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IL-22RA2 Associates with Multiple Sclerosis and Macrophage Effector Mechanisms in Experimental Neuroinflammation

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Multiple sclerosis (MS) is an inflammatory neurodegenerative disease of the CNS. Recent advances in whole-genome screening tools have enabled discovery of several MS risk genes, the majority of which have known immune-related functions. However, disease heterogeneity and low tissue accessibility hinder functional studies of established MS risk genes. For this reason, the MS model experimental autoimmune encephalomyelitis (EAE) is often used to study neuroinflammatory disease mechanisms. In this study, we performed high-resolution linkage analysis in a rat advanced intercross line to identify an EAE-regulating quantitative trait locus, *Eae29*, on rat chromosome 1. *Eae29* alleles from the resistant strain both conferred milder EAE and lower production of proinflammatory molecules in macrophages, as demonstrated by the congenic line, DA.PVG-*Eae29* (Dc1P). The soluble IL-22R α2 gene (*Il-22ra2*) lies within the *Eae29* locus, and its expression was reduced in Dc1P, both in activated macrophages and splenocytes from immunized rats. Moreover, a single nucleotide polymorphism located at the end of *IL-22RA2* associated with MS risk in a combined Swedish and Norwegian cohort comprising 5019 subjects, displaying an odds ratio of 1.26 ($p = 8.0 \times 10^{-4}$). IL-22 and its receptors have been implicated in chronic inflammation, suggesting that IL-22RA2 regulates a central immune pathway. Through a combined approach including genetic and immunological investigation in an animal model and large-scale association studies of MS patients, we establish *IL-22RA2* as an MS risk gene. *The Journal of Immunology*, 2010, 185: 000–000.

Multiple sclerosis (MS) is a demyelinating, inflammatory, and neurodegenerative disease of the CNS. Most immune cells have been attributed a role in MS pathogenesis, demonstrating the importance of both innate and adaptive immune mechanisms (1). Innate immune cells include CNS-resident microglia and infiltrating macrophages that can act as effector cells, causing and mediating destruction of myelin and axons, through both direct damage and production of proinflammatory molecules, thereby increasing the disease severity (2).

MS is considered a complex disease resulting from an interaction between many genes and factors in the environment (3). For many years, the only unequivocally established MS risk locus was the HLA complex (4), but the advent of whole-genome screening tools has now enabled discovery of new MS risk genes, including *IL-7R* and *IL-2RA*, *CD58,* and *CLEC16A* (5, 6). Future genome-wide scans in very large cohorts will likely identify even more MS risk genes. These risk genes show heavy bias toward immune-related functions (5), and understanding the biology of MS risk genes will better define different pathogenic mechanisms, although the low accessibility of target tissue samples from patients make functional studies difficult.

The MS model, experimental autoimmune encephalomyelitis (EAE), is often used to study neuroinflammatory disease mechanisms. The similarities between MS and various EAE models are established at many levels, including disease course (7), histological observations (8), immune mechanisms (1), and identified risk genes (9, 10). One translational approach is to establish intercrosses between rodent strains, and through linkage mapping to identify genomic regions harboring EAE risk genes, so-called quantitative trait loci (11–14). Once experimental candidate genes have been confirmed, they can be tested for association in human disease cohorts or further evaluated in the animal model. For example, crosses between the highly polymorphic DA and PVG.AV1 rat strains, which demonstrate differential susceptibilities to EAE, have led to successful identification of several candidate genes that have also been translated as MS risk genes, including *MHCIITA*, *VAV1, IL-21R,* and *CCL* chemokines (13, 15–17). Importantly, congenic lines can be established for risk genes and thereby offer a tool in further studying their functional relevance. In congenic lines, a disease-regulating genomic fragment from one strain is transferred onto the background of the other strain

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Abbreviations used in this paper: AIL, advanced intercross line; B, B cells; EAE, experimental autoimmune encephalomyelitis; FPRP, false-positive report probability; Inn., innate cells; LN, lymph node; LOD, logarithm of odds; Mb, megabases; MS, multiple sclerosis; MSSS, MS severity score; NOR, Norwegian; p.i., postimmunization; rMOG, recombinant myelin oligodendrocyte glycoprotein; SNP, single nucleotide polymorphism; SWE, combined Swedish; T, T cells.

through backcrossing (18), enabling the genetic region of interest to be studied in isolation.

In this study, we used this combined approach to identify an MS risk gene, the soluble IL-22R α2 (*IL-22RA2*). Our findings target a mechanism in macrophages previously not described for *Il-22ra2*. The role of *IL-22RA2* in regulating effector mechanisms of innate immunity merits further investigation.

Materials and Methods

Ethical statement

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) under the ethical permit N332/06 entitled "Genetic Regulation, Pathogenesis and Therapy of EAE, an Animal Model for Multiple Sclerosis," which was approved by the North Stockholm Animal Ethics Committee (Stockholms Norra djurförsöksetiska nämnd). Rats were tested according to a health-monitoring program at the National Veterinary Institute (Statens Veterinärmedicinska Anstalt) in Uppsala, Sweden. The local ethical committees in Stockholm, Sweden, and South and Eastern Norway Regional Health Authority in Norway approved the human association study experiments.

