Interleukin 22 is a candidate gene for Tmevp3, a locus controlling Theiler’s virus induced neurological diseases

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After intracerebral inoculation, Theiler’s virus induces in its natural host, the mouse, an acute encephalomyelitis followed, in susceptible animals, by chronic inflammation and primary demyelination. Susceptibility to demyelination among strains of laboratory mice is explained by the capacity of the immune system to control viral load during persistence. Also, differences of susceptibility to viral load between the susceptible SJL strain and the resistant B10.S strain are mainly due to two loci, named Tmevp2 and Tmevp3, located close to the Ifng locus on chromosome 10. In this article, we show that the Tmevp3 locus controls both mortality during the acute encephalomyelitis and viral load during persistence. Most probably, two genes located in the Tmevp3 interval control these two different phenotypes with efficiencies that depend on the age of the mouse at inoculation. Il22, a member of the IL-10 cytokine family, is a candidate gene for the control of mortality during the acute encephalomyelitis.
INTRODUCTION

Theiler’s virus is a picornavirus whose natural host is the mouse. After intracerebral inoculation, the DA strain of Theiler’s virus induces an acute encephalomyelitis in all strains of laboratory mice. The virus is cleared from the grey matter of the brain and the spinal cord after two weeks regardless of the mouse genotype. Some mice suffer sequelae such as flaccid paralysis of hind legs. In susceptible mice, the virus persists in the white matter of the spinal cord for the lifetime of the animal inducing chronic inflammation and primary demyelination (BRAHIC et al. 2005; LIPTON et al. 2005). Spastic paralysis is common. Lesions are very similar to those of the human disease multiple sclerosis. During persistence there is a great variability of viral load, measured as the quantity of viral genomes present in the spinal cord, among strains of laboratory mice (BUREAU et al. 1992). A major locus controlling viral load, the H2-D class I gene, has been characterized on chromosome 17 (AUBAGNAC et al. 2001; AZOULAY et al. 1994; AZOULAY-CAYLA et al. 2000; MENDEZ-FERNANDEZ et al. 2005). However during persistence, the SJL strain is infected at a higher level than the B10.S strain, although both bear the same H2^a haplotype. Two non-H2 loci controlling viral load, named Tmevp2 and Tmevp3, have been located on chromosome 10 close to the Ifng locus by studying an (SJL/J x B10.S)F1 x B10.S backcross and SJL lines congenic for different B10.S genetic intervals of chromosome 10 (BIHL et al. 1999; BUREAU et al. 1993). The B10.S allele of the Tmevp3 locus confers resistance to viral load. The Tmevp3 locus has been finely mapped. The smallest B10.S Tmevp3 interval among those of the different SJL congenic strains is borne by the SJL.Tmevp3^{B10S} line and is about 4.1 megabases long. A cluster of cytokines with at least three genes Il22, Ifng and Tmevpg1 is located in the most telomeric part of this interval just telomeric to the Mdm1 gene. This gene, which codes for a putative transcription factor, is amplified in 3T3 transformed cell line (SNYDER et al. 1988). The Il22 gene, which is a member of the IL-10 cytokine family, is expressed predominantly by memory CD4^+ Th17 lymphocytes and is involved with innate immunity (CHUNG et al. 2006; LIANG et al. 2006; NAGEM et al. 2006; ZHENG et al. 2007). The Ifng gene has well known antiviral and immunoregulatory activities (HARARI et al. 2006; NOVELLI and CASANOVA 2004; VAN DEN BROEK et al. 1995). The Tmevpg1 gene codes for a non-coding mRNA expressed in non-stimulated CD4^+, CD8^+ and NK cells (VIGNEAU et al. 2003).

Surprisingly, study of bone marrow chimeras between SJL.Tmevp3^{B10S} congenic mice and the parental strains revealed that 13-14-week-old SJL.Tmevp3^{B10S} mice are infected at a higher level than SJL mice (AUBAGNAC et al. 2002), in contrast to the infection of three to four-week-old mice in which the SJL.Tmevp3^{B10S} mice are infected at a lower level than the SJL mice. These opposite phenotypes for mice inoculated at different ages suggest that the effect of the Tmevp3 locus is due to the action of two genes: one responsible for the susceptibility of three to four-week-old mice, when
it bears the SJL allele, and the other one responsible for the susceptibility of 13-14-week-old mice, when it bears the B10.S allele. The study of bone marrow chimeras showed that the second gene is expressed in radio-resistant cells. However, in spite of the high number of backcrosses used to develop the SJL.Tmevp3B10S congenic strain, we cannot formally rule out that another B10.S interval was introgressed into its genome or that an intragenic crossing over occurred. The analysis of a congenic strain developed independently of the SJL.Tmevp3B10S strain, if it confirmed that phenotype(s) segregate differently depending on the age of the mouse at the time of inoculation, would exclude this possibility.

