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J Immunol. Author manuscript; available in PMC 2013 September 15.

Published in final edited form as:
J Immunol. 2012 September 15; 189(6): 2897–2908. doi:10.4049/jimmunol.1103087.

Myelin Basic Protein-Specific TCR/HLA-DRB5*01:01Transgenic Mice Support the Etiologic Role of DRB5*01:01 in Multiple Sclerosis **

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Abstract

Genetic susceptibility to multiple sclerosis (MS) has been linked to the HLA-DR15 haplotype consisting of DRB1*15:01(DR2b)- and DRB5*01:01(DR2a) alleles. Given almost complete linkage disequilibrium of the two alleles, recent studies have suggested differential roles in susceptibility (DR2b) or protection from MS (DR2a). Our objective was to assess the potential contribution of DR2a to disease etiology in MS using a humanized model of autoimmunity. To assess the potential contribution of DR2a to disease etiology, we created DR2a humanized transgenic (Tg) mice and subsequently crossed them to Tg mice expressing TL3A6, an MS patient-derived myelin basic protein (MBP)83-99 -specific T cell receptor (TCR). In TL3A6/DR2a Tg mice, CD4 Tg T cells escape thymic and peripheral deletion and initiate spontaneous experimental autoimmune encephalomyelitis (EAE) at low rates depending on the level of DR2a expression. The ability to induce active EAE was also increased in animals expressing higher levels of DR2a. Inflammatory infiltrates and neuronal damage were present throughout the spinal cord consistent with a classical ascending EAE phenotype with minor involvement of the cerebellum, brainstem and peripheral nerve roots in spontaneous as well as actively induced disease. These studies emphasize the pathologic contribution of the DR2a allele to the development of autoimmunity when expressed as the sole MHC class II molecule, and strongly argue for DR2a as a contributor to CNS autoimmunity in MS.

**This work was supported by the National Institutes of Health (NIH) intramural program and in part by an NIH grant (R21 AI067474 to K. Ito) with additional support from the Multiple Sclerosis National Research Institute. JQ was supported by fellowships from the MS Society of Canada and the National MS Society.

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Keywords

EAE; MS; myelin basic protein; humanized transgenic mice

Introduction

The major histocompatibility complex (MHC, Human leukocyte antigen, HLA, in humans) region and particularly the HLA-DR15 haplotype are long known as the major genetic susceptibility determinant in multiple sclerosis (MS)(1-5). An estimated 10-60% of the genetic risk appears to be conferred by the DR15 haplotype (6), while approximately 3% or less are attributed to recently identified cytokine receptor genes (IL7RA, IL2RA) and other quantitative trait loci (7-10). The HLA-DR15 haplotype is comprised of two DR beta chain genes, HLA-DRB1*15:01 and HLA-DRB5*01:01(3), and these are in virtually complete linkage disequilibrium. The two DR beta chains are expressed together with DR alpha and form the heterodimeric membrane proteins DR2a (DRA1*01:01/DRB5*01:01) and DR2b (DRA1*01:01/DRB1*15:01), respectively. Whether both or only one of the two alleles and resulting DR molecules contribute to MS etiopathogenesis is one of the most important questions in MS research. A role for both alleles in MS has been postulated earlier based on the ability of both DR2a and DR2b molecules to support myelin autoreactivity in MS (11, 12). Each allele can serve as restriction element for MBP-specific CD4 T cells, which are postulated to drive autoimmunity in MS (13-17). Furthermore, both DR2a and DR2b bind the immunodominant MBP peptide (83-99), probably the best examined autoantigen in humans (17-20).

Different from the above early studies, a series of observations during recent years pinpoints MS risk to the DR2b allele. Genetic association studies in a small number of African Americans, who show greater haplotype diversity in the HLA region, indicated that MS risk may be separated from DR2a (21). MBP (84-101)/DR2b complexes could be shown with a monoclonal antibody in MS brains (22), suggesting that they are targets for autoimmune T cell responses in the target tissue. Furthermore, the occurrence of spontaneous autoimmune disease in humanized Tg mice expressing a MBP (84-102)-specific T cell receptor (TCR) and DR2b (23, 24) have lent support to the functional role of DR2b and its contribution to MS. Research into the relationship between vitamin D, a confirmed environmental risk factor for MS [(25),(26)], and MS has indicated the involvement of DR2b in disease based on the identification of vitamin D-responsive elements in the transcriptional regulatory regions controlling expression levels of DR2b, which, according to Ramogapalan et al. (27), are not conserved in several other alleles. Particularly the findings that HLA-DRB5*null subjects are at an increased risk for the more debilitating secondary progressive (SP) form of MS in African Americans (28), and a disease-ameliorating effect of DR2a in protecting from severe MBP-specific EAE in DR2b Tg mice (29) have been interpreted as evidence that evolutionary pressure to counterbalance the negative influence of DR2b by DR2a underlies the linkage disequilibrium of the two alleles. Gregersen et al. therefore have argued for an epistatic interaction such that the risk to develop autoimmune disease that is conferred by one allele (DR2b) is offset by the linked second allele (DR2a) (29).

A number of observations primarily from our laboratory argue, however, that the situation may be more complex and that both alleles are indeed jointly involved in MS etiopathogenesis. When characterizing in detail the MBP-specific CD4 T cell response in MS patients and healthy controls, T cell lines (TCL) and clones (TCC) restricted by DR2b or DR2a can both easily be established (11, 15, 17, 30). As mentioned above, the immunodominant MBP peptide (83-99) binds strongly to both DR2b and DR2a (17, 20). Perhaps more importantly, DR2b binds only one MBP peptide, (83-99), while DR2a binds at

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least three (83-99, 131-145/139-153, 76-91)(11, 12). Furthermore, MBP-specific DR2a- and DR2b-restricted CD4 TCL/TCC show distinct phenotypes. DR2a-restricted TCC are often capable of perforin-mediated and very efficient target cell lysis and more frequently show a Th1 phenotype (15). In contrast, DR2b-restricted TCL/TCC lyse target cells relatively inefficiently by Fas/Fas-L-mediated mechanisms (15, 31, 32). When examining the relative expression of DR2b versus DR2a in different cells and tissues, the DR2a heterodimers are expressed at higher levels in almost all cells and tissues when compared with DR2b heterodimers at the mRNA- and protein level (33).

The above discrepancies led us to address whether DR2a contributes to autoimmune inflammation in the central nervous system (CNS) in a humanized transgenic EAE model. Toward this end, we chose a well-characterized MBP (83-99)-specific and DR2a-restricted TCR, TL3A6, which we had isolated from a MS patient (30) and which has been co-crystallized with MBP (83-99)/DR2a (34). The present study characterizes humanized transgenic mice carrying the DRA1*01:01- and DRB5*01:01 alleles to form the heterodimer DR2a together with the TCR-alpha and -beta chains of the TL3A6 TCR. The TL3A6/DR2a Tg mice develop spontaneous autoimmunity with associated demyelination and axonal loss at low rates, similar to those described in a DR2b/MBP-TCR Tg mouse (23) and following immunization develop more severe clinical disease. The predisposition for disease is increased in animals expressing higher levels of DR2a supporting a role for this molecule in the pathogenesis of autoimmune disease. These observations show that DR2a like DR2b can restrict pathogenic T cell responses in humanized mice and that both alleles are likely to contribute to the etiopathogenesis of MS.

Materials and Methods

Generation of TL3A6/DR2a Tg Mice

The HLA-DRB5*01:01-IEβ gene construct was generated by replacing the HLA-DRB1*04:01 exon 2 gene of HLA-DRB1*04:01-IEβ fusion gene (35) with exon 2 of the HLA-DRB5*01:01gene. Briefly, the *Eco*RI fragment containing HLA-DRB1*04:01 exon 2 and I-E intron genes was subcloned into a pWS vector (Stratagene) and was used to replace HLA-DRB1*04:01 exon 2 with HLA-DRB5*01:01 exon 2 with a Seamless cloning kit (Stratagene). The HLA-DRA-I-E^d α and HLA-DRB5*01:01-I-E^d β human/mouse chimeric MHC class II gene (DR2a) constructs were microinjected into fertilized eggs of C57BL/6 mice to generate Tg mice expressing these chimeric MHC class II genes. The DR2a Tg mice were crossed with MHC class II (I-A and I-E)-deficient mice (Jackson laboratory, Bar Harbor, ME)(36).

The TL3A6 TCR Tg mice were generated as follows. HLA DRB5*01:01-restricted MBP (83-99) -specific TL3A6 TCC was established from peripheral blood mononuclear cells isolated from an MS patient by a limiting dilution split well technique (32, 37). TL3A6 expresses Vα9.2-Jα12-Cα and Vβ5.1-D-Jβ1.2-Cβ (34) thus, PCR products encoding Vα9.2-Jα12 were synthesized with a primer located in the 5’ flanking region of the Vα9.2 gene (5’-GGGAGTGGCTTCCTAACACA-3’) and a primer located in the 3’flanking region of the Jα12 gene (5-ACACTTTGGCAGAGGATGGA-3’), while PCR products encoding Vβ5.1-D-Jβ1.2 were synthesized with a primer located in the 5’flanking region of the Vβ5.1 gene (5’-TGCTGTGCAGAACAGAGAGC-3’) and a primer located in the 3’flanking region of the Jβ1.2 gene (5’-GAGACCCCCAGCCTTACCTA-3’). These Vα9.2-Jα12 and Vβ5.1-D-Jβ1.2 PCR products were then subcloned into pTα and pTβ cassette expression vectors (38), respectively. TCR functionality was confirmed in TCR-/- hybridomas prior to microinjection of chimeric TL3A6 TCR α and β gene constructs into fertilized eggs of C57BL/6 mice. Offspring were screened with Southern blotting and PCR, and crossed to DR2a/MHC class II KO mice to generate TL3A6/DR2a/MHC class II KO mice. These mice

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were crossed again to Rag-1KO mice (Jackson Laboratories) and screened to maintain only mice devoid of Rag-1 and mouse Class II-IA/IE. Animals were maintained in a specific pathogen free (SPF) facility at the NIH and results were duplicated in a SPF facility at UMDNJ.

