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Genetic Interactions in Eae2 Control Collagen-Induced Arthritis and the CD4+/CD8+ T Cell Ratio

Jenny Karlsson,* Martina Johannesson,* Therese Lindvall,* Patrik Wernhoff,† Rikard Holmdahl,* and Åsa Andersson²*

The Eae2 locus on mouse chromosome 15 controls the development of experimental autoimmune encephalomyelitis (EAE); however, in this study we show that it also controls collagen-induced arthritis (CIA). To find the smallest disease-controlling locus/loci within Eae2, we have studied development of CIA in 676 mice from a partially advanced intercross. Eae2 congenic mice were bred with mice congenic for the Eae3/Cia5 locus on chromosome 3, previously shown to interact with Eae2. To create a large number of genetic recombinations within the congenic fragments, the offspring were intercrossed, and the eight subsequent generations were analyzed for CIA. We found that Eae2 consists of four Cia subloci (Cia26, Cia30, Cia31, and Cia32), of which two interacted with each other, conferring severe CIA. Genes within the other two loci independently interacted with genes in Eae3/Cia5. Investigation of the CD4/CD8 T cell ratio in mice from the partially advanced intercross shows that this trait is linked to one of the Eae2 subloci through interactions with Eae3/Cia5. Furthermore, the expression of CD86 on stimulated macrophages is linked to Eae2. The Journal of Immunology, 2005, 174: 533–541.

C hronic inflammatory diseases such as rheumatoid arthritis (RA)¹ and multiple sclerosis (MS) are characterized by a complex etiology including both genetic and environmental factors (1). Studies of autoimmune diseases in human cohorts and experimental animal models have shown that autoimmune diseases are polygenic, and it is believed that small contributions from many genes determine the disease outcome. Due to the genetic heterogeneity in the human population and the relatively low sample number in the investigations, the identification of genes significantly linked to susceptibility to autoimmune disease in humans has been limited to date (2, 3). For this reason, animal models resembling human disease are very important tools for studying complex genetic traits.

RA affects the wrists and small joints of the hands and feet, leading to deformity and disability. Susceptibility is associated with genes in the MHC (4, 5). Collagen-induced arthritis (CIA), a frequently studied model for RA, can be induced in genetically susceptible mouse strains. Immunization with collagen type II (CII) results in polyarthritis, and the disease process is dependent on both cell-mediated and humoral immune mechanisms (6–11). Studies of CIA development, followed by genetic linkage analyses on gene-segregating crosses between susceptible and resistant MHC-congenic mouse strains, have revealed a large number of non-MHC CIA susceptibility loci (http://informatics.jax.org). The B10.RIII mouse strain (H-2r) is susceptible to chronic arthritis upon immunization with bovine CII. In an F2 intercross between B10.RIII and the CIA-resistant MHC congenic strain RIIS/J (H-2ª), two loci on chromosome 3 (Cia5) and chromosome 13 (Cia10) were significantly linked to the development of CIA (12). Cia5 overlaps with the Eae3 locus, which was previously demonstrated to interact with chromosome 15 (Eae2) in controlling susceptibility to experimental autoimmune encephalomyelitis (EAE) (13). EAE is an experimental model of MS, an inflammatory demyelinating disease in the CNS. B10.RIII mice are susceptible to chronic EAE induced by immunization with the myelin basic protein (MBP) peptide 89–101, whereas the RIIS/J mouse strain is resistant to disease induction. In addition to Eae3, development of EAE with this immunization protocol has been linked to several gene regions (13–15). The Eae2 locus is located on the centromeric part of chromosome 15. In the previous F2 intercross between B10.RIII and RIIS/J, the increased incidence of EAE was linked to an interaction between the Eae2 and the Eae3 quantitative trait loci (QTL). It was observed that mice heterozygous in the Eae2 region and homozygous for B10.RIII in Eae3 were the most affected (13). To confirm these data, Eae2 congenic mice were produced and studied in an EAE experiment (14). These data showed a nonsignificant tendency toward increased severity in the Eae2 heterozygous mice (14).