In the present work, we have developed a new congenic line, named B10S.Tmevp3SJL, with a B10.S background and a Tmevp3SJL interval. Using the two parental strains and the two congenic lines, B10S.Tmevp3SJL and SJL.Tmevp3B10S, the goals of the present study were to reduce the interval of the Tmevp3 locus, to define which phenotypes are controlled by the Tmevp3 locus, and the number of genes affecting these phenotypes, lastly, to find candidate gene(s).
MATERIALS AND METHODS

**DNA and genotyping.** Eleven segments of genomic DNA from 14 mouse strains, C57BL/6J, FVB/NCrI, SWR/OLaHsd, 129S2/SvPas, DBA/2J, BALBc/ByJ, C3H/HeJc, DBA/1JPas, STF/Pas, NZB/OLaHsd, MAJ/Pas, MBT/Pas, SJL/JOrlCrI, and B10.S-H2S/SgMcdJ, have been PCR amplified using 9700 GeneAmp PCR System (Applied Biosystems), cloned in TOPO vector (invitrogen, Cergy Pontoise, France) and sequenced. These fragments have been amplified from the 8 exons of *Tmevpg1*, intron 1 of *Ifng*, intron 11 of *Mdm1*, intergenic region between *Mdm1* and *Rap1b*, intron 8 of *Mdm2*, concatenated in a 3.9kb sequence that has been used to construct of phylogenetic tree (Li 2003; Schmidt et al. 2002). Four polymorphic microsatellites were discovered in the sequence alignment of the *Il22* gene and its pseudogene (ID number: AJ294727, and AJ294728 respectively). Duplication of this region has been studied in the 14 mouse strains using these four microsatellites and confirmed by amplifying, cloning and sequencing a region covering the 603bp deletion found in the promoter and the first exon of the pseudogene. In all cases of duplications, two bands were amplified for the four microsatellites and the 603bp deletion was recovered. In all the other cases, one band was amplified without recovering the deletion.

**Mice and viral infection.** SJL mice were purchased from Charles River (L’Arbresle, France). SJL.*Tmevp3* and B10S.*Tmevp3* congenic mice and B10.S-H2S/SgMcdJ mice were bred at the Pasteur Institute animal facility. The SJL.*Tmevp3* congenic strain was named SJL.*B10.D10Mit180-D10Mit74* in a previous paper (BiHL et al. 1999). B10S.*Tmevp3* congenic mice are a new congenic strain stabilized after 10 backcrosses towards the B10.S strain and selection of the progeny with the *D10Mit68*, *D10Mit233*, *D10Mit180*, *D10Mit237* and *D10Mit271* markers. Its exact name is B10.S.SJL.*D10Mit233-D10Mit271*. The DA strain of Theiler’s virus was produced by transfection of BHK-21 cells with the pTM762 plasmid as described elsewhere (McAllister et al. 1989; Michiels et al. 1997). Anesthetized mice were inoculated intracerebrally with 10^6 PFU of the DA strain in 40 µl of phosphate-buffered saline. Mice were sacrificed at seven, 21 and 45 days post-inoculation (p.i.).

**Bone marrow reconstitution.** Three-to-four-week-old mice were irradiated with a 137Cs source at 9.5 Gy. Mice were reconstituted with syngeneic or allogeneic bone marrow cells that had been harvested from the tibia and femurs of age- and sex-matched mice. For the reconstitution, we used 2.0 X 10^6 to 4.0 X 10^6 bone marrow cells from the donor strain. Reconstituted and 12-14-week-old control mice were anesthetized and inoculated intracerebrally as described above. The efficiency of the reconstitution was assessed with peripheral blood lymphocytes at the time of sacrifice for each mouse reconstituted with allogeneic bone marrow cells using PCR with *D10Mit180* marker and or FACScan and anti-Ly-9.1 antibodies as previously described (Aubagnac et al. 2002). The degree of
chimerism varied from 75 to 100%. The mean of chimerism is higher than 94% in each of the 6 groups of mice. We also analyzed complete blood counts and the degree of chimerism on CD3⁺, CD4⁺, CD8⁺, B220⁺ cells with FACScan for reconstituted mice at 10 weeks post-reconstitution and control mice of the same age. No difference was detected for all these parameters between these two groups of mice (Table 1S).

**RT-PCR assays.** Anesthetized mice were perfused through the left ventricle with phosphate-buffered saline. The spinal cord was dissected out and stored at –80°C. Total RNAs were extracted using TRI-REAGENT (Molecular Research Center, Cincinnati, OH) and reverse-transcribed as previously described (Vigneau et al. 2003). Hprt amplification was used to normalize the efficiency of the reverse transcription. Forward and reverse primers were chosen in different exons of the studied gene. Both PCR products of the cDNA to quantify, and of the Hprt cDNA were cloned in bluescript vector. The insert containing both PCR products was digested from the vector using appropriate restriction enzymes, eluted, quantified by spectrophotometry and 10-fold diluted from 10⁷ to 10² molecules per µl. This dilution was used to construct a standard curve in each Taqman RT-PCR assay. This curve allowed the determination of the number of copies of the gene to quantify and that of the Hprt gene in each sample. Square root of the ratio of the two quantities was used in the different Figures of that paper. Real-time PCR was performed using qPCR MasterMix (Eurogentec, Seraing, Belgium) according to the manufacturer conditions on a 7000 Sequence Detection System (Applied Biosystems). The Hprt primers were Forward CTGGTGAAAGACCTCCTCG and Reverse TGAAGTACTCTTATAGTCAAGGGCA and probe Yakima Yellow-TGTGGATACACAGCCAGACTTTTGGGAT-DDQ1. The TMEV primers were Forward GCCGCTCTCTCACCCCAT and Reverse AGCAGGGCAGAAAGCATCAC and the probe 6FAM-CGACGTGGTTGGAGAT-DDQ1. The Il22 primers were Forward AGAAGGCTGAAAGGACAGT Reverse GACATAAACAGCAGGCATCCAGT and the probe 6FAM-AAAAGCTTGGAGAGAGTGGA-DDQ1. The probe specific for the B10.S allele was 6FAM-CAATCGCCTTGGATCTC-DDQ1 and that for SJL allele is Yakima Yellow-CCAATCGCCTTGGATCTC-DDQ1. Concentration of the primers and the probe were chosen to optimize the amplification and the detection of each PCR product. Each assay was validated only if the slope of the standard curve is between 3.32 and 3.8. Allelic discrimination of Il22 gene used the Allelic discrimination technology develops by Applied Biosystems.