Proteins, peptides and Positional Scanning Combinatorial Libraries (PSCL)

MBP (83-99): (ENPVVHFFKNIVTPRTP) was synthesized to 95% purity (Stanford Pan Facility, Stanford CA) for use in both animal immunizations and cell culture assays. A synthetic N-acetylated, C-amide L-aa decapeptide combinatorial library in a positional scanning format (PS-SCL; 200 mixtures in the O_1X_9 to X_9O_1 format, where O stands for one of the twenty defined L-amino acid and X for a randomized position containing all 20 L-amino acids except cysteine to avoid generation of secondary structures) was prepared as previously described (39). Each OX_9 mixture consists of 3.2×10^{11} (19^9) different decamer peptides at approximately equimolar concentration. Both combinatorial libraries and individual 10- or 20-mers of MBP and agonists of MBP were synthesized by Torrey Pines Institute for Molecular Studies (TPIMS, Torrey Pines, CA). Individual peptides were synthesized using solid phase Fmoc technology while peptide libraries were synthesized using the simultaneous multiple peptide synthesis approach. Purity and identity were assessed by mass spectrometry. Whole human MBP was prepared as previously described (40, 41).

Flow cytometric analysis and T cell proliferation

Thymi, spleens or lymph nodes (axillary, brachial, and inguinal) were aseptically removed from mice immediately after euthanasia. To isolate CNS-cell infiltrates, mice were anaesthetized and perfused intracardially through the left ventricle with 30 ml ice-cold PBS. CNS-cell infiltrates were purified with a 30/70% percoll density gradient after digestion of the homogenized brain and spinal cord with Neural Tissue Dissociation kit (Miltenyi Biotec, Auburn, CA). Tissues were passed through a 40μm nylon mesh prior to create a single cell suspension for subsequent red blood cell (RBC) lysis of spleen cells. For FACS analysis, 1×10^6 cells were washed twice in staining buffer (0.1% NaN_3, 2% FCS in PBS), pre-incubated with Fc block (Pharmingen, San Jose, CA) and stained with biotinylated-, PE-, APC- or FITC-labeled antibodies to murine CD4, CD8, CD3, CD69 (BD Pharmingen, Franklin Lakes, NJ), HLA-DR, mouse Foxp3, IFN-γ, IL-17A, GM-CSF (eBiosciences, San Diego, CA) or human Vβ5.1 (Beckman Coulter, Brea, CA). The cells stained with biotinylated mAb were further incubation with Streptavidin-Cychrome (BD Pharmingen). Intracellular staining kit (eBioscience) was used to fix and permeabilize the cells for staining with anti-Foxp3 mAb. To examine the expression of IL-17A, GM-CSF and IFN-γ, cells were cultured with MBP83-99 at 10 μg/ml for 5 hrs in the presence of brefeldin A (10 μg/ml) and then stained with FITC-anti IL-17A, PE-anti GM-CSF, APC-IFN-γ mAbs. FACS analysis was performed on a BD FACSCalibur using CellQuest software (BD Pharmingen) and a Cytomics FC 500 using CXP Analysis Software (Beckman Coulter). Production of IFN-γ, IL-17, and GM-CSF was also measured by ELISA (R&D Systems, Minneapolis, MN).

Spleen and LN populations were tested for proliferative responses at 4×10^6 cells/ml in 96 well round bottom plates in the presence of dose titrations of antigens. Bare lymphocyte syndrome (BLS) patient cells devoid of MHC class II expression and transfected to express HLA-DR2a were kindly provided by G. Nepom and W. Kwok (University of Washington, Seattle, WA). TL3A6 Tg T cell lines (TCL) were prepared by repeated stimulation of TL3A6/DR2a/Rag-1KO mouse spleen cells with irradiated DR2a Tg spleen cells (2×10^5 per well, 3000 rad). 3×10^4 rested TL3A6 TCC or TL3A6 Tg TCL were co-cultured with 9×10^4 irradiated (25,000 rad) DR2a-transfected BLS cells or DR2a Tg spleen cells in serum-

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free X-Vivo-15 media (BioWhittaker, Walkersville, MD), respectively. PSCL mixtures or peptides were added at various concentrations in 96 well round bottom plates for 48 h at 37°C. 1 μCi of [^{3}H]-thymidine (Perkin Elmer, Waltham, MA) was added to each well for an additional 16 h before harvesting on a Tomtec IIIM harvester (Tomtec, Hamden, CT) and measuring the incorporated radioactivity on a Wallac Trilux Microbeta Scintillation Counter (Perkin Elmer).

Immunization for epitope mapping or the induction of active or passive EAE

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources and approved by the NINDS and UMDNJ Animal Care and Use Committee. 6-10 week old male and female mice were immunized with 500 μg human MBP or 200, 100, or 50 μg of MBP 83-99 or agonist peptide in 1 or 4mg/ml Mycobacterium H37Ra in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) for epitope mapping or disease induction, respectively. For active EAE induction, 200ng of pertussis toxin (List Biologicals, St. Louis, MO) was administered i.p. on days 0 and 2 to initiate disease.

Animals were similarly immunized for passive EAE and ten days later the inguinal, brachial and axillary lymph nodes (LN) were removed. Tissues were dissociated on steel mesh and single cell suspensions seeded at 4×10^6 cells/ml in T cell media (TCM) consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% Fetal calf serum (FCS, Invitrogen) with glutamine, non essential amino acids, penicillin/ streptomycin (Biowhittaker) and 5 μg/ml MBP (83-99). Four days later cells suspensions were washed well in TCM and resuspended in PBS. $1\text{-}2\times10^7$ cells were delivered i.v. to each recipient and pertussis toxin administered as for active disease. Animals were weighed and monitored daily; clinical signs were assessed according to the following scale: 0: no disease,1: limp tail, 2: hindlimb paresis-mild; 2.5: severe paresis, 3: single hindlimb paralysis; 3.5: 2 limbs paralyzed; 4: hindlimb paralysis, forelimb paresis and 5: no mobility/moribund.

Histology

For histological analysis, deeply anesthetized mice were perfused with 50 ml cold PBS followed by 50 ml 4% paraformaldehyde in PBS. The brain and spinal column were removed, and placed in 10% PFA for 5-10 days before systematic processing. Brains were cut into 3mm slices coronally and embedded in paraffin; after decalcification spinal cords were cut and divided into upper and lower columns. Each was cut into 5 mm cross sections and 8 mm longitudinal sections; paraffin embedded tissue was cut at 6 μm followed by hematoxylin and eosin (H&E) staining or Bielschowsky silver impregnation stain to detect axons (Histoserve, Gaithersburg, MD). To prepare frozen sections, the paraformaldehyde-fixed brain and spinal cord were soaked in 30% sucrose/PBS for 3 days. The frozen sections (thickness: 20 μm) were generated with a HM505E cryostat (Microm) and stained with anti-CD4, -Mac 3, and -Ly-6G mAbs (BD Bioscience) and rhodamine- and fluorescein-labeled secondary Abs (Jackson Immunoresearch, West Grove, PA). Nuclei were stained with Hoechst-33258 (Sigma Aldrich). The stained sections were analyzed with an Axiovert 200 microscope (Carl Zeiss, Thornwood, NY).

Results

TL3A6 TCR Tg myelin-reactive T cells are positively selected in HLA-DR2a Tg animals

HLA-DR plays an essential role in the development of autoreactive CD4 T cells including myelin-specific CD4 T cells. Thus, we first examined whether HLA-DRB5*01:01 can promote the development of MBP-specific CD4 T cells. Transgenic mice expressing HLA-DRA1*01:01 and HLA-DRB5*01:01genes were created and crossed with MHC class II KO

