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*J Immunol* 2009; 182:5131-5139; doi: 10.4049/jimmunol.0803918
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HLA-DQ8 (DQB1*0302)-Restricted Th17 Cells Exacerbate Experimental Autoimmune Encephalomyelitis in HLA-DR3-Transgenic Mice

Ashutosh Mangalam,* David Luckey,* Eati Basal,* Megan Jackson,2* Michele Smart,* Moses Rodriguez,*† and Chella David3*

Among all of the genetic factors associated with multiple sclerosis (MS) susceptibility, MHC class II molecules have the strongest association. Although a direct role of DR alleles in MS have been confirmed, it has been difficult to understand the role of DQ alleles in disease pathogenesis due to strong linkage disequilibrium with certain DR alleles. Population studies have indicated that DQ alleles may play a modulatory role in progression of MS. Using HLA class II transgenic (Tg) mice, we investigated gene complementation between DR and DQ genes in the disease process. Previously, using single Tg mice (expressing HLA-DR or DQ gene), we showed that PLP91–110 peptide induced experimental autoimmune encephalomyelitis (EAE) only in DR3/DR4 mice, suggesting that DR3 (DRB1*0301) is a disease susceptibility gene in the context of PLP. We also showed that DQ6 protects development of EAE in DQ6/DR3 double Tg mice by production of anti-inflammatory IFN-γ. In this study, we investigated the ability of DQ8 to modulate disease in DR3/DQ8 double Tg mice. Introduction of DQ8 onto DR3 Tg mice led to higher disease incidence and increased disease severity on immunization with PLP91–110, indicating that DQ8 had an exacerbating effect on the development of EAE. Increased susceptibility in DR3/DQ8 Tg mice was due to increased production of proinflammatory cytokine IL-17 by DQ8-restricted T cells. HLA-DR3/DQ8 mice with EAE also demonstrated increased inflammation and demyelination in CNS as compared with single DR3 Tg mice. Thus double Tg mouse provides a novel model to study epistatic interactions between HLA class II molecules in inflammatory and demyelinating disease.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS characterized by the infiltration of immune cells such as T cells, B cells, and macrophages, resulting in demyelination of axons (1). The pathogenic CD4 T cells in the CNS are mainly restricted to components of self-myelin such as proteolipid protein (PLP), myelin basic protein, and myelin oligodendrocytic glycoprotein (MOG). PLP, a major component of CNS myelin, has emerged as an important target Ag in experimental autoimmune encephalomyelitis (EAE) and MS. PLP peptide-reactive T cells have been identified in MS patients (2–4).

Since myelin-Ag specific CD4 T cells are restricted by MHC class II molecules, it is not surprising that the strongest genetic factors influencing development of MS have been reported with the HLA locus on chromosome 6p21. Population studies have reported that individuals with certain HLA class II haplotypes such as HLA-DR2/DQ6, DR3/DQ2, and DR4/DQ8 have increased frequency of MS. The class II linkage in MS differs in various populations with highest association with HLA-DR2 (DRB1*1501)/DQ6 (DQB1*0602) (5–8), followed by DR3/DQ2 and DR4/DQ8 genes (8–13). Elegant studies by Dymet et al. (14) have shown that beside DRB1*15, the DRB1*17 (DR3) allele shows a clear association with MS susceptibility. A similar finding on association of DR3 with MS has been shown in non-Northern European, Canadian, Mexican, and Sardinian MS patients (11, 12, 15, 16). Recent studies have shown that disease outcome might be decided by a complex interaction among different class I and class II present in a haplotype, suggesting that a haplotype might be the basic immunogenetic unit of susceptibility or resistance (14, 17–20). The strong linkage disequilibrium among HLA-DR, HLA-DQ, and other genes within the HLA region makes it difficult to identify the role of individual genes in the immunopathogenesis of MS. To understand the role of class II molecules in inflammatory autoimmune diseases, transgenic (Tg) mice expressing human HLA-DR or -DQ genes on mouse endogenous class II-negative backgrounds were generated. Using these class II Tg mice, we and others have previously shown that PLP91–110 peptide can induce MS-like neurological disease in HLA-DR3 (21) and HLA-D4 Tg mice (22), while HLA-DR2 Tg mice were susceptible to MOG- and myelin basic protein-induced EAE (23, 24). Neither PLP nor MOG Ags were able to induce any disease in Tg mice expressing the human DQ6 or DQ8 gene (25). Thus, current data from HLA class II Tg mice suggest that HLA-DR genes such as HLA-DR2, DR3, and DR4 are responsible for predisposition and susceptibility to demyelinating disease. Population studies in MS indicate that other DR, DQ, as well as HLA class I alleles on disease-susceptible haplotypes influence frequency, progression, and severity of...
disease in human patients. Although HLA-DRB1*01, -DRB1*11, -DRB1*14, -DQB1*0601, and -DQB1*0603 protect MS (14, 17, 18, 26, 27), DQB1*0602 and DQB1*0302 alleles can increase disease susceptibility (7, 26, 28–30). Thus, it is hypothesized that the epistatic interaction between HLA molecules on disease-susceptible haplotypes plays an important role in final disease outcome in MS. We undertook this study to investigate the role of the HLA-DQ8 (DQB1*0302) gene in disease-susceptible HLA-DR3 Tg mice. We generated double Tg mice expressing DQB1*0302 (DQ8) on the disease-susceptible HLA-DR3 background to determine whether the presence of DQ8 allele can modulate development of EAE. Since these HLA class II Tg mice express human class II in the absence of endogenous mouse class II molecules, all of the T cell responses are restricted to human class II molecules. Previously, using double Tg mice, we reported that DQ6 (DQB1*0601) can protect DR3 (DRB1*0301).ABαβ mice from development of EAE (31). In the present study, we report that the presence of DQ8 on the DR3 background led to increased disease severity and CNS pathology as compared with DR3 single Tg mice. The synergistic effect of DQ8 on EAE might be due to increased production of IL-17 by DQ8-restricted CD4 T cells in DR3DQ8 double Tg mice.