Experimental animals

DA rats were originally obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). MHC-identical PVG rats were originally obtained from Harlan UK Limited (Blackthorn, U.K.). From these, colonies have then been established at Karolinska Institutet (DA/Kini and PVG.1AV1/Kini; Stockholm, Sweden). The generation of the advanced intercross line (AIL) has been described (19). The Dc1P congenic strain was established using a speed-congenic approach with marker-assisted selection (18). DA female rats were mated with male offspring selected for PVG alleles within *Eae29* and against PVG background contamination at 96 microsatellite markers equally spaced throughout the genome. DA female rats were used throughout the breeding program to ensure that mitochondrial DNA was inherited from DA. A breeding pair selected from the N7 generation was intercrossed to produce homozygous DA.PVG-*Eae29* (Dc1P) congenic rats, carrying PVG alleles from beginning of chromosome 1 to D1Rat10 (0–25.4 Mb). All animals were bred and housed at the Karolinska University Hospital (Stockholm, Sweden) in polystyrene cages containing aspen wood shavings and with free access to water and standard rodent chow with regulated 12-h light/dark cycles.

EAE induction and evaluation

Recombinant myelin oligodendrocyte glycoprotein (rMOG; aa 1–125 from the N terminus) was expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography, as previously described (20). The purified protein, dissolved in 6 M urea, was then dialyzed against PBS. Rats between 10 and 14 wk of age were anesthetized with isoflurane (Forane; Abbott Laboratories, Abbot Park, IL) and immunized with a single s.c. injection at the dorsal tail base with 200 μl inoculum containing rMOG in saline emulsified in a 1:1 ratio with IFA (Sigma-Aldrich, St. Louis, MO). The rats were monitored daily for clinical signs of EAE from day 8 until 35 postimmunization (p.i.). The clinical score was graded as follows: 0, no clinical signs of EAE; 1, tail weakness or tail paralysis; 2, hind-limb paraparesis or hemiparesis; 3, hind-limb paralysis or hemiparalysis; 4, tetraplegia or moribund; and 5, death.

Genotyping

Genomic DNA was prepared from tail tips, as described previously (21). For genotyping of the AIL and the Dc1P congenic line, six polymorphic microsatellite markers were used, spanning the first 25 Mb of rat chromosome 1 (Supplemental Table I). DNA amplification was performed with PCR using forward primers end-labeled with a fluorescent dye (VIC, NED, FAM, or PET). Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) or Applied Biosystems (Foster City, CA). PCR products were run in an ABI 3730 capillary sequencer and analyzed using GeneMapper v3.7 (Applied Biosystems) (22). All genotypes were evaluated manually and double-checked.

Immunohistochemistry

At day 35 p.i., 10 Dc1P rats and 10 DA control animals were euthanized by asphyxiation using CO_2 and perfused with PBS followed by 4% paraformaldehyde. Immunohistochemical analysis of dissected brains and spinal cords was performed on paraformaldehyde-fixed paraffin-embedded sections. Sections 0.4 mm thick were stained with hematoxylin and contrastained in eosin solution (Mayer's Hämalaun staining solution; Merck, Vienna, Austria) and Luxol fast blue to detect CNS inflammation and demyelination, respectively. The inflammatory index was determined from the number and size of inflammatory lesions on an average of 20 complete cross sections of the spinal cord and the brain of each animal, as previously described (8). The lesion activity was defined as a percentage of the total surface size under lesion (in mm^2) containing $Ox6^+$ (MHC class II^+) cells.

Staining was performed with Abs against the following targets: mouse monoclonal α-CD8 (1:200; Serotec, Oxford, U.K.), rabbit monoclonal α-CD3 (1:150; Thermo Scientific, U,K.), purified mouse anti-rat CD45R mAb, (1:100; Pharmingen International), purified mouse anti-rat MHC class II RT1B, Ox6 clone (1:200; AbD Serotec), and goat polyclonal Iba-1 Ab (1:3000; Abcam). Incubation with specific primary Abs diluted in 10% FCS/DAKO buffer solution was performed overnight at 4°C. Incubation with biotinylated secondary Ab lasted for 1 h at room temperature followed by incubation with avidin–HRP complex (Sigma-Aldrich) for 1 h at room temperature. The visualization of bound biotinylated secondary Abs was performed through addition of 3,3′ diaminobencidine/H_2O_2-solution and microscopical analysis using a Leica DRMBE light microscope.

