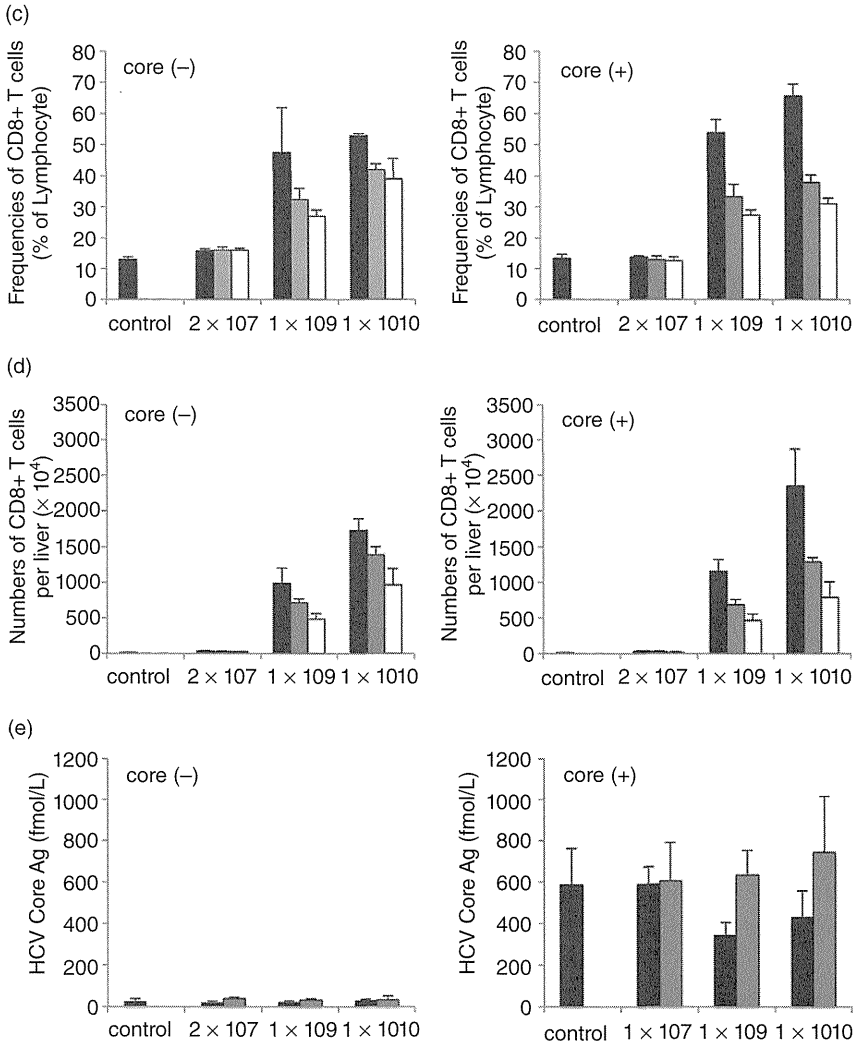

Figure 1 *Continued*

T-cell responses and their effector function in the liver of mice infected with 2×10^7, 1×10^9 and 1×10^{10} PFU Ad-HCV-NS3.

In histological studies, we observed Ad-infection-mediated hepatic inflammation in mice injected with 1×10^9 and 1×10^{10} PFU. Especially, infection with 1×10^{10} PFU caused drastic infiltrations of inflammatory cells (Fig. 1a). We also observed that CD8 lymphocytes infiltrated into the lobular areas of the infected liver in mice injected with 1×10^{10} PFU (Fig. 1b). At 7 days post-infection, we found by flow cytometric assay that the numbers and the frequencies of CD8 T cells in the liver were markedly increased after infection with 1×10^9 PFU and 1×10^{10} PFU, and the increased CD8 T cells decreased with time (Fig. 1c). We did not find significant differences between the number of CD8 T cells of core (+) and core (–) at each time point and infectious dose.

In addition, we evaluated core protein expression in the liver in each infectious dose at 7 and 14 days post-infection; there was no significant difference in core protein expression between Ad-infected and non-infected livers (Fig. 1e).