Materials and Methods

Tg mice

The HLA-DQ8 (DQA1*0103, DQB1*0302), HLA-DR3 (DRB1*0301), and HLA-DR3/DQ8 Tg mice were produced as previously described (28, 32, 33). Briefly, HLA class II transgenes were introduced into (B6 × SWR/JF hybrids, a fertile eggs. Positive offspring were backcrossed to B110 M mice for several generations. HLA Tg mice were then mating over male mice. We undertook this study to investigate the role of the HLA-DQ8 (DQB1*0302) gene in disease-susceptible HLA-DR3 Tg mice. We generated double Tg mice expressing DQB1*0302 (DQ8) on the disease-susceptible HLA-DR3 background to determine whether the presence of DQ8 allele can modulate development of EAE. Since these HLA class II Tg mice express human class II in the absence of endogenous mouse class II molecules, all of the T cell responses are restricted to human class II molecules. Previously, using double Tg mice, we reported that DQ6 (DQB1*0601) can protect DR3 (DRB1*0301).ABαβ mice from development of EAE (31). In the present study, we report that the presence of DQ8 on the DR3 background led to increased disease severity and CNS pathology as compared with DR3 single Tg mice. The synergistic effect of DQ8 on EAE might be due to increased production of IL-17 by DQ8-restricted CD4 T cells in DR3DQ8 double Tg mice.

Flow cytometry

Expression of HLA-DR and HLA-DQ molecules on PBLs, lymph node cells (LNCs), and splenocytes were analyzed by flow cytometry using mAbs specific for VαM mice for several generations. HLA Tg mice were then mated to class II-negative mice from depletion of the T cell responses is restricted to human class II molecules. Previously, using double Tg mice, we reported that DQ6 (DQB1*0601) can protect DR3 (DRB1*0301).ABαβ mice from development of EAE (31). In the present study, we report that the presence of DQ8 on the DR3 background led to increased disease severity and CNS pathology as compared with DR3 single Tg mice. The synergistic effect of DQ8 on EAE might be due to increased production of IL-17 by DQ8-restricted CD4 T cells in DR3DQ8 double Tg mice.

Disease induction

For disease induction, 12- to 14-wk-old Tg mice were immunized s.c. in both flanks with 100 µg of PLP1-110 emulsified in CFA containing M. tuberculosis H37Ra (400 µg/mice). Pertussis toxin (100 ng; Sigma-Aldrich) was injected i.v. at days 0 and 2 after immunization. Mice were observed daily for clinical symptoms and disease severity was scored as follows: 0: normal; 1: loss of tail tone; 2: hind limb weakness; 3: hind limb paralysis; 4: hind limb paralysis and forelimb paralysis or weakness; and 5: moribundity/death. Mice of both sexes were used.

Cytokine production

Draining lymph nodes (LNs) were collected 10 days after immunization and stimulated with PLP1-110. Peptide was analyzed using real-time PCR. RNA was extracted from cells using RNaseasy columns (Qiagen). cDNA was prepared using reverse transcriptase (Invitrogen). cDNA was analyzed by real-time quantitative PCR in triplicates by using a SYBR GreenER qPCR Reagent System (Invitrogen). The expression level of each gene was quantified using the threshold cycle (Ct) method normalized for the housekeeping gene β-actin.

Neutralization of IFN-γ and IL-17 (anti-cytokine) treatment of EAE

HLA-DQ8 or DR3DQ8 Tg mice were injected i.p. either with 250 µg of anti-IFN-γ (clone H22, mouse IgG) or 200 µg of anti-IL-17 (clone TC11-18H10, BD Biosciences) or isotype control mouse IgG. Anti-IFN-γ was given at days 1 and 5 after immunization (both anti-IFN-γ and isotype control Abs were a gift from Dr. R. Schreiber, Washington University, School of Medicine, St. Louis, MO) while anti-IL-17 was administered at 4, 8, 12, and 16 days after immunization as published previously (35).

In situ apoptosis detection

Apoptotic cells were detected by TUNEL using an In Situ Cell Death Detection Kit (fluoresein; Roche Applied Science) according to the manufacturer’s directions. Apoptosis (as evidenced by intense nuclear TUNEL staining) was evaluated using a Zeiss Axiosvert 510 M confocal laser-scanning microscope.