Cell collection and in vitro stimulation

Splenocytes and lymph node (LN) cells were collected from extracted spleens/LNs, followed by mechanical separation through a mesh screen, followed by erythrocyte lysis using 0.84% NH_4Cl (Sigma-Aldrich), pH 7.3 (for splenocytes), and washing with complete medium plus 5% heat-inactivated FCS (Life Technologies-BRL, Rockville, MD). i.p. macrophages were collected from euthanized rats by lavage of the peritoneal cavity with 50 ml sterile PBS. The fluid was collected and centrifuged for 10 min at 330 × *g* before resuspension in complete medium plus 10% FCS. Cells were plated in 70-cm^2 flasks (Nunc, Naperville, IL) and media changed at days 1 and 3 to remove nonadherent cells. The remaining macrophages were detached by adding prewarmed (37°C) 1× trypsin-EDTA (Life Technologies-BRL) and mechanical scraping. Macrophages were stimulated with LPS/IFN-γ (100 ng/ml and 100 U/ml, respectively). For recall experiments, LN cells were stimulated for 72 h with rMOG (20 μg/ml) or Con A (2.5 μg/ml). Splenocytes were stimulated for 72 h with the following stimuli: LPS/IFN-γ, Con A (both with concentrations as above) and PMA/ionomycin (5 ng/ml; 50 ng/ml). Supernatants from in vitro experiments were analyzed in duplicates by ELISA using commercial kits for TNF (BioSite, Paris, France), IL-6 (BioSource International, Camarillo, CA), or IL-22 (R&D Systems, Minneapolis, MN), respectively, or using a colorimetric assay for NO (Griess reagent, from Sigma-Aldrich).

Expression analysis

mRNA expression was performed using RNeasy mini kit (Qiagen, Hilden, Germany), including on-column DNA digestion. For macrophage and LN experiments, cells were lysed directly according to the manufacturers' protocol. For ex vivo expression experiment, spleens from immunized rats were collected and snap-frozen at the end of the experiment and disrupted using Lysing Matrix D tubes (MP Biomedicals, Irvine, CA) with a FastPrep homogenizer (MP Biomedicals). Reverse transcription was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers used for quantitative real-time PCR are listed in Supplemental Table II. Quantitative PCR was performed using a Bio-Rad iQ5 iCycler Detection System with SYBR green as fluorophore (Bio-Rad). Relative expression levels, corrected for amplification efficiency, were recorded using iQ5 v2.0 software (Bio-Rad). The geometrical mean of *Gapdh*, *Hprt*, and *18S* expression was used as reference.

Flow cytometry

LN cells were washed with cold PBS and resuspended in 100 μl PBS. Cells were stained for 20 min at 4°C. The following Ab combinations were used: CD3-allophycocyanin, CD8-PE, CD4-PE:Cy5 and CD25-FITC, or CD11B-FITC and CD45RA-PE:Cy5 (all from BD Biosciences, San Jose, CA). Stainings were visualized using a FACSort (BD, Franklin Lakes, CA) with CellQuest (version 3.2.1f1; BD) and analyzed using FlowJo v8.8.2 software (Tree Star, Ashland, OR). For splenocyte experiments, CD3-allophycocyanin, CD4-PE, and CD45RA-PE:Cy5 were used, and CFSE was added according to the manufacturers' protocol (Invitrogen, Carlsbad, CA). For expression on sorted cells, splenocytes were sorted into three categories using a MoFlo cell sorter (Dako, Fort Collins, CO): T cells

(CD3-allophycocyanin), B cells (CD45RA-PE:Cy5), and innate cells (the negative, non-B, non-T cell fraction).

Human association studies

The association study was conducted in two independent MS cohorts. All patients fulfilled either the Poser or the McDonald criteria for MS (23, 24). Information about the study and questions regarding previous participation and ethnic origin of parents was presented to donors both orally and in written form. Oral informed consent was obtained from all patients and control subjects. Anonymity was maintained throughout material handling and analysis. The first study consisted of a Swedish cohort of 2158 (1546 female) MS patients and 1759 (1154 female) blood donor control subjects. For further details of the Swedish MS cohort, see Ref. 25.

A follow-up study was then performed using a Norwegian cohort including 548 patients (396 female patients) and 554 control subjects (323 female subjects) (15). All patients of the Norwegian cohorts gave written informed consent to participate. The local ethics committees in Sweden and Norway approved the study. For further details of the Norwegian MS cohort, see Ref. 25.

Marker selection

Single nucleotide polymorphism (SNP) markers were selected for genotyping by running the TAGGER algorithm with $r^2 \geq 0.9$ permitting aggressive two and three SNP tagging (26), as implemented in Haploview 3.32 (27) based on data from the HapMap CEU population (release 21/phase II, NCBI B35 assembly, dbSNP b125). Allelic discrimination was performed with either TaqMan SNP genotyping assays using a 7900 HT Fast Real-time PCR system (Applied Biosystems) as described in Ref. 28, or with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, San Diego, CA), as described previously (25).

Genotyping of human material

In total, 29 SNPs were analyzed and manually checked by two independent persons using SpectroTYPER RT 3.3.0 software (Sequenom; Supplemental Table III). On each 384-well plate analyzed as a unit, half of the samples were controls and the remainder was cases. The quality-control requirements were: Hardy–Weinberg $p > 0.01$ among controls, a concordance rate >95% with known HapMap individual genotypes, as well as duplicate DNA and a success rate >80%. The success of each SNP genotyping assay was calculated as the number of genotypes retrieved over the possible number of genotypes. Two SNPs in *IFNGR1* and one SNP in *IL-22RA2* were excluded because of monomorphism and Hardy–Weinberg disequilibrium. Differences in allele frequencies between MS patients and control subjects were then tested through single marker association analysis using the model command in PLINK v 1.04 (29).

