

Spontaneous and inducible CD8 T cell lesions in the brain and spinal cord of HLA-DR15-positive MS PBMC humanized mice

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Abstract

Summary

Autoimmune diseases of the central nervous system such as multiple sclerosis (MS) are only partially represented in current experimental models and the development of humanized immune mice is crucial for better understanding of immunopathogenesis and testing of novel therapeutics. We describe a humanized mouse model with several key MS features. Severely immunodeficient B2m-NOG mice were transplanted with peripheral blood mononuclear cells (PBMC) from MS and healthy (HI) donors and showed rapid engraftment by human T and B lymphocytes. DR13-positive MS PBMC mice developed low levels of graft versus host disease (GVHD) and no CNS inflammation. Both DR15 MS and DR15 HI mice developed spontaneous and EAE-inducible infiltration of CNS barriers and parenchyma by CD8⁺ and CD4⁺ T cells. DR15 MS mice uniquely developed spontaneous T cell lesions in brainstem and spinal cord grey matter, and large EAE-inducible lesions in the brain corpus callosum, with relatively low GVHD levels compared to DR15 HI mice. Main limitations of this model for further development are poor monocyte engraftment, lack of demyelination and of lymph node organization and IgG responses. These results show that PBMC humanized mice represent promising experimental tools for MS immunopathology and for testing experimental immunotherapeutics in a patient-specific approach.

eLife assessment

This manuscript describes a unique humanized model of MS/EAE-like pathology and provides a **valuable** new model that incorporates enhanced susceptibility related to HLA type. The data are modestly **incomplete** to **solid** in terms of supporting their claims that this model improves upon prior models. Overall, these claims could be further supported by a more comprehensive quantification of the immune infiltrate, a better assessment of peptide sensitization requirement, and an assessment of CNS/spinal cord pathology.

Introduction

Multiple sclerosis (MS) is a complex chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with autoimmune characteristics. Pathological hallmarks are focal plaques of immune-mediated demyelination in the white matter, inflammation at CNS borders and diffuse demyelination and neurodegeneration of the grey and white matter of the brain and spinal cord. Genome wide association studies show that the vast majority of MS risk genes are related to immune activation and function, thereby strongly supporting the role of adaptive immunity in MS pathogenesis. These include the strongest MS associated genetic risk factor, human leukocyte antigen (HLA) DR15 haplotype, encoded by two DRB* genes, *DRB1*15:01* (DR2b) and *DRB5*01:01* (DR2a), of the MHC class II that restricts CD4⁺ T cells (1,2). Pathological analyses of autopsy tissues reveal CD8⁺ lymphocytes and monocytes as the main CNS infiltrating cells, with B cells accumulating in meningeal and perivascular spaces, and relatively few infiltrating CD4⁺ T cells (3). Studies in animal models, mainly experimental autoimmune encephalomyelitis (EAE), showing that myelin-reactive T cells are both necessary and sufficient to induce EAE, as well as the effectiveness of current immunotherapies in MS patients that work mainly by targeting peripheral immune responses, further support an autoimmune pathogenesis for MS.

EAE and MS share many clinical, immunological, and pathological similarities (4,5), but they also present significant differences (6,7). One difference is the predominance of CD8⁺ T cells in MS lesions, while these cells are scarce in the CNS of EAE mice (8). In addition, the inflammatory process in EAE primarily targets the spinal cord, whereas in MS it also targets the brain. Moreover, while some MS drugs were discovered from research in the EAE model (natalizumab, glatiramer acetate), there is a relatively low or moderate success rate of drugs that showed high efficacy in both the EAE model and the human disease (9). These differences infer that different pathways or mechanisms are prevalent in MS versus EAE (10,11). Differences are further highlighted by recent clinical data showing the critical importance of B cells to MS pathology, a cell population that does not show functional participation in most EAE models (12,13). The development of animal models capable of developing human immune system is crucial to the study of human immune-mediated diseases such as MS, and for evaluating the safety and efficacy of human immunotherapies.

Most current studies with humanized mouse models use immunodeficient non-obese diabetic (NOD) - severe combined immunodeficiency disorder (*scid*) mice deficient or mutant for the common cytokine receptor γ -chain gene (*IL2rg*) (named NSG or NOG mice, respectively) that lack B, T cells and NK cells and have defective innate immune responses (14–17). These mouse strains readily engraft with human peripheral blood monocytes (PBMC) without prior irradiation, as murine MHC molecules support the proliferation of both CD4⁺ and CD8⁺ T cells, with CD8⁺ T cells predominating. Recent variants of these strains lacking MHC class I molecules (B2m) show

increased CD4 to CD8 ratios and a longer time window before onset of xenogeneic graft versus host disease (GVHD) and represent improved strains for the study of human immune responses (18 [↗](#), 19 [↗](#)). Previous studies showed that PBMC humanized NSG mice with cells from healthy donors are susceptible to EAE, exhibiting subclinical disease, with infiltration of the brain and spinal cord by CD4⁺ and CD8⁺ T cells, but not monocytes (20 [↗](#)).

To investigate the potential of PBMC from MS patients to induce CNS-directed immunopathology in humanized mice, we engrafted B2m-NOD/scid-IL2Rγ^{null} (B2m-NOG) mice with PBMC from 3 human donors based on HLA-DRB1 genotype and RRMS diagnosis, and monitored CNS inflammation that developed spontaneously or after immunization with myelin antigens. We show that PBMC from MS and healthy (HI) donors rapidly engraft B2m-NOG mice, but with marked differences towards the proportions of immune cell populations engrafted and propensity to develop CNS inflammation between donors. Mice transplanted with PBMC from HLA-DR15-positive MS and HI donors engrafted with human (h)CD4⁺ and hCD8⁺ T cells and showed prominent hCD8⁺ T cell accumulation at CNS borders and infiltration of brain and spinal cord parenchyma associated with locally activated microglia and astrocytes. Overall, CNS inflammation tended to be more severe in DR15 MS mice and uniquely showed spontaneous hCD8⁺ T cell lesions in the spinal cord grey matter and brainstem, and prominent corpus callosum white matter lesions in mice immunized with myelin peptides. Our results show that the PBMC humanized B2m-NOG mouse model partially reproduces MS immunopathology and can be used to investigate human immune responses towards CNS in a simple, fast, and personalized manner.

Results

Reconstitution of human adaptive immune system in B2m-NOG mice engrafted with PBMC from MS patient and HI donors

Two long-term RRMS patients, one HLA-DR15-positive (DR15 MS) and one HLA-DR13-positive (DR13 MS), both presenting with highly active disease following immunomodulatory treatment with natalizumab, were selected as PBMC donors (Table 1 [↗](#)). An HLA-DR15-positive healthy individual (DR15 HI) was selected as a control donor. Fresh blood samples were used for immunoprofiling by flow cytometry with a panel of standard human immune cell marker antibodies (Supplementary Table 1A, B), for screening of plasma antibody responses to viruses (Table 1 [↗](#); and Supplementary Table 1C for interpretation of EBV results), and for the isolation of fresh PBMC. Groups of 8 B2m-NOG mice were injected intravenously with freshly prepared PBMC (1×10^7 /mouse) from each donor (Supplementary Table 2, protocol 1). The transplanted mice were monitored for human immune cell engraftment by flow cytometry analysis of small blood samples recovered from the tail vein at different time points and all showed progressive engraftment by human CD45⁺ (hCD45⁺) leukocytes from day 7 (Fig. 1A [↗](#)). At day 14 post-transplantation (dpt 14), 5 mice from each group were immunized for EAE using a mixture of immunodominant T cell myelin peptide antigens. The PBMC donors used in this study show T cell proliferation responses to myelin peptides (Table 1 [↗](#)), as shown in a previous study (21 [↗](#)). Non-immunized mice showed steadily increasing levels of blood hCD45⁺ leukocytes up to sacrifice at dpt 42. Immunized mice showed reduced levels of blood hCD45⁺ leukocytes following immunization (Fig. 1A [↗](#)). Further FACS analysis showed preferential expansion of hCD4⁺ T lymphocytes compared to hCD8⁺ T lymphocytes in blood and spleens of all mice (Fig. 1B [↗](#), C), a finding consistent with a previous report describing the B2m-NSG model (18 [↗](#)).

