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Vra4 Congenic Rats with Allelic Differences in the Class II Transactivator Gene Display Altered Susceptibility to Experimental Autoimmune Encephalomyelitis[1]

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Presentation of Ag bound to MHC class II (MHC II) molecules to CD4$^+$ T cells is a key event in adaptive immune responses. Genetic differences in MHC II expression in the rat CNS were recently positioned to allelic variability in the CIITA gene (*Mhc2ta*), located within the *Vra4* locus on rat chromosome 10. In this study, we have examined reciprocal *Vra4*-congenic strains on the DA and PVG^{av1} backgrounds, respectively. After experimental nerve injury the strain-specific MHC II expression on microglia was reversed in the congenic strains. Similar findings were obtained after intraparenchymal injection of IFN-γ in the brain. Expression of MHC class II was also lower on B cells and dendritic cells from the DA.PVG^{av1}-*Vra*4- congenic strain compared with DA rats after in vitro stimulation with IFN-γ. We next explored whether *Vra4* may affect the outcome of experimental autoimmune disease. In experimental autoimmune encephalomyelitis induced by immunization with myelin oligodendrocyte glycoprotein, DA.PVG^{av1}-*Vra4* rats displayed a lower disease incidence and milder disease course compared with DA, whereas both PVG^{av1} and PVG^{av1}.DA-*Vra4* rats were completely protected. These results demonstrate that naturally occurring allelic differences in *Mhc2ta* have profound effects on the quantity of MHC II expression in the CNS and on immune cells and that this genetic variability also modulates susceptibility to autoimmune neuroinflammation. *The Journal of Immunology,* **2008, 180: 3289–3296.**

Presentation of Ag to T cells is a key process in the initiation and propagation of immune responses. The MHC class I and II molecules present on APCs are thus of critical importance in infection, inflammation, and autoimmunity. The human HLA locus is genetically linked to complex inflammatory diseases like multiple sclerosis (MS)[4] (1–3), rheumatoid arthritis (RA) (4, 5), and type 1 diabetes (6, 7). The presence of disease-specific risk and protective HLA alleles supports the notion of a qualitative effect of the HLA on susceptibility to autoimmune diseases. In addition, there are early reports on the role of increased local HLA expression in endocrine autoimmunity (8).

The CIITA protein, encoded by the *MHC2TA* gene (*Mhc2ta* in rat) is a key transcriptional regulator of MHC class II (MHC II) (9–11). We recently demonstrated an association between a single nucleotide polymorphism (SNP) in the regulatory region of *MHC2TA* and differential expression of *MHC2TA* and MHC II genes in peripheral blood cells (12). Furthermore, the SNP (rs3087456), located in the pIII *MHC2TA* promoter, was associated to increased risk of three inflammatory diseases, MS, RA, and myocardial infarction (12). Taken together, these findings argue that also genetically determined quantitative differences in MHC II could influence susceptibility to inflammatory diseases, although this effect may be relatively weaker than the risk modulating effects conferred by qualitative aspects of the HLA complex.

The candidate gene status of *MHC2TA* was based on experimental work in the rat using ventral root avulsion (VRA), a model of mechanical nerve injury. The VRA lesion, in which lumbar ventral roots are unilaterally avulsed, results in a very proximal axotomy of motoneurons, in turn leading to a reproducible retrograde reaction with glial activation, up-regulation of immunological surface markers, such as MHC II, on microglia and neurodegeneration (13, 14). Initial studies identified differential expression of MHC II as a genetically regulated phenotype segregating a panel of inbred rat strains into high and low responders (14, 15). A full genome scan in an F_2 intercross between a high (DA) and low (PVG) responder strain subsequently identified the *Vra4* locus as regulating MHC II in the CNS after mechanical nerve injury (16). Finally, the *Vra4* locus was fine-mapped in the G8 and G10 generations of an advanced intercross line and *Mhc2ta* identified as the candidate gene, with differential expression of MHC II segregating with a haplotype spanning the promoter regions of the gene (12).

In this study, we have isolated the *Vra4* locus in congenic rat strains to study its effect on neuroinflammation. Congenic strains were bred reciprocally by transferring *Vra4* from PVG^{av1} to the DA background and vice versa, creating the DA.PVG^{av1}-*Vra4* and

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[4] Abbreviations used in this paper: MS, multiple sclerosis; RA, rheumatoid arthritis; MHC II, MHC class II; SNP, single nucleotide polymorphism; VRA, ventral root avulsion; EAE, experimental autoimmune encephalomyelitis; rMOG, recombinant myelin oligodendrocyte glycoprotein; Aif1, allograft inflammatory factor; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; lm, littermate control.

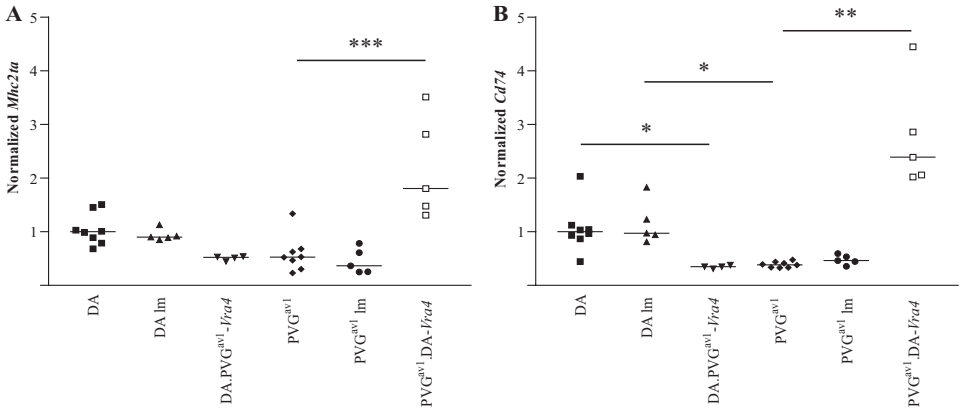

FIGURE 1. Expression of *Mhc2ta* (*A*) and *Cd74* (*B*) at 21 days after VRA. Congenic strains differ from their respective parental strains, while the lm retain the parental phenotype (*, $p < 0.05$; **, $p < 0.01$; **, $p < 0.001$).