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mice to replace mouse MHC class II genes with the human MHC class II genes (referred to as DR2a Tg mice). To examine the influence of expression levels of DR2a gene on the development of MBP-specific CD4 T cells, two lines of DR2a Tg mice were selected: one with higher ($DR2a^{hi}$) and one with lower ($DR2a^{lo}$) levels of HLA-DRB5*01:01 expression (Fig. 1A). Expression levels of HLA-DR in the $DR2a^{hi}$ mice were similar to that of human PBMCs, while the transgene expression in the $DR2a^{lo}$ mice was roughly 10 times lower as compared with $DR2a^{hi}$ mice (Fig. 1A). The latter DR2a Tg animals were immunized with whole human or bovine MBP and draining LN cells were tested *ex vivo* against overlapping 20-mer MBP peptides for myelin reactivity. However, proliferative responses were low, and MBP (83-99)-specific T cells could not be detected (data not shown), suggesting that DR2a-restricted MBP (83-99)-specific Type A T cells, which can recognize naturally processed MBP peptides, are largely deleted in DR2a mice as reported previously (42). In the same study, MBP-specific T cells are also deleted in DR2b Tg mice. In contrast to this observation, DR2a- and DR2b-restricted MBP (83-99)-specific Type A T cells, including TL 3A6 TCC, have been isolated from humans expressing DR15. This difference could be due to distinct TCR diversity when comparing mice and humans as indeed, T cell diversity in humans is 10-20 times higher than that in mice (43, 44). Thus, Tg mice expressing human TCR (TL3A6) specific for MBP (83-99)/DR2a were created and crossed with $DR2a^{hi}$ and $DR2a^{lo}$ Tg mice (referred to as TL3A6/DR2a mice) to examine whether MBP-specific human TCR Tg T cells can develop in DR2a Tg mice. To our surprise, CD4+TL3A6 TCR Tg T cells were positively selected in the thymus and efficiently developed in the periphery of TL3A6/$DR2a^{hi}$ mice (Fig.1B). Although CD8+ TL3A6 TCR Tg T cells developed as well as CD4+ TL3A6 TCR Tg T cells in the thymus of TL3A6/$DR2a^{lo}$ mice, increased expression of DR2a in TL3A6/$DR2a^{hi}$ mice preferentially promoted the development of CD4+ TL3A6 TCR Tg T cells without significant reduction of the thymic cellularity (Fig. 1B). Poor development of CD4 T cells in the TL3A6/$DR2a^{lo}$ mice could be due to inefficient positive selection because expression of CD69 on $CD4^+CD8^+$ DP thymocytes was reduced in the TL3A6/$DR2a^{lo}$ mice (Fig.1C) as observed in other TCR Tg mice in which inefficient positive selection occurs (45, 46). To eliminate the effect of endogenous mouse TCRs on thymic and peripheral development of TL3A6 Tg T cells, TL3A6/DR2a mice were crossed to Rag-1 KO mice to create TL3A6/DR2a/ mouse MHC class II KO/ Rag-1KO mice (referred to as TL3A6/DR2a/Rag-1KO). CD4+TL3A6 TCR Tg T cells were still positively selected in the thymus and efficiently develop in the periphery of TL3A6/ $DR2a^{hi}$/Rag-1KO mice. In contrast, development of CD4+TL3A6 Tg T cells is inefficient in the thymus and spleen of the TL3A6/$DR2a^{lo}$/Rag-1KO mice (Fig. 1D). These data suggest that DR2a-restricted MBP (83-99)-specific CD4+ T cells can develop in DR2a Tg mice, however, their development is dependent on the expression level of DR2a. We also examined the development of TL3A6 Tg $Foxp3^+$ regulatory T cells. 5.2 % of CD4+TL3A6 Tg T cells express Foxp3 in the spleen of TL3A6/$DR2a^{hi}$ mice, while the percentage of $Foxp3^+$ CD4+ T cells increased to 22.5 % in the spleen of TL3A6/$DR2^{lo}$ mice (Supplementary Fig.S1). In accordance with other MBP-TCR Tg mice (47), development of $Foxp3^+$ regulatory T cells was not detected in TL3A6/DR2a/Rag-1 KO mice (Supplementary Fig. S2).

Specificity and HLA-restriction of the human TCC TL3A6 are preserved in TL3A6 Tg mice

To examine whether TL3A6 Tg T cells can recognize naturally processed MBP peptides as well as exogenously added MBP (83-99) peptide in the context of DR2a, splenocytes from TL3A6 Tg mice expressing high or low levels of DR2a were cultured with whole MBP or MBP (83-99) peptide. Splenocytes isolated from TL3A6/$DR2a^{hi}$ and TL3A6/$DR2a^{lo}$ mice proliferated to MBP and MBP (83-99), yet cells from TL3A6/$DR2a^{hi}$ mice responded more strongly than those from TL3A6/$DR2a^{lo}$ mice (Fig. 2A and B). Lower proliferative responses of spleen cells isolated from the TL3A6/$DR2a^{lo}$ mice correlated with lower

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expression of DR and less efficient development of CD4+ T cells expressing the TL3A6 Vbeta chain Vβ5.1^{+} in TL3A6/DR2a^{lo} mice (Fig. 1A and B). Importantly, MBP (83-99)-specificity (Type A) was preserved in the TL3A6 Tg T cells that had been selected in TL3A6/DR2a mice.

We next examined whether the fine specificity of TL3A6 TCR is also preserved in this humanized animal. We previously demonstrated systematically by using positional scanning synthetic combinatorial peptide libraries (ps-SCL) and bioinformatics approaches that TL3A6 recognizes a large number of peptides in addition to MBP (83-99) and with higher, similar or lower affinities (i.e. at lower, similar or higher antigen concentrations). In order to assess whether the TL3A6 TCR/HLA-DR2a transgene constructs consisting of human-mouse chimeric TCR and human-mouse chimeric HLA-DR2a maintained this reactivity pattern, we tested the human TL3A6 TCC and TL3A6 TCR Tg cells with ps-SCL. To eliminate any expression of endogenous mouse TCR, the TL3A6 Tg T cells were established from TL3A6/DR2a/Rag-1 KO mice. When comparing the amino acids in each of the 10 positions of ps-SCL that activated the human TL3A6 TCC (Fig. 3A) or a murine TL3A6 TCR Tg TCL (Fig. 3B), the patterns are comparable confirming that the peptide specificities of the two TCRs (human versus mouse-human chimeric construct) are close to identical. Minor differences exist in only a few positions, (only Gly in position 2 stands out for the TL3A6 TCR Tg TCL), while the primary TCR contact (Lys in position 3) and other important residues are recognized by both TCRs (48). These data show that the fine specificity of TL3A6 TCR was preserved in this humanized animal, which was further supported by altered MBP (83–99) peptides. The human TL3A6 TCC has been characterized extensively for recognition of numerous altered peptides derived from MBP (83–99) as well as other molecular mimics (30, 49-51). Superagonists of the native antigen MBP (83-99) for TL3A6 were predicted previously by screening TCC TL3A6 systematically for recognition of amino acid exchanges in the MBP (83-99) peptide (50). These peptides with up to 6 log lower half-maximal stimulatory effective concentration (EC_{50}) values activated the Tg T cells in a similar hierarchy relative to MBP (83-99), confirming that the specificity of the interactions between DR2a and TL3A6 Tg humanized constructs reproduced data obtained with the original TL3A6 TCC (Fig. 4). Notably, mouse Tg TCL from these mice respond to MBP 81-99 and 83-99 peptides when presented by human APCs expressing DR2a (BLS cells expressing DR2a). They do not, however, respond to MBP(83-99) or MBP(81-99) when presented by BLS cells transfected with DR2b. The original human T cell clone demonstrated an EC_{50} of between 0.7 to 1 ug/ml when cultured with either DR2-matched PBMC or DR2a-transfected BLS cells and MBP (83-99). The mouse TL3A6 Tg TCL responded to MBP(83-99) and DR2a-transfected human BLS cells with a similar EC_{50} of 0.8 ug/ml, but demonstrated a one log lower EC_{50} (0.08ug/ml) when cultured with DR2a Tg spleen cells (data not shown).

TL3A6 Tg T cells are encephalitogenic

We examined whether TL3A6 Tg T cells possess encephalitogenic potential and how DR-expression levels affect the development of EAE. We first examined the induction of active EAE in TL3A6/DR2a double Tg mice. Disease could not be readily induced in DR2a single Tg animals with MBP (83-99), nor could it be induced in TL3A6 TCR single Tg mice (Table I). TL3A6/DR2a double Tg mice rarely exhibited disease when immunized with CFA or pertussis alone, yet immunization with MBP (83-99) in CFA with pertussis elicited disease in 47% and 90% in TL3A6/DR2a^{lo} versus TL3A6/DR2a^{hi} animals, respectively, suggesting that TL3A6/DR2a^{hi} Tg mice are more susceptible to active EAE compared to TL3A6/DR2a^{lo} Tg mice. We next examined whether a stronger stimulus by using superagonist peptides increases the incidence of active EAE in TL3A6/DR2a^{lo} Tg mice. This was not the case (Table II), however, the superagonist induced clinical disease at lower

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doses (50 μg) and with slightly higher clinical scores compared to MBP (89-98) (Table II). In contrast to active EAE, adoptive transfer of TL3A6 Tg TCL established from MBP (83-99) -immunized TL3A6/DR2a^{lo} Tg mice induced disease in both lines of DR2a Tg mice at 100% (Fig. 5). While the severity of disease was not significantly different between these two groups, the DR2a^{lo} Tg mouse line showed delayed onset of disease (day 12 vs. day 7, $p<0.05$) and a tendency to recover with less severe disease than DR2a^{hi} animals (Fig. 5). These data suggest that TL3A6 Tg T cells are potentially encephalitogenic and that a high expression of DR2a increases the development of induced EAE.

TL3A6 Tg TCR mice develop spontaneous EAE

We next examined the occurrence of spontaneous EAE in TL3A6/DR2a Tg mice. TL3A6/DR2a Tg mice developed spontaneous disease with differing incidences (Table III), from 4.5% in TL3A6/DR2a^{hi} Tg mice to less than 1% in TL3A6/DR2a^{lo} Tg mice. In addition to increased disease incidence, TL3A6/DR2a^{hi} Tg mice showed a trend towards earlier onset of disease (10 vs 21 weeks of age at onset), but numbers were insufficient to reach statistical significance ($p=0.08$). To examine the level of DR2a expression required for the development of spontaneous EAE, TL3A6/DR2a^{hi} Tg mice were crossed with TL3A6/DR2a^{lo} Tg mice to generate TL3A6/DR2a Tg mice expressing various levels of DR2a (Fig. 6). Expression of HLA-DR in these animals was normalized to that observed on the HLA-high expressing TL3A6/DR2a^{hi} Tg mice and expressed as % of the maximal mean fluorescence identity (MFI). The onset of spontaneous EAE was greatly decreased in mice expressing HLA-DR at low levels, i.e. less than 50% of that observed in TL3A6/DR2a^{hi} Tg mice (Fig.6). 14% of TL3A6/DR2a Tg mice that express higher levels of DR (over 50% of the TL3A6/DR2a^{hi} Tg DR-expression level) developed spontaneous EAE.

Since Rag-1-deficiency increases the frequency of spontaneous EAE in MBP-TCR Tg mice due to the lack of CD4+CD25+ regulatory T cells (52, 53), we also examined this aspect in TL3A6/DR2a/Rag-1KO mice. As expected, the incidence of spontaneous EAE increased in TL3A6/DR2a^{hi}/Rag-1KO mice (Table III), however, not in the TL3A6/DR2a^{lo}/Rag-1KO mice.