The human diseases RA and MS as well as the corresponding animal models CIA and EAE are considered to be T cell-mediated diseases. Ag-specific Th1 cells, activated in the peripheral lymphoid organs, migrate into the target organ and become reactivated by resident APCs (16–19). To make a proper Ag response, T cells need a costimulatory signal. The CD80 and CD86 costimulatory molecules expressed on different APCs interact with the CD28/CD152 molecules on T cells. The important role for this interaction in the induction of autoimmunity in CIA and EAE has been

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; AUC, area under the curve; CIA, collagen-induced arthritis; CII, collagen type II; EAE, experimental autoimmune encephalomyelitis; LOD, likelihood of odds; LRS, likelihood ratio statistics; MBP, myelin basic protein; MS, multiple sclerosis; PAI, partially advanced intercross; QTL, quantitative trait locus/loci; wt, wild type.

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reported (20, 21). Although the main focus in studies of experimental models for autoimmune diseases has been on CD4⁺ T cells, it is suggested that the CD8⁺ T cells take part in the pathogenesis of EAE (22, 23). The specificities of the T cell repertoire in the periphery, including clones reactive toward autoantigens, are selected in the thymus. In addition, the relative numbers of CD4⁺ and CD8⁺ T lymphocytes in the lymphoid organs are genetically controlled and differ between mouse strains (15, 24). Comparison between the CIA/EAE-resistant RIIS/J and -susceptible B10.RIIII strains with regard to the ratio between CD4⁺ and CD8⁺ T cells in the spleens of naive animals reveals a higher CD4⁺/CD8⁺ T cell ratio in the RIIS/J strain. This trait was mapped in a gene-segregating backcross between the two strains. The CD4⁺/CD8⁺ T cell ratio was linked to three genomic regions in this study, most interestingly to the Eae2 region on chromosome 15 (15).

In this study we report that the Eae2 congenic fragment harbors four sub-QTL (denoted Cia26, Cia30, Cia31, and Cia32) controlling CIA. The data are based on CIA experiments with mice derived from a partially advanced intercross (PAI) between Eae2 and Eae3/Cia5 bicongenic mice. The intercrossed bicongenic animals and single-congenic mice, altogether comprising 1051 animals, have been investigated for CIA development. We found that genes within the Eae2 region control CIA development in a complex interplay between genes inside the Eae2 fragment as well as genes in the Eae3/Cia5 region on chromosome 3. Additionally, the CD4⁺/CD8⁺ T cell ratio in PBL was investigated and found to be controlled by interactions between genes within Eae2 on chromosome 15 and Eae3/Cia5 on chromosome 3. Moreover, based on a genetic linkage analysis on a backcross between the B10.RIIII and RIIS/J strains, we investigated the expression of costimulatory molecules on activated macrophages from Eae2 single-congenic mice, and we conclude that genes within the Eae2 locus, in addition, control CD86 expression.

Materials and Methods

Mice

B10.RIIII mice were originally provided by J. Klein (Max-Planck-Institut für Biologie) and were kept in our breeding colony. RIIS/J mice were purchased from The Jackson Laboratory. Eae2 and Eae3/Cia5 congenic mouse strains were bred in our colony at Lund University. RIIS/J (donor) genes were bred onto the B10.RIIII (recipient) background. The mice were bred in our colony at Lund University. RIIIS/J (donor) mouse strains were bred in our colony at Lund University. RIIIS/J (donor) genes were bred onto the B10.RIIII (recipient) background. The mice were genotyped and typed for CIA. By association studies between genotype and disease outcome, the desired genetic regions for breeding the next generation were chosen. In this way, recombinations in each generation were collected.

(B10.RIIII × RIIS/J)F₁ × B10.RIIII animals were produced in our colony at the Section for Medical Inflammation Research, Lund University, and studied in an EAE experiment (15). Eighty animals were analyzed for the expression of CD86 on peritoneal macrophages at the end of the experiment.

Disease induction and scoring

Bovine CIA was prepared from calf nasal cartilage by pepsin digestion and was purified as previously described (25). CIA was induced in 10- to 16-wk-old mice by intradermal immunization with 100 µg of CIA emulsified in IFA (Difco) in the base of the tail. The mice were boosted with 50 µg of bovine CIA emulsified in IFA on day 35. The clinical arthritis severity was measured every second day by scoring the swelling and erythema in the joints of the paws. This scoring system is based on the number of inflamed joints, ranging from one to 15 for each affected paw, with a maximum score of 60/mouse. Each affected ankle/wrist was scored as 5, and each inflamed knuckle and toe was given 1 point.