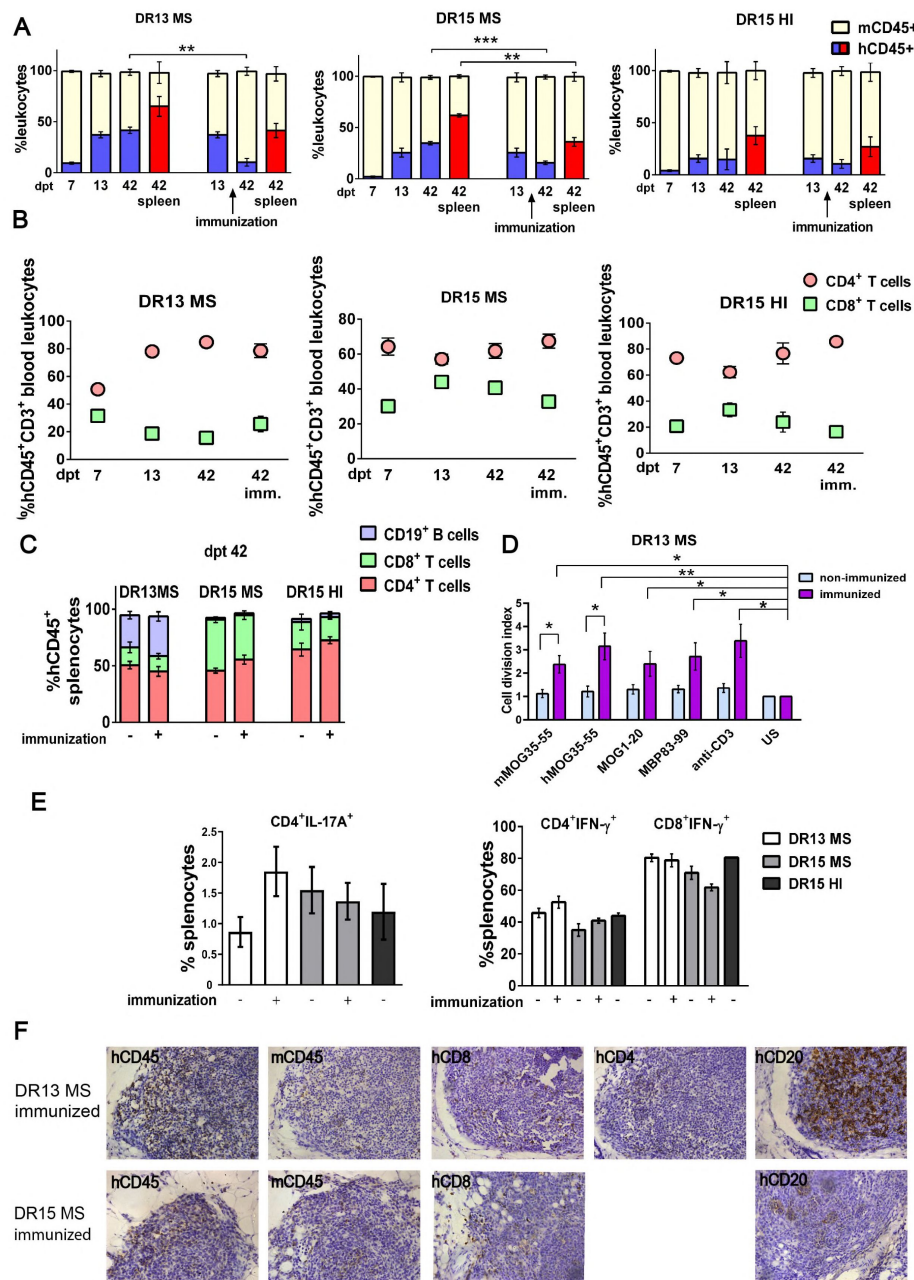


Figure 1

Reconstitution of a human adaptive immune system in B2m-NOG mice engrafted with PBMC from MS patient and healthy donors.

(A) Progressive engraftment of human (h) CD45+ leukocytes from donors in groups of B2m-NOG mice, non-immunized (left) and immunized using an EAE protocol (right), measured in peripheral blood samples taken at different time points, and in spleen recovered at sacrifice 42 days post-transplantation (dpt 42). **(B)** Proportions of hCD45+ and hCD8+ T cells in blood hCD45+CD3+ T cells at different time points. **(C)** Proportions of human immune cell subpopulations in spleens of immunized and non-immunized mice at dpt 42 (see also Supplementary Table 3). **(D)** Antigen-specific T cell proliferation responses to the immunizing antigens (mMOG35-55, hMOG35-55, MOG1-20, MBP83-99), anti-hCD3 (positive control) and medium (unstimulated; US), in splenocytes recovered from non-immunized and immunized DR13 MS PBMC humanized mice at dpt 42. Results are expressed as a cell division index. **(E)** Proportions of IL-17A-producing CD4+ (left graph), and interferon- γ -producing (right graph) CD4+ and CD8+ T cells in splenocytes recovered from mice in the different groups at dpt 42. **(F)** Draining lymphoid structures in inguinal fat of mice engrafted with DR13 MS and DR15 MS PBMC and immunized with myelin peptides, recovered at dpt 42. Immunohistochemistry revealed the presence of hCD45- and mCD45-positive leukocytes, hCD4- and hCD8-positive T cells, and hCD20-positive B cells (latter only DR13 MS mice). All results are depicted as mean $\pm$ SEM. Statistical significance is shown after pairwise comparisons between groups using Student's t test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Genotype Diagnosis	G	Age	MS duration (y)	Therapies	Relapse (12/24 m)	MRI Brain/Gd	MRI Spine/Gd	EDSS	Viral infections (titre)	T cell responses (21)
DR13 1302/1303 RRMS	F	42	27	Azathioprine Interferon beta-1a Interferon beta-1b Fingolimod Natalizumab (#5) Cortisone (1 m prior to sampling)	1/1	(+) stable	(+) GD (+)	2.5	CoV2 ⁺ JC ⁺ (1.24) EBV VCA IgG ⁺ EBV EA IgG ⁺ EBV EBNA1 IgG ⁺ EBV EBNA1 IgM ⁺	MBP83-99 hMOG35-55 (Patient 17)
DR15 0402/1501 RRMS	F	38	15	Interferon beta-1a Fingolimod Natalizumab (#24) Cladribine	1/1	(+) stable	(+) stable	2.5	CoV2 ⁺ JC ⁺ (1.47) EBV VCA IgG ⁺ EBV EA IgG ⁺ EBV EBNA1 IgG ⁺ EBV EBNA1 IgM ⁺	MBP13-32 MBP83-99 hMOG35-55 (Patient 11)
DR15 04/15 Healthy	F	28	None	None	None	ND	ND	ND	CoV2 ⁺ JC ND EBV VCA IgG ⁺ EBV EA IgG ⁺ EBV EBNA1 IgG ⁺ EBV EBNA1 IgM ⁺	None (Normal 1)

KEY: EDSS, expanded disability status scale; Gd, gadolinium; NA, not applicable; ND, not done; RRMS, relapse-remitting MS.

Successful engraftment of mice by hCD45⁺ leukocytes was confirmed in the spleen recovered from non-immunized and immunized mice at sacrifice on dpt 42 (**Fig. 1A**). Further analysis of splenocytes revealed robust engraftment of hCD4⁺ and hCD8⁺ T cells in all mice, and of hCD19⁺ B cells in DR13 MS PBMC mice (**Fig. 1C**; Supplementary Table 3). Human immune cells other than T and B cells, notably monocytes, were undetectable in dpt 42 spleen. Analysis of cytokine production by intracellular staining showed high proportions of IFN- γ -producing hCD4⁺ and hCD8⁺ splenocytes, and IL-17A-producing hCD4⁺ in both immunized and non-immunized mice (**Fig. 1E**).

To estimate the contribution of mouse myeloid cell populations in the humanized mice, we analyzed peripheral blood from a parental NOD-*scid* mouse strain and wild-type C57BL/6 (B6) mice, non-immunized (naïve) or day 8 post-immunization (dpi 8) with CFA and intraperitoneal (i.p.) injections with *Bordetella pertussis* toxin (PTx) following the EAE protocol, by flow cytometry. Immature Ly6C^{hi} monocytes and Ly6G⁺ cells are known to massively expand in the periphery of wild-type B6 mice following immunization with adjuvants and are critical for the development of EAE in wild-type mice (22, 23). Unlike B6 mice, very high proportions of mouse (m) CD11b⁺, mCD11b⁺Ly6C^{hi} and mCD11b⁺Ly6G⁺ myeloid cells were already present in the blood of non-immunized NOD-*scid* mice, and these did not increase significantly following immunization (Supplementary Table 4).

Functionality of the human immune system in B2m-NOG mice was investigated using an *ex vivo* proliferation assay for T cell responses in splenocytes (23), and a cell-based assay for anti-MOG antibody responses in plasma. Briefly, splenocytes recovered from non-immunized and immunized mice at sacrifice were stimulated *ex vivo* individually with the 4 myelin peptides used for immunization (mMOG35-55, hMOG35-55, MOG1-20, MBP83-99). T cell proliferation responses were measured using a CFSE dilution assay. Splenocytes isolated from DR13 MS mice (**Fig. 1D**), not DR15 MS or DR15 HI mice (data not shown), showed significant T cell proliferation responses to all myelin peptides, equal to control splenocytes stimulated with anti-hCD3 antibody. The difference in responses between donors might be explained by the significant engraftment of

human B cells in DR13 MS mice, which can act as antigen-presenting cells in the *ex vivo* T cell proliferation assay. Anti-hMOG IgG antibody responses were not detectable in the plasma of any mice at any time point.

We next investigated whether draining lymph nodes (LN) structures could be identified in the immunized mice at sacrifice. Mice lacking the common cytokine receptor γ -chain gene are known to have poor lymphoid organ structure and functioning, because NK cells are needed for the formation of LN inducer cells, which in turn are needed for LN organogenesis (24). Nevertheless, the inguinal fat tissue from several immunized mice showed accumulations of leukocytes containing mCD45- and hCD45-positive leukocytes, CD8-, CD4- and CD20-positive lymphocytes by immunohistochemistry, although lacking obvious LN organization (Fig. 1F). Together the results show that human T and B lymphocytes are efficiently engrafted in B2m-NOG mice by PBMC from MS patients, that functional T cell responses can be detected, depending upon the PBMC donor, while lack of organized LN and germinal centers prevents the generation of anti-MOG IgG antibody responses.

Human T cells accumulate at CNS borders in non-immunized DR15 MS and DR15 HI mice, and form spontaneous parenchymal lesions in brainstem and spinal cord of DR15 MS mice

For the period of the study, none of the engrafted mice showed clinical symptoms of neuroinflammation or EAE using criteria commonly used for scoring EAE (21), or other neurological deficits (25). Mild symptoms of GVHD, specifically fur ruffling and reduced mobility (26), were recorded in few mice independent of group close to the time of sacrifice. To determine whether human immune cells enter CNS tissues in PBMC humanized B2m-NOG mice, we performed immunohistochemical analyses of serial sections from brain and spinal cord recovered from non-immunized mice at sacrifice using marker antibodies for human and mouse immune cells as well as for mouse microglia and astrocytes. To further monitor GVHD development we also performed standard histological analyses of lung and liver, two main tissues targets of GVHD.