**Statistical analysis.** The Statview Version 4.5 package was used for all the analysis. Each comparison of mRNA expression among the four groups of mice was performed using non-parametric test of Kruskal Wallis. If this first test was significant, groups were merged according to the allele at the Tmevp3 locus or the origin of the background and distributions were compared using
the non-parametric test of Mann Whitney. Interactions between the Tmeyp3 locus and the background were tested by comparing the distributions of the parental strains and that of the congenic strains. Mortality between two groups of mice was compared using the exact Fisher Test. Survival data was analyzed using the non-parametric Kaplan-Meier model with stratification. Differences were considered significant if the probability $P < 0.05$ (*). Two higher level of significance was also used $P < 0.01$ (**) and $P < 0.001$ (***) . Non-significant $P$ values lower than 0.1 were also reported.
RESULTS

Refining the localization of the Tmevp3 locus

The smallest Tmevp3\textsuperscript{B10S} interval among those of the different SJL congenic strain is borne by the SJL.Tmevp3\textsuperscript{B10S} strain and is about 4.1 megabases long (Figure 1). The crossing over defining the telomeric border of this interval has been located at less than 2kb from the D10Mit74 marker (Chr10: 117,957,802-117,958,312. UCSC Mouse Feb 2006). The centromeric border is located between the D10Mit233 marker (Chr10: 113,785,306-435), the last marker with an SJL genotype, and the D10Mit180 marker (Chr10: 117,553,604-737), the first marker with a B10.S genotype. They are separated by 3.7 megabases in a region poor in polymorphisms between the two parental strains. An extensive search of polymorphic microsatellites and Single Nucleotide Polymorphisms (SNP) in this region was negative (Table 1). The first marker with a B10.S genotype (Chr10: 117,496,216-117,496,364. UCSC Mouse Feb 2006) most probably defines the centromeric border of the Tmevp3 interval. Interestingly, this region of \(\approx400\) kb long is rich in polymorphisms between the two parental strains: a polymorphism is found every 100-200bp. This result suggests that this \(\approx400\) kb region is most likely to contain the Tmevp3 locus.

To confirm this hypothesis, the sequence of 11 DNA segments covering the \(\approx400\) kb region was compared between 14 inbred mouse strains. Eleven were laboratory mouse strains of known susceptibility to Theiler's virus persistent infection, including the B10.S and the SJL strains and three were wild derived strains (MAI, MBT, STF). A phylogenetic tree of the region was constructed using the TREE-PUZZLE program (Figure 2). The 14 inbred strains are divided in three groups: The first one contains only the STF, a strain from the \textit{spretus} subspecies; the second one contains 8 strains of laboratory mice and the B10.S strain; the third one contains two wild derived strains from the \textit{Mus musculus musculus} sub-species, MAI and MBT, and two strains of laboratory mice, the SJL and the NZB strains. Therefore, the SJL and NZB strains are the two strains of laboratory mice that received the entire \(\approx400\) kb region from a mouse of the \textit{Mus musculus musculus} sub-species. The nine other strains of laboratory mice received this region from a mouse of the \textit{Mus musculus domesticus} sub-species (data not shown). There is a good correlation between the haplotype of this \(\approx400\) kb region and the susceptibility to persistent infection by Theiler's virus. The SJL strain is the only strain of laboratory mouse among the 11 studied in which susceptibility to viral load is not predicted by the H2 haplotype suggesting that it is the only strain of laboratory mice bearing a susceptible allele at the Tmevp3 locus. The only exception to the correlation between this prediction and the haplotype at the \(\approx400\) kb region is the NZB strain that bears the same Tmevp3 haplotype as the SJL strain but for which susceptibility to the persistent infection is predicted by its H2 haplotype. These results confirmed that the Tmevp3 locus is located within this \(\approx400\) kb region.
Genetic characteristics of the congenic strains

A new congenic strain, B10S.Tmevp3SJL, was constructed for the present work. The strain was stabilized after 10 backcrosses towards the B10.S parent and selection of the offspring with the D10Mit68, D10Mit233, D10Mit180, D10Mit237 and D10Mit271 markers. Figure 1 shows the genetic map of the telomeric region of chromosome 10 for the two parental strains, the B10S.Tmevp3SJL strain and the previously described SJL.Tmevp3B10S strain. Four hundred and thirty three polymorphic SNPs from the MMDAP panels (MORAN et al. 2006), and 61 polymorphic microsatellites were used to verify that the interval of each congenic strain on chromosome 10 was the only one that had been introgressed during backcrossing. No other introgressed interval than that selected for was detected in the genome of the two congenic strains.

