These responses were considered to be advantageous effects in cellular immune response to inserted Ag85B *versus* rhPIV2 vector. To confirm this advantageous response, cells from immunized mice were re-stimulated *in vitro* with syngeneic spleen cells infected with rhPIV2 or rhPIV2–Ag85B. Although responses to both Ag85B and rhPIV2 vector were observed, Ag85B-specific responses were clearly seen, especially in pLN and BAL cells after single immunization (Fig. 1D). After performing immunization twice, Ag85B-specific responses were also seen in spleen cells as booster effects more than responses to the vector virus (Fig. 1E). These results indicated that rhPIV2–Ag85B immunization elicited inserted Ag85B-specific immune responses without being hidden by vector responses.

3.2. Intranasal administration of rhPIV2–Ag85B prevents infection with Mtb in mice

To investigate the ability of intranasal administration of rhPIV2–Ag85B to elicit a protective effect against pulmonary TB, rhPIV2–Ag85B-immunized mice were aerosol-infected with highly pathogenic Mtb kurono strain [13]. One group of mice were intranasally immunized with rhPIV2–Ag85B 4 times at 2-week intervals, and another group of mice were intranasally immunized with rhPIV2–Ag85B twice following intramuscular immunization with Ag85B DNA twice (Fig. 2A). Intranasal administration of rhPIV2–Ag85B resulted in a decreases in granulomatous lesions and inflammatory area. However, there were no apparent histopathological differences, such as infiltrating cell types, between the each group of mice, and these results are similar to the results of another study focusing on TB vaccine [14]. On the other hand, these vaccine effects were clearly seen by staining for acid-fast bacillus. Mice immunized with rhPIV2–Ag85B showed a substantial reduction in the infiltration of bacteria, and this inhibitory effect on bacterial expansion was correlated with the number of rhPIV2–Ag85B intranasal administrations (Fig. 2B). CFU of Mtb in spleens from both groups of immunized mice was also significantly lower than those in mice immunized with the control vector (Fig. 2C). As for a preventive effect on Mtb infection in the lung, the mice immunized with rhPIV2–Ag85B clearly showed a substantial reduction in CFU.

3.3. Ag85B-specific immune response is elicited by rhPIV2–Ag85B administration

The capacity of rhPIV2–Ag85B intranasal immunization to elicit effector cells that recognize endogenously expressed Ag85B was assessed. Spleen, pLN, and BAL cells obtained from immunized mice were re-stimulated *in vitro* with syngeneic spleen cells infected with the recombinant vaccinia virus expressing Ag85B, and endogenously expressed Ag85B-specific cellular immune response was examined by ELISPOT assays. Both $CD4^+$ and $CD8^+$ splenocytes exhibited Ag85B-specific responses, and $CD8^+$ T cells showed much stronger responses than those of $CD4^+$ T cells in splenocytes from mice immunized with rhPIV2–Ag85B (Fig. 3A). Ag85B-specific responses were also seen in both $CD4^+$ and $CD8^+$ T cells at almost the same levels in pLN and BAL cells (Fig. 3B and C).

3.4. Analysis of Ag-specific effector cells and immune responses in pLN cells and the lung

Delayed initial activation of effector cells in lungs has been reported in the case of Mtb infection [15]. To control bacterial expansion in the early phase of infection, rapid Mtb Ag-specific $CD4^+$ T cell responses are required. Thus, we next analyzed recruitment of Ag85B-specific IFN-γ^+ $CD4^+$ T cells in pLN and BAL cells in mice immunized with rhPIV2–Ag85B. Mice were intranasally immunized with rhPIV2–Ag85B or the control vector virus 3 times

