areas in adjacent sections. (p-r) A pre-active lesion characterised by numerous activated MHC class II⁺ microglia (p) and still preserved myelin [inset in p: immunostaining of the same area for myelin-oligodendrocyte glycoprotein (MOG)], contains two small blood vessels, each containing a single perivascular EBER⁺ cell (q and r, respectively). This finding suggests early pathogen recognition by the brain's innate immune system. Bars: 10 μ m in the insets in b, i and k; 20 μ m in b, f, k, q and r; 50 μ m in a, c-e, g-j, l-p and insets in l, m, o and p. . (s) Statistically significant correlation between the number of CD20⁺ and EBER⁺ cells in the white matter (left panel) and meninges (right panel) of EBV-low (n = 12) and EBV-high (n = 8) MS cases. EBER⁺ and CD20⁺ cells were counted as indicated in the legend of Figure 7.

Figure 2

Detection of EBV latent and lytic proteins in the MS brain. (a-d) Expression of the EBV latent proteins EBNA2 (a, b) and LMP1 (c, d) in the brain of EBV-high MS cases. An acute (a) and a chronic (b) white matter lesion containing several EBNA2⁺ cells in a perivascular position are shown. The inset in b shows that EBNA2 staining is confined to the cell nucleus. A sparse meningeal infiltrate (c) and an intrameningeal ectopic B-cell follicle (d) containing LMP1⁺ cells (membrane staining) are shown; the inset in d shows the same follicle stained for EBER. (e, f) Perivascular EBNA2⁺ and LMP1⁺ cells in chronic active lesions from an EBV-low MS case. (g-m) Expression of the EBV early lytic protein BFRF1 in EBV-high MS cases. Numerous BFRF1⁺ cells are present in an intrameningeal ectopic follicle (g). In two different ectopic follicles (h, i), double immunofluorescence staining for CD20 (green) and BFRF1 (red) shows that BFRF1 is expressed in a substantial proportion of CD20⁺ B-cells but its intensity is much stronger in CD20-negative cells (arrows). The insets in g and h highlight the typical perinuclear localisation of BFRF1 immunoreactivity. (j-m) Double immunofluorescence staining for Ig (green) and BFRF1 (red). The lytic cycle-associated protein is strongly expressed in a proportion of Ig⁺ plasma cells in the same

ectopic follicle shown in i (j; the asterisks in i and j mark the Ig-negative B-cells with lower BFRF1 expression in the centre of the follicle). In an acute intraparenchymal lesion, BFRF1 is mainly expressed in Ig⁺ plasma cells located at the periphery of the large perivascular inflammatory cell infiltrate (k). Two double labelled Ig⁺/BFRF1⁺ plasma cells in the same infiltrate are shown at higher magnification (l, m). (n) Absence of BFRF1 immunoreactivity in the B-cell area of a control human lymph node. (o) One productively infected cell expressing gp350/220, a protein of the viral envelope, in the perivascular cuff of a chronic active lesion in an EBV-high MS case. Bars: 5 µm in l, m and insets in g and h; 20 µm in a-j, n, o and inset in b; 50 µm in k and inset in d.

Figure 3

Relationship of CD8⁺ T-cells and plasmacytoid dendritic cells to EBV-infected cells in the MS brain. (a-f) Prominent accumulation of CD8⁺ T-cells in the brain of EBV-high MS cases. Double immunofluorescence staining for CD8 (red) and CD20 (green) shows the presence of numerous CD8⁺ T-cells in an intrameningeal ectopic follicle (a) and an impressive accumulation of CD8⁺ T-cells in a large perivascular cuff of an acute white matter lesion (b) which also contains several BFRF1⁺ plasma cells (see Fig. 2k; double BFRF1/Ig immunostaining was performed in an adjacent section). The same perivascular cuff contains numerous proliferating cells some of which were identified as B-cells (double positive CD20⁺/Ki67⁺ cells in c, arrows) and some as CD8⁺ T-cells (double positive CD8⁺/Ki67⁺ cells in the inset in c). Double immunostainings for CD8 (red) and CD20, CD8 (red) and Ig (green), and CD8 (green) and BFRF1 (red) show that CD8⁺ T cells with an activated, lymphoblastoid morphology cluster around and extend cytoplasmic processes that contact CD20⁺ B-cells (d), Ig⁺ plasma cells (e) and BFRF1⁺ cells (f and insets). (g) The presence of perforin granules (green) in a CD8⁺ T-cell (red) identified in the same BFRF1⁺ perivascular cuff shown in panels b and c and in Fig. 2k confirms the cytotoxic activity of CD8⁺ T-cells. (h) Two scattered CD8⁺ T-cells in the meninges of an EBV-low MS case are shown, of which one contacts a CD20⁺ B-cell. (i, j) Accumulation of BDCA2⁺ plasmacytoid dendritic cells in an ectopic B-cell follicle (i; the inset shows the same follicle stained for CD20), in a sparse meningeal inflammatory cell infiltrate (j) and in a perivascular cuff of a chronic active lesion (inset in j) from 3 different EBV-high MS cases, respectively. (k) Statistically significant correlation between the number of CD8⁺

and EBER⁺ cells in the white matter (left panel) and meninges (right panel) of EBV-low (n = 12) and EBV-high (n = 8) MS cases (see also Figure 7). CD8⁺ and EBER⁺ cells were counted as indicated in the legend of Figure 7. Bars: 10µm in d, e, g, h and insets in f and j; 20µm in f, i, j and inset in c; 50µm in a-c and inset in i.

