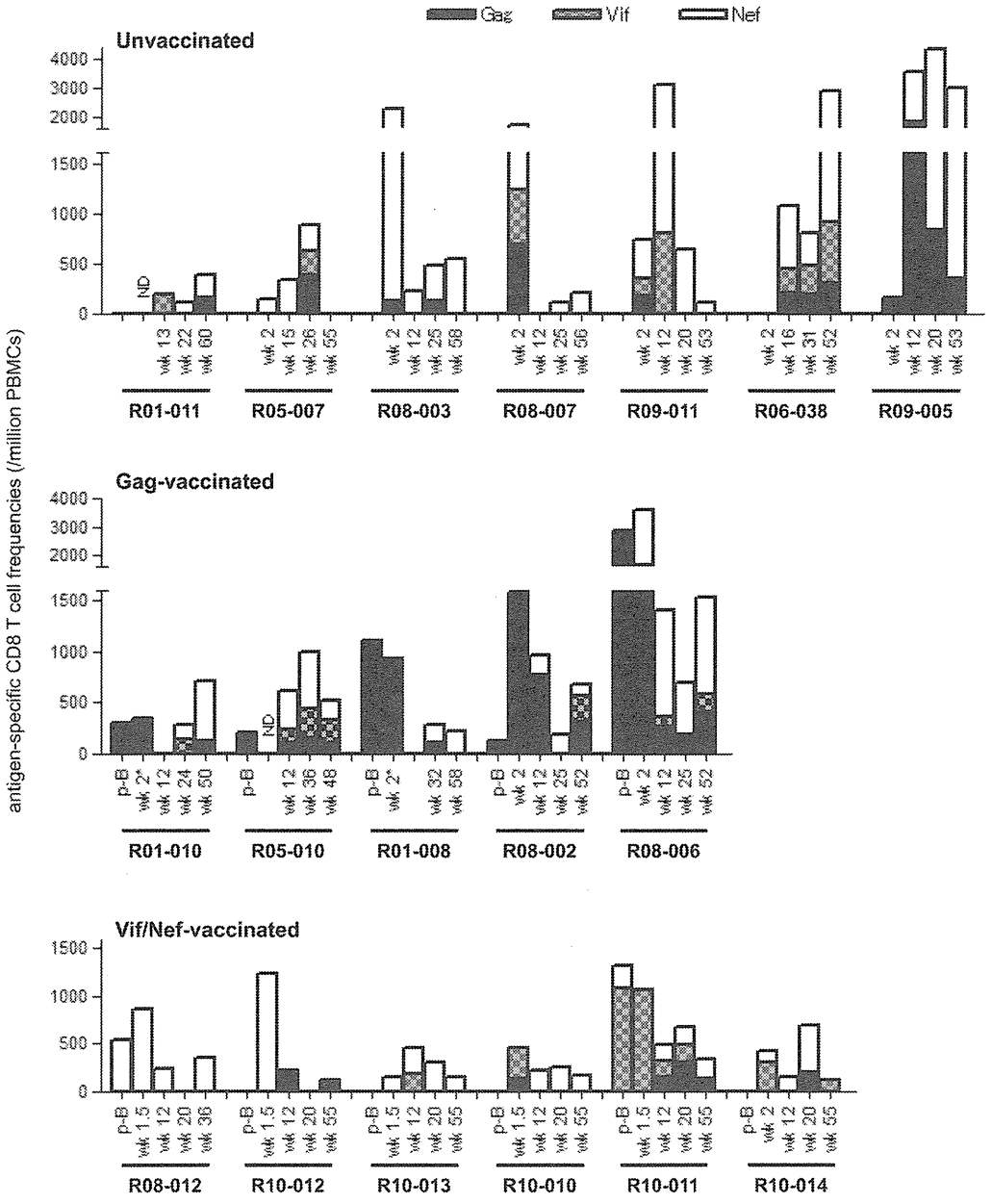

FIG 3 SIV Gag/Vif/Nef-specific CD8$^+$ T-cell responses in macaques. We examined CD8$^+$ T-cell responses specific for Gag, Vif, and Nef 1 week after SeV-Gag boost (p-B) and approximately 2 weeks, 3 months, 6 months, and 1 year after SIV challenge in unvaccinated (top), Gag-vaccinated (middle), and Vif/Nef-vaccinated (bottom) animals. We examined only Gag-specific CD8$^+$ T-cell responses but not Vif- or Nef-specific ones at week 2 in macaques R01-010 and R01-008 (indicated by asterisks). ND, not determined.

quencies in the acute phase (data not shown). The sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase, however, was significantly higher in the controllers than in the noncontrollers ($P = 0.0031$ by Mann-Whitney U test) (Fig. 4A). Indeed, the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase was inversely correlated with postpeak plasma viral loads ($P = 0.0268$, $R = -0.5205$ with viral loads at 3 months [data not shown]; $P = 0.0017$, $R = -0.6849$ with viral loads at 1 year [Fig. 4B] by Pearson test). When we focused on seven unvaccinated and five Gag-vaccinated animals, three Gag-vaccinated controllers showed significantly higher Gag-specific CD8$^+$ T-cell frequencies in the acute phase than the remaining nine noncontrollers ($P = 0.0045$ by Mann-Whitney U test) (Fig. 4C). Also, in the analysis of seven unvaccinated and six Vif/Nef-vaccinated animals, Vif-specific CD8$^+$ T-cell frequencies in the acute phase were significantly higher in three Vif/Nef-vaccinated controllers than in the remaining 10 noncontrollers ($P = 0.0140$ by Mann-Whitney U test) (Fig. 4D). These results suggest that efficient Gag- or Vif-specific CD8$^+$ T-cell responses in the acute phase can result in SIV control.

Viral *gag*, *vif*, and *nef* mutations in vaccinated animals. We then tried to define the CD8$^+$ T-cell responses that might be contributing to the vaccine-based SIV control, although we were not able to map all of the CD8$^+$ T-cell epitopes because of sample

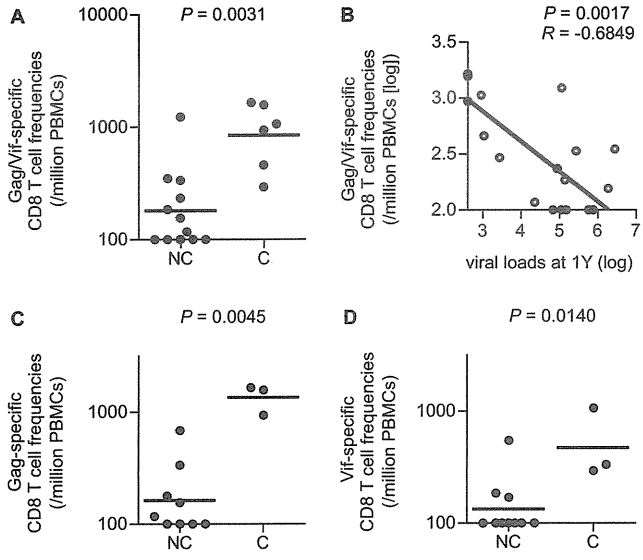

FIG 4 Comparison of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase between SIV controllers (C) and noncontrollers (NC). Data on Gag- and Vif-specific $CD8^+$ T-cell frequencies around week 2 postchallenge, which are shown in Fig. 3, were used. In macaques R01-011 and R05-010, samples at week 2 were unavailable, and data at week 12 were used. (A) Comparison of the sum of Gag- and Vif-specific $CD8^+$ T-cell frequencies (Gag/Vif-specific $CD8^+$ T-cell frequencies) between the controllers (three Gag-vaccinated and three Vif/Nef-vaccinated animals) and the noncontrollers in seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals ($n = 18$). The controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0031$ by Mann-Whitney U test). (B) Correlation analysis of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase with plasma viral loads at 1 year. The frequencies were inversely correlated with the viral loads ($P = 0.0017$, $R = -0.6849$ by Pearson test). (C) Comparison of Gag-specific $CD8^+$ T-cell frequencies in seven unvaccinated and five Gag-vaccinated animals ($n = 12$). The three Gag-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0045$ by Mann-Whitney U test). (D) Comparison of Vif-specific $CD8^+$ T-cell frequencies in seven unvaccinated and six Vif/Nef-vaccinated animals ($n = 13$). The three Vif/Nef-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0140$ by Mann-Whitney U test).

limitation. Among three Gag-vaccinated controllers, R01-008, R08-002, and R08-006, our previous study found $Gag_{367-381}$-specific $CD8^+$ T-cell responses at week 5 in macaque R01-008 (5). This animal showed rapid selection of a mutation leading to an isoleucine (I)-to-threonine (T) change at the 377th aa (I377T) in SIV Gag, which results in escape from $Gag_{367-381}$-specific $CD8^+$ T-cell recognition. This suggests that these $Gag_{367-381}$-specific $CD8^+$ T-cell responses may have played an important role in SIV control. Analysis in the present study found $Gag_{385-400}$-specific $CD8^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to an I-to-T change at the 391st aa (I391T) in Gag in macaque R08-006 (Fig. 5A). We confirmed that this I391T substitution results in escape from $Gag_{385-400}$-specific $CD8^+$ T-cell recognition (data not shown), suggesting a contribution of these $Gag_{385-400}$-specific $CD8^+$ T-cell responses to the control of SIV. Macaque R08-002 mounted $Gag_{273-292}$-specific $CD8^+$ T-cell responses but showed no *gag* mutation in the early phase. None of the noncontrollers selected *gag* mutations at week 5 or 6.