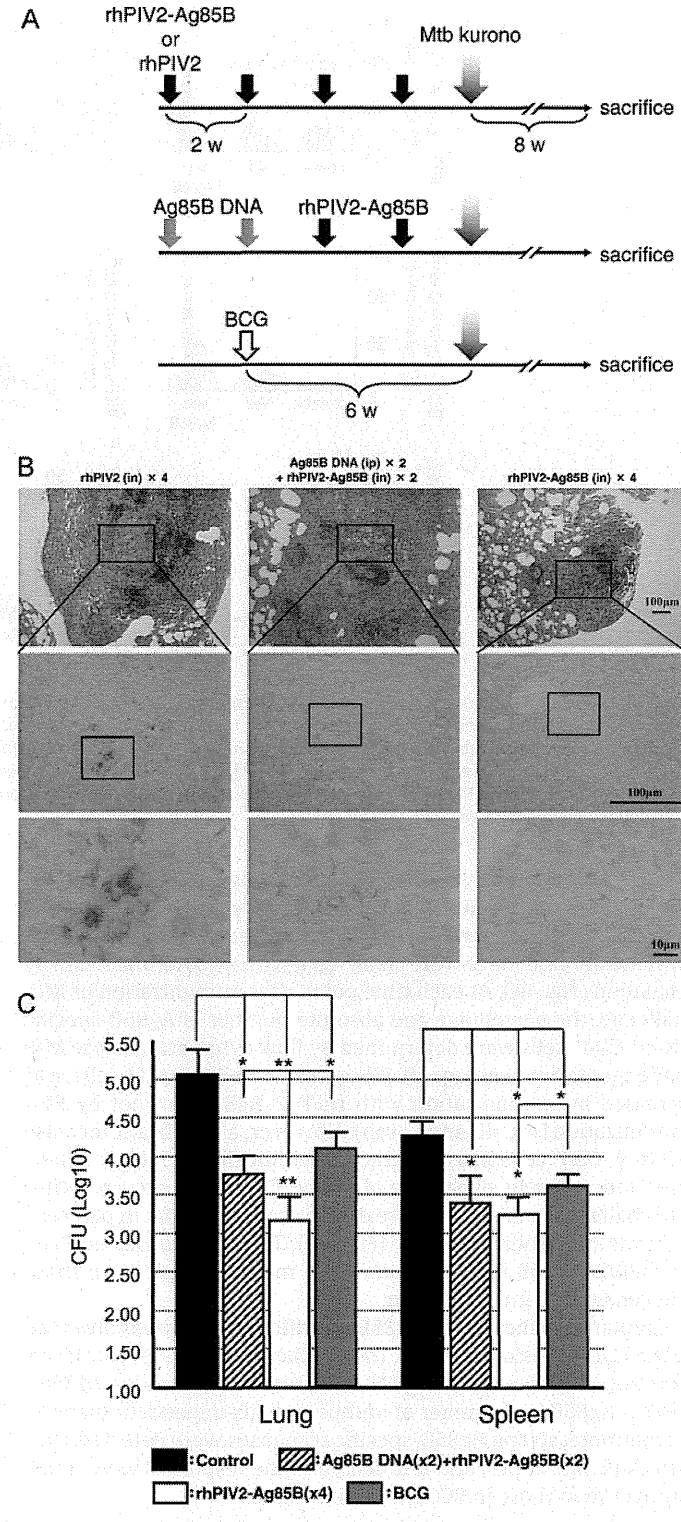

Fig. 2. Repeated immunization with rhPIV2–Ag85B results in protection from TB. (A) Groups of mice were vaccinated in this schedule. (B) Histological images of the lungs of Mtb-infected mice. Groups of mice ($n = 10$) immunized 4 times with rhPIV2 (left panel), 2 times with Ag85B DNA vaccine and 2 times with rhPIV2–Ag85B (middle panel) or 4 times with rhPIV2–Ag85B (right panel) were challenged by Mtb infection. Arrows point to tubercles. Lower panels in (B) show magnified images of images in the middle panels. (C) Inhibition of bacterial growth by immunization with rhPIV2–Ag85B in the lung and spleen. Groups of mice immunized 2 times with Ag85B DNA vaccine and 2 times with rhPIV2–Ag85B or immunized 4 times with rhPIV2–Ag85B or BCG were challenged by Mtb infection. The numbers of Mtb CFU in the lung and spleen were determined by a colony enumeration assay. The bacterial load is represented as mean $\log_{10}$ CFU per organ. Error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.05$, **, $P < 0.005$).

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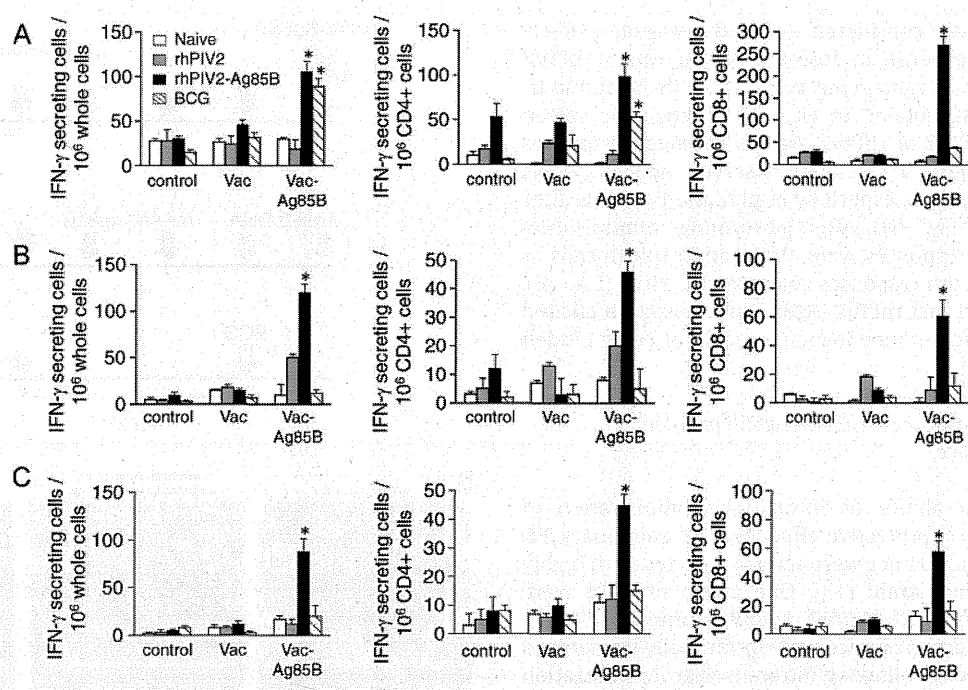

Fig. 3. Induction of Ag85B-specific cellular immune responses in rhPIV2–Ag85B-immunized mice. Mice were immunized with rhPIV2, rhPIV2–Ag85B, or BCG ($n = 5$ per group) according to the schedule shown in Fig. 2A. Two (rhPIV2 or rhPIV2–Ag85B) or 4 weeks (BCG) after the final immunization, the spleen, pLN, and BAL were collected. Isolated cells from the spleen (A), pLN (B), or BAL (C) were separated into whole (left panels), $CD4^+$ (middle panels), and $CD8^+$ (right panels) T cells and examined for IFN-γ production in an ELISPOT assay. These cells were stimulated *in vitro* with syngeneic spleen cells infected with control vaccinia virus (Vac) or recombinant vaccinia virus carrying the Ag85B gene (Vac-Ag85B) for 24 h. Error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.01$ compared to the group stimulated with Vac).

at 2-week intervals. Another group of mice were immunized with BCG by subcutaneous injection. Two weeks (rhPIV2–Ag85B-immunized mice) or 6 weeks (BCG-immunized mice) after the final immunization, all mice were challenged with Mtb Kurono strain by inhalation (Fig. 4A). At each time point after immunization or Mtb challenge, the percentage and absolute number of Ag85B-specific IFN-γ^+ $CD4^+$ cells were determined by flow cytometry. Before Mtb challenge, the percentage of IFN-γ^+ $CD4^+$ cells in pLN cells was increased by immunization with rhPIV2–Ag85B but not by BCG immunization (Fig. 4B and C, top). However, a significant increase in IFN-γ^+ $CD4^+$ cells was not detected in BAL cells (Fig. 4B and C, bottom). Interestingly, expansion of IFN-γ^+ $CD4^+$ cells occurred after Mtb challenge in BAL cells more dramatically than that in pLN cells in terms of absolute number (Fig. 4C). These responses induced by rhPIV2–Ag85B immunization were much stronger than those induced by BCG immunization.

Similarly, an increase in Ag85B-specific responses was observed by the ELISPOT assay (Fig. 4D). The number of Ag85B-specific IFN-γ secreting cells increased in pLN cells from mice immunized with rhPIV2–Ag85B in a number of immunizations-dependent manner. Furthermore, strong Ag85B-specific responses were detected after Mtb challenge in pLN and BAL cells, and the responses were much stronger than those in BCG immunized mice.

