Verification of genetic loci responsible for the resistance/susceptibility to the Sendai virus infection using congenic mice

コンジェニックマウスを用いたセンダイウイルス感染抵抗性／感受性遺伝子座の証明

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Abbreviations

B6	C57BL/6
BALF	Broncho-alveolar lavage fluid
Chr	Chromosome
cM	Centimorgan
d.p.i.	Days post infection
D2	DBA/2
EDTA	Ethylenediaminetetraacetic acid
IFN	Interferon
IL	Interleukin
Mb	Mega base pairs
min	Minutes
MMDBJ	Mouse Microsatellite Marker Database of Japan
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SD	Standard deviation

SE	Standard error
SeV	Sendai virus
SPF	Specific pathogen-free
$TCID_{50}$	50% tissue culture infection dose
TNF-α	Tumor necrosis factor-alpha

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1. Introduction

Sendai virus (SeV) is the prototype of paramyxovirus, known as the murine parainfluenza virus type 1. Moreover, SeV is the counterpart of human parainfluenza virus type 1 (HPIV1) causing the respiratory infection in children. SeV is an enveloped virus belonging to the *Paramyxoviridae* family and *Respirovirus* genus with a negative sense single-stranded 15-16 kb genomic RNA (15, 21, 11).

Heterogeneity of inbred mouse strains regarding the resistance or susceptibility to SeV infection was reported in several previous studies (6, 8, 9, 14). Resistant mouse strains seem to suppress the initial viral replication than susceptible strains. Moreover, resistant strains abolish the infection by confining infected regions primarily to the airways. On the other hand, the susceptible strains allow the infection to spread from the airway into the lung parenchyma with severe pathological outcomes. Moreover, following the respiratory tract infection, SeV proliferates extensively in the lungs reaching its peak titers on the fourth to fifth day post infection (d.p.i.) (3). Subsequently, in resistance phenotypes, virus titers decline rapidly after the 10 to 11 d.p.i. and no longer recovered (3, 4). Moreover, it was indicated that regardless of the phenotype either resistant or susceptible, the degree of SeV replication in lungs of both mouse phenotypes was similar (8).

The immune traits are known to be a complex trait controlled by polygenes with different mechanisms. The use of inbred mouse strains to investigate the resistant and susceptible phenotypes as well as the different responses against various pathogens provides a useful tool to determine the genetic basis of these different phenotypes. It is valuable to examine the crosses, backcrosses, and recombinant inbred strains to map the responsible gene(s) for these phenotypes (5).

Recently, the genetic mapping using the DBA/2 (D2) and C57BL/6 (B6) as a representative for susceptible and resistant strains, respectively, was carried out (17). One major quantitative trait locus (QTL) with highly significant linkage was mapped to the distal portion of chromosome (Chr) 4 (*Sen1*) as well as two other QTLs showing statistically suggestive linkage on Chrs 8 and 14 (*Sen3*). In addition, a highly significant epistatic interaction was found between a locus on Chr 3 (*Sen2*) and Chr 14 (*Sen3*). Moreover, it was found that D2 mice exhibited a dysregulated hyper-inflammatory cytokine/chemokine cascades, so-called "cytokine storm" as the cause for the susceptibility (18).

Therefore, the current study was conducted to verify that the three previously identified QTLs were solely responsible for resistance or susceptibility to SeV infection by producing congenic mice, in which B6 alleles of each *Sen1*, *Sen2*, and *Sen3* were introgressed into D2-genetic background and *vice versa*. Further, to examine the epistatic interaction between *Sen2* and *Sen3*, both *Sen2* and *Sen3* were introgressed from counterparts into B6- or D2-genetic background. Furthermore, triple-congenic line carrying *Sen1*, *Sen2*, and *Sen3* was generated with each genetic background. Then, these congenic mice were subjected to the SeV infection experiments. In addition, the immune responses to SeV infection in D2-triple congenic mice were analyzed and compared to that of D2 and B6 mice to understand more the function of these three QTLs.

2. Materials and methods

2.1. Mice

Eight-week-old specific pathogen-free (SPF) B6 and D2 mice were purchased from SLC Japan (Hamamatsu, Japan). Mice from both strains as well as all generated congenic lines were bred under SPF conditions and infection experiment was conducted in the bio-safety level 3. Animal use protocol was approved by President of Hokkaido University after reviewing by the Institutional Animal Care and Use Committee, Hokkaido University (approved protocol No. 11-0057, 11-0117, and 14-0158). Laboratory animal care and use program was conducted to adhere the AAALAC International-approved program in the Graduate School of Veterinary Medicine, Hokkaido University.

2.1.1. Single-congenic mice

To produce single-congenic mice, in which B6 alleles of each *Sen1*, *Sen2*, or *Sen3* was introgressed into D2-genetic background and *vice versa*. Female (B6 x D2)F_1 mice were backcrossed to male parental strain to produce backcrosses, (B6 x D2)F_1 x B6 or (B6 x D2)F_1 x D2. The backcrossing was repeated for 10 generations to produce heterozygous B6- or D2-congenic line. These heterozygous mice were intercrossed to get homozygous B6 and D2 single-congenic lines.

2.1.2. Double-congenic mice

To produce double-congenic mice, both *Sen2* and *Sen3* were introgressed from B6 into D2-genetic background and *vice versa*. In the production of B6-congenic mice, B6.D2-*Sen2* and B6.D2-*Sen3* were mated to produce B6.D2-*Sen2Sen3* heterozygous mice. These heterozygous mice were intercrossed to get B6.D2-*Sen2Sen3* homozygous mice. To get B6.D2-*Sen2Sen3* homozygous mice, the intercrossing was repeated for 7 generations. The same method was used to generate double-congenic mice in the D2-genetic background. To get D2.B6-*Sen2Sen3* homozygous mice, the intercrossing was repeated for 9 generations.

2.1.3. Triple-congenic mice

To produce B6 triple-congenic mice carrying D2 alleles of all *Sen1*, *Sen2*, and *Sen3* QTLs, B6.D2-*Sen2Sen3* and B6.D2-*Sen1* were mated to produce heterozygous mice. Heterozygous mice were intercrossed to get B6.D2-*Sen1Sen2Sen3* homozygous mice. To get B6.D2-*Sen1Sen2Sen3* homozygous mice, the intercrossing was repeated for 9 generations. To produce D2 triple-congenic mice, D2.B6-*Sen2Sen3* and D2.B6-*Sen1* were mated to produce D2.B6-*Sen1Sen2Sen3* heterozygous mice, then, the heterozygous mice were intercrossed to get D2.B6-*Sen1Sen2Sen3* homozygous mice. To get D2.B6-*Sen1Sen2Sen3* homozygous mice, the intercrossing was repeated for 11 generations.

2.2. Genotyping

Genomic DNA was prepared from ear snips of parental strains and congenic lines. Ear snips were incubated at 54 °C for 3 h in 500 μl of lysis buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 1% sodium dodecyl sulfate (SDS)] containing 10 mg/ml proteinase K and 10 mg/ml RNase. Genomic DNA was extracted by the standard phenol chloroform extraction method, purified by ethanol precipitation in the presence of 0.3 M sodium acetate, and finally dissolved in TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)].

Informative microsatellite markers that show difference between B6 and D2 were used for genotyping (Fig. 1). The genetic map positions (cM) of markers were obtained from the Mouse Microsatellite Database of Japan (MMDBJ). Fig. 2 shows haplotypes of introgressed Chrs in 6 lines of single-congenic mice. Replacement by recipient genomes in other Chrs was all verified using microsatellite markers shown in Fig. 1.

2.3. Amplification of microsatellite markers

The touchdown PCR was performed with *Taq* DNA polymerase (Ampliqon A/S, Stenhuggervej 22, Odense M, Denmark) as follows; denaturing at 95 °C for 1 min, followed by 10 cycles of denaturing at 95 °C for 30 sec, primer annealing at 65 °C for 30 sec (-2 °C in 2 cycles), and extension at 72 °C for 30 sec, and then, 35 cycles of denaturing at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, and final extension at 72 °C for 1 min. The amplified

samples were electrophoresed in 12% polyacrylamide gels in TBE solution (89 mM Tris, 89 mM boric acid and 2 mM EDTA), and then, stained with ethidium bromide. The stained samples were visualized and photographed under ultraviolet lamp. The results of PCR amplification of representative microsatellite markers were shown in Fig. 3.

2.4. Virus and infection experiment

The MN strain of SeV stock in modified Eagle medium (Sigma, MO, USA) with 1% bovine serum albumin was inoculated into 10-day-old embryonated chicken eggs and incubated at 35 °C for 72 h. The allantoic fluid was recovered from the inoculated eggs, centrifuged at 2,500 xg for 20 min, and then the supernatant was collected and stored at -80 °C until used for infection experiment. Virus titer in the allantoic fluid was determined by hemadsorption assay using a monkey kidney cell line, LLC-MK2, and chicken red blood cells. The values of virus titer were indicated as median tissue culture infectious dose ($TCID_{50}$).

Eight-week-old mice of parental strains as well as all congenic lines were used for infection experiment. First, mice were anesthetized by inhalation with ISOFLURANE (Pfizer Japan Inc., Tokyo, Japan), followed by intra-peritoneal injection of 200 µl of anesthetic mixture [0.75 mg/kg medetomidine hydrochloride (Domitol, Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 4 mg/kg midazolam (Dormicum, Astellas Pharma Inc., Tokyo, Japan), and 5 mg/kg butorphanol (Vetorphale, Meiji Seika Pharma Co., Ltd.)] as described previously (10). Then, mice were infected with 10^3 $TCID_{50}$ SeV as described previously (17). A volume of 25 µl of the viral inoculum was slowly

injected intranasally into the left side. Mice were monitored by visual inspection twice a day to figure out the main symptoms for SeV virus infection, such as hunched posture, lethargy, anorexia, respiratory distress, and ruffled fur. Moreover, the body weight of mice was measured on a daily basis during the experimental period. When the animals showed severe symptoms with morbidity, mice were immediately euthanized with overdose of ISOFLURANE. Some mice were dead not showing severe symptoms with morbidity at the observation twice a day. Both euthanized and dead mice were counted as non-survival mice.

2.5. Analysis of the broncho-alveolar lavage fluid (BALF)

Mice were euthanized with overdose of ISOFLURANE before and after (at 8 d.p.i.) the infection. Then, the trachea was cannulated through the mouth and larynx and lungs were lavaged slowly with warm phosphate-buffered saline (PBS, pH 7.2).

2.5.1. Cytokine analysis

BALF samples were collected from all congenic lines as well as parental strains (n=3 mice in each time-point). Lungs were lavaged three times with warm PBS (pH 7.2, 500 µl/time). BALF samples were centrifuged at 1,000 ×g for 10 min, and then the supernatants were stored at -80 °C until used for cytokine analysis.

Both TNF-α and IL-6 expression in BALF samples were measured using Mouse TNF-α Singleplex Bead Kit (Invitrogen, Frederick, MD, USA) and Mouse IL-6 Singleplex Bead Kit (Invitrogen, Flynn Rd, Camarillo, CA, USA) according to the manufacturer's protocol.

2.5.2. Cytology Analysis

Freshly collected BALF samples were centrifuged at 300 ×g for 5 min at 4°C and then, supernatants were discarded. The collected BALF cells were suspended in 1.5 ml of distilled water and then, incubated for 10 sec followed by adding 500 µl of 0.6 M KCl and mixing by inverting. After that, samples were centrifuged at 300 ×g for 5 min at 4°C and then, supernatants were discarded. A volume of 500 µl of saline containing 2.6 mM EDTA was added to the cell pellets and mixed by inverting. After that, 200 µl of the BALF cell suspensions were loaded onto a Shandon^{TM} EZ Single Cytofunnel (Thermo Fisher Scientific, Cheshire, UK) and centrifuged for 10 min at 400 rpm. Finally, the slides were dried at room temperature and stained with a Diff-Quick Staining Kit according to the manufacturer's protocol.

2.6. SeV titration

A FastStart Essential DNA Green Master Mix (Roche Diagnostic GmbH, Mannheim, Germany) was used to quantify SeV replication level in the lung tissue of infected B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Briefly, total RNA was extracted from the right lung before and after

(at 8 d.p.i.) the infection using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Two μg of extracted RNA were transcribed using Rever-Tra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol and then, 200 ng of synthetized cDNA were used to titer the SeV. The primers used are as follow: SeV-F CAGAGGAGCACAGTCTCAGTGTTC and SeV-R TCTCTGAGAGTGCTGCTTATCTGTGT for SeV. Nucleotide sequence was determined based on the National Center for Biotechnology Information (NCBI) data base (accession no. M30202).

2.7. Lung histopathological analysis

The left lungs were collected from infected B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. All lungs were fixed in 10% formalin solution and embedded in paraffin blocks. Nine sections were cut with 5-μm thickness in each lung, stained with hematoxylin-eosin, and the pathological scores were evaluated under the light microscope. Five fields of view were randomly observed in each slide and evaluated their pathological scores categorized as follows; 0; intact, 1; few inflammatory cells in the interstitium, 2; mild edema surrounding blood vessels and few inflammatory cells, 3; mild edema surrounding blood vessels and moderate numbers of inflammatory cells and/or bronchial epithelial cell necrosis, 4; moderate edema surrounding blood vessels, moderate to high numbers of inflammatory cells, and bronchial epithelial cell necrosis, 5; moderate to high edema, high numbers of inflammatory cells, and bronchial epithelial cell necrosis.