Among three Vif/Nef-vaccinated controllers, R10-010, R10-011, and R10-014 (Fig. 5B), macaque R10-010 mounted Vif_{65-76}-specific $CD8^+$ T-cell responses in the acute phase that resulted in the rapid selection of a mutation leading to a histidine (H)-to-tyrosine (Y) change at the 66th aa (H66Y) in Vif. Macaque R10-011 mounted $Vif_{113-132}$-specific and $Vif_{134-148}$-specific $CD8^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to a Y-to-cysteine (C) change at the 143rd aa (Y143C) in Vif. We confirmed that this Y143C substitution results in escape from $Vif_{134-148}$-specific $CD8^+$ T-cell recognition (data not shown). None of the noncontrollers selected *vif* mutations at week 5 or 6. These suggest that Vif_{65-76}-specific and $Vif_{134-148}$-specific $CD8^+$ T-cell responses contributed to SIV control in macaques R10-010 and R10-011, respectively. Macaque R10-014 mounted $Vif_{113-132}$-specific $CD8^+$ T-cell responses but showed no *vif* mutation in the early phase.

In E^+ macaques, $CD8^+$ T-cell responses specific for Nef_{38-66} and $Nef_{101-138}$ regions were frequently observed (see Fig. S2 in the supplemental material). In all three Gag-vaccinated controllers, we confirmed both Nef_{38-66}-specific and $Nef_{101-138}$-specific $CD8^+$ T-cell responses in the chronic phase, although we did not have available samples for analysis of these responses in the acute phase. In five Vif/Nef-vaccinated animals, we confirmed Nef_{38-66}-specific $CD8^+$ T-cell responses in the acute phase, followed by $Nef_{101-138}$-specific $CD8^+$ T-cell induction. Nef_{38-66}-specific $CD8^+$ T-cell responses became undetectable at week 12 in all the three noncontrollers but were maintained at detectable levels in controllers R10-010 and R10-011.

Further mapping defined the Nef_{45-53} $CD8^+$ T-cell epitope. Mutations in the Nef_{45-53}-coding region were selected after 1 year in five of seven unvaccinated E^+ animals. Rapid selection of mutations at this Nef_{45-53}-coding region in a month after SIV challenge was observed in both Gag-vaccinated noncontrollers and all three Vif/Nef-vaccinated noncontrollers (Fig. 5C). In contrast, out of six Gag-vaccinated or Vif/Nef-vaccinated controllers, only one animal (R10-010) rapidly selected a mutation in this region. We confirmed that the leucine (L)-to-proline (P) substitution at the 53rd aa (L53P) in Nef results in escape from Nef_{45-53}-specific $CD8^+$ T-cell recognition (data not shown). Thus, Nef_{45-53}-specific $CD8^+$ T-cell responses may have exerted strong suppressive pressure on SIV replication in the acute phase in Gag-vaccinated or Vif/Nef-vaccinated noncontrollers.

DISCUSSION

In this study, we examined efficacy of prophylactic DNA-prime/SeV-boost vaccines against SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype E. Our previous study indicated that unvaccinated E^+ animals show typical courses of SIV infection and AIDS progression (39). However, three of five Gag-vaccinated and three of six Vif/Nef-vaccinated E^+ animals controlled SIV replication, indicating a possibility of virus control by prophylactic vaccination.

Unvaccinated E^+ animals showed high-frequency Nef-specific $CD8^+$ T-cell responses, particularly specific for the Nef_{38-66} and $Nef_{101-138}$ regions, after SIVmac239 challenge. The Nef_{45-53} region is a candidate for a $CD8^+$ T-cell target associated with MHC-I haplotype E, and the NefL53P mutation resulting in escape from Nef_{45-53}-specific $CD8^+$ T-cell recognition was often selected in E^+ animals. These results imply suppressive pressure on SIV replication by Nef-specific $CD8^+$ T-cell responses in macaques sharing this MHC-I haplotype.

Gag-vaccinated animals elicited detectable Gag-specific $CD8^+$ T-cell responses after SeV-Gag boost. All three Gag-vaccinated

A

Gag-vaccinated controllers	Gag CD8 T cell targets	gag mutations at wk 5
R08-002	Gag273-292	none
R08-006	Gag385-400	I391T

B

Vif/Nef-vaccinated controllers	Vif CD8 T cell targets	vif mutations at wk 6
R10-010	Vif65-76	H66Y
R10-011	Vif113-132 & 134-148	Y143C
R10-014	Vif113-132	none

C

		Nef45-53 GLDKGLSSL			Nef45-53 GLDKGLSSL
Unvaccinated					
R01-011	1 mo	--------F	R09-011	1 mo	---------
	6 mo	-S-------		6 mo	--------P
	1 yr	----C----		1 yr	--G-----P
R05-007	1 mo	---------	R06-038	1 mo	---------
	6 mo	---------		6 mo	---------
	1 yr	---------		1 yr	---------
R08-003	1 mo	---------	R09-005	1 mo	---------
	6 mo	--G------		6 mo	--G------
	1 yr	--------R		1 yr	--G-----H
R08-007	1 mo	---------			
	6 mo	-S-------			
	1 yr	-S-------			
Gag-vaccinated non-controllers			controllers		
R01-010	1 mo	--------P	R01-008	1 mo	---------
	6 mo	--G-----P	R08-002	1 mo	---------
	1 yr	--G------		6 mo	E--------
R05-010	1 mo	E--------		1 yr	E--------
	6 mo	E-G------	R08-006	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------
Vif/Nef-vaccinated non-controllers			controllers		
R08-012	1 mo	--------P	R10-010	1 mo	A--------
	6 mo	--------P		6 mo	E-------P
R10-012	1 mo	E----S---		1 yr	E--------
	6 mo	----D---P	R10-011	1 mo	---------
	1 yr	-----L--P		6 mo	---------
R10-013	1 mo	--------R		1 yr	---------
	6 mo	--------R	R10-014	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------

FIG 5 Predominant nonsynonymous mutations in CD8$^+$ T-cell target-coding regions. (A) Gag target regions for CD8$^+$ T-cell responses in the acute phase in Gag-vaccinated controllers. Macaque R01-008 induced $Gag_{367-381}$-specific CD8$^+$ T-cell responses and selected I377T mutation in 5 weeks as described before (5). (B) Vif target regions for CD8$^+$ T-cell responses in the acute phase in Vif/Nef-vaccinated controllers. (C) Nonsynonymous mutations in Nef_{45-53} CD8$^+$ T-cell epitope-coding regions of viral cDNAs at 1 month (1 mo), 6 months (6 mo), and 1 year (1 yr). Amino acid substitutions are shown.

controllers showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge. In particular, macaques R01-008 and R08-006 showed rapid SIV control without detectable plasma viremia after week 5. $Gag_{367-381}$-specific CD8$^+$ T-cell responses with rapid selection of a $Gag_{367-381}$-specific CD8$^+$ T-cell escape mutation, I377T, were observed in R01-008, whereas $Gag_{385-400}$-specific responses were associated with an escape mutation, I391T, in R08-006. Our results suggest that the prophylactic Gag vaccination results in the efficient induction of these Gag-specific CD8$^+$ T-cell responses in the acute phase, which then played an important role in the control of primary SIV replication. The MHC-I haplotypes other than E (see Table S1 in the supplemental material) may be associated with these effective Gag epitope-specific CD8$^+$ T-cell responses. Nef-specific CD8$^+$ T-cell responses became predominant after 3 or 6 months.

Vif/Nef-vaccinated animals induced Vif- or Nef-specific CD8$^+$ T-cell responses in the acute phase after SIVmac239 challenge. Before challenge, detectable Vif-specific CD8$^+$ T-cell responses were elicited after SeV-Vif/Nef boost only in macaque R10-011. It should be noted, however, that all three Vif/Nef-vaccinated controllers showed high-frequency Vif-specific CD8$^+$ T-cell responses in the acute phase, while the three noncontrollers exhibited Nef-specific CD8$^+$ T-cell responses. In particular, our results implicate Vif_{65-76}-specific and $Vif_{134-148}$-specific CD8$^+$ T-cell responses in the control of primary viral replication in macaques R10-010 and R10-011, respectively. These CD8$^+$ T-cell responses may be associated with the second MHC-I haplotypes (see Table S1 in the supplemental material). Even Vif/Nef-vaccinated controllers inducing Vif-specific CD8$^+$ T-cell responses in the acute phase showed predominant Nef-specific CD8$^+$ T-cell responses in the chronic phase.