PVG^{av1}.DA-*Vra4* congenics, respectively. Congenic animals displayed a reversal of the strain-specific expression pattern of MHC II in the CNS both after nerve injury and intraparenchymal IFN-γ injections, demonstrating the overriding effect of allelic difference in the *Vra4* locus on this particular phenotype. In contrast, there were no discernible effects on other markers of microglial activation. MHC II expression was also lower on B cells and bone marrow-derived dendritic cells from DA.PVG^{av1}-*Vra4*-congenic rats compared with DA rats upon IFN-γ stimulation in vitro. Furthermore, DA.PVG^{av1}-*Vra4* congenics displayed a lower susceptibility to experimental autoimmune encephalomyelitis (EAE) induced by active immunization with rat recombinant myelin oligodendrocyte glycoprotein (rMOG) protein compared with DA rats, suggesting that *Vra4* allele-dependent quantitative differences in MHC II expression modulate susceptibility to autoimmune neuroinflammation.

Materials and Methods

Animals

DA, PVG^{av1} (PVG.DA-*RT1^{av1}*), DA.PVG^{av1}-*Vra4*, and PVG^{av1}.DA-*Vra4* adult rats were used. The congenics were bred by reciprocal crossing of DA (provided by Prof. Hans Hedrich, Medizinische Hochschule, Hannover, Germany) and PVG^{av1} (Harlan Breeders) rats. The original *Vra4* donors were males selected from the G8 generation of a DA× PVG^{av1} advanced intercross line, chosen to transfer a relatively short and well-defined fragment harboring the *Vra4* (RNO10: 0-*D10Rat184*). Repeated backcrossing to the respective recipient strain was performed for an additional nine generations to create congenics with theoretically <0.1% of the donor genome outside the *Vra4* locus. Littermate controls (lm) from the breeding step leading to homozygous congenics were used to exclude effects from possible genetic contamination. Animals were kept in a barrier animal facility under specific pathogen-free and climate-controlled conditions with 12-h light/dark cycles, housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water ad libitum. All experiments in this study were approved and performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and the European Community Council Directive (86/609/EEC).

Genotyping

Genomic DNA was extracted from tail tips or ear clippings using a standard protocol. PCR primers for polymorphic simple sequence length polymorphisms were selected from available internet databases (Rat Genome Database (rgd.mcw.edu), Center for Genomic Research, Whitehead Institute/MIT (www-genome.wi.mit.edu/rat/public/), Ensembl (www.ensembl.org), and UniSTS at NCBI (www.ncbi.nlm.nih.gov). The primers were purchased from PROLIGO. One primer in each pair was labeled with [γ-^{33}P]ATP (PerkinElmer), genomic DNA was amplified with a standard PCR protocol, and the amplified fragments were separated on 6% polyacrylamide gels. Genotypes were recorded manually from autoradiographic films independently by two investigators. DNA from DA and PVG^{av1} rats were included for each marker.

Nerve lesion

Five rats each from the DA.PVG^{av1}-*Vra4* and PVG^{av1}.DA-*Vra4* strains were subjected to unilateral avulsion of the left L3-L5 ventral roots under standardized conditions and in deep isoflurane anesthesia at an age of 8–10 wk, with a postoperative survival time of 21 days. Ten rats from the parental strains DA and PVG^{av1} and five littermates from each respective congenic strain were used as controls. Animals were sacrificed with CO_2 and perfused with cold PBS. Spinal cords were carefully examined in a dissection microscope to verify the completeness of the lesion and to exclude signs of hemorrhage, necrotic zones, or direct damage to the spinal cord. The meninges were removed and the L3 segment was dissected. That is, after bisection at the midline, a horizontal section at the level of the central canal was made to isolate the ipsilateral ventral quadrant of the cord. The L4-L5 segments were kept intact for histological analysis. The tissue was subsequently snap frozen and stored at −80° C until further use.

RT-PCR

Total RNA was isolated from homogenized tissues using a RNeasy total RNA extraction kit (Qiagen). Each spinal cord sample consisted of the ipsilateral ventral quadrant from the L3 segment. RNA samples underwent 15 min on-column DNase digestion (27 Kunitz units; Qiagen) before cDNA synthesis to avoid amplification of genomic DNA. Reverse transcription was performed with 10 μl of total RNA, random hexamer primers (0.1 μg; Invitrogen Life Technologies), and superscript reverse transcriptase (200 U; Invitrogen Life Technologies). The following primers were used for RT-PCR: *Aif1* (allograft inflammatory factor) forward 5′-GGAGGCCTTCAAGACGAAGTAC, reverse 5′-AGCATTCGCTTCAAGGACATAATA; *B_2m* forward 5′-TGCTTGCCATTCAGAAAACTC, reverse 5′-TATTTGAGGTGGGTGGAACTG, *Cd11b* forward 5′-CATCTTTCCCGCTAATTCTG, reverse 5′-TTCTCGCTGGTCACAACG; *Cd74* forward 5′-GTGATGCACCTGCTTACGAAGT, reverse 5′-CTCCGGGAAGCTCCCCT; *Cd86* forward 5′-GCTCTCAGTGATCGCCAAC, reverse 5′-TCTTTGTAGGTTTCGGGTATCC; *Gapdh* forward 5′-TCAACTACATGGTCTACATGTTCCAG, reverse 5′-TCCCATTCTCAGCCTTGACTG; *Hprt* forward 5′-CTCATGGACTGATTATGGACAGGAC, reverse 5′GCAGGTCAGCAAAGAACTTATAGCC; and *Mhc2ta* forward 5′-CATACTCTGTGTGCCACCATGG, reverse 5′-AGTTCGATCTCTTCCTCCCCA using Qiagen QuantiTect SYBR green according to the manufacturer's instructions. Amplification was performed on an iQ5 real-time PCR detection system (Bio-Rad). All primers, except *B_2m*, were designed with Primer Express software (PerkinElmer). *B_2m* was designed using Beacon Designer software (Bio-Rad). Primer specificity was assessed by analyzing amplicon dissociation curves in each sample. Relative amounts of mRNA levels were calculated using the standard curve method, constructed by using serial dilutions of cDNA. All samples were analyzed in duplicates. The transcript level in each sample was calculated as the ratio between the relative amount of the specific marker investigated to the endogenous control, *Hprt*. Using *Gapdh* as control gave similar results for *Mhc2ta* and *Cd74* (data not shown). For glial markers, the two reference genes were combined. Samples without template or with template where the reverse transcription step had been omitted served as controls for contamination and amplification of genomic DNA, respectively. The relative expression for each sample was normalized to the DA median value. Groups were compared using the Kruskal-Wallis test followed by Dunn's post test with the following comparisons; DA vs DA lm, DA vs