Susceptibility to persistent infection of mice inoculated at three to four-weeks of age

Three to four week old B10.S, SJL, B10S.Tmevp3SJL, and SJL.Tmevp3B10S mice were inoculated intracerebrally with 10^4 pfu of Theiler’s virus. The mice were sacrificed 45 days later and viral load in the spinal cord was measured using the Taqman real-time RT-PCR assay. Figure 3 shows the result of the experiment. A significant difference of viral load is detected among the four groups of mice (P < 0.01). The viral load depends on the allele of the Tmevp3 locus (P < 0.001) and not on the background (P: NS) or on an interaction between the two (P: NS). Mice with the Tmevp3SJL allele were infected at a higher level than mice with the Tmevp3B10S allele.

Susceptibility of three to four-week-old mice to acute encephalomyelitis.

When three to four week old B10.S, SJL, B10S.Tmevp3SJL, and SJL.Tmevp3B10S mice were inoculated intracerebrally with 10^5 pfu of Theiler’s virus (see previous section), some mortality from acute encephalomyelitis was observed in mice with a B10.S background (18 out 125) whereas no mortality occurred in mice with an SJL background (0 out of 104). The difference is highly significant (P < 0.001). Interestingly, mortality was significantly higher in male B10.S mice (9 out of 30) than female B10.S mice (1 out of 39; P < 0.01). In contrast, there was no difference of mortality between male and female B10.Tmevp3SJL mice (4 out of 26 for males versus 4 out of 30 for females). These results show a clear effect of the B10.S versus SJL background on mortality from acute encephalomyelitis. They suggest that, on the B10.S background, the Tmevp3 locus, which was identified because of its effect on viral load during persistent infection, also controls the effect of sex on mortality.
Susceptibility of bone marrow chimeras

Figure 3 confirms previously published results (BIHL et al. 1999) showing that SJL \( Tmevp3^{B10S} \) mice are less susceptible to the persistent infection than SJL mice when both strains are inoculated at three to four weeks of age. In contrast we showed in another publication that SJL \( Tmevp3^{B10S} \) mice are more susceptible to persistent infection than the SJL parents when the mice are inoculated at 13-14-weeks of age (AUBAGNAC et al. 2002). Study of bone marrow chimeras showed that this difference of viral load depends on the \( Tmevp3 \) allele of recipient strains with an SJL background. These opposite phenotypes strongly suggest that at least two genes control this phenotype, and that at least one of them is located in the \( Tmevp3 \) interval. Therefore, we decided to inoculate 13-14-week-old B10.S and B10S\( Tmevp3^{SJL} \) mice and bone marrow chimeras to look for a different segregation of mortality during the acute encephalomyelitis and/or viral load during persistence. Since in our previous experiments, the \( Tmevp3 \) allele of the recipient conferred the phenotype, this new study used the B10.S and B10S\( Tmevp3^{SJL} \) mice as bone marrow recipients.

Three to four week old B10.S and B10S\( Tmevp3^{SJL} \) mice were irradiated and reconstituted with bone marrow from B10.S, B10S\( Tmevp3^{SJL} \), SJL\( Tmevp3^{B10S} \), or SJL mice. Ten weeks later the mice were inoculated with \( 10^4 \) pfu of Theiler’s virus and observed for 45 days. Some mortality from early encephalomyelitis was expected, and observed, since the mice had a B10.S genetic background. Surprisingly, more mortality was observed in B10S\( Tmevp3^{SJL} \) than in B10.S recipient mice (Figure 4). The level of mortality depended also on the nature of the bone marrow donor strain. B10S\( Tmevp3^{SJL} \) mice reconstituted with bone marrow from the B10.S and SJL strains showed the highest mortality. All surviving mice were sacrificed at 45 days p.i. Survival data (Figure 4) was analyzed according to the non-parametric Kaplan-Meier model. Even when using a stratification strategy to take into account the effect of the donor strain, the effect of the recipient strain on mortality is highly significant (Logrank test = 7.985 [1ddl] \( P = 0.0047 \)). The reconstitution itself did not significantly affect mortality (Table 2). In conclusion, these experiments showed that, on a B10.S background, the \( Tmevp3^{SJL} \) allele increases mortality from early encephalomyelitis in bone marrow chimeras. The \( Tmevp3 \) locus must be expressed in radio-resistant cells since the effect was seen in B10S\( Tmevp3^{SJL} \) recipients.

Viral load in the spinal cord of bone marrow recipients was measured 45 days p.i. using a Taqman real-time RT-PCR assay (data not shown). Because up to 50% mortality was observed between 10 and 21 days p.i. in some groups and not in others, as described above, and because mortality might be linked to viral load, the results were difficult to interpret. No major effect of the \( Tmevp3 \) locus on viral load in survivors was observed. B10S\( Tmevp3^{SJL} \) recipient mice were infected at a higher level than B10.S recipient mice but the difference was not statistically significant (\( P = \)
0.0546). The effect of the donor strain on viral load was also not significant ($P = 0.0902$). These results contrast with those obtained with mice inoculated at three to four weeks of age in which mortality was lower, about 15% in both B10.S and B10S.$Tmep3^{SJL}$ mice, and the difference of viral load was larger and statistically significant.