Estimation of false-positive report probability

We have estimated the probability that the association between MS and *IL-22RA2* is false by estimating the false-positive report probability (FPRP). The FPRP depends on the prior probability of association, the power of the study, and the significance threshold: FPRP = 1/(1 + posterior odds for true association). Posterior odds for true association = prior odds × power/significance threshold.

The estimation and details of these measures are included in Supplemental Table IV.

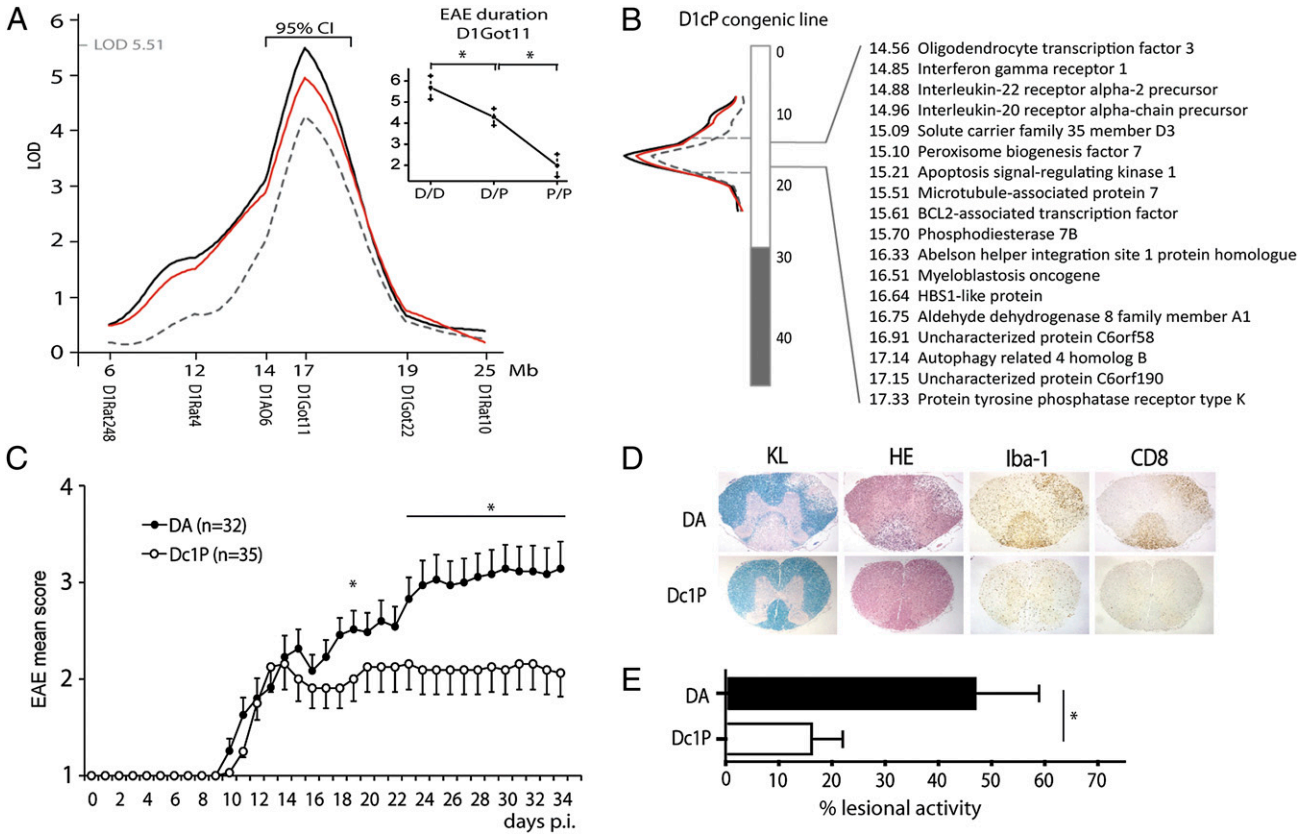

FIGURE 1. *Eae29* regulates EAE severity and contains 18 annotated genes. *A*, AIL mapping reduces *Eae29* confidence interval to 4.5 Mb. *y*-Axis depicts probability of linkage as LOD. Black line denotes LOD score for EAE duration; red line denotes EAE cumulative score; and dashed line denotes EAE maximum score. DA alleles at the peak marker D1Got11 worsen disease compared with PVG alleles in an additive manner (small box). *B*, Schematic overview of the congenic line Dc1P, carrying PVG alleles in *Eae29,* with the 18 annotated genes in *Eae29* and numbers indicating position (Mb). *C*, Dc1P displays less EAE compared with DA rats. Female (n = 12 DA and 18 Dc1P) and male rats (n = 23 DA and 14 Dc1P) were immunized with 20 and 40 mg rMOG, respectively. *D*, Spinal cord and brain sections of DA and Dc1P rats. Sections were stained with Klüver (KL; row 1) and Hämalaun-eosin (HE; row 2), ionized calcium binding adaptor molecule 1 (Iba-1; row 3), and CD8 (row 4), respectively. Dc1P rats showed fewer signs of CNS inflammation and demyelination, whereas DA rats exhibited severe loss of myelin and cell infiltration at the site of the lesion in the spinal cord white matter. Dc1P rats also revealed lower recruitment of Iba-1$^+$ and CD8$^+$ macrophages (CD3$^-$) compared with DA. *E*, Lower percentage of active lesions in Dc1P (9.7% compared with 21.3% in DA; also see Supplemental Fig. 1). LOD, logarithm of odds; Mb, megabases.