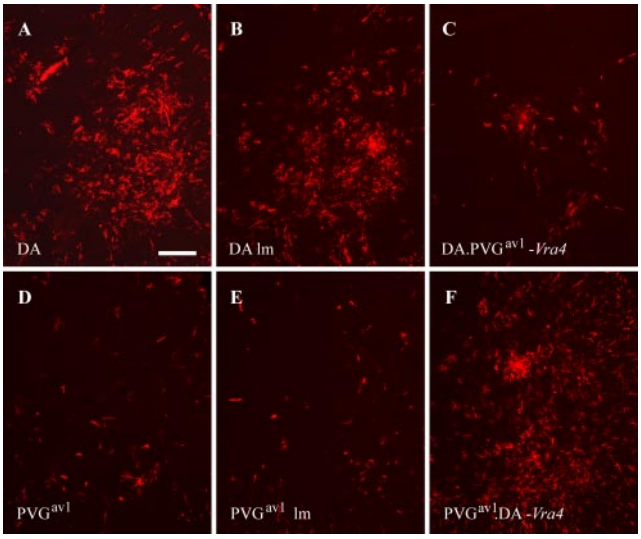

FIGURE 2. Micrographs displaying immunolabeling of MHC II (OX-6) in the left ventral horn of the lumbar spinal cord in parental strains (*A* and *D*) lm (*B* and *E*), and congenics (*C* and *F*) at 21 days after VRA. The MHC II expression follows the pattern of the *Vra4* origin. Scale bar, 200 μm.

DA.PVG^{av1}-*Vra4*, DA vs PVG^{av1}, PVG^{av1} vs PVG^{av1} lm, and PVG^{av1} vs PVG^{av1}.DA-*Vra4*.

Immunohistochemistry

The immunohistochemistry methods used have been described previously (17, 18). Cryosections (14 μm) were cut at the level of the L4-L5 segment of the spinal cord for VRA and surrounding the injection site for intraparenchymal injections, respectively. Sections were fixed in 80% ice-cold acetone and 20% methanol (5 min) and incubated overnight at 4°C in the following primary antisera/Abs diluted in PBS (pH 7.4), goat anti-rat CD3ε (polyclonal IgG; 1/100; Santa Cruz Biotechnology), mouse anti-rat Cd11b (clone OX-42, 1/200; BD Pharmingen), mouse anti-rat macrophage Ag (clone ED-1, 1/200) (Serotec), or mouse anti-rat Ia Ag (clone OX- 6, 1/200; Serotec. After serial washings in PBS, sections were incubated for 45 min at room temperature (20–22°C) with Alexa Fluor 488-conjugated donkey anti-goat Ab (1/250; Molecular Probes),or biotin-conjugated donkey anti-mouse (1/250; Jackson ImmunoResearch Laboratories). The biotin-labeled antiserum was visualized by subsequent incubation with Cy3-conjugated streptavidin (1/1000; Jackson ImmunoResearch Laboratories) for 45 min at room temperature. The sections were analyzed in a Leica DM RBE microscope. The specificity of the immunostainings was tested in control slides by omission of the primary Ab and incubation with unrelated isotype-matched Ab controls.

Intraparenchymal injections

Stereotactic intraparenchymal injections (coordinates anterior-posterior, −0.3; medial-lateral, +2.5; dorsoventral, −4.3) were given to isoflurane-anesthetized 8- to 10-wk-old male DA and DA.PVG^{av1}-*Vra4* rats using a Hamilton syringe (type 701 RN, gauge 26s). Total injection volume was 2 μl over 2 min, containing a total of 50 U of IFN-γ (BD Pharmingen) in PBS. Animals were sacrificed 3 days later and brains were removed and kept at −70°C until sectioned at 14 μm.

Splenocyte preparation and culture

Untreated female DA and DA.PVG^{av1}-*Vra4* rats (n = 5/group) were used for in vitro studies. Spleens were dissected and splenocytes were filtered through a 70-μm filter. Cells were cultured at a density of 2×10^6/ml in 12-well plates (Nunc) in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg L-glutamine (DMEM complete, all from Invitrogen Life Technologies) with 0, 10, or 100 U/ml IFN-γ, respectively, for 8 h at 37°C in 5% CO_2.

Dendritic cell differentiation

Bone marrow from untreated female DA and DA.PVG^{av1}-*Vra4* rats (n = 5/group) was flushed from femur bones with DMEM complete using a 21-gauge needle and filtered through a 70-μm filter. Cells were cultured at a density of 1×10^6/ml in DMEM complete, as described above, in 24-well plates (Nunc) for 6 days. Cells were differentiated into myeloid dendritic cells (mDC) through exposure to 5 ng/ml GM-CSF and 25 ng/ml IL-4, or differentiated into plasmacytoid dendritic cells (pDC) through exposure to