Pathology

Mice were perfused via intracardiac puncture with 50 ml of Trump’s fixative. Spinal cords and brains were removed and postfixed for 24–48 h in Trump’s fixative in preparation for morphological analysis. All grading was performed without knowledge of the experimental group. Spinal cords were cut into 1-mm coronal blocks and every third block was postfixed in osmium and embedded in glycol methacrylate. Two-micrometer sections were stained with a modified erichrome/cresyl violet stain. Morphological analysis was performed on 12–15 sections per spinal cord. Briefly, each quadrant from every coronal section from each spinal cord was graded for the presence or absence of inflammation and demyelination. The score was expressed as the percentage of spinal cord quadrants examined with the pathological abnormality. A maximum score of 100 indicated that there was a particular pathological abnormality in every quadrant of all spinal cord sections. Brain pathology was assessed following perfusion. Two coronal cuts were made in the intact brain (one section through the optic chiasm and a second section through the infundibulum). This resulted in three blocks that were embedded in paraffin. This allowed for analysis of the cortex, corpus callosum, hippocampus, brainstem, striatum, and cerebellum. The resulting slides were stained with H&E. Each area of the brain was graded on a 4-point scale: 0, no pathology; 1, no tissue destruction but minimal inflammation; 2, early tissue destruction, demyelination, and moderate inflammation; 3, moderate tissue destruction (neuronal loss, demyelination, parenchymal damage, cell death, neurophagia, neuronal vacuolation); and 4, necrosis (complete loss of all tissue elements with associated cellular debris). Meningeal inflammation was graded as follows: 0, no inflammation; 1, no inflammation; 2, one cell layer of inflammation; 3, two cell layers of inflammation; and 4, four or more cell layers of inflammation. The area with maximal extent of tissue damage was used for assessment of each brain region.
Class II expression in single and double Tg mice. Normal expression of HLA-DR and/or HLA-DQ was observed in PBLs (A), spleen (B), and LNCs (C) of single and double Tg mice. PBLs, splenocytes, and LNCs were isolated from MHC class II-deficient control mice (Aβ), DQ8.Aβ, DR3.Aβ, or DR3.DQ8.Aβ Tg mice and analyzed for cell surface markers (HLA-DR/HLA-DQ alone or along with B cells/DCs markers) by flow cytometry. Numbers in histograms indicate the percentage of cells positive for the HLA-DR/HLA-DQ marker. B cells showed maximum class II expression in all of the tissue samples analyzed. Data represent one of three experiments performed at different time points.

Statistical analysis
The statistical significance of the differences in functional and histological scores between groups was assessed by a one-way ANOVA on ranks (Kruskal-Wallis test) when comparing more than two groups and by the Mann-Whitney U test when only two groups were analyzed. The Student’s t test was used when more than two groups were analyzed or by Student’s t test when comparing only two groups. The statistical significance of the differences in proliferation or in cytokine levels between groups was assessed by a one-way ANOVA with multiple comparisons of the means when more than two groups were analyzed or by Student’s t test when only two groups were analyzed if their data were normally distributed.

Results
Characterization of HLA-DR3.DQ8 Tg mice
All Tg lines developed normally and showed no gross phenotypic abnormalities. Both HLA-DR and DQ were expressed on 35–50% of cell populations in PBLs and splenocytes (Fig. 1, A and B). HLA-DR or DQ expression was also detected on 14–21% of LN cell populations in DR3, DQ8, or DR3DQ8 mice (Fig. 1C). Expression of HLA-DR and DQ was observed on B cells, macrophages, and DCs (data not shown). No endogenous class II expression was seen in class II knockout Aβ mice (Fig. 1). Thus, both HLA-DR and -DQ molecules were expressed at similar levels in DR3.Aβ, DQ8.Aβ, and DR3.DQ8.Aβ Tg mice.

Table 1. *PLP* 91–110-induced EAE in HLA Tg mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Disease Incidence (%)</th>
<th>Mean Onset of Disease ± SD</th>
<th>No. of Mice with Maximum Severity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10</td>
<td>0/15 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ</td>
<td>0/15 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQ8.Aβ</td>
<td>0/20 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR3.Aβ</td>
<td>23/35 (66%)</td>
<td>13 ± 1.4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>DR3.DQ8.Aβ</td>
<td>32/35 (91%)</td>
<td>9.3 ± 0.9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

a Mice were immunized with 100 μg of PLP peptide/400 μg of *M. tuberculosis* in CFA and pertussis toxin was administered at 0 and 48 h after immunization. Mice were scored daily for disease as mentioned in Materials and Methods. The data are from three experiments combined.

p < 0.01 Mann-Whitney U rank-sum test, DR3 compared to other mice.
mice (91% vs 66%, p < 0.05). DR3.DQ8.Aβ mice also showed increased disease severity compared with DR3.Aβ mice (mean clinical score, 3.4 ± 0.2 vs 2.3 ± 0.3, p < 0.05). Disease severity in DR3.DQ8.Aβ mice was characterized by weight loss (data not shown) accompanied by paralysis of the hind limb (15 of 32), as well as paralysis of the forelimb (10 of 32), and 5 of 32 mice also became moribund. Thus, the above data indicated that DQ8 plays a modulatory role in DR3DQ8 double Tg mice by causing more severe EAE in disease-susceptible DR3 Tg mice.