Figure 4

Analysis of total and EBV-specific oligoclonal IgG bands in post-mortem cerebrospinal fluid from MS cases. The graphs show: (a) significantly higher numbers of oligoclonal IgG bands (OCBs) in the CSF of MS cases with a high frequency of EBV-positive cells (EBV-high; n = 7) versus those with a lower frequency of EBV-positive cells (EBV-low; n = 9) in MS lesions and meninges; and (b) no difference in the number of EBV-specific OCBs between EBV-high and EBV-low MS cases. Dot points represent values for each MS case; the bars represent median values for each group; *p* values, calculated by the Mann-Whitney U-test, are indicated where statistically significant. (c) Affinity-mediated immunoblotting on EBV antigen-coated nitrocellulose paper of isoelectrofocussed CSF from two EBV-low MS cases (lanes 1 and 2, MS154 and MS102, respectively), anti-EBV EA-D monoclonal (mo) antibody used as positive control (lane 3), and serum (ser) from a patient with monoclonal IgG used as control for binding specificity (lane 4). An additional control for binding specificity included CSF from the MS102 case that was blotted onto casein-coated nitrocellulose paper (absence of reactivity) (lane 5). Faint (lane 1) and both faint and strong (lane 2) EBV-specific OCBs are present in MS154 and MS102 cases, respectively (arrows indicate OCBs). Of note, MS102 was also positive for EBV DNA and had the highest frequency of EBER⁺ and CD8⁺ cells in both white matter lesions and meninges among the EBV-low MS cases.

Figure 7

Quantification of CD20⁺, EBER⁺ and CD8⁺ cells in MS brain sections. MS cases with and without ectopic follicles were *a posteriori* classified as EBV-high and EBV-low, respectively. The graphs show significant differences in the number of CD20⁺, EBER⁺ and CD8⁺ cells in the white matter (A) and meninges (B) between the two groups. Dot points represent values for each MS case. Each value represents the mean of cell counts performed in adjacent brain sections (2 sections for each marker)

for each brain tissue block; 1-2 blocks for each MS case were analysed. The bars represent median values for each group (n = 8 for EBV-high MS cases; n = 12 for EBV-low MS cases).

EXAMPLE 2

Following on from the above discovery of huge deposits of Epstein-Barr virus (EBV)-infected B cells and plasma cells in the brain of multiple sclerosis patients (as also post-published as Serafini³² et al. 2007), we have asked whether an abnormal accumulation of EBV-infected cells in the target organ was also a feature of other autoimmune diseases characterized by dysregulated humoral immunity. We decided to address this issue in two diseases, autoimmune thyroiditis and myasthenia gravis (MG), in which tissue-specific autoantibodies have been unequivocally identified and play a role in disease pathogenesis. Moreover, both in the thyroid of patients with Hashimoto's thyroiditis (HT) and Grave's disease and in the thymus of myasthenic patients, B-cell hyperplasia and well organized ectopic B-cell follicles have been described (reviewed in Aloisi and Pujol-Borrell³¹, 2006; see also Fig. 5 A-B for HT and Fig. 6 A-C for MG), raising the suspect that EBV could be involved in these processes.

To evaluate the extent and status of EBV infection in bioptic tissues from patients with HT and MG, both in situ hybridization for EBER and immunohistochemical stainings for viral latency and lytic cycle-associated proteins were performed, as described above (and subsequently published in Serafini et al., 2007). Hashimoto's thyroiditis: tissues were provided by Dr Ricardo Pujol-Borrell Laboratory of Immunology (LIRAD), Banc de Sang i Teixits; Institute for Research in Health Sciences, Germans Trias i Pujol, 08916 Badalona, Barcelona, Spain; thyroid samples from 5 HT cases were examined. Myasthenia gravis: tissues were provided by Dr Renato Mantegazza, Istituto Neurologico Besta, Milan, Italy. Thymus samples from 8 MG cases were examined.

The following results were obtained. Proteins expressed during the EBV latency phase (EBNA2, growth programme, and LMP1, growth and default programmes) were observed in all thyroids examined from HT cases (Fig. 5 C-E) and in all the thymus samples from MG patients (Fig. 6 F, G) (representative cases are shown). LMP1+ cells

were much more numerous than EBNA2+ cells and were detected mainly within ectopic B-cell follicles. The few cells expressing EBNA2 were found at the periphery of the follicles and within the hyperplastic areas. These data are in line with previous findings in multiple sclerosis brain tissue (and described above and in Serafini et al., 2007). In some MG thymus samples, the presence of EBV-infected cells was confirmed by in situ hybridization for EBV-associated small RNAs (EBERs) (Fig. 6 D, E). In all cases examined, the distribution of EBV markers in thyroid or thymus overlapped with that of the B cell marker CD20.

In all 5 HT thyroids analysed we also found expression of the replication factor BALF2 (Fig. 5 F) and of two early lytic antigens BMRF1 and BFRF1 associated with viral reactivation (Fig. 5 G and H). Numerous cells expressing these viral antigens were located both around and inside the ectopic follicles. Only rare cells in the hyperplastic areas were found positive for p160 and gp350/220, two EBV structural proteins that are expressed during the late phase of the lytic cycle (Fig. 5 I, J). Similar data was obtained in the MG thymus, where EBV-infected cells expressing BMRF1 and BFRF1 colocalized with B cell aggregates (Fig. 6 H and J) and cells expressing gp350 were present at a much lower frequency in the same areas (not shown).

Although a detailed analysis of the activation state and relationship to EBV-infected cells of CD8+ T cells infiltrating the HT thyroid and MG thymus has not been performed yet, both organs contain a large amount of CD8+ T cells, which suggests involvement of an EBV-targeted cytotoxic immune response in tissue damage.