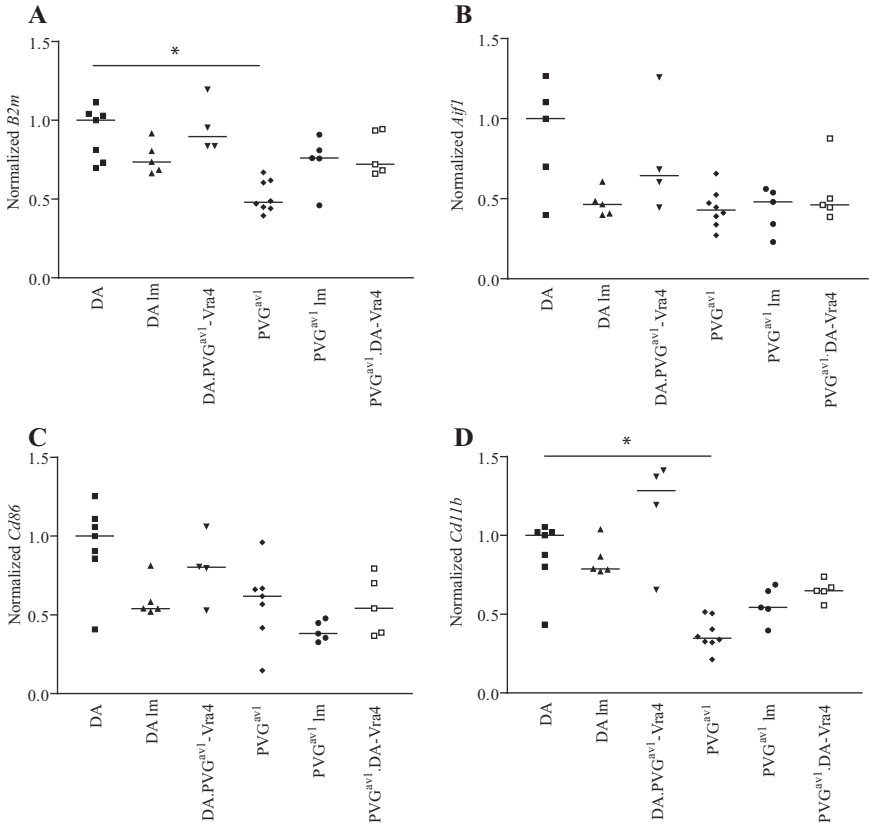

FIGURE 3. Expression of B_2m (*A*), *Aif1* (*B*), *Cd86* (*C*), and *Cd11b* (*D*) at 21 days after VRA. No significant effect on expression could be attributed to the *Vra4* region.

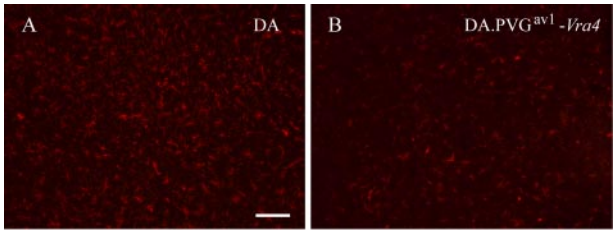

FIGURE 4. Micrographs displaying immunolabeling of OX-6 (MHC II) in the striatum of rats receiving intraparenchymal injections with 50 U of IFN-γ. DA (*A*) expresses substantially higher levels of MHC II than the congenic strain DA.PVG^{av1}-*Vra4* (*B*). Scale bar, 200 μm.

human FMS-related tyrosine kinase ligand (all growth factors from R&D Systems). The medium was changed every 3 days. Cells were subsequently stimulated with 0, 10, or 100 U/ml IFN-γ (BD Pharmingen), respectively, for 8 h at 37°C in 5% CO_2.

Flow cytometry

Cells were stained with the following FACS Abs: anti- CD11b-R-PE, anti-CD11c-FITC (both from Serotec), anti-B220-PE, and anti-OX6-PerCP (both from BD Biosciences). Splenocytes were stained with either anti-CD11c-FITC or anti-OX6-PerCP to identify macrophages or with anti-B220-PE and anti-OX6-PerCP to identify B cells. Bone marrow-derived mDC and pDC were stained with anti-CD11c-FITC, anti-B220-PE, and anti-OX6-PerCP. The stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using Summit software (DakoCytomation). To quantify MHC II expression, we measured the mean fluorescence intensity of MHC II^+ cells for each cell subset. Statistical analysis was performed using the Mann-Whitney *U* rank sum test.

Experimental autoimmune encephalomyelitis

rMOG, aa 1–125 from the N terminus, was expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography (19). Two separately titrated batches of rMOG were used in the two EAE experiments and thus doses are not directly comparable. In the first experiment, rats of both sexes (five in each group) from the DA.PVG^{av1}-*Vra4* and DA strains, respectively, were anesthetized with isoflurane and injected with a 200-μl inoculum at the tail base containing 60 or 80 μg of rMOG emulsified in PBS (Invitrogen Life Technologies) diluted (1/1) in IFA (Sigma-Aldrich). In the second experiment, female DA.PVG^{av1}-*Vra4* and DA rats (15 in each group) were immunized with 10 μg from the second rMOG batch as described above. Animals were weighed and scored daily from day 7 (exp 1) or 10 (exp 2) postimmunization. Clinical score was evaluated as follows: 1, limp tail; 2, hind leg paraparesis (wobbling gait); 3, hind leg paralysis; 4, tetraplegia, and 5, moribund state or death. Rats who suffered severe disease (score ≥4) for >1 day and/or lost >25% of their body weight were sacrificed. The score at sacrifice was used for the remaining days. The following clinical parameters were recorded: occurrence/incidence of EAE, i.e., signs of EAE present for more than 1 day; onset of EAE, i.e., first day of clinical signs; duration of EAE, i.e., number of days rats showed clinical signs; cumulative EAE score, i.e., sum of the EAE scores obtained from day 10 until the end of the experiment; and the weight loss index, calculated as (1– [minimum weight during the experiment]/[weight at day 10 postimmunization]) × 100. The statistical tests used were: Fischer's exact test for incidence (number of affected animals), Mann-Whitney *U* rank sum test for cumulative score and median day of onset, and unpaired *t* test for weight loss (phenotype passed normality test).