**DR3.DQ8.Aβ mice produce increased levels of IL-17 and IFN-γ**

We previously showed that both DR3 as well as DQ8 molecules recognize the PLP91–110 peptide (21). Although DQ8 Tg mice are resistant to PLP91–110-induced EAE, double Tg DR3DQ8 mice showed more severe EAE. Therefore, we analyzed the T cell proliferative response and levels of different inflammatory cytokines in PLP91–110-immunized single and double Tg mice in an attempt to identify the mechanism for this increased severity. LNCs from PLP91–110-immunized DR3.Aβ°, DQ8.Aβ°, and DR3.DQ8.Aβ° Tg mice were stimulated in vitro with PLP peptide in the presence or absence of blocking Abs to HLA-DR (L227) or HLA-DQ (IVD12) and the T cell proliferation was measured using a standard thymidine incorporation assay. T cells from DR3.DQ8.Aβ° Tg mice showed a higher T cell response to PLP91–110 peptide as compared with the T cell response observed in single Tg DR3 or DQ8 mice (Fig. 3A). As expected HLA-DR Ab inhibited T cell proliferation of DR3.Aβ° but had no effect on T cell proliferation from DQ8.Aβ° mice, and similarly anti-DQ Ab IVD12 inhibited the T cell response only in DQ8 mice (data not shown) but not in DR3-specific T cell cultures. Although the presence of anti-DR Ab inhibited T cell proliferation completely in DR3 mice, only partial inhibition was observed in T cell cultures from double Tg DR3.DQ8.Aβ° mice. Similarly, anti-DQ Ab inhibited T cell proliferation completely in DQ8 but partially in DR3.DQ8.Aβ° mice. Thus, both DR- and DQ- specific T cells were recognizing PLP peptide in double Tg mice.

Disease-susceptible DR3.Aβ° Tg mice produced moderate to high levels of IFN-γ, TNF-α, IL-2, IL-6, and IL-12 cytokines (Fig. 3B), showing classical Th1 phenotype. Although mononuclear cells from DQ8 mice did not produce IFN-γ, they produced significantly higher levels of IL-17 (p < 0.01), recently discovered proinflammatory cytokine, and associated with development of autoimmune diseases. Double Tg DR3DQ8 mice also produced higher levels of IL-17 as well as IFN-γ, besides producing moderate to high levels of TNF-α, IL-2, IL-6, and IL-12 cytokines. IL-4 levels were below detection limits in all samples from single and double Tg mice. DR3.Aβ° Tg mice also produced moderate amounts of IL-17, IL-21, and IL-23 (Fig. 3C); however, levels were significantly less (p < 0.01) as compared with DQ8 or DR3DQ8 mice. Both DR3 and DR3DQ8 mice produced comparable levels of IL-27. Thus, disease-susceptible DR3.DQ8 mice produced higher levels of IL-17, IL-21, IL-23, and IFN-γ as compared with DR3 mice.

**Increased levels of IFN-γ is produced by DR3-specific T cells, while IL-17 is produced by DQ8-specific T cells**

Thus, cytokine data suggested that the exacerbating effect of the DQ8 molecule on disease severity in double Tg DR3.DQ8.Aβ° mice might be due to high levels of IL-17 or IFN-γ, which can be produced by either DQ- and/or DR- specific T cells. Therefore, we performed an ELISPOT assay (a standard assay for analyzing Ag-specific cytokine levels) to analyze the source of T cells producing IL-17 and IFN-γ. LNCs from PLP91–110-immunized
DR3.Aβ₀, DQ8.Aβ₀, and DR3.DQ8.Aβ₀ Tg mice were stimulated in vitro with PLP peptide and levels of IL-17 and IFN-γ were analyzed in the presence or absence of blocking Abs to HLA-DR (L227) or HLA-DQ (IVD12) using a cytokine-specific ELISPOT assay. Analysis of IFN-γ spots showed that T cells from DR3DQ8 mice produced slightly higher levels of IFN-γ (275 ± 30 vs 200 ± 25, p < 0.01) compared with T cells from DR3 Tg mice (Fig. 4A). Ab-blocking experiments confirmed that most of the IFN-γ was produced by DR-specific T cells as it was significantly inhibited by anti-DR Ab in DR3DQ8 as well as DR3-specific cultures, while anti-DQ Ab had no effect on IFN-γ spots (Fig. 4A). Furthermore, T cells from DQ8 as well as DR3DQ8 showed significantly higher IL-17 spots compared with those from DR3 Tg mice (240 ± 27 vs 62 ± 20 (DR3), p < 0.001 and 320 ± 37 (DR3DQ8) vs 62 ± 20 (DR3), p < 0.001). Although blocking with anti-DR Ab suppressed IFN-γ-producing T cells in DR3.DQ8.Aβ₀-specific cultures, it had minimal effect on IL-17-producing cells (Fig. 4B). In contrast, anti-DQ-blocking Ab suppressed most of the IL-17 spots in DR3DQ8-specific cultures but had no effect on IFN-γ-producing T cells. The presence of anti-DQ Ab in cultures

Table II. Neutralization of IFN-γ and IL-17 in HLA Tg mice immunized with PLP91–110α