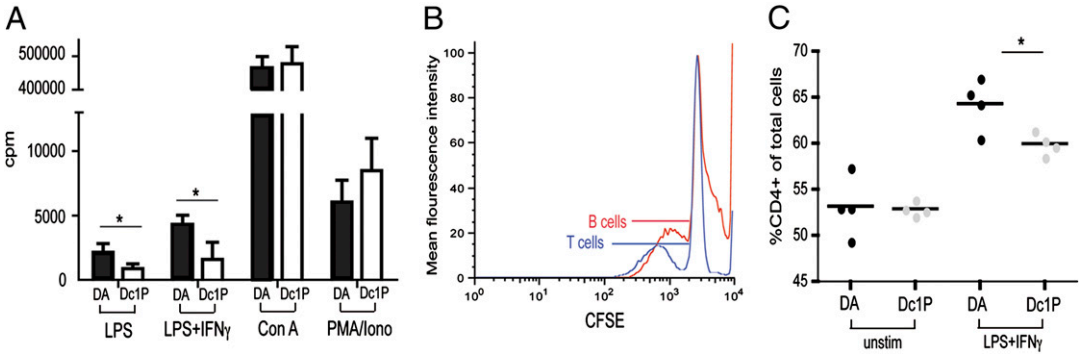

FIGURE 2. LPS/IFN-γ–induced Dc1P splenocytes activate lymphocytes weaker than DA splenocytes. *A*, Splenocytes were activated for 72 h and assayed for proliferation using the ^{3}Thymidine incorporation assay. *B*, LPS/IFN-γ activation of splenocytes preferentially expanded T cells over B cells, as shown by CFSE staining. Blue line denotes T cells; red line denotes B cells. *C*, LPS/IFN-γ–activated DA splenocytes have an increased CD4 T cell proportion compared with Dc1P, as shown by flow cytometry.

Statistical analysis of experimental data

Linkage analysis was performed using the statistical software R/qtl v2.7.1. Disease phenotypes were tested for linkage using the Haley–Knott regression model. The 95% confidence interval for *Eae29* was arbitrarily defined by the flanking markers of a 1.8 logarithm of odds decline, based on simulation experiments performed previously (30). Data from in vitro studies were analyzed using either the nonparametric Kruskal–Wallis test followed by a Dunn's post hoc test or Mann–Whitney *U* test using Prism v4 software (GraphPad, San Diego, CA). Mean values in graphs are presented with SEs unless stated otherwise.

Results

Eae29 regulates EAE severity

A region on rat chromosome 1 was previously linked to EAE in three F2 rat intercrosses (11, 12, 31, 32). We have now redefined this locus, *Eae29,* using a 10th generation (G10) AIL between the EAE-susceptible DA and EAE-resistant PVG.AV1 rat strains. *Eae29* displayed linkage to several disease phenotypes, with strongest linkage to severity phenotypes including duration, and cumulative and maximum disease score (Fig. 1*A*). For all phenotypes, DA alleles drove disease in an additive manner (Fig. 1*A*). The 95% confidence interval of *Eae29* harbors 18 annotated genes (Fig. 1*B*). To confirm and further study the role of *Eae29,* we created a congenic line, DAc1PVG-*Eae29* (hereby named Dc1P), in which PVG alleles at *Eae29* were transferred onto the DA background (Fig. 1*B*).

Dc1P rats developed milder EAE compared with DA rats (Fig. 1*C*), also reflected in histopathological analyses of the brain and spinal cord cross sections. Dc1P rats developed less severe CNS inflammation and demyelination than did the DA rats (Fig. 1*D*). In accordance with this finding, Dc1P rats also had a lower percentage of active lesions (9.7% compared with 21.3% in DA; Fig. 1*E*), represented by MHC class II-expressing microglia cells/macrophages within the lesions (Fig. 1*E* and Supplemental Fig. 1). When assessing the cell types infiltrating CNS lesions, we observed no proportional differences in $CD8^{+}$ macrophages, $CD3^{+}$ T cells, or $CD45RA^{+}$ B cell ratios between the two strains, indicating that the composition of infiltrating cells is similar. Compared with macrophages that highly infiltrated the lesions (Fig. 1*D*), T and B cells were present in only very low numbers in both strains ($\leq$40 and $\leq$10 cells/mm^2 spinal cord, respectively; data not shown). With these data, we confirmed that *Eae29* regulates severity of EAE.

Eae29 locus confers lower innate immunity activation

To identify candidate genes within *Eae29,* we investigated disease-related immune mechanisms in DA and Dc1P. We began studying disease-priming events by investigating draining LNs from immunized rats. The proportions of LN cell populations were similar between the strains (Supplemental Fig. 2). Moreover, LN cells from immunized rats displayed similar proliferative capacities in DA and Dc1P on recall with myelin autoantigen (Supplemental Fig. 3). A similar result was observed when stimulating LN cells with the T cell superantigen Con A. Thus, T and B cells, which are major expanding cells during priming, responded similarly in both strains.