3.5. *rhPIV2–Ag85B induces innate immune responses*

We explored innate immune responses induced by rhPIV2–Ag85B infection. We confirmed that Ag85B did not affect the viability of rhPIV2–Ag85B infected cells (Supplemental Fig. 1) [44–46]. Type I IFNs were assessed after infection with rhPIV2–Ag85B in NHBE and BEAS cells as an indication of innate immune responses. Both types of cells showed mRNA expression of type I IFNs after infection with rhPIV2–Ag85B but not after addition of recombinant Ag85B protein (Fig. 5A). Production of IFN-β was also detected in the culture supernatant by ELISA (Fig. 5B). The mRNA expression of intracellular receptors, RIG-I, MDA5, and TLR3, and the induction of cytokines, IL-6 and IL-15 were also enhanced by infection with rhPIV2–Ag85B, whereas these effects were not observed with the addition of recombinant Ag85B protein (Fig. 5C and D). Furthermore, the expression of ICAM-1 was induced by infection with rhPIV2–Ag85B (Fig. 5E). Similar results were obtained after infection with rhPIV2 vector alone or rhPIV2-GFP (Supplemental Fig. 2). Other co-stimulation molecules, CD80, CD86, ICAM-2 and selectin, were not detected (data not shown).

To further investigate the participation of these receptors in innate immune activation induced by rhPIV2–Ag85B infection, expression of these receptors was knocked down by transfecting siRNA. At 48 h after transfection with siRNA, expression levels of these receptors were reduced by approximately 90% or expression was no longer detectable (Fig. 5F). IFN-β production induced by rhPIV2–Ag85B infection was inhibited when the cells were treated with RIG-I siRNA. For other receptors, MDA5 and TLR3, siRNA treatment did not result in inhibition of IFN-β production induced by rhPIV2–Ag85B infection (Fig. 5G). This result was confirmed by phosphorylation of IRF3, which is a downstream molecule of RIG-I in epithelial cells. The phosphorylation of IRF3 induced by rhPIV2–Ag85B infection was inhibited when epithelial cells were treated with siRNA of RIG-I (Fig. 5H).

4. Discussion

In the present study, we demonstrated the effectiveness of hPIV2 vectors for TB vaccines to induce systemic and mucosal immune responses. The rhPIV2 vector is a weak immunogenic; however, intranasal immunization with rhPIV2–Ag85B showed more potent protection against pulmonary TB in BALB/c mice than did conventional BCG vaccination. The rhPIV2–Ag85B shows a vaccine effect by itself alone, and this effect is more useful than the effects of other vectors for TB vaccines.

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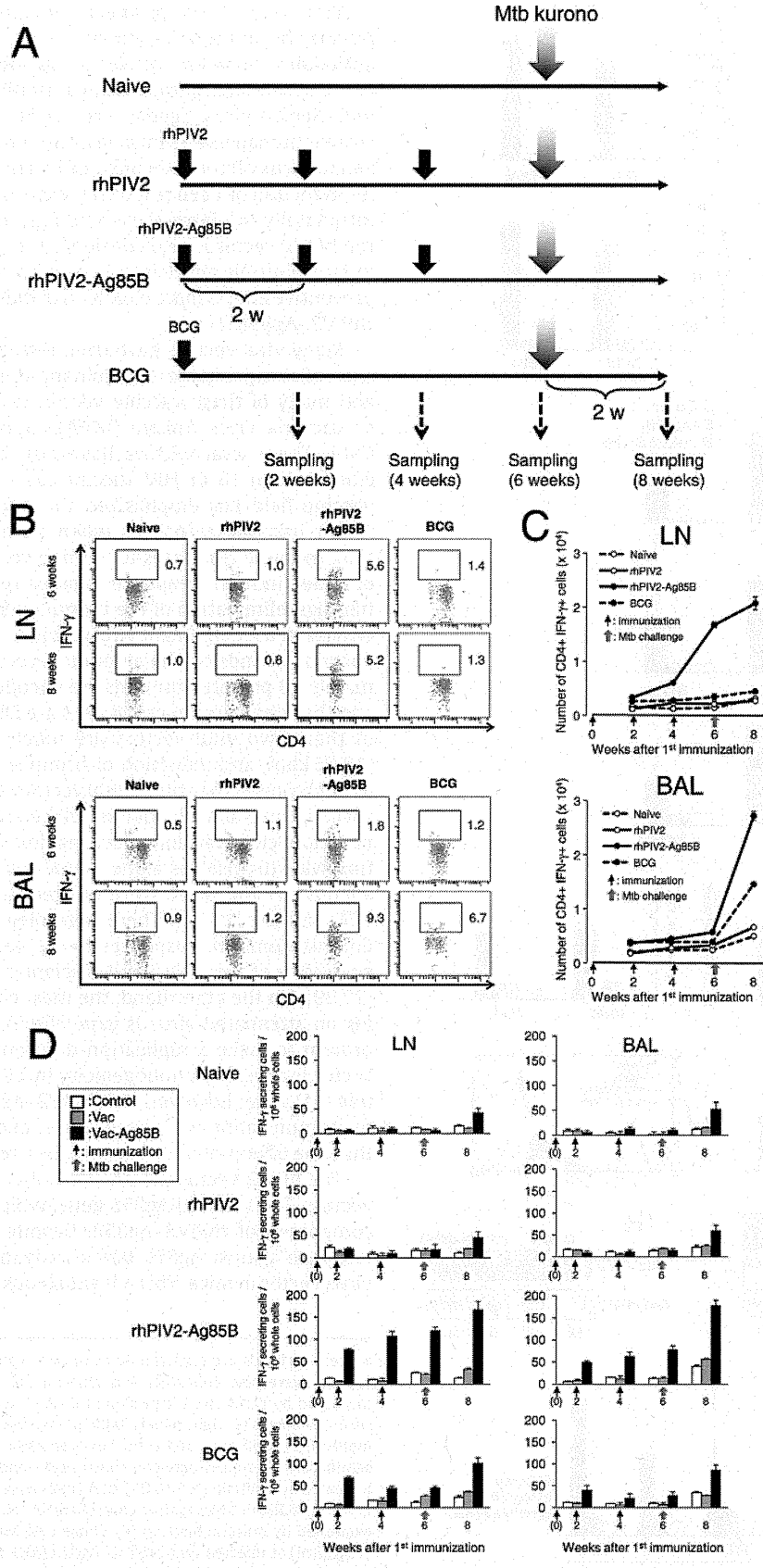