Using major histocompatibility complex (MHC) class I tetramer complexed with the H2-Db-binding HCV-NS3 GAVQNEVTL epitope, we found that i.v. infection with 2×10^7 PFU generally elicited only a weak expansion of HCV-NS3 tet^+ $CD8^+$ IHL (Fig. 2a,b) and IFN-γ^+ HCV-NS3 tet^+ $CD8^+$ IHL (Fig. 2a,c). In contrast, infection with 1×10^9 PFU induced a significant proliferation

of HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,b) and IFN-γ$^+$ HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,c).

In each infectious dose, HCV-NS3 tet$^-$ CD8 IHL did not show the diminution of elicited IFN-γ production (Fig. 2a). In contrast, HCV-NS3 tet$^+$ CD8 IHL showed the dose-dependent diminution of elicited IFN-γ production (Fig. 2d). Especially, infection with 1×10^{10} PFU led to a dramatic diminution of the elicited IFN-γ production in HCV-NS3 tet$^+$ CD8$^+$ IHL (Fig. 2a,d). These indicate that high infectious dose of Ad-HCV-NS3 cause NS3 Ag-specific immunosuppression.

As shown in Figure 2(c), the number of IFN-γ-producing HCV-NS3 tetramer$^+$ CD8 T cells in the liver of core (+) mice was lower than that of core (−) mice following PMA/ionophore stimulation. In addition, the percentage of IFN-γ-producing CD8 lymphocytes in tetramer$^+$ CD8 IHL of core (+) mice was suppressed as compared with core (−) mice following PMA/ionophore stimulation (Fig. 2d). These suggest that the presence of HCV core gene significantly impair antiviral effector CD8 T-cell responses in the liver.