Cell preparation, in vitro stimulation, and FACS analyses

Peritoneal cell exudates were prepared by injecting 10 ml of DMEM (In-vitrogen Life Technologies) supplemented with 5% FCS into the peritoneal cavity and were subsequently dispensed into a polypropylene tube. The cells were washed and resuspended in 1 ml of DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, 0.1 M HEPES, and 2-ME (cell culture medium). The cells were seeded (10⁵ cells/well) in a 48-well plate. The macrophages were allowed to adhere for 30–60 min and then washed with DMEM to remove nonadherent cells before culture overnight in cell culture medium. Subsequently, the cells were washed and stimulated with IFN-γ (100 U/ml) and LPS (10 ng/ml) for 48 h. The stimulated peritoneal cells were removed from the cell culture plate by gently scraping off the cells. Subsequently, the cells were incubated with normal rat serum and supernatant from the 2-4G2 hybridoma (anti-CD16/CD32, our hybridoma collection) to reduce non-AP-specific binding of IgG to FcRs. Abs against CD11b (Mac-1) conjugated with FITC (clone M1/70) and CD86 (B7.2) conjugated with PE (clone GL1; BD Pharmingen) were used for staining the peritoneal cells. Negative controls were stained with the anti-CD11b-FITC only. Cells expressing high levels of CD11b were electronically gated for analysis of CD86 expression.

RBC were lysed with 0.8% ammonium chloride, and the cells were washed with staining buffer (PBS, 3% FCS, and 0.01% NaN₃) and incubated with supernatant from the 2-4G2 hybridoma. The cells were subsequently stained with anti-CD4-PE (clone H129.19) and anti-CD8-CyChrome (clone 53-6.7; BD Pharmingen) and analyzed on a FACScan (BD Biosciences).

Genotyping

DNA was prepared from toe or tail biopsies according to a standard protocol (26). Alternatively, tissues were dissolved in 500 µl of 50 mM NaOH for 1–2 h at 95°C, and subsequently neutralized with 100 µl of 1 mM Tris buffer (pH 8). Seventeen and 18 informative fluorescence-labeled microsatellite markers (Interactiva and MWG Biotech) were used for genotyping the Eae2 and Eae3/Cia5 congenic fragments, respectively. PCR and genotyping analyses were performed as previously described (15).

Statistics and linkage analysis

The significance value for interactions between phenotype and two markers was calculated by two-way ANOVA. The Mann-Whitney U test was used to calculate the significance between two groups. Two-locus interaction analyses were also performed using R (copyright 2004, The R Foundation for Statistical Computing, version 2.0.0) and R/qtl (27). The analysis was performed using regression of phenotypes on multiple genotype probabilities (Haley-Knott regression). The two-QTL model compares a full model in the presence of covariates (y = μ + β₁q₁ + β₂q₂ + β₃q₃ + q₂ + A + Y + Zβ₃q₂ + Zβ₄q₃ + Zβ₅q₂q₃ + ε) to a null model (y = μ + A + Y + ε), e.g., likelihood of odds (LOD) joint values, where q₁ and q₂ represent QTL genotypes at two different locations, A is a matrix of covariates, and Z is a matrix of QTL interacting covariates. For the interaction, epistasis LOD scores, the full model is compared with an additive model (y = μ + β₁q₁ + β₂q₂ + A + Y + ε), e.g., LODint values. The experimental significance thresholds for the two-locus interactions were subsequently determined using permutation tests (n = 1000), where p < 0.05 was considered significant. One-locus linkage analysis and permutation tests were conducted using the R packages QTLkr (http://www.qtlkr.sourceforge.net) and Qtltools (http://www.qtltools.org). The marker map was generated using the Kosambi map function. Permutation tests were performed to establish the empirical significance thresholds on a genome-wide level (p < 0.05) for the phenotype. Interval mapping was conducted at 1-cM increments under the additive regression model to calculate the test statistics (likelihood ratio statistic (LRS)).