Based on these findings, we conclude that abnormal accumulation of latently and lytically EBV-infected B cells and plasma cells with very limited production of infectious virus in the target, inflamed organ is a feature shared among several putative autoimmune diseases. We interpret these data as evidence that, similarly to what proposed for multiple sclerosis; also in autoimmune thyroiditis and myasthenia gravis, EBV could play a dual role: on the one hand, induce abnormal humoral immunity, including production of disease-specific autoantibodies, and on the other hand stimulate an EBV-specific cytotoxic immune response that, in the attempt to eliminate EBV deposits in the target organ (thyroid and thymus, respectively), would sustain the immunopathological process. These findings support the view that not only patients

with MS, but also those with autoimmune thyroiditis and myasthenia gravis, could benefit from the treatments indicated herein.

Figure Legends for Example 2

Figure 5

Expression of EBV latent and lytic proteins in the thyroid of patients with Hashimoto's thyroiditis

Ectopic B cell follicles stained with the pan B-cell marker CD20 in the thyroid of patients affected by Hashimoto's thyroiditis (A, B). Numerous cells expressing the EBV latency protein LMP1 were observed inside ectopic B follicles (C and inset; the same follicle shown in C is circled in red in panel B), whereas immunopositivity for EBNA2 was restricted to a small number of cells distributed at the edge of the follicle and in areas of hyperplasia (D, E). Numerous cells expressing the replication factor BALF2 (F) and the early lytic cycle-associated proteins BMRF1 and BFRF1 (G, H) were found within and around the B cell follicles. Conversely, cells expressing the most abundant protein of the viral envelope, gp350/220, and the viral capsid antigen p160 were observed only occasionally (I, J, respectively).

Figure 6

Expression of EBV latent and lytic proteins in the thymus of patients with *myasthenia gravis*

Ectopic B cell follicles stained with the pan B-cell marker CD20 in the thymus of patients affected by myasthenia gravis (A-C). In situ hybridization for EBER reveals the presence of numerous EBV-infected cells inside ectopic follicles (D) and as well as in sparse B-cell infiltrates (E). Rarely, isolated EBNA2+ cells were observed at the edge of ectopic follicles (F); the latter comprised numerous cells positive for the EBV latency protein LMP1 (G and inset. Numerous cells expressing the early lytic cycle-associated proteins BMRF1 and BFRF1 were found within and outside the B cell follicles (H-J).

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CLAIMS:

1. The use of at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent, in the manufacture of a medicament for the treatment of an autoimmune disease.
2. Use according to claim 1, wherein the autoimmune disease is systemic lupus erythematosus, Hashimoto's thyroiditis, Grave's disease, multiple sclerosis, Sjögren's syndrome, rheumatoid arthritis, myasthenia gravis and inflammatory myopathies.
3. Use according to claim 1 or 2, wherein the autoimmune disease is multiple sclerosis.
4. Use according to claim 2, wherein the inflammatory myopathy is dermatomyositis, inclusion body myositis, and polymyositis.
5. Use according to any preceding claim, wherein the medicament comprises no B cell cidal agents, and the autoimmune disease is associated with regions of the body not restricted to the CNS.
6. Use according to claim 5, wherein the medicament is for the treatment of systemic lupus erythematosus or rheumatoid arthritis, and comprises one or more anti-EBV substances, and no B cell cidal agents.
7. Use according to any preceding claim, wherein the anti-EBV substance is selected from drugs, antibodies and siRNA.
8. Use according to any preceding claim, wherein the anti-EBV substance is one or more of acyclovir, valacyclovir, and a Type-1 interferon.
9. Use according to claim 7, wherein any antibody is selected from antibodies against EBV gp350/220 surface glycoprotein, the HLA B8 restricted epitope from EBNA-3A, Epstein Barr Nuclear Antigen 2, latent membrane protein 1 (LMP1), and BFRF1.
10. Use according to claim 7, wherein the siRNA targets the EBNA2 gene.
11. Use according to any preceding claim, wherein the medicament is for intrathecal administration.

12. Use according to claim 11, wherein the autoimmune disease is multiple sclerosis.
13. Use according to claim 11 or 12, wherein the medicament comprises at least one B cell cidal agent.
14. Use according to claim 13, wherein the B cell cidal agent is a monoclonal antibody targeting a B-cell surface protein to activate antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity and or/apoptosis.
15. Use according to claim 14, wherein the B cell cidal agent is Rituximab.
16. Use according to any preceding claim, wherein the medicament comprises at least one each of substance (a) and agent (b).
17. Use according to claim 16, wherein the medicament comprises Rituximab and valacyclovir.
18. Use according to claim 16, wherein the medicament comprises Rituximab and acyclovir.
19. Use according to any of claims 13 to 18, wherein the medicament further comprises a plasma cell cidal agent.
20. Use according to any of claims 13 to 19, wherein the medicament is for intrathecal administration.
21. Use according to any preceding claim, wherein the medicament is for intrathecal administration and comprises, additionally to, or alternatively to, one of substance (a) and agent (b), a T cell inhibitory, blocking, suppressing, or cidal substance.
22. Use according to claim 21, wherein the further substance is targeted at CD8⁺ T cells.
23. At least one of (a) an anti-EBV substance, and (b) a B cell cidal agent for the treatment of an autoimmune disease.
24. A pharmaceutical composition comprising at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent, for the treatment of an autoimmune disease.

25. A method for treating an autoimmune disease, comprising administering, to a patient in need thereof, a therapeutically effective amount of at least one of (a) an anti-EBV substance, and (b) a B cell cidal agent.
26. A vaccine for the prevention of multiple sclerosis, or any other chronic, inflammatory, autoimmune disease, comprising at least one epitope characteristic of EBV.

