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**Involvement of CD8⁺ T cells in the development of renal hemorrhage in a mouse model of
hemorrhagic fever with renal syndrome**

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Running title: Role of CD8⁺ T cells in HFRS

Abstract

Hemorrhagic fever with renal syndrome (HFRS) is caused by hantavirus infection. Although host immunity is thought to be involved in the pathogenesis of HFRS, the mechanism remains to be elucidated. A mouse model of HFRS, which showed renal hemorrhage similar to that seen in patients, has been developed previously. In this study, we aimed to clarify whether CD4⁺ and CD8⁺ T cells are involved in the development of renal hemorrhage in the mouse model. At 2 days before virus inoculation, CD4⁺ or CD8⁺ T cells in 6-week-old BALB/c mice were depleted by administration of antibodies. The CD4⁺ T cell-depleted mice developed signs of disease such as transient weight loss, ruffled fur and renal hemorrhage as in non-depleted mice. In contrast, the CD8⁺ T cell-depleted mice showed no signs of disease. After determination of CTL epitopes on the viral glycoprotein in BALB/c mice, the quantity of virus-specific CTLs was analyzed using an MHC tetramer. The quantity of virus-specific CTLs markedly increased in spleens and kidneys of virus-infected mice. However, the quantity in high-pathogenic clone-infected mice was comparable to that in low-pathogenic clone-infected mice. We previously reported that the high-pathogenic clone propagated more efficiently than the low-pathogenic clone in kidneys of mice during the course of infection. Therefore, there is a possibility that the balance between quantities of the target and effector is important for disease outcome. In conclusion, this study showed that CD8⁺ T cells are involved in the development of renal hemorrhage in a mouse model of HFRS.

Introduction

Hantaviruses, which were recently taxonomically classified into the order *Bunyavirales*, family *Hantaviridae*, genus *Orthohantavirus* [1], are spherical enveloped viruses with a tri-segmented single-strand negative-sense RNA genome. The segments, designated as small, medium and large segments, encode the nucleocapsid protein, glycoprotein (GP) and RNA-dependent RNA polymerase, respectively. GP is co-translationally cleaved into Gn and Gc.

Hantaviruses cause two types of disease in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). HFRS is caused by Old World hantaviruses such as Hantaan virus (HTNV), Seoul virus, Dobrava-Belgrade virus and Puumala virus and is characterized by renal dysfunction and hemorrhage. HPS is caused by New World hantaviruses such as Andes virus and Sin Nombre virus (SNV) and is characterized by acute respiratory failure. Although the major symptoms and targeted organs in HFRS and HPS are different, immune responses are thought to be involved in the pathogenesis of HFRS and HPS [2]. However, the mechanism for this remains to be elucidated, and specific treatment is not yet available. In order to elucidate the mechanism and develop countermeasures, an animal model that mimics human disease is necessary.

In general, hantaviruses cause no disease in adult mice. In contrast, hantaviruses cause lethal disease in suckling mice. However, the symptoms are different from those in humans [3]. However, we have recently found that HTNV strain Korean hemorrhagic fever virus (KHFV) clones exceptionally cause renal hemorrhage in adult mice [4]. This is the first animal model showing renal hemorrhage similar to that seen in HFRS patients. Renal hemorrhagic lesions in the mice were macroscopically

limited to the medullary region, which is one of the characteristic pathological changes in severe HFRS patients. Histopathological changes such as widespread capillary engorgement, interstitial hemorrhage, flattened tubular epithelium and dilated lumina were observed in the mice as in HFRS patients. In this mouse model, cells infiltrating the kidney were observed. This result raises the possibility that host immunity is related to disease development in this mouse model, as in HFRS patients.

In this study, we examined whether CD4⁺ and CD8⁺ T cells are involved in the development of hemorrhagic disease in the mouse model by depleting CD4⁺ and CD8⁺ T cells. In addition, we determined the major CTL epitopes on GP in BALB/c mice and synthesized an MHC tetramer to analyze the GP-specific CTL in the mouse model.