Vif/Nef-vaccinated noncontrollers showed no Vif-specific CD8$^+$ T-cell responses but mounted Nef-specific CD8$^+$ T-cell responses in the acute phase. All three noncontrollers rapidly se-

lected *nef* mutations in the Nef_{45-53}-coding regions, and Nef_{45-53}-specific $CD8^+$ T-cell responses were undetectable after 3 months postchallenge. Interestingly, both Gag-vaccinated noncontrollers also showed rapid selection of *nef* mutations in the Nef_{45-53}-coding regions. We speculate that, in these Gag-vaccinated or Vif/Nef-vaccinated noncontrollers, dominant Nef_{45-53}-specific $CD8^+$ T-cell responses may have exerted strong suppressive pressure on primary SIV replication without the help of other vaccine antigen-specific, effective $CD8^+$ T-cell responses, leading to failure in virus control with rapid selection of escape mutations. Unvaccinated macaque R08-007 elicited Gag- and Vif-specific as well as Nef-specific $CD8^+$ T-cell responses in the acute phase but failed to control SIV replication. The high magnitude of responses may reflect the highest peak viral loads (1.4×10^7 copies/ml) at day 10 in this animal among the unvaccinated. These naive-derived Gag- and Vif-specific $CD8^+$ T-cell responses may have been less functional and insufficient for SIV control. In contrast, in vaccinated controllers, prophylactic vaccination resulted in effective Gag- or Vif-specific $CD8^+$ T-cell responses postexposure, leading to primary SIV control, followed by Nef-specific $CD8^+$ T-cell responses possibly contributing to maintenance of virus control. Induction of $CD8^+$ T-cell responses specific for dominant Nef epitopes by prophylactic vaccination may not be good for SIV control in E^+ animals. Several studies have indicated contribution of subdominant $CD8^+$ T-cell responses to HIV or SIV suppression (51–53). Thus, induction of $CD8^+$ T-cell responses specific for subdominant but not dominant epitopes by prophylactic vaccination may be a promising AIDS vaccine strategy resulting in effective, broader $CD8^+$ T-cell responses postexposure.

In summary, this study demonstrates SIV control by prophylactic vaccination in hosts possessing MHC-I alleles associated with dominant non-Gag antigen-specific $CD8^+$ T-cell responses. Our results suggest that prophylactic vaccination resulting in effective subdominant Gag/Vif epitope-specific $CD8^+$ T-cell responses in the acute phase postexposure can lead to primary HIV control. This may imply a rationale of altering the hierarchy of postexposure $CD8^+$ T-cell immunodominance toward HIV control.

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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 ($CN2\text{-}29^{(+/-)}/MxCre^{(+/-)}$) 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 *XbaI*/*XhoI* 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

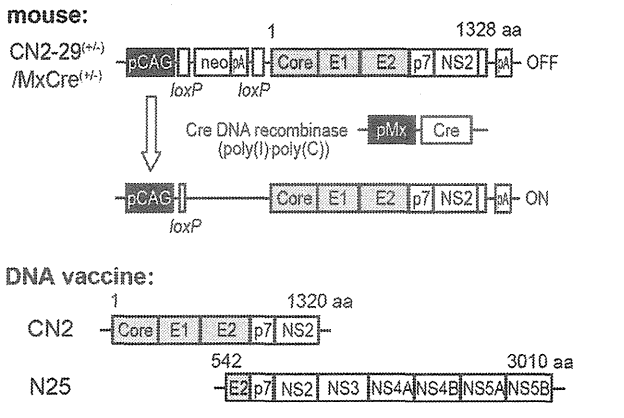

Fig. 1. Generation of HCV DNA vaccines. HCV gene structure in CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice and schematic diagram of plasmid constructs expressing an HCV structural protein (CN2) and a non-structural protein (N25). CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express partial (nucleotides 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

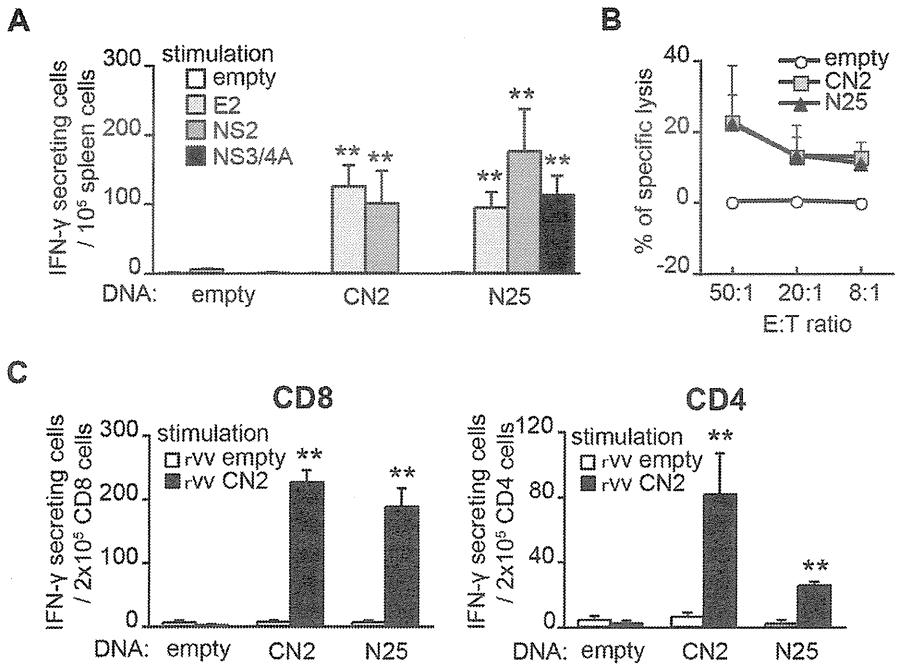

Fig. 2. Immunization of C57BL/6 mice with DNA vaccine induced strong HCV-specific cellular immune responses. C57BL/6 mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins (A), and ^{51}Cr release assay using EL-4 target cells expressing HCV NS2 protein at indicated ratios (B). CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2) (C). Data are shown as means ± SEM, $n = 3$ (A and B); $n = 5$ (C). Data shown in A–C are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

that use live-attenuated or killed pathogens, proteins, or synthetic peptides [35]. Moreover, DNA vaccine against HIV and malaria showed high levels of safety and good tolerability profile in human clinical trials [36,37]. DNA vaccines can induce cytoplasmic expression of encoded antigens (Ags) that more closely resemble native conformation of pathogens than can immunization with proteins. A DNA vaccine induced immunity against encoded Ags, whereas cytoplasmic expression using a viral vector delivery system induced immune responses to not only vaccine Ags but also vector organisms [38]. Infection with a vaccine vector virus might be an obstacle by induction of unneeded immune responses to vectors as side effects [38]. A DNA vaccine might be

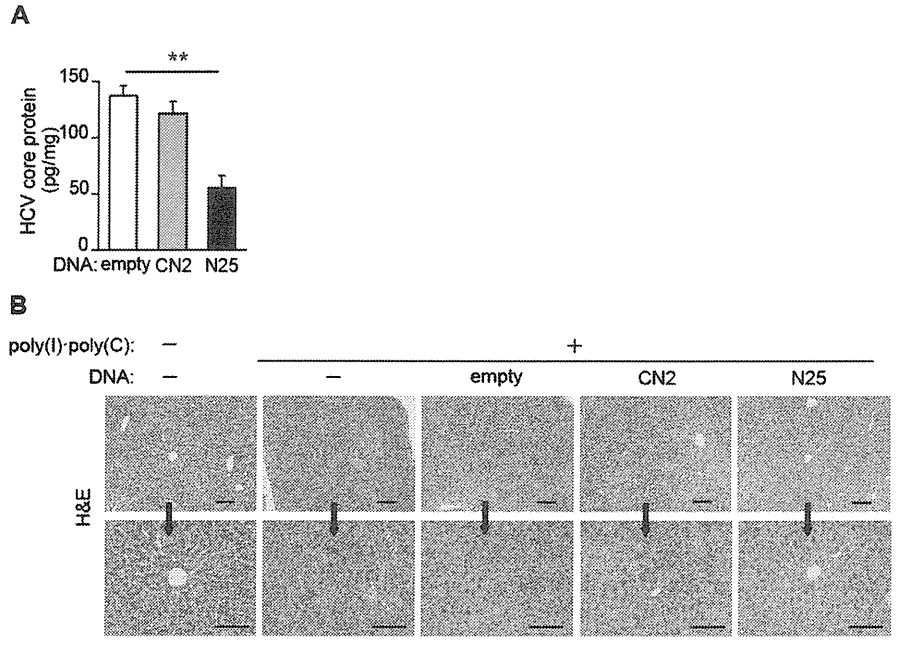

Fig. 3. Immunization with N25 DNA vaccine showed therapeutic effects in the liver of HCV transgenic mice. (A) Three months after induction of HCV protein by poly(I)·poly(C) injection, CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, livers were isolated and examined for HCV core protein expression. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. $^{**}P < 0.01$. (B) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice before (–) and after poly(I)·poly(C) injection (+). Three months after induction of HCV protein, mice were immunized three times with the DNA vaccine at intervals of 2 weeks. At 4 weeks after the final immunization, livers were isolated and stained with hematoxylin and eosin (H&E). Scale bars: 100 μm. Data shown in A and B are representative of at least 3 repeated experiments.