DR13 MS humanized mice showed very few hCD45-positive leukocytes at CNS borders, specifically meninges, and none in CNS parenchyma, so no further analysis was made (data not shown). DR15 HI mice showed an accumulation of human immune cells at CNS borders, particularly the meninges and some in the choroid plexus of the interventricular foramen between lateral and third ventricles (Fig. 2A, C, D, arrowheads). In the brain, individual hCD45-positive leukocytes were scattered throughout the parenchyma and comprised mainly hCD8-positive T cells (Fig. 2C) and fewer hCD4-positive T cells (data not shown). Mild activation of Iba1-positive microglia and GFAP-positive astrocytes was seen at sites of immune cell accumulation at CNS borders (Fig. 2C). In spinal cord, hCD3-positive T cells accumulated at borders and few individual cells infiltrated parenchyma of white and grey matter (Fig. 3A, B). Sparse small T cell lesions (≥ 3 adjacent cells) were detected in the white matter of the spinal cord (Fig. 3A, left panel, arrowhead). Mouse CD45-positive cells were sparse at barriers, absent from parenchyma, and demyelination was not observed in the brain or spinal cord of DR15 HI mice.

DR15 MS mice showed more severe pathology than HLA-DR15 HI mice, with accumulation of human immune cells at CNS borders, particularly the meninges and choroid plexus of the interventricular foramen between lateral and third ventricles (Fig. 2A, C, D, arrowheads), widespread immune cell infiltration of the parenchyma, and small T cell lesions in the brainstem and spinal cord. In the brain, hCD45-positive leukocytes comprised mainly hCD8-positive T cells (Fig. 2C, arrows) and fewer hCD4-positive T cells, (data not shown). Numbers of brain parenchymal hCD8-positive T cells tended to increase in the brain parenchyma of DR15 MS mice compared to control HI mice, but differences were not significant due to variation within the groups (Fig. 2E). Unlike control HI mice, DR15 MS mice showed parenchymal T cell lesions (≥ 3

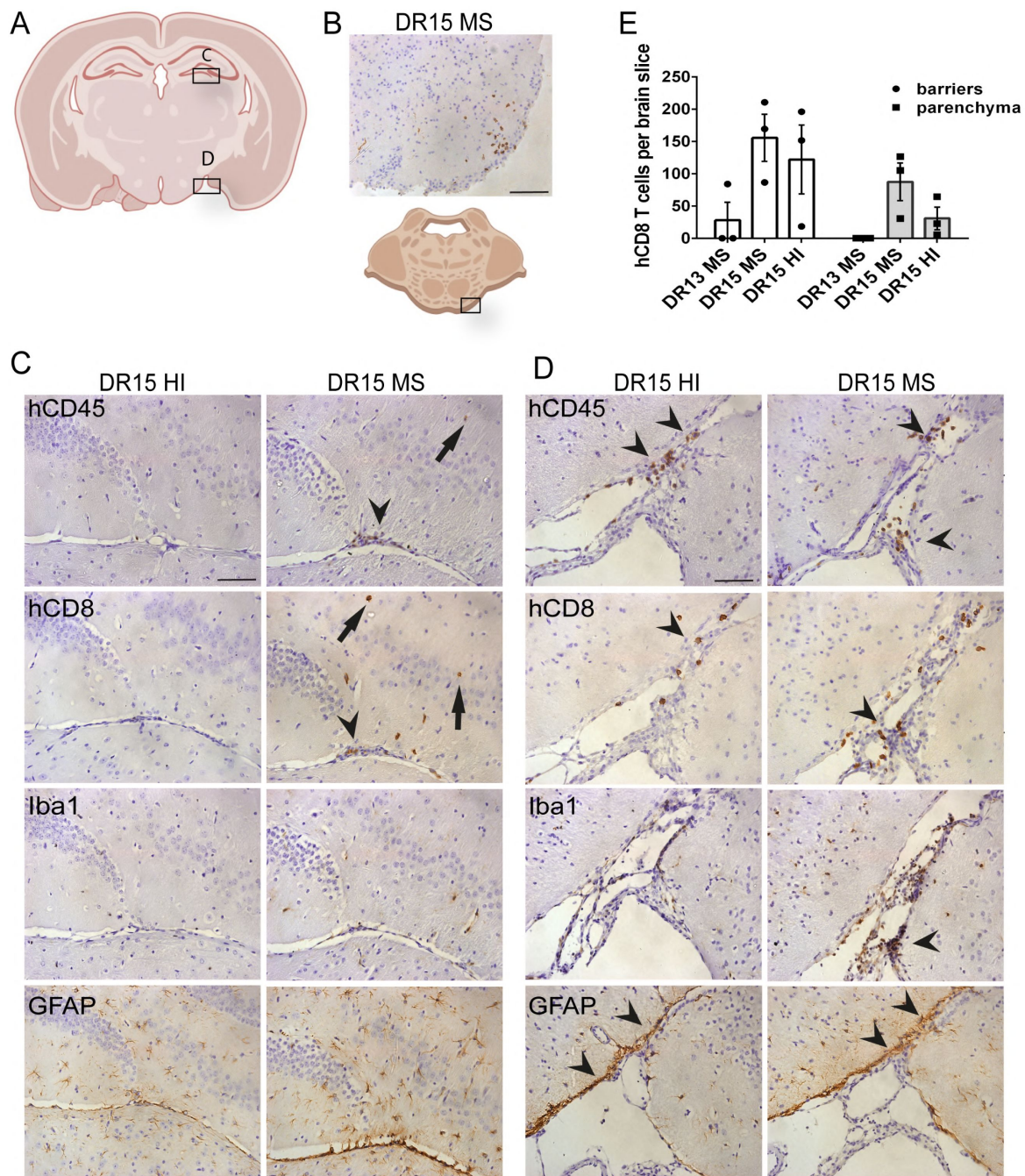


Figure 2

Human T cells accumulate at brain borders in non-immunized DR15 MS and DR15 HI mice, and form parenchymal lesions in brain and spinal cord of DR15 MS mice

Immunohistochemical analysis of the brain from non-immunized PBMC B2m-NOG mice showing infiltration by human (h) and mouse (m) CD45-positive leukocytes, and hCD8-positive T cells, in brain border regions (denoted in diagram **A**) and in brainstem parenchyma (denoted in diagram **B**). **(B)** Parenchymal lesion containing hCD8-positive T cells in brainstem of a DR15 MS mouse. **(C, D)** Accumulation of hCD45- and mCD45-positive leukocytes and hCD8-positive T cells, together with local activation of Iba1-positive microglia and GFAP-positive astrocytes, at border regions in the brains of DR15 HI and DR15 MS mice, specifically in the connective tissue of the interventricular foramen joining the lateral and third ventricles (**C**, arrowheads), and at meninges close to the optic tract (**D**, arrowheads). Scattered hCD45-positive leukocytes and hCD8 T cells in brain parenchyma of DR15 MS mice (**C**, arrows). **(E)** Counting of barrier-associated and parenchymal hCD8-positive T cells in whole coronal sections of brain (shown in **A**) from DR13 MS, DR15 MS and DR15 HI mice. Scale bar 100 μ m; x20 objective (**C, D**). All results are depicted as mean $\pm$ SEM. Statistical analysis was performed by pairwise comparisons between different groups of mice using Student's t test.

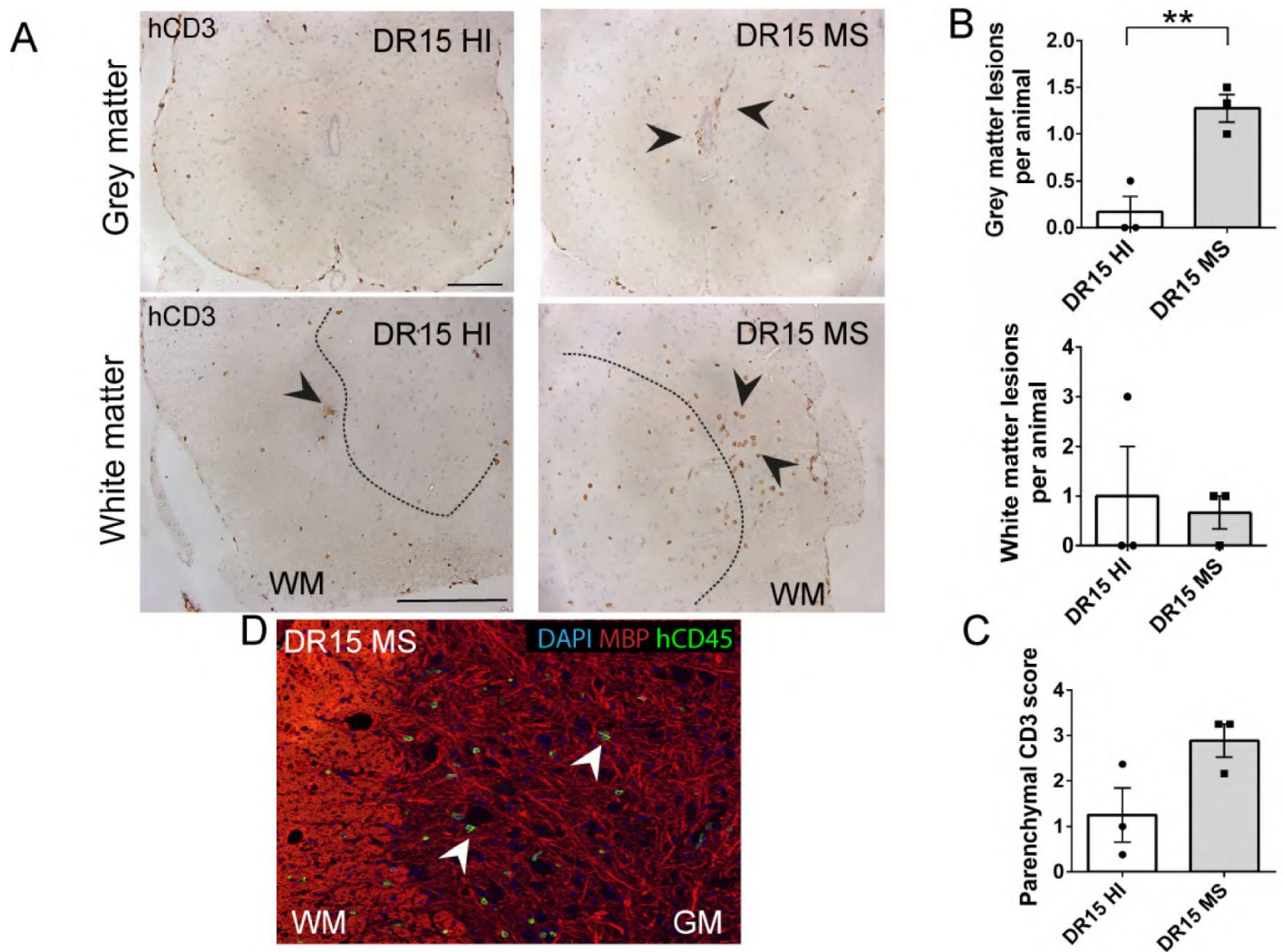


Figure 3

Human T cells infiltrate spinal cord white matter in non-immunized DR15 MS and DR15 HI mice, and form grey matter lesions in DR15 MS mice