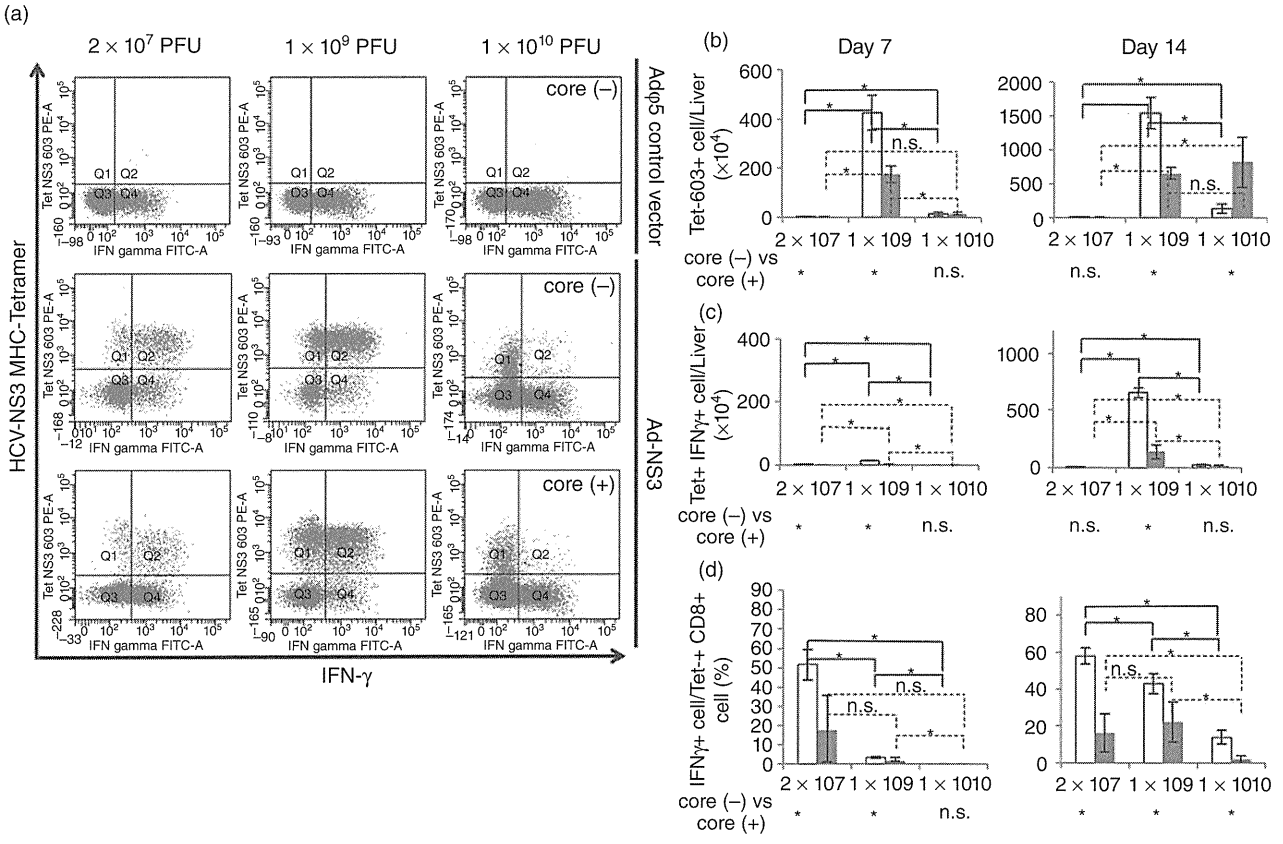

Figure 2 Impaired CD8$^+$ T-cell responses in the livers of high infectious doses. (a) Flow cytometric dot gram gating on the CD8 lymphocyte at day 14 post-infection. Graded doses of adenovirus (Ad)-hepatitis C virus (HCV)-NS3 were administrated i.v and NS3-specific intrahepatic cytotoxic T lymphocytes (CTL) were analyzed using major histocompatibility complex (MHC) class I tetramer and intracellular interferon (IFN)-γ staining method. Data show one representative mouse per group ($n = 3$). (b) The number of MHC tetramer$^+$ CD8 lymphocytes in the liver of core (−) and core (+) mice at day 7 and day 14 following Ad-HCV-NS3 infection ($^*P < 0.05$; n.s., not statistically significant). (c) The number of tetramer+ intracellular IFN-γ + CD8 lymphocytes in the liver of core (−) and core (+) mice at day 7 and day 14 following Ad-HCV-NS3 infection. Intrahepatic lymphocytes (IHL) were restimulated with phorbol myristate acetate (PMA)/ionophore for 5 h and IFN-γ production was determined by intracellular cytokine staining ($^*P < 0.05$; n.s., not statistically significant). (d) The percentage of intracellular IFN-γ$^+$ CD8 lymphocytes in tetramer$^+$ CD8 IHL of core (−) and core (+) mice on day 7 and day 14 following Ad-HCV-NS3 infection. IHL were restimulated with PMA/ionophore for 5 h and IFN-γ production was determined by intracellular cytokine staining ($^*P < 0.05$; n.s., not statistically significant). □, core (−); ▩, core (+).

The existence of HCV core gene cause higher expression of suppression molecules

The PD-1 and Tim-3 inhibitory pathways have been reported to play important roles in the dysfunction of effector T-cell response during viral infection. For instance, the expression of PD-1 is increased on functionally exhausted CD8 T cells during chronic viral infection.[15] To investigate the relation between the viral infectious doses or the expression of HCV core gene in the liver and suppression marker expression of antiviral CD8 IHL, we examined the expression for both PD-1 and Tim-3 in the CD8 IHL and PD-L1 in the intrahepatic APC of core (+) and core (−) following various doses Ad-HCV-NS3 infection.

We found that i.v. infection with 1×10^{10} PFU induced a significant expression of PD-1 and Tim-3 by Ad-HCV-NS3 specific intrahepatic CD8 T cells (Fig. 3).