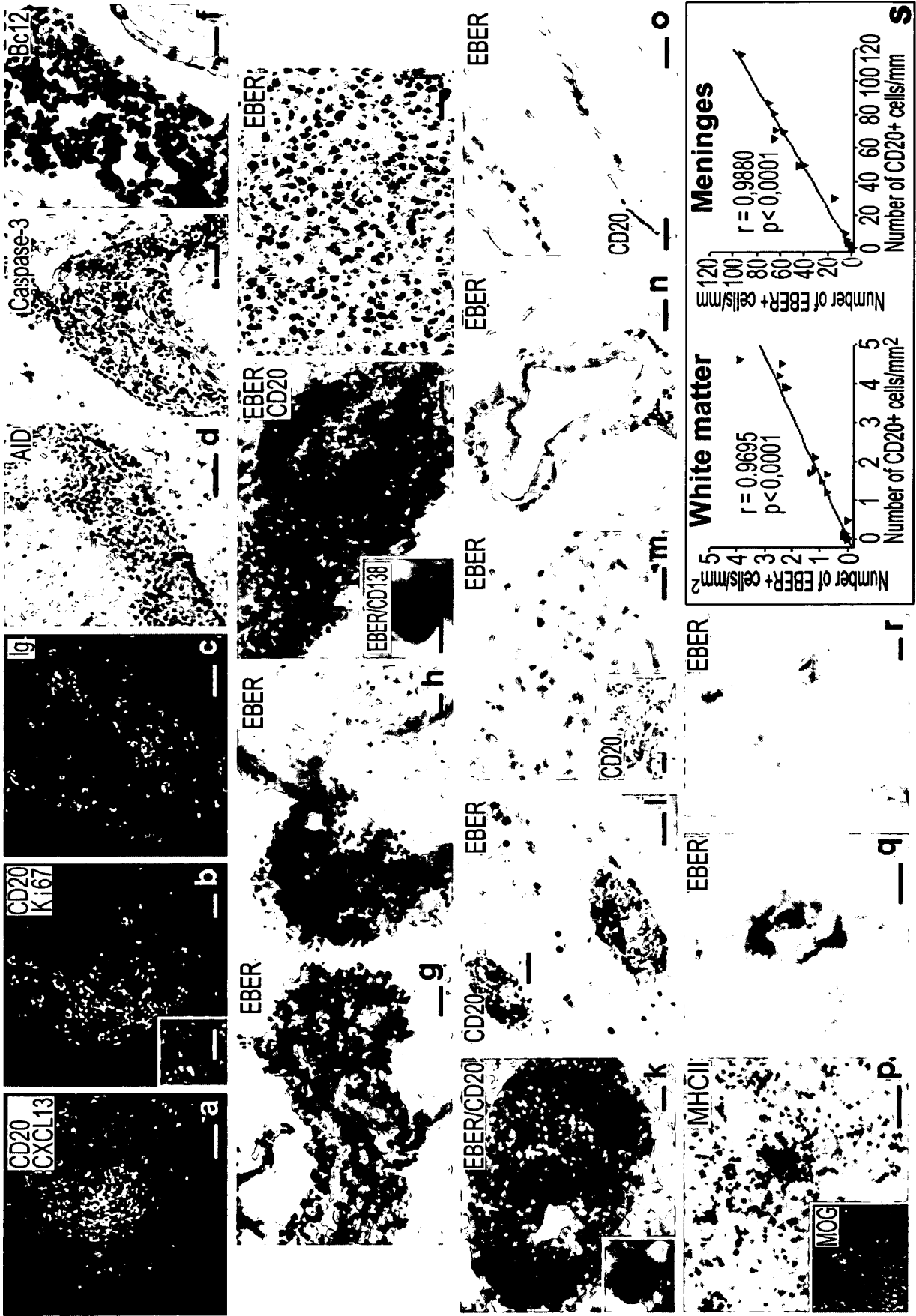


Fig.1

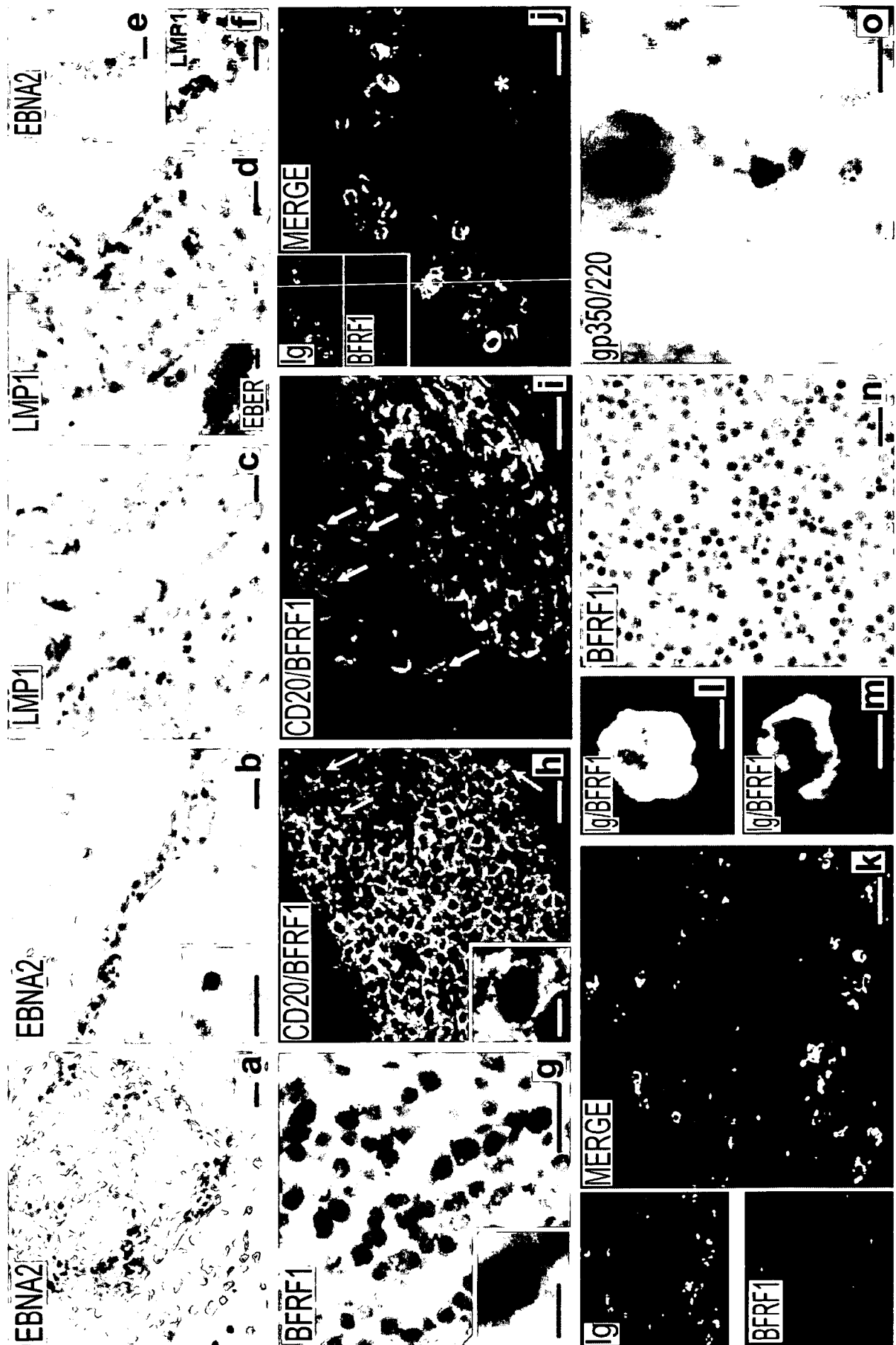