We next investigated the effect of various stimuli that also activate innate immune responses on splenocytes. LPS-stimulated Dc1P splenocytes proliferated less well compared with DA, an effect that was enhanced when IFN-γ was added (Fig. 2*A*). The major proliferating cell subsets were B and T cells, with a preferential expansion of T cells, demonstrated by CFSE incorporation (Fig. 2*B*). T cells from Dc1P rats expanded less prolifically compared with DA (Fig. 2*C*). No differences were evident when directly stimulating lymphocytes with either Con A or a combination of the calcium-releasing agents PMA and ionomycin. Collectively, these data suggested that defects in the innate response regulated by *Eae29* control the expansion of adaptive immune cells.

Dc1P macrophages are less active and indicate cytokine receptors as candidate genes

We hypothesized that the effects observed in splenocytes originated from macrophages, and that Dc1P macrophages were less activated during EAE, leading to reduced disease severity. We therefore assayed isolated i.p. macrophages for production of effector molecules on proinflammatory LPS/IFN-γ stimulation. Dc1P macrophages produced less TNF, IL-6, and NO compared with DA macrophages (Fig. 3). These clear differences in macrophage

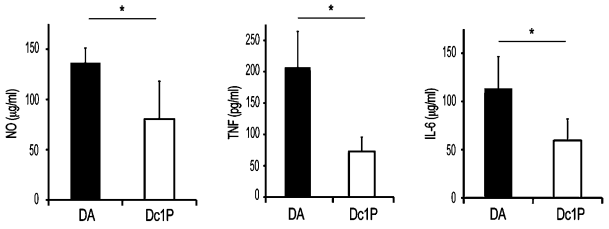

FIGURE 3. Dc1P macrophages produce less proinflammatory effector molecules on activation. Isolated i.p. macrophages were stimulated for 18 h with LPS/IFN-γ, and protein levels of TNF, IL-6, and NO reactive intermediates from supernatants were quantified by ELISA/colorimetric assay.

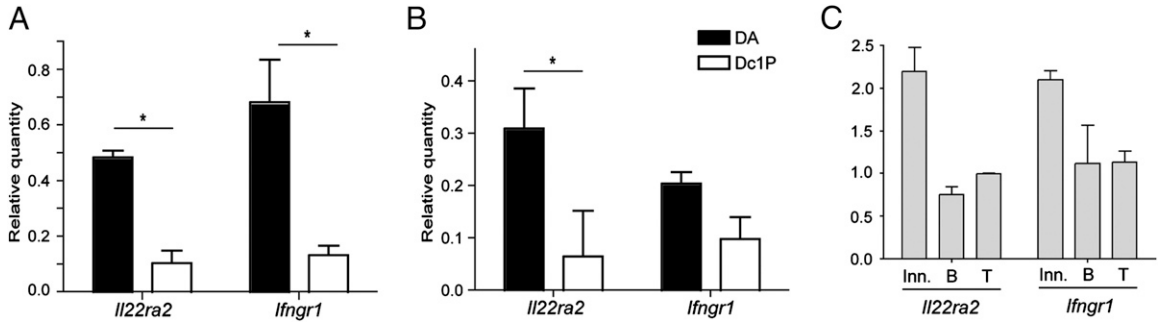

FIGURE 4. Gene expression levels indicate *Il-22ra2* and possibly *Ifngr1* as *Eae29* candidate genes. *A*, Quantitative expression of *Il-22ra2* and *Ifngr1* in LPS/IFN-γ–stimulated i.p. macrophages. *B*, Expression in splenocytes collected day 35 p.i. *C*, Expression in sorted pooled cells from splenocytes collected day 27 p.i. B, B cells; Inn., innate cells (non-B cell, non-T cell fraction); T, T cells.

activation phenotype, together with the lower numbers of active lesions, prompted us to scrutinize the candidate genes in *Eae29*. Of the 18 genes, both IFN-γR1 (*Ifngr1*) and *Il-22ra2* are implicated in macrophage function (33). The two genes are juxtaposed in the genome and are located within a cytokine receptor cluster (Fig. 1*B*). All exons of both *Ifngr1* and *Il-22ra2* were sequenced; however, PVG.AV1 and DA had identical sequences (data not shown). Nevertheless, expression of both genes was reduced in Dc1P macrophages on LPS/IFN-γ stimulation (Fig. 4*A*). In splenocytes from the late-stage EAE, when there is clinical difference between DA and Dc1P rats, only *Il-22ra2* was significantly downregulated in Dc1P, although a trend was also observed for *Ifngr1* (Fig. 4*B*). We also observed increased expression of both *Il-22ra2* and *Ifngr1* in the innate cell compartment, as compared with in B and T cells (Fig. 4*C*). These findings establish *Il-22ra2* and possibly *Ifngr1* as EAE candidate genes. Both genes were then tested for association in MS cohorts.