Results

The Eae2 region confers sensitivity or resistance to CIA depending on the inheritance of RIIS/J alleles

In contrast to the Eae3/Cia5 locus, the Eae2 QTL has not previously been linked to CIA in any cross. Because genes within the Eae3 and the Eae2 QTL were postulated to interact in EAE (13), we hypothesized that this might also be true for CIA. First, the Eae2 single-congenic strain was immunized for CIA to investigate whether the Eae2 fragment alone controls CIA when background genes are neutralized, as in a congenic strain. The Eae2 locus was
not linked to CIA development in a previous experiment (12), which might have been due to background genes. In an altered genetic environment in which only the Eae2 region differs from B10.RIII wild-type (wt), genes within the Eae2 region might be unraveled. The disease phenotype area under the curve (AUC) was used to assess disease severity. The AUC is the sum of scores for each individual mouse during a defined test period. The scoring started on day 18, mice were then boosted on day 35, and the experiment was ended on day 92. In addition to the whole test period, days 18–92 (AUC d18–92), we separated the early phase of the test period, days 18–35 (AUC d18–35) from the acute phase, days 53–92 (AUC d53–92). Other disease traits analyzed were the mean day of onset and the incidence of disease along with the mean maximum score. As shown in Fig. 1, we could demonstrate that the Eae2 locus indeed controlled arthritis, because an exacerbated disease was observed when mice had one allele from each parental strain, and protection from disease was conferred when mice had two RIIIS/J alleles. These data are in line with the previous F2 intercross (13) and support our hypothesis that the same genes operate in EAE and CIA. RIIIS/J homozygous alleles in the Eae2 fragment protected against disease development in both males and females (Fig. 1), but the exacerbating effect from heterozygous alleles was found only in female mice (Fig. 1A). The incidence of CIA in female Eae2 congenic mice with a heterozygous fragment was 88% compared with 47% in B10.RII female and 41% in congenic females with a RIIIS/J homozygous Eae2 fragment.

Dissection of the Eae2 congenic fragment in a PAI reveals four loci controlling different phases of arthritis

From the finding that the Eae2 congenic fragment indeed controls arthritis, the next step was to pinpoint the QTL for the disease-causing genes. The aim was to produce mice with multiple recombinations in the fragment, followed by investigation of CIA to define the genetic linkage region(s). By crossing the single Eae2 and Eae3 congenic strains (Fig. 2A) and by further intercrossing the bicongenic strain, we could investigate the previously postulated genetic interaction between the Eae2 and Eae3 regions (13) and, in addition, obtain recombinations in the fragments. The heterozygous B10.RIIIRIIIS/J Eae2 × Eae3 F1 offspring were subsequently bred in a PAI for eight generations, and the progeny was investigated for CIA (Fig. 2A). In this way, only genes within the congenic fragments segregated, because the genetic background from B10.RIII was identical in these mice. Altogether 1051 congenic mice were investigated for CIA, including wt control mice.

Different phases of CIA were analyzed in the Eae2/Eae3 single- and bicongenic mice. In this experiment it became clear that different genes operate in the early phase, before boost, compared with the acute phase of the disease. These traits were differently inherited and linked to different subloci within the congenic fragments (Table I). We found that the development of CIA was linked to four regions within the Eae2 congenic region. We have denoted the novel CIA QTL within Eae2 on chromosome 15 as Cia26, 30, 31, and 32. The Eae3/Cia5 on chromosome 3 was similarly split into three sub-QTL (Cia5, Cia21, and Cia22), controlling different phases of CIA (44).

Cia30 confers protection from CIA

The Eae2 single-congenic mice with a homozygous RIIIS/J fragment developed CIA with lower severity than B10.RIII wt mice (Fig. 1). By separating the congenic fragments in the PAI, we could show that suppression of disease development originated from homozygous RIIIS/J alleles in Cia30. The protective effect from Cia30 was only observed when Cia5 on chromosome 3 had homozygous B10.RIII alleles, because one or two RIIIS/J alleles in Cia5 conferred protection from disease (Fig. 2B). Fig. 2C shows the development of disease in mice with different genotypes in Cia30, but homozygous for B10.RII alleles in Cia5. Thus, in the PAI we could show that the gene(s) responsible for the development of milder CIA is located in the uppermost part of the original Eae2 congenic fragment, Cia30.

Intrachromosomal interaction within the Eae2 fragment controls severe development of CIA

Many genes that have relatively small effects on the disease phenotype control the development of autoimmune disease. Epistatic interactions between gene products are therefore believed to be an important feature for the development of a full-blown disease. Moreover, genes within the same biological pathway tend to be clustered in the genome (28–30). Because we had collected mice with many recombinations in the Eae2 region, we wanted to investigate the possibility for gene interactions between genes within