These data suggest that TL3A6 Tg T cells are able to differentiate into pathogenic T cells spontaneously, however, incidence of spontaneous EAE is dependent on expression levels of DR2a.

Spontaneous development of Th1, Th17, and GM-SCF-producing CD4 T cells in TL3A6/DR2a^{hi} mice

We examined the production of IFN-γ, IL-17, and GM-CSF in response to MBP83-99 in the splenocytes isolated from healthy TL3A6/DR2a^{lo} and TL3A6/DR2a^{hi} mice. Production of these cytokines is higher in TL3A6/DR2a^{hi} mice than TL3A6/DR2a^{lo}mice (Fig.7A), and greater numbers of Th1, Th17 and GM-CSF+ expressing CD4 T cells exist in TL3A6/DR2a^{hi} mice (Fig.7B). Intracellular flow cytometric analysis indicated that GM-CSF is predominantly produced by Th1/Th17 cells that co-produce IFNγ and IL-17 (Fig.7B).

We next examined the production of IFN-γ, IL-17, and GM-CSF in mice that had spontaneously developed EAE. Although the development of Th1, Th17, and GM-CSF +CD4 T cells in the spleen was comparable between healthy and spontaneous EAE mice (Fig.7A and B), an increased production of these cytokines was detected in the CNS of spontaneous EAE mice (Fig.7A). In accordance with the cytokine profile, greater numbers of MBP-specific Th1, Th17, and GM-CSF+ CD4+ T cells were detected in the CNS during the course of spontaneous EAE (Fig.7B). Infiltration of these pathogenic T cells was not detected in healthy TL3A6/DR2a ^{hi} mice (data not shown).

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Histological analysis of demyelinating disease in both spontaneous as well as induced EAE

Both induced and spontaneous EAE mice develop typical EAE with ascending motor deficits, and induced- and spontaneous disease in the double Tg mice were indistinguishable. Histological differences were also not apparent between TL3A6/DR2a^{hi} vs TL3A6/DR2a^{lo} Tg mice that developed induced EAE. Inflammation predominated within the spinal cord with lesser involvement of the brainstem and relative sparing of the cerebrum and cerebellum (Fig. 8 A-F and Table SI). Infiltrates contained predominantly CD4+ T cells and mononuclear cells, but also significant numbers of polymorphonuclear (PMN) leukocytes/granulocytes (Fig 8 A,B (arrows) and Supplementary Fig.S3). Swollen axons and degenerating neurons could be detected throughout the spinal cord (arrows, arrow heads Fig. 8C) including evidence of axonal loss (Fig. 8E). Similar to our previous humanized MBP TCR Tg model (54) and also in other models of MBP-induced EAE, there was minor involvement of the cranial and spinal nerve roots and thus involvement of the proximal peripheral nervous system (Fig 8 G,H and Supplementary Table SI). These data indicate that TL 3A6 Tg T cells can induce neuroinflammation and subsequently neurodegeneration in TL3A6/DR2a Tg mice.

Discussion

Almost thirty years ago, DR2, later termed DR15, and now the DR15 haplotype consisting of DR2a and DR2b, was identified as the DR type most consistently linked to MS (55). In the majority of studies the focus of attention has been on DR2b, and the existence of the second DR15 gene, DR2a, which is in almost absolute linkage disequilibrium with DR2b, has often not been considered. Several lines of evidence seemed to suggest that only DR2b is pathogenic, i.e. the above mentioned genetic data in a small number of African Americans with MS (21), but also the demonstration that Tg mice expressing the MBP (84-102)-specific and DR2b-restricted TCR Ob.1A12 develop spontaneous EAE and are highly susceptible to actively induced disease (23). The effect of DR2a on the development of spontaneous EAE was examined using a. DR2a-resticted Epstein-Barr virus (EBV) peptide specific TCR (Hy.2E11), which also recognizes MBP (85-99) in the context of DR2b (56). Hy.2E11 TCR/DR2b Tg mice and a second Tg mouse recognizing MBP (83-99) in the context of DR2b, Ob.1A12-TCR/DR2b Tg mice, both develop spontaneous EAE. These observations are the main reason for the currently prevailing assumption that DR2b confers disease risk in MS. However, development of spontaneous EAE is reduced in the Hy.2E11-TCR Tg mice that are bred to co-express DR2b and DR2a genes, and this was attributed to the induction of DR2a-mediated apoptosis in Hy.2E11TCR Tg T cells in the periphery (29). Based on these data the authors concluded that DR2a plays a protective role in the development of spontaneous EAE by deleting autoreactive Tg T cells in the periphery. Since these mice are not infected with EBV, Hy.2E11-TCR Tg T cells may cross-react with an unknown self-antigen associated with DR2a in the Hy.2E11-TCR/DR2b/DR2a Tg mice. In contrast to Hy.2E11-TCR/DR2a/DR2b Tg mice, development of spontaneous EAE is not affected by expression of DR2a in Ob.1A12-TCR/DR2a/DR2b Tg mice (29). Since the authors have not identified DR2a-restricted peptides recognized by the Ob.1A12-TCR, and therefore one may conclude that a DR2a-presented self-peptide mediates suppressive effects or e.g. acts as co-inhibitor, only on Hy.2E11-TCR-carrying MBP-specific T cells. However, the pathogenic role of DR2a-restricted MBP-specific T cells has not been addressed. We show here that MBP-specific, DR2a restricted T cells are indeed encephalitogenic, and that an increase in DR2a expression is associated with the development of spontaneous EAE in TL3A6/DR2a Tg mice.

Our and others groups' prior data also indicated that it is more likely that both alleles jointly confer risk. The main observations in that direction are that DR2a-restricted,

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proinflammatory MBP-specific T cells can readily be generated (11, 12), that DR2a is expressed at higher levels in most cells and tissues (33) and binds more MBP peptides (17), and that the two alleles, due to similarities of their binding pockets, bind overall similar or overlapping sets of peptides (17-19). The most important example for the latter is the immunodominant MBP peptide (83-99) that has been described as a target epitope for both DR2a- and DR2b-restricted T cell clones. These observations combine with our TL3A6/DR2a Tg mouse data to suggest that DR2a-restricted MBP-specific CD4+ T cells are likely to contribute to the pathogenesis of MS.

Thymic and peripheral development of autoreactive T cells are dependent on the avidity of TCR to self-antigen/MHC class II complex. MBP-specific T cells are largely deleted in the thymus and periphery, and only those expressing a low avidity TCR can escape the tolerance-inducing mechanisms (57). Immunization of DR2a Tg mice with whole MBP and subsequent attempts to map DR2a-restricted MBP epitopes identified very low numbers of responsive cells. As a consequence disease is difficult to induce by immunization with MBP suggesting that DR2a-restricted, MBP (83-99)-specific Type A T cells, which recognize naturally processed MBP antigens, largely undergo deletion and/or anergy in DR2a Tg mice. Similarly, MBP (83-99)-specific Type A T cells also undergo tolerance in DR2b Tg mice (42). The observation that MBP(82-100) specific Type A T cells can be positively selected in DR2a Tg and DR2b Tg mice that are deficient in MBP-expression (42), suggests that MBP (82-100)-specific Type A T cells are deleted in the thymus and/or undergo anergy in the periphery due to the expression of endogenous MBP. In contrast, MBP (82-100) specific Type B T cells that recognize a cryptic MBP (82-100) epitope can escape immune tolerance in DR2a Tg mice and possess encephalitogenic activity (42). Although thymic negative selection and peripheral tolerance may occur more frequently in MBP-specific Type A T cells than Type B T cells, studies have shown MBP-specific Type A T cells that escape thymic and peripheral tolerance are more encephalitogenic than Type B T cells (57). Despite the tolerance induction in MBP (83-99)-specific Type A T cells in DR2a and DR2b Tg mice, DR2a- and DR2b-restricted MBP-specific Type A T cells are frequently isolated in humans. This difference may be due to higher TCR diversity in DR15+ humans compared with DR2a Tg mice. The total TCR repertoire in the human blood and mouse spleen is estimated as 2.5×10^7 and $1\text{-}2 \times 10^6$, respectively (43, 44). Thus, T cell diversity in humans is 10-20 times higher than that in mice. Our study examines such a DR2a-restricted MBP83-99-specific Type A human TCR (TL3A6) that escaped tolerance in a patient and is capable of driving disease. Tg mice that express DR2a positively select the TL3A6 Tg CD4+Type A T cells, and these cells can escape thymic and peripheral deletion in the TL3A6/DR2a^{hi} Tg mice and go on to initiate spontaneous disease.

The escape from thymic and peripheral tolerance could be due to a low avidity of TL3A6TCR to MBP (83-99)/DR2a. Indeed, the crystal structure of the TL3A6/MBP (83-99)/HLA-DR2a complex shows relatively few TCR contact residues available for recognition and an overall low affinity for the peptide-MHC complex with no high affinity bonds (salt bridges, hydrogen bonds), but only van der Waals bonds contributing to TCR-MHC/peptide interaction(34). In addition, the crystal structure analysis revealed a non-traditional nesting of the peptide within the DR2a molecule with a slight shift to the N-terminus (34). The bound peptide's sitting slightly off center in the MHC likely limits optimum availability to the TCR. Such unconventional recognition of the N-terminal portion of the peptide has been demonstrated in another autoreactive TCR and may contribute to escaping thymic deletion (58).

Spontaneous EAE develops in TL3A6/DR2a^{hi} mice in about 4.5% of animals, i.e. roughly 45 times more frequently than the prevalence observed in MS in Northern European and North American populations and similar to the prevalence in first-degree relatives of MS

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patients (59), while spontaneous autoimmunity was detected in TL3A6/DR2a^{lo} Tg mice at considerably reduced levels. Development of spontaneous EAE was significantly reduced in TL3A6/DR2a Tg mice when DR2a-expression fell below 50% of that in DR2a^{hi} mice. This higher incidence of spontaneous EAE, the earlier disease onset and more severe score in the DR2a^{hi}-expressing animals are all consistent with the increased risk of MS in DR15 homozygote individuals (60). Since HLA genes are expressed codominantly, DR15 homozygote individuals display roughly twice the amount of DR2a and DR2b molecules than heterozygote individuals, who express DRB1- and DRB3-, 4-, or 5- alleles from another haplotype.