Fig. 4. Analysis of Ag-specific effector cells and these immune responses in pLN and BAL. (A) Groups of mice were immunized with rhPIV2, rhPIV2–Ag85B, or BCG ($n = 10$ per group) and challenged by Mtb infection in this schedule. (B) Representative flow cytometry plots of IFN-γ^+ cells on gated CD4$^+$ cells from pLN (top panels) and BAL (bottom panels) are shown. Numbers shown beside the gates represent the percentages within CD4$^+$ T cells. (C) Kinetics of recruitment of Ag85B-specific IFN-γ^+ cells in pLN (top panel) and BAL (bottom panel). Absolute numbers of IFN-γ^+ CD4$^+$ cell populations at each time points are shown. Error bars represent standard deviations. (D) Isolated cells from the pLN and BAL at each time point were examined for IFN-γ production in an ELISPOT assay. These cells were stimulated *in vitro* with syngeneic spleen cells infected with control vaccinia virus (Vac) or recombinant vaccinia virus carrying the Ag85B gene (Vac-Ag85B) for 24 h. Error bars represent standard deviations.

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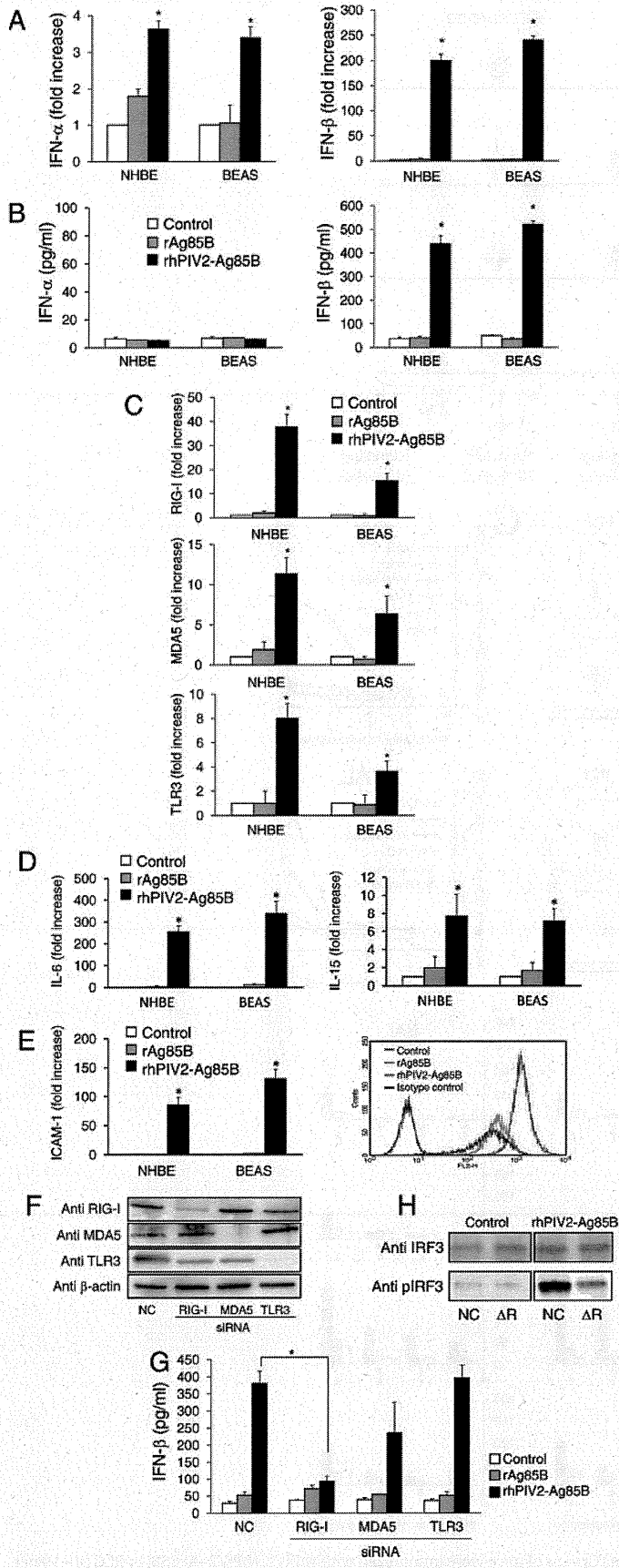

Fig. 5. Evaluation of adjuvant activity of rhPIV2–Ag85B *in vitro*. NHBE and BEAS cells were treated with rAg85B protein (10 μg/ml) or infected with rhPIV2–Ag85B (MOI of 10) for 24 h, and the increases in mRNA levels of IFN-α, IFN-β (A), RIG-I, MDA5, TLR3 (C), IL-6, IL-15 (D), and ICAM-1 (E, left panel) were determined by real-time PCR. Fold increase of each target gene was normalized to β-actin, and the expression levels are represented as relative values to the control. Culture supernatants were also collected, and amounts of secreted IFN-α and IFN-β were measured by ELISA (B). Expression of ICAM-1 was also confirmed by FACS analysis in BEAS cells (E, right panel). Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations. Statistically significant differences between control cells and rhPIV2–Ag85B-infected cells are indicated by asterisks (*, $P < 0.01$). BEAS cells were treated with siRNA targeting RIG-I, MDA5, TLR3, or the negative control siRNA (NC) for 48 h. Depletion of them was examined by immunoblotting (F). Those cells were stimulated by rAg85B protein (10 μg/ml) or infected with rhPIV2–Ag85B (MOI of 10) and then production of IFN-β was measured by ELISA (G). Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.01$ compared to NC). The effects of depletion of RIG-I on IRF3 phosphorylation were tested. BEAS cells treated with NC or siRNA targeting RIG-I (ΔR) for 48 h were infected with rhPIV2–Ag85B or not infected (control). Whole IRF3 and phosphorylated IRF3 (pIRF3) were detected by immunoblotting 6 h after infection (H).