Materials and methods

Viruses and cells

The HTNV strain KHFV was isolated from the blood of a patient with Korean hemorrhagic fever by Dr. H. W. Lee and was passaged 10 times in the brains of newborn ICR mice [5]. Five clones of KHFV (KHFV cl-1 to -5) were cloned by plaque purification on Vero E6 cells (ATCC CRL-1586) [4]. KHFV cl-1, 2, 3 and 5 cause transient body weight loss, ruffled fur and renal hemorrhage in adult mice after intravenous (i.v.) inoculation, while KHFV cl-4 did not cause disease in mice [4]. In this study, KHFV cl-5 and cl-4 were used as representatives of high-pathogenic and low-pathogenic clones, respectively. A stock of KHFV clones was prepared in Vero E6 cells and stored at -80°C until use. The infectivity titers of the stock of KHFV clones were measured by counting infected cell foci detected by

immunostaining, as described previously [6]. Experiments involving virus infections were performed in BSL-3 and ABSL-3 facilities under national and institutional regulations.

Flow cytometry

Suspensions of spleen cells were obtained by homogenizing spleens in RPMI 1640 medium supplemented with 10% FBS and 2-mercaptoethanol (50 mM). Suspensions of kidney cells were obtained by treatment of kidney tissue with RPMI 1640 medium supplemented with 1 mg/ml Collagenase and 30 µg/ml DNase I (Sigma) for 30 min at 37°C. Erythrocytes were lysed with 10 mM Tris-HCl (pH 7.4) containing 0.83% NH₄Cl. Flow cytometric data were collected on a BD FACSCant II (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc.). Cells were stained using FITC-conjugated anti-mouse interferon (IFN)-γ (Caltag Laboratories), PE-conjugated anti-mouse CD4, allophycocyanin-conjugated anti-mouse CD8, FITC-conjugated anti-mouse CD44 (eBioscience) and PE-conjugated MHC class-I tetramer (Medical and Biological Laboratories) as described previously [7]. Lymphocyte populations were identified based on forward scatter and side scatter characteristics.

Depletion of CD4⁺ and CD8⁺ T cells

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan Inc. In order to deplete CD4⁺ and CD8⁺ T cells in mice, rat anti-mouse CD4 IgG2B (clone GK1.5) and rat anti-mouse CD8 IgG2A (clone 53-6.7) (R&D Systems) were inoculated via the intraperitoneal (i.p.) route, respectively. Rat IgG2B and rat IgG2A (R&D Systems) were used as isotype controls. Depletion of CD4⁺ and CD8⁺ T

95 cells was evaluated by flow cytometry using spleen cells at 9 days after administration of antibodies.

96 Mice were euthanized by inhalation of sevoflurane.

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98 **Virus inoculation to mice**

99 At 2 days after administration of 100 µg of each of the antibodies against CD4 and CD8, the mice were

100 inoculated with 10⁵ focus-forming units (f.f.u.) of KHFV cl-5 via the i.v. route. Body weight of the mice

101 was measured daily, and the mice were euthanized at 12 days post-inoculation (dpi) by inhalation of

102 sevoflurane. Kidneys were collected for pathological examination and quantification of viral RNA,

103 while serum samples were collected for detection of antibodies. Quantity of viral RNA in kidneys was

104 determined by real-time PCR analysis as described previously [4]. IgG antibodies to HTNV were

105 detected by an immunofluorescence assay as described previously [8, 9]. In another experiment, female

106 BALB/c mice (6 weeks old) were inoculated with 10⁵ f.f.u. of KHFV cl-5 and KHFV cl-4 via the i.v.

107 route, and spleen and kidney samples were collected at 9 dpi to examine the numbers of CD4⁺ and

108 CD8⁺ T cells and GP-specific CTLs.

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110 **Peptides**

111 In total, 225 15-mer peptides with 10 aa overlaps encompassing the entire amino acid sequence of GP of

112 the HTNV strain 76–118 and 51 10-mer peptides with 9 aa overlaps encompassing the amino acid

113 region 141 to 200 of GP were purchased from Mimotope. The peptides LGPYRVQVV and

114 SYCMTGVLI, which were identified as GP-specific CTL epitopes in this study, were purchased as

highly purified products from Hokkaido System Science. Stock solutions of these synthetic peptides were prepared in 100% DMSO at concentrations of 5 mg/mL.