Immunohistochemical analysis of spinal cord from non-immunized PBMC B2m-NOG mice showing infiltration by human (h) CD3-positive T cells in the grey and white matter regions. **(A)** Infiltrating hCD3-positive T cells were scattered individually throughout spinal cord grey and white matter and formed small lesions in the white matter (WM) of both DR15 MS and DR15 HI mice (lower panels, arrowheads), and grey matter lesions only in DR15 MS mice (upper panels, arrowheads). The dotted lines mark the boundary between grey matter and white matter. **(B)** Counting of hCD3-positive T cell lesions (≥ 3 adjacent cells) in grey and white matter in whole spinal cord sections from DR15 HI and DR15 MS mice. **(C)** Semi-quantitative estimation of hCD3-positive T cells in whole spinal cord sections from DR15 HI and DR15 MS mice. **(D)** Double immunofluorescence staining for hCD45-positive leukocytes (green, arrowheads) and MBP-positive myelin (red), with DAPI counterstained nuclei (blue), in spinal cord white matter (WM) of DR15 MS mice. Grey matter (GM). Scale bars 100 μm ; x20 objective (A, upper panels); x40 objective (A, lower panels). All results are depicted as mean $\pm$ SEM. Statistical analysis was performed by pairwise comparisons between different groups of mice using Student's t test.

adjacent cells) in brainstem (**Fig. 2A** [↗](#), B). Locally activated Iba1-positive microglia and GFAP-positive astrocytes were observed at sites of immune cell accumulation (**Fig. 2C** [↗](#), D, arrowheads). In the spinal cord, hCD3-positive T cells accumulated at borders and infiltrated both white and grey matter parenchyma (**Fig. 3A** [↗](#)). T cell lesions were present in spinal cord white and grey matter (**Fig. 3A** [↗](#), right panels, arrowheads). Numbers of parenchymal hCD3-positive T cells in whole spinal cord sections tended to be increased in DR15 MS mice compared to control HI mice, but differences were not significant (**Fig. 3C** [↗](#)). However, unlike control HI mice, DR15 MS mice showed numerous small T cell lesions in the grey matter, often close to the central canal suggesting this is a main site of human T cell entry into the spinal cord grey matter (**Fig. 3A** [↗](#), right panel, arrowheads, B). Mouse CD45-positive cells were rare at barriers, absent from parenchyma, and demyelination was not observed in the brain or spinal cord of DR15 MS mice.

To determine whether differences in the severity of CNS immune infiltration in the immunized mice was secondary to levels of GVHD inflammation in the periphery, we performed semi-quantitative analysis of H&E stained liver and lung sections, which are main targets of GVHD responses ([26](#) [↗](#)). All mouse groups showed equal levels of inflammation in both liver and lung, revealing a dissociation between peripheral GVHD and CNS inflammation (Supplementary Fig 1). These results show that non-immunized DR15 MS humanized mice have increased infiltration of brain and spinal cord by human T cells compared to non-immunized DR15 HI mice, and uniquely develop hCD8⁺ T cell lesions in spinal cord grey matter and brainstem parenchyma.

Immunization with myelin peptides increases T cell infiltration of CNS parenchyma and promotes development of brain and spinal cord lesions in both DR15 MS and DR15 HI mice

To further investigate whether human immune cells have the potential to induce CNS immunopathology in PBMC humanized B2m-NOG mice, we immunized mice following an EAE protocol using a mixture of immunodominant myelin peptides. In a previous study, we found that humanized HLA-DR2b transgenic mice lacking all mouse MHCII genes required greater amounts of peptide antigen to induce clinical EAE than wild-type B6 mice ([21](#) [↗](#)). We tested several EAE protocols using wild-type B6 mice, mainly to exclude the possibility of toxicity at high peptide doses. Briefly, 8-week-old female B6 mice were immunized by subcutaneous (s.c.) tail-base injection of 37 µg (standard EAE protocol in B6 mice) or 200 µg mMOG35-55 (protocol used in HLA-DR2b transgenic mice), or a myelin peptide antigen cocktail containing 200 µg each of mMOG35-55, hMOG35-55, MOG 1-20 and MBP83-99, dissolved in 100 µl saline and emulsified in an equal volume of complete Freund's adjuvant (CFA), together with i.p. PTx injections. Mice receiving high dose peptide also received an identical booster immunization 7 days after the first. All mice developed clinical symptoms of EAE, albeit with delayed disease onset in the high peptide groups possibly due to induction of T cell tolerance in B6 mice, with no evidence of increased morbidity or mortality at high peptide doses (Supplementary Fig. 2). For this reason, the high-dose peptide cocktail was chosen for immunization of groups of 12-14-week-old humanized B2m-NOG mice on dpt 14. Mice were monitored for engraftment by human immune cells (see above, and **Fig. 1** [↗](#)) and clinical symptoms at different time points, and brain and spinal cord tissues were recovered at sacrifice for immunohistochemical analysis using marker antibodies for human and mouse immune cells as well as for mouse microglia and astrocytes. In agreement with a previous study in NSG mice humanized with PBMC from healthy donors ([20](#) [↗](#)), none of the mice immunized with myelin antigens developed typical clinical symptoms of EAE.

To investigate whether immunization increased immune cell infiltration of the brain and spinal cord we performed immunohistochemical analysis of tissues recovered from the mice at sacrifice at dpt 42. Immunized DR13 MS mice, like their non-immunized group, showed very few hCD45-positive leukocytes at CNS borders and infiltrating spinal cord parenchyma, so no further analysis was made (data not shown). Immunized DR15 HI mice, like their non-immunized group, showed an accumulation of human immune cells at CNS borders, particularly the meninges and choroid

plexus. In the brain, individual hCD45-positive leukocytes were scattered throughout the brain parenchyma and comprised mainly hCD8-positive T cells (**Fig. 4A** [↗](#), B) and fewer hCD4-positive T cells (not shown). In one of three immunized mice, small T cell lesions were present in the optic tract (**Fig. 4C** [↗](#), arrowhead). Locally activated Iba1-positive microglia and GFAP-positive astrocytes were present at sites of immune cell accumulation at borders and in optic tract lesions (**Fig. 4C** [↗](#)). In spinal cord, hCD3-positive T cells accumulated at borders and individual cells infiltrated grey and white matter (**Fig. 5A** [↗](#)), forming small T cell lesions in the white matter (**Fig. 5A** [↗](#), B). Mouse CD45-positive cells were rare at barriers, absent from parenchyma, and demyelination was not observed in the brain or spinal cord of immunized DR15 HI mice.

Immunized DR15 MS mice showed prominent accumulation of human immune cells at CNS borders, widespread immune cell infiltration of the parenchyma and development of T cell lesions in the brain and the spinal cord (Figs. **4** [↗](#) and **5** [↗](#)). Brain infiltrates comprised mainly hCD8-positive T cells, and fewer hCD4-positive T cells (**Fig. 4D** [↗](#), arrowheads). Accumulation at borders and infiltration of parenchyma by hCD8-positive cells in immunized DR15 MS mice was increased compared to immunized DR15 HI mice, but differences in numbers of hCD8-positive T cells did not reach significance (**Fig. 4E** [↗](#)). A unique feature of immunized DR15 MS mice was the presence of large T cell lesions in the corpus callosum white matter (Figs. **4D** and **4G** [↗](#), arrowheads), and small lesions in other brain regions (e.g. hypothalamus, cortex, periventricular regions; data not shown). Locally activated Iba1-positive microglia and GFAP-positive astrocytes were present at sites of immune cell entry at CNS borders and in T cell lesions (**Fig. 4D** [↗](#)). In the spinal cord, like in immunized DR15 HI mice, hCD3-positive T cells accumulated at borders and individual cells infiltrated grey and white matter (**Fig. 5A** [↗](#), right panels), but forming small T cell lesions only in the white matter (WM) (**Fig. 5A** [↗](#), B). Numbers of hCD3-positive T cells counted in whole spinal cord sections were equal to those in immunized HLA-DR15 HI mice (**Fig. 5D** [↗](#)). Again, mCD45-positive cells were rare at barriers, absent from parenchyma, and demyelination was not observed in the brain (**Fig. 4G** [↗](#)) or spinal cord (**Fig. 5C** [↗](#)) of immunized DR15 MS mice.