Viral vectors are promising vaccine candidates for eliciting Ag-specific immune responses [16,17]. Pre-existing anti-vector antibodies, however, constitute an obstacle for use in humans [18–20]. Although antibodies against hPIV are known to cross-react with Sendai virus, Sendai virus vector is considered to be effective for human use by intranasal administration [21]. Additionally, Sendai virus vector is not affected by antibodies against Sendai virus for induction of T cell responses, especially when it is administered intranasally [4]. From these findings, intranasal administration of the hPIV2 vector is also considered to be effective for human use. In fact, multiple administrations with rhPIV2–Ag85B also showed preventive effects more clearly than did immunization 2 times with rhPIV2–Ag85B (Fig. 2).

Many viral vectors have been tested as recombinant viral vaccines eliciting suitable recombinant Ag-specific immune responses, and many of these vaccine vectors are not vaccine viruses such as vaccinia virus Ankara (MVA), adenovirus, Sendai virus, and CMV. These viral vectors have also been used in several vaccine trials in TB or HIV vaccine [22–24]. Experience in the HIV vaccine field has emphasized the importance of avoiding anti-vector immune responses when developing a vectored vaccine [25]. Immune responses to vaccine vectors prevent the induction of aimed immune responses against recombinant Ag. From these findings, elimination of the immunogenicity of a vaccine vector is critical for a recombinant viral vaccine. The immunogenicity of viral vectors depends on the amount of vector viral proteins. Approximately 80 poxvirus proteins are encoded by its over 130–300 kbp and the adenovirus genome sizes are 26–45 kbp. The genome sizes of these two viral vectors are much larger than that of hPIV2 (15.65 kbp), and induction of immune responses to hPIV2 vector might be lower than other viral vectors. In TB vaccines, recombinant vaccinia virus and adenovirus, which are immunogenic viruses, did not show clear vaccine effects against TB infection by immunization with themselves alone. These two recombinant TB vaccines, adenovirus and MVA, were utilized as boost immunization after BCG priming [26,27]. These heterologous prime-boost strategies diminish immune responses to the vector virus and indicate the possibility of a practical and efficient strategy for prevention of TB [28,29]. On the other hand, the most common method for obtaining an attenuated virus is gene elimination of the viral construct protein to make a replication-deficient virus *in vivo*. The rhPIV2 vector is a weak immunogenicity by elimination of structural protein (M) gene; however, the rhPIV2–Ag85B shows a vaccine effect by immunization with itself alone, and this effect is more useful than the effects of other vectors for a recombinant TB vaccine.

The hPIV2 vector has an additional advantage over other viral vectors. The inserted Ag85B gene, which is only 978 bp, is a minor component of rhPIV2–Ag85B. Despite that, the cellular immune response against Ag85B had an advantage over that against the virus vector in mice. This advantageous effect is thought to depend

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on Ag85B expression mechanisms. The frequency with which viral RNA polymerase reinitiates the next mRNA at gene junctions is imperfect, and this leads to a gradient of mRNA abundance that decreases according to distance from the genome 3′ end [30]. Insertion of the Ag85B gene into the 3′ proximal first locus between the leader sequence and the NP gene results in the highest level of gene expression. Ag85B is transcribed earlier and more abundantly than other viral products (Fig. 1B and C). This property of rhPIV2–Ag85B leads to elicit stronger Ag85B-specific immune responses than vector-specific responses in our system (Fig. 1D and E), although recombinant virus vaccine immunization usually induces overwhelming viral-specific immune responses compared with an inserted gene product [31,32]. We also demonstrated that intranasal administration of the rhPIV2 vector had no adverse effects and provided sufficient immunogenicity and a sufficient vaccine effect against Mtb in mice. These results suggest that intranasal administration of rhPIV2–Ag85B does not cause functional failure as a vaccine by multiple administrations, and these features of the rhPIV2 vector are definitely advantages for clinical use.

Another major feature of rhPIV2–Ag85B is effective prevention of TB by intranasal administration. Vaccination in the respiratory tract may enhance protection against Mtb infection, since Mtb initially establishes infection on mucosal surfaces of the respiratory tract. Indeed, a number of recombinant TB vaccines have been developed and evaluated for respiratory mucosal immunization [33–35]. It is important to note that lack of Ag-specific effector cells persists even up to about 21 days after pulmonary Mtb infection caused by a bacterial component [15,36]. In the present study, the arrival of Ag-specific T cells was detected in lung and pLN by rhPIV2–Ag85B immunization, and this arrival of effector cells was recognized faster than BCG immunization after Mtb challenge (Fig. 4B and C). We were able to establish a novel intranasal vaccine, rhPIV2–Ag85B, against TB by utilizing various advantages of intranasal administration. Nasal administration of a vaccine to induce mucosal and systemic immune responses has several advantages other than the induction of effective immune responses. It is even possible that intranasal administration of replication-incompetent rhPIV2–Ag85B limits the areas of infection in respiratory organs and induces a respiratory tract mucosal immune response in addition to a systemic immune response against TB. Our study suggested that intranasal administration of rhPIV2–Ag85B, which can induce both mucosal and systemic immune responses against Mtb, has a great advantage as a TB vaccine.

Attempts have been made to use various types of adjuvants for enhancing an immune responses to vaccines, including vaccines against TB [37]. In fact, a protein-based TB vaccine required the addition of an adjuvant to induce effective immune responses [38–41]. For the generation of adaptive immune responses, induction of innate immunity is crucial for vaccines to elicit potent Ag-specific immune responses. Pattern recognition receptors have been studied as potential targets for an adjuvant. dsRNA is a dominant activator of innate immunity because viral dsRNA is recognized by TLR3, RIG-I, and MDA5 [42,43]. As a result, it was demonstrated that the rhPIV2 vector had a potent adjuvant activity as dsRNA recognized by the RIG-I receptor and enhanced not only local innate immunity but also systemic adaptive immunity. It is possible that no extra addition of an adjuvant is required to prevent TB by vaccination with rhPIV2–Ag85B. Furthermore, the inhibitory effects on the growth of rhPIV2–Ag85B *in vivo* by IFN through the innate receptor are not required to consider since the rhPIV2 vector is replication-incompetent *in vivo* by elimination of the M gene (Fig. 1A).

In summary, our results provide evidence for the possibility of rhPIV2–Ag85B as a novel intranasal vaccine for eliciting Mtb-specific mucosal immunity. Immunization with rhPIV2–Ag85B showed significant protection against TB without any prime vaccine or addition of an adjuvant in mice. Further studies will contribute to the ultimate goal of establishing a new vaccine strategy that can definitely prevent Mtb infection.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.11.108.