Determination of the GP-specific CTL epitope and MHC restriction

CTL epitopes were determined by detecting intracellular IFN- γ after stimulation of spleen cells with peptides as described previously [7]. MHC restriction was predicted by the Bioinformatics and Molecular Analysis Section (BIMAS) algorithms [10]. MHC restriction was evaluated by an inhibition-of-peptide-stimulation assay using anti-H-2D^d and anti-H-2K^d antibodies (BD Biosciences) as described previously [7].

Statistical analysis

Student's *t* test was used to determine statistical significance, with a *P* value of <0.05 being considered statistically significant.

Results

Depletion of CD4⁺ and CD8⁺ T cells

In order to determine the quantity of antibodies sufficient for depletion of CD4⁺ and CD8⁺ T cells in BALB/c mice, increasing amounts of rat anti-CD4 IgG2B and rat anti-CD8 IgG2A (25, 50, 100 and 200 μ g) were administered via the i.p. route. Depletion of CD4⁺ and CD8⁺ T cells was evaluated by flow cytometry using spleen cells at 9 days after administration of antibodies. In a normal mouse, CD4⁺ and

CD8⁺ T cells accounted for about 21.7% and 9.5% of lymphocytes in the spleen, respectively (Fig. 1a). When 100 µg of anti-CD4 or anti-CD8 antibody was administrated, CD4⁺ and CD8⁺ T cells decreased to 0.2% and 1.1% of lymphocytes in the spleen, respectively (Fig. 1a). Fig. 1b shows the dose-dependency of the effect of antibody administration. The quantity of CD4⁺ T cells and that of CD8⁺ T cells in the spleen of a normal mouse were set to 100%. Antibody administration caused marked decreases of CD4⁺ and CD8⁺ T cells in the spleen, and the effect reached a plateau around the dose of 100 µg. We repeated experiments at a dose of 100 µg of antibodies and confirmed the reproducibility (Fig. 1c). We therefore considered that 100 µg of the antibody is sufficient to deplete the two types of cells.

Virus inoculation to CD4⁺ and CD8⁺ T cell-depleted mice

In order to evaluate the role of CD4⁺ and CD8⁺ T cells in disease development in the mouse model, CD4⁺ and CD8⁺ T cells were depleted by administration of 100 µg of anti-CD4 antibody and 100 µg of anti-CD8 antibody, respectively, 2 days before inoculation with high-pathogenic KHFV cl-5. The CD4⁺ T cell-depleted mice showed transient body weight loss, ruffled fur and reduced activity at 6 to 9 dpi as non-depleted mice did (Fig. 2, left). Pathological examination of kidneys at 12 dpi showed macroscopic hemorrhagic lesions in the renal medulla in CD4⁺ T cell-depleted mice and non-depleted mice. In contrast, CD8⁺ T cell-depleted mice showed no signs of disease including renal hemorrhage (Fig. 2, right). These results indicate that CD8⁺ T cells are involved in disease development in this mouse model.

We also investigated the antibody titer and quantity of viral RNA in serum and kidneys at 12 dpi (Table 1). Antibody titers in CD8⁺ T cell-depleted mice were elevated to a level comparable to those in non-depleted mice. In contrast, antibody titers in CD4⁺ T cell-depleted mice were lower than those in non-depleted mice. The quantity of viral RNA in kidneys of CD8⁺ T cell-depleted mice was comparable to that in kidneys of CD4⁺ T cell-depleted mice and was slightly larger than that in kidneys of non-depleted mice. These results indicate that levels of antibody production and viral growth are not correlated with disease development in the mouse model.

Quantities of CD4⁺ and CD8⁺ T cells in kidneys of mice inoculated with high-pathogenic and low-pathogenic clones

Next, we examined the quantities of CD4⁺ and CD8⁺ T cells in kidneys of high-pathogenic KHFV cl-5 and low-pathogenic KHFV cl-4-infected mice by flow cytometry. A previous study has shown that KHFV cl-4 did not cause disease in mice [4]. The quantity of lymphocytes in the kidneys of KHFV cl-5-infected mice slightly increased compared to that seen in normal mice. However, there was no significant difference in the quantity of kidney lymphocytes between KHFV cl-5 and KHFV cl-4-infected mice (Fig. 3a). The proportion of CD8⁺ T cells in lymphocytes in the kidneys of KHFV cl-5-infected mice markedly increased compared to that in normal mice and became larger than the proportion of CD4⁺ T cells (Fig. 3b). In addition, the proportion of CD8⁺ T cells in lymphocytes in the kidneys of KHFV cl-5-infected mice was significantly larger than that in KHFV cl-4-infected mice (Fig. 3b).