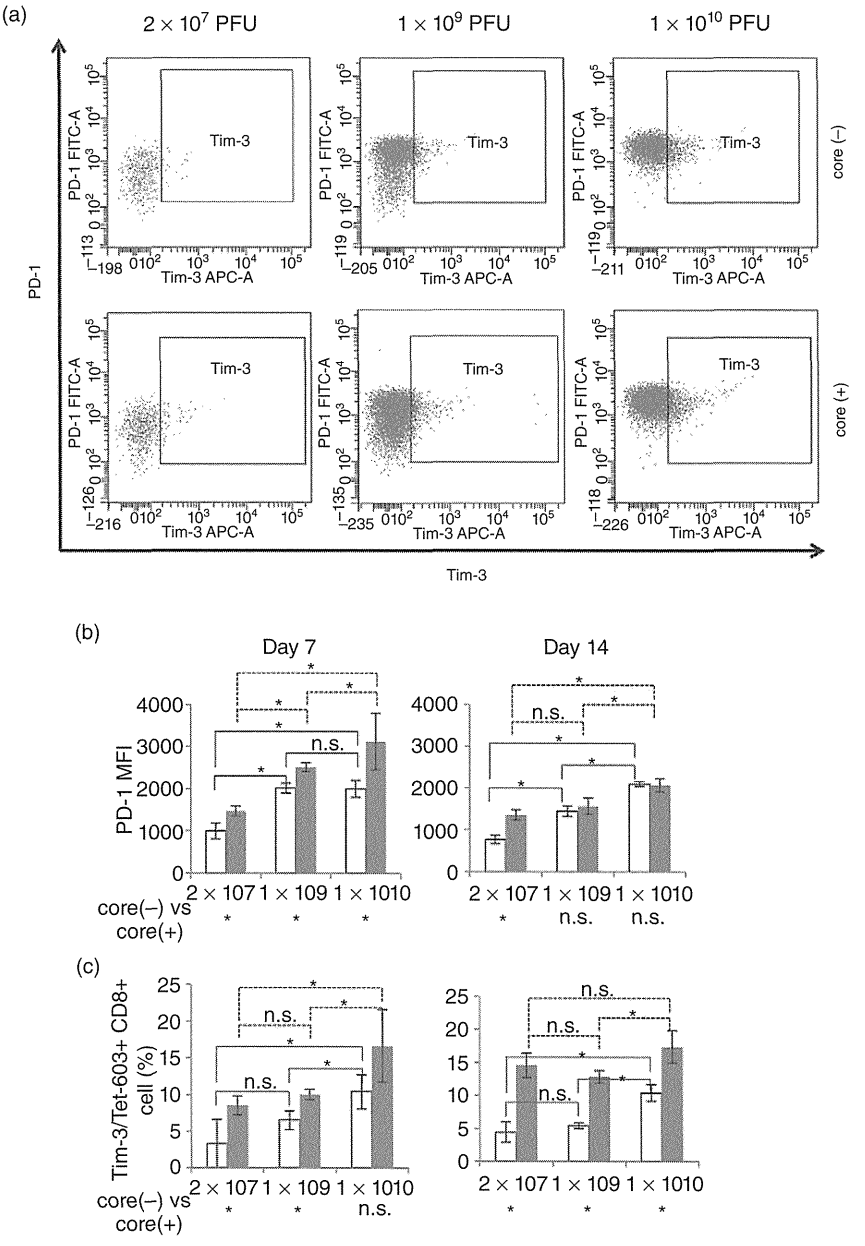

Figure 3 Differential suppression marker expression on NS3-specific CD8 lymphocyte in various infectious doses. (a) Flow cytometric dot gram gating on the hepatitis C virus (HCV)-NS3-tetramer$^+$ CD8 lymphocyte at day 14 post-infection. Graded doses of adenovirus (Ad)-HCV-NS3 were administrated i.v and NS3-specific intrahepatic cytotoxic T lymphocytes (CTL) were analyzed using major histocompatibility complex (MHC) class I tetramer and anti-PD-1 and anti-Tim-3 monoclonal antibody. Data show one representative mouse per group ($n = 3$). (b) The median fluorescence index (MFI) value of PD-1 expressed on HCV-NS3-specific CD8 intrahepatic lymphocytes (IHL) from core (−) and core (+) mice at 14 days following Ad-HCV-NS3 infection. (*$P < 0.05$; n.s., not statistically significant). (c) The number of Tim-3$^+$ HCV-NS3-specific CD8 IHL from core (−) and core (+) mice at 14 days following Ad-HCV-NS3 infection. (*$P < 0.05$; n.s., not statistically significant). □, core (−); ■, core (+).