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Treatment IFN-γ/IL-17 or Isotype Control</th>
<th>Disease Incidence (%)</th>
<th>Mean Onset of Disease ± SD</th>
<th>No. of Mice with Maximum Severity Score</th>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>DQ8.Aβ₀</td>
<td>IFN-γ</td>
<td>0/10 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQ8.Aβ₀</td>
<td>Isotype</td>
<td>0/10 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR3.DQ8.Aβ₀</td>
<td>IFN-γ</td>
<td>9/10 (90%)</td>
<td>9.5 ± 1.2</td>
<td>1</td>
</tr>
<tr>
<td>DR3.DQ8.Aβ₀</td>
<td>Isotype</td>
<td>9/10 (90%)</td>
<td>9.2 ± 0.9</td>
<td>2</td>
</tr>
<tr>
<td>DQ8.Aβ₀</td>
<td>IL-17</td>
<td>0/10 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DQ8.Aβ₀</td>
<td>Isotype</td>
<td>0/10 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR3.DQ8.Aβ₀</td>
<td>IL-17</td>
<td>6/10 (60%)</td>
<td>11 ± 2.0</td>
<td>3</td>
</tr>
<tr>
<td>DR3.DQ8.Aβ₀</td>
<td>Isotype</td>
<td>9/10 (90%)</td>
<td>9.5 ± 1.2</td>
<td>5</td>
</tr>
</tbody>
</table>

α Mice were immunized with 100 μg of PLP peptide/400 μg of M. tuberculosis in CFA and pertussis toxin was administered at 0 and 48 h after immunization. Anti-IFN-γ (250 μg/mice) or isotype control was administered at days −1 and 10 after immunization. In the IL-17 group, mice were treated with anti-IL-17 Ab (200 μg/mice) or isotype control Ab at days 4, 8, 12, and 16 after immunization. Mice were scored daily for disease (as stated in Materials and Methods) and the daily mean clinical score is presented for each group. The data are from two experiments combined.

β p < 0.05 Mann-Whitney U rank-sum test, DR3.DQ8 treated with anti-IL-17 Ab compared to DR3.DQ8 treated with isotype control Ab mice.
suppressed (>80%) IL-17 spots in both DQ8-specific and DR3DQ8-specific cultures, while anti-DR Ab suppressed only 15–20% of IL-17 spots in DR3DQ8-specific T cell cultures. Anti-DQ Ab had no effect on IL-17 spots observed in cultures from DR3 mice. Thus, higher levels of IL-17 in double Tg DR3DQ8.A° mice was produced by PLP-restricted DQ8-specific CD4 T cells, while most of the IFN-γ was produced by DR-specific CD4 T cells.

Neutralization of IL-17 but not IFN-γ in DR3DQ8.A° Tg mice abolished the synergistic effect of DQ8 molecule

Data from previous experiments suggested that either high levels of IL-17 produced by PLP-specific DQ8-restricted T cells or IFN-γ produced by DR3-specific T cells might be responsible for the synergistic effect of DQ8, leading to increased disease incidence and severity. Therefore, we performed in vivo neutralization of either IL-17 or IFN-γ, with a rationale that blocking of cytokines responsible for increased disease severity would lead to a decrease in disease incidence and severity. Single and double Tg mice were immunized with PLP\textsubscript{91–110} and treated with either neutralizing IFN-γ Ab (clone H-22) or neutralizing IL-17 Ab (clone TC11-18H10) at time points mentioned in Materials and Methods. Five animals in each group were also treated with respective control isotype Ab. Treatment with anti-IFN-γ had no effect on disease incidence or severity in double Tg DR3DQ8.A° mice (Table II and Fig. 5A). However, treatment with neutralizing IL-17 Ab led to a decrease incidence and severity in double Tg DR3DQ8.A° mice compared with mice treated with isotype control Ab (Table II and Fig. 5B). Thus, the increased disease severity observed in double Tg DR3DQ8.A° mice was due to high levels of IL-17 produced by DQ8-specific T cells. Blocking of IFN-γ or IL-17 in DQ8.A° mice by neutralizing Ab had no effect on disease phenotype.

FIGURE 6. CNS-infiltrating inflammatory cells. A, DR3DQ8 mice with EAE showed a higher number of CNS-infiltrating cells compared with DR3 mice with EAE. EAE was induced in HLA Tg mice and CNS (brain and spinal cord)-infiltrating mononuclear cells were isolated using a Percoll gradient. B and C, High levels of IFN-γ as well as IL-17 was detected on culturing CNS-infiltrating cells from DR3 mice and DR3DQ8 mice; however, levels of both cytokines were significantly higher in DR3DQ8 Tg mice compared with DR3 Tg mice. Stimulation with PLP\textsubscript{91–110} led to a 2- to 3-fold increase in the levels of IL-17 and IFN-γ. For cytokine analysis, CNS-infiltrating cells from Tg mice (with EAE) were stimulated with or without PLP\textsubscript{91–110} for 48 h in in vitro cultures and cytokines levels were determined as described previously.