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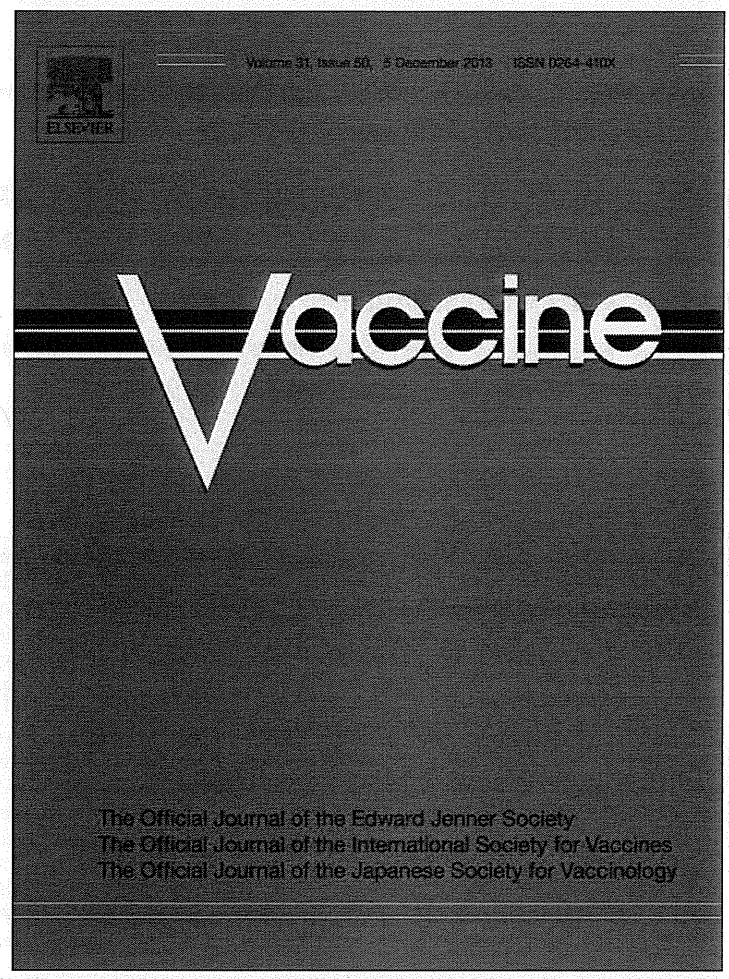

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DNA vaccine expressing the non-structural proteins of hepatitis C virus diminishes the expression of HCV proteins in a mouse model

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ABSTRACT

Most of the people infected with hepatitis C virus (HCV) develop chronic hepatitis, which in some cases progresses to cirrhosis and ultimately to hepatocellular carcinoma. Although various immunotherapies against the progressive disease status of HCV infection have been studied, a preventive or therapeutic vaccine against this pathogen is still not available. In this study, we constructed a DNA vaccine expressing an HCV structural protein (CN2), non-structural protein (N25) or the empty plasmid DNA as a control and evaluated their efficacy as a candidate HCV vaccine in C57BL/6 and novel genetically modified HCV infection model (HCV-Tg) mice. Strong cellular immune responses to several HCV structural and non-structural proteins, characterized by cytotoxicity and interferon-gamma (IFN-γ) production, were observed in CN2 or N25 DNA vaccine-immunized C57BL/6 mice but not in empty plasmid DNA-administered mice. The therapeutic effects of these DNA vaccines were also examined in HCV-Tg mice that conditionally express HCV proteins in their liver. Though a reduction in cellular immune responses was observed in HCV-Tg mice, there was a significant decrease in the expression of HCV protein in mice administered the N25 DNA vaccine but not in mice administered the empty plasmid DNA. Moreover, both $CD8^+$ and $CD4^+$ T cells were required for the decrease of HCV protein in the liver. We found that the N25 DNA vaccine improved pathological changes in the liver compared to the empty plasmid DNA. Thus, these DNA vaccines, especially that expressing the non-structural protein gene, may be an alternative approach for treatment of individuals chronically infected with HCV.

1. Introduction

Infection with hepatitis C virus (HCV) can lead to chronic hepatitis and ultimately death through liver failure or onset of carcinoma [1]. Although various immunotherapies against the progressive disease status of HCV infection have been studied, a preventive or therapeutic vaccine against this pathogen is still not available. Therefore, the development of effective vaccines, especially therapeutic vaccines, is needed to control the progressive disease of HCV.

Acute infections are characterized by high frequencies of HCV-specific CD8 and CD4 T cell responses that can persist for a long time after the clearance of viremia and recovery from the infection [2,3]. On the other hand, individuals who remain chronically infected have weak or undetectable cellular immune responses to HCV antigens [4–6]. It has been reported that HCV evades immune responses by suppression of the activity of effector T cells and establishes persistent infection [7,8]. Therefore, activation of cellular immune responses to HCV might lead to improvement of the pathological condition caused by HCV.

The use of a DNA vaccine is an attractive approach for generating antigen-specific immunity to various pathogens because of its stability and simplicity of delivery. Many studies on DNA vaccines against HCV infection have been performed in mouse systems [9–22]. On the other hand, there have been a few studies in which the therapeutic effect of DNA vaccines was investigated in chronic HCV carrier model mice [23–25]. Furthermore, a conventional transgenic mouse model was used as a chronic HCV carrier status in those studies. Unfortunately, those transgenic mice were immunotolerant to their expressed HCV protein, and the immune status of the mice was therefore different from that of patients with chronic HCV infection.

To overcome the limitation of these mice, we used novel genetically modified (CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$) mice that conditionally

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express HCV cDNA, using Mx promoter-driven Cre recombinase with poly(I)·poly(C) induction. These mice expressed the HCV core protein consistently for at least 600 days and developed chronic active hepatitis, steatosis, lipid deposition, and hepatocellular carcinoma [26,27]. Since these pathological findings in the transgenic mice are very similar to those in humans with chronic HCV infection [27], it was thought that this mouse model of HCV would be useful for analyzing the immune responses to chronic hepatitis.