HLA-DR2a may affect the development of spontaneous EAE in several ways. Interestingly, the incidence and disease severity were not significantly affected by the level of DR2a expression in adoptive transfer EAE (Fig.5), suggesting that a high expression of DR2a is not required during the effector stages of the disease. Since spontaneous development of Th17 cells depends on the strength of the interaction between the TCR and self Ag/MHC class II complex (61), low expression of DR2a may not be sufficient for the development of Th17 T cells in TL3A6/DR2alo Tg mice. Indeed, development of MBP-specific Th1 and Th17 cells is low in TL3A6/DR2a^{lo} Tg mice and their development increases in the TL3A6/DR2a^{hi}Tg mice (Fig.7). In addition, development of GM-CSF-producing Th1/Th17 cells, which play an essential role in the development of EAE (62, 63), was increased in TL3A6/DR2a^{hi} Tg mice (Fig. 7). Thus, expression levels of DR is one of the factors involved in the differentiation of encephalitogenic T cells. In addition, a decrease in the development of MBP-specific Foxp3 Tregs in the TL3A6/DR2a^{hi} mice (Supplementary Fig. S1) may be contributing to the spontaneous development of encephalitogenic T cells. Nevertheless, only a small percentage (4.5-14%) of TL3A6/DR2a^{hi}Tg mice develops spontaneous EAE although MBP-specific Th1, Th17, and GM-CSF+CD4 T cells develop in the mice. Thus, further experiments are required to investigate other factors influencing the development of spontaneous EAE.

In summary, data from this novel Tg mouse model documents that a DR2a-restricted, MBP (83-99)-specific TCR causes EAE spontaneously, upon active induction with MBP and following adoptive transfer. Therefore, we conclude that DR2a independently or together with the other HLA-DR15 risk allele DR2b serves as etiologic risk factor for MS rather than as an epistatic modulator that lowers MS risk. These data are probably not only relevant for MS and its major HLA risk haplotype DR15, but also for other autoimmune diseases, for which an association with a specific HLA-DR haplotype has been demonstrated including rheumatoid arthritis, type I diabetes, myasthenia gravis and others.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Bruno Gran, MD, Division of Clinical Neurology, Nottingham University Hospitals, UK, and Mireia Sospedra, PhD, Institute for Neuroimmunology and Clinical MS Research, University Medical Center Hamburg-Eppendorf, Germany, for helpful suggestions and critical reading of the manuscript. We would like to dedicate this manuscript to the memory of Marco Vergelli, MD, who first isolated and characterized the TL3A6 T cell clone.

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Abbreviations used in this paper

EAE	Experimental Autoimmune Encephalomyelitis
EC_{50}	half maximal effective concentration
MBP	Myelin Basic Protein
MS	Multiple Sclerosis
PSCL	Positional Scanning Combinatorial Libraries

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Rag	Recombinase-Activating Gene
TCR	T cell receptor
Tg	Transgenic

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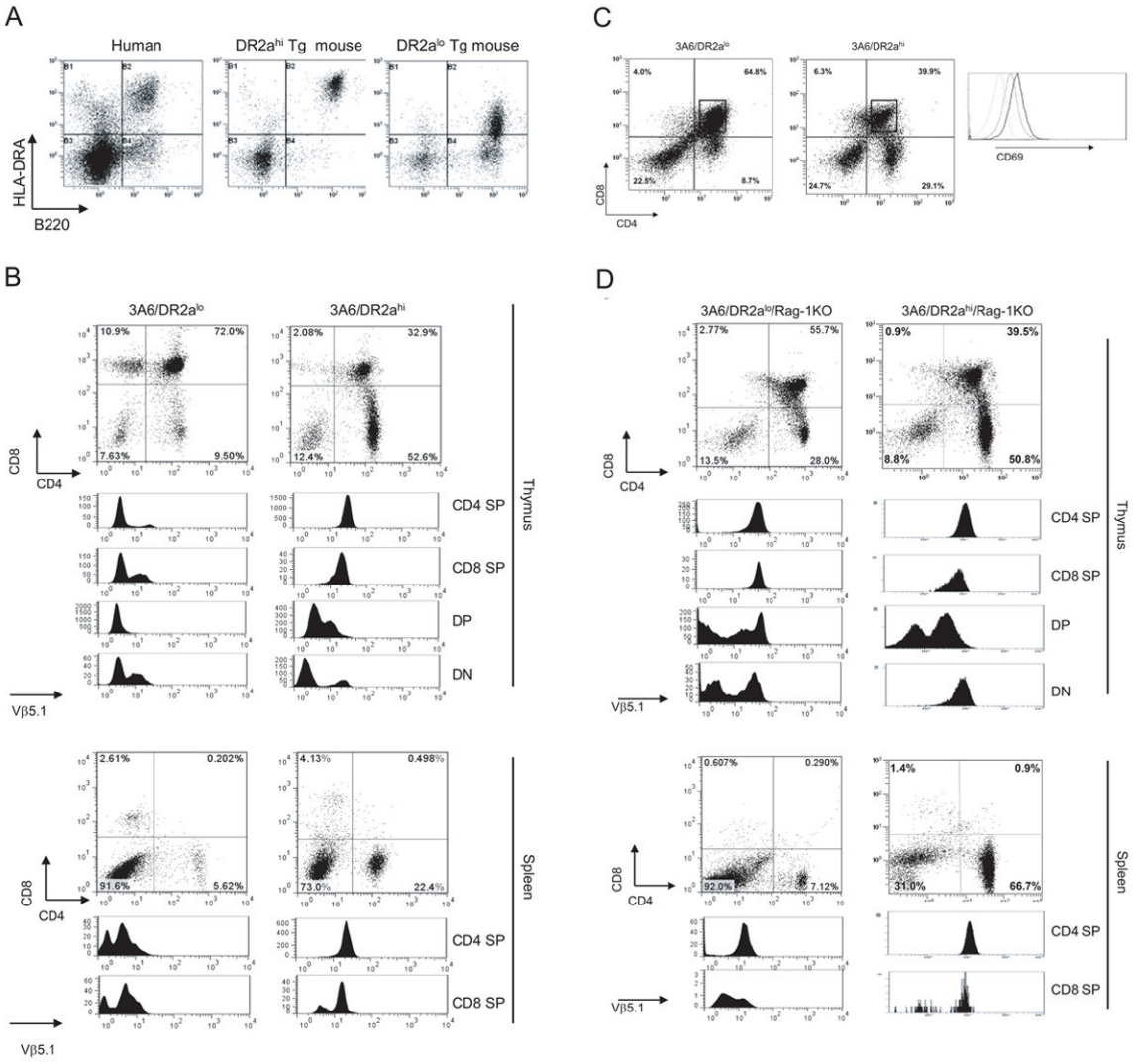

Figure 1.

TL3A6 Tg T cells are positively selected in the thymus and efficiently develop into the periphery. **(A)** Comparative expression of HLA-DR in DR2a Tg mice and humans. Peripheral blood cells isolated from two founder DR2a Tg mouse lines, DR2a^{hi} and DR2a^{lo}, and healthy donors carrying DR15 haplotype were stained with anti-HLA-DR and -B220 mAb. **(B)** Greater DR2a-expression promoted the development of TL3A6 Tg CD4 T cells in TL3A6/DR2a double Tg mice. Thymocytes and splenocytes isolated from TL3A6/DR2a double Tg mice at 6 weeks of age were stained with anti-CD4, CD8, and Vβ5.1 mAbs. The number of thymocytes and splenocytes are as follows: TL3A6/DR2a^{lo} mice (n=3); 9.4+/-4.0 × 10e7 (thymocytes) and 11.0+/-2.6 × 10e7 (splenocytes), TL3A6/DR2a^{hi} mice (n=5); 7.4+/-2.8 × 10e7 (thymocytes) and 7.9 +/- 4.4 × 10e7 (splenocytes). **(C)** Inefficient positive selection of TL3A6 Tg CD4 T cells in TL3A6/DR2a^{lo} mice. Thymocytes isolated from TL3A6/DR2a^{lo} and TL3A6/DR2a^{hi} mice were stained with anti-CD4, -CD8, and -CD69 mAbs. CD4+CD8+ DP cells are gated for analysis of CD69 expression. ——— ; IgG control, ▪▪▪▪▪▪ ; TL3A6/DR2a^{lo} mouse, ▬▬▬ ; TL3A6/DR2a^{hi} mouse. Data shown are representative of three experiments. **(D)** Development of Vβ5.1+ Tg CD4 T cells is less efficient in TL3A6/DR2a^{lo}/Rag-1KO mice compared with TL3A6/DR2a^{hi}/Rag-1KO mice. TL3A6/DR2a^{hi}/Rag-1KO mice (n=4); 2.9+/- 1.2 × 10e7 (thymocytes) and 2.3+/-0.8 × 10e7

(splenocytes). TL3A6/DR2a^{lo}/Rag-1KO (n=3); 4.9+/-0.4 × 10e7 (thymocytes) and 11.1+/-0.6 × 10e7 (splenocytes).