Fig. 2

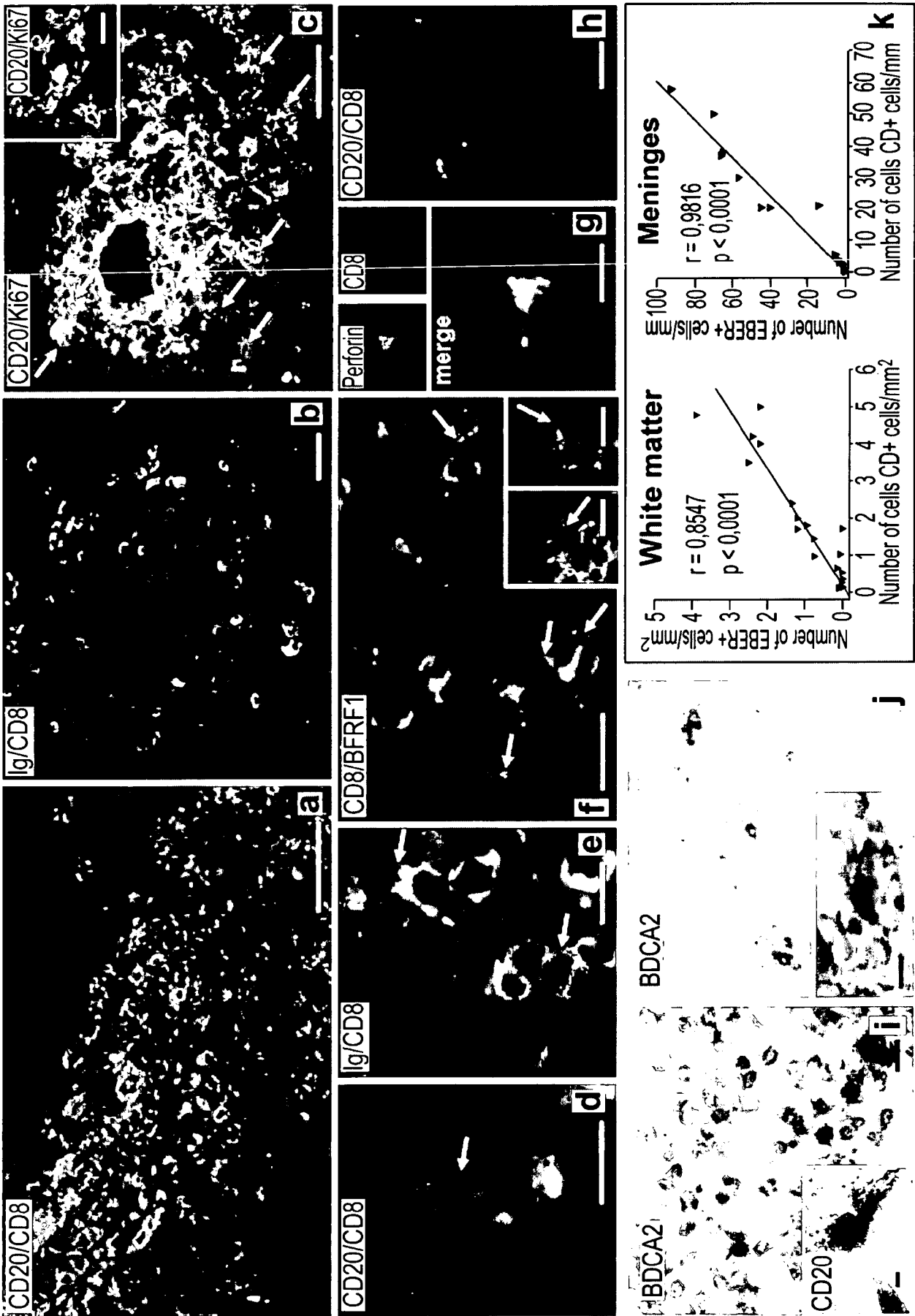
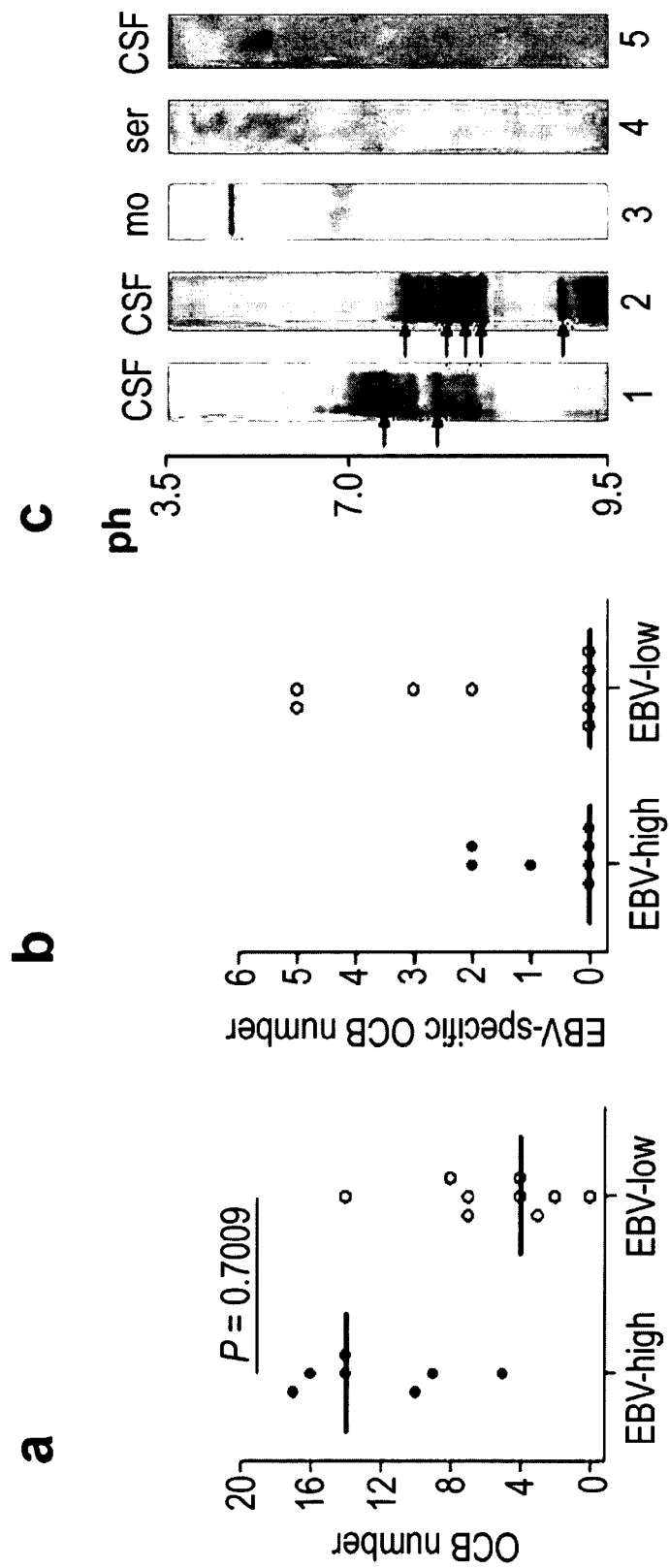