Determination of GP-specific CTL epitopes and MHC restriction

In order to examine the quantity of virus-specific CTLs, we searched for major CTL epitopes on HTNV GP in BALB/c mice. In total, 225 15-mer peptides with 10 aa overlaps, encompassing the entire amino acid sequence of HTNV GP, were divided into 15 pools and screened for their capacity to stimulate IFN- γ production in spleen cells of HTNV-infected BALB/c mice. Flow cytometric analyses showed that peptides corresponding to the amino acid region 141 to 200 of GP had the capacity to stimulate IFN- γ production (data not shown). To identify the minimum sequence of the T cell epitope, we additionally examined 51 10-mer peptides with 9 aa overlaps encompassing the amino acid region 141 to 200 of GP. As a result, we identified two HTNV GP-specific epitopes, LGPYRVQVV (GP161-169) and SYCMTGVLI (GP173-181). The GP173-181 peptide had strong activity to stimulate IFN- γ production in spleen CD8⁺ T cells (0.89%), while the GP161-169 peptide had weak activity (0.24%) compared to the activity of the NP335-343 peptide, which has been identified as a major CTL epitope on the nucleocapsid protein of HTNV [7] (Fig. 4). We predicted the MHC restriction of these peptides by the BIMAS algorithms [10]. The score for the GP161-169 peptide was high in H-2D^d (600), while the score for the GP173-181 peptide was high in H-2K^d (2400) (Table 2). We also confirmed the MHC restriction by an inhibition-of-peptide-stimulation assay [7]. Antibodies to the class I molecules D^d and K^d inhibited the IFN- γ production stimulated by GP161-169 and GP173-181, respectively (Table 2). We therefore considered GP173-181 to be a major CTL epitope on GP, and we generated an MHC tetramer using GP173-181 peptides and H-2K^d molecules.

GP-specific CTLs in the spleens and kidneys of mice inoculated with high-pathogenic and low-pathogenic clones

We examined the quantities of GP-specific CTLs in the spleens of high-pathogenic KHFV cl-5 and low-pathogenic KHFV cl-4-infected mice by flow cytometry using the MHC tetramer. The tetramer-positive CD8⁺ T cells were detected in 1.4% of spleen lymphocytes from KHFV cl-5-infected mice at 9 dpi. However, there was no significant difference between KHFV cl-5 and KHFV cl-4-infected mice (Fig. 5a). Next, we examined the quantity of GP-specific CTLs in the kidney, where a number of CD8⁺ T cells migrated after virus infection. It is noteworthy that the proportion of tetramer-positive CD8⁺ T cells in kidney lymphocytes of KHFV cl-5-infected mice was 23.5%, which was considerably larger than in the spleens (Fig. 5b). However, the proportion of tetramer-positive CD8⁺ T cells in kidney lymphocytes from KHFV cl-5-infected mice was comparable to that in KHFV cl-4-infected mice.

Discussion

The relevance of CD8⁺ T cells in the pathogenesis of HFRS and HPS has been examined previously. An increased number of CD8⁺ T cells and a decrease in the ratio of CD4⁺ T cells to CD8⁺ T cells in HFRS patients have been reported [11]. Kilpatrick et al. reported that the frequency of SNV-specific CD8⁺ T cells in PBMCs was higher in severe HPS patients than in moderately ill HPS patients [12]. In this study, we confirmed that depletion of CD8⁺ T cells prevented the development of hemorrhagic disease in mice,