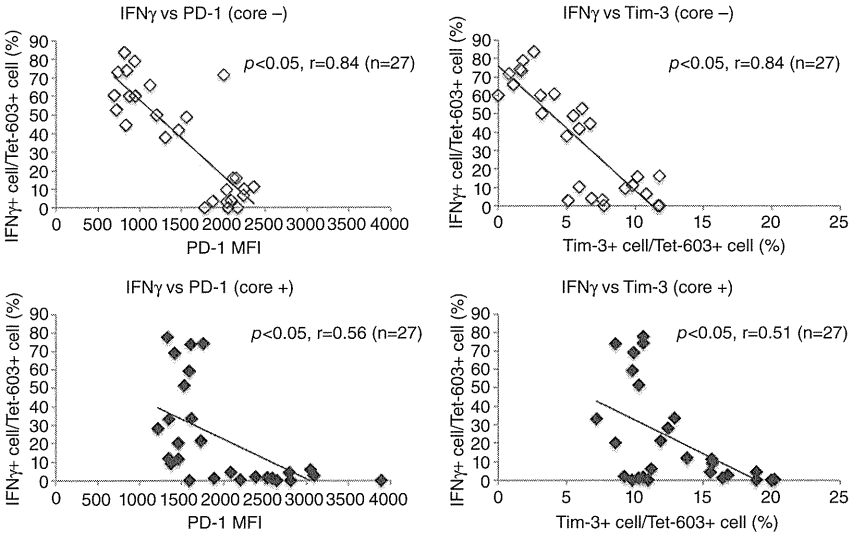

Figure 4 Inverse correlation between the percentages of interferon (IFN)-γ-producing cells and expression of regulatory molecules in antigen-specific intrahepatic CD8 T cells.

When core (+) and core (−) mice were compared, the expression of PD-1 and Tim-3 by Ad-HCV-NS3-specific intrahepatic CD8 T cells was significantly higher in core (+) than core (−) at various time points following Ad-HCV-NS3 infection. Furthermore, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells (Fig. 4).

To determine whether suppression ligand expression by intrahepatic APC is altered in core (+) mice, the intensity of PD-L1 expressed by $CD11^+$ cells was analyzed at 7 and 14 days post-infection. Intrahepatic APC showed the infectious dose-dependent augmentation of PD-L1 expression. We observed elevated expression of PD-L1 by APC in core (+) mice infected with 10^{10} PFU at both time points (Fig. 5a,b). In PD-L1 expression, we did not find a significant difference between Ad-HCV-NS3 infection and Adψ5 control vector infection (Fig. 5c,d).

Taken together, these data suggest that the existence of HCV core gene suppress T-cell-mediated immune response by causing higher expression of suppression molecules.

Ag persistence after Ad-HCV-NS3 infection

To determine the Ag persistence after Ad-HCV-NS3 infection, we analyzed the expression of FLAG-tagged HCV-NS3 protein in the liver by IP-western blot after administration of 2×10^7, 1×10^9 or 1×10^{10} PFU of the virus. The Ag expression in the liver could be found in both core (+) and core (−) mice on 21 days after infection with 1×10^{10} PFU. When 1×10^9 PFU of Ad-HCV-NS3 was administrated, HCV NS3-protein was almost cleared from the liver of core (−) mice at day 21 post-infection, whereas the Ag expression persisted in the liver of core (+) mice until day 21 post-infection (Fig. 6).

It is important to note that the loss of Ag expression in the liver of core (−) mice after infection with 1×10^9 PFU coincided with the high HCV-NS3-specific CD8 T-cell response at 14 days post-infection (Fig. 2c), whereas Ag persistence in the liver of core (+) mice after infection with 1×10^9 PFU or the liver of core (−) and core (+) mice after infection with 1×10^{10} PFU was associated with strongly diminished Ag-specific CD8 T-cell response (Fig. 2c). It is likely that the expression of core protein and the high amount of Ag in the liver contributed to the functional exhaustion of HCV-NS3-specific CD8 T cells.

DISCUSSION

IN THIS STUDY, we found an impaired response of HCV-NS3-specific intrahepatic CD8 T cell in a high dose setting (1×10^{10} PFU) of Ad-HCV-NS3 infection. Furthermore, higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and PD-L1 by intrahepatic APC were observed in HCV core Tg mice and the expression increased dependent on infectious dose. In addition, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules