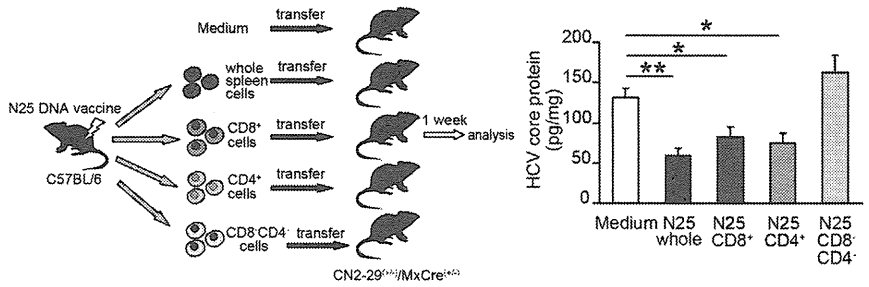

Fig. 4. CD8⁺ and CD4⁺ T cells are required for the decrease of HCV protein in the liver. Whole spleen cells, purified CD8⁺ T cells or CD4⁺ T cells from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, and livers were isolated from the recipient mice 1 week later. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. Data are representative of 3 repeated experiments. $^{**}P < 0.01$; $^{*}P < 0.05$.

one of the best candidates for a therapeutic vaccine for infectious disease.

In the case of viral persistence resulting in chronic disease, HCV-specific CD4 and CD8 T cell responses appeared to be diminished [39]. Various hypotheses have been proposed to explain the dysfunctional T cell response in HCV infection, including viral escape mutations, exhaustion of the T cell compartment, induction of regulatory T cells and impaired DC function [40]. DCs play an important role in triggering the primary antiviral immune responses. Therefore, modulation of the function of DCs has been suggested as one of the mechanisms used by persistent viruses to evade the immune system. Several studies have demonstrated impairment of DC function in HCV-infected individuals [32,34]. On the other hand, some studies have shown that HCV proteins did not impair DC function [41–44]. In the present study, the ability of DCs to induce HCV-specific CD8⁺ T cells in HCV transgenic mice was not impaired (Fig. 6B), therefore, another mechanism that suppressed the generation of HCV-specific CD8 T cells might exist in this mouse model.

HCV conventional transgenic mice have been used as surrogate models for chronic HCV infection in humans. In a previous study, when FVB/n Tg mice expressing HCV structural proteins (core, E1 and E2) and WT FVB/n mice were intramuscularly immunized with plasmid DNA encoding core/E1/E2, CTL activities against E2 were detected in WT mice but not in Tg mice [23], and either CD4 or CD8 T cell responses against the envelope proteins appeared to be

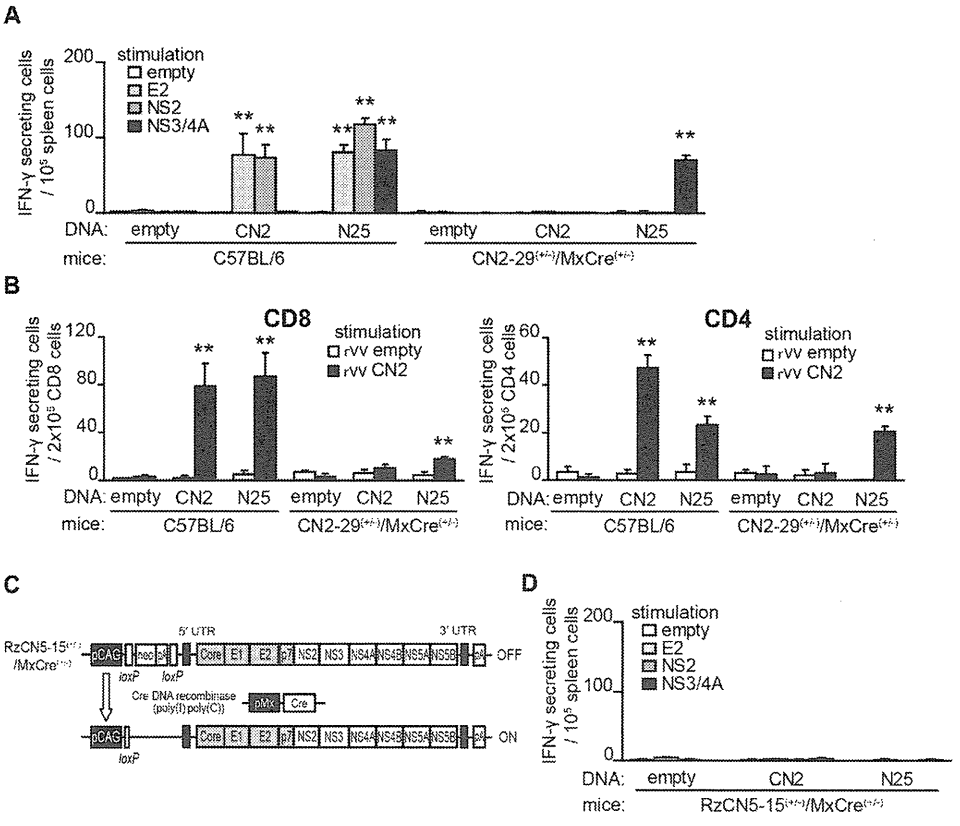

Fig. 5. Immunization of HCV-Tg mice with DNA vaccine failed to induce strong HCV-specific cellular immune responses. CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized with the DNA vaccine as in the experiment for which results are shown in Fig. 3A. (A and D) Spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2). (C) HCV gene structure in RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice. RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express full length of HCV genotype 1b cDNA. Data are shown as means ± SEM, $n = 3$ (A, B and D). Data shown in A, B and D are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

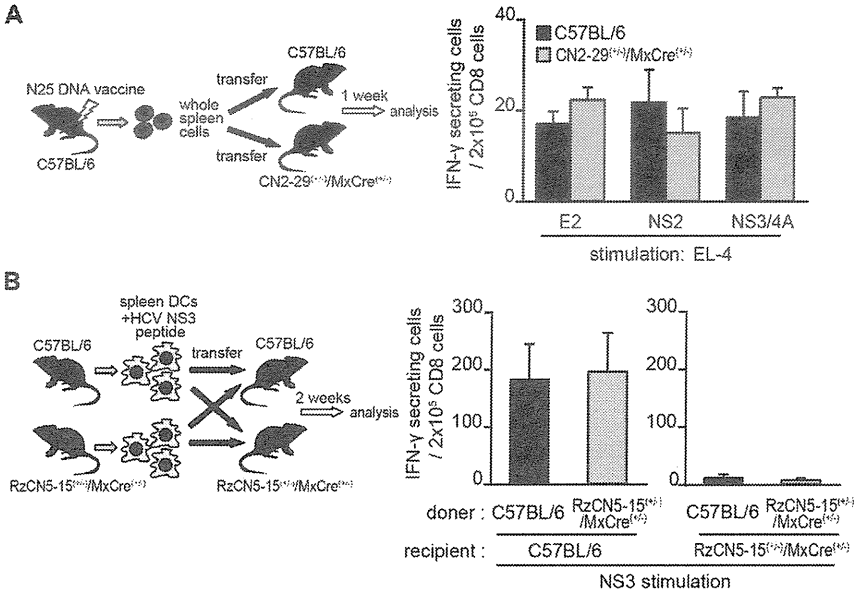

Fig. 6. Ability of DCs to induce HCV-specific $CD8^+$ T cells in HCV transgenic mice was not impaired. (A) Whole spleen cells (1×10^8 cells) from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into C57BL/6 or CN2-$29^{(+/-)}$/MxCre$^{(+/-)}$ mice, and spleen cells were isolated from the recipient mice 1 week later. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) HCV NS3 peptide-pulsed spleen DCs (2×10^5 cells) derived from C57BL/6 or RzCN5-$15^{(+/-)}$/MxCre$^{(+/-)}$ mice were adoptively transferred to C57BL/6 or RzCN5-$15^{(+/-)}$/MxCre$^{(+/-)}$ mice. Two weeks later, spleen cells were isolated from the recipient mice. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using HCV NS3 peptide. Data are shown as means ± SEM, $n = 3$ each. Data shown in A and B are representative of at least 3 repeated experiments.

immunologically tolerant and could not overcome this tolerance by DNA immunization in the Tg mice [23]. These observation are consistent with our findings that cellular immune responses to several HCV structural and non-structural proteins were abolished in the CN2 DNA vaccine-immunized HCV-Tg (CN2-$29^{(+/-)}$/MxCre$^{(+/-)}$) mice, unlike in WT mice (Fig. 5A and B). On the other hand, the N25 DNA vaccine induced HCV-specific CD4 and CD8 T cell responses in HCV-Tg mice, and the level of CD4 T cell responses were equivalent to those in WT mice. However, the activities of CD8 T cell responses were not high compared with those in WT mice (Fig. 5B). This difference in the efficacy of CN2 and that of N25 may be caused by the difference of the expression site of HCV construct. The CN2 DNA construct contained the HCV core protein-encoding region. Several studies have demonstrated that the HCV core protein has the immunomodulatory function of suppressing host immune responses [45]. It has been reported that HCV core protein could suppress host immune responses by inhibiting antiviral CTL activity in mice infected with recombinant vaccinia virus expressing the core protein [7], and it has also been reported that Tg mice in which the HCV core protein was expressed in T cells under the control of the CD2 promoter showed significantly reduced T cell responses, including the production of IFN-γ and IL-2, compared to those in non-Tg mice [46]. The N25 DNA construct did not contain the HCV core protein-encoding region, and the N25 DNA vaccine may therefore not be susceptible to these immunosuppressive factors derived from DNA vaccine's own self.