To determine whether CNS immune infiltration in the immunized mice was secondary to levels of GVHD inflammation in the periphery, we performed semi-quantitative analysis of H&E stained liver and lung sections. Immunized DR13 MS mice showed the lowest levels of immune cell infiltration in liver and lung, DR15 MS mice intermediate levels, and DR15 HI highest levels (Supplementary Fig. 1). Results revealed an inverse relationship between peripheral and CNS inflammation in the DR15 HI and MS mice, with DR15 HI mice showing highest peripheral and relatively lower CNS involvement, while DR15 MS mice showed the highest CNS and relatively lower peripheral inflammation. These results show that immunization of DR15 HI and DR15 MS mice with myelin peptides equally induced spinal cord white matter inflammatory lesions, and uniquely induced large confluent corpus callosum white matter lesions in DR15 MS mice. Taken together, our results show that PBMC from a DR15-positive MS patient show increased propensity to induce inflammatory CNS lesions compared to PBMC from a DR13-positive MS patient or a DR15-positive healthy individual.

Discussion

Here we show that PBMC from RRMS and healthy donors with different HLA genotypes have the potential to efficiently engraft B2m-NOG mice with human T and B lymphocytes and to induce sub-clinical CNS inflammation dominated by hCD8⁺ T cells in the brain and the spinal cord. Specifically, mice engrafted with PBMC from DR15-positive MS and healthy donors, and not from a DR13-positive MS donor, showed spontaneous brain and spinal cord infiltration by hCD8⁺ and hCD4⁺ T cells which tended to be more severe in DR15 MS mice. DR15 MS mice uniquely developed prominent T cell lesions in several brain regions including the corpus callosum following

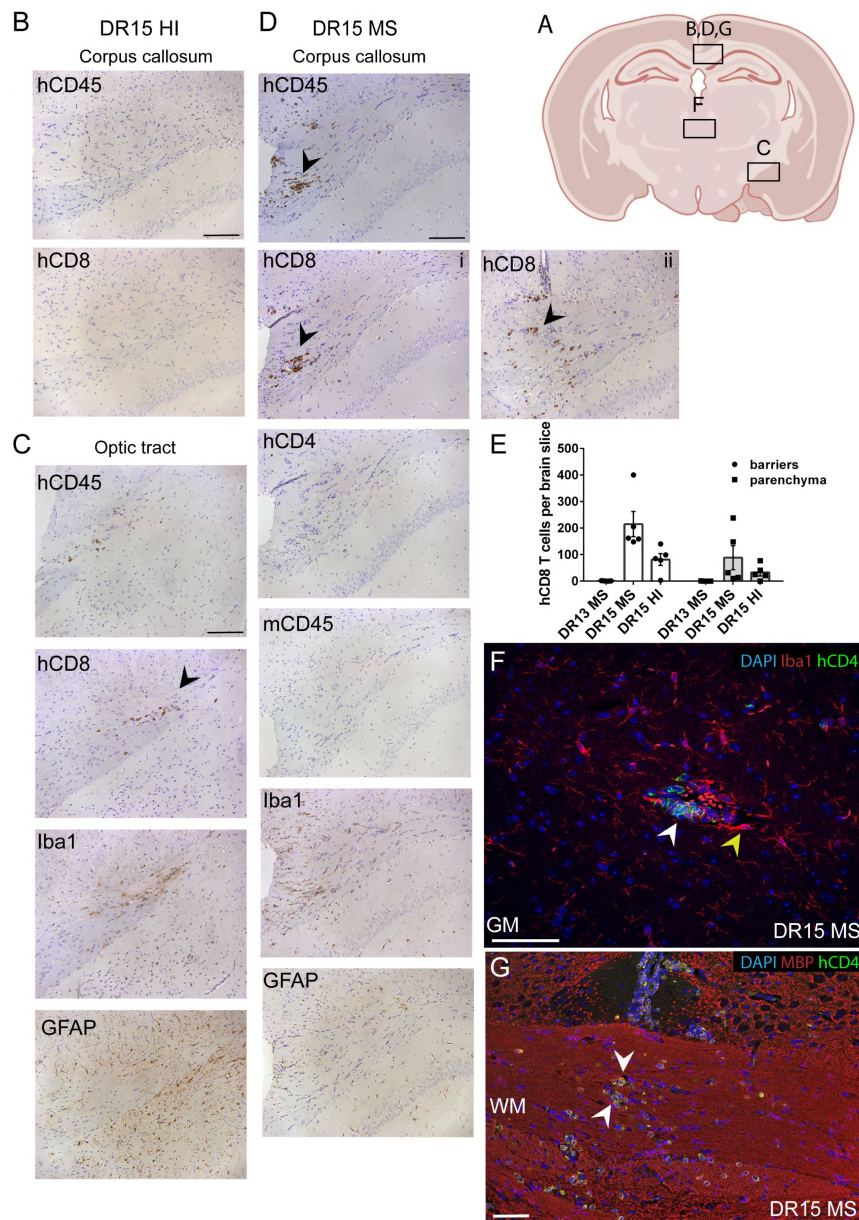


Figure 4

Immunization with myelin peptides increases T cell infiltration of brain parenchyma and promotes development of brain lesions in both DR15 MS and DR15 HI mice

Immunohistochemical analysis of the brain from PBMC B2m-NOG mice immunized for EAE showing infiltration by human (h) and mouse (m) CD45-positive leukocytes, hCD8- and hCD4-positive T cells, and local activation of Iba1-positive microglia and GFAP-positive astrocytes, in brain regions denoted in the diagram (A). (B, C) Individual hCD45-positive leukocytes and hCD8-positive T cells scattered in the corpus callosum white matter (B) and small lesions in the optic tract (C, arrowhead) in the presence of locally activated Iba1-positive microglia and GFAP-positive astrocytes in immunized DR15 HI mice. (D) Prominent lesions in the corpus callosum white matter of two of the three DR15 MS mice (i, ii, arrowheads), containing hCD45- and mCD45-positive leukocytes, hCD4- and hCD8-positive T cells and locally activated Iba1-positive microglia and GFAP-positive astrocytes. (E) Counting of barrier-associated and parenchymal hCD8-positive T cells in whole coronal sections of brain from DR13 MS, DR15 MS and DR15 HI mice. (F) Double immunofluorescence staining for hCD45-positive leukocytes (green, arrowhead) and Iba1-positive microglia/border macrophages (red, yellow arrowhead; non-specific red staining of erythrocytes in vessel lumen), with DAPI counterstained nuclei (blue), showing a small perivascular lesion in the thalamus grey matter (GM) of immunized DR15 MS mice. (G) Double immunofluorescence staining for hCD45-positive leukocytes (green, arrowheads) and MBP-positive myelin (red), with DAPI counterstained nuclei (blue), in corpus callosum in immunized DR15 MS mice, showing inflammatory lesion without demyelination. Scale bars 100 μ m; x20 objective (B, C, D, G); x40 objective (F). All results are depicted as mean $\pm$ SEM. Statistical analysis was performed by pairwise comparisons between different groups of mice using Student's t test.

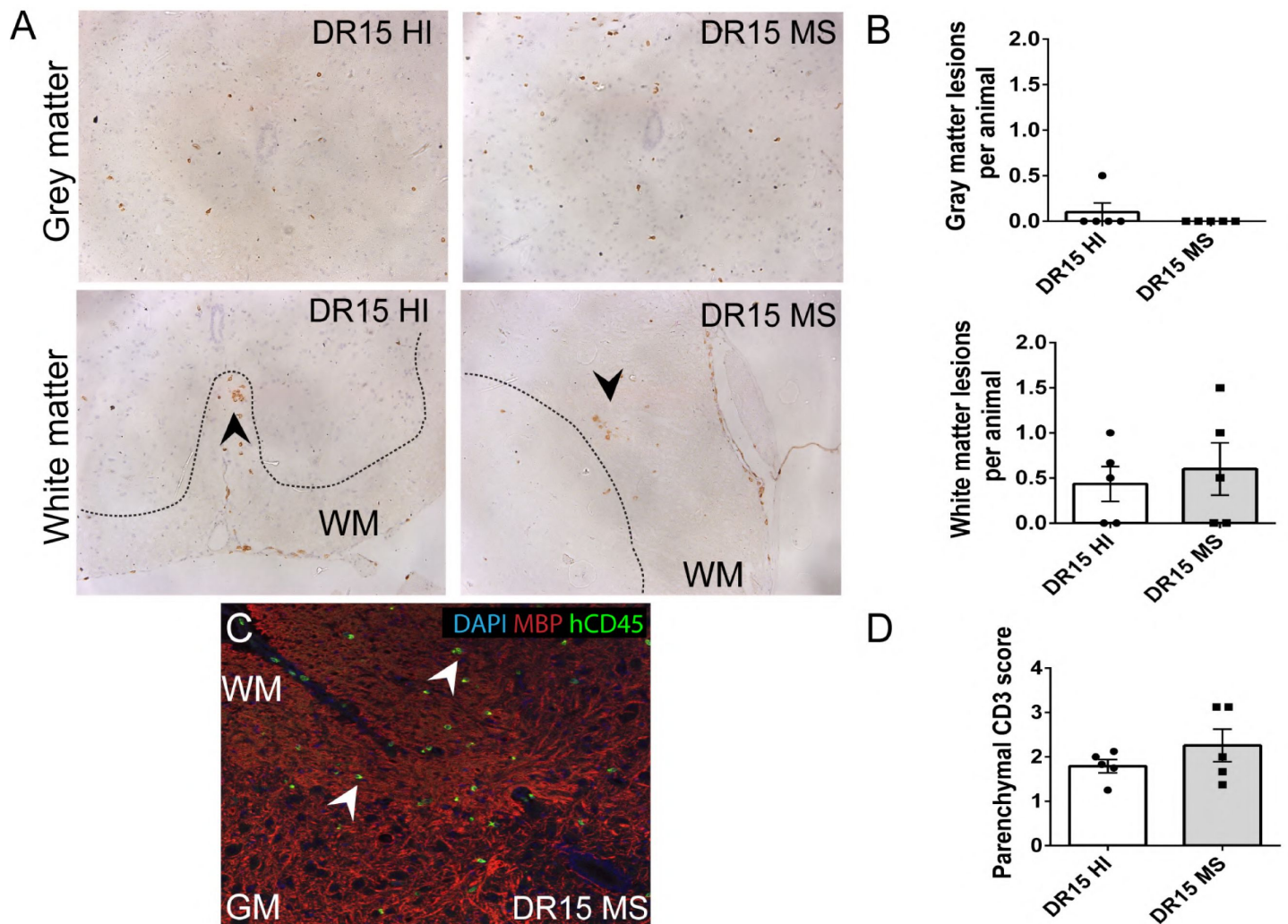


Figure 5

Immunization with myelin peptides induces T cell lesions in spinal cord white matter of both DR15 MS and DR15 HI mice