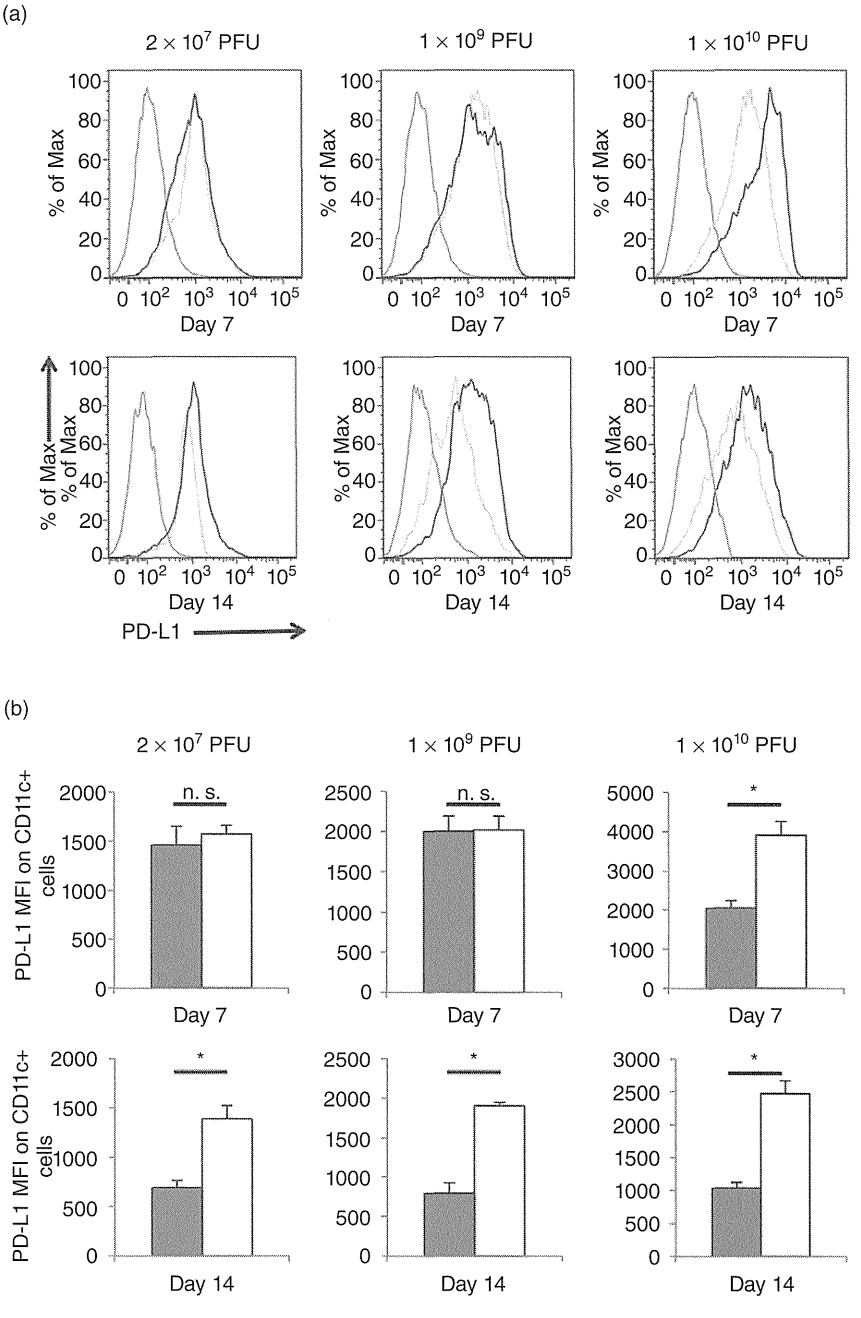

Figure 5 PD-L1 expression in the liver of core (+) and core (−) mice. Core (+) and core (−) mice were injected with 2×10^7, 1×10^9 and 1×10^{10} plaque-forming units (PFU) of adenovirus (Ad)-hepatitis C virus (HCV)-NS3 or Adψ5 control vector. (a) PD-L1 expression by intrahepatic antigen-presenting cells (APC) from core (+) and core (−) mice infected with Ad-HCV-NS3. The % of Max is the number of cells in each sample divided by the number of cells in the sample that contains the largest number of cells. (b) The median fluorescence index (MFI) expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 ($^*P < 0.05$; n.s., not statistically significant). (c) PD-L1 expression by intrahepatic APC from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector. (d) The MFI expression of PD-L1 by intrahepatic $CD11c^+$ leukocyte from core (+) and core (−) mice infected with Ad-HCV-NS3 or Adψ5 control vector (n.s., not statistically significant). (a) ┈┈, isotype; ┈┈, core (−); ——, core (+); (b) ■, core (−); □, core (+); (c) ┈┈, isotype; ┈┈, Adψ5; ——, Ad-NS3; (d) ■, Adψ5; □, Ad-NS3.

in Ag-specific intrahepatic CD8 T cells. These results indicated that high infectious dose and the presence of HCV core gene were strongly involved in ineffective CD8 T-cell responses.