Immunization of HCV-Tg mice with N25 DNA vaccine resulted in improvement in pathological changes in the liver. Sekiguchi et al. [27] reported that immunization with recombinant vaccinia virus strain (rVV-N25), which encoded the same non-structural HCV proteins as those encoded by N25 DNA vaccine in this study, alleviated the symptoms of pathological changes in the liver of HCV-Tg mice. They showed that TNF-α and IL-6 are responsible for the pathological symptoms in HCV-Tg mice and that immunization with rVV-N25 rapidly suppressed the inflammatory responses. The mechanism of action of N25 DNA vaccine may be similar to that of rVV-N25, though further examination is required.

In the present study, using novel HCV conditional transgenic mice to overcome the problem of immune tolerance in HCV conventional transgenic mice, the efficacy of a candidate HCV vaccine was evaluated. The use of DNA vaccines, especially the N25 DNA vaccine, expressing a non-structural protein gene resulted in reduced expression of HCV protein and improved pathological changes in the liver. Our findings may provide new avenues toward the development of an alternative approach for the treatment of individuals chronically infected with HCV, although further studies are needed.

Acknowledgments

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Intranasally Administered Antigen 85B Gene Vaccine in Non-Replicating Human *Parainfluenza* Type 2 Virus Vector Ameliorates Mouse Atopic Dermatitis

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Abstract

Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Various factors including heredity, environmental agent, innate and acquired immunity, and skin barrier function participate in the pathogenesis of AD. T-helper (Th) 2-dominant immunological milieu has been suggested in the acute phase of AD. Antigen 85B (Ag85B) is a 30-kDa secretory protein well conserved in *Mycobacterium* species. Ag85B has strong Th1-type cytokine inducing activity, and is expected to ameliorate Th2 condition in allergic disease. To perform Ag85B function in vivo, effective and less invasive vaccination method is required. Recently, we have established a novel functional virus vector; recombinant human *parainfluenza* type 2 virus vector (rhPIV2): highly expressive, replication-deficient, and very low-pathogenic vector. In this study, we investigated the efficacy of rhPIV2 engineered to express Ag85B (rhPIV2/Ag85B) in a mouse AD model induced by repeated oxazolone (OX) challenge. Ear swelling, dermal cell infiltrations and serum IgE level were significantly suppressed in the rhPIV2/Ag85B treated mouse group accompanied with elevated IFN-γ and IL-10 mRNA expressions, and suppressed IL-4, TNF-α and MIP-2 mRNA expressions. The treated mice showed no clinical symptom of croup or systemic adverse reactions. The respiratory tract epithelium captured rhPIV2 effectively without remarkable cytotoxic effects. These results suggested that rhPIV2/Ag85B might be a potent therapeutic tool to control allergic disorders.

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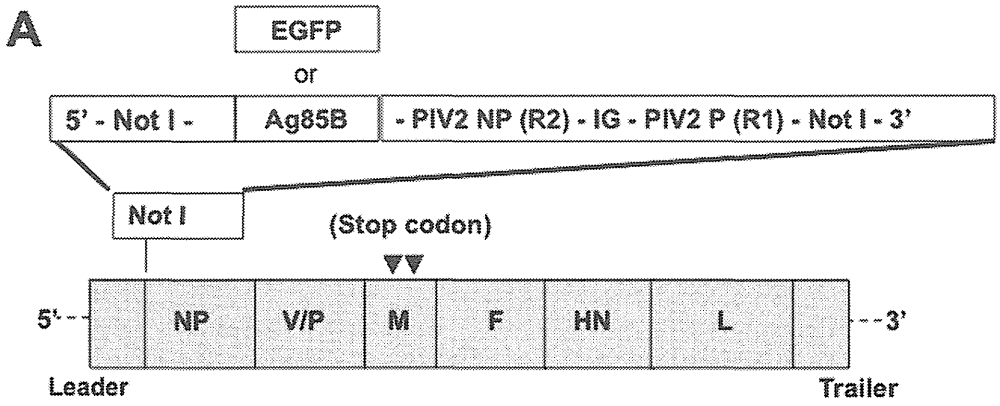
Introduction

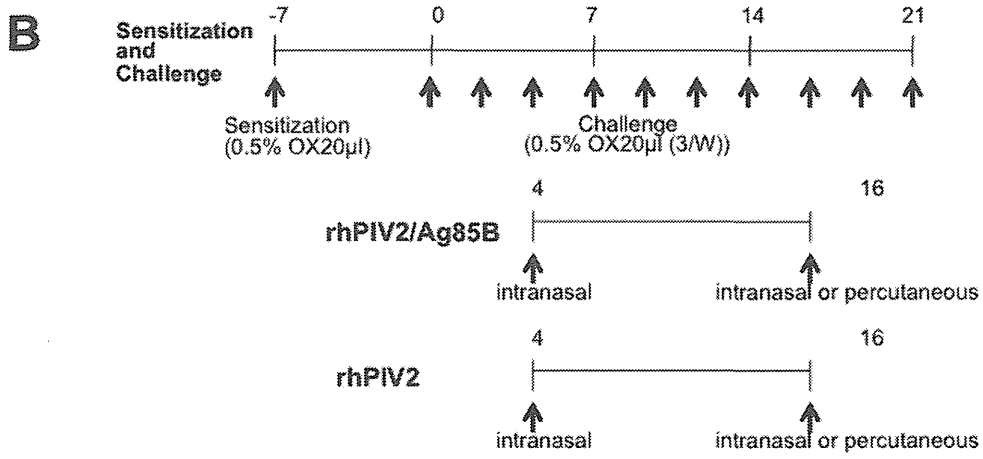
Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Heredity, environmental agent, immunity, and skin barrier function participate in the pathogenesis of AD. AD symptoms are triggered by various non-specific or specific allergic reactions. The cytokine pattern of AD, especially in the acute phase skin lesion is Th2-type cytokine dominant [1]. The barrier disrupted skin in AD is easily permitted the percutaneous entry of environmental allergens that strongly promotes Th2 immunological responses [2]. Th2 cells as well as T regulatory cell (Treg) subsets play key roles in development of AD. Patients with AD have significantly increased numbers of peripheral blood Treg compared with healthy controls, which is correlated with disease activity in AD [3,4]. This suggests involvement of some self regulation system in immune responses in AD [5].

Repeated elicitation with hapten such as oxazolone (OX) on the ear of BALB/c mice develops immediate type responses with late phase reactions followed by delayed type hypersensitivity responses. This accompanied with balance shift of cytokines in the lesional skin from Th1 to Th2 type [6], and has been utilized as mouse AD.

Ag85B is 30-kDa major secretory protein well conserved in *Mycobacterium* species [7]. The studies for the tuberculosis vaccine revealed strong activities of Ag85B in priming naïve T cells for Th1 effector cells under the appropriate conditions, and induction of strong Th1-type immune responses in mice as well as in humans [8,9]. Recently we reported that plasmid DNA vaccination encoding Ag85B derived from M. *kansasii* inhibits immediate-type hypersensitivity responses with Treg induction in skin [10], and a combined vaccination with heat-killed BCG followed by Ag85B also suppressed skin eczematous reactions in AD model mice by inducing Treg [11].

Human parainfluenza type 2 virus (hPIV2) is one of the human respiratory pathogens and a member of the genus Rubulavirus of the family Paramyxoviridae in the order Mononegavirales,





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groups	Day4	Day16
PBS	PBS	PBS
Ag ear	rhPIV2/Ag85B	rhPIV2/Ag85B (percutaneous)
Ag nasl	rhPIV2/Ag85B	rhPIV2/Ag85B (intranasal)
PIV2 ear	rhPIV2	rhPIV2 (percutaneous)
PIV2 nasal	rhPIV2	rhPIV2 (intranasal)

Figure 1. Schematic diagram of constructs and strategy used in this study. A. The constructs of recombinant hPIV2/EGFP and hPIV2/Ag85B. The EGFP or Ag85B gene open reading frame was engineered to be flanked by hPIV2-specific gene end of NP gene (R2), intergenic sequence (IG), and gene start (R1) transcriptional signal of V/P gene. It was inserted into a cloned cDNA of the hPIV2 antigenome at a Not I site that had been engineered to be at 5′-noncoding region of NP gene. A genomic nucleotide length divisible by six (the rule of six) was maintained. For generating of replication-deficient virus, two stop codons (▼) were introduced on the M gene. **B.** Schedule for the development of a hapten-induced atopic dermatitis model and vaccination of rhPIV2/Ag85B. Mice were initially sensitized with 20 μl of 0.5% OX solution to their right ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the right ear 3 times per week from day 0 until day 21. Mice were inoculated intranasally with 20 μl (5×10^6 $TCID_{50}$) of rhPIV2/Ag85B or rhPIV2 on day 4. rhPIV2 vector or phosphate buffered saline (PBS) were also applied as controls. On day 16, mice were vaccinated again intranasally or subcutaneously with PBS, rhPIV2 or rhPIV2/Ag85B. **C.** Summarized schedule of the experimental groups.
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possessing a non-segmented and negative-stranded RNA genome of 15,654 nucleotides. The genome of hPIV2 encodes 7 mRNAs [12–14] and has about 60-nt leader sequence at 3′ end and about 20-nt noncoding trailer sequence. The gene order is 3′ (leader)-NP-V/P-M-F-HN-L-(trailer)-5′. The coding proteins are the nucleocapsid (NP), the V (V) and phospho (P), the matrix (M), the fusion (F), the haemagglutinin-neuraminidase (HN), and the polymerase protein (L). The genomic RNA of the virus: viral RNA (vRNA) is encapsidated with the NP proteins, and the nucleocapsids are associated with the P and L proteins to form the

ribonucleoprotein complex. In paramyxovirus particles, vRNA is enclosed by the viral envelope composed of a cellular lipid bilayer and two envelope glycoproteins, HN and F, which are integral transmembrane proteins mediating virus attachment and cell fusion, respectively [15]. M protein underlies the lipid bilayer to ensure the structural integrity of the viral particles and is essential for interactions between the viral envelope and the RNP complex [15]. This association leads to the budding and release of viral particles from the cell surface [15].