In conclusion, the study of mortality from early encephalomyelitis and of viral load during persistent infection showed that the $Tmep3$ locus influences both phenotypes. The difference of segregation in the four mouse strains depending both on age and on the phenotype studied (Table 3), proved that more than one gene control these phenotypes and that at least one of them is located in the $Tmep3$ interval. In the simplest model, two genes are located in the $Tmep3$ interval. One affects mortality from early encephalomyelitis in mice inoculated at 13-14 weeks of age. The other one affects viral load during persistent infection in mice inoculated at three to four weeks of age. The $SJL$ allele confers susceptibility over the $B10.S$ allele in both cases. Previous experiments have suggested that the $Tmepg1$ gene is a candidate gene to control viral load during persistence of mice inoculated at three to four weeks of age (Vigneau et al. 2003). Finding a candidate gene for the control of mortality during the acute encephalomyelitis in the $\approx 400$kb region would substantiate this model.

**$Il22$ is a candidate gene for the control of mortality during acute encephalomyelitis**

$Il22$ is one of the four genes present in the $Tmep3$ interval. Because the IL22 cytokine is involved with innate immunity, the $Il22$ gene is an interesting candidate for the control of mortality during the acute encephalomyelitis. A comparison of the cDNA sequences of the $Il22^{SJL}$ and $Il22^{B10.S}$ alleles showed only one non-synonymous mutation at position three of the protein (I3V), three synonymous mutations, and four polymorphisms in the 3’non-coding sequence (Table 4). None of the 3’NC polymorphisms is located in the ATTTA rich region conserved in mammals. Three polymorphisms were also found in the 340bp long promoter region of the gene suggesting that its expression could be regulated differently in SJL and B10.S mice. It was not possible to study other regulatory elements due to a genomic duplication containing the $Il22$ gene. This duplication was detected in seven strains of laboratory mice, B10.S, FVB, SWR, C57BL/6, 129/Sv, C3H, DBA/1, using four microsatellites. One of the two $Il22$ copies is most probably a pseudogene since all these seven strains have a 603bp deletion of its putative promoter and its first exon. No duplication was detected in the seven other mouse strains including the SJL strain.

The levels of $Il22$ expression in the spinal cord depends on viral load. His level is high only in a small fraction of mice with a low viral load (Figure 1S). Therefore, to compare the levels of $Il22$ expression in mice with different $Il22$ alleles, it was necessary that each parental strain and its
congenic carried similar viral load (i.e. that there was no effect of the Tmevp3 locus on viral load between the four strains of mice). This condition was met at 21 days p.i. (Figure 5A). At this time, differences of viral load between B10.S, SJL, B10S.Tmevp3SJL, and SJLTmevp3B10S mice do not depend on the Tmevp3 locus (P: NS) but only on the genetic background (P < 0.001). Mice with an SJL background were infected at higher level than mice with a B10.S background. The level of expression of the II22 gene was determined in the spinal cord of these mice using TaqmanR real-time RT-PCR assay. Figure 5B shows the results. The level of II22 expression is significantly different among the four strains (P < 0.001). It depends on the allele at the Tmevp3 locus (P < 0.001) and not on the genetic background (P: NS) or on an interaction between these two factors (P: NS). The II22SJL allele is expressed at higher level than the II22B10S allele. Interestingly, the difference of II22 expression is not limited to the central nervous system as similar results were obtained when comparing the level of II22 expression in resting CD4+ T splenocytes (data not shown).

To confirm these results, the level of II22 expression was measured in the spinal cord and the thymus of (B10S.Tmevp3SJL x B10.S) F1 mice 21 days p.i. using Taqman probes specific for each allele and labeled with different fluorophores. Figure 6A shows the level of expression of the II22SJL and the II22B10S alleles measured at the same time, and in the same sample. Whereas in thymus both alleles were expressed at the same level, the SJL allele was expressed at higher level than the B10.S allele in the infected spinal cords.

According to the results of the bone marrow chimera experiments described above, the candidate gene must be expressed in radio-resistant cells of the spinal cord. Interestingly, it was reported that the II22 gene is expressed in uninfected mouse brain (DUMOUTIER et al. 2000a). We confirmed this finding for the spinal cord (Figure 2S). To test if, in bone marrow chimera, the II22 gene was expressed or not by radio resistant cells, the expression of both alleles was measured in the spinal cord of bone marrow chimeras 45 days p.i. Figure 6B shows that the II22SJL allele was expressed at higher level in the spinal cord of B10S.Tmevp3SJL recipients of B10.S bone marrow than in the spinal cord of B10.S recipients of B10S.Tmevp3SJL bone marrow. The converse was true for the II22B10S allele. Therefore, the II22 allele expressed in the spinal cord of infected bone marrow chimera was predominantly that of the recipient.

In conclusion, the results reported in this section are congruent with the hypothesis that II22 is the gene of the Tmevp3 interval, which is responsible for mortality during early encephalomyelitis.