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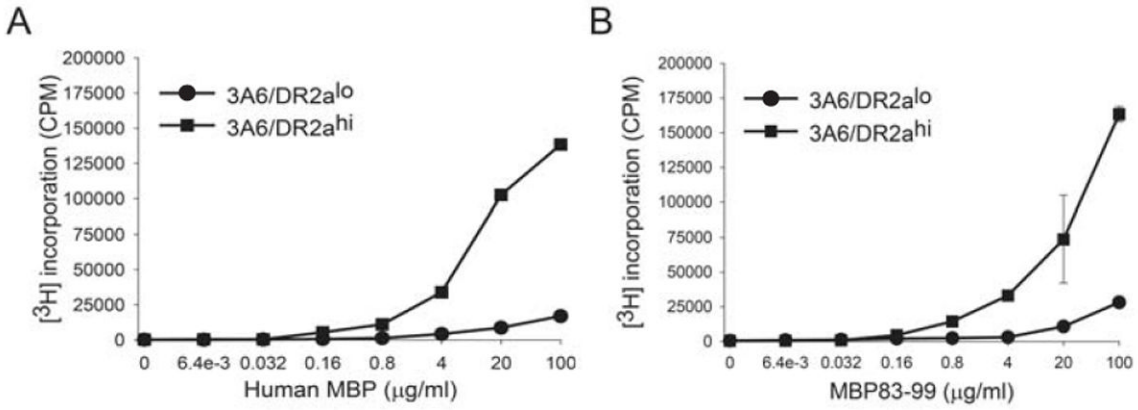

Figure 2.

TL3A6 Tg T cells recognize naturally processed MBP peptides as well as MBP 83-99 peptide. Splenocytes isolated from 6-8 wk healthy TL3A6/DR2a^{lo} and TL3A6/DR2a^{hi} Tg mice were cultured with whole human MBP (A) and MBP_{83-99} peptide (B) and their proliferation was examined by [^{3}H]-thymidine-uptake. In A and B the average of 2 and mean of 3 mice per group respectively are shown, error bars in B indicate SEM.

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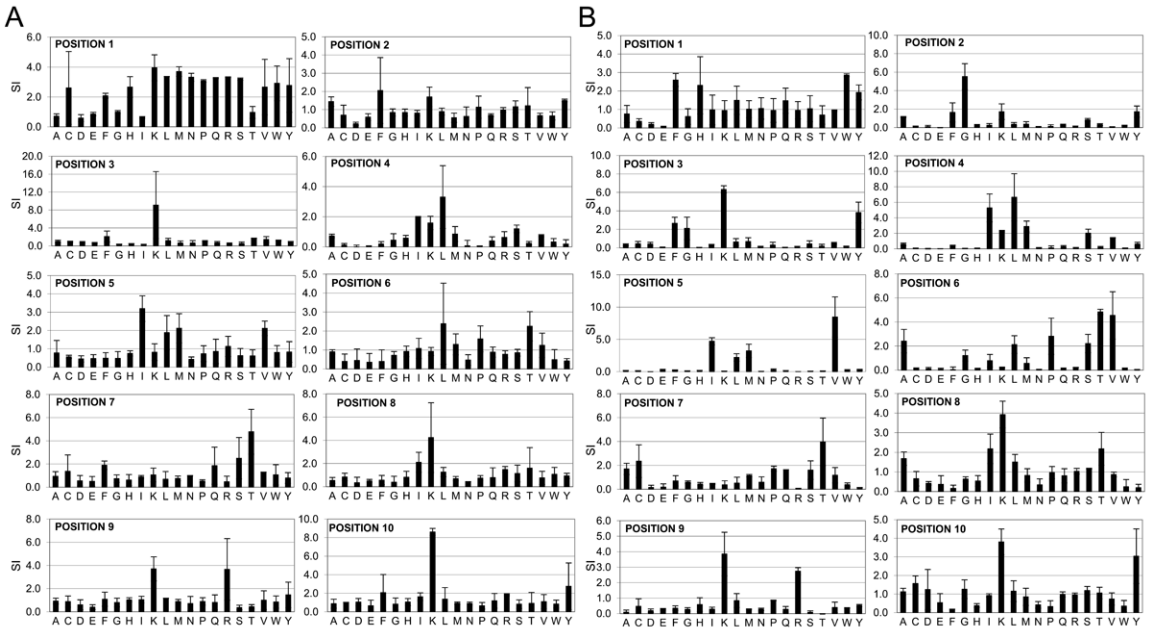

Figure 3.

PSCL comparison of TL3A6 TCC to TL3A6 Tg TCC antigen recognition in the context of DR2a. (A) TL3A6 TCC was tested with BLS-DR2a transfectants against PSCL at 100 μg/ml to identify stimulatory amino acids throughout scanning 10-mers and compared against to distinguish recognition in the context of DR2a. (B) TL3A6 Tg TCC established from a TL3A6/DR2a/Rag-1KO animal was tested with DR2a Tg spleen cells. The stimulation index (SI) is the ratio of CPM of duplicate wells to controls without antigen. Data represent the mean SI from 2 experiments, error bars represent the standard deviation.

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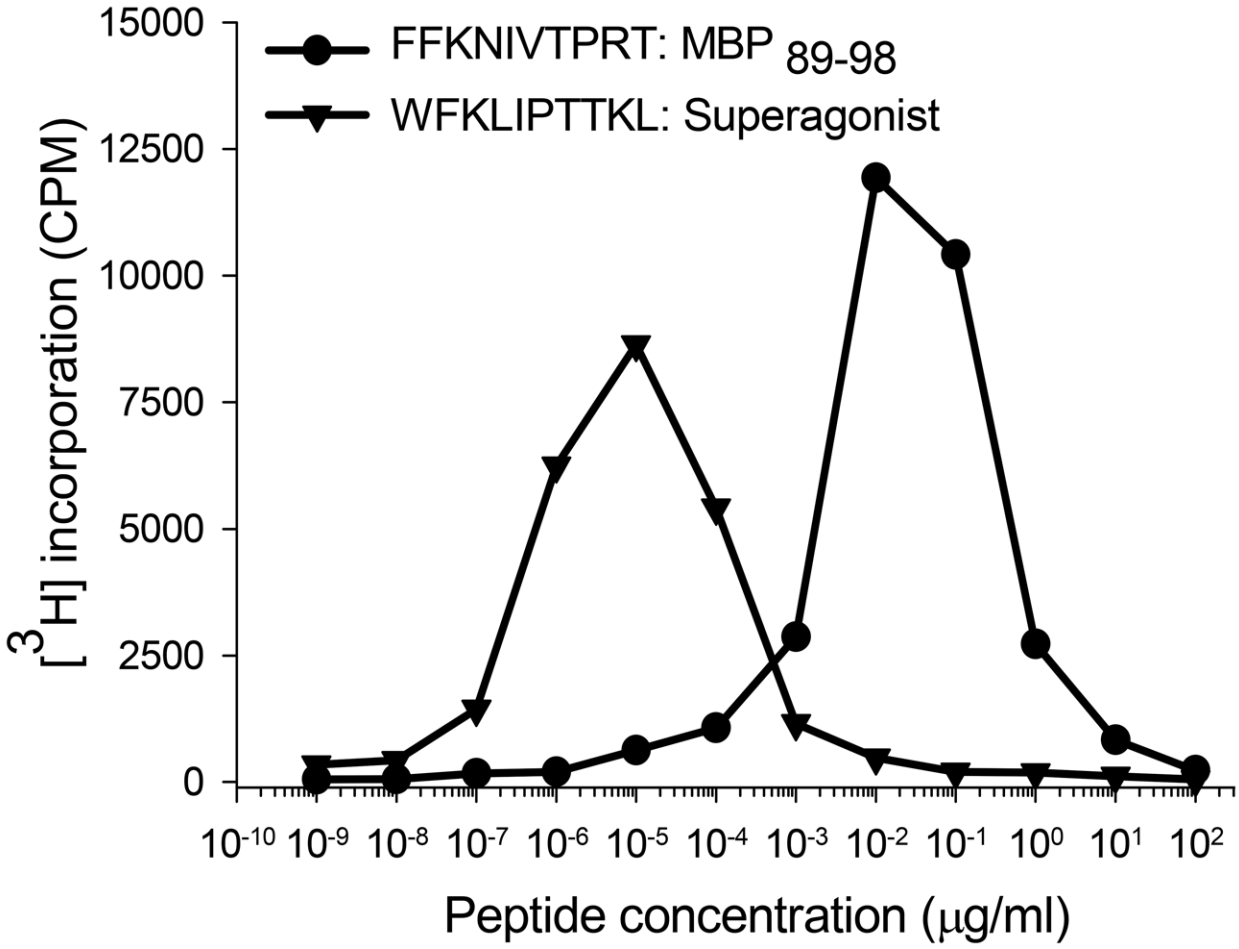

Figure 4.

Proliferation of TL3A6 Tg TCL in response to native MBP89-98 and a relative TL3A6 TCC superagonist. TL3A6 Tg TCL were established from TL3A6/ DR2a Tg mice and cultured with DR2a Tg spleen cells with MBP 89-98 (FFKNIVTPRT: EC_{50}= 2.1 × 10^{-3}μg/ml) and superagonist (WFKLIPTTKL: EC_{50}=3.7 × 10^{-7}μg/ml) for 48hrs and [^{3}H]-thymidine-uptake measured over an additional 16 hours. Data represent the average of 2 independent experiments.