In this study, we constructed a DNA vaccine expressing an HCV structural protein (CN2) and non-structural protein (N25) and evaluated the efficacy of the vaccines as a candidate HCV vaccine in novel transgenic mice that conditionally express HCV cDNA.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from CLEA Japan. HCV-Tg mice CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ were previously described [27]. Mice used in this study (n=460) were 6-week-old males at the start of the experiment. HCV-Tg mice were injected intraperitoneally (i.p.) with 300 μg polyinosinic acid–polycytidylic acid (poly(I)·poly(c)) (GE Healthcare) three times at 48-h intervals to induce the expression of HCV protein 3 months before using the mice for experiments. Injection of CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice with poly(I)·poly(C) induces IFN production and the expression of CN2-29 or RzCN5-15 gene products in hepatocytes, hematopoietic cells (mainly in Kupffer cells and lymphocytes), and spleens but not in most other tissues [26]. All animals were cared for according to ethical guidelines approved by the institutional Animal Care and Use Committee of the National Institute of Biomedical Innovation.

2.2. Cells, virus, peptide

EL-4 transformants that expressed E2 (EL-4/E2), NS2 (EL-4/NS2) or NS3/4A (EL-4/NS3/4A) of HCV protein and LC16m8, a highly attenuated strain of vaccinia virus (VV), and a recombinant vaccinia virus (rVV) that encoded mainly structural proteins (core/E1/E2/NS2; amino acids (aa) 1–1320) (rVV-CN2) were previously described [27].

HCV NS3$_{1629–1637}$ peptide (GAVQNEITL) was synthesized by Toray Research Center (Tokyo).

2.3. DNA immunization

For DNA immunization, two different plasmids, CN2 and N25 expressing the HCV core/E1/E2/NS2 (aa 1–1320) and E2/NS2/3/4/5 (aa 542–3010) polyproteins under the control of the CAG promoter were constructed (Fig. 1). The PCR product of HCV cDNA from a type 1b strain (R6) cDNA containing the plasmid vector pBMSF7C [27] as an *Xba*I/*Xho*I fragment was cloned into a CAG expression plasmid, pCAGGS [28]. All plasmid DNAs were purified with an endotoxin-free plasmid extraction kit (Qiagen).

Mice were intramuscularly injected with 100 μg of the plasmid DNA in 25 μl PBS, and then the site of inoculation was immediately given an electric pulse by an Electric Square Porator (T820; BTX) as previously described [29]. One group of mice was boosted with the same amount of DNA at 2 weeks for ELISPOT and cytotoxicity assay. Another group of mice were also boosted with the same amount of DNA at 2 and 4 weeks for histopathological examination.

2.4. ELISPOT assay

IFN-γ ELISPOT assay was performed according to the manufacturer's protocol (Mabtech). Briefly, total spleen cells

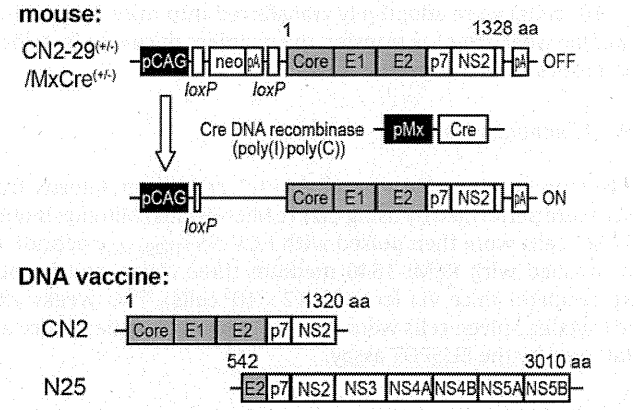

Fig. 1. Generation of HCV DNA vaccines. HCV gene structure in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and schematic diagram of plasmid constructs expressing an HCV structural protein (CN2) and a non-structural protein (N25). CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express partial (nucleotides 294–3435, including the viral genes that encode the core, E1, E2, and NS2 proteins) of HCV genotype 1b cDNA. The expression of HCV proteins is regulated by the Cre/loxP switching expression system. pCAG, CAG promoter; pMx, Mx1 promoter; neo, neomycin-resistant gene; pA, poly A signal.

(1×10^5 cells/well), CD8$^+$ T cells or CD4$^+$ T cells (2×10^5 cells/well) were incubated with mitomycin C-treated EL-4 cells expressing HCV protein (1×10^4 cells/well) or with mitomycin C-treated syngenic splenocytes infected with an rVV carrying the cDNA of HCV Core-NS2 antigens (4×10^4 cells/well) or HCV NS3$_{1629–1637}$ peptide (0.1 μg/ml) at 37 °C for 48 h in a 96-well plate coated with anti-mouse IFN-γ mAb. Cells were removed and the plate was stained with biotinylated anti-mouse IFN-γ mAb, streptavidin-HRP, and a DAB Substrate kit for peroxidase (Vector Laboratories). Test wells were assayed in duplicate and antigen-specific T cells was calculated after subtracting the mean number of spots obtained in the absence of stimulation.

2.5. Generation of CTL effector cells and cytotoxicity assay

Spleen cells (1×10^7 cells) were co-cultured with mitomycin C-treated EL-4/NS2 cells (2×10^6 cells). The effector cells generated were harvested after 5 days of culture. ^{51}Cr-labeled target cells (EL-4/NS2) were incubated for 4 h with effector cells. Specific lysis was calculated as previously described [29].

2.6. Quantification of HCV core proteins

HCV core protein concentrations were determined with ELISA kit (Ortho-Clinical Diagnostics) as previously described [27]. The HCV protein concentration in the tissue samples was divided by the total protein concentration and expressed as pg/mg of total protein.

2.7. Histopathological examination

Liver tissues were fixed in 10% phosphate-buffered formalin. Sections of paraffin-embedded tissue were cut at 4 μm in thickness and stained with hematoxylin and eosin (H&E).

2.8. Adoptive transfer of cells

For adoptive transfer experiments, spleen cells were isolated from C57BL/6 mice that had been immunized twice with N25 DNA vaccine. CD8$^+$, CD4$^+$, and CD8$^-$CD4$^-$ cells were prepared by using CD8a (Ly-2) and CD4 (L3T4) MicroBeads according to manufacturer's instructions (Miltenyi Biotec). Whole spleen cells (1×10^8 cells) or purified CD8$^+$, CD4$^+$ or CD8$^-$CD4$^-$ cells

(1×10^7 cells) were adoptively transferred into mice by i.p. injection. One week after the transfer, the recipient mice were sacrificed and tissues were analyzed.