Results

Ventral root avulsion

Quantitative differences in *Mhc2ta* expression 21 days after VRA were assessed by RT-PCR, which demonstrated a significant influence by *Vra4* in the congenics (Fig. 1*A*). Furthermore, a similar expression pattern was present for transcript levels of *Cd74*, the MHC II-associated invariant chain (Fig. 1*B*). The lm rats were included for both congenics to assess the possible effect of contaminating background genome of the donor strain outside the

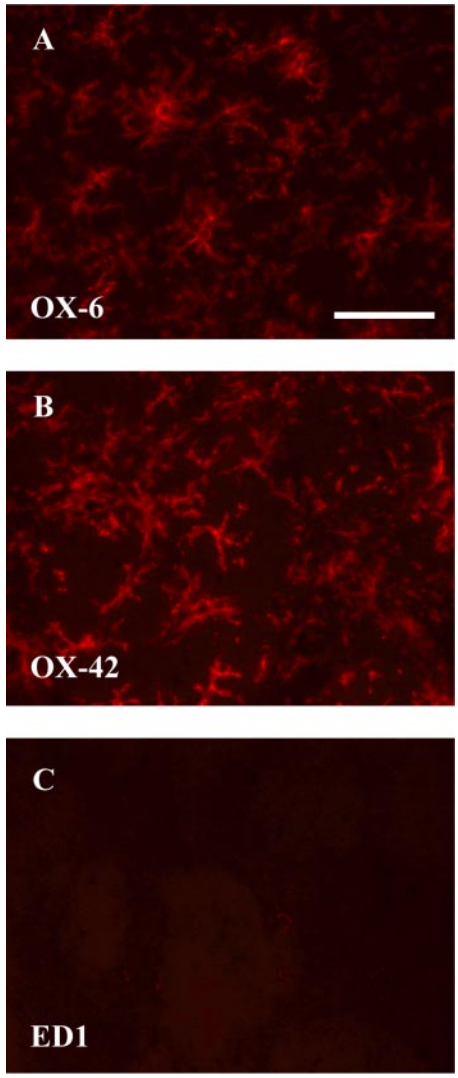

FIGURE 5. Micrographs displaying immunolabeling of OX-6 (MHC II; *A*), OX-42 (CD11b; *B*), and ED1 (CD68; *C*) in serial sections of the striatum of a DA rat receiving an intraparenchymal injection of 50 U of IFN-γ. OX-6 and OX-42 immunolabeling display a very similar pattern, although not all OX-42-positive structures are labeled with OX-6. Furthermore, the labeling pattern of both OX-6 and OX-42 resembles the ramified morphology typical of activated microglia. ED1 labeling is almost absent in the striatum 3 days following injection of IFN-γ. Scale bar, 100 μm.

Vra4 region. The phenotype of the lm corresponded well to the recipient strain (Fig. 1), thus attributing the phenotypic effect in the congenics to the *Vra4* region proper. Interestingly, levels of *Mhc2ta* and *Cd74* in PVG^{av1}.DA-*Vra4* were even higher than in DA rats, suggesting possible interactive genetic effects between *Vra4* DA alleles and the PVG^{av1} background genome present in the congenics. Similar differences were seen when comparing the congenics with the DA lm.

MHC II expression was studied with immunolabeling using OX-6. A strong up-regulation of MHC II was present in the ventral horn on the lesioned side 21 days after VRA in DA and DA lm rats, while PVG^{av1} and PVG^{av1} lm rats displayed a much weaker OX-6-labeling pattern (Fig. 2). The OX-6-labeling pattern in the DA.PVG^{av1}-*Vra4*-congenic strain was similar to PVG^{av1}, while the PVG^{av1}.DA-*Vra4*-congenic strain showed an up-regulation similar to that of the DA strain (Fig. 2). The degree of MHC II expression was thus determined by the *Vra4* origin.

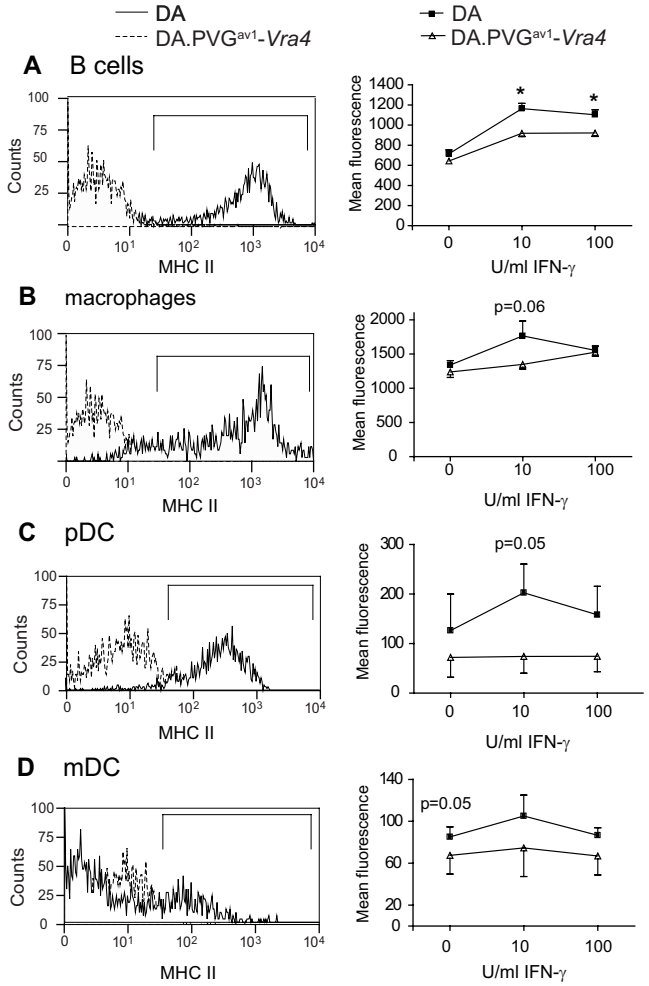

FIGURE 6. MHC II expression is lower in B cells from DA.PVG^{av1}-*Vra4* rats than in B cells from DA rats. Expression of MHC II was measured by flow cytometry in splenic B220$^+$MHC II$^+$ B cells (*A*), CD11b$^+$MHC II$^+$ macrophages (*B*), bone marrow-derived CD11c^{low}B220$^+$MHC II$^+$ pDC (*C*), and CD11c$^+$ B220$^-$ MHC II$^+$ mDC (*D*) after stimulation for 8 h with 0, 10, or 100 U/ml IFN-γ in vitro. Data are represented as mean ± SEM (n = 5/group; ∗, $p < 0.05$.