Common gene variants in IL-22RA2 *associate with MS*

The *Eae29* locus is homologous to a 10-Mb region on human chromosome 6, 6q23. The experimental findings indicated *IL-22RA2* and *IFNGR1* as plausible risk genes; a total of 29 tagging SNPs located across *IL-22RA2* and *IFNGR1* were therefore tested for association with MS (Supplemental Table III). Of these, three SNPs did not fulfill defined requirements and were thus removed (Supplemental Table III). Association was first tested with a Swedish MS cohort consisting of a total of 2158 patients (female/male ratio = 2.53) and 1759 control subjects (female/male ratio = 1.91). The SNP rs276474, located at the very end of *IL-22RA2*, displayed the strongest association with MS (Fig. 5*A*). No significant association was apparent for any marker or haplotype within *IFNGR1* (Fig. 5*A*). The rs276474 AA genotype was next tested for interaction with the HLA locus, but no signs of interaction were detected (data not included). We next stratified patients for the rs276474 genotype and compared MS severity score between the groups. Patients with the disease-predisposing AA-genotype also experienced more severe MS (Fig. 5*B*). However, because the association was still relatively modest, we sought to type rs276474 in an independent MS cohort. For this purpose, a Norwegian cohort consisting of 548 patients (female/male ratio = 2.6) and 554 control subjects (female/male ratio = 1.4) was tested for association. The rs276474 AA genotype displayed a similar trend as in the Swedish cohort, with the combined analysis yielding an odds ratio of 1.26 ($p = 8.0 \times 10^{-4}$; Fig. 5*C*).

The association of *IL-22RA2* with both EAE and MS strengthens its potential role in disease progression. Taken together, our experimental and human studies establish *IL-22RA2* as a disease risk gene associating with macrophage activation.

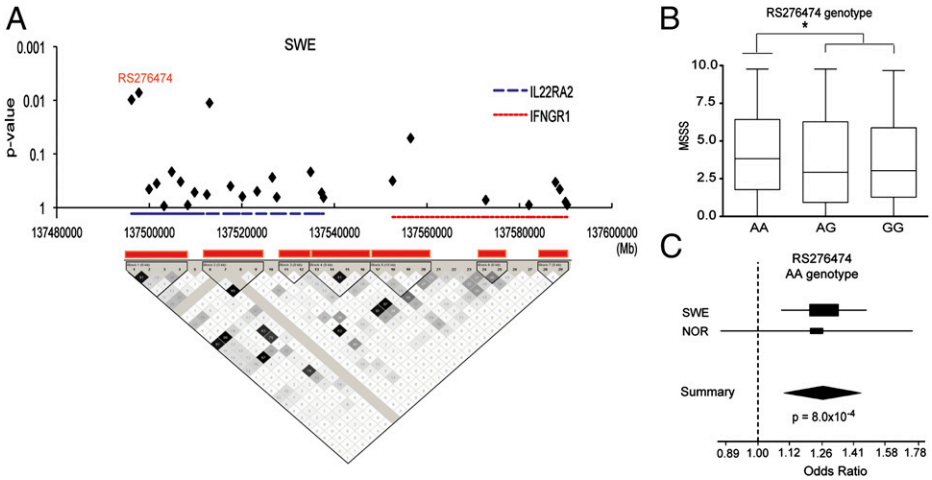

FIGURE 5. *IL-22RA2* associates with MS, whereas *IFNGR1* does not. *A*, Twenty-six markers in *IL-22RA2* and *IFNGR1* were tested for association in a Swedish MS cohort consisting of total 3917 patients and control subjects. The strongest association with MS was observed for rs276474 located at the end of *IL-22RA2*. $p = 2.6 \times 10^{-3}$. No association was detected for *IFNGR1*. Red bars indicate seven linkage disequilibrium blocks within *IL-22RA2* and IFNGR1. *B*, Multiple sclerosis severity scores (MSSS) for patients stratified on rs276474 genotype. Patients with the disease-predisposing AA-genotype also displayed more severe MS. *C*, The SWE and Norwegian (NOR) MS cohorts yield an odds ratio of 1.26. NOR, Norwegian; SWE, combined Swedish.

Discussion

The use of genetically distinct rodent strains to map risk genes of common human disorders relies on central immune pathways being conserved across species. We have used this translational approach to establish *IL-22RA2* as a risk gene in EAE and MS. The created Dc1P line confirmed the genetically regulated differential expression of *IL-22ra2* and also shed light into the functional role of *Il-22ra2*, involving macrophage effector mechanisms.

IL-22RA2 was only recently discovered and encodes a natural receptor for IL-22 that has not been extensively studied (34, 35). The cellular source of IL-22RA2 is not fully established, but tissues expressing IL-22RA2 include placenta, spleen, and skin (36). In particular, the observed expression of both IL-22RA2 and its ligand IL-22 in alveolar macrophages and neutrophils attracted our interest (33). Importantly, the IL-22RA2 protein lacks a transmembrane domain and is therefore secreted. It also prevents the structurally similar surface-bound IL-22RA1 from binding IL-22 (37). The biological relevance of soluble cytokine receptors remains unclear. On the one hand, they could function as neutralizing agents, thereby blocking the function of the respective cytokine. Conversely, they could serve as carrier proteins both prolonging cytokine half-life and enhancing long-distance signaling, as in the case of soluble IL-4Rs and TNFRs (38–40). Our data suggest an IL-22–enhancing role of IL-22RA2, as *Il-22ra2* expression was higher in the susceptible DA strain, but this requires further investigation.

