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Immune cellular responses to Sendai virus infection in D2.B6-*Sen1Sen2Sen3* congenic mice, of which three quantitative trait loci responsible for the resistance to infection were introgressed from C57BL/6 mouse into DBA/2 mouse

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Abstract

It has been reported that C57BL/6 (B6) mice are resistant to the Sendai virus (SeV) infection, whereas DBA/2 (D2) mice are susceptible, the cause of susceptibility in D2 mice is hyper-inflammatory cytokine production, and three quantitative trait loci (QTLs), *Sen1*, *Sen2*, and *Sen3* are identified to be responsible for this difference. We previously have verified that the introgression of B6-derived these three QTLs into D2-genetic background increases survival rate and suppresses cytokine production by generating D2.B6-*Sen1Sen2Sen3* congenic mice. In this study, we investigated immune cellular responses of D2.B6-*Sen1Sen2Sen3* mice after SeV infection. Body weight loss, viral load, immune cells in broncho-alveolar lavage fluid (BALF), and histopathological index of SeV-infected male D2.B6-*Sen1Sen2Sen3* mice were comparable to those of B6 mice except for the number of neutrophils in BALF. In contrast, female D2.B6-*Sen1Sen2Sen3* mice were divided into survived and non-survived mice after SeV infection. Viral load and macrophage number in BALF in SeV-infected female D2.B6-*Sen1Sen2Sen3* 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.B6-*Sen1Sen2Sen3* mice. These results indicate that 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.

Key Words: Immune cellular response; Sendai virus infection; Congenic mouse; Quantitative trait locus

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Introduction

Sendai virus (SeV), known as the murine parainfluenza virus type 1, is the natural respiratory pathogen of laboratory mice and the prototype of paramyxovirus[4]. SeV is an enveloped virus with 15–16 kb of a negative sense single-stranded genomic RNA and belongs to the *Paramyxoviridae* family and *Respirovirus* genus[10].

Several previous studies have reported that the susceptibility to SeV infection varies in mouse inbred strains[5,6,7,9]. The susceptible strains allow the infection to spread from the airway into the lung parenchyma with severe pathological outcomes, while resistant mouse strains suppress the initial viral replication and limit the infection by confining infected regions predominantly to the airways and then, virus titers decline rapidly after the 10^{th} to 11^{th} days post infection (d.p.i) and no longer recover[2,3].

The genetic mapping using the DBA/2 (D2) and C57BL/6 (B6) as representatives of susceptible and resistant strains, respectively, revealed quantitative trait loci (QTLs) responsible for susceptibility and resistance to SeV infection, a significant QTL on chromosome (Chr) 4 (*Sen1*) as well as two suggestive QTLs on Chrs 8 and 14 (*Sen3*). In addition, a highly significant epistatic interaction was found between QTLs on Chr 3 (*Sen2*) and Chr 14 (*Sen3*)[11]. Moreover, the immune response analysis in B6 and D2 mouse strains revealed that the D2 mouse exhibited a dysregulated hyper-inflammatory cytokine/chemokine cascades, so-called "cytokine storm" after SeV infection. This cytokine storm was considered to be the main cause of D2 mice susceptibility[12].

Recently, we produced a total of 10 congenic mice lines, five B6-congenic lines that were introgressed D2 alleles of these three QTLs (singly each and different combinations) and five D2-congenic lines that were introgressed B6 alleles of them (singly each and different combinations) to verify that these three QTLs are responsible for susceptibility or resistance to SeV infection. Infection experiments with SeV in these congenic mice revealed that introgression of these three QTLs singly or with combinations in the D2-genetic background showed significant improvement in survival rate and hyper-production of cytokines, in particular, triple introgression of B6 alleles of these three QTLs altered the phenotype of D2 mice to the similar level in B6 mice with respect to survival rate and cytokine production[1]. However, other immune responses in D2 triple-congenic mice to SeV infection were not well studied. Therefore, in this study, 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.

Materials and Methods

Mice: Eight-week-old B6 and D2 mice purchased from SLC Japan (Hamamatsu, Japan) and D2.B6-*Sen1Sen2Sen3* triple-congenic mice were used for SeV infection experiment. Animal experimentation was conducted under the AAALAC International-accredited program and 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. 14-0158).