2.8. Statistical analysis

Collected data were subjected to one-way analysis of variance (ANOVA) using a SPSS 16.0 software. All values were represented as means ± SD. Statistical differences were analyzed with Tukey *HSD* test. Values of $p<0.05$ and $p<0.01$ were considered to be significant.

3. Results

3.1. Survival rates of congenic lines after SeV infection

Table 1 and Fig. 4 show the survival rates of congenic mice after SeV infection. In congenic lines with B6-genetic background, introgression of D2-derived *Sen1* reduced survival rate by a limited extent, 16.0% for males and 22.2% for females, whereas introgression of D2-derived *Sen2* and *Sen3* did not change survival rate. Furthermore, double introgression of D2-derived *Sen2* and *Sen3* and triple introgression of D2-derived *Sen1*, *Sen2*, and *Sen3* did not change survival rate, either. In contrast, in congenic lines with D2-genetic background, introgression of B6-derived *Sen1* greatly increased survival rate from 0% to approximately 50%, 40% for males and 50% for females. Introgression of B6-derived *Sen2* also slightly increased survival rate, 29.4% for males and 9.1% for females, but introgression of *Sen3* did not. In D2-genetic background, epistatic interaction between *Sen2* and *Sen3* was observed in male mice, where survival rate of double-congenic male mice increased to 50%. In D2-genetic background, triple introgression of B6-derived *Sen1*, *Sen2*, and *Sen3* greatly increased survival rate. Remarkably, survival rate of male triple-congenic mice was 100% as seen in parental B6 strain. These results indicate that in D2-genetic background, B6-derived *Sen1* can alter survival rate partially by itself and combination of B6-derived three QTLs, *Sen1*, *Sen2*, and *Sen3* can alter D2 mice to be resistant to SeV infection in the same level as in B6 mice. In contrast, in B6-genetic background, D2-derived all QTLs could not exert their effect. The reason for it is

unknown; however, it might be the reason for it that there may be other dominant QTLs not detected by the previous QTL analysis in the B6 mouse.

Another interesting results in the survival rates of congenic mice were that males were more resistant than females in D2.B6-*Sen2* congenic mice. Survival rates were 29.4% and 9.1% for males and females, respectively. This difference was potentiated by the addition of *Sen3*, which showed epistatic interaction with *Sen2* in the previous QTL analysis (17). Survival rates of D2.B6-*Sen2Sen3* were 50% and 0% for males and females, respectively. Although this difference was dampened by further addition of *Sen1*, this tendency was still remained in triple-congenic, D2.B6-*Sen1Sen2Sen3*. Survival rates of D2.B6-*Sen1Sen2Sen3* were 100% and 66.7% for males and females, respectively. It is quite interesting phenomenon, because there is no difference in the susceptibility by the sex in parental strains, both B6 and D2. *Sen2* may be positively interacting with D2 male genetic background; however, this is fully speculation because it was not investigated in this study.

3.2. The BALF cytokine analysis

3.2.1. IL-6

In a previous report (18), the cellular and humoral immunity was investigated in SeV-infected B6 and D2 mice and it was concluded that susceptibility of D2 mice to the SeV infection was attributed to hyper-response of cytokines in the lung. IL-6 and TNF-α were cytokines that mostly up-regulated in

SeV-infected D2 mice compared to that of B6 mice. Therefore, in the current research the IL-6 and TNF-α levels in BALF in SeV-infected congenic mice were measured.

IL-6 level of D2 mice remarkably increased at 8 d.p.i. compared to that of B6 mice (Fig. 5A and B), which was consistent with the previous report (18). However, introgression of each B6-derived QTL did not suppress this increase. On the contrary, the introgression of B6-derived *Sen3* further increased IL-6 level remarkably, suggesting that B6-derived *Sen3* contains gene(s) up-regulating IL-6 production in the D2-genetic background. However, when all three B6-derived QTLs were introgressed into D2-genetic background, IL-6 production was suppressed to the same level of B6 mice, which is consistent with the survival data.

3.2.2. TNF-α

B6 single-congenic lines (B6.D2-*Sen1*, B6.D2-*Sen2*, and B6.D2-*Sen3*) showed a tendency to express high TNF-α in the basal level before SeV infection (0 day); however, this tendency was suppressed in both B6 double- and triple-congenic lines (B6.D2-*Sen2Sen3* and B6.D2-*Sen1Sen2Sen3*) (Fig. 6A). This up-regulation of basal level of TNF-α may be due to the genetic interaction between D2-derived genes and B6-genetic background and suppression may be due to the interaction between B6-derived QTLs in the D2-genetic background. Similarly, the D2.B6-*Sen2* and D2.B6-*Sen3* showed high level of TNF-α expression before SeV infection (0 day) in D2-congenic lines (Fig. 6A). This up-regulation was decreased by co-introgression with other QTLs in D2.B6-*Sen2Sen3* and D2.B6-*Sen1Sen2Sen3*. These results suggest that the up-regulation may be

due to the genetic interaction between B6-derived QTLs and D2-genetic background and suppression may be due to genetic interaction between D2-derived QTLs in the D2-genetic background.

At 8 d.p.i. (Fig. 6B), TNF-α levels were not statistically different among each congenic line in B6-genetic backgrounds, whereas B6-derived QTLs suppressed TNF-α levels in the D2-genetic background except for single introgression of *Sen2*. Particularly, co-introgression with B6-derived three QTLs in the D2-genetic background mostly suppressed TNF-α level, consistent with the survival data.

3.3. Body weight changes in D2 triple-congenic mice after SeV infection

Body weight changes after SeV infection were recorded on daily basis (Fig. 7). Body weight loss continued and reached death or humane endpoints in both male and female D2 mice. In contrast, both male and female B6 mice showed loss of body weights after SeV infection and then, recovered body weights after 8 d.p.i. Male D2 triple-congenic mice showed similar course of body weight changes to B6 mice. However, female D2 triple-congenic mice showed two different patterns. Approximately two third of female D2 triple-congenic mice showed similar pattern to female B6 mice (survived), whereas remaining one third mice showed similar pattern to female D2 mice (non-survived).

3.4. Virus titers of D2 triple-congenic mice after SeV infection

The virus replication load in the mouse lung was measured at 8 d.p.i. using quantitative PCR method. Fig. 8 showed the viral copy number per lung in infected mice. Both male and female D2 mice showed significantly (p<0.01) higher viral copy numbers in comparison with B6 and D2 triple-congenic mice and there was no statistical difference in viral copy numbers between B6 and D2 triple-congenic mice for both males and females, suggesting that the B6 alleles of three QTLs introgressed into D2-genetic background resulted in suppression of virus replication as seen in B6 mice.

3.5. Cytology analysis in the BALF of D2 triple-congenic mice after SeV infection

BALF cells collected from the right lungs from all mice before infection and at 8 d.p.i. were stained and observed under the light microscope (Fig. 9). The number of BALF cells in both male and female D2 mice was much higher than that of B6 mice. The number of BALF cells in male D2 triple-congenic mice was comparable to that of B6 male mice. In contrast, the number of BALF cells in female D2 triple-congenic mice showed two patterns; BALF cells in mice showing mild symptoms were comparable to that of B6 mice, whereas BALF cells in mice showing severe symptoms were comparable to that of D2 mice. Next, BALF cells were subjected to count total cells, macrophages, neutrophils, and lymphocytes (Fig. 10). Total cell counts as well as counts of macrophages,

neutrophils, and lymphocytes in infected D2 mice were all higher than those of infected B6 mice in consistence with the previous report (18). Total cell counts as well as macrophages, neutrophils, and lymphocytes in infected male D2 triple-congenic mice were statistically less than those of infected male D2 mice, indicating that introgression of B6 alleles of these three QTLs into male D2-genetic background reduced immune reaction in D2-genetic background. In contrast, total cell counts as well as neutrophils and lymphocytes except for macrophages in infected female D2 triple-congenic mice were not significantly different from those of infected female D2 mice, suggesting that introgression of B6 alleles of these three QTLs was limited to suppress immune reaction in female D2-genetic background.

3.6. Lung histopathology and pathological index score of D2-triple congenic mice

Fig. 11 shows histopathology of lungs in B6, D2, and D2 triple-congenic mice. There were no lung lesions in B6, D2, and D2 triple-congenic lungs before SeV infection. However, both male and female D2 mice showed moderate to high edema surrounding blood vessels and moderate to high number of inflammatory cells as well as bronchial epithelial cell necrosis. In contrast, both male and female B6 mice showed low to mild edema surrounding blood vessels and few inflammatory cells. It is most interesting that male D2 triple-congenic mice showed similar pathological scores to that of male B6 mice. In contrast, female D2 triple-congenic mice exhibited two different patterns; mice showing mild symptoms showed similar features to B6 mice, whereas mice showing severe

symptoms showed similar features to D2 mice. In both males and females, the pathological index scores of D2 mice were much higher than that of B6 mice, whereas that of D2 triple-congenic mice was comparable to that of B6 mice.

3.7. Correlation between body weight loss and cellular responses in female D2 triple-congenic mice

Since female D2 triple-congenic mice were categorized into survival and non-survival mice and it could be predicted by the extent of body weight loss (Fig. 7), the correlation between body weight loss and immune cellular responses at 8 d.p.i. were examined (Fig. 12). Immune cellular responses such as viral load, BALF total cells, macrophages, neutrophils, and lymphocytes, and lung pathological index were all well correlated with body weight loss in female D2 triple-congenic mice. These results indicate that the effect of B6-derived three QTLs are limiting in female D2-genetic background and suggest that hyper immune cellular responses cause body weight loss in female D2 and D2 triple-congenic mice and then leading to death in case of extremely hyper immune cellular responses.

Table 1. Survival rates (%) of congenic lines after SeV infection.

Mouse	Sex	Background of congenic lines	No. of survived / No. of infected mice	Survival rate (%)
B6	♀		**11 / 11**	**100.0 %**
B6.D2-*Sen1*	♂	**B6**	**21 / 25**	**84.0 %**
B6.D2-*Sen1*	♀	**B6**	**14 / 18**	**77.8 %**
B6.D2-*Sen2*	♂	**B6**	**13 / 13**	**100.0 %**
B6.D2-*Sen2*	♀	**B6**	**10 / 10**	**100.0 %**
B6.D2-*Sen3*	♂	**B6**	**33 / 33**	**100.0 %**
B6.D2-*Sen3*	♀	**B6**	**28 / 28**	**100.0 %**
B6.D2-*Sen2Sen3*	♂	**B6**	**20 / 20**	**100.0 %**
B6.D2-*Sen2Sen3*	♀	**B6**	**14 / 14**	**100.0 %**
B6.D2-*Sen1Sen2Sen3*	♂	**B6**	**6 / 6**	**100.0 %**
B6.D2-*Sen1Sen2Sen3*	♀	**B6**	**8 / 8**	**100.0 %**
D2	♀		**0 / 14**	**0.0 %**
D2.B6-*Sen1*	♂	**D2**	**6 / 15**	**40.0 %**
D2.B6-*Sen1*	♀	**D2**	**5 / 10**	**50.0 %**
D2.B6-*Sen2*	♂	**D2**	**5 / 17**	**29.4 %**
D2.B6-*Sen2*	♀	**D2**	**2 / 22**	**9.1 %**
D2.B6-*Sen3*	♂	**D2**	**0 / 13**	**0.0 %**
D2.B6-*Sen3*	♀	**D2**	**0 / 12**	**0.0 %**
D2.B6-*Sen2Sen3*	♂	**D2**	**4 / 8**	**50.0 %**
D2.B6-*Sen2Sen3*	♀	**D2**	**0 / 7**	**0.0 %**
D2.B6-*Sen1Sen2Sen3*	♂	**D2**	**5 / 5**	**100.0 %**
D2.B6-*Sen1Sen2Sen3*	♀	**D2**	**4 / 6**	**66.7 %**