FIGURE 7. CNS pathology in DR3.Aβ° and DR3DQ8.Aβ° Tg mice with EAE. Double Tg DR3DQ8.Aβ° mice with EAE show severe inflammation and demyelination in CNS compared with single Tg DR3.Aβ° mice with EAE. Representative photomicrographs of inflammatory lesion in brain (A) and spinal cord (B) of DR3 and DR3DQ8 Tg mice immunized with PLP Ag. A, Brain samples were embedded in paraffin and stained with H&E. Brain pathology was characterized with widespread inflammation and demyelination in DR3.DQ8.Aβ° compared with mild meningeal inflammation in DR3.Aβ° mice with EAE. B, The photographs of 2-μm-thick spinal cord sections show increased inflammation and demyelination in DR3.DQ8.Aβ° mice compared with DR3.Aβ° mice. These figures are representative of one of four experiments. Quantitative analysis of brain (C) and spinal cord (D) pathology also showed that double Tg DR3.DQ8.Aβ° mice with EAE have a higher pathology score compared with DR3.Aβ° mice. C, Pathology scores in brain (each bar represents the histological score for each mouse) and D, percentage of spinal cord quadrants showing inflammation and demyelination (mean ± SD) as described in Materials and Methods. Tg mice and control mice were immunized with 100 μg of PLP\textsubscript{91–110} peptide emulsified in CFA containing 400 μg of Mycobacterium. Pertussis toxin (100 ng) was administered at 0 and 48 h after immunization and Tg mice were sacrificed on day 25 after immunization.
Plastic-embedded sections from DR3DQ8.A and demyelination in DR3.DQ8 mice was also observed in the spinal cord with increased inflammation. A similar pattern of pathology was observed in all parts of the brain tissue including cerebellum, brainstem, and meninges (Fig. 7). To determine whether the presence of DQ8 on disease-susceptible DR3.A also had an effect on CNS pathology, we analyzed brain and spinal cord tissue of single Tg DR3.A mice but not from DR3.DQ8.A mice. Although DR3.DQ8.A mice showed demyelination only in 20 ± 5% of spinal cord quadrants, 40 ± 6% of the quadrants from double Tg DR3.A mice showed loss of myelin (p < 0.001, Fig. 7, B and D). Thus, double Tg mice showed severe CNS pathology which was similar to the pathology observed in MS.

Discussion

MS, like other putative autoimmune diseases, has a strong genetic component associated with certain HLA-DR/DQ haplotypes such as DR2/DQ6, DR3/DQ2, and DR4/DQ8. Population studies in MS as well as EAE studies done in HLA class II Tg mice have confirmed that HLA-DR alleles such as DR2, DR3, and DR4 are major susceptibility genes. The role of the HLA-DQ allele in disease pathogenesis has not been well understood due to strong linkage disequilibrium between DR and DQ genes; prevailing data suggest that the HLA-DQ allele might play a modulatory role. Using double HLA class II Tg mice expressing both HLA-DR3 and HLA-DQ8, we showed that whereas HLA-DR3 is the main disease predisposing gene in EAE in the context of PLP91–110, the HLA-DQ8 molecule plays a modulatory role. Although, DQ8 (DQB1*0302) Tg mice were resistant to PLP91-110-induced EAE, the presence of the DQ8 gene on disease-susceptible DR3 (DRB1*0301) Tg mice led to increased incidence and disease as well as severe CNS pathology, suggesting an exacerbating role for the DQ8 molecule in inflammation and demyelination. This increased severity observed in DR3DQ8 double Tg mice might be due to high levels of the 

Increased apoptosis of inflammatory cells in CNS of single Tg DR3.DQ8 mice

Next, we investigated why double Tg mice expressing both DR3 and DQ8 show more pathology. It has been suggested previously that apoptosis of CNS-infiltrating inflammatory cells play an important part in recovery from EAE. We hypothesized that less pathology in DR3 mice was the result of CNS-infiltrating inflammatory cells undergoing increased apoptosis. To investigate this hypothesis, TUNEL staining was performed on brain sections. Inflammatory cells in the CNS of DR3 mice with EAE showed a higher percentage of TUNEL-positive cells compared with DR3DQ8 double Tg mice with EAE (Fig. 8). The TUNEL-stained cells were observed in the inflamed meninges. No TUNEL staining was observed in control mice without EAE. Thus, inflammatory cells in the CNS of double Tg mice had a longer survival than in DR3 Tg mice with EAE.

High levels of IL-17 led to increased T cell migration into CNS of DR3.DQ8.Aβ° mice

In recent years, IL-17 has emerged as an important inflammatory cytokine in the development of autoimmune diseases and it can exert its proinflammatory role through a number of pathways. We investigated whether Ag-specific T cells from DR3DQ8 mice can cross blood-brain barrier more efficiently then those from single Tg DR3 mice. Brain- and spinal cord-infiltrating cells were isolated at different time points from mice immunized with PLP91-110 peptide. At all time points, DR3DQ8 mice with EAE showed an increased cellular infiltration in the CNS compared with DR3 single Tg mice (Fig. 6A). CNS-infiltrating cells from DR3 as well as DR3DQ8 produced 5- to 6-fold higher levels of IFN-γ (Fig. 6B) and IL-17 (Fig. 6C) without any stimulation in ex vivo cultures suggesting highly activated cells. Ex vivo stimulation of CNS-infiltrating cells with PLP91-110 peptide lead to a 2- to 3-fold increase in the levels of IFN-γ (Fig. 6B) and IL-17 (Fig. 6C), indicating the presence of Ag-specific T cells in CNS. Levels of both cytokines were higher in DR3DQ8 mice compared with DR3 mice (3000 ± 440 vs 1150 ± 200, p < 0.01). When stained for T cell activation markers, we found that CD4+ T cells showed activated phenotype, as they were CD44high and CD45RBlow (data not shown). Thus, the above findings indicated that PLP91-110-specific, HLA-DR/DQ-restricted CD4+ T cells were present in the CNS of both DR3 and DR3DQ8 Tg mice with EAE.