Infection experiments: Infection experiments with SeV in mice were conducted as described previously[1,11,12]. Briefly, 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.)][8]. Then, mice were infected with 10^3 median tissue culture infectious dose ($TCID_{50}$) of SeV, MN

strain. A volume of 25 μl of the viral inoculum was slowly injected intranasally into the left side. Mice were monitored twice a day to examine the main symptoms of SeV virus infection, as well as measuring the body weight of mice on a daily basis during the experimental period. When mice showed severe symptoms with moribundity, mice were immediately euthanized with overdose of isoflurane. Some mice were dead not showing severe symptoms with moribundity at the observation twice a day. Both euthanized and dead mice were counted as non-survival mice.

Cytology in broncho-alveolar lavage fluid (BALF): Mice were euthanized with overdose of isoflurane before and after (at 8 d.p.i.) the infection and then, BALF samples were collected from the right lung, where the left bronchus was tied up using surgical suture. The trachea was cannulated through the mouth and larynx and the right lung was lavaged slowly three times with warm phosphate-buffered saline (PBS, pH 7.2, 500 μl injection and 300 μl collection in each time). 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 ethylenediaminetetraacetic acid was added to the cell pellets and mixed by inverting. After that, 200 μl of the BALF cell suspensions were loaded onto a Shandon™ 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 manufacture's protocol.

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 mice. Briefly, total RNA was extracted from the right lung of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after (at 8 d.p.i) the infection using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol. Two μg of extracted RNA were transcribed using Rever-Tra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacture'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 NCBI database (accession no. M30202).

Lung histopathological analysis: The left lungs were collected from all 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.

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 $P \leq 0.05$ and $P \leq 0.01$ were considered to be significant.

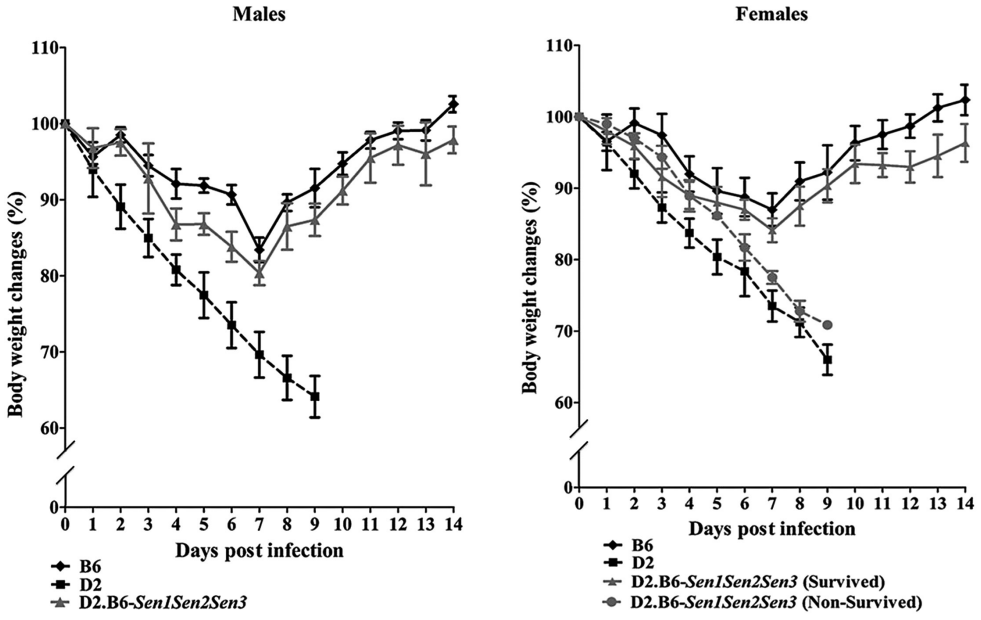


Fig. 1. Body weight changes of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice after SeV infection. Five males and six females were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Body weight was presented as a percentage of the initial body weight on day 0 until the 14^{th} day post SeV infection.

Results

Body weight changes after SeV infection: Body weight changes after SeV infection were recorded on daily basis (Fig. 1). 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).