The up-regulation of MHC II was accompanied by a modest infiltration of T cells. DA rats display higher numbers of infiltrating T cells compared with PVG^{av1}; however, no discernible congenic phenotype was evident for this parameter (data not shown).

To study the *Vra4* effect on the general inflammatory activation pattern in the congenics, transcript levels of B_2m and the microglial activation markers *Aif1*, B7-2 (*Cd86*), and integrin α_M (*Cd11b*) were assessed in spinal cord by RT-PCR at 21 days after VRA. Significant differences in expression levels of *Cd11b* and B_2m ($p < 0.05$) could be detected between the parental strains, while there were no differences that could be attributed to Vra4 in the congenics for any of the markers (Fig. 3).

*Intraparenchymal injections of IFN*γ

To study the effect of *Vra4* in a classic model for inducing MHC II expression, 50 U of IFN-γ was injected into the striatum of DA.PVG^{av1}-*Vra4* and DA rats and OX-6 immunolabeling was analyzed at 3 days after injections. A strong up-regulation of MHC II expression on microglia was observed in DA rats, while a much weaker induction was present in DA.PVG^{av1}-*Vra4* rats (Fig. 4). In adjacent sections, the morphology and distribution of OX-6-labeled cells corresponded to a subpopulation of OX-42-positive microglial cells (Fig. 5, *A* and *B*). In contrast, ED1 immunolabeling was very low at the site of injection, indicating absence of infiltrating macrophages (Fig. 5*C*). A difference was also evident adjacent to the needle tract through the cortex, where rounded OX-6-positive cells were more numerous in the DA rat compared with DA.PVG^{av1}-*Vra4*. Some of these cells were stained by ED1, suggesting infiltrating macrophages (data not shown).

*Lower MHC II expression in APCs from DA.PVG^{av1}-*Vra4 *rats*

MHC II-mediated Ag presentation by APCs is essential for initiation of CD4$^+$ T cell responses. Therefore, the expression of MHC II on APC subsets from DA.PVG^{av1}-*Vra4*- congenic rats compared with DA rats was investigated. Splenocytes derived from untreated DA.PVG^{av1}-*Vra4*-congenic rats or DA rats were cultured with or without IFN-γ in vitro, after which surface MHC II expression in B220$^+$MHC II$^+$ B cells and CD11b$^+$MHC II$^+$ macrophages was determined by flow cytometry. The expression of

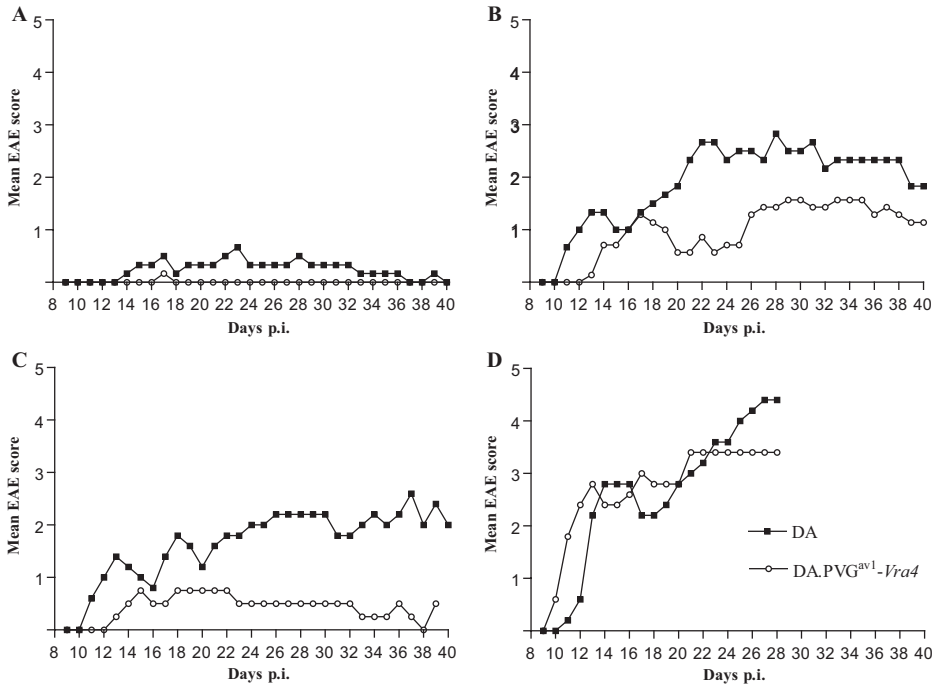

FIGURE 7. Mean EAE score in male and female DA and DA.PVG^{av1}-*Vra4*-congenic rats upon immunization with rMOG at different doses. *A*, Males, 60 μg of rMOG; *B*, males, 80 μg of rMOG; *C*, females, 60 μg of rMOG; and *D*, females, 80 μg of rMOG. p.i., Postimmunization.