![FIGURE 1. Development of CIA in Eae2 congenic mice. A. Disease development in female mice heterozygous or homozygous for RIIIS/J alleles in the Eae2 fragment and in B10.RIII wt mice. The AUC is the sum of the mean score per group over the test period. The AUC d18–92 in Eae2 heterozygous mice is compared with that in B10.RIII wt mice (p = 0.005, by Mann-Whitney U test). Two RIIIS/J alleles in Eae2 reduced disease severity compared with that in female B10.RIII wt mice (p = 0.02, Mann-Whitney U test). The mean maximum score was 25.6 in heterozygous mice compared with 10.6 in B10.RIII females (p < 0.0001, by Mann-Whitney U test) and 7.5 in RIIIS/J homozygous Eae2 congenic females. aa, RIIIS/J. B. Mean arthritis score of male mice. The effect of heterozygous Eae2 alleles was female specific, because male Eae2 heterozygous mice did not develop more severe disease than male B10.RIII wt mice. aa, RIIIS/J homozygous alleles; ab, heterozygous alleles; bb, B10.RIII homozygous alleles.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
the locus. Surprisingly, we found that RIIIS/J homozygous alleles (aa) in the telomeric region of the Eae2 congenic fragment, Cia30, interacted with homozygous B10.RIII alleles (bb) in Cia5 to promote severe disease development and a higher incidence of CIA (Fig. 3, A and B, and Table I). The effect was most prominent in female mice, which had 90% incidence of disease (data not shown).

To further investigate the possibilities of intrachromosomal interactions, we used the R/qtl software to calculate and visualize the results. Fig. 3C demonstrates an interaction plot created from calculations in the R/qtl analysis program (27). The color diagram shows the significance values for the trait AUC (d18–92), where the colors indicate the significance of the interactions according to the scale. As shown in the figure, there are several intrachromosomal interactions between loci in Eae2. The Cia30 locus interacted with both Cia32 and Cia26. Additionally, an interaction between Cia31 and Cia26 was observed (Fig. 3C). Taken together, the Cia30 locus influenced the severity of CIA through interactions with genes on chromosome 3 and with genes located in the nearby region on chromosome 15.

Disease-promoting heterozygous alleles in Cia31 and Cia32

In female Eae2 heterozygous single-congenic mice, we observed an exacerbating effect on CIA development compared with that in wt mice (Fig. 1A). When investigating the heterozygous effect in the PAI, mice heterozygous in Cia31 (D15Mit21) and homozygous B10.RIII alleles (bb) in Cia5 developed more severe disease before boost (AUC d18–35) than mice with other genotypes in the two loci and B10.RIII wt (Table I). In addition, disease onset was earlier in these mice (Table I). We observed aggressive development of disease in female mice heterozygous in Cia32 (D15Mit101) with homozygous B10.RIII alleles (aa) in Cia30 (Fig. 1C). This trait was specific to females (data not shown), which is in line with the findings in Eae2 single-congenic mice (Fig. 1). From these data we conclude that the heterozygous effect on disease severity is due to genes within the
Cia31 and Cia32 loci. A summary of the novel QTL within the Eae2 region and the postulated interactions with Eae3/Cia5 controlling the development of CIA is shown in Fig. 4.

Proportions of CD4+ and CD8+ T cells in peripheral blood are controlled by genes in the Cia30 locus that interact with genes in Eae3/Cia5

Subphenotypes are important tools in the process of finding genes controlling complex traits. In a linkage study with 200 Subphenotypes are important tools in the process of finding genes controlled by genes in the Cia30 locus that interact with genes markers (D15Mit80 and D15Mit150) within the CD4/CD8 T cell ratio in spleen lymphocytes is linked to two (Fig. 5).

AUCORT (days 53–92)

Cia26 166 ± 27 (aa) (bb) (bb) D15Mit182 (42.1 Mb) 0.3
Cia30 166 ± 27 (aa) (bb) (bb) D15Mit111*D15Mit182 0.0003

Incidence CIA

Cia26 79% (aa) (ab) (bb) D15Mit182 (42.1 Mb) 0.04
Cia30 44% (aa) (bb) (bb) D15Mit11 (6.5 Mb) 0.4

AUC (days 18–35)

Cia31 4 ± 1 (aa) (ab) (bb) D15Mit21 (26.4 Mb) 0.0004
Cia5 5 ± 2 (aa) (ab) (bb) D15Mit11 (6.5 Mb) <0.0001

Day of onset

Cia31 77 ± 3 (aa) (ab) (bb) D15Mit21 (26.4 Mb) <0.0001
Cia5 72 ± 3 (aa) (ab) (bb) D15Mit21*D3Mit50 0.13