DR3DQ8 double Tg mice show severe CNS pathology compared with DR3 Tg mice

To determine whether the presence of DQ8 on disease-susceptible DR3 mice also had an effect on CNS pathology, we analyzed brain and spinal cord tissue from mice with EAE. Pathological analysis of CNS tissue showed that double Tg DR3DQ8.Aβ° mice had increased inflammation and demyelination compared with DR3.Aβ° mice (Fig. 7). Although single Tg DR3.Aβ° showed primarily inflammation localized to the meninges of the spinal cord and brain, double Tg DR3DQ8.Aβ° mice showed more widespread brain pathology with severe inflammation and demyelination in all parts of the brain tissue including cerebellum, brainstem, cortex, corpus callosum, stratum, and meninges (Fig. 7, A and C). Plastic-embedded sections from DR3DQ8.Aβ° mice but not from DR3.Aβ° also showed typical parenchymal white matter loss, the classical pathology observed in MS. A similar pattern of pathology was also observed in the spinal cord with increased inflammation and demyelination in DR3DQ8.Aβ° mice compared with DR3.Aβ° mice (Fig. 7B). Quantitative analysis of spinal cord tissues showed that on average 44 ± 6% of the spinal cord quadrants from DR3DQ8.Aβ° mice showed inflammation compared with only 23 ± 7% in single Tg DR3.Aβ° mice (p < 0.01; Fig. 7D). Although DR3.Aβ° showed demyelination only in 20 ± 5% of spinal cord quadrants, 40 ± 6% of the quadrants from double Tg mice showed loss of myelin (p < 0.001, Fig. 7, B and D). Thus, double Tg mice showed severe CNS pathology which was similar to the pathology observed in MS.
proinflammatory cytokine IL-17 produced by DQ8-restricted LPF90-110-specific T cells.

A disease-enhancing role for DQ8 is in agreement with human linkage studies. Population studies have shown that the DQ8 (DQB1*0302) allele is associated with more severe forms of MS (9, 13, 36, 37). Olerup et al. (36) and Marrou et al. (37) showed that the DQ8/DR4 haplotype was associated with MS in Sardinia and since then a number of other studies have confirmed the association of DQ8 with MS in different ethnic populations. Using a high-resolution HLA genotyping analysis, Zivadinov et al. (13) showed that beside DR2/DR6, the presence of the DQ8 allele was associated with more severe damage on inflammatory and neurodegenerative magnetic resonance imaging measures. We previously showed that DR3DQ8 double Tg mice develop severe disease on immunization with whole myelin extract compared with DR3 single Tg mice (28). Similarly, MOG induced a more severe form of EAE in DR2/DQ8 double Tg mice compared with DR2 single Tg mice (24). In this study, we show that the presence of DQ8 on the DR3 background increases disease incidence and severity in PLP-induced EAE, indicating a modulating role of DQ8. Since DQ8 can worsen disease severity in both DR2/DQ8 and DR3/DQ8 double Tg mice, we hypothesize that the presence of DQ8 with any of the disease-susceptible DR alleles such as DR2, DR3, or DR4 might lead to more severe demyelinating disease.

How certain HLA class II molecules such as DQ8 modulates disease when present with the disease-susceptible HLA-DR allele is not understood. There are several explanations for this modulatory effect of the DQ8 molecule. The first possibility is that there is a reduced number of CD4+CD25+FoxP3+ Treg cells in double Tg DR3DQ8 mice compared with DR3 single Tg mice. However, we observed a similar number of regulatory T cells in all three strains of mice (data not shown), ruling out the role of regulatory T cells in modulating disease. Another possibility is that DR3DQ8 Tg mice show a stronger T cell response to the encephalitogenic epitope of PLP, which leads to production of increased levels of proinflammatory cytokines resulting in increased inflammation and severe disease. Analysis of T cell responses indicated that although DQ8 showed a moderate T cell response to PLP90-110, T cells from immunized double Tg DR3DQ8 mice showed 1.5-fold stronger T cell responses compared with the T cell response observed in DR3 Tg mice. Because it is well established that both Th1 as well as Th17 play an important role in immunopathogenesis of EAE, we next analyzed the cytokine(s) responsible for this severe disease and CNS pathology. Disease-susceptible DR3 mice produce only moderate amounts of IFN-γ and IL-17, whereas DR3DQ8 double Tg mice produce significantly higher levels of IFN-γ (1180 ± 150 vs 650 ± 90, p < 0.05) and IL-17 (1200 ± 150 vs 215 ± 40, p < 0.001). DR3DQ8 mice also produced higher levels of IL-6 and IL-23, cytokines associated with the IL-17 and Th17 pathways. However, levels of TNF-α were similar between DR3 and DR3DQ8 double Tg mice. Disease-resistant DQ8 mice also produced high levels of IL-17 compared with DR3 Tg mice, but no IFN-γ. The failure of DQ8 Tg mice to produce IFN-γ in response to PLP90-110 might be a reason for the allele being disease resistant despite producing high levels of IL-17. Thus, both proinflammatory IFN-γ and IL-17 may be required for increased inflammation and demyelination of the CNS.