Virus titers: The virus replication load in the mouse lung was measured at 8 d.p.i. using quantitative PCR method. Fig. 2 showed the viral copy number per lung in infected mice. Both male and female D2 mice showed significantly ($P \leq 0.01$) 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.

Cytology analysis in the BALF: 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. 3). 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. 4). Total cell counts as well as counts of

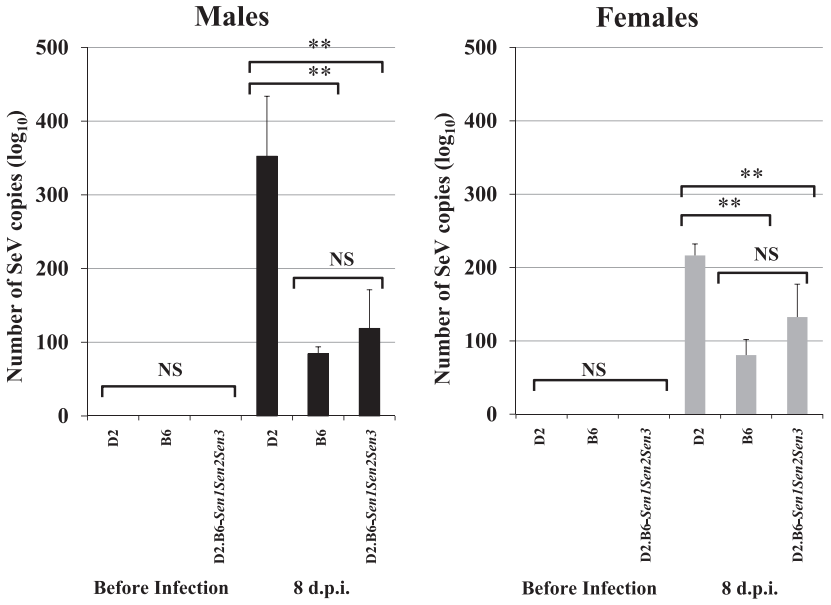


Fig. 2. SeV replication load in lung tissue of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Quantitative real time PCR (qPCR) was performed to quantify the viral copy number in lung tissue. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Right lungs 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.

macrophages, neutrophils, and lymphocytes in infected D2 mice were all higher than those of infected B6 mice in consistence with the previous report[12]. 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.

Lung histopathology and pathological index score: Fig. 5 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.

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. 1), we examined correlation between body