2.9. DC immunization

Isolation and purification of $CD11c^+$ cells from spleens from mice were performed by using CD11c MicroBeads (Miltenyi Biotec). $CD11c^+$ cells were then pulsed with HCV $NS3_{1629-1637}$ peptide for 5 h, washed with RPMI-1640 medium three times, and injected into recipient mice via footpads (2×10^5 cells). Two weeks after the transfer, spleen cells were isolated from the recipient mice and examined by the ELISPOT assay.

2.10. Statistics

Statistical significance ($P < 0.05$) was determined by 2-tailed Student's *t* test or ANOVA followed by Ryan's test.

3. Results

3.1. Immunization of C57BL/6 mice with DNA vaccine induces strong HCV-specific cellular immune responses

To analyze the cellular immune responses induced by the DNA vaccine, C57BL/6 mice were immunized twice with the DNA vaccine at a 2-week interval. Strong cellular immune responses to several HCV structural and non-structural proteins characterized by IFN-γ production (Fig. 2A) and cytotoxicity (Fig. 2B) were observed in CN2 or N25 DNA vaccine-immunized mice but not in mice injected with the empty plasmid DNA. We next assessed the activity of $CD8^+$ and $CD4^+$ T cells in mice immunized with the DNA vaccine. Purified $CD8^+$ or $CD4^+$ T cells from the spleen were re-stimulated in vitro with syngenic splenocytes infected with an rVV carrying the cDNA of HCV Core-NS2. Significant IFN-γ production in $CD8^+$ and $CD4^+$ T cells was observed in CN2 and N25 DNA-immunized mice but not in mice injected with the empty plasmid DNA, and the responses of $CD8^+$ T cells were much stronger than those of $CD4^+$ T cells (Fig. 2C).

3.2. Immunization with N25 DNA vaccine showed therapeutic effects in the liver of HCV transgenic mouse

We next assessed the expression of HCV protein in the liver after immunization with DNA vaccine using HCV-Tg mice. Three months after induction of HCV protein by poly(I)·poly(C) injection, $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice were immunized twice with the DNA vaccine at a 2-week interval. Immunization of mice with the N25 DNA vaccine resulted in reduced expression of HCV protein in the liver of $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice compared with the expression in mice injected with the empty plasmid DNA (Fig. 3A). Pathological changes in the liver after immunization with the DNA vaccine were also examined. Pathological changes, including swelling of hepatocytes and abnormal architecture of liver cell cords were observed in both empty plasmid DNA-immunized or CN2 DNA-immunized mice. However, these pathological changes in the liver were improved by the N25 DNA vaccine (Fig. 3B). These results suggested that the N25 DNA vaccine has a potential as a therapeutic vaccine for HCV infection.

3.3. $CD8^+$ and $CD4^+$ T cells are required for the decrease of HCV protein in the liver

We next searched for effector cells having the ability to reduce the expression of HCV protein in the N25 DNA-immunized mice. Whole spleen cells, $CD8^+$ T cells and $CD4^+$ T cells were obtained from spleens of C57BL/6 mice immunized with the N25 DNA vaccine. These cells were adoptively transferred into $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice that expressed HCV proteins. The adoptive transfer of unfractionated spleen cells, $CD8^+$ T cells or $CD4^+$ T cells decreased the expression HCV protein in the liver (Fig. 4). These results indicated that both $CD8^+$ and $CD4^+$ T cells were required for the decrease of HCV protein in the liver.

3.4. Immunization of $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice with DNA vaccine failed to induce strong HCV-specific cellular immune responses

Cellular immune responses induced by the DNA vaccine in $CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$ mice were also assessed. Unlike in WT (C57BL/6) mice, cellular immune responses to several HCV structural and non-structural proteins were reduced in the N25 DNA vaccine-immunized HCV-Tg ($CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$) mice (Fig. 5A). CD4 or CD8 T cell responses to HCV antigens were abolished in the CN2 DNA vaccine-immunized HCV-Tg mice. On the other hand, the N25 DNA vaccine elicited HCV-specific CD4 and CD8 T cell responses in HCV-Tg mice. The levels of CD4 T cell responses were equivalent to those in WT mice; however, CD8 T cell responses were weak compared with those in WT mice (Fig. 5B). These reductions of cellular immune responses were also observed in another strain of HCV-Tg ($RzCN5\text{-}15^{(+/-)}/MxCre^{(+/-)}$) mice that possessed the full-length cDNA of HCV (Fig. 5C and D).

3.5. Ability of DCs to induce HCV-specific CD8 T cells in HCV transgenic mice was not impaired

In the present study, the activity of effector cells induced by the DNA vaccine was thought to be suppressed in HCV-Tg mice. To explore these inhibitory effects on HCV-specific cellular immune responses by the DNA vaccine in HCV-Tg mice, adoptive transfer of effector cells was performed. Spleen cells were isolated from C57BL/6 mice that had been immunized with the N25 DNA vaccine. When these cells were transferred into C57BL/6 or HCV-Tg mice, nearly equal levels of CTL activities were detected in the recipient mice (Fig. 6A), suggesting that immunosuppressive mechanisms of IFN-γ production by mature CTLs did not exist in HCV-Tg mice.

Dendritic cells (DCs) play a critical role in the induction of immune responses by DNA vaccination [30]. Moreover, several studies have demonstrated that HCV impaired the function of DCs [31–34]. To assess DC function in HCV-Tg mice, DCs were freshly purified from spleens of WT and HCV-Tg ($RzCN5\text{-}15^{(+/-)}/MxCre^{(+/-)}$) mice, loaded with NS3 peptide, and transferred into WT and HCV-Tg mice. Two weeks later, the functional status of CD8 T cells in the recipient mice was evaluated. DC functions to induce HCV-specific CD8 T cell responses were not different in WT and HCV-Tg mice; however, NS3-specifc CD8 T cell responses in HCV-Tg mice that had been injected with DCs of either WT or HCV-Tg mice were much weaker than those in WT mice (Fig. 6B). These results indicated that the ability of DCs to induce HCV-specific CD8 T cells in HCV-Tg mice was not impaired.

4. Discussion

It has been reported that DNA vaccines elicited strong and long-lasting humoral and cell-mediated immune responses against pathogenic agents such as HBV, HIV, tuberculosis and malaria and that they had many advantages over traditional vaccines