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3 215 indicating that CD8⁺ T cells are involved in disease development in this mouse model, as in humans.
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6 216 In general, hantaviruses have no or only a weak cytopathic effect on infected cells [13-16].
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9 217 Therefore, it is thought that viral growth itself is not sufficient to cause disease. Indeed, despite the
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12 218 efficient viral growth in kidneys of CD8⁺ T cell-depleted mice, the mice showed no signs of disease
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15 219 after infection. Hayasaka et al. reported that the coexistence of SNV-infected cells and SNV-specific
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18 220 CD8⁺ T cells increased cellular permeability, which is a characteristic change thought to be involved in
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21 221 the pathogenesis of HFRS and HPS, but infection or CD8⁺ T cells alone did not increase the
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24 222 permeability [17]. These results suggest that interaction between virus-infected cells and virus-specific
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27 223 CD8⁺ T cells is important for disease development.
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29 224 A previous study showed that KHFV cl-4 caused no apparent disease in mice, in contrast to
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32 225 KHFV cl-5 [4]. We therefore speculated that CD8⁺ T cells in KHFV cl-5-infected mice were excessively
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35 226 activated. However, although the quantities of CD8⁺ T cells and virus-specific CTLs considerably
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38 227 increased after virus infection, there were no significant differences in the quantities of virus-specific
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41 228 CTLs between KHFV cl-5 and cl-4-infected mice. We previously reported that the quantities of viral
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47 230 mice at any time point (3, 6, 9 and 12 dpi) [4]. Therefore, there is a possibility that the balance between
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50 231 quantities of the target and effector determines the disease outcome.
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52 232 Alternatively, there is a possibility that the effector function of virus-specific CTLs was different
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55 233 in KHFV cl-5-infected mice and KHFV cl-4-infected mice. Zhu et al. reported that the frequency of
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58 234 regulatory T cells (Treg) was reduced in the acute phase of HFRS compared to that in the convalescent
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phase and that **seen** in healthy controls and was inversely correlated with disease severity [18]. Chen et al. reported that the activity of Treg was decreased in the early stage of HFRS and that there was a negative correlation between the suppression level and the proportion of CD8⁺ T cells [11]. In contrast, in the natural reservoir, Treg may play an important role in limiting immunopathology [19]. In this study, we examined the number of CD8⁺ T cells reacting to the MHC tetramer, but we did not examine the effector function of the cells. Further studies are needed to determine whether immune suppressive functions are related to the difference between the pathogenicity of KHFV cl-5 and that of KHFV cl-4.

We identified two GP-specific CD8⁺ T cell epitopes. Both epitopes were proximally located within the amino acid sequence 161-181 of the Gn region. The capacity of GP173-181 peptide to stimulate IFN- γ production in spleen cells was higher than that of GP161-169 peptide as well as that of NP335-343 peptide, which was identified as a major CTL epitope on the nucleocapsid protein of HTNV in a previous study [7]. The results suggest that GP173-181 epitope is a major epitope for HTNV-specific CTLs. CTL epitopes on hantavirus protein have been identified in humans [12, 20-22]. However, it is difficult to analyze the detailed mechanism in patients. Determination of CTL epitopes in BALB/c mice will be helpful for further analysis of the role of CTL response **s** in disease development. We previously reported that the amino acid at position 417 in Gn is the sole amino acid difference in viral proteins between KHFV cl-5 and cl-4 [4]. **This** amino acid position **is** not included **in** the two GP-specific CD8⁺ T cell epitopes. Further studies are needed to elucidate the mechanism by which the amino acid at position 417 is involved in pathogenesis.

In conclusion, we showed the involvement of CD8⁺ T cells in disease development in a mouse

model of HFRS. CD8⁺ T cells may be a possible target for therapeutic measures for HFRS.

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Compliance with Ethical Standards

Conflicts of interest: The authors declare that they have no conflict of interest.

Ethical statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of National

University Corporation Hokkaido University (13-0115).

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Figure legends

Figure 1: Depletion of CD4⁺ and CD8⁺ T cells by administration of antibodies. The quantity of antibodies sufficient for depletion of CD4⁺ and CD8⁺ T cells in mice was evaluated using spleen cells at 9 days after administration of antibodies. CD4⁺ and CD8⁺ T cells in spleen were detected using flow cytometry. (a) Representative data from normal mice and mice administered **with** 100 µg of anti-CD4 or anti-CD8 antibody. The gates were set for lymphocytes, and the values are percentages of CD4⁺ and CD8⁺ cells. (b) Dose-dependency of the effect of antibody administration. Increasing doses of anti-CD4 or anti-CD8 antibody (25, 50, 100, 200 µg) intraperitoneally administered to mice. The quantity of CD4⁺ T cells and the quantity of CD8⁺ T cells in spleens of normal mice were set to 100%. (c) Proportion of CD4⁺ and CD8⁺ T cells in spleens of normal mice and mice administered 100 µg of anti-CD4 or CD8

antibody. There was 1 mouse per group in (b) and 3 mice per group in (c)