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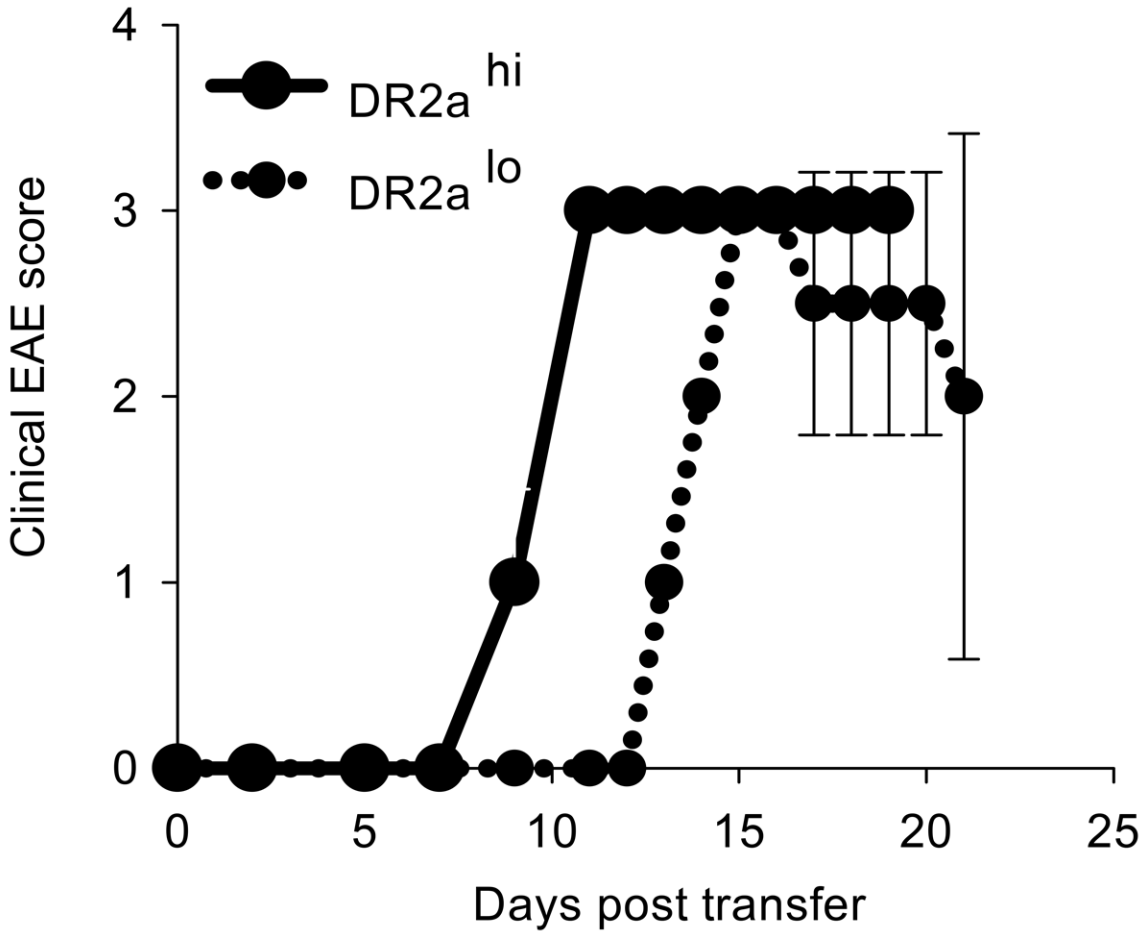

Figure 5.
Induction of passive EAE in DR2a Tg mice. TL3A6 Tg TCL raised from an immunized TL3A6/DR2a^{lo} mouse were transferred into 8 week-old DR2a^{lo} (n=4) and DR2a^{hi} (n=5) mice for induction of passive disease as outlined in Material & methods. Data indicate the mean and error bars the standard deviation.

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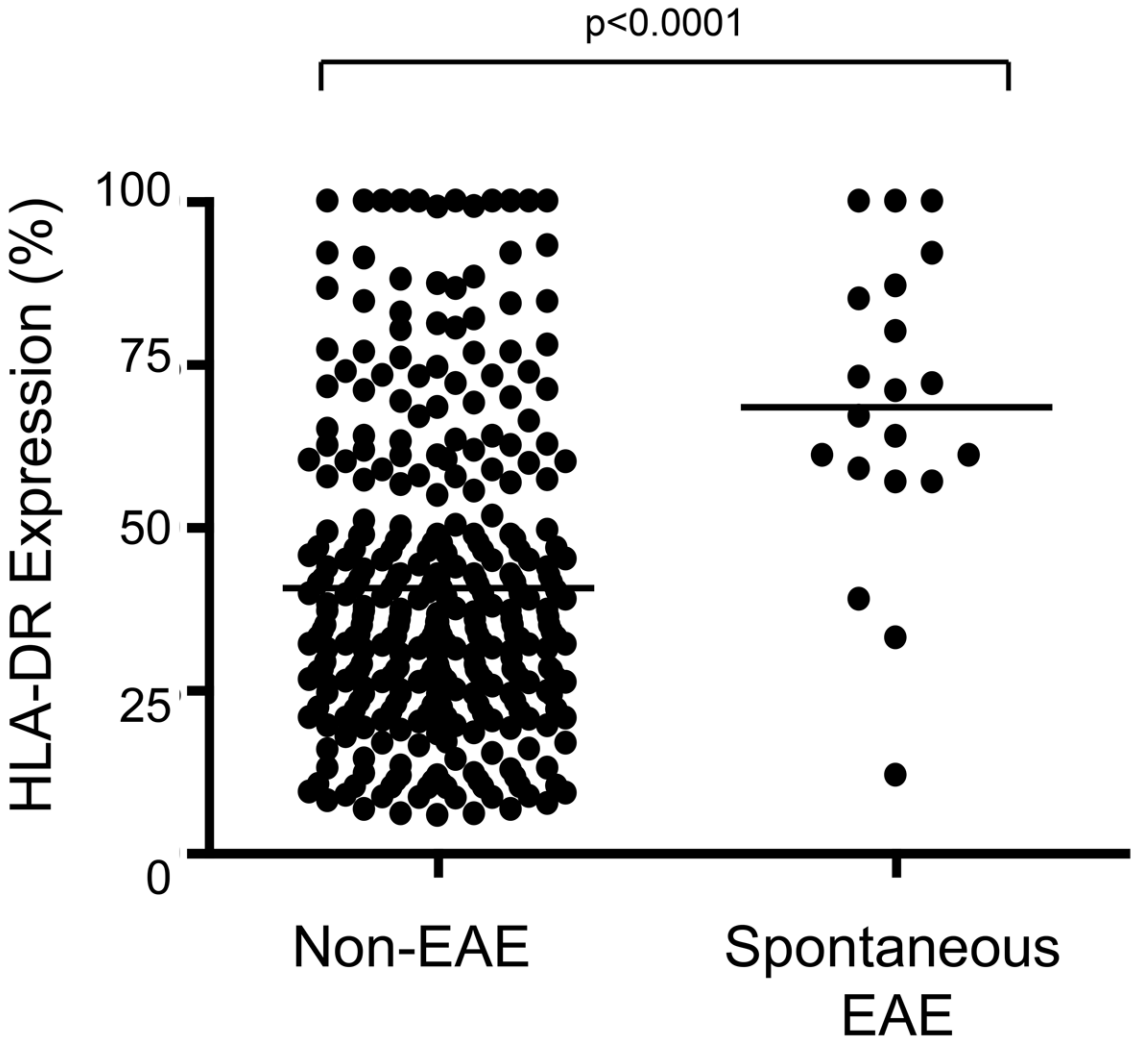

Figure 6.

Correlation between expression level of DR2a transgene and development of spontaneous EAE. Homozygous TL3A6/DR2a^{hi} Tg mice (HLA-DR mean fluorescence intensity (DR-MFI) = 100%) were crossed with homozygous TL3A6/DR2a^{lo} Tg mice (DRA-MFI ~10%) and the offspring were intercrossed several times to generate TL3A6/DR2a Tg mice expressing various levels of DR. Expression of HLA-DR on PBMC was examined by staining with anti-HLA-DR mAb at the age of 4-5 week-old and development of EAE was monitored until 25-30 weeks of age. Numbers are given as relative HLA-DR levels in percent compared with the HLA-DR-expression in TL3A6/DR2a^{hi} Tg mice. Twenty out of 346 mice developed spontaneous EAE. The clinical score of spontaneous EAE was 3.3±1.0 and onset of disease was 7.2±2.4 wks.

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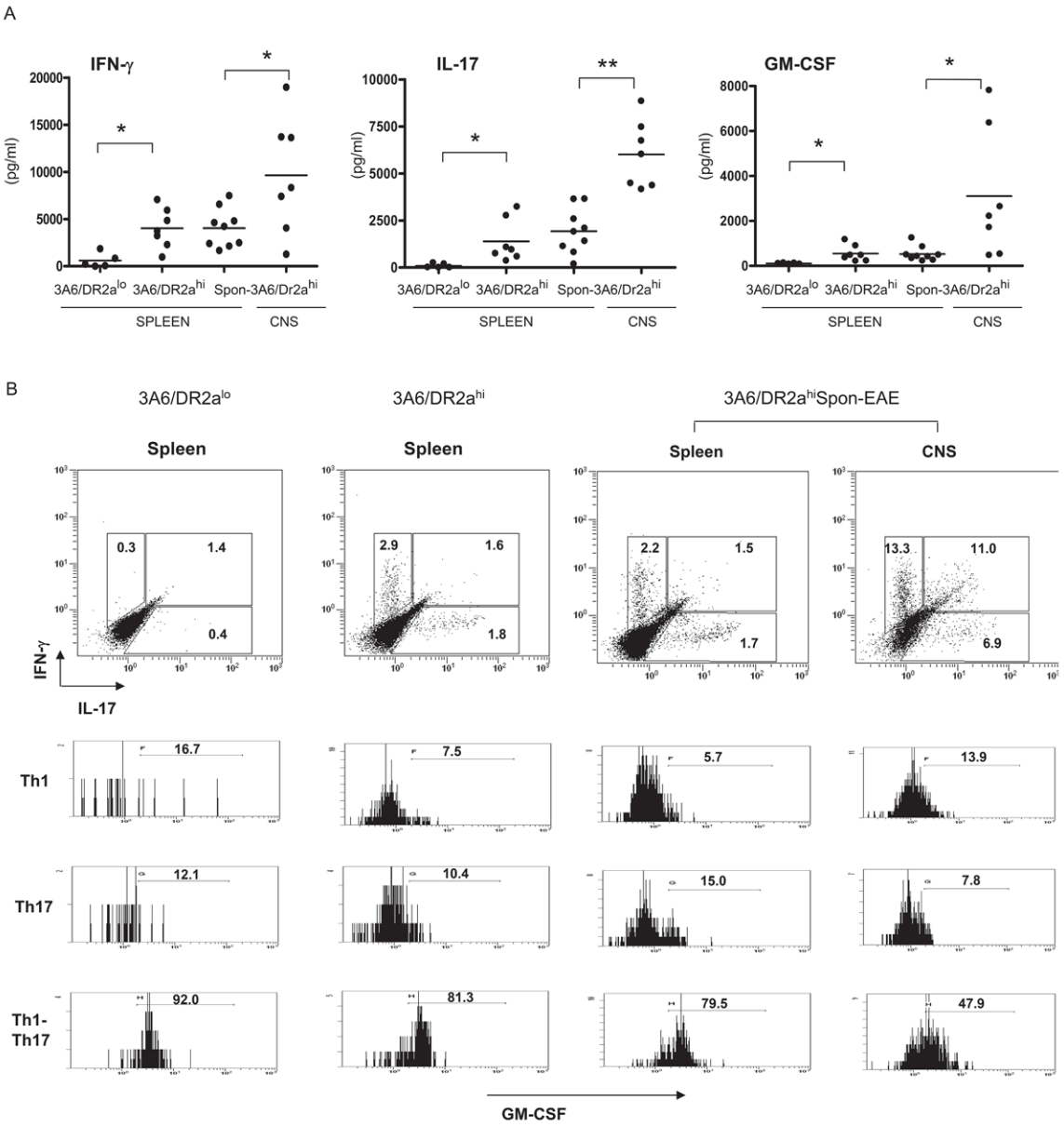