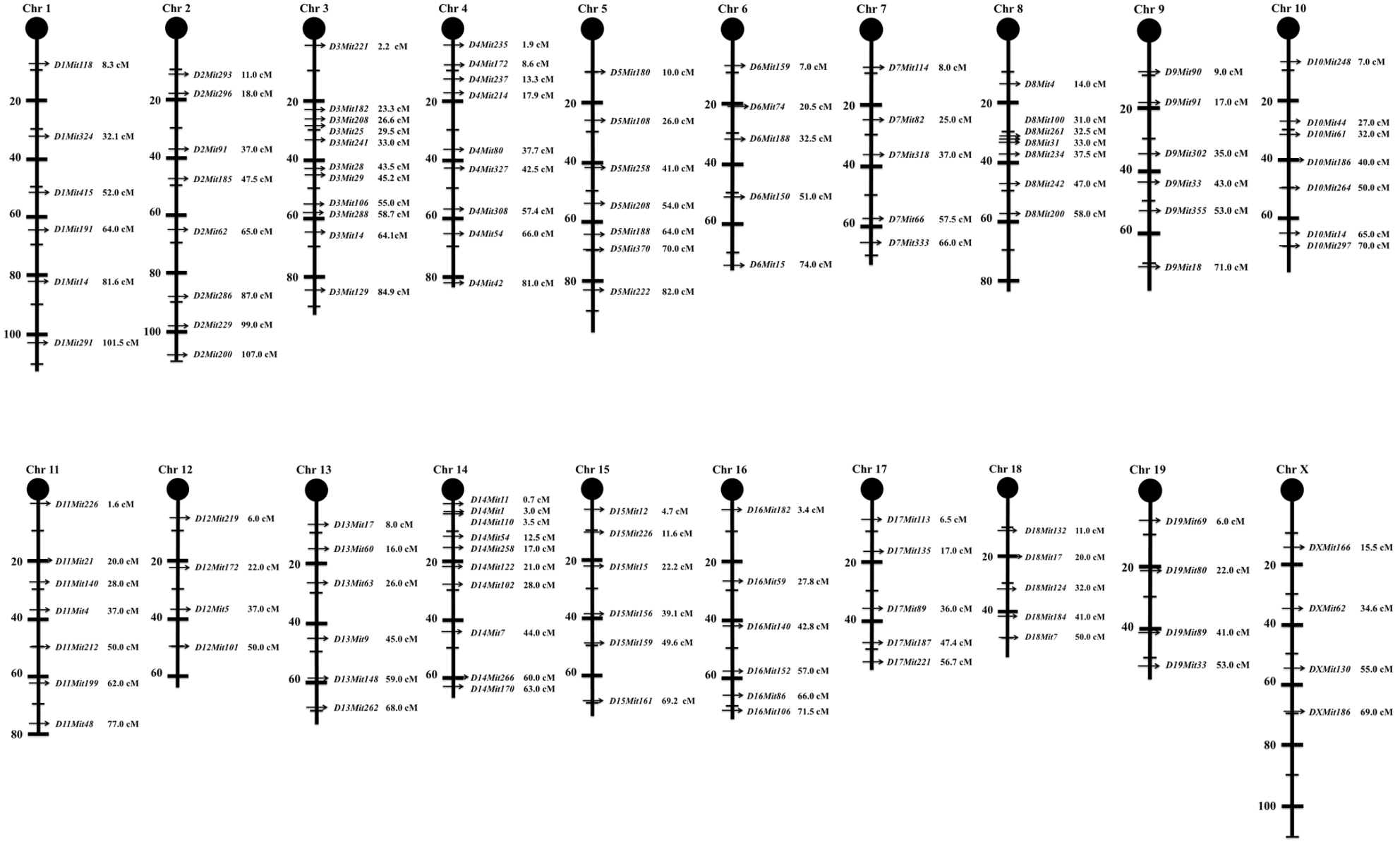


Fig. 1. Microsatellite markers used for generation of congenic mice.

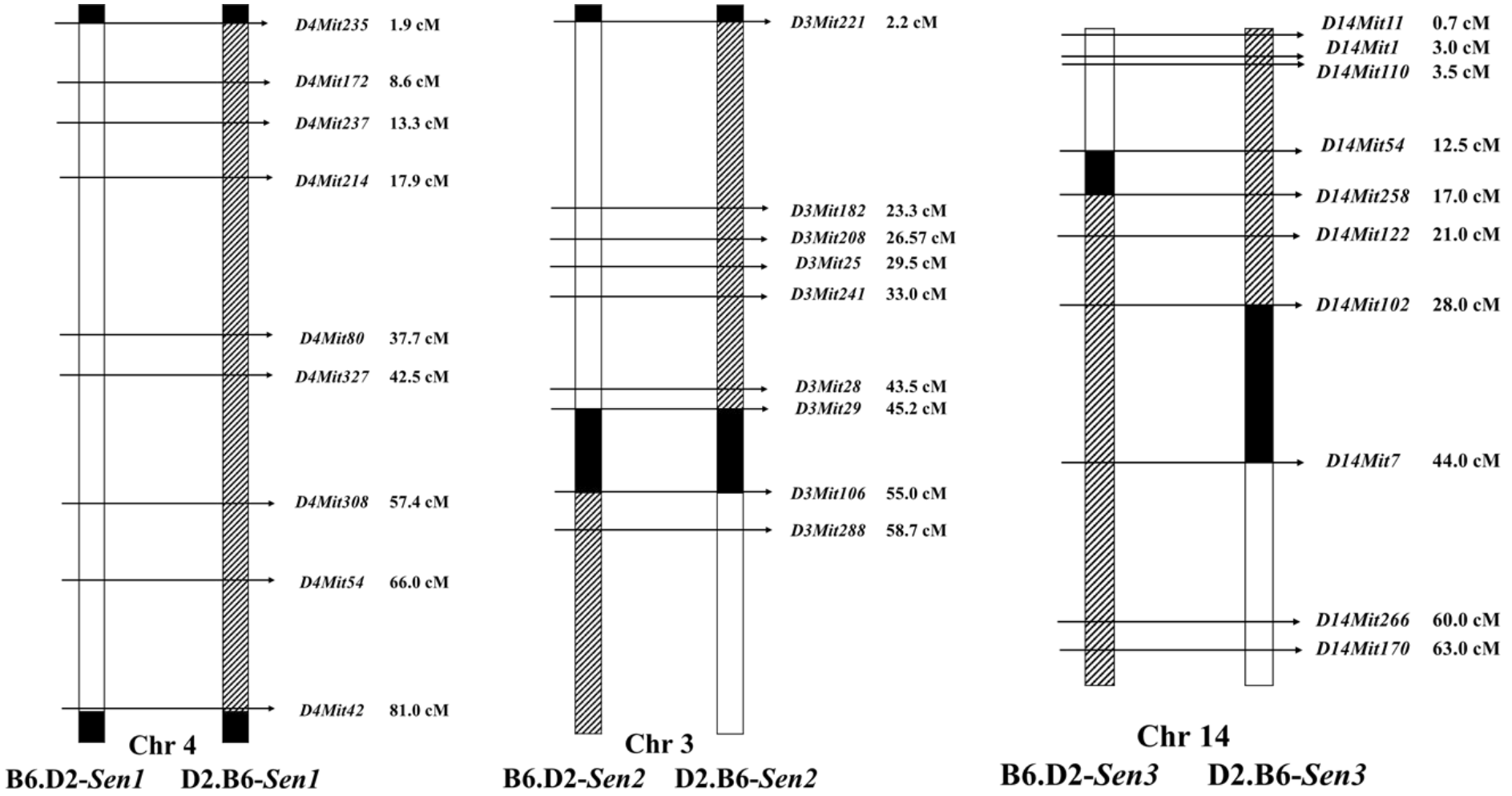


Fig. 2. Schematic illustration of Chr 4 (*Sen1*), Chr 3 (*Sen2*), and Chr 14 (*Sen3*) in congenic lines. Shaded, open, and black columns indicate B6-derived, D2-derived, and unknown chromosomal portions, respectively.

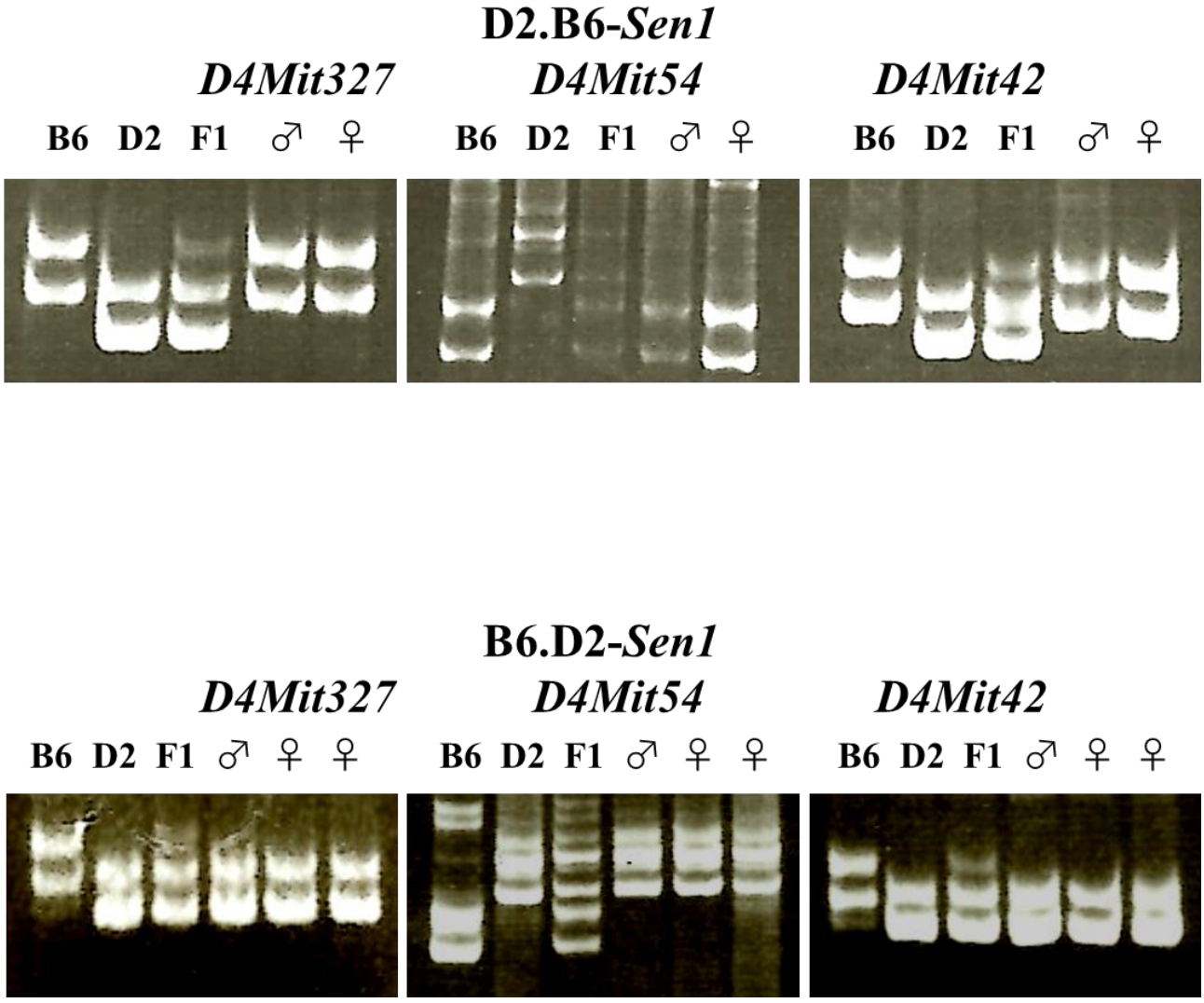
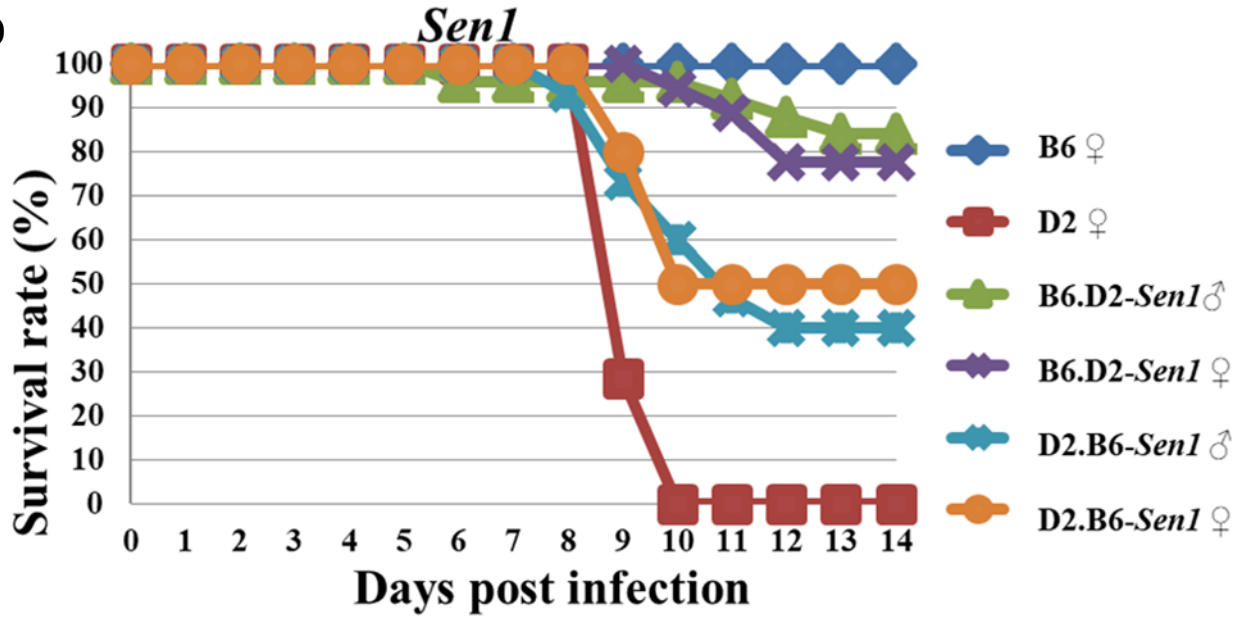
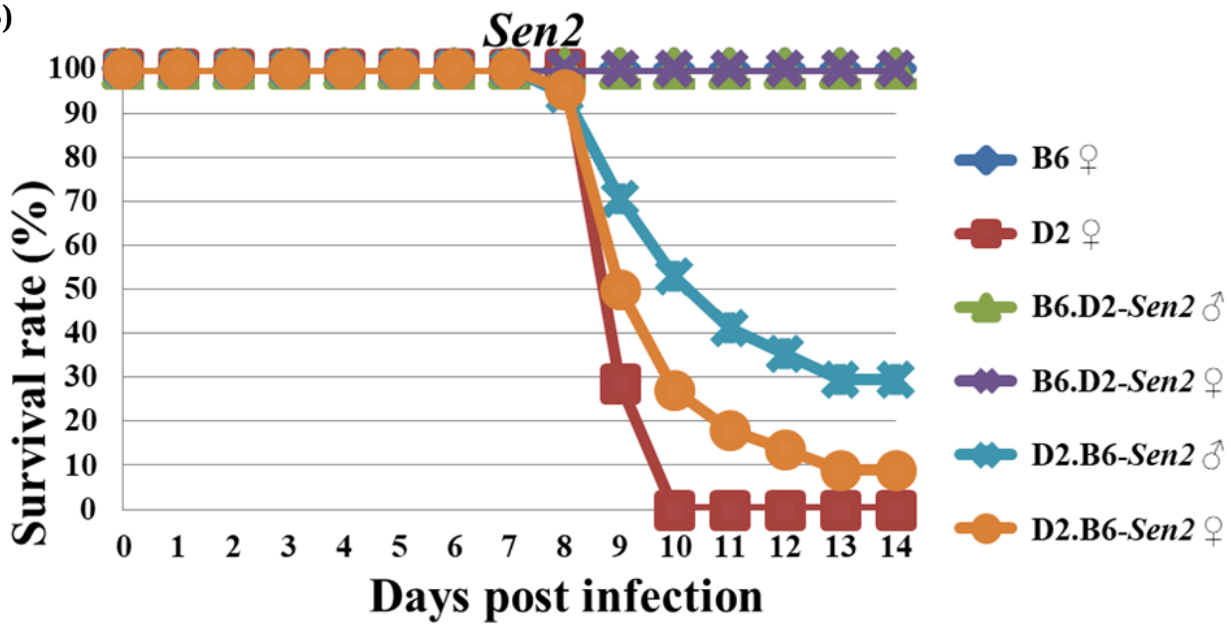


Fig. 3. The results of PCR amplification of representative microsatellite markers, *D4Mit327*, *D4Mit54*, and *D4Mit42*.