AUC (days 18–92)

Cia32 52 ± 13 (aa) (ab) (bb) D15Mit111 (29.6 Mb) <0.0001
Cia22 51 ± 13 (aa) (ab) (bb) D3Mit103 (80.7 Mb) <0.0001

Table I. CIA loci on proximal mouse chromosome 15

<table>
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<tr>
<th>Phenotype</th>
<th>QTL</th>
<th>Phenotype Value</th>
<th>Linkage Markers (Mb)</th>
<th>p</th>
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<tr>
<td>AUC (days 18–92)</td>
<td>Cia30</td>
<td>82 ± 15 (aa)</td>
<td>D15Mit10 (7.5 Mb)</td>
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<tr>
<td>AUC (days 53–92)</td>
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<tr>
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<tr>
<td>Incidence CIA</td>
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<td>79% (aa)</td>
<td>D15Mit182 (42.1 Mb)</td>
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<td></td>
<td>Cia30</td>
<td>44% (aa)</td>
<td>D15Mit11 (6.5 Mb)</td>
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<tr>
<td></td>
<td></td>
<td>44% (bb)</td>
<td>D15Mit111*D15Mit182</td>
<td>&lt;0.0001</td>
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<tr>
<td>AUC (days 18–35)</td>
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<td>Day of onset</td>
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<td>AUC (days 18–92)</td>
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<td>Cia22</td>
<td>51 ± 13 (aa)</td>
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<td>51 ± 13 (bb)</td>
<td>D15Mit111*D3Mit103</td>
<td>&lt;0.0001</td>
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</table>

AUCORT (days 18–92) in bold. Cia5 and Cia22 are located within the Eae3/Cia5 locus on chromosome 3.4

Phenotype value is the mean ± SE of the phenotype values for the respective genotypes.

The Mb positions are according to the Celerag mouse genome database.

The p value is calculated with the ANOVA statistical method.

Mean AUC days 18–92 in the different groups.

The genotype for the indicated QTL. aa = homozygous RIIIS/J alleles; ab = heterozygous alleles; bb = homozygous B10.RIII alleles.

Denotes an interaction between the indicated genetic markers calculated with two-way ANOVA statistical method.

Mean day of disease onset in the different groups.

Female specific.

Expression of CD86 on stimulated macrophages is linked to Eae2

It has been recently shown that subphenotypes, such as relative numbers of splenic lymphocyte populations, are genetically linked to disease loci (15, 24). To compare the macrophage compartment between B10.RIII and RIIIS/J wt mice, we investigated macrophage expression of cell surface molecules ex vivo or upon stimulation in vitro. We did not observe a difference in the expression of MHC class I, MHC class II, or CD80 on peritoneal macrophages from B10.RIII and RIIIS/J/wt mice (data not shown). Similarly, the expression of CD86 ex vivo or after activation with IFN-γ and LPS for 24 h revealed the same expression levels in the two strains (data not shown). However, after 48 h of activation in vitro, macrophages from B10.RIII mice continued to have high expression of CD86, whereas the expression on RIIIS/J macrophages was at levels comparable to nonstimulated cells (Fig. 6A). The difference in CD86 levels after activation was investigated in animals from a B10.RIII × RIIIS/J/F1 × B10.RIII backcross. A complete genome scan showed that this trait was significantly linked to chromosome 17 in male mice with a peak on the marker.
D17Mit20 (by Mann-Whitney U test, $p = 0.0001$; LRS, 14.3; LRS significance level, 12.6). The Eae2 region, peaking on D15Mit21 (in this study denoted Cia31) on chromosome 15 (by Mann-Whitney test, $p = 0.0017$; LRS, 10.9; LRS significance level, 12.1), was suggestively linked to this trait. To confirm the linkage to chromosome 15, we investigated the expression of CD86 on stimulated macrophages from Eae2 congeneric and B10.RIII wt mice. The results showed a difference in CD86 expression level between mice with one or two RIIIS/J alleles in the Eae2 region compared with mice homozygous for B10.RIII genes (Fig. 6B) and confirmed that there is one or more genes in this region that control this trait. However, in the Eae2 congeneric mice with one or two RIIIS/J alleles, the expression of the CD86 molecule was higher compared with the B10.RIII mice (Fig. 6B). This is in contrast to what was