Immunohistochemical analysis of spinal cord from PBMC B2m-NOG mice immunized for EAE showing infiltration by human (h) CD3-positive T cells in the grey and white matter regions. **(A)** Infiltrating hCD3-positive T cells scattered throughout spinal cord grey and white matter and forming small lesions in the white matter (WM) of both DR15 MS and DR15 HI mice (lower panels, arrowheads). The dotted lines mark the boundary between grey matter and white matter. **(B)** Counting of hCD3-positive T cell lesions (≥ 3 adjacent cells) in grey and white matter in whole spinal cord sections from DR15 HI and DR15 MS mice. **(C)** Double immunofluorescence staining for hCD45-positive leukocytes (green, arrowheads) and MBP-positive myelin (red), with DAPI counterstained nuclei (blue), in white matter (WM) of immunized DR15 MS spinal cord. Grey matter (GM). **(D)** Semi-quantitative estimation of hCD3-positive T cells in whole spinal cord sections from DR15 HI and DR15 MS mice. All results are depicted as mean $\pm$ SEM. Scale bars 100 μ m; x40 objective (A). Statistical analysis was performed by pairwise comparisons between different groups of mice using Student's t test.

immunization with myelin peptides using an EAE protocol. T cell lesions were associated with locally activated microglia and astrocytes, but notably lacked infiltrating monocytes and demyelination, and did not lead to clinical symptoms of EAE or other neurological defects.

PBMC humanized B2m-NOG mice developed several hallmark features of MS that are not readily reproduced in conventional animal models, such as EAE. First, brain immune infiltrates in DR15 MS engrafted mice were enriched for hCD8⁺ over hCD4⁺ T cells, even though the B2m-NOG model is characterized by increased engraftment of CD4⁺ over CD8⁺ T cells (18) (Figure 1B). The preferential infiltration of the CNS parenchyma by CD8⁺ T cells, and the presence of inflammatory lesions in the brain white matter in our model are both features of MS (3,8,27). Second, T cell lesions and locally activated microglia and astrocytes, were observed in brain white matter regions, particularly the corpus callosum. Lesions in the corpus callosum are important findings in MRI of MS patients and are correlated with cognitive problems as well as disability in the upper limbs (28). Third, human leukocytes were concentrated on the meninges and choroid plexus, notably in the interventricular foramen connecting the lateral and third ventricles. The choroid plexus is an important immunological site for immune surveillance and a site of CD8⁺ T cell and granulocyte involvement in progressive MS (29).

Nevertheless, MS is a complex disease and immunopathology was only partially reproduced in the brain and the spinal cord of PBMC humanized B2m-NOG mice. Parenchymal lesions contained both hCD4⁺ and hCD8⁺ T cells associated with locally activated microglia and astrocytes, but there was poor engraftment by human monocytes in the blood, spleens or CNS of both immunized and non-immunized B2m-NOG mice. Blood myeloid cells are short-lived and constantly renewed by the bone marrow, so that human myeloid cells are nearly absent in the commonly used PBMC-NSG model (18). In contrast, mouse CD45⁺ cells comprising very high proportions of CD11b⁺ myeloid cells, Ly6C^{hi} monocytes and Ly6G⁺ cells, were present in the blood of immunized and non-immunized NOD-*scid* mice, which are a parental strain for B2m-NOG mice. Nevertheless, mouse CD45⁺ myeloid cells did not infiltrate the CNS of the humanized mice, even after EAE induction. The absence of CNS-infiltrating monocytes in the PBMC humanized B2m-NOG mice NOG mice likely underlies the absence of clinical symptoms and demyelination in this system. Our results are consistent with a previous study in which NSG mice were humanized with PBMC from healthy donors and immunized for EAE (20). In that study, mice developed CNS inflammation with infiltrating CD4⁺ and CD8⁺ T cells but did not show clinical symptoms or demyelination (20). CNS-infiltrating Ly6C^{hi}CCR2⁺ monocytes, which differentiate into inflammatory macrophages locally in the CNS after being activated by T cells, are critical effector cells in EAE (22,30). These cells are massively recruited into the periphery from the bone marrow following immunization (23), and are likely to compete for space with engrafted human immune cells following EAE induction. The use of immunodeficient NOG/NSG models expressing human transgenes that would enhance the engraftment of human monocytes, such as GM-CSF, or facilitate communication of human T cells with mouse inflammatory monocytes (31,32), should allow a more comprehensive modeling of MS immunopathology in humanized mice.

Another limitation for MS modeling in the B2m-NOG mice used in this study is the poor LN structure and functioning that results from depletion of the common cytokine receptor γ -chain gene (24). This deficiency was confirmed in this study by the absence of organized LN and germinal follicles, and of anti-MOG IgG antibodies in the PBMC humanized B2m-NOG mice immunized for EAE. Again, further development of available immunodeficient mouse strains to promote LN organogenesis will be an important step for modeling MS immunopathology in mice. In a previous study, NSG mice injected directly in the CNS with CSF cells from MS patients (33) developed clinical symptoms, immune infiltration of the CNS and demyelination, although differences in the route of administration of the transplanted cells might explain the differences between our study and that of others (20).

Our observation that mice engrafted with PBMC from a DR15-positive active RRMS patient showed both spontaneous and EAE-inducible T cell lesions in the brainstem and spinal cord is highly relevant in the context of previous data concerning this haplotype. HLA-DR2 has long been associated with MS (34), with the DR15 haplotype being linked to earlier disease onset (1,35). MHCII molecules present antigens to CD4⁺ T cells, and DR15 is strongly associated with the autoproliiferation of peripheral CD4⁺ T cells in MS patients (36). T cell autoproliiferation is mediated by memory B cells in a HLA-DR-dependent manner, and the autoproliiferating T cells are increased in MS patients treated with natalizumab and enriched for brain-homing T cells (37). In a recent study with humanized NSG mice, CD34⁺ hematopoietic progenitor cells (HSC) from DR15-positive donors induced higher peripheral T cell responses and alloreactivity compared to HSC from DR15-negative donors, and increased CD8⁺ T cell responses after Epstein-Barr virus (EBV) infection (38). There is now strong evidence that EBV infection plays a major role in MS pathogenesis (39), acting synergistically with DR15 to increase the likelihood of developing MS (34). DR15 individuals with specific antibody reactivity to EBNA1 (amino acid 385–420) have 24-fold increased risk for MS (40). Anti-EBNA-1 antibodies present high homology and react with several CNS antigens, such as αB-crystallin, MBP, anoctamin 2 and more recently glial cell adhesion molecule (41–44). In our study, the DR15 MS and HI donors were both positive for IgG and IgM anti-EBNA-1 antibodies, which can be a sign of autoimmune disease, whereas the DR13 MS patient was positive for IgG anti-EA (early antigen) antibodies, which indicates either primary EBV infection or possible reactivation (Table 1, and Supplementary Table 1C for interpretation). In the PBMC humanized mice, we only found immune system infiltration and brain lesions with the DR15 MS and DR15 HI donors and not with the DR13 MS donor. This could be indicative of the presence of autoproliiferating and brain-homing CD4⁺ and CD8⁺ T lymphocytes in the blood of the DR15 MS and HI donors, particularly following treatment with natalizumab, prior to transplantation in B2m-NOD mice, increasing the likelihood of cross-reactivation upon encounter of similar mouse CNS antigens. Nevertheless, positive correlations between the DR15 haplotype, T cell autoproliiferation, EBV and the development of brain lesions in humanized mice remain to be formally tested in a larger study using greater numbers of DR15-positive MS donors against DR15 negative MS and DR15-positive healthy controls, together with the measurement of basal levels of T cell proliferation in the peripheral blood of the PBMC donors.

In summary, PBMC humanized B2m-NOD mice show partial representation of MS immunopathology that is not reproduced in conventional animal models such as EAE, particularly the development of hCD8⁺ lesions in the grey and white matter of the brain and spinal cord. New immunodeficient mouse strains developed to promote human monocyte engraftment and the development of organized LN therefore hold great promise for comprehensive modelling of MS immunopathology. An important outcome of this study is that PBMC humanized B2m-NOD mice highlight the variability between the different human PBMC donors, and therefore represent valuable research tools for generating personalized immune models for drug screening.

Materials & Methods

MS patients and healthy subjects

Blood donors were selected from a Hellenic MS patient cohort attending the MS outpatient clinic at Aeginition University Hospital of the National and Kapodistrian University of Athens (NKUA) School of Medicine, supervised by Dr. Maria Anagnostouli and genotyped for the HLA-DRB1 allele, at the Research Immunogenetics Laboratory, of the same Hospital, as previously described (21) (Table 1). Two patients with long-term RRMS recently presenting with highly active disease since the last dose of immunomodulatory therapy (natalizumab) were selected. Natalizumab treatment was chosen as a criteria for this study because it blocks lymphocyte migration to the brain, thereby increasing circulating T and B lymphocytes (45). One patient was DR15-positive (DR15 MS), the other DR13-positive (DR13 MS). The DR13-positive patient additionally received

cortisone treatment 1 month prior to sampling because of high disease activity. A healthy DR15-matched healthy individual (DR15 HI) was selected as control. Donor characteristics, including viral infections and EBV status are listed in **Table 1** [↗](#). Blood samples were obtained from MS patients and healthy individuals under signed informed consent in accordance with the Declaration of Helsinki and approval from the Institutional Ethics Committee of Aeginition Hospital, NKUA (Protocol No: 7BSH46Y8N2-B66, 13/05/2015).