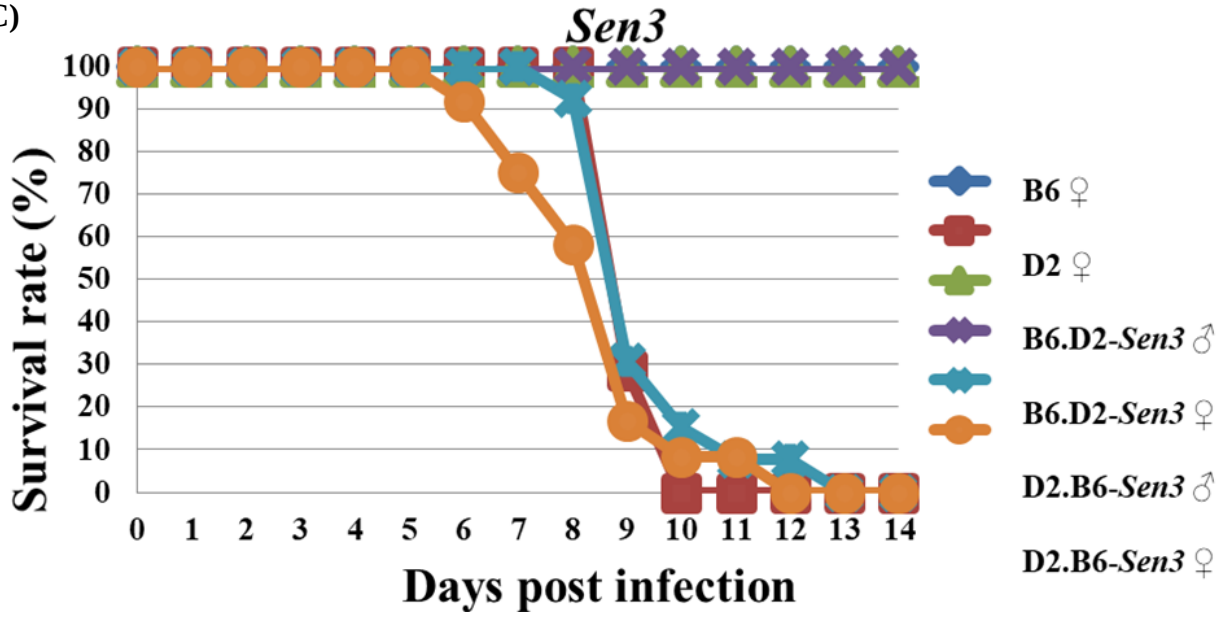
A)

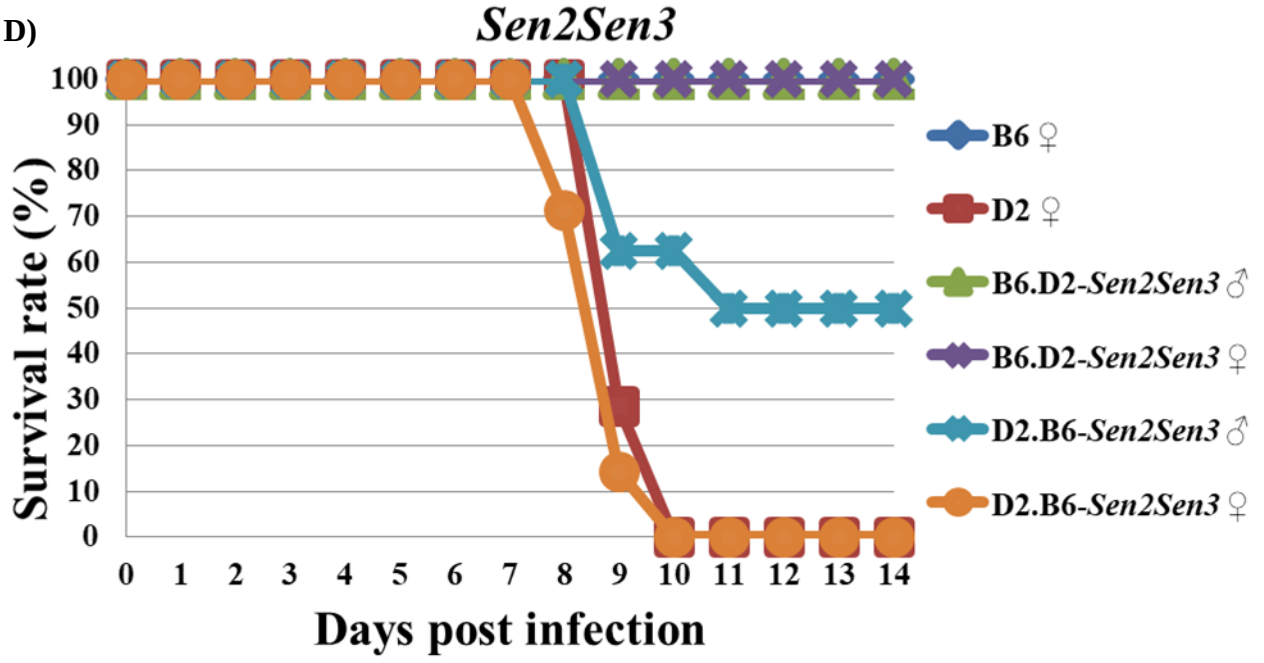

Sen1
Survival rate (%)
Days post infection
B6 ♀
D2 ♀
B6.D2-Sen1 ♂
B6.D2-Sen1 ♀
D2.B6-Sen1 ♂
D2.B6-Sen1 ♀

B)


Sen2
Survival rate (%)
Days post infection
B6 ♀
D2 ♀
B6.D2-Sen2 ♂
B6.D2-Sen2 ♀
D2.B6-Sen2 ♂
D2.B6-Sen2 ♀

C)


Sen3
Survival rate (%)
Days post infection
B6 ♀
D2 ♀
B6.D2-Sen3 ♂
B6.D2-Sen3 ♀
D2.B6-Sen3 ♂
D2.B6-Sen3 ♀



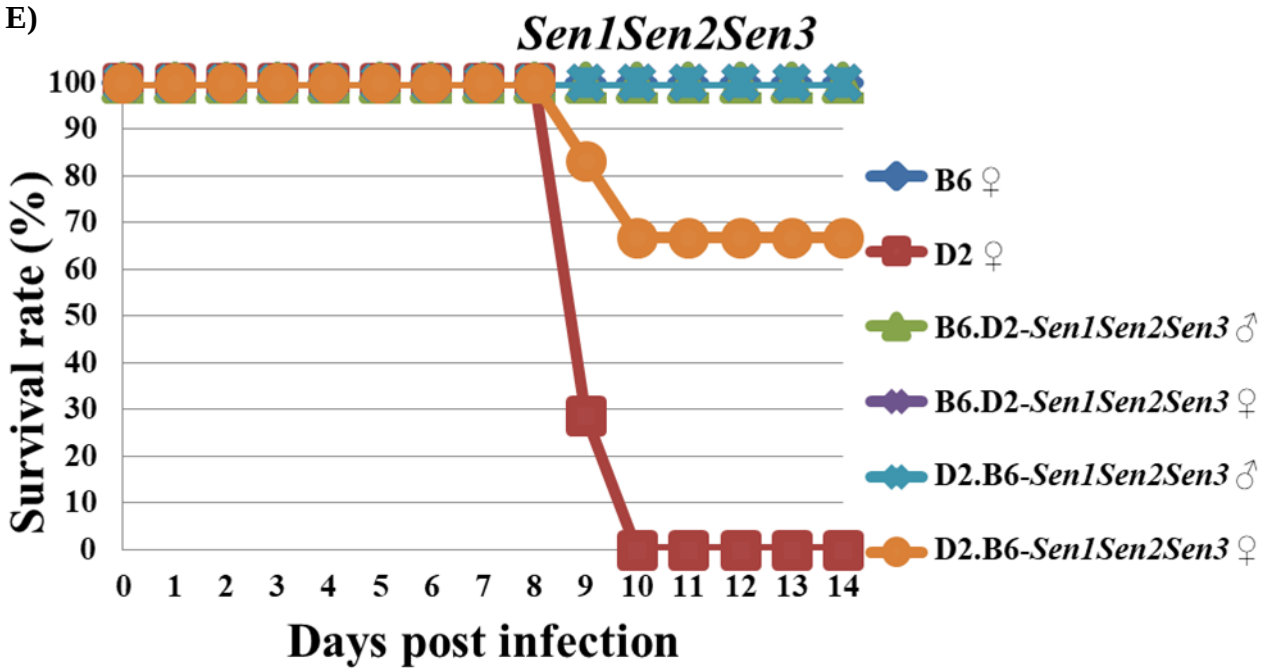


Fig. 4. Survival rates of congenic lines after SeV infection. Survival rates of single-congenic lines for *Sen1*, *Sen2*, and *Sen3* are shown in A, B, and C, respectively. (D); survival rates of double-congenic lines for *Sen1* and *Sen2*. (E); survival rates of triple-congenic lines for *Sen1*, *Sen2*, and *Sen3*.

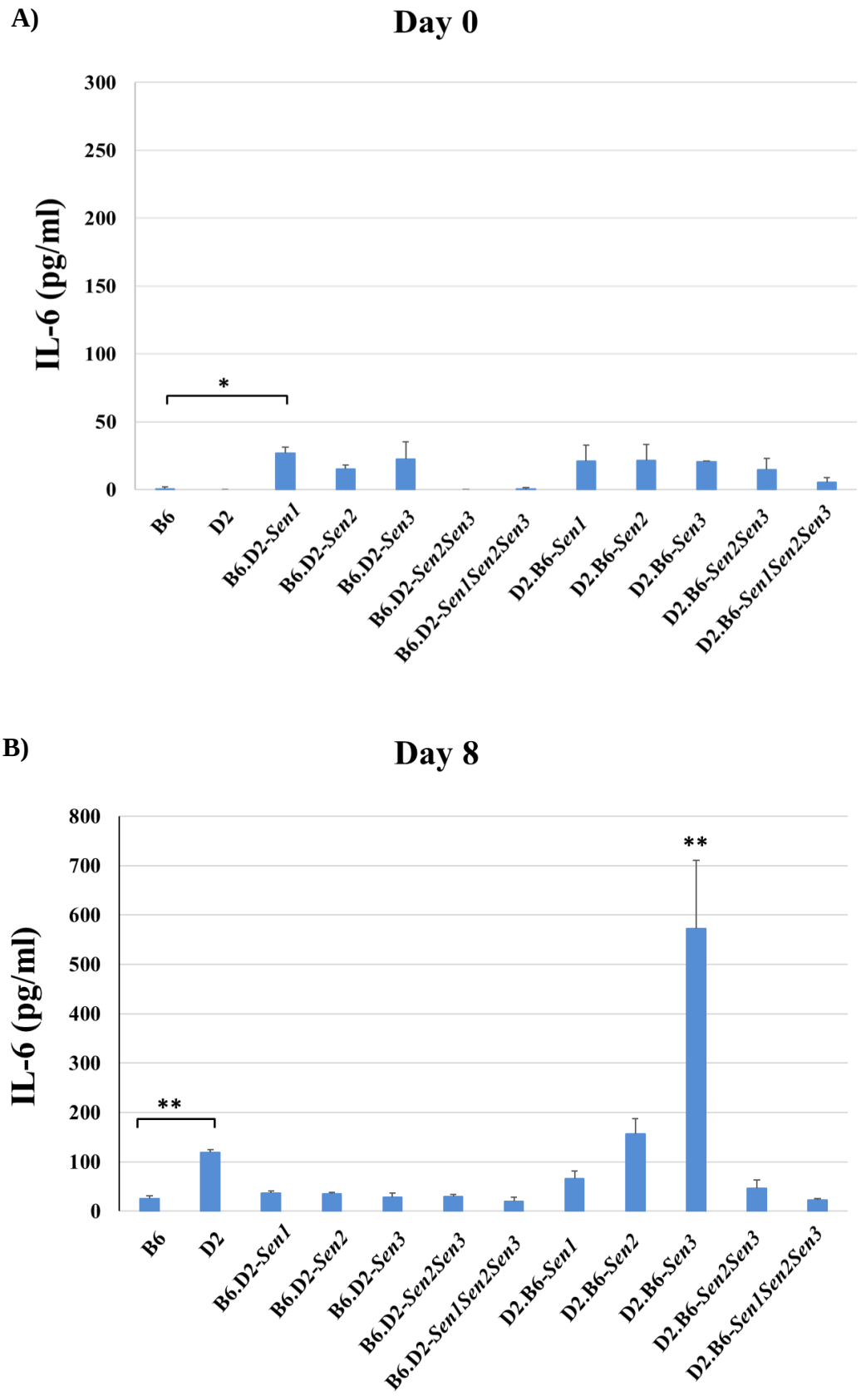


Fig. 5. IL-6 concentrations in broncho-alveolar lavage fluid. (A); IL-6 concentrations before infection in B6, D2, and congenic lines. (B); IL-6 concentrations at 8 d.p.i. in B6, D2, and congenic lines. Statistical analysis was performed in congenic lines with B6-genetic background including parental strains, B6 and D2. Statistical analysis was also performed in congenic lines with D2-genetic background including parental strains, B6 and D2. ** and * indicate p <0.01 and p <0.05, respectively. D2.B6-*Sen2* was significantly different from all mice in the same group.