Interestingly, IL-22 and its receptors have been implicated in both MS/EAE and other chronic inflammatory diseases. For example, IL-22 is the candidate gene for a locus controlling mortality in a mouse EAE model (41) and associates with increased disease priming in our rat EAE model (9, 41). IL-22 expression also clearly associates with severity of psoriasis, rheumatoid arthritis, and Crohn's disease (37, 42, 43). Moreover, the surface-bound IL-22RA1 mediates breakage of the blood–brain barrier in MS patients and is also a risk gene for chronic rhinosinusitis (44, 45). Most of these disease associations relate to Th17-mediated effects of tissue cells such as epithelial cells. The ligand IL-22 is predominantly produced by Th17 and NK cells (46), and is known to act on nonimmune cells including skin, but other functions are not fully understood (46). Although it is likely that IL-22RA2 also regulates these mechanisms, our data demonstrate a more complex role that also involves innate responses. Because IL-22RA2 has been poorly studied, more experiments are needed to fully characterize its function in biology and disease. Possibly, *IL-22RA2* is a common risk gene for several immune disorders by regulating shared central pathways.

We identified *Il-22ra2* as an EAE risk gene through defining the phenotype of the Dc1P congenic line, both clinically and with in vitro immunoassays. Dc1P rats developed milder EAE than did DA rats, as reflected histologically by reduced inflammation and demyelination. The only proportional difference we observed within CNS lesions and infiltrates was the lower lesion activity in Dc1P. Lesion activity referred to the percentage of MHC class II^+ macrophages and microglia. CNS-infiltrating lymphocytes and blood-borne monocytes may also contribute to disease regulation, and we observed negligibly low numbers of CNS infiltrating T cells at day 35 p.i. in both strains. The lesion-restricted CD8 upregulation therefore predominantly reflects invading macrophages (47), and we suggest that they likely regulate CNS inflammation in concert with brain-resident microglia. The more inactive lesions in DA spinal cord sections indicate a failure in DA to resolve chronic inflammation, concordant with an increased role of innate immune responses in immunoregulation. Indeed, we have previously determined phenotypic differences between proinflammatory activated macrophages derived from different strains of rat and have correlated innate immune phenotypes with specific genotypes (48).

We next investigated what possible mechanisms could control these differences. Our Ag recall stimulation experiments of draining LN cells revealed no significant differences in expansion between the strains. During recall, most expanding cells are adaptive immune cells (mainly T and B cells). We therefore hypothesized that the differential effect of *Eae29* resided within an innate immune cell, possibly macrophages. In EAE priming, dendritic cells are the predominant APCs (49), which could possibly mask the differential effect from macrophages in the recall. We therefore investigated splenocytes and observed only phenotypic differences when stimulating with LPS or LPS/IFN-γ. Isolated Dc1P macrophages also produced lower levels of the effector molecules TNF, IL-6, and NO when stimulated with LPS/IFN-γ. Collectively, our data suggest that macrophage/microglial activation is the primary mechanism regulating disease in *Eae29*.

Considering the genes included within *Eae29,* eight genes are associated with described immune functions. Of these, we focused on genes with possible roles in innate immunity, even though no gene within *Eae29* can be formally excluded as being disease regulating. Many of the genes within *Eae29* are plausible candidates in both MS and other inflammatory diseases. It is also conceivable that several genes in *Eae29* interact to regulate EAE, especially if they are involved in the same pathway. This is also consistent with the apparent clustering of immune genes (50). However, we chose to focus on one such mechanism involving innate responses. This highlighted *Ifngr1* and *Il-22ra2* as interesting candidate genes, both being preferably expressed in macrophages. Of the two genes, *Il-22ra2* was also expressed at significantly lower levels in Dc1P spleens collected during late-stage disease, a time point when DA and Dc1P rats differ in their clinical disease severity.

The human association study identified variants in *IL-22RA2* associating with disease. We observed p values in the range of 10^{-4}, which does not reach the arbitrarily defined p value of 5×10^{-7} applied in genome-wide studies, in which the number of comparisons is far greater. However, the addition of experimental evidence strengthens the case for *IL-22RA2* as an MS risk gene. Moreover, many now replicated risk genes for common disorders have often displayed p values in the range we observe or lower. For example, in an Australian/Finnish replication study of confirmed MS risk genes, both *IL-2RA* and *CD58* displayed p values in the range of 10^{-2}, and were confirmed only in larger meta-analyses (51). Upcoming genome-wide screens of very large cohorts may more clearly define the association of *IL-22RA2* with MS.

Soluble cytokine receptors have been particularly attractive as therapeutics in inflammatory diseases, with soluble TNF-binding agents in treatment of RA being the most successful example (52). Our findings therefore highlight IL-22 neutralizing agents as potential drugs, not only for MS but for other chronic inflammatory diseases. In conclusion, our stepwise identification of *IL-22RA2* as an EAE and MS risk gene illustrates the value of comparative genetics in complex diseases. The approach also allowed us to identify an Il-22ra2–mediated mechanism important for innate immune effector mechanisms.

Disclosures

The authors have no financial conflicts of interest.

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