DISCUSSION

For the present work, we developed a new congenic line, B10S.Tmevp3SJL, which bears the SJL allele of the Tmevp3 locus on a B10.S background. Extensive screening with SNPs did not detect
any other SJL interval, besides \textit{Tmevp3}, in the genome of this strain. The converse is true for the SJL.\textit{Tmevp3}^{\text{B10S}} strain. Therefore, the phenotypes of the SJL.\textit{Tmevp3}^{\text{B10S}} and B10S.\textit{Tmevp3}^{\text{SJL}} strains must result from genes located respectively in the \textit{D10Mit233-D10Mit74} and \textit{D10Mit73-D10Mit151} intervals. For the SJL.\textit{Tmevp3}^{\text{B10S}} strain, an analysis of polymorphisms between parental strains allowed us to reduce the \textit{Tmevp3} interval to approximately 400kb (Figure 1; http://genome.ucsc.edu/cgi-bin/hgGateway). This region contains on average one polymorphism every 200bp. The exact size of the region is difficult to determine because it contains a duplication, which has not been sequenced yet, in most strains of laboratory mice (DUMOUTIER et al. 2000b)(http://genome.ucsc.edu/cgi-bin/hgGateway). However, we cannot rule out that a recent mutation in the centromeric region to the \approx 400kb \textit{Tmevp3} interval is responsible for the \textit{Tmevp3} phenotype, although this seems very unlikely. A cluster of cytokines, which covers the most important part of the \approx 400kb region, contains two genes of cytokine, \textit{Ifng} and \textit{Il22}, and \textit{Tmevpg1}, a gene coding for a non-coding mRNA. The three genes are candidate for the \textit{Tmevp3} locus. However, we cannot rule out that the gene which controls mortality during acute encephalomyelitis in mice of B10.S background inoculated at 13-14-weeks of age is located outside the \approx 400 kb region at another location of the \textit{D10Mit73-D10Mit151} interval (see Figure 1). Even if it was the case, the gene controlling viral load at 45 days p.i. in 13-14-week-old mice of SJL background would be still located within the \approx 400kb region.

Our work shows that the \textit{Tmevp3} locus affects two distinct phenotypes related to Theiler’s virus pathogenesis: mortality during early encephalomyelitis and viral load during persistent infection. The effect of the \textit{Tmevp3} locus on mortality during the acute encephalomyelitis was not reported previously because the SJL.\textit{Tmevp3}^{\text{B10S}} congenic mice bears an SJL background which does not confer susceptibility to the early disease. This complex phenotype (Table 3) is due to the action of more than one gene, one of them being located in the \textit{Tmevp3} interval. The simplest model to explain our data is to locate two genes in the \textit{Tmevp3} interval. The first gene controls viral load at 45 days p.i. in mice inoculated at three to four weeks of age. Its resistant B10.S allele not only decreases viral load during the persistent infection but also increases the difference of mortality between males and females in mice with a B10.S background. We showed previously that the \textit{Tmevpg1} gene which codes for a non-coding mRNA is a candidate gene to control viral load of mice inoculated at three to four weeks of age (VIGNEAU et al. 2003). \textit{Tmevpg1} could control the expression of the \textit{Ifng} gene whose inactivation increases susceptibility to viral load during persistence (FIETTE et al. 1995; PULLEN et al. 1994; RODRIGUEZ et al. 1995). Interestingly, the activity of the \textit{Ifng} pathway increases with age (ADKINS et al. 2004; HÄRTEL et al. 2005; NEWPORT et al. 2004; SACK et al. 1998). This could explain that the phenotypes we described depend on age. The second gene controls mortality
and viral load during the persistent infection of 13-14 week old mice. Its SJL allele decreases viral load at 45 days p.i. in mice with an SJL background and increases mortality during the acute encephalomyelitis in mice with a B10.S background.

Data from the literature and the study of the \textit{Il22} gene are in agreement with this model. \textit{Il22} is a good candidate gene for the control of mortality during acute encephalomyelitis in mice inoculated at 13-14-weeks of age, since the \textit{Il22}^{SJL} allele is expressed at higher level than the \textit{Il22}^{B10.S} allele in the CNS of these mice and mortality is higher in mice with a \textit{Tmevp3}^{SJL} allele than in mice with a \textit{Tmevp3}^{B10.S} allele. Interestingly, strains whose immune responses control viral load after the acute encephalomyelitis, such as the C57BL/6 and C57BL/10 strains, are also strains which show neurological symptoms and mortality during the early encephalomyelitis. Also, it has been shown that viral clearance from the gray matter during the acute encephalomyelitis is immune mediated (DRESCHER et al. 1999; NJENGA et al. 1997). This suggests that the cost of an efficient immune response, which controls the infection, might be clinical disease and sometimes death. IL22 induces the expression of genes involved with the innate immunity (WOLK et al. 2004). Therefore, it is conceivable that an increased expression of IL22 induces the efficient immune response responsible for the phenotype of mice inoculated at 13-14 weeks of age. However, we cannot rule out a role of the non–synonymous polymorphism (I3V) present in the gene. In this model, the \textit{Il22}^{B10.S} allele is responsible for an increase of viral load in mice with an SJL background and inoculated at 13-14 weeks of age. This late effect for a gene involved in innate immunity would be probably the consequence of an event that occurred during the early encephalomyelitis. The second gene, which controls viral load during persistence in mice inoculated at three to four weeks of age shows the same relationship between susceptibility to acute encephalomyelitis and resistance to viral load during persistence but in this case, it is the B10.S allele which is associated with an efficient immune response. Interestingly, the two parental strains received the $\approx$400kb region from two allopatric sub-species, \textit{Mus musculus domesticus} and \textit{Mus musculus musculus}, that were separated 700-800 thousand years ago (GUÉNET and BONHOMME 2003). Since these two genes in the \textit{Ifng} cluster of cytokines might have been differently selected in these two sub-species, admixture of that cluster in strains of laboratory mice would be responsible for the appearance of this complex phenotype. Search for signature of either positive or balancing selection in candidate genes might help to characterize the causal polymorphism.