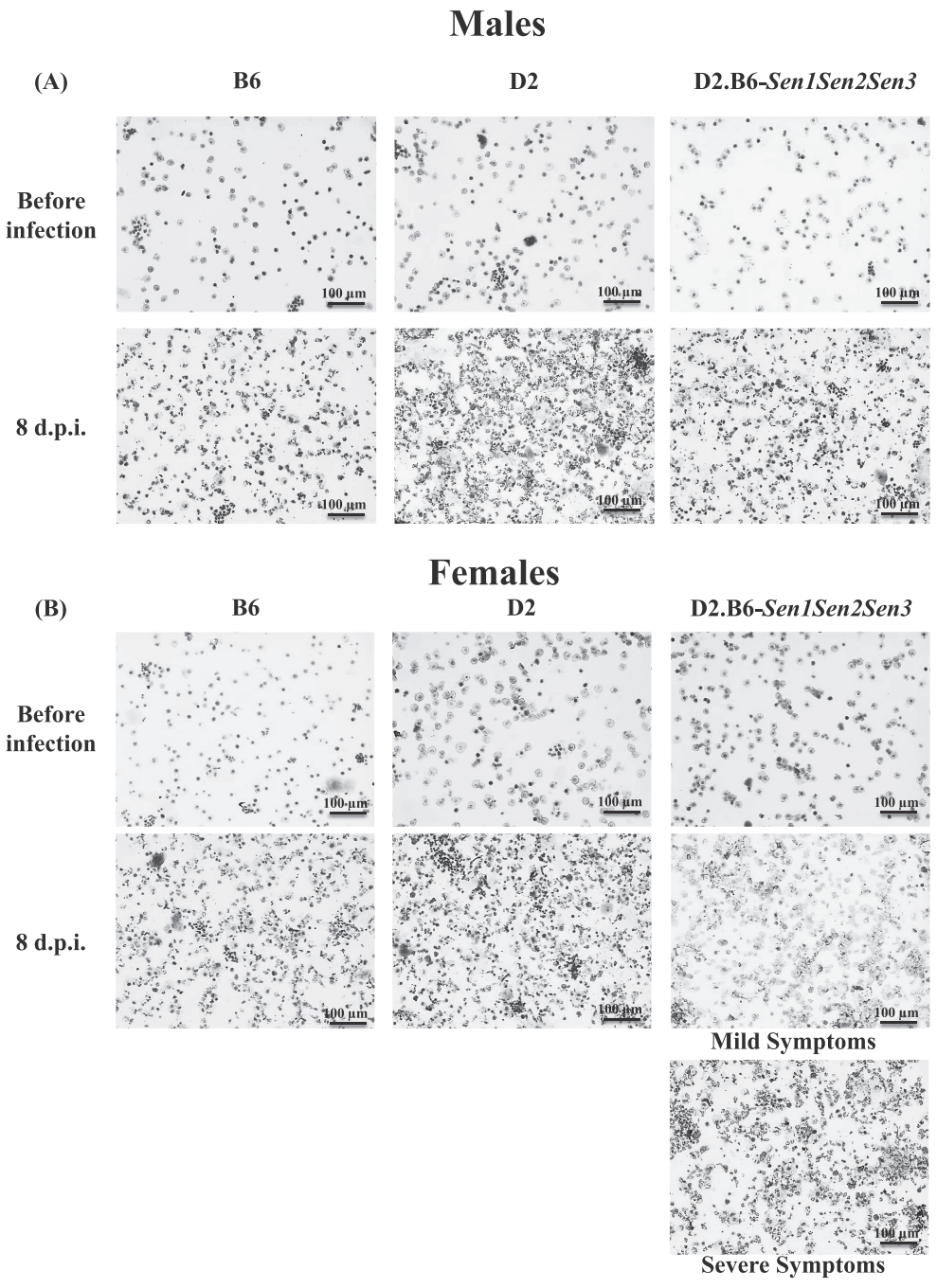


Fig. 3. Photomicrograph of cytospin of BALF samples collected from B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. BALF samples were collected from mice before and at 8 d.p.i.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

weight loss and immune cellular responses at 8 d.p.i. (Fig. 6). 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