To confirm the source of IL-17 and IFN-γ in DR3 DQ8 mice, we performed an ELISPOT assay in the presence of anti-DQ- or anti-DR-blocking Abs. Blocking with anti-DR Ab suppressed IFN-γ levels in both DR3 and DR3DQ8 mice, indicating that DR-specific T cells are the major source of the IFN-γ in double Tg mice. However, most of the IL-17 in DR3DQ8 mice was produced by DQ-specific T cells as anti-DQ but not anti-DR Ab-suppressed IL-17 levels.

Thus, DR3DQ8 mice have higher levels of IFN-γ (produced by DR-specific T cells) as well as high levels of IL-17 (produced by DQ-specific T cells). Either IFN-γ or IL-17 or both of these proinflammatory cytokines might be responsible for severe disease observed in DR3DQ8 mice. To confirm the role of these cytokines, we conducted in vivo neutralization of IFN-γ and IL-17 using blocking Abs. Of interest, neutralization of only IL-17 led to a decrease in disease incidence and severity in DR3DQ8 mice, whereas anti-IFN-γ had no effect on disease incidence and severity. Thus, IL-17 produced by DQ8-specific T cells appears to be responsible for increased disease incidence and severity in DR3DQ8 mice. We also observed that T cells from DQ8 as well as DR3DQ8 mice produced high levels of IL-23. Our results that IL-17 neutralization decreased disease incidence and severity in DR3DQ8 support the hypothesis that the IL-23/IL-17 axis plays an important role in immunopathogenesis of EAE in DR3DQ8 mice. Although neutralization of IFN-γ had no effect on the disease course in our animal model, we cannot completely rule out the role of the Th1 cells in pathogenesis of EAE. Recent data also support the idea that both Th1 and Th17 cells are capable of inducing autoimmunity. Generation of IL-17 and IFN-γ knockout on the DR3DQ8 background in the future will help us in defining a clear role of the Th1 and Th17 responses in our animal model.

IL-17A is a member of the IL-17 family (IL-17A-F) and stimulates various types of cells, such as epithelial cells, endothelial cells, and fibroblasts, to produce proinflammatory cytokines and chemokines (38, 39). IL-17 can modulate disease through multiple pathways such as CNS recruitment of inflammatory cells, induction of proinflammatory mediators inside the CNS, or direct injury to CNS tissue. DR3DQ8 mice with higher disease incidence and severity also had a higher frequency of CNS-infiltrating cells, suggesting that IL-17 might help to recruit cells into the CNS. Recently, Carlson et al. (40) showed that the Th17-induced chemokine pathway is essential for blood-brain barrier breakdown and CNS infiltration of inflammatory cells. These CNS-infiltrating cells also produced high levels of IL-17 and IFN-γ, suggesting an important role in CNS pathology. Because a high IL-17 level in DR3DQ8 mice was associated with increased inflammation and demyelination in both spinal cord and brain, it is possible that IL-17 might be responsible for severe pathology observed in these mice. Earlier, Lees et al. (41) showed that IL-17-deficient mice develop less severe EAE on adoptive transfer of encephalitogenic T cells. Beside IL-17 and IFN-γ, other inflammatory cytokines, chemokines, and chemical mediators can also play an important role in disease pathogenesis (1). It is possible that severe CNS pathology in DR3DQ8 double Tg mice may be due to less apoptosis of inflammatory cells in the CNS of these mice compared with DR3 single Tg mice. Our TUNEL staining data support this hypothesis since there were more TUNEL-positive cells in CNS tissue sections from DR3 mice compared with DR3DQ8 mice. Apoptotic elimination of encephalitogenic CD4+ T cells is a well-documented early step in recovery from EAE (42, 43).

Our study shows that the DQ8 molecule in trans modulates the disease incidence and severity in HLA-DR3,DR4 of Tg mice. The modulatory effect of DQ8 might be due to production of the proinflammatory cytokine IL-17. IL-17 might exert its effect through multiple pathways such as increased recruitment of inflammatory cells into the CNS and/or increased tissue injury inside the CNS. We previously reported that the DQ6 molecule produces anti-inflammatory IFN-γ to protect DR3DQ6 double Tg mice from development of EAE (31). Our studies imply that incidence, progression, severity, and modulation of EAE are dependent on epistatic interactions in trans between MHC class II molecules. Similarly, interactions between HLA genes on disease-susceptible haplotypes in humans may determine the
course of the demyelinating diseases. Linkage analysis from MS patients indicate that there is complex interaction among MHC haplotypes in MS. MHC genes within a haplotype such as HLA-DR,-DP,-DQ, or HLA class I genes can influence disease outcome either through cis- or trans-interactions. Some protective HLA-DR alleles such as DRB1*11 and DRB1*14 act in cis, while other protective HLA-DR alleles DRB1*01 and DRB1*10 act in trans (20). Similarly, HLA class I alleles might influence the risk of MS susceptibility by their cis-interaction with HLA-DRB1*15 (17). Knowledge of such interactions could aid in designing individualized therapy for MS patients.

Acknowledgments

We thank Julie Hanson and the staffs for mouse husbandry. We also thank Lauri Zoecklein, Louiza Papke, and Mable Peirce for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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