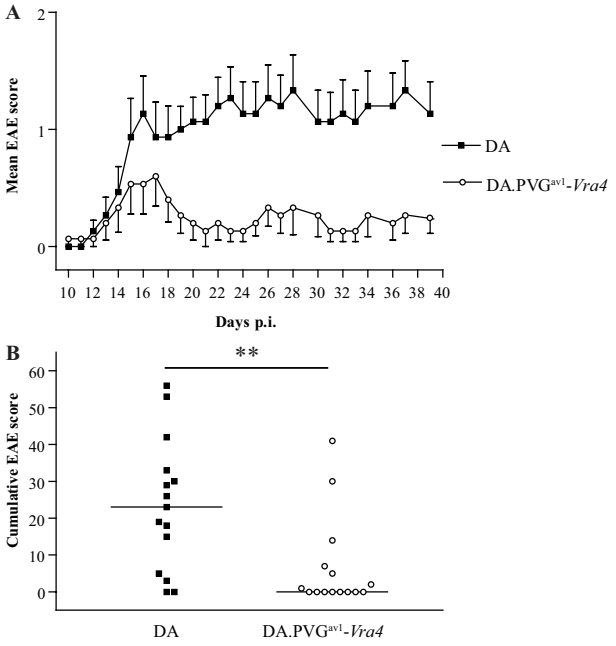

FIGURE 8. *A*, Mean EAE score in DA.PVG^{av1}-*Vra4* rats compared with DA rats. *B*, Significant difference in the cumulative EAE score between DA.PVG^{av1}-*Vra4* and DA rats. Error bars, SEM (**, $p < 0.01$).

MHC II in response to IFN-γ stimulation was significantly lower in B cells from DA.PVG^{av1}-*Vra4*-congenic rats compared with DA rats (Fig. 6*A*). In macrophages, there was a tendency toward lower MHC II levels in DA.PVG^{av1}-*Vra4*-congenic rats, however, not achieving statistical significance ($p = 0.06$ at 10 U/ml IFN-γ; Fig. 6*B*).

To assess MHC II expression on dendritic cells, bone marrow cells from untreated DA.PVG^{av1}-*Vra4*-congenic rats and DA rats were isolated and cultured with FMS-related tyrosine kinase ligand to generate pDC or with IL-4 and GM-CSF to generate mDC. Levels of MHC II expression in pDC (CD11c^{low}B220^{+}) and mDC (CD11c^{+}B220^{-}), respectively, were determined by flow cytometry. The expression of MHC II in response to IFN-γ stimulation tended to be lower and at the threshold level of statistical significance in both subsets of dendritic cells derived from DA.PVG^{av1}-*Vra4* rats compared with DA rats (both $p = 0.05$; Fig. 6, *C* and *D*). Taken together, these results suggest that allelic differences at the *Vra4* locus regulate levels of MHC II also in APCs, but that differences were greater in the B cell subset.

Experimental autoimmune encephalomyelitis

To study the in vivo effects of the *Vra4* locus in regulation of susceptibility to autoimmune neuroinflammation, we induced EAE in rats by immunizing with rMOG in IFA, a reproducible model with many similarities to MS (20). In a first experiment using a mild to moderate induction protocol, PVG^{av1} and PVG^{av1}.DA-*Vra4* rats were completely protected from EAE. In contrast, DA.PVG^{av1}-*Vra4* congenics of both sexes displayed a dose-dependent protection from EAE (Fig. 7). Thus, DA.PVG^{av1}-*Vra4* males immunized with 80 μg of rMOG and females immunized with 60 μg of rMOG demonstrated a milder disease course compared with parental DA controls. This effect was, however, not seen in females immunized with the higher dose of rMOG, suggesting that the protective effect of *Vra4* could be overcome by a stronger induction protocol. These findings were reproduced in a larger experiment using a relatively mild induction protocol, in which a protective effect of the *Vra4* locus in female DA.PVG^{av1}-*Vra4* rats compared with DA controls was confirmed (Fig. 8*A*). Severity of disease, reflected by the cumulative EAE score and weight loss, was significantly lower in animals with PVG^{av1} alleles at the *Vra4* locus (Fig. 8*B*). There was also a significantly lower incidence rate in the congenic animals compared with the DA parental animals. However, there was a trend toward an earlier onset of disease in the DA.PVG^{av1}-*Vra4* rats (Table I). In conclusion, the *Vra4* locus affects both the susceptibility to EAE and the severity of disease.

Table I. *DA.PVG^{av1}-Vra4-congenic animals show a significantly lower incidence, lower degree of weight loss, and less severe disease course compared to parental DA animals*[a]

	DA	DA.PVG^{av1}-*Vra4*	*p*
Total no.	15	15	
Affected	13	6	0.0209
Median cumulative EAE score	23	0	0.0061
Mean weight loss	6.89	2.63	0.0415
Median day of onset (affected)	17.5	13.5	0.0822

[a] The median day of onset does not differ significantly between strains.

Discussion

We demonstrate here that the *Vra4* locus, comprising naturally occurring alternative alleles of the *Mhc2ta* gene, exerts a pivotal regulatory effect on the expression of MHC II in the CNS of congenic rats and that these allelic differences also affect MHC II expression on immune cells in the periphery. These findings corroborate and complement the mapping of differences in MHC II expression to the *Vra4* locus obtained in previous intercross experiments (12, 16). By using congenics, that is, isolating *Vra4* from a donor strain on a recipient background, we obtained the tools to study the effects in a well-controlled and reproducible model. Interestingly, the up-regulation of *Mhc2ta* and *Cd74* in the CNS of PVG^{av1}.DA-*Vra4*-congenic strain was increased compared with the *Vra4* donor strain DA. Possible explanations could be effects from the PVG^{av1} genome acting upstream of *Mhc2ta* transcription or an interactive effect of the DA *Mhc2ta* allele with other gene regions present in the PVG^{av1} background. In the original genome-wide scan of differences in MHC II expression in an F_2 DA × PVG intercross, the *Vra4* locus was the only region displaying genome-wide significance (16). However, after correction for genotype at the *Vra4* locus, linkage to the MHC complex on chromosome 20 is also evident (O. Lidman, M. Swanberg, T. Olsson, and F. Piehl, unpublished observation). We have here used MHC-congenic rats and the discrepancy in expression of *Cd74* between DA (RT1.1AV1) and PVG^{av1}.DA-*Vra4*-congenic animals can thus not be explained by an effect of the MHC complex. However, there is evidence for the existence of additional gene regions outside of the MHC complex that affect expression of MHC II. In the BN rat, a higher ratio of constitutive MHC II-positive microglia has been documented and the up-regulation of MHC II after VRA is more rapid compared with other strains despite the fact that BN carries the same *Mhc2ta* allele as the LEW and DA strains (12, 15, 21). Additional support for this hypothesis is provided by a whole genome scan in an F_2 intercross between the BN (RT1.1N) and LEW^{n} (LEW.BN-RT1^{n}) strains revealing two quantitative trait loci outside of the MHC complex linked to differences in MHC II expression (M. Diez, N. Abdelmagid, K. Harnesk, M. Ström, O. Lidman, M. Swanberg, T. Olsson, and F. Piehl, unpublished data). In one of these quantitative trait loci, the MHC II expression was driven by the allele originating from the LEW^{n} strain, the original slow and

low responder. This exemplifies that such effects may go undetected in the original inbred strain, but become unmasked in an intercross or congenic experiment. However, it is strikingly clear from the findings obtained here that the *Vra4* locus on its own is the main determinant for DA and PVG^{av1} strain-specific differences in MHC II expression in the CNS.