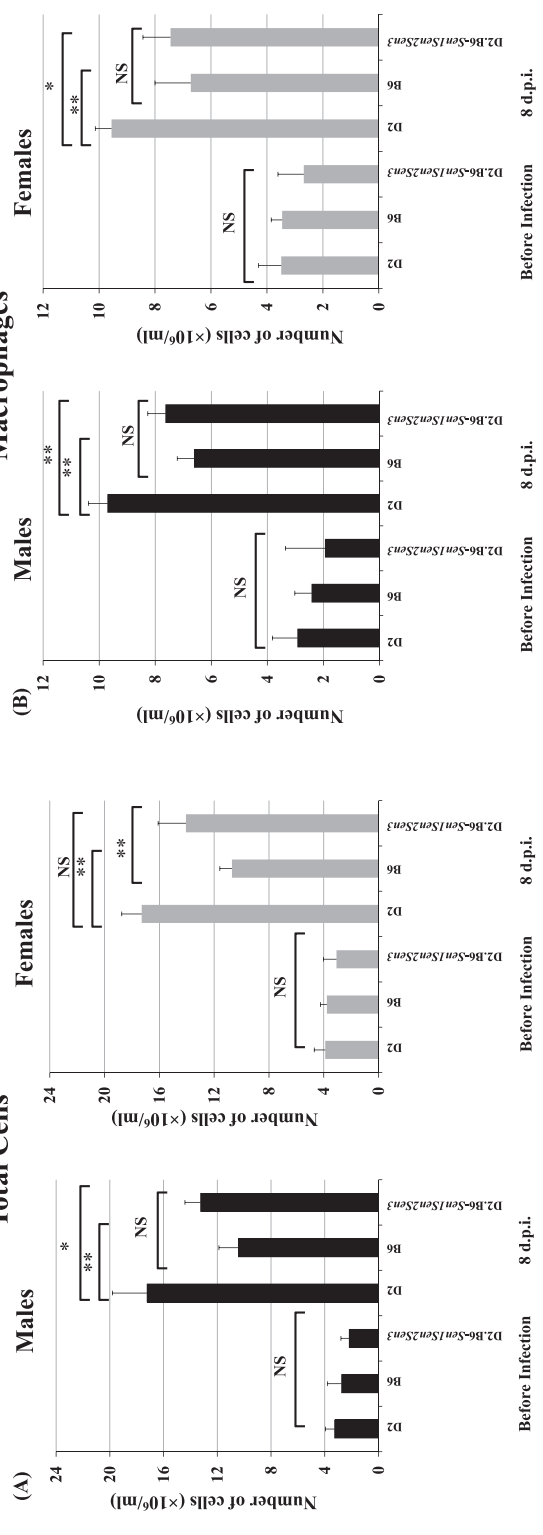


Fig. 4. Cell counts of various immune cells in BALF collected from B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. BALF samples were collected from mice before and at 8 d.p.i. A; total cells, B; macrophages, C; neutrophils, and D; lymphocytes. ** and * indicate $P < 0.01$ and $P < 0.05$, respectively, NS; not significant.

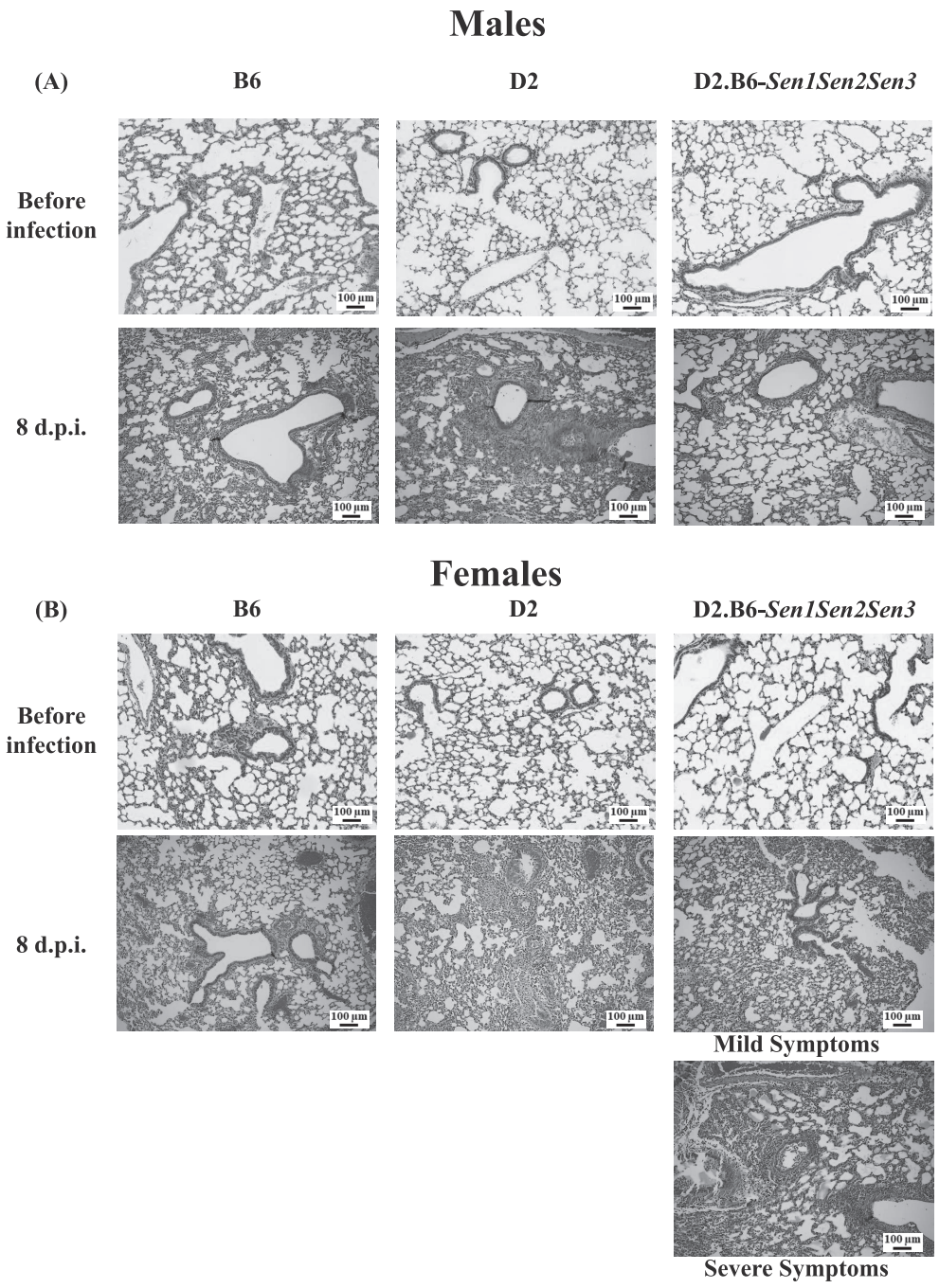
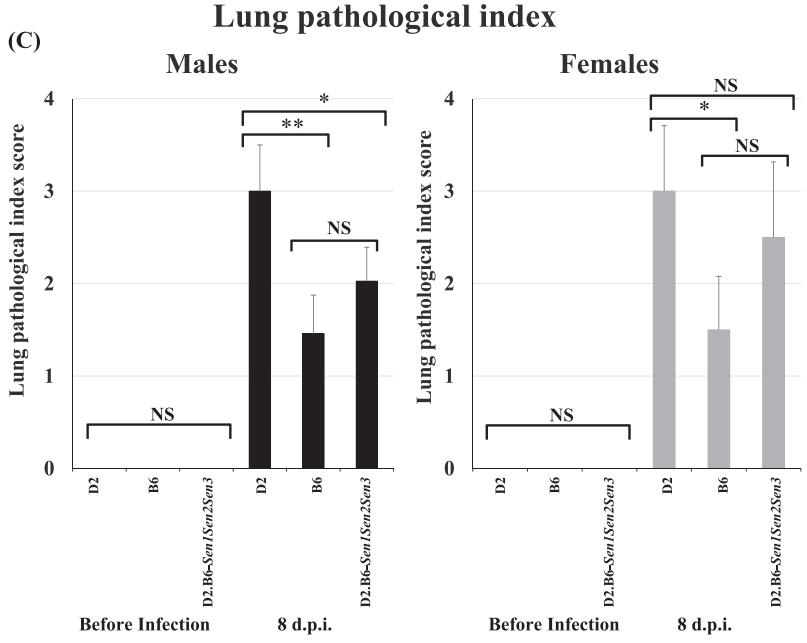