Recently, as technology advances in reverse genetics [16], hPIVs offer several advantages as a vaccine vector. hPIVs efficiently infect the respiratory tract but don't spread far beyond it, which is an important safety factor. hPIV-based vectors have proven the effect in inducing local and systemic immunity against a number of foreign antigens [17]. hPIVs infect to various cell types and cause little cytopathic effects. Moreover, they replicate exclusively in the cytoplasm of infected cells, don't have a DNA phase during their life cycle and can thus avoid the possibility of integration of foreign genes into the host DNA genome [18].

In the present study, we utilized newly engineered rhPIV2: replication-deficient rhPIV2 vector. rhPIV2 lacks M gene that is an essential gene for virus particle formation by insertion of two stop codons. This alteration might support much safer application to animals than original proliferating virus vector. We first

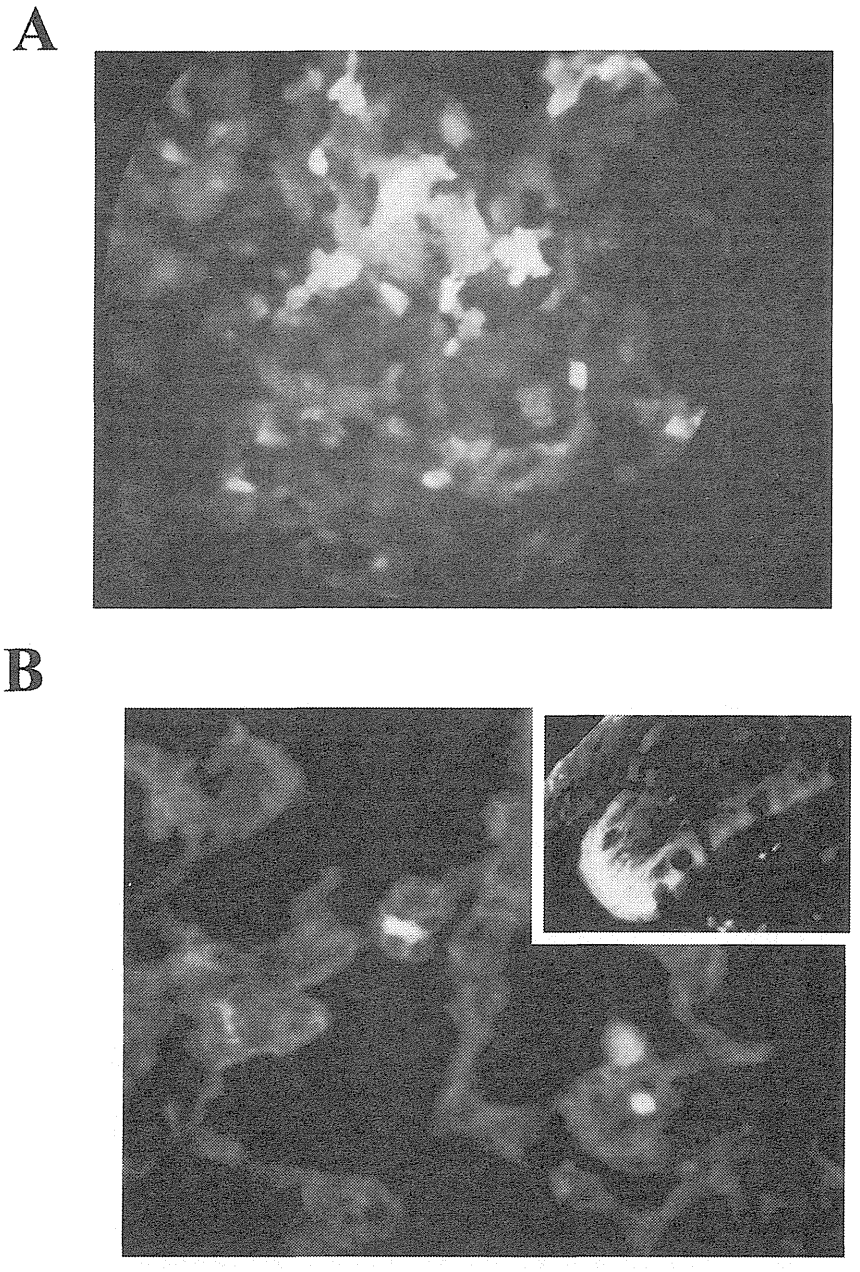

Figure 2. Expression of EGFP from rhPIV2/EGFP. A. HaCat cells were infected with rhPIV2/EGFP at an MOI of 0.5. Three days after, EGFP was clearly visualized using a fluorescence microscopy (x100). **B.** The rhPIV2/EGFP (5×10^6 $TCID_{50}$) were administered to a wild type BALB/c mice intranasally EGFP was visualized clearly in the airway epithelial cells 4 days after administration (x200, upper right box, x400).
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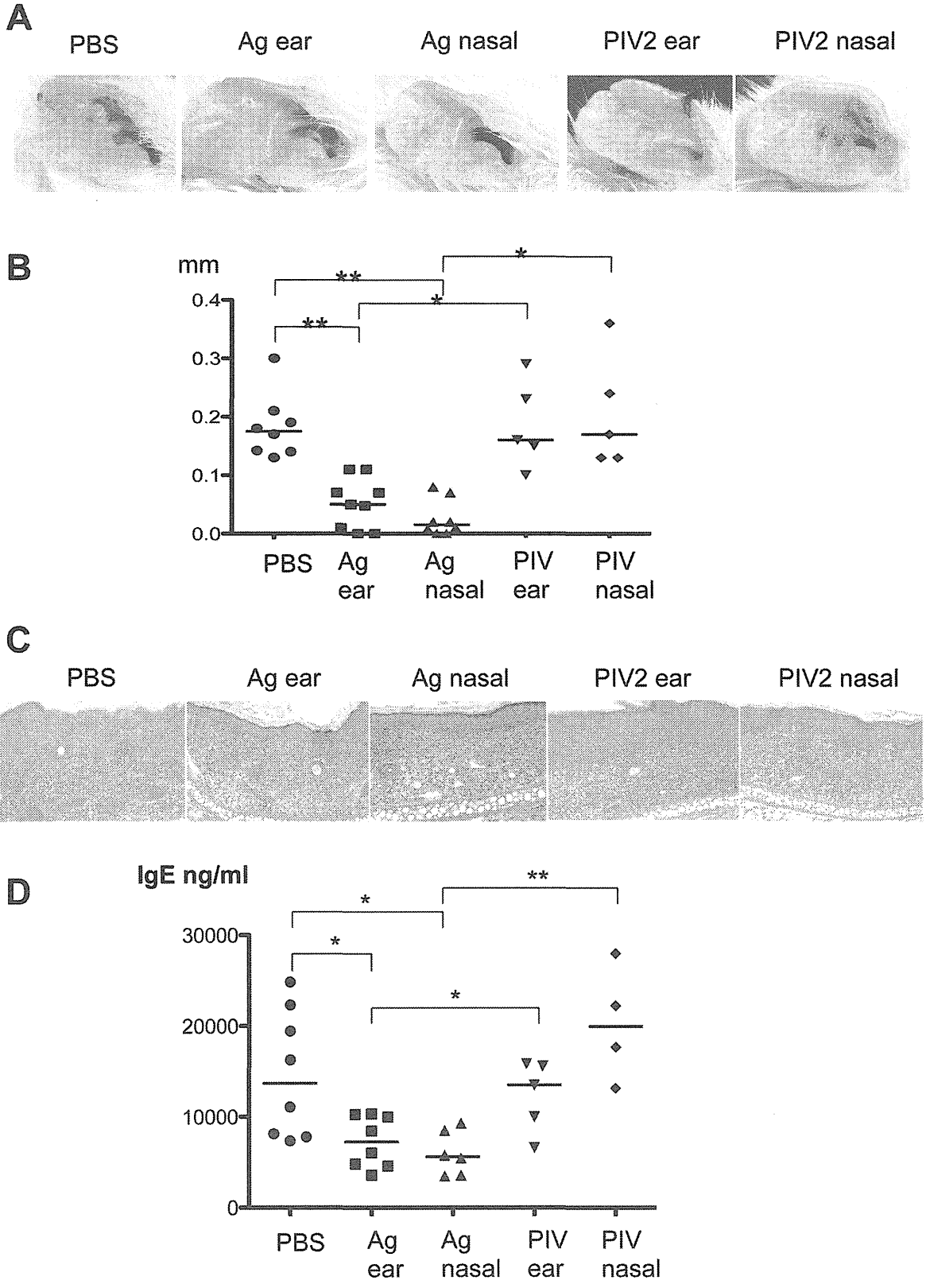