PBMC isolation

Fresh peripheral blood (50 ml) was collected in EDTA-treated polypropylene tubes and PBMC were isolated under Ficoll-Histopaque®-1077 gradient centrifugation (Sigma-Aldrich). Buffy coats containing blood leukocytes were washed with 2% FBS in PBS. Erythrocytes were removed using ACK erythrocyte lysis buffer (BioLegend) and leukocytes were washed with 2% FBS in PBS. Before transfer of PBMC into mice, we compared several protocols for preparation of human PBMC using blood from a healthy individual, specifically, 1) PBMC isolated from fresh blood on the day of transfer, 2) PBMC isolated from blood after 3 days' storage at 4°C, 3) PBMC cultured for 24 h in complete RPMI-1640 after snap freezing of fresh PBMC and thawing. Cell viability and analysis of immune cell populations by flow cytometry showed that freshly isolated PBMC gave the best results and were used for transfer in this study (Supplementary Table 2).

EBV antibody responses in human blood

Blood plasma from the DR13 MS, DR15 MS and DR15 HI donors was analyzed for EBV antibody responses using the following ELISA tests: ELISA-VIDITEST anti-VCA EBV IgG (ODZ-265), ELISA-VIDITEST anti-EA (D) EBV IgG (ODZ-006), ELISA-VIDITEST anti-EBNA-1 EBV IgM (ODZ-002) and ELISA-VIDITEST anti-EBNA-1 EBV IgG (ODZ-001) (Vidia). Standardized criteria for the clinical interpretation of EBV antibody responses are shown in Supplementary Table 1C.

Mice

Ten-to-twelve-week-old female immunodeficient B2m-NOD/Shi - *scid IL2rg^{null}* (B2m-NOG) mice (stock number 14957-F: NOD.Cg-B2m Prkdc <SCID>IL2rg<TM1SUG>JicTac; Taconic Biosciences) were used for transfer of freshly isolated human PBMC. These mice lack mature T, B, and NK cells and lack MHC class I molecules. Previous studies show that an equivalent mouse strain, *NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}B2m^{tm1Unc}/Sz* (NSG β2m^{null}; The Jackson laboratory) facilitates the engraftment of CD4⁺ over CD8⁺ T cells and exhibits delayed onset of graft versus host disease ([17](#) [↗](#), [18](#) [↗](#)). *NOD.CB17-Prkdc scid/NCrHsd* (Envigo) were used for investigation of peripheral mouse myeloid cell populations in a related severely immunodeficient mouse strain by flow cytometry.

Mice were transplanted via tail vein injection with 10x10⁶ PBMC in 200 μL PBS on the same day as cell isolation from fresh peripheral human blood. Mice were assessed daily for symptoms of GVHD, such as fur ruffling, hunched posture, reduced mobility and tachypnea ([26](#) [↗](#)), as well as for any spontaneous neurological deficits ([25](#) [↗](#)).

All experiments were performed under sterile conditions in the Department of Animal Models for Biomedical Research of the Hellenic Pasteur Institute. Mice were housed in a specific pathogen-free facility in microisolator cages and given *ad libitum* UV-sterilized standard chow, acidified water and gels for hydration. The experiments complied with ARRIVE guidelines and were in accordance with the local Ethical Committee guidelines on the use of experimental animals at the Hellenic Pasteur Institute, were approved by the national authorities and complied to EU Directive 2010/63/EU for animal experiments.

Immunization with myelin peptides

Peptides MBP83-99, MOG1-20, and mMOG35-55 and hMOG35-55 (S42 in mMOG, P42 in hMOG), were synthesized as previously described (46). In a previous study, we found that humanized HLA-DR2b transgenic mice lacking all mouse MHCII genes required greater amounts of peptide antigen to induce clinical EAE than wild-type B6 mice (21). Due to limited numbers of B2m-NOG mice available for this study, several EAE protocols were first tested using wild-type B6 mice, mainly to exclude the possibility of toxicity at high peptide doses. Briefly, 8-week-old female B6 mice were immunized by subcutaneous (s.c.) tail-base injection of 37 µg or 200 µg mMOG35-55, or a myelin peptide antigen cocktail containing 200 µg each of mMOG35-55, hMOG35-55, MOG1-20 and MBP83-99, dissolved in 100 µl saline and emulsified in an equal volume of Freund's complete adjuvant (FCA) (Sigma-Aldrich). FCA was supplemented with 400 µg/injection of H37Ra *Mycobacterium tuberculosis* (Difco). All mice, except those immunized using the standard protocol (37 µg mMOG35-55), received an identical boost immunization 7 days later. The immunized mice also received intraperitoneal (i.p.) injections of 200 ng of *Bordetella pertussis* toxin (PTx) (Sigma-Aldrich) at the time of each immunization and 48 h later. All mice developed clinical symptoms of EAE with no evidence of increased morbidity or mortality at high peptide doses. For this reason, the high-dose peptide cocktail was chosen for immunization of groups of 12-14-week-old PBMC humanized B2m-NOG mice on dpt 14. Mice were monitored daily for the clinical symptoms of EAE according to criteria commonly used for scoring EAE: 0, normal; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; 5, moribund or dead (0.5 gradations represent intermediate scores) (21). Mice were also monitored for signs of other neurological deficits (25).

MOG antibody responses in PBMC humanized mouse blood

Peripheral blood was collected from immunized and non-immunized mice at sacrifice on dpt 42, and sera collected for analysis of antibody responses. Samples were screened for IgG antibodies against MOG, using indirect immunofluorescence assays (IFA). Sera were analyzed at a starting dilution of 1:10, on EU 90 cells transfected with MOG protein and on EU 90 control transfected cells, according to the manufacturer instructions (Euroimmun, Lubeck, Germany). Visualization and evaluation of IFA results were performed under a fluorescence microscope (Zeiss, Axioskop 40).

Flow cytometry

Fresh peripheral blood samples from the MS and HI donors were analyzed by FACS using a panel of immune cell marker antibodies. To monitor immune cell populations, 3 ml fresh peripheral blood was collected in EDTA tubes and a 100 µl aliquot was used for flow cytometry. Erythrocyte lysis was achieved with with Quicklysis™ (Cytognos) for 25 min. Cells were washed and incubated with an antibody mixture for human immune cell markers BV-510-hCD45 (clone 2D1), APC-Cyanine 7-hCD3 (clone HIT3a), PE-Cyanine 7-hCD4 (clone A161A1), PerCP-hCD8 (clone SK1), PE-hCD19 (clone 4G7), FITC-hCD56 (clone 5.1H11), APC-hCD66b (clone G1OF5), BV-421-hCD14 (clone HCD14) (all from Biolegend), for 30 min at 4 °C (Supplementary Table 1A). To monitor engraftment of human immune cells in B2m-NOG mice, peripheral blood was collected in heparin tubes from the tail vein at dpt 7, 13 and 42. Erythrocyte lysis and incubation with antibody mixture was performed as for fresh human blood above, except that the APC-hCD66b antibody was replaced by mouse APC-CD45 (clone 30-F11) (Biolegend) (Supplementary Table 1A). Data was acquired with FACSCelesta™ or FACSCanto™ II cytometer and analyzed with FACSDiva™ (BD) and FlowJo software (Tree Star, Inc.).

For intracellular staining of IFN-γ, splenocytes were isolated at sacrifice at dpt 42 as described above, and cells were stimulated with phorbol 12-myristate 13 acetate (PMA) (20 ng/ml, Sigma-Aldrich) and ionomycin (1µg/ml, Sigma-Aldrich) and treated with brefeldin-A (5µg/ml, Sigma-Aldrich) for 3 h at 37°C/5% CO2 in order to disrupt Golgi-mediated transport. Cells were fixed with 2% PFA in FBS, permeabilized using 0.5% wt/vol saponin, and stained with fluorochrome-

conjugated antibodies for APC-CD4 (clone RPA-T4, BD Biosciences), CD8-PerCP (clone SK1, Biolegend), and PE-IFN- γ (clone B27; BD Biosciences) and PE-IL-17A (clone eBio64DEC17, eBioscience). Data acquisition was performed using a FACSCaliburTM cytometer and analyzed with FlowJo software (Tree Star, Inc.).

T cell proliferation assay

Spleens were isolated at sacrifice and homogenized in RPMI (Invitrogen Life Technologies) containing 10% heat-inactivated FCS. To obtain single cell suspensions, the washed homogenate was passed through a 70 μ m cell strainer and erythrocyte lysis was performed with Gey's erythrocyte lysis buffer for 5 min. The reaction was stopped with RPMI 1640/FCS. Washed splenocytes at a concentration of 10^7 cells/mL in PBS were incubated with 5 μ M carboxylfluorescein succinimidyl ester (CFSE, V12883, Thermofisher) for 15 min at 37°C. Cells were washed and PBS/2% FCS was applied for 30 min at 37°C to stop the reaction. Cells were washed and resuspended in RPMI 1640/FCS with 50 μ M 2-mercaptoethanol (Sigma-Aldrich) at concentration of 10^6 cells/mL. The cells were stimulated with in triplicates in round-bottom 96-well plates with a myelin peptide antigen cocktail containing 30 μ g/mL each mMOG35-55, hMOG35-55, MOG1-20 and MBP83-99 for 120 h. Negative control cells were incubated with medium only, and positive control cells were incubated in plates coated with anti-CD3 (1 μ g/mL) (clone HIT3a, BD Biosciences). Results were expressed as cell division index which is the ratio of %CFSE^{low} splenocytes (proliferating splenocytes) cultured with peptides or anti-CD3 to the of %CFSE^{low} splenocytes cultured with medium only. Data acquisition was performed using a FACSCaliburTM cytometer and analyzed with FlowJo software (Tree Star, Inc.).