Recently, a novel mechanism of T-cell dysfunction was demonstrated in a murine model of chronic LCMV infection.[24] It was found that the expression of PD-1 was upregulated on dysfunctional LCMV-specific CD8 T cells in mice.[24] *In vivo* blockade of PD-1/PD-L1 interaction restored the functions of LCMV-specific CD8 T cells and reduced the viral titer.[24] More recently, other inhibitory receptors such as Tim-3 have also been studied as the factors that can cause T-cell impairments in chronic viral infections.[25] These influential discoveries led to

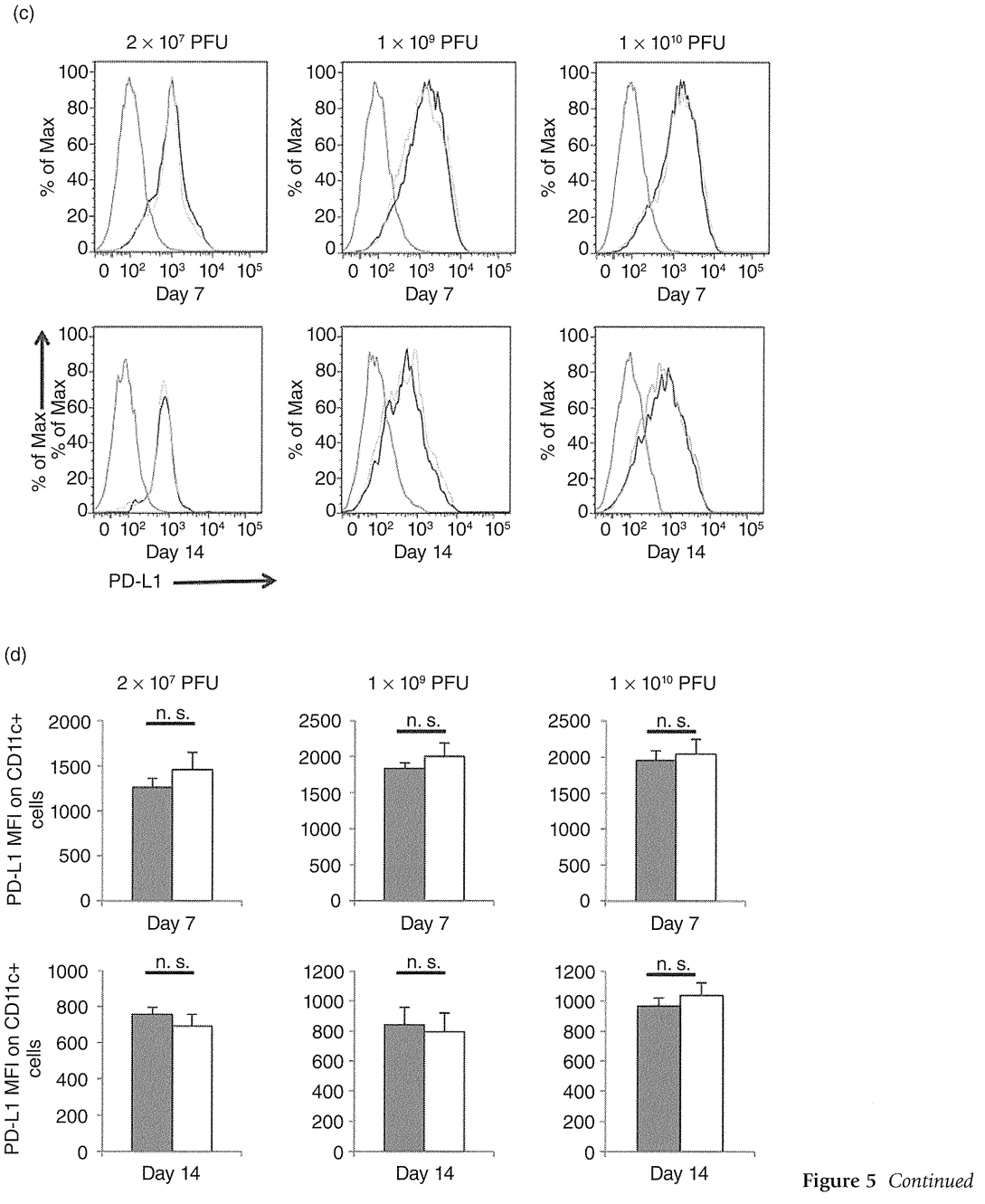

Figure 5 *Continued*

extensive investigations of inhibitory receptors in the regulation of T cells in human chronic viral infections.[25,26]