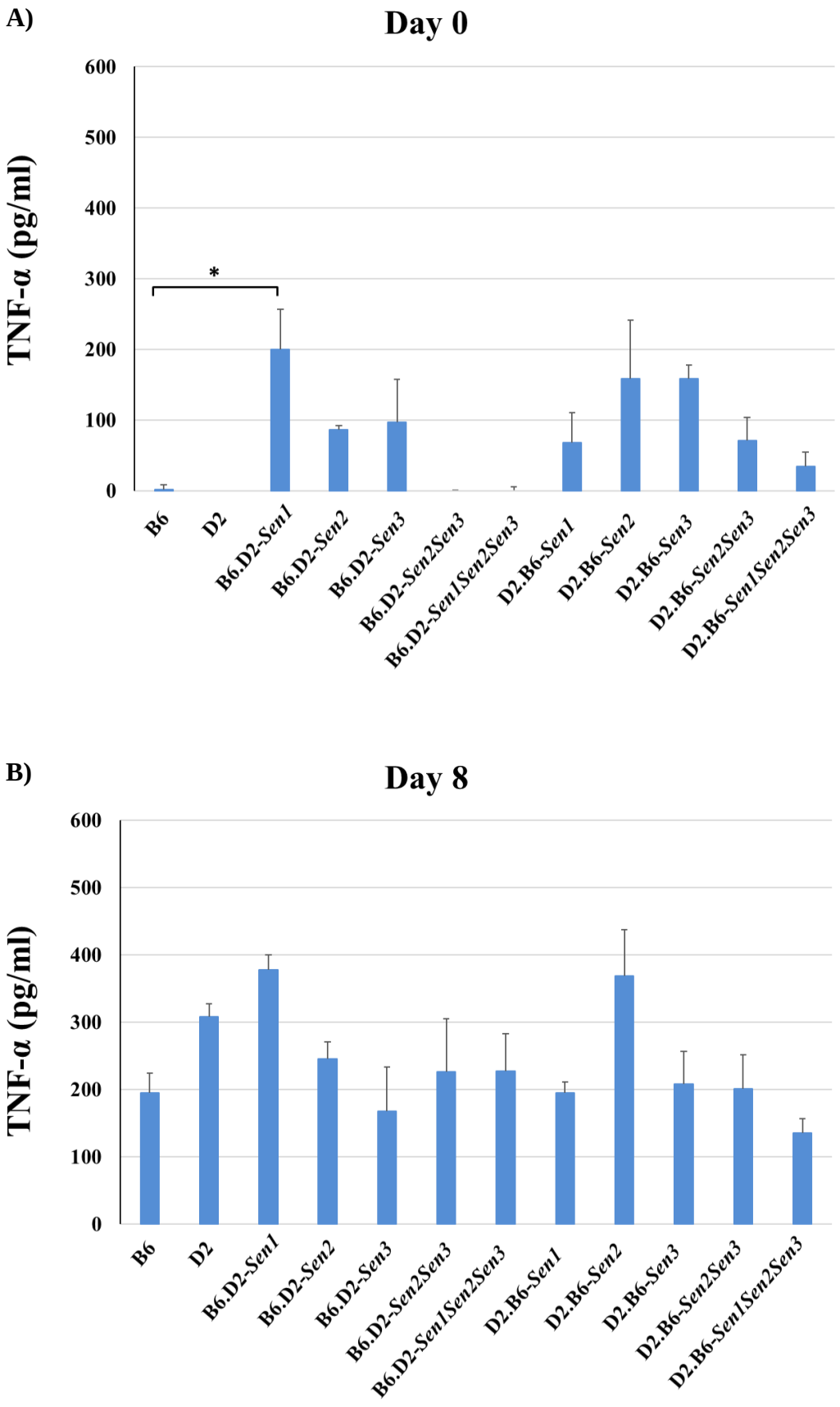


Fig. 6. TNF-α concentrations in broncho-alveolar lavage fluid. (A); TNF-α concentrations before infection in B6, D2, and congenic lines. (B); TNF-α concentrations at 8 d.p.i. in B6, D2, and congenic lines. Statistical analysis was performed in congenic lines with B6-genetic background including parental strains, B6 and D2. Statistical analysis was also performed in congenic lines with D2-genetic background including parental strains, B6 and D2. * indicates p <0.05.

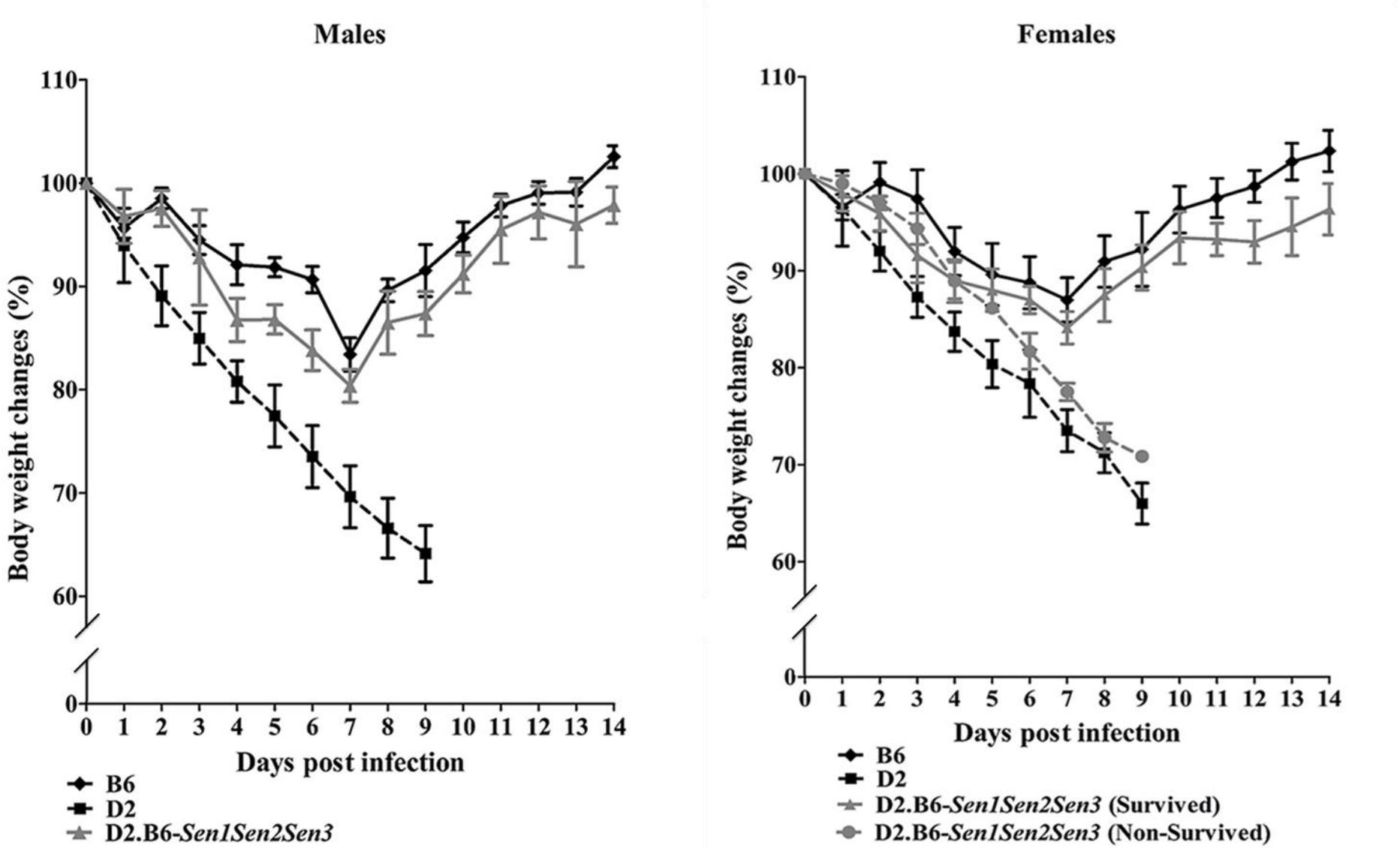


Fig. 7. Body weight changes in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice after SeV infection. Five males and six females were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Body weight was presented as a percentage of the initial body weight on day 0 until the 14th day post SeV infection.

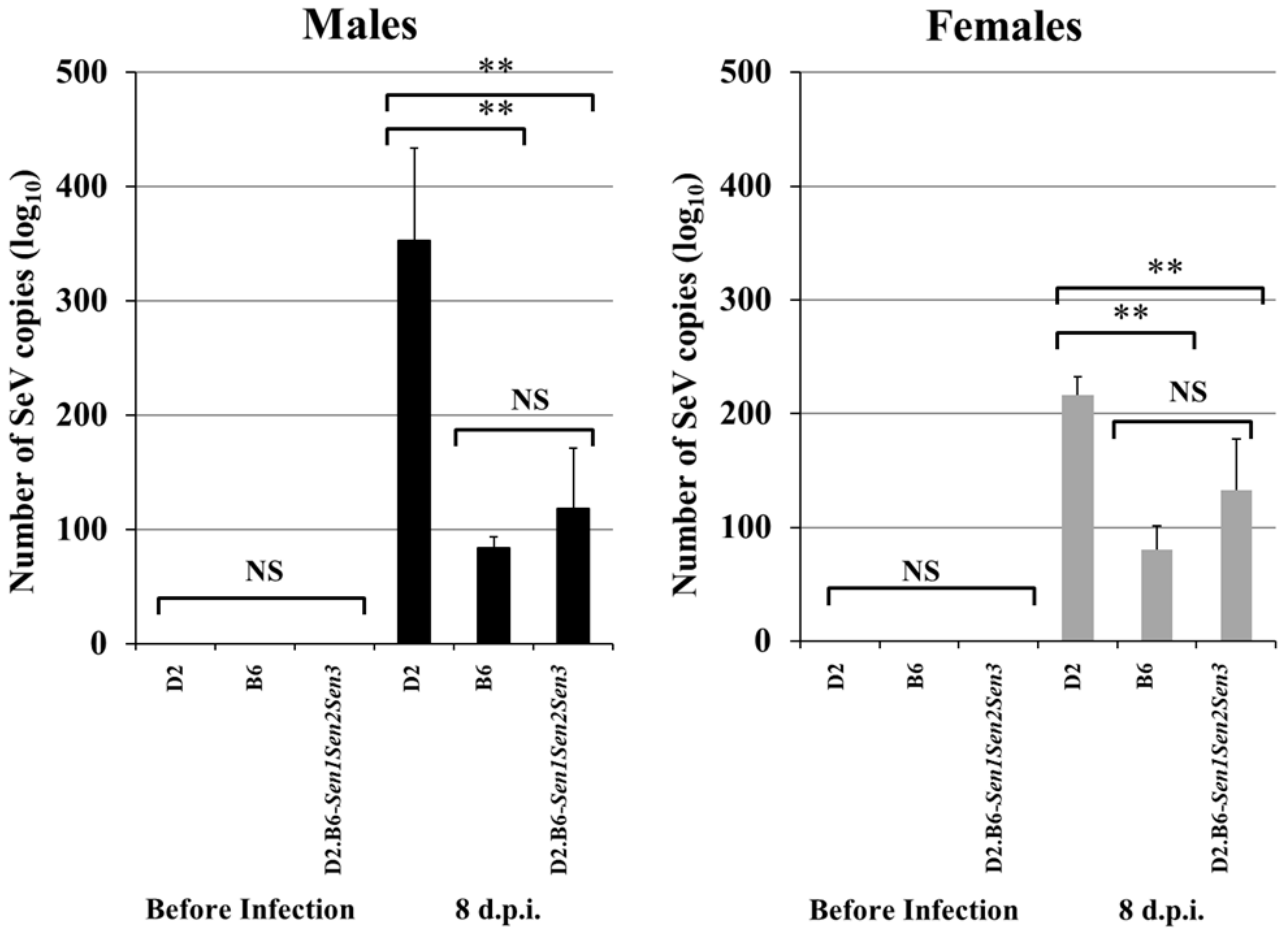


Fig. 8. SeV replication load in lung tissue of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Quantitative real time PCR (qPCR) was performed to quantify the viral copy number in lung tissue. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Right lungs were collected from mice before and at 8 d.p.i. Total RNA was extracted from lung tissue and used for qPCR. Data were represented as mean ± SD. ** and * indicate p <0.01 and p <0.05, respectively. NS; not significant.