Fig.3



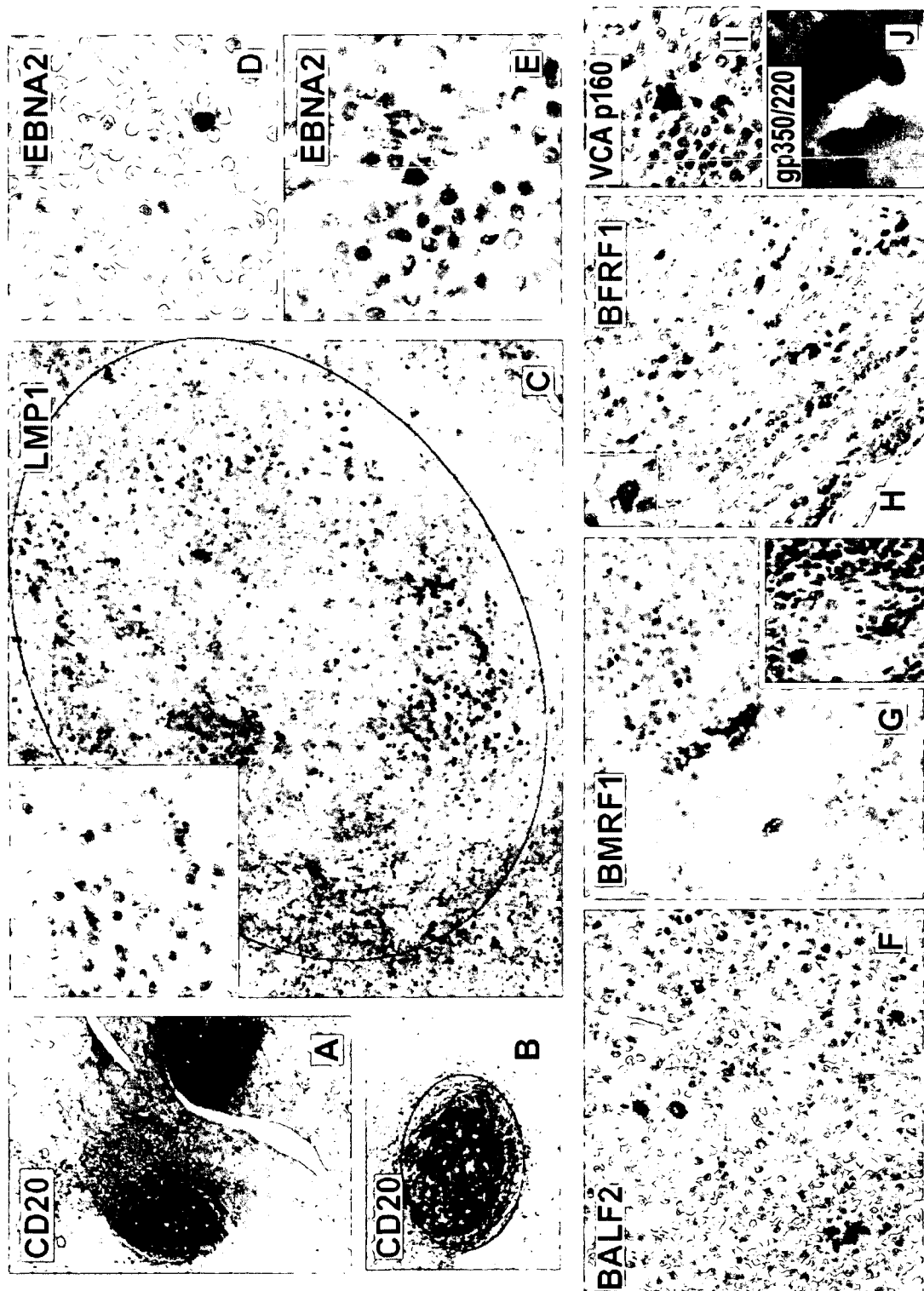


Fig. 5

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