Fig. 5. Lung histopathology of B6, D2, and D2.B6-*Sen1Sen2Sen3* mice before and after SeV infection. Four male and female mice were infected with 10^3 $TCID_{50}$ SeV in B6, D2, and D2.B6-*Sen1Sen2Sen3* mice. Left lungs were collected before and at 8 d.p.i. Nine sections were made in each mouse, 5 random fields of view were observed and scored their pathological index in each slide. Photo represents the field of view scored highest 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.



loss in female D2 and D2 triple-congenic mice and then leading to death in case of extreme-hyper immune cellular responses.

Discussion

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[5,6,7,9]. We previously performed QTL analysis using backcrosses from B6 and D2 mice and found three QTLs, *Sen1*, *Sen2*, and *Sen3* to control resistance/susceptibility to SeV infection[9]. Recently, we verified that introgression of B6-derived these three QTLs into D2-genetic background increased survival rate and reduced cytokine production after SeV infection by generating D2.B6-*Sen1Sen2Sen3* congenic mice, in which all three QTLs were introgressed into D2 mice[11]. We also investigated the immune cellular responses in B6 and D2 mice after SeV infection and concluded that the cause of susceptibility was dysregulated hyper-inflammatory cytokine/chemokine cascades, so-called "cytokine storm" resulting from hyper immune cellular responses[12]. Therefore, in this study, we investigated immune cellular responses in D2.B6-*Sen1Sen2Sen3* congenic mice.