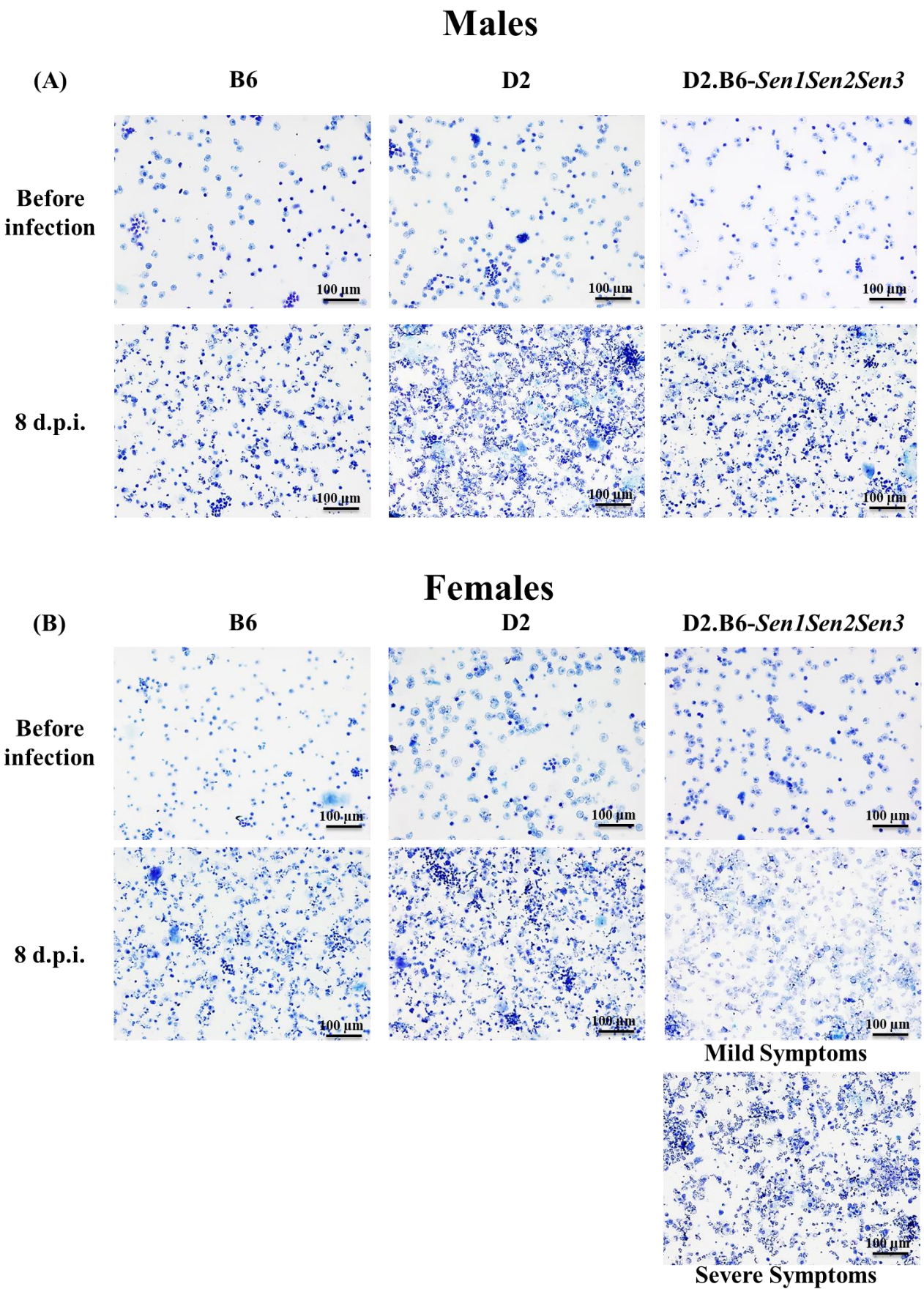
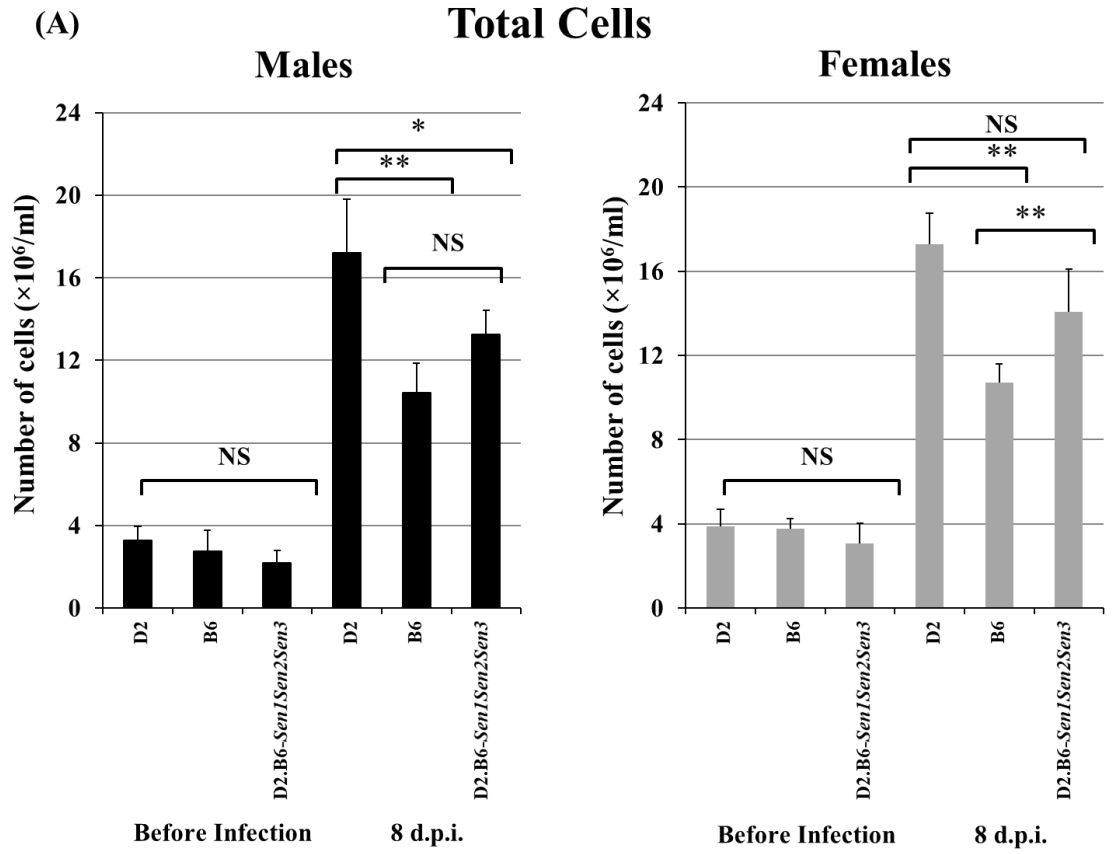
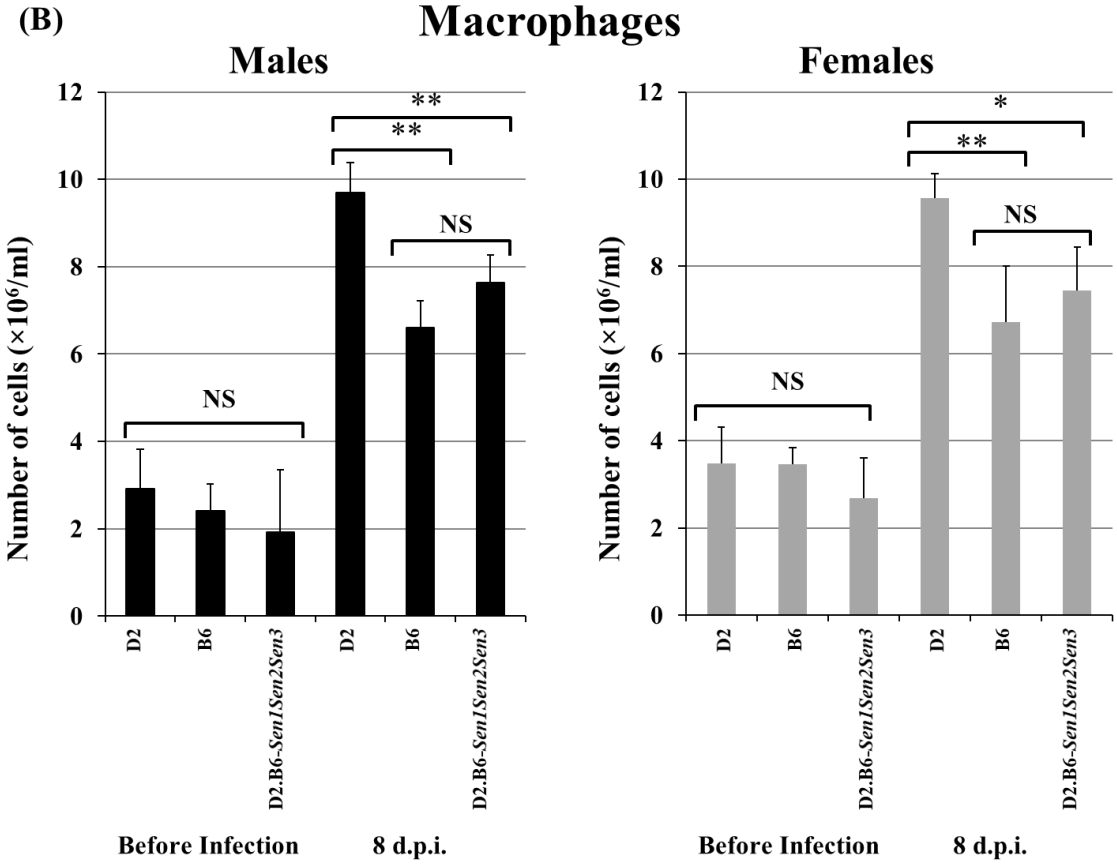


Fig. 9. Photomicrograph of cytospin of BALF samples collected from B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. BALF samples were collected from mice before and at 8 d.p.i. A; males and B; females.


(A)
Total Cells
Males
Females
Number of cells (×10⁶/ml)
0
4
8
12
16
20
24
*
**
NS
D2
B6
D2.B6-Sen1Sen2Sen3
Before Infection
8 d.p.i.


(B)
Macrophages
Males
Females
Number of cells (×10⁶/ml)
0
2
4
6
8
10
12
*
**
NS
D2
B6
D2.B6-Sen1Sen2Sen3
Before Infection
8 d.p.i.