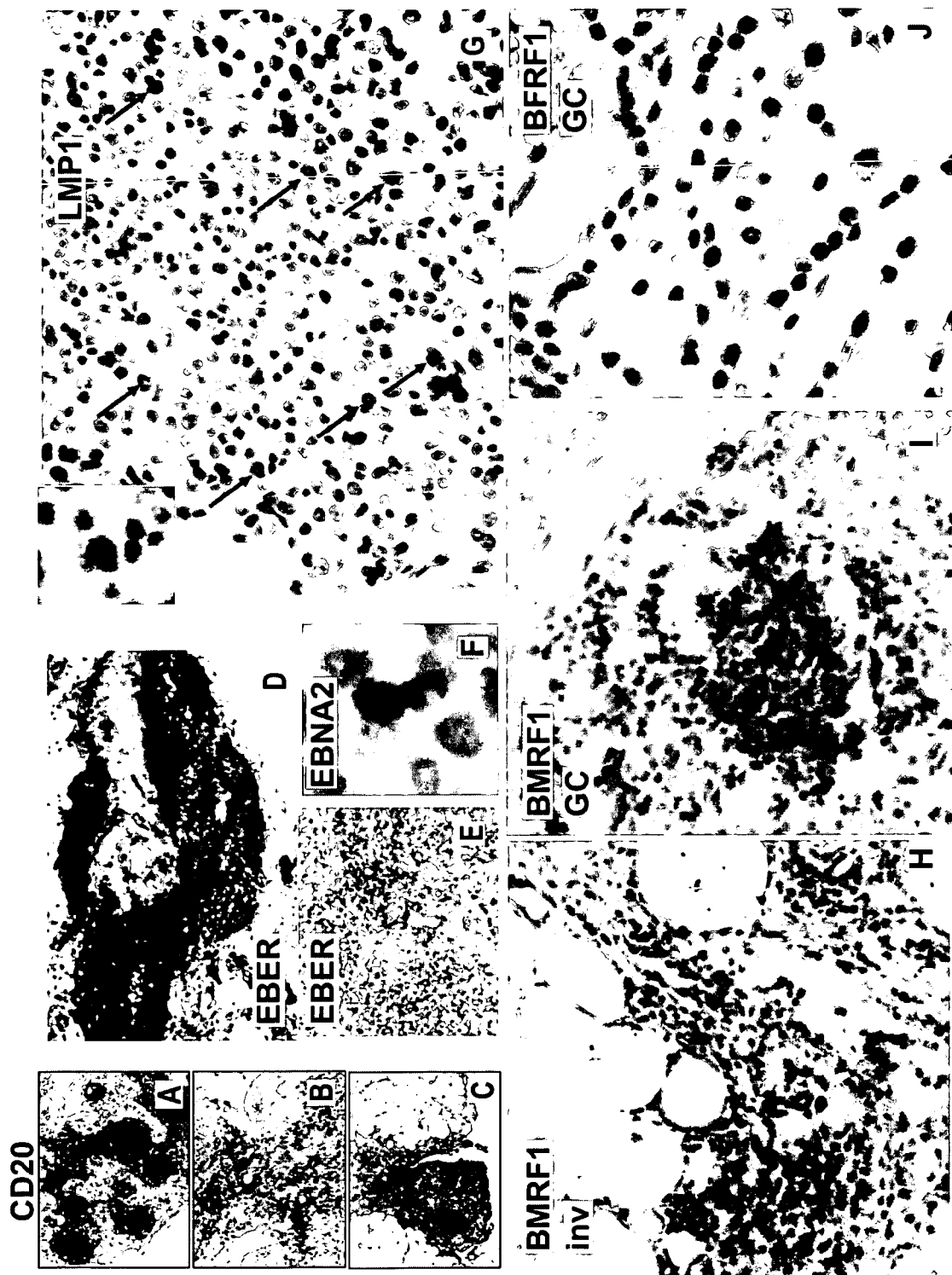
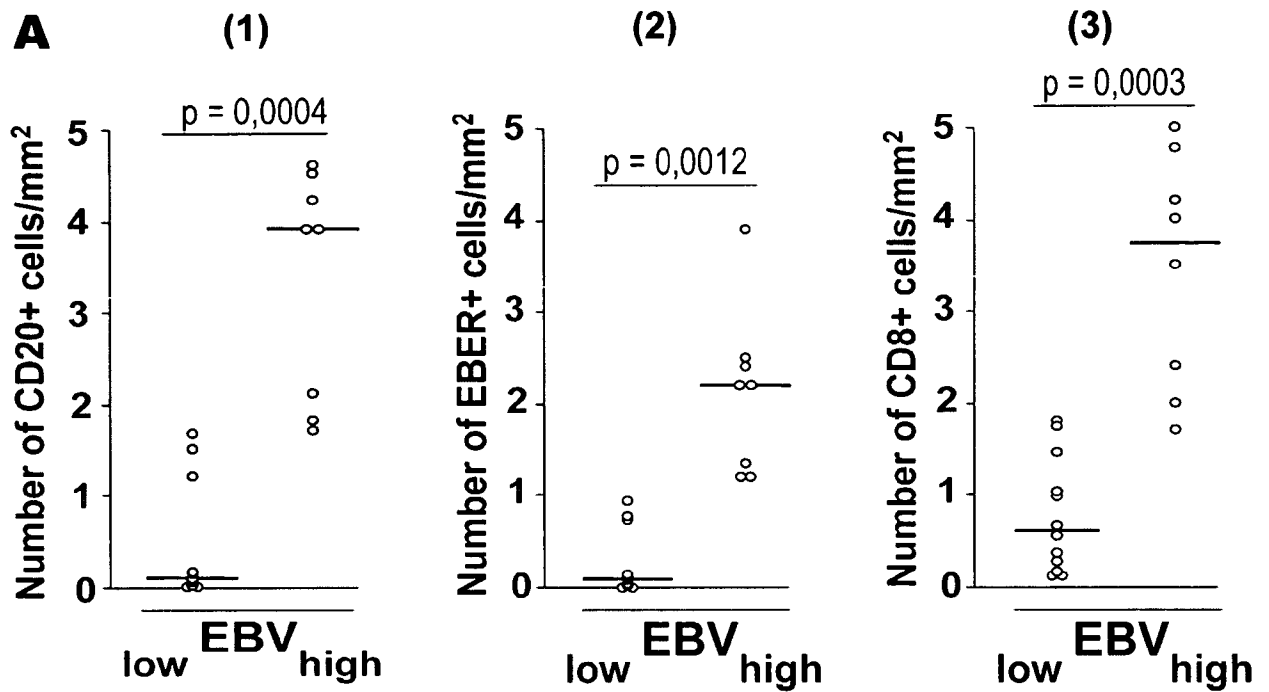