Figure 3. Anti-inflammatory effects of vaccination with rhPIV2/Ag85B. A. Clinical manifestation of the ear skin at 6 hours after OX challenge on day 21. The control groups (PBS, PIV2 ear, and PIV2 nasal on the panel) showed severe edema with erythema, however the intranasal and/or subcutaneous administration of the rhPIV2/Ag85B (Ag nasal and Ag ear on the panel, respectively) clearly reduced skin reactions in OX-sensitized mice. **B.** Ear thickness measured before and 6 hours after each OX application on day 21. The ear swelling was suppressed significantly in rhPIV2/Ag85B treated groups in two ways compared to those in the placebo treated groups. (*$P<0.05$, **$P<0.01$.) **C.** Histopathological changes of the ear skin obtained on day 21 in paraffin embedded sections stained with hematoxylin and eosin. The placebo treated groups (PBS, PIV2 ear and PIV2 nasal

on the panel) revealed marked inflammatory reactions with acanthosis and ulceration in epidermis, and marked edema with cellular infiltration including mononuclear cells and neutrophils in the dermis. The skin infiltration of inflammatory cells and epidermal thickness were decreased in rhPIV2/Ag85B treated group (Ag85B ear and Ag85B nasal on the panel). Original magnification × 100. **D.** Plasma IgE levels on day 21. Plasma IgE level was decreased in rhPIV2/Ag85B treated groups (Ag ear and Ag nasal). *P<0.05, **P<0.01.
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investigated efficiency of rhPIV2 vaccine vector expressing enhanced green fluorescence protein (EGFP) gene (rhPIV2/EGFP) in infection and expression in vitro and in vivo. Then, we evaluated effectiveness of the vaccination pathways: subcutaneous or intranasal administration of rhPIV2 expressing Ag85B gene (rhPIV2/Ag85B) in a mice AD model induced by repeated hapten challenge.

Materials and Methods

Animals

BALB/c 6-week old male mice were purchased from Japan SLC Co. (Shizuoka, Japan) and used at 7-week. Animal care was performed according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University.

Construction of rhPIV2/Ag85B and rhPIV2/EGFP

rhPIV2/Ag85B and rhPIV2/EGFP was constructed according to the method reported previously, except for methods of the supply of T7 and hPIV2 RNA polymerases (NP, P, L). In brief, to generate replication-deficient rhPIV2 vector, two nucleotides change [ATG to TAG (position of 89aa) and AAG to TAG (259aa)] were introduced into the M frame of the plasmid pPIV2, a full-length cDNA copy of hPIV2 anti-genome [19] (Fig. 1A). Consequently, the 6 n length cDNA of Ag85B or EGFP, followed by transcriptional end sequence of NP gene (R2), intergenic sequence (IG), and transcriptional start signal of V/P gene (R1) ([20] was synthesized by PCR using appropriate primers), was inserted into a Not I site of the plasmid DNA encoding the replication-deficient rhPIV2 genome described above. Then, the viruses (rhPIV2/Ag85B and rhPIV2/EGFP) were recovered by co-transfection of each anti-genomic plasmid and plasmids expressing the NP, P, M and L, each cloned in a mammalian gene expression vector (pCAGGS) [21] into BSR7/5 cells expressing T7 RNA polymerase [22]. The cells were harvested, and then co-cultured with fresh Vero cells every 48 hr. Approximately 90% of the cells showed syncytia formation in the 10^{th} co-cultured cells, and its state was maintained in further co-culture. Furthermore, for virus propagation, Cos7 cells were transfected with the plasmid expressing M and co-cultured with above-mentioned 10^{th} cells. The supernatant was centrifuged at 9,000 g for 12 h at 4°C. The virus pellet was suspended in Opti-MEM (Invitrogen, Carisbad, CA, USA). The virus titers were determined by CPE method using Vero cells, and were expressed as 50% tissue culture infectious dose ($TCID_{50}$).

In vitro and in vivo Infection of rhPIV2 Vector Expressing EGFP

HaCat cells (Cell Line Service, Eppelheim, Germany) were cultured in Dulbecco's MEM supplemented with 5% (v/v) FBS, 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. HaCat cells were seeded one day before the transduction at 1×10^6 cells/ml (1 ml/well) in 6-well culture plates (Costar, NY, USA). The cells were incubated for 8 hours at 37°C in a 5% CO_2 atmosphere. The next day, the media was removed and 1 ml of the rhPIV2/EGFP viruses were added to the cells to be adjusted to 1×10^6 $TCID_{50}$. Two hours after infection, the media was removed and fresh culture media was supplemented to the cells. After 3 days culture, each well was observed by fluorescence microscopy.

At the next step, 20 μl of concentrated rhPIV2/EGFP (5×10^6 $TCID_{50}$) were administered into the cavity of the nose of the mice. Four days after infection, the respiratory tract and lung were sampled, embedded in Tissue-Tek OCT compound (Miles, Elkhart, USA), frozen in liquid nitrogen, and cut into 7 μm-thick sections. Sections were examined and recorded by fluorescence microscopy.

Sensitization and Challenge Schedule

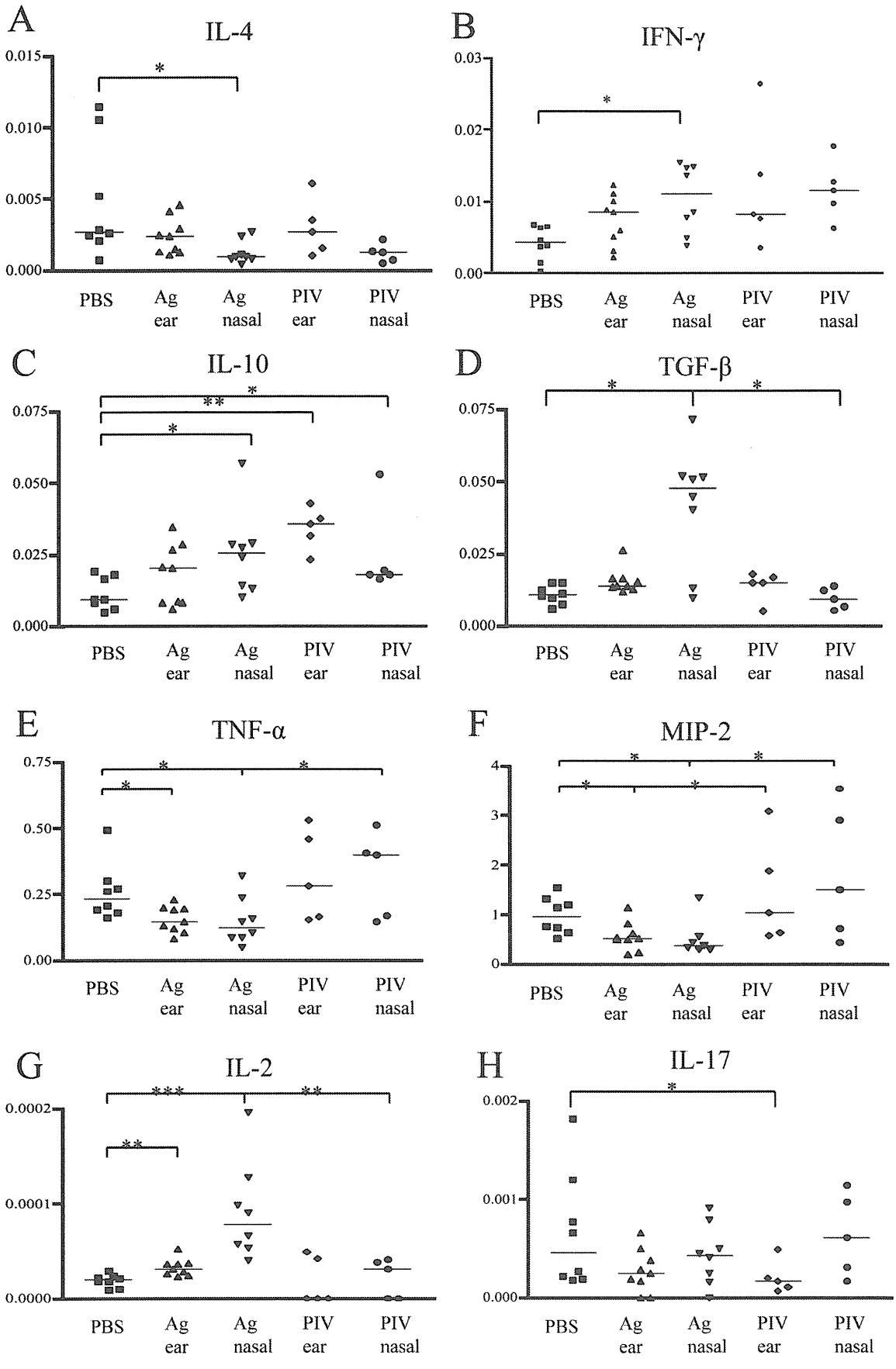
Repeated hapten sensitization and challenge system was introduced in this experiment. OX (Sigma, St. Louis, MO) was dissolved in acetone/olive oil (1:1). As shown in Fig. 1B, mice were initially sensitized by pasting 20 μl of 0.5% OX solution to their right ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the right ear 3 times per week from day 0 until day 21. Repeated application of OX causes delayed type hypersensitivity followed by immediate-type and late phase reaction. For vaccination, mice were infected intranasally under general anesthesia with 5×10^6 $TCID_{50}$ of the virus in a 20 μl inoculum or phosphate-buffered saline (PBS) on day 4. On day 16, mice were vaccinated again with PBS, rhPIV2/Ag85B or control rhPIV2 vector intranasally or subcutaneously to the pinna skin (Fig. 1B,C). Ear swelling was measured with thickness gauge calipers before and 6 hours after last OX challenge on day 21. Blood and pinna skins were also sampled.