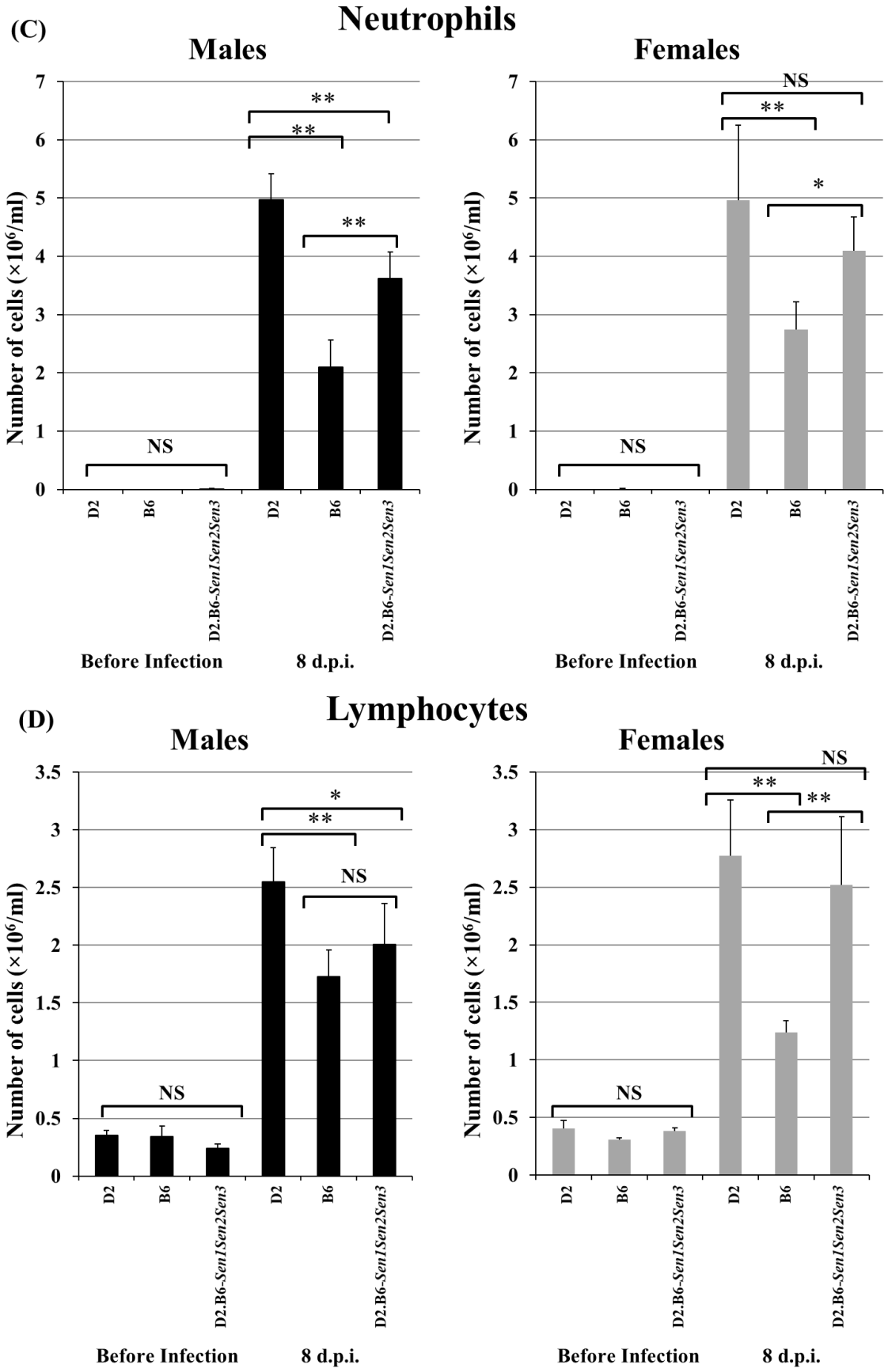
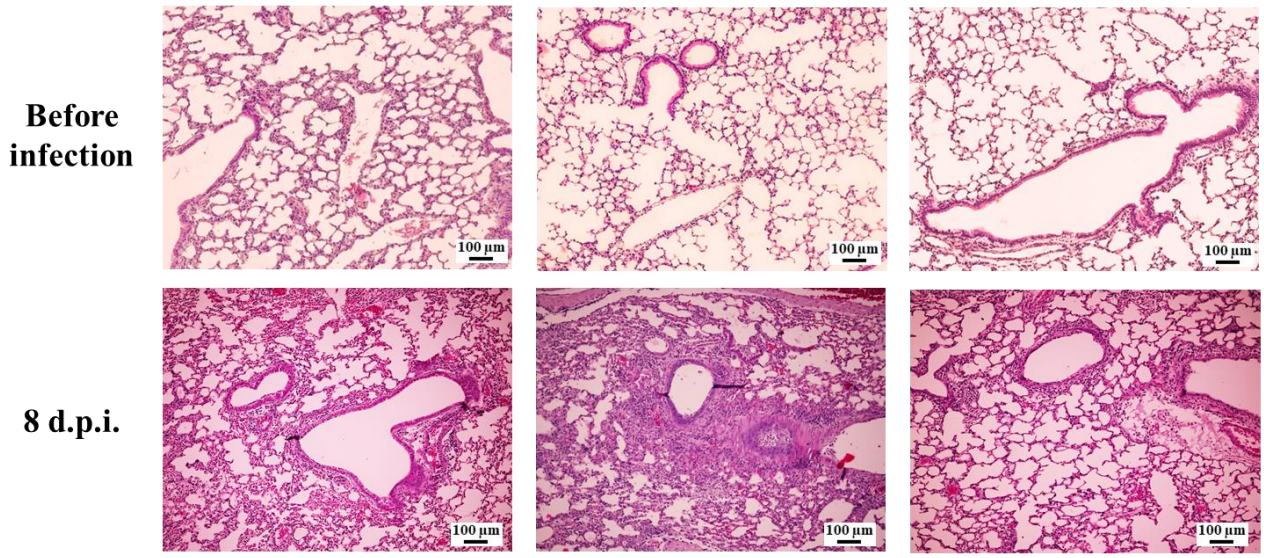


Fig. 10. Cell counts of various immune cells in BALF collected from B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. BALF samples were collected from mice before and at 8 d.p.i. A; total cells, B; macrophages, C; neutrophils, and D; lymphocytes. ** and * indicate $p < 0.01$ and $p < 0.05$, respectively, NS; not significant.

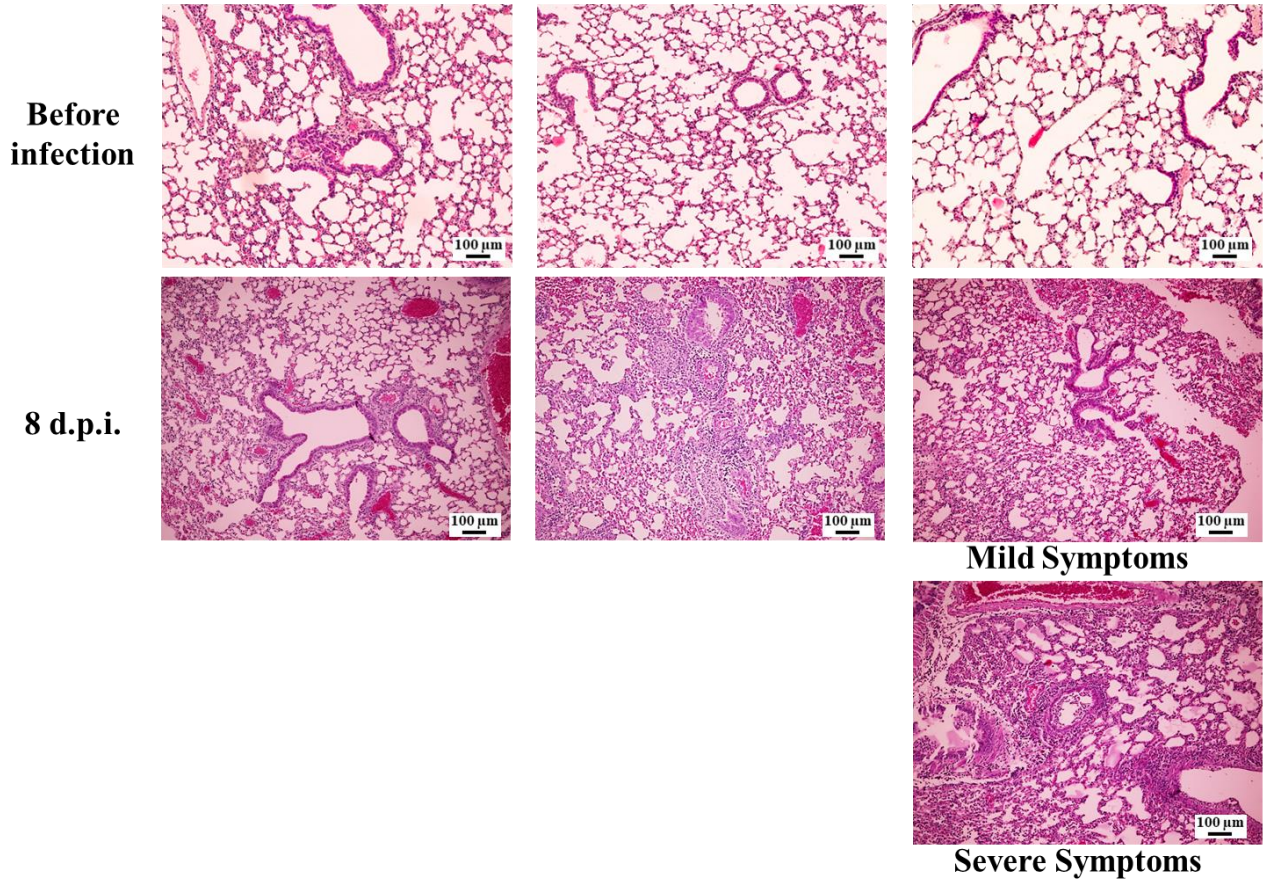
Males

(A)



Females

(B)



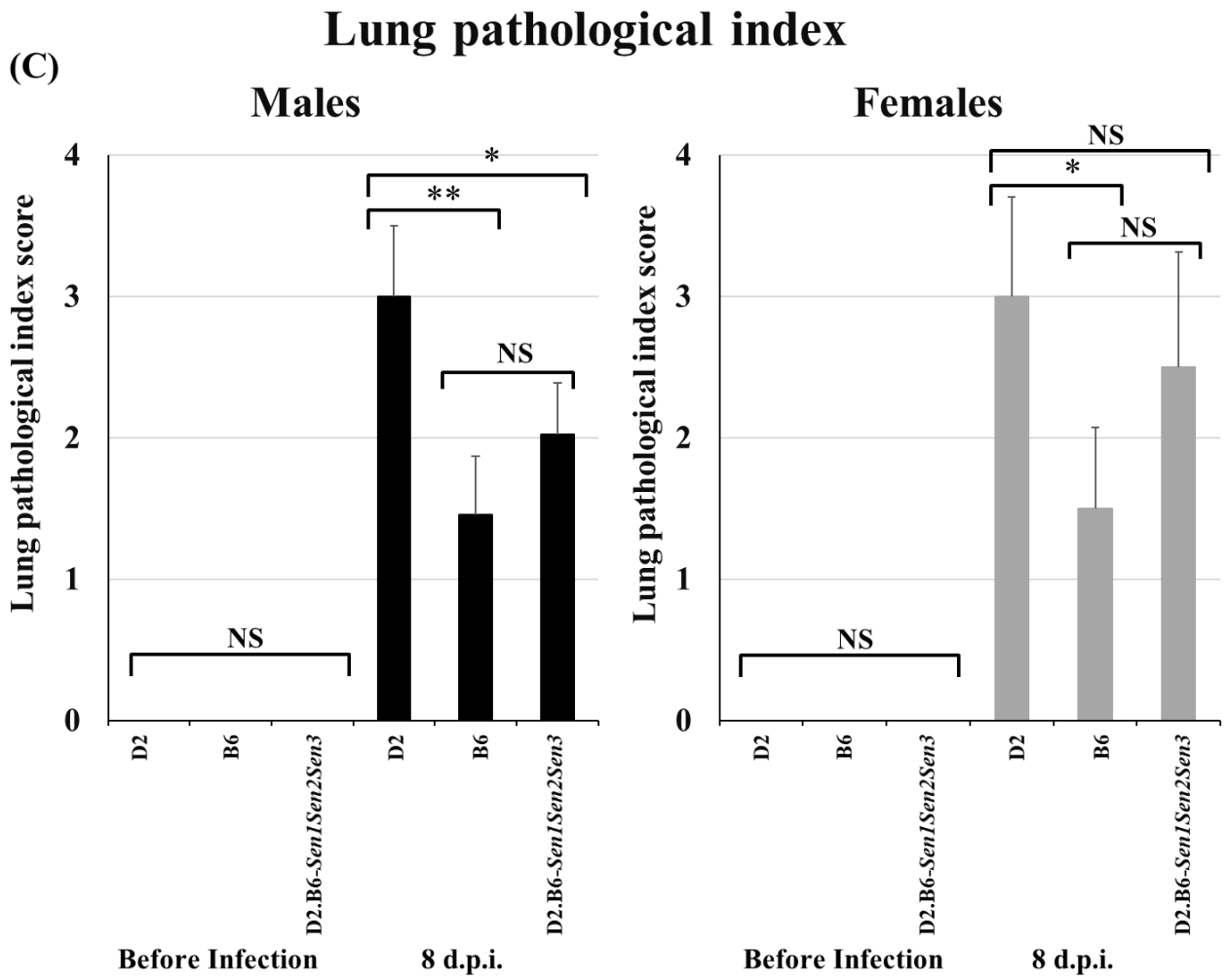


Fig. 11. Lung histopathology of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Left lungs were collected before and at 8 d.p.i. Nine sections were made in each mouse, 5 random fields of view were observed and scored their pathological index in each slide. Photo represents the field of view scored heist values of pathological index. A; males and B; females. C; the lung pathological index score of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and at 8 d.p.i. Lung pathological index was calculated as the mean of view showing highest and secondly highest values of pathological index for each mouse. Then, mean values of pathological index was calculated from the date for four mice in each group.