In conclusion, we postulate that two genes in the \textit{Tmevp3} interval are responsible for the phenotypes that we studied. Their efficiency depends on the age of the mouse at the time of inoculation. For both genes, the allele inducing an efficient immune response increases mortality during the acute encephalomyelitis and decreases viral load during persistence. But for one of them,
the SJL allele is efficient and for the second one, it is the B10.S allele. These two genes affect predominantly one of the two phenotypes, susceptibility to mortality during the acute encephalomyelitis or to viral load during persistence. This level of dissection of complex traits can be performed only in animal models at present. Characterizing such loci at the molecular level in mouse models will help to develop strategy that can be applied to susceptibility to infections of humans. The frequent organization of immune genes in clusters suggests that such a level of complexity may be common in human diseases. Il22, a member of the IL-10 family, could be an interesting candidate gene for several infectious diseases of mouse and humans (MISSE et al. 2007; ZHENG et al. 2007).

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TABLES
TABLE 1: Microsatellite and SNP diversity between the B10.S and SJL strains

<table>
<thead>
<tr>
<th>Region</th>
<th>Size (kb)</th>
<th>changes</th>
<th>percent</th>
<th>differences (nt)</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10Mit233-D10Mit180</td>
<td>3700</td>
<td>1/21a</td>
<td>5</td>
<td>1/4300</td>
<td>0.02</td>
</tr>
<tr>
<td>D10Mit180-D10Mit74</td>
<td>400</td>
<td>19/28</td>
<td>68</td>
<td>68/6000</td>
<td>1.13</td>
</tr>
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</table>

* This microsatellite defines the centromeric border of the *Tmevp3* interval
TABLE 2: Mortality during early disease of bone marrow chimeras and control mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mortality at 21 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.S</td>
<td>1/14</td>
</tr>
<tr>
<td>B10S.\textit{Tmevp}^3\textit{SJL}</td>
<td>1/14</td>
</tr>
<tr>
<td>SJL</td>
<td>0/12</td>
</tr>
<tr>
<td>B10.S -&gt; B10.S\textsuperscript{a}</td>
<td>2/14</td>
</tr>
<tr>
<td>B10S.\textit{Tmevp}^3\textit{SJL} -&gt; B10S.\textit{Tmevp}^3\textit{SJL}</td>
<td>1/12</td>
</tr>
</tbody>
</table>

\textsuperscript{a} donor->recipient
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age at inoculation</th>
<th>Recipient strain</th>
<th>Age at inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>three to four weeks of age</td>
<td>mortality</td>
<td>viral load</td>
</tr>
<tr>
<td>B10.S</td>
<td>14% with a sex effect</td>
<td>low</td>
<td>B10.S</td>
</tr>
<tr>
<td>B10S.Tmevp3SJL</td>
<td>14% without sex effect</td>
<td>high</td>
<td>B10S.Tmevp3SJL</td>
</tr>
<tr>
<td>SJL.Tmevp3B10S</td>
<td>0%</td>
<td>low</td>
<td>SJL.Tmevp3B10S</td>
</tr>
<tr>
<td>SJL</td>
<td>0%</td>
<td>high</td>
<td>SJL</td>
</tr>
</tbody>
</table>

Mortality during the acute encephalomyelitis. Viral load was measured at 45 days p.i.

<sup>a</sup> Results from S. Aubagnac et al. (2002).
### TABLE 4: Polymorphisms in the *Il22* gene

<table>
<thead>
<tr>
<th>Position in the <em>Il22</em> gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B10.S</th>
<th>SJL</th>
</tr>
</thead>
<tbody>
<tr>
<td>-62</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>-46</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>-19</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>159</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>193</td>
<td>A</td>
<td>G</td>
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<tr>
<td>266</td>
<td>T</td>
<td>C</td>
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<tr>
<td>539</td>
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<tr>
<td>603</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>693</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>697</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>975</td>
<td>T</td>
<td>C</td>
</tr>
</tbody>
</table>

<sup>a</sup> Identification number of the *Il22* reference sequence: NM_016971
**FIGURE LEGENDS**

**Figure 1**: Genotype of the B10S.*Tmevp3SJL* and SJL.*Tmevp3B10S* strains on chromosome 10. Microsatellite markers are listed on the left. Distances are arbitrary and do not reflect physical distances. Black square: B10.S genome interval. White square: SJL genome interval. Gray square: interval in which the crossing over occurs.