FIGURE 3. Splitting up the Eae2 congeneric fragment reveals an intrachromosomal gene interaction that controls the development of severe CIA in the acute phase (AUC d53–92). A, The distal part of the Eae2 congeneric region, Cia26, harbors disease-promoting genes inherited from the resistant RIIIS/J strain (aa; ○). The interaction plot shows that to unravel the disease-promoting effect from RIIIS/J homozygous alleles in Cia26, the Cia30 locus should be homozygous for B10.RIII (bb; x-axis). B, CIA disease curve in mice homozygous for B10.RIII (bb; x-axis). C, The color diagram, generated by R/qtl software, visualizes the complex intrachromosomal interactions among the different QTL on chromosome 15. In addition to the Cia30 and Cia26 interaction, an interaction between Cia31 and Cia26 was found. Cia30 interacts with a broad fragment, including Cia32. The experimental significance thresholds for the two-locus interactions were determined using permutation tests ($n = 1000$), where $p < 0.05$ was considered significant. The color scale represents the LODjoint scores, where LODjoint $= 6.54$ is $p = 0.05$. The cM positions (genetic distance) shown in this figure are calculated on the genetic material in the cross and are dependent on recombinations. This means that the positions of the markers in this figure cannot be compared with the Mb positions from the physical map shown in Fig. 4.
observed in the parental RIIIS/J strain (Fig. 6A) and in the backcross animals, where macrophages from the heterozygous mice had lower expression of the CD86 molecule (data not shown).

Discussion

From this and other studies one can conclude that the centromeric part of mouse chromosome 15 harbors several genes that control the development of inflammatory disease in different experimental models, such as CIA (Cia26 and Cia30–32) (in the present study), EAE (Eae2) (13, 15), progression of autoimmune arthritis in MRL mice (Pam1) (33), Borrelia burgdorferi-associated arthritis (Bbap14) (34), and Theiler’s murine encephalomyelitis virus-induced demyelination (Tmevd8) (35). Moreover, the homologous region on human chromosome 5 has been associated with MS susceptibility (36). To date, >20 Cia loci have been reported, including the four novel loci defined in the present study. Similarly, >25 Eae loci have been described. Approximately half the QTL for EAE overlap with Cia loci or with loci linked to disease in other experimental models of arthritis (http://informatics.jax.org). It has been discussed that this would reflect that the same pathways are operating in different autoimmune diseases. Another point of view is that genes potentially involved in the development of autoimmunity are clustered in the genome, and this would explain linkages to the same genetic regions in different diseases (1, 37).

The two original QTL discussed in this paper, Eae2 and Eae3, were defined in a genetic linkage analysis on data from an EAE experiment with mice from an F2 intercross between the B10.RII and RIIIS/J mouse strains (13). Subsequently, Eae3 was also linked to the development of CIA in a cross between the two strains, and was denoted Cia5 (12). CIA development was not linked to the Eae2 region in the F2 intercross, but as demonstrated in the present study, CIA development is controlled by genes within the Eae2 loci in the context of an Eae2 congenic mouse strain. In addition, we have demonstrated that the original Eae2 fragment harbors genes that counteract each other. The fact that Eae2 was not linked to CIA development in the Jirholt et al. (12) cross could be explained by a low number of animals that did not allow the necessary recombinations needed to obtain the genetic context for these genes to operate in a disease-promoting way.
In the CIA experiments performed in this study, we observed an inhibitory effect on disease development from homozygous RiIS/J genes in the uppermost QTL of the novel Cia loci on chromosome 15, Cia30. However, the incidence and severity could be restored to the wt levels in the presence of one or two B10.RIII alleles. Moreover, the promotion of disease in mice with one or two B10.RIII alleles in Cia30 was only observed when genes in Cia5 were B10.RIII homozygous. The presence of one or two RiIS/J alleles in Cia5 promoted protection that was stronger than the disease-inducing genes in Cia30. This could be found with the finding in the EAE experiment on the original F2 intercross, in which none of the mice with homozygous RiISJ alleles in Eae2 developed disease (13). Interestingly, the protection from CIA in mice with homozygous RiIS/J alleles in Cia30 and Cia5 was accompanied by a high CD4+/CD8− T cell ratio in peripheral blood. From studies of a backcross between B10.RIII and RiIS/J, we recently reported genetic linkage of the CD4+/CD8− T cell ratio in spleen to chromosomes 2, 6, and 15 (Eae2) (15). There was no linkage of the CD4+ /CD8− T cell ratio to the Eae3/Cia5 region; this could be explained by the fact that in the backcross there were no RiIS/J homozygous alleles. In the backcross, however, the Eae3/Cia5 locus was linked to the relative numbers of CD4+ and CD8− T cells, which now has been confirmed in the PAI animals as being confined to the same genetic marker (D3Mit103).