Fig. 6

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White matter



Meninges

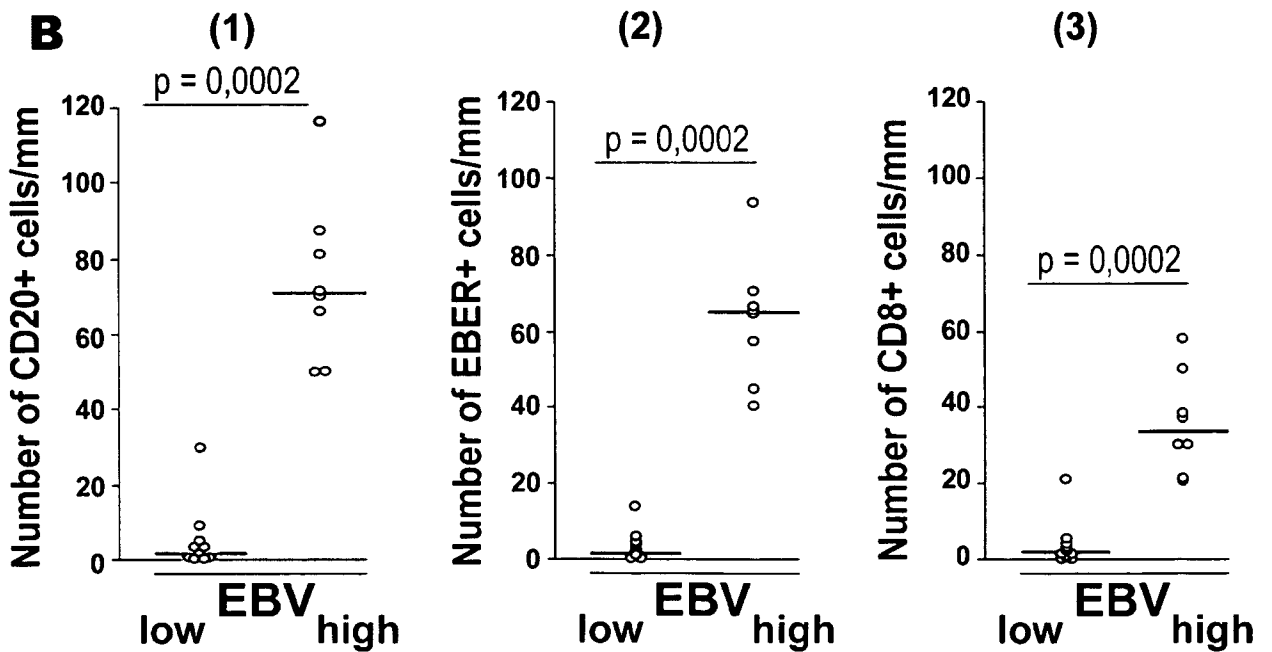


Fig.7