In contrast, we were not able to detect any other glial expression phenotype in the congenic strains. This includes B_2m as a marker of MHC I expression as well as *Cd86*, *Cd11b*, and *Aif1*, all markers of microglial activation. Other studies have demonstrated that the CIITA protein exerts a regulatory effect on the transcription of specific gene products other than MHC II, including MHC I (22), IL-4 (23), and cathepsin E (24) and have effects on global transcription as measured by microrarrays (11, 25). However, these studies were conducted in vitro, with cells deficient of *MHC2TA* and/or transfected with *MHC2TA* expression constructs. The results obtained here suggest that naturally occurring genetic variants of rat *Mhc2ta* studied in vivo under physiological conditions have limited effects on the VRA phenotype, except for MHC II-associated transcripts. This conclusion is also supported by in vivo findings in the mouse using transgenic animals (26, 27). In this context it is of interest that expression of MHC II is often used as a marker of microglial activation, although it can be dissociated from other markers of activation as judged from our results. This is supported by findings in the E3 rat that in the VRA model displays prominent microglial activation, but very sparse MHC II expression (15).

In this study, we provide the first experimental data showing that naturally occurring allelic variability in *Mhc2ta* modulates susceptibility to autoimmune disease. Previous studies using mouse knockouts with impaired MHC II expression demonstrate reduced susceptibility to EAE (26, 28, 29). Although these results are interesting mechanistically, knockout models do not provide information about the genetic influence in complex diseases such as MS. In the first set of experiments, rats of both sexes with the PVG^{av1} *Mhc2ta* allele on the DA background displayed a milder disease course of rMOG-induced EAE than DA controls. However, the difference between congenics and DA female rats disappeared at the higher dose of immunogen, suggesting that the protective effect could be overcome by a stronger induction protocol. The disease modulating effect of *Vra4* was subsequently reproduced in a larger experiment using a relatively mild induction protocol. As expected, DA rats displayed a mild, but chronic disease during the entire experiment. In contrast, DA.PVG^{av1}-*Vra4*- congenic rats were largely protected and in the minority of immunized rats displaying clinical signs, the disease course was milder than in DA controls.

Lower expression of MHC II was demonstrated for peripheral APCs from the DA.PVG^{av1}-*Vra4*-congenic compared with the DA strain when stimulated in vitro with increasing concentrations of IFN-γ. Our data may indicate that APCs from DA.PVG^{av1}-*Vra4*-congenic rats have a reduced capacity to present autoantigens to encephalitogenic Th cells in the periphery, which could explain the altered susceptibility to EAE. It may also be that B cells, mDC, and/or pDC are involved in the priming of encephalitogenic Th cells in EAE. Further studies of the immune mechanisms involved are warranted and ongoing in the laboratory.

We previously found a weak, but significant association of a *MHC2TA* SNP (rs3087456) to risk of disease in three separate clinical materials, comprising patients with MS, RA, and myocardial infarction. This observation spurred a number of studies attempting to reproduce the original finding, where both negative (30–34) and positive (35, 36) results have been reported. Linkage to the 16p.13 region harboring the *MHC2TA* gene was also found in three RA linkage meta-analyses (37–39). In addition, positive association results have been reported for other autoimmune diseases (40, 41). A problem is that most studies published so far have been underpowered to detect a possible low to moderate effect on disease susceptibility. In some cases, the lack of proper population-based control materials may have interfered with the interpretation of the results. Of note, the largest study published so far reproduced the findings of an association between the rs3087456 SNP in the *MHC2TA* type III promoter to risk of mortality of cardiovascular disease and the metabolic syndrome (42). Confirmation of the candidate gene status of *MHC2TA* in human complex diseases thus awaits studies in larger clinical materials. In our view, the rationale for such studies is increased by the finding of a clinical effect on EAE in *Vra4* congenics. However, it is intriguing that the lowered expression of MHC II in DA.PVG^{av1}-*Vra4* animals was associated with partial protection from EAE, while in the human the SNP genotype linked to lower MHC II expression was associated with increased risk of disease (12). This discrepancy may depend on a number of reasons. Most important, extrapolating data from acute animal models should be done cautiously when trying to understand lifelong, chronic diseases. In addition, the haplotypes detected in rats and humans, respectively, may be functionally different in other aspects than in the regulation of MHC II in microglia and peripheral blood cells. This underscores the need for further studies aimed at understanding the mechanisms of how allelic variations in *MHC2TA* modulate disease susceptibility. For example, it is possible that MHC2TA exerts effects on Th cell differentiation (43) or affects the response to environmental factors such as infectious agents (44). The congenic strains reported here can serve as valuable tools for such studies.

In conclusion, we demonstrate here that reciprocal *Vra4* congenics on the DA and PVG^{av1} background, respectively, display a reversed strain-specific pattern of expression of MHC II molecules on peripheral immune cells and on microglia, but not of other markers of microglial activation. Furthermore, *Vra4* congenics on an EAE permissive background, i.e., DA, are partly protected from rMOG-induced EAE in the settings of a mild immunization protocol. These data support the notion that naturally occurring allelic variants of *Mhc2ta* are the main determinants for strain-specific expression of MHC II, in turn of importance for susceptibility to autoimmune neuroinflammation.

Disclosures

The authors have no financial conflict of interest.

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