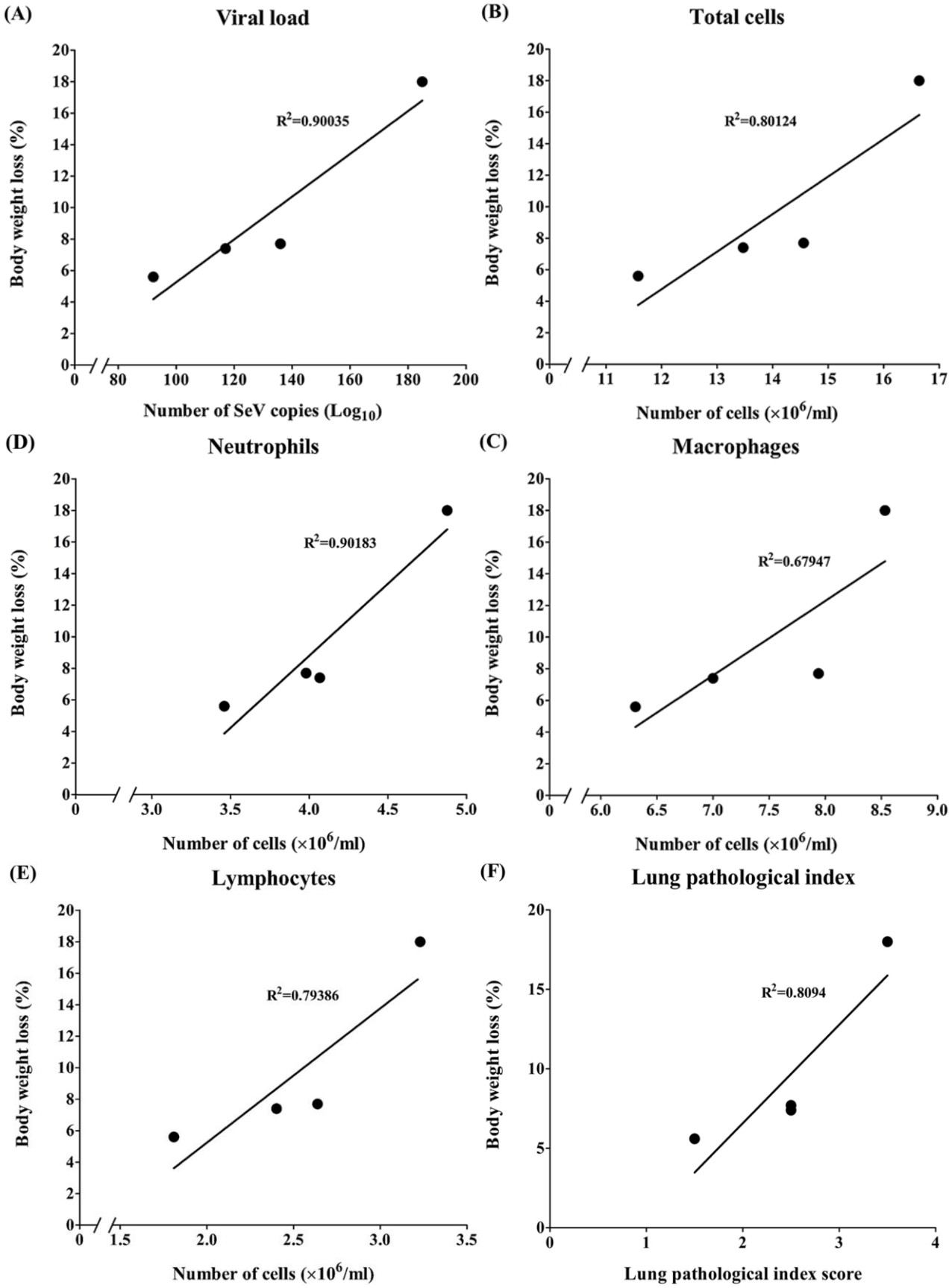


Fig. 12. Correlation between body weight loss and immune cellular responses in female D2.B6-*Sen1Sen2Sen3* mice. A; viral load, B; BALF total cells, C; BALF macrophages, D; BALF neutrophils, E; BALF lymphocytes, and F; lung pathological index.

4. Discussion

SeV, also known as murine parainfluenza virus type 1 or hemagglutinating virus of Japan, is a negative sense, single-stranded RNA virus that belongs to the genus *Respirovirus* and the family *Paramyxoviridae*. SeV is one of the most important pathogens in the SPF rodents (7). The primary targets of SeV are epithelial cells of the upper respiratory tract, from which SeV moves through the trachea into the lungs. Symptoms are very variable from mild snuffles to severe pneumonia with breathing difficulties and even death.

Following respiratory tract infection, SeV proliferates extensively in the lungs reaching its peak titers on the fourth to fifth day of the infection (3, 7). Subsequently, virus titers decline rapidly with infectious virus no longer recoverable after the 10 to 11 d.p.i. (4). Several reports indicated that, regardless the phenotype whether resistance or susceptible, the degree of SeV replication in lungs seems to be similar in the both mouse phenotypes (9, 14).

It has been well known that there is variable susceptibility among mouse inbred strains and B6 and D2 mice are representatives of resistant and susceptible strains, respectively (6, 8, 9, 14). Recently, the genetic mapping using the D2 and B6 as a representative for susceptible and resistant strains, respectively, as well as F_1 and backcrosses, were carried out, and it was found that one major QTL with highly significant linkage was mapped to the distal portion of Chr 4 (*Sen1*) as well as two other QTLs showing statistically suggestive linkage on Chr 8 and Chr 14 (*Sen3*) (17). In addition, a highly significant epistatic interaction was found between a locus on Chr3 (*Sen2*) and Chr 14 (*Sen3*).

Moreover, it was found that D2 mice exhibited a dysregulated hyper-inflammatory cytokine/chemokine cascades, so-called “cytokine storm” as the cause for the susceptibility (18).

Therefore, current study has conducted to verify that the three previously identified QTLs are responsible for resistance or susceptibility to the SeV infection by producing congenic mice, in which B6 alleles of each *Sen1*, *Sen2*, and *Sen3* was introgressed into D2-genetic background and *vice versa*. Further, to explore the epistatic interaction among these three QTLs, double-congenic mice for *Sen2* and *Sen3*, as well as triple-congenic mice carrying *Sen1*, *Sen2*, and *Sen3* in the B6- or D2-genetic background were generated and subjected to the SeV infection experiments. And then survival rate as well as the immune cellular responses were investigated.

In this study, the B6-derived *Sen1* was most effective in D2-genetic background. Further, the epistatic interaction was observed between B6-derived *Sen2* and *Sen3* in the D2-genetic background as shown in the previous QTL analysis (17). Furthermore, triple introgression of B6-derived *Sen1*, *Sen2*, and *Sen3* into D2-genetic background could change D2 mice from susceptible to resistant phenotype to the SeV infection with the similar level of B6 mice. However, this was not the case for D2-derived QTLs in B6-genetic background. The reason for it is unknown; however, strongly dominant and resistant genetic background in B6 mice might be the reason for it.

In the previous report (18), the cellular and humoral immunity in SeV-infected B6 and D2 mice were investigated and it was concluded that susceptibility of D2 mice to the SeV infection was attributed to hyper-response of cytokines, so-called “cytokine storm” in the lung. IL-6 and TNF-α were cytokines that mostly up-regulated in BALF of the SeV-infected D2 mice compared to that of B6 mice. Therefore, in this research IL-6 and TNF-α levels were measured in BALF in SeV-infected

congenic mice. Although introgression of each QTL resulted in somewhat unexpected results, co-introgression of all three QTLs into D2-genetic background suppressed both IL-6 and TNF-α levels, which is consistent with the survival data.

The IL-6 expression level was significantly increased up to 10-fold at 24 h after respiratory syncytial virus infection, member of *Paramyxoviridae*, in peripheral blood monocytes, suggesting that the IL-6 expression may be partially dependent on cytokine priming (1). The elevated level of IL-6 has damaging effects and has been proposed as a reliable marker for functional decline and as a predictor of morbidity and mortality (12). High IL-6 serum levels influence the onset of frailty, poor physical performance, loss of muscle strength, cognitive decline, and cardiological, neurological, and vascular events. It is also closely linked to the genesis of cancers, with cardiac remodeling in heart failure and with the risk of community-acquired pneumonia. Therefore, the obtained results in the current study may explain the underlying reason for the mortality observed in D2 strain.

The effects of SeV infection and importance of the TNF-α has been studied in BN and F344 rats as a representative to susceptible and resistant rat strains to SeV-induced bronchiolar fibrosis, respectively (22). It was found that the TNF-α had clear contradictory effects. The excessive pulmonary TNF-α expression was associated with pathological airway fibrosis, airway dysfunction, and mortality in BN rats. In contrast, inhibition of TNF-α activity was associated with massive respiratory viral infection and increase in the mortality in BN rats. These results suggest that TNF-α is a two-edged sword in the pulmonary host-virus interaction, where sufficient TNF-α activity is critical for termination of viral replication and inhibition of lung injury, whereas the TNF-α overexpression results in an excessive repair response marked by airway scarring and permanent dysfunction.

Moreover, it was indicated that high TNF-α expression level was associated with the pathophysiological consequences in a number of pulmonary diseases, where the duration and TNF-α expression level have significant effects on its diverse functions as well as the genetic background and TNF-α expression time-point determines its function and diversity of the immune response (13). These findings collectively with the obtained results in the current study could explain the mortality observed in the D2 strain.

The immune cellular responses in D2.B6-*Sen1Sen2Sen3* congenic mice were investigated. The body weight loss, viral load, immune cells in BALF, and histopathological index of male D2 triple-congenic mice were comparable to that of B6 mice except for the number of neutrophils in BALF, suggesting that B6-derived three QTLs were almost enough to alter susceptibility to SeV infection in male D2-genetic background. However, the result not reducing the number of neutrophils in BALF from male D2 triple-congenic mice to the level of male B6 mice may raise the possibility that there are other loci to differentiate the resistance or susceptibility to SeV infection between B6 and D2 mice.

In contrast, in female D2 triple-congenic mice, the effect of B6-derived three QTLs were limited. Most remarkable result was survival or not. As shown in Fig. 7, female triple-congenic mice were divided into survived and non-survived mice after SeV infection and it could be predicted by the extent of weight loss. Further, some parameters for immune cellular response such as viral load and number of macrophages in BALF in female D2 triple-congenic mice were comparable to those of B6 mice, whereas other parameters such as the number of total cells, neutrophils and lymphocytes in BALF were remained in the level of D2 mouse. Furthermore, Fig. 12 shows a correlation between

body weight loss and these parameters for immune cellular response, indicating that hyper immune cellular responses cause body weight loss and further leads to death if these immune cellular responses were extremely high. It is unknown at present the reason for the difference between males and females. However, these results suggest that there are further additional loci to differentiate susceptibility to SeV infection in females between B6 and D2 mice.

The susceptibility and resistance in D2 and B6 mice, respectively, are seen in other pathogens. The infection experiments with influenza virus, *Orthomyxoviridae*, using seven inbred mouse strains concluded that D2 and A/J mice were susceptible, whereas other 5 inbred strains including B6 mice were resistant (2, 19). Basically, laboratory inbred strains including D2 and B6 mice are all susceptible to the infection of influenza virus due to the deficiency in *Mx* gene (20). Moreover, both B6.A2G-*Mx1*$^{r/r}$ and D2.A2G-*Mx1*$^{r/r}$ congenic lines being introgressed with the functional *Mx1* gene derived from the feral mouse-derived inbred strain, A2G, were infected with lethal H1N1 influenza virus, and it was found that B6.A2G-*Mx1*$^{r/r}$ mice were resistant, whereas D2.A2G-*Mx1*$^{r/r}$ mice were susceptible to the influenza virus infection (16). These results suggest that differential genetic factor**(**s) are present between D2 and B6 mice other than the *Mx* gene and such factors may include, at least partially, three QTLs found as the resistant or susceptible factors to the SeV infection.

The obtained results in the current study indicated that the three QTLs, *Sen1*, *Sen2*, and *Sen3* were indeed responsible for the resistance/susceptibility phenotype in B6 and D2 strains. Moreover, both IL-6 and TNF-α played a crucial role to control the lethal outcome of SeV infection. In addition, the introgression of the B6 alleles of three QTLs into the D2 genetic background resulted in resistance to SeV infection by optimizing the aggressive immune cellular responses that seen in D2 mice,

although the effect of these three QTLs were different between males and females. Further investigation is still required to uncover the underlying mechanism to resist SeV infection in D2 triple-congenic mice and identify the genes responsible for the resistance/susceptibility to SeV infection.

5. Summary

Sendai virus (SeV) is one of the most important pathogens in the specific-pathogen free rodents. It is known that there are some inbred mouse strains susceptible or resistant to SeV infection. The C57BL/6 (B6) and DBA/2 (D2) mice are representatives of the resistant and susceptible strains, respectively. Previous study with the quantitative trait locus (QTL) analysis identified three QTLs responsible for resistance or susceptibility to SeV infection on different chromosomes and indicated that resistance or susceptibility to SeV infection was almost predicted by genotypes of these three QTLs. To verify the above hypothesis, congenic lines were generated as follows; B6-congenic lines carrying one of the D2 alleles of three QTLs and combination of these three QTLs, and D2-congenic lines carrying single or combination of B6 alleles of three QTLs. All these congenic lines were then challenged with SeV infection and survival rate as well as the immune cellular responses were investigated to verify that these three QTLs were responsible for the difference in resistance/susceptibility to the SeV infection between B6 and D2 mice.

D2 congenic lines introgressed single or combination of B6 alleles of QTLs changed to resistance to SeV infection. Especially, a D2 triple-congenic line became resistant as similar level to B6-parental strain. However, B6-congenic lines introgressed single or combination of D2 alleles of QTLs all remained to be resistant to SeV infection. Both IL-6 and TNF-α in broncho-alveolar lavage fluid (BALF) of D2 triple-congenic line decreased to the similar level of B6 mice, suggesting that this is a part of factors that D2 triple-congenic line became resistant to the similar level of B6 mice. In addition, the body weight loss, viral load, immune cells in BALF, and histopathological index of

SeV-infected male D2 triple-congenic mice were comparable to those of B6 mice except for the number of neutrophils in BALF. In contrast, female D2 triple-congenic mice were divided into survived and non-survived mice after SeV infection and it could be identified by the extent of body weight loss. Viral load and macrophage number in BALF in SeV-infected female D2 triple-congenic mice were comparable to those of B6 mice, whereas the number of total cells, neutrophils, and lymphocytes in BALF were remained in the level of D2 mouse. There was a correlation between body weight loss and these immune cellular responses in SeV-infected female D2 triple-congenic mice.

Data obtained from these congenic mice verified that three QTLs identified previously were indeed responsible for the resistance/susceptibility to SeV infection in B6 and D2 mice. As well, the introgression of B6 alleles of these three QTLs into D2-genetic background resulted in resistance to SeV infection by optimizing the aggressive immune cellular responses that seen in D2 mice, although there may be other loci responsible for difference between B6 and D2 mice.

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