Histopathology and Immunohistochemistry

Mice were transcardially perfused with ice-cold PBS at sacrifice on dpt 42 by carbon dioxide inhalation. Brains, spinal cords and peripheral tissues (including lung, liver and inguinal fat tissue) were dissected and post-fixed in 4% paraformaldehyde fixative (PFA) overnight at 4°C. Tissues were embedded in paraffin and 5 μ m sections and used for immunohistochemistry and histopathology. Inflammation in peripheral tissues was visualized by haematoxylin and eosin (H&E) using standard techniques. Immune cell infiltration and inflammation of brain and spinal cord was visualized by immunohistochemistry using antibodies specific for hCD45, hCD4, hCD8, mCD45, and mouse Iba1 and GFAP followed by biotinylated secondary Abs, horseradish peroxidase-labeled avidin-biotin complex, and signal development using 3'3'-diaminobenzidine. Nuclei were counterstained with hematoxylin. Images were captured with an Olympus DP71 microscope digital camera using cell^A imaging software (Soft Imaging System GmbH). High resolution immunofluorescence imaging was performed by confocal microscopy using paraffin sections immunostained with marker antibodies for myelin (MBP), microglia (Iba1) followed by anti-rat CF-647-(Biotrium) and anti-rabbit AlexaFluor 568-(Invitrogen) labelled secondary antibodies, respectively. Nuclei were counterstained with DAPI. A Leica TCS SP8 confocal microscope was used to acquire fluorescent images.

Statistical Analysis

Data were processed using Microsoft Excel, and statistical analysis was performed with Graphpad Prism 8. Figures were made using Adobe Illustrator V24.3 Data was analyzed with Student's t test and results are presented as means $\pm$ SEM. Results were considered statistically significant when $p \leq 0.05$. Graphical abstract was created using BioRender.com

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Conflict of interest statement

The authors report no conflict of interest.

Ethics approvals

The studies involving human participants were reviewed and approved by the Scientific Committee of Aeginition Hospital, National and Kapodistrian University of Athens, Greece. Licence 283/13-05-2015ADA: 7BΣH46Y8N2-B66. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Committee for Evaluation of Experimental Procedures, Department of Experimental Animal Models, Hellenic Pasteur Institute (Presided by Dr P Andriopoulos pandriopoulos@patt.gov.gr for the Hellenic Republic, General Secretariat for Agricultural Economy, Veterinary and Licenses). Licence number 770851/27-11-2019.

Patient consent statement

Written informed consent was obtained from the human individual(s) for the publication of any potentially identifiable images or data included in this article.

Authors contributions

IP designed, set-up and performed experimental protocols for PBMC preparation and humanization of B2m-NOG mice, monitored mice for clinical symptoms and performed immunohistochemical analysis of CNS tissues, analysed results and prepared figures; MK performed quantitative immunohistochemical analysis of CNS tissues, analysed results and prepared figures; AD designed and performed EAE experiments, monitored mice for clinical symptoms, performed immunohistochemistry of LN tissues and FACS analyses of humanized mice, analysed results and prepared figures; LD designed human FACS antibody panel, performed analyses of human and mouse blood and analysed results; VG performed FACS analysis and EBV ELISA on fresh human donor blood samples; NM collected and co-ordinated delivery of human donor blood samples; FB performed histological analysis of peripheral humanized mouse tissues and evaluated GVHD; MB and MA designed and performed cell-based anti-MOG antibody assays; TT designed, synthesized, purified and characterized myelin peptide analogues; MA performed HLA genotyping, selected patients for this study, and supervised Hellenic MS patient cohort; LP was responsible for overall experimental design and management of project, analysed and interpreted data, wrote paper. All authors contributed to the article, edited, and approved the submitted version.

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Reviewer #1 (Public Review):

Papazian et al. demonstrate that human peripheral blood mononuclear cells (PBMCs) can be successfully and stably grafted into immunodeficient mice. They demonstrate that the adoptive transfer of PBMCs from multiple sclerosis (MS) patients is capable of inducing damage to the central nervous system (CNS). Furthermore, they demonstrate that the CNS inflammatory properties of these transferred cells are more dependent on HLA restriction rather than the disease status of the donor. Specifically, T cells restricted by HLA-DR15 (from both an MS patient and a healthy control) showed a greater propensity to induce neuroinflammation than T cells transferred from an MS patient that are restricted by a different MHC haplotype. This observation suggests that the differences in the peptide repertoire presented by this MHC haplotype biases adaptive immune responses toward encephalitogenic T cell generation. The conclusions of this paper are partially supported by their data, but the lack of important considerations and various controls limits the overall impact of this study. Major weaknesses of this study include:

1. The extent to which various immune cell quantification is performed. Two of the reasons the authors cite for the use of this model rather than a traditional EAE model are: i) the lack of involvement of CD8 T cells in the pathogenesis of EAE and ii) the marginal importance of B cells in EAE pathogenesis. However, throughout their paper, the authors never quantify the difference in CD4 vs CD8 T cell infiltration into the CNS. While repeatedly claiming that there are fewer CD4 T cells present than CD8 T cells within the CNS, this data is not included. Further, spinal cord numbers of CD4 and CD8 are not provided in lieu of CD3 T cell characterization. Given that there are far more hCD4 T cells in the periphery in these mice than CD8 T cells as well as the fact that the lack of B2m expression in this mouse model biases cells towards a CD4 fate, the omission of these data is concerning. Additionally, B cells don't make up any significant component of the cells transferred from HLA-DR15 donors. While the cells transferred from the HLA-DR13 donor are composed of a considerable number of B cells, the mice that received these cells didn't develop any signs of neurologic disease.

2. Incomplete exploration of potential experimental autoimmune encephalomyelitis (EAE) modeling. The authors justify the use of an extremely high amount of myelin peptide when immunizing their mice by citing that another humanized mouse model had such a requirement to induce clinical EAE. However, a demonstration of this technical requirement in their own model is not provided. Rather, they show that C57BL/6 mice get milder disease when such large doses of peptides are administered, leading to speculation that this is due to a tolerizing immune response that occurs at such high doses. Comparison of the susceptibility of B2m-NOG mice to EAE dependent on various peptide doses would be highly informative. Given that the number of hCD45⁺ in the periphery of NOG mice decreases following this immunization it would be prudent for the authors to determine if such a high peptide dose is truly ideal for EAE development in this mouse model.
3. The degree of myelin injury is not presented. The statement is repeatedly made that "demyelination was not observed in the brain or spinal cord" but no quantification of myelin staining is shown. A central feature of multiple sclerosis and related diseases is demyelination of the CNS. Hence, while compartmentalized inflammatory responses are detailed in this report, the utility of the humanized model for the exploration of human CNS demyelinating diseases remains unclear and in doubt.

Minor points:

- Method of quantification (e.g. cells per brain slice in figures 2E; 4E) is not very quantitative and should be justified or more appropriately updated to be more rigorous in methodology.
- Fig. 4 data should be shown from un-immunized DR15 MS and DR15 HI mice.

The premise of this work carries great potential. Namely, developing a humanized mouse system in which features of adaptive immunity that contribute to inflammatory demyelination can be interrogated will allow for traction into therapeutics currently unavailable to the field. Immediate questions stemming from the current study include the potential effect of ex vivo activation of PBMCs (or individual T and B cells) in vitro prior to transfer as well as the TCR and BCR repertoire of CNS vs peripheral lymphocytes before and after immunization. This group has been thoughtful and clever about their approach (e.g. use of subjects treated with natalizumab), which gives hope that fundamental aspects of pathogenesis will be uncovered by this form of modeling MS disease. Overall, while the current study makes several unique observations, the data collection is incomplete and the impact of this study could be greatly improved by addressing the limitations noted.

Reviewer #2 (Public Review):

Multiple sclerosis is an inflammatory and demyelinating disease of the central nervous system where immune cells play an important role in disease pathobiology. Increased incidence of disease in individuals carrying certain HLA class-II genes plus studies in animal models suggests that HLA-DRB1*15 restricted CD4 T cells might be responsible for disease initiation, and other immune cells such as B cells, CD8 T cells, monocytes/macrophages, and dendritic cells (DC) also contribute to disease pathology. However, a direct role of human immune cells in disease is lacking to a lag between immune activation and the first sign of clinical disease. Therefore, there is an emphasis on understanding whether immune cells from HLA-DR15⁺ MS patients differ from HLA-DR15⁺ healthy controls in their phenotype and pro-inflammatory capacity. To overcome this, authors have used severely immunodeficient B2m-NOG mice that lack B, T cells, and NK cells and have defective innate immune responses and engrafted PBMCs from 3 human donors (HLA-DR15⁺ MS and HI donors, HLA-DR13⁺ MS donor) in these B2m-NOG mice to determine whether they can induce CNS inflammation and demyelination like MS.

The study's strength is the use of PBMCs from HLADRB1-typed MS subjects and healthy control, the use of NOG mice, the characterization of immune subsets (revealing some interesting observations), CNS pathology etc. The major weaknesses are i) lack of sufficient sample size (n=1 in each group) to make any conclusion, ii) lack of phenotype in mice, iii) no disease phenotype even in humanized mice immunized for disease using standard disease induction protocol employed in an animal model of MS, and iv) mechanistic data on why CD8 T cells are more enriched than CD4+ T cells. The last point is very important as postmortem human MS patients' brain tissue had been shown to have more CD8+ T cells than CD4+ T cells.

Thus, this work is an important step in the right direction as previous humanized studies have not used HLA-DRB1 typed PBMCs however the weaknesses as highlighted above make the findings incremental to the field.