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. 1, 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

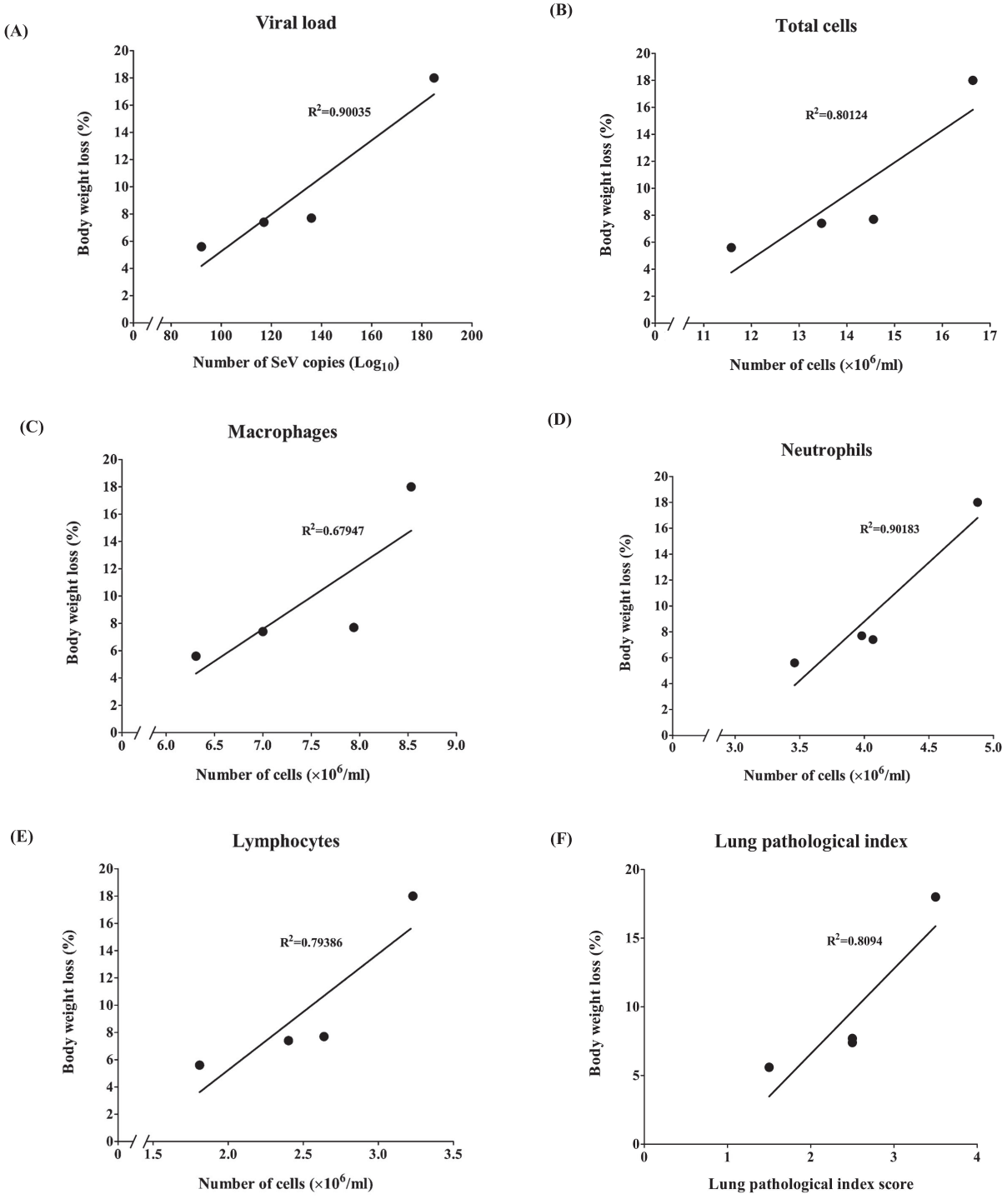


Fig. 6. Correlation between body weight loss and immune cellular responses in female D2. B6-*Sen1Sen2Sen3* mice. A; viral load, B; BALF total cells, C; BALF macrophages, D; BALF neutrophils, E; BALF lymphocytes, and F; lung pathological index.

lymphocytes in BALF were remained in the level of D2 mouse. Furthermore, Fig. 6 shows 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.

In conclusion, the obtained results in the current study indicated that the introgression of the B6 alleles of three QTLs responsible for the resistance to SeV infection 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.

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