Figure 2: Changes in body weight of CD4⁺ and CD8⁺ T cell-depleted mice after inoculation of high pathogenic KHFV cl-5. Mice were inoculated with 10⁵ f.f.u. of KHFV cl-5 at 2 days after administration of 100 µg of anti-CD4 IgG2B (filled circle), anti-CD8 IgG2A (filled square) or isotype control antibody (open circle and square). Body weight of mice at the time of antibody administration was set to 100%. Body weight of mock-inoculated normal mice (open triangle) is shown as a reference. The values are averages and standard deviations of relative weight. There were 7 mice per group except for the control groups (n=3)

Figure 3: Quantities of lymphocytes, CD4⁺ T cells and CD8⁺ T cells in kidneys of high pathogenic KHFV cl-5 and low pathogenic KHFV cl-4 infected mice. (a) Proportion of lymphocytes in kidneys of mice inoculated with 10⁵ f.f.u. of KHFV cl-5 and cl-4. Kidneys were collected at 9 dpi. Values are percentages of lymphocyte in kidney determined by flow cytometry. (b) Proportions of CD4⁺ T cells and CD8⁺ T cells in lymphocytes in kidneys of mice inoculated with 10⁵ f.f.u. of KHFV cl-5 and cl-4. Kidneys were collected at 9 dpi. CD4⁺ and CD8⁺ T cells in kidney were detected by flow cytometry. The gates were set for lymphocytes, and the values are percentages of CD4⁺ and CD8⁺ T cells in kidney lymphocytes. There were 7 mice per group except for the control group (n=2). Asterisk indicates statistical difference ($P<0.05$)

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3 355 **Figure 4:** IFN- γ production in spleen CD8⁺ T cells after stimulation with NP335-343, GP161-169 and
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6 356 GP173-181 peptides. Spleen cells were obtained from BALB/c mice 10 days after HTNV inoculation.
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9 357 Spleen cells were stimulated with peptides in the presence of brefeldin A and IL-2. IFN- γ -producing
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12 358 CD8⁺ T cells were detected by flow cytometry. Values are percentages of IFN- γ ⁺ CD8⁺ T cells.
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17 360 **Figure 5:** Proportions of GP173-181 peptide-specific CD8⁺ T cells in spleens and kidneys of mice
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20 361 inoculated with 10⁵ f.f.u. of KHFV cl-5 and cl-4. Spleen and kidney samples were collected at 9 dpi.
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23 362 The MHC tetramer generated from the GP173-181 peptide was used. MHC tetramer-binding CD8⁺ T
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26 363 cells in spleen (a) and kidney (b) were detected by flow cytometry. The gates were set for lymphocytes,
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29 364 and values are percentages of MHC tetramer-positive cells. There were 7 mice per group in (a) and 6
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32 365 mice per group in (b).
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Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5

Table 1. Antibody titers and quantities of viral RNA in CD4⁺ and CD8⁺ T cell-depleted mice at 12 days after inoculation of KHFV cl-5.

Antibodies administered	Signs of disease	IgG titer	Viral RNA in kidney (copy/ μ g RNA)
Anti-CD4 IgG2B	+	:80 to :320	6.6×10^5
IgG2B	+	:1280 to :2560	5.5×10^4
Anti-CD8 IgG2A	–	:1280 to :2560	9.5×10^5
IgG2A	+	:1280 to :2560	7.2×10^4

Table 2. MHC restriction of **the** GP161-169 and GP173-181 peptides.

Peptide	Sequence	Scores in BIMAS analysis			MHC restriction determined by inhibition-of-peptide-stimulation assay
		D ^d	K ^d	L ^d	
GP161-169	LGPYRVQVV	600	10	3	D ^d
GP173-181	SYCMTGVLI	0.6	2400	1	K ^d