Figure 7.

Development of Th1, Th17, and GM-CSF-producing CD4 T cells.

(A) Splenocytes isolated from TL3A6/DR2a^{lo} mice, TL3A6/DR2a^{hi} mice (neither exhibiting clinical disease), and spontaneous EAE mice (TL3A6/DR2a^{hi} mice), and CNS mononuclear cells isolated from spontaneous EAE mice (TL3A6/DR2a^{hi} mice) were cultured with MBP(83-99) at 10 μg/ml for 3 days and production of IFN-γ, IL-17, and GM-CSF was measured by ELISA. (B) Flow cytometric analysis of the production of IFN-γ, IL-17, and GM-CSF in CD4$^+$ T cells by stimulation with MBP83-99 at 10 μg/ml for 5 hrs. CD4$^+$ cells are gated for production of IFN-γ and IL-17. IFNγ$^+$ (Th1), IL-17$^+$ (Th17), and IFNγ$^+$IL-17$^+$ (Th1-Th17) cells are further gated for analysis of GM-CSF production. Data are from one representative of more than three independent experiments. *; $p<0.05$, **; $p<0.0001$

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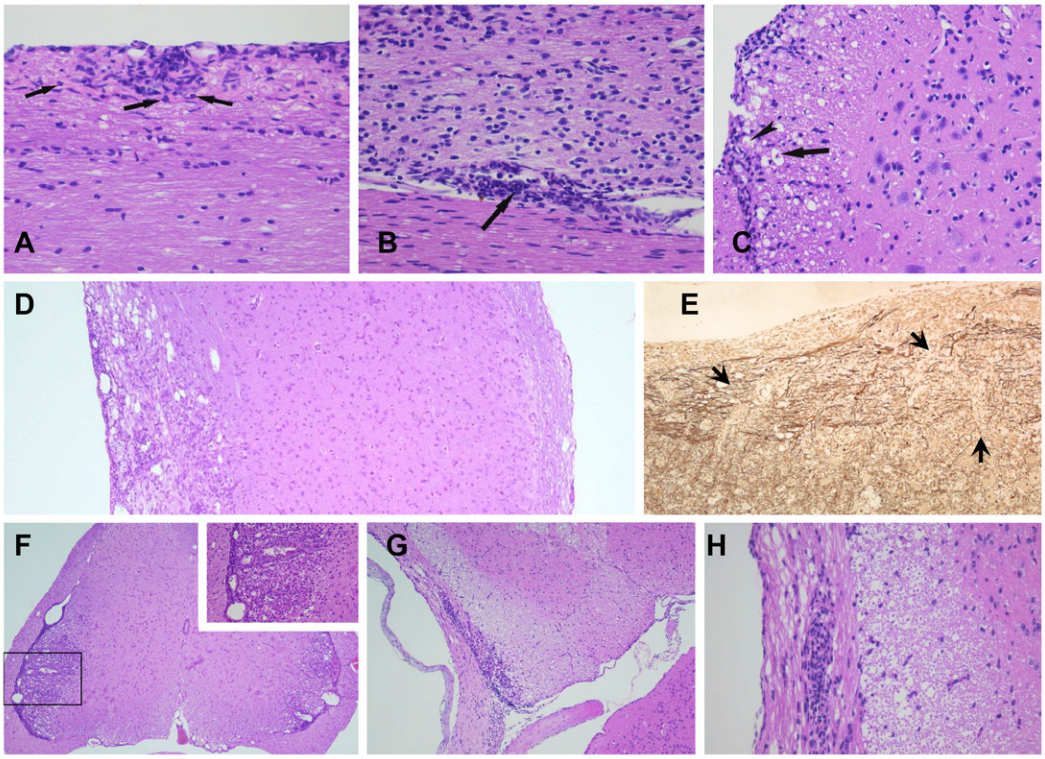

Figure 8.
Histopathological analysis of induced and spontaneous disease. Inflammatory infiltrates and axonal degeneration were observed primarily in the spinal cord of MBP 83-99-immunized TL3A6/DR2a Tg mice with relative sparing of the cerebrum or cerebellum. (A) Longitudinal section of the spinal cord reveals both granulocytic (arrows) and lymphocytic infiltrates similar to those shown in (B) at the margin of the spinal cord adjacent to the meninges. (C) Cross section of the spinal cord and spinal nerves of the cauda equina depict a swollen axon (arrow head) and a degenerated axon (arrow) (A-C, X400). Animals exhibiting spontaneous disease (in the absence of immunization or adjuvant) showed a similar pattern of primarily spinal cord involvement (D-H, inset). (D) Longitudinal section of the cord reveals severe white matter infiltration with relative gray matter sparing (X200). (E) Bielschowsky silver stained specimens indicate axonal injury/loss in spinal cord with several interrupted longitudinal fibers/loss of axonal staining (arrowheads) (X200). (F) Spontaneous meningomyelitis in the lumbar spinal cord with numerous infiltrates and swollen/fragmented axons (X100, inset X200). (G) Spontaneous EAE animals also showed evidence of neuritis where lymphocytic infiltration associated with cranial nerves (X100) (higher magnification in H: X200).

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Table I

MBP 83-99 immunization induces EAE in TL3A6/DR2a Tg mice.

Transgenes	Antigen	Incidence of disease (%)			Average disease severity
		Females	Males	Total	
MHC Class II (Lo)					
DR2a	MBP83-99/CFA	1/15 (7)	0/3	1/18 (6)	2.0
TL3A6/DR2a	MBP83-99/CFA	8/17 (47)	7/15 (47)	15/32 (47)	1.9±0.5
TL3A6/DR2a	CFA	1/3 (33)	0/3	1/6 (17)	2.0
MHC Class II (Hi)					
DR2a	MBP83-99/CFA	1/4 (25)	0/3	1/7 (14)	2.0
TL3A6/DR2a	MBP83-99/CFA	6/7 (86)	3/3 (100)	9/10 (90)	2.0±0.9
TL3A6/DR2a	CFA	1/6 (17)	1/5 (20)	2/11 (18)	1.0
MHC Class II -/-					
TL3A6	MBP83-99/CFA	0/2	0/2	0/4	

6 to 8 week-old mice were immunized with various combinations of peptide, CFA, and pertussis toxin to induce disease in DR2a-single, TL3A6-single, and double TL3A6/DR2a Tg mice. Mice were weighed and assessed daily for clinical disease.

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Table II

Induction of active EAE in TL3A6/DR2a^{lo} Tg mice with superagonist and native ligand MBP 83-99 peptide

Peptide EC_{50} (μg/ml)	Dose (μg) per mouse	Incidence of disease (%)	Day of onset	Peak severity
WFKLIPTTKL 3.7×10E-7	200	5/9 (56)	10.8 ± 2.1	2.6 ± 0.5
	100	5/9 (56)	10.6 ± 6.1	2.1 ± 1.0
	50	5/8 (63)	11.6 ± 5.8	2.3 ± 0.4
	25	1/4 (25)	32	1.0
FFKNIVTPRT 2.1×10E-3	200	5/9 (56)	13.0 ± 2.7	2.2 ± 0.3
	100	3/9 (33)	12.6 ± 2.9	1.7 ± 0.6
	50	0/9 (0)		
	25	0/9 (0)		

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Table III

Spontaneous disease in TL3A6/DR2a Tg mice.

Strain	Incidence of disease (%)	Age of onset (wks)	Peak severity	Gender	
				F	M
TL3A6/DR2a^{lo}	2/360 (0.6)	21 ± 13	3.0 + 0.7	1	1
TL3A6/DR2a^{hi}	5/110 (4.5)	10 ± 3	2.7 + 1.1	3	2
TL3A6/DR2a^{lo}/Rag-1KO	1/20 (5.0)	12	2.5	0	1
TL3A6/DR2a^{hi}/Rag-1KO	7/12 (58)	6 ± 1	2.1 + 1.0	2	5

Animals were monitored twice weekly for signs of clinical disease. Animals displaying clinical symptoms were either monitored for disease course or euthanized for histological analysis.