**Figure 2**: Phylogenetic tree of 14 inbred strain of mouse. From each 11 strains of laboratory mice and three wild derived strains, MAI, MBT, and STF, sequences from 11 DNA segments covering the \( \approx 400\text{kb} \) region were concatenated in a 3.9kb long sequence. These concatenated sequences were aligned according to CLUSTALW program. A phylogenetic tree was constructed using the TREE-PUZZLE program with the HKY model of substitution. Parameters were estimated from the data. The tree was drawn by the DRAWTREE program. Italics: strains of laboratory mice in which viral load is predicted by the \( H2 \) haplotype. Underlined: laboratory mouse strain in which viral load is not predicted by the \( H2 \) haplotype. Bold type: wild-derived strains.

**Figure 3**: Viral load in the spinal cords of the four groups of mice inoculated at three to four weeks of age, at 45 days post-inoculation. SQRT: Square root. Rectangle: 90% interval of viral load around median. Error bar: 95% interval around median. Viral load among the four groups of mice is significantly different (\( P < 0.01 \)). Effect of the *Tmevp3* locus, the background and the interaction between both factors is shown below the figure. Significant differences between two groups of mice are also shown inside the figure. \( P \): NS, non significant difference; * : \( P < 0.05 \); **: \( P < 0.01 \); ***: \( P < 0.001 \). \( n = \) number of mice.

**Figure 4**: Mortality during the acute encephalomyelitis of bone marrow chimeras. Cumulative survival curve for B10S.*Tmevp3SJL* recipient strain (A) and B10.S recipient strain (B) are obtained using the Kaplan Meier model stratified according to the donor strain (Circle: B10.S; Square: B10S.*Tmevp3SJL*; Triangle: SJL.*Tmevp3B10S*; Diamond: SJL). \( n = \) number of mice. The strain before the arrow is the donor strain and that after the recipient strain.

**Figure 5**: Viral load (A) and expression of *Il22* gene (B) in the four groups of mice at 21 days p.i. SQRT: Square root. Rectangle: 90% interval of viral load around median. Error bar: 95% interval around median. Viral load is not significantly different between each parental strain and the congenic strain of the same background. Expression of *Il22* gene among the four groups of mice is significantly different (\( P < 0.01 \)). Effect of the *Tmevp3* locus, the background and the interaction...
between both factors on Il22 expression is shown below the figure. Significant differences of Il22 expression between two groups of mice are also shown inside the figure. P: NS, non significant difference; *: P < 0.05; **: P < 0.01; ***: P < 0.001. n = number of mice.

**Figure 6**: Allelic expression of Il22 gene in (B10S.Tmevp3SJL x B10.S) F1 animals at 21 days p.i. (A) and in bone marrow chimeras at 45 days p.i. (B). For each sample, fluorescence (in arbitrary unit) of the SJL allele (x-axis) and that of the B10.S allele (y axis) are measured using allelic specific probe after 40 cycles of amplification by Taqman RT-PCR. The strain before the arrow is the donor strain and that after the recipient strain. Samples of spinal cord at 21 or 45 days p.i. from B10.S and SJL mice with very different level of Il22 expression were chosen as positive controls. Each sample of a B10.S mouse was matched with one of an SJL one according to their level of Il22 expression. In all positive control, the PCR amplification reached a plateau and the specificity of detection of each Il22 allele was always verified.

**Figure 1S**: Relationship between the expression of the Il22 gene and the viral load in the spinal cord at 21 days p.i. (A) and 45 days p.i. (B) for mice of the four strains. Expression of the Il22 gene and the viral load were normalized using the expression of Hprt as standard in a Taqman RT-PCR assay. Each circle corresponds to an individual mouse.

**Figure 2S**: Expression of the Il22 gene in the four non-infected strains of mice. Legend is similar to that of Figure 3. SQRT: Square root. Rectangle: 90% interval of Il22 expression around median. Error bar: 95% interval around median.
SQRT(copies of TMEV/copies of HPRT)

n = 21  n = 23  n = 17  n = 26

Tmevp3 locus  Background  Interaction

P < 0.001  P : NS  P : NS

Mice

B10.S  B10S.Tmevp3SJL  SJL  SJL.Tmevp3B10S

*  *  *  **

Tmevp3 locus  Background  Interaction

P < 0.001  P : NS  P : NS
Cumulative survival curve

- B10.S → B10.S.Tmevp3SJL
  n = 25; Censored data n = 12
- B10.S.Tmevp3SJL → B10.S.Tmevp3SJL
  n = 12; Censored data n = 11
- SJL.Tmevp3B10S → B10.S.Tmevp3SJL
  n = 26; Censored data n = 21
- SJL → B10.S.Tmevp3SJL
  n = 20; Censored data n = 13

Cumulative survival curve

- B10.S → B10.S
  n = 14; Censored data n = 12
- B10.S.Tmevp3SJL → B10.S
  n = 26; Censored data n = 22
- SJL.Tmevp3B10S → B10.S
  n = 29; Censored data n = 28
- SJL → B10.S
  n = 21; Censored data n = 18