By splitting the Eae2 locus in the context of the PAI, an intrachromosomal interaction between Cia30 and Cia26 was revealed. As mentioned above, homozygous RiIS/J alleles in the centromeric Cia30 locus suppressed disease development and overcame the disease-promoting genes in Cia26. However, in mice with B10.RIII homozygous genes in Cia30 and RiIS/J homozygous genes in Cia26, the incidence and severity of CIA were very high. Statistical analyses suggested an interaction between the two loci to promote disease. A similar effect was reported in studies of the genetic contribution to susceptibility to autoimmune ovarian dysgenesis (38). By using recombinant congenic lines, it could be concluded that different alleles on the same chromosome influenced disease development in opposite directions. The clustering of genes within an original QTL, that in different ways contribute to disease development, has been shown in models for systemic lupus erythematosus and type I diabetes (28–30). This demonstrates that complex diseases are controlled by multiple genetic interactions that can only be dissected out by breeding recombinant congenic lines or advanced intercrosses with congenic lines.

The genetic linkage results from an EAE experiment on an F2 intercross between B10.RIII and RiIS/J indicated an interactive effect of homozygous B10.RIII alleles on Eae2, and heterozygous alleles on Eae2 (13). Studies of Eae2 congenic mice could, however, not confirm that genes within this locus control EAE development (14). In the present study we observed that females with a heterozygous congenic Eae2 fragment had a higher incidence and severity of CIA compared with heterozygous males as well as with female B10.RIII wt mice. In the PAI, we reproduced the disease severity phenotype in females and located the genetic linkage to the Eae2 sub-QTL Cia32. Furthermore, we demonstrated that B10.RIII homozygous alleles on chromosome 3 indeed interacted with heterozygous alleles in Cia32. Thus, we have confirmed the confidence interval for the disease-exacerbating gene within the Eae2 region on chromosome 15 from ~40 Mbp down to 7 Mbp.

The Cia31 locus in the heterozygous state promoted significantly greater severity in the early phase of disease along with early onset of disease. Statistical calculations showed that this trait was also the result of a true interaction between Cia31 and a locus on chromosome 3 (D15Mit21*D3Mit187), thus confirming the Sundvall data (13), in addition to supporting the hypothesis that common genes control different inflammatory disorders. Interestingly, the ank locus, encoding for a trans-membrane protein that controls pyrophosphate levels in cells, is located in the Cia31 region. Mutations in the ank gene result in unregulated calcification, leading to inflammation and arthritis (39).

During the initiation of EAE, CD4+ T cells infiltrate the CNS accompanied by macrophages. It was recently demonstrated that the invading macrophages express MHC class II and B7 molecules, a prerequisite to function as APCs (40). The role for the CD86 molecule in the development of EAE, however, has revealed somewhat contradictory results (41–43). In mice from a backcross between B10.RIII and RiIS/J, we investigated the expression of the costimulatory molecule CD86 on stimulated macrophages. A complete genome screen, followed by linkage analysis showed linkage to chromosome 17 in male mice and to the Cia31 region on chromosome 15 in all mice. In line with the RiIS/J wt mice, the backcross mice heterozygous in the Cia31 region down-regulated CD86 on 48-h stimulated macrophages, indicating that the genes were dominantly inherited. In Eae2 congenic mice, it was confirmed that genes in this locus control the expression of CD86. The inheritance was, however, opposite to the backcross and wt data. It is likely that the different inheritance is due to genetic interactions.
within Eae2, in line with the complex genetic interactions controlling CIA that were observed in this study. It can be speculated that the Ciaa20-Ciaa26 genetic interactions or the effect of Ciaa1 also control this trait, but this has to be further investigated in mice with smaller congenic fragments.

In this study we have elucidated a complex genetic interplay between disease loci on two different chromosomes as well as between loci closely located within one chromosome. It can be assumed that additional loci on other chromosomes will also contribute to the disease trait through interactions with the disease loci on chromosome 15. However, by combining genetic regions on chromosome 15 and 3 to obtain the strongest phenotype, it will be possible to further limit the QTL and eventually come closer to the responsible genes.

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