Histopathological Study

The ear skins were sampled at six hours after last OX challenge on day 21. Samples were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Histological sections were of 6 mm thickness and stained with hematoxylin & eosin (H&E).

Analysis of Cytokine mRNA Expression in Mouse Ear

The mRNA was extracted from the mouse ear using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions: One ml of homogenate was mixed with 200 μl of chloroform, and then centrifuged. The aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifuging, the precipitate was washed with 70% ethanol (Nacalai Tesque) and the RNA was suspended in 40 μl of RNase-free water. The concentration of RNA was measured at 260 nm absorbent, and the quality was confirmed by electrophoresis. cDNA was synthesized from 2 μg of mRNA using an archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure transcriptional activity in skin lesions. A 25 μl reaction mixture containing 1 μg of cDNA, 900 nM of each primer, and 250 nM of TaqMan probe was mixed with 12.5 μl of TaqMan Master Mix (AB). Quantitative RT-PCR for cytokine transcripts was performed using prequalified primers and probes for IL-2, IL-4, IL-10, IL-17A, MIP2, TNF-α, TGF-β, IFN-γ and GAPDH (AB). The


A
IL-4
0.015
0.010
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*
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Ag ear
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Figure 4. Changes in cytokines mRNA expression levels in the ears of AD mice by vaccination of rhPIV2/Ag85B. Cytokines: IL-4 (panel A), IFN-γ (panel B), IL-10 (panel C), TGF-β (panel D), TNF-α (panel E), MIP2-α (panel F), IL-2 (panel G), IL-17 (panel H), mRNA expression in the ear lesions measured with Quantitative RT-PCR. Expressions of IL-4, TNF-α and MIP2-α mRNA were significantly decreased in the ear skin treated with intra-nasally rhPIV2/Ag85B treated group compared to those of control groups. Meanwhile, the expression levels of mRNA of IFN-γ, IL-10, TGF-β and IL-2 were significantly elevated in rhPIV2/Ag85B intra-nasally treated group compared to those of control groups. *P<0.05, **P<0.01, ***P<0.001.
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ΔCt method was used to standardize the transcripts to GAPDH, and the ratio to that of control mice was calculated.

Immunohistochemistry

The ear skins sampled on day 21 were snap-frozen, and the frozen sections prepared at 7 μm thickness were subjected to a blocking procedure with 5% normal goat serum (Vector Laboratories, Burlingame, CA). Sections were then incubated with FITC-conjugated rat anti-mouse CD4 antibody (Beckman Coulter) and PE conjugated anti-mouse FoxP3 antibody (BioLegend), and examined under Fluoview FV1000 laser scanning confocal microscopy (Olympus, Tokyo, Japan). Skin infiltrating $CD4^+$ T cells and $FoxP3^+CD4^+$ T cells were counted at x100 field, and the numbers in 10 randomly chosen fields of five samples were evaluated.

Measurement of Serum IgE

Serum IgE level was determined by a sandwich enzyme-linked immunosorbent assay (Yamasa, Tokyo, Japan) according to the manufacturer's instructions. Optical density of each well was determined by using a microplate reader (Multiscan JX, Thermo Electron, Yokohama, Japan).

Statistical Analysis

Statistical analysis was performed using Mann-Whitney U-test. $P<0.05$ was considered as significant.

Results

rhPIV2/EGFP Infection in vitro and in vivo

To investigate expression levels of the inserted gene in rhPIV2 in vitro, HaCat cells were infected with rhPIV2/EGFP at an MOI of 0.5 and were examined directly using a fluorescence microscopy. The EGFP from rhPIV2/EGFP was highly expressed in HaCat cells, and remarkable fluorescence extended to nearly all the cells in spite of low MOI (Fig. 2A). Then, to evaluate the gene expression in vivo, mice were intra-nasally inoculated with rhPIV2/EGFP (5×10^6 $TCID_{50}$), and the intense EGFP expression was revealed in the lung epithelium of the mice (Fig. 2B).

Cutaneous Manifestations

To evaluate the clinically relevant therapies, mice were treated following the strategy shown in Fig. 1B. Ear lobes of the rhPIV2 (vector alone) or PBS-treated mice developed severe edematous erythema with exudation and erosion at 6 hours after OX challenge on day 21. However, rhPIV2/Ag85B-treatment reduced dermatitis in both of the intra-nasal and subcutaneous application groups (Fig. 3A). Ear swelling was dramatically suppressed in both of the rhPIV2/Ag85B-treated mice compared to PBS or rhPIV2 treated mice (Fig. 3B).

Histopathological Findings

PBS or rhPIV2-treated mice showed marked inflammatory reactions with acanthosis and ulceration in epidermis, and marked edema with cellular infiltration including mononuclear cells and neutrophils in the dermis. Both of the intra-nasal and subcutaneous rhPIV2/Ag85B application successfully reduced inflammatory cell infiltration and epidermal thickness (Fig. 3C).

Serum IgE Levels

High levels of IgE were detected in sera from PBS or rhPIV2-treated mice. On the other hand, the IgE levels in the sera from Ag85B-treated mice by two ways were suppressed significantly (Fig. 3D).

Cytokines mRNA Expression in the Ear Skins

Expression of IL-4 mRNA was significantly decreased in the ear skin of intra-nasally rhPIV2/Ag85B treatment group compared to that of PBS treated mice (Fig. 4A). In clear contrast, IFN-γ mRNA expression was significantly increased in rhPIV2/Ag85B intra-nasally treated group (Fig. 4B). As expected, IL-10 levels were significantly increased in intra-nasally treated with rhPIV2/Ag85B and rhPIV2-vector groups (Fig. 4C). Surprisingly, TGF-β expression is remarkably elevated in the intra-nasal rhPIV2/Ag85B group (Fig. 4D). mRNA expressions of TNF-α and MIP-2 were significantly decreased in both of intra-nasally and subcutaneously rhPIV2/Ag85B treated groups compared with PBS or vector treated group (Fig. 4E, F). The expression of IL-2 mRNA was also significantly elevated in both of the rhPIV2/Ag85B intra-nasally and subcutaneously treated groups (Fig. 4G). No obvious suppression in IL-17 mRNA expression was detected in rhPIV2/Ag85B group (Fig. 4H).

Immunostaining for Tregs in the Inflamed Ear Skin Lesions

As shown in Fig. 5A, $CD4^+$ T cells are displayed with green fluorescence, and $Foxp3^+$ T cells are with red. Merged yellow color means $Foxp3^+CD4^+$ T cells. The skin infiltrating $CD4^+$ T cells are significantly decreased in Ag nasal group and Ag ear group compared to that of PBS-treated group. Although it does not reach the significance, the $CD4^+$ T cells number is less in Ag nasal group compared to Ag ear group (Fig. 5B). The $Foxp3^+CD4^+$ T cells are significantly increased in both of Ag nasal and Ag ear groups (Fig. 5C).

Discussion

Immune system is finely controlled on the balance of four main subsets of Th1, Th2, Th17, and Treg [5] cells. AD, especially in its acute phase, is a disease that Th2 cells are dominantly involved in the pathogenesis. In fact PBMCs from patients with AD have increased production of IL-4, IL-5, and IL-13 with limited capacity in production of IFN-γ [23–25]. Repeated elicitation of OX on mice ear shifts the cutaneous Th1 cytokine milieu to Th2, which represents the characteristic immunological features of AD. Immunotherapy for AD has some different options to correct the imbalance of the shifted cytokine profile. In the present study, we investigated effects of vaccination using replication-deficient rhPIV2 vector expressing Ag85B gene to mouse AD model. BCG is known as a strong Th1 response modifier; however, it has a risk for granuloma formation. To avoid granuloma formation, non-wax protein antigen is required. Ag85B is a conserved protein in mycobacterial species and can elicit a strong Th1-type immune