Chronic HCV infection in humans is characterized by CD8 T-cell exhaustion and dysfunction.[27] As in chronic LCMV infection, the expression of PD-1 is similarly upregulated on the virus-specific CD8 T cells in chronic HCV infection, and HCV-specific PD-1^{high} T cells are functionally impaired.[28–30] Also, Tim-3 is overexpressed on HCV-specific dysfunctional CD8 T cells.[25] In addition, a blockade of PD-1/PD-L1 or Tim-3/galectin9 (Gal9) interaction restores T-cell functions such as proliferation, cytolytic activity and cytokine (IFN-γ and tumor necrosis factor-α) production.[25,28–30] As was

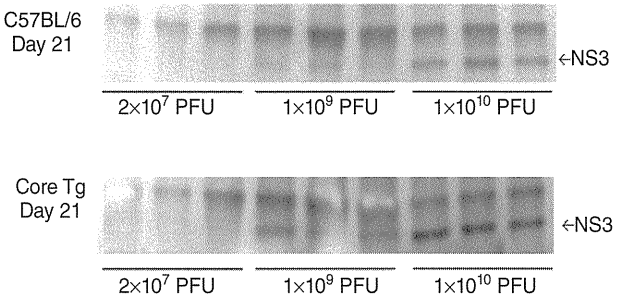

Figure 6 Persisting hepatitis C virus (HCV)-NS3 antigen detection was performed on the liver sections isolated 21 days post-infection. Liver sections were analyzed by IP-western blot assay using anti-FLAG antibody.

mentioned above, it has been reported that increased expression of inhibitory receptors is associated with the impaired HCV-specific CD8 T cells observed in chronic HCV patients. However, the underlying mechanisms for HCV-mediated impaired CD8 T-cell responses have yet to be determined. Based on our finding that lower level of activation and higher levels of expression of regulatory molecules, Tim-3 and PD-1, by intrahepatic CD8 T cells and higher levels of expression of PD-L1 by intrahepatic APC were observed in core (+) mice in comparison with core (−) mice, it is possible that HCV core-induced T-cell dysfunction is one of the viral factors that contributes to impaired CD8 T-cell responses as seen in chronic HCV patients. Our speculation is in accordance with the study by Lukens *et al.*[31]

Suppression of CTL responses via highly expressed Ag was found in chronic HCV infection. Inverse relationships between HCV viral titer and HCV-specific T cells have been reported.[7,32,33] In this study, we found higher levels of expressions of PD-L1 by intrahepatic APC and an impaired intrahepatic CD8 T-cell response in high infectious dose setting. Moreover, we found a significant inverse correlation between the percentages of IFN-γ-producing cells and expression of regulatory molecules in Ag-specific intrahepatic CD8 T cells. It is likely that the PD-1/PD-L1 or Tim-3/Gal9 pathway play a major inhibitory role in our model. High-dose Ad-HCV NS3 infection may inhibit the NS3-specific CD8 T-cell responses not at the induction phase but at the effector phase because Ag-specific-MHC tetramer$^+$ T cells were observed, and most Ag-specific MHC tetramer$^+$ T cells was anergic to PMA/ionophore stimulation and these T cells expressed PD-1 and Tim-3. The role of PD-1/PD-L1 as mechanism for liver tolerance has been well established. PD-1 expression by T cells has been shown to inhibit intrahepatic antiviral immune responses at the effector phase.[34–36]

Hepatitis C virus infection affects approximately 170 million people in the world and is a major global health problem because infected individuals can develop liver cirrhosis and hepatocellular carcinoma. Pegylated interferon and ribavirin therapy, although beneficial in approximately half of treated patients, are expensive and associated with significant side-effects.[37] In this clinical context, there is an urgent need for the development of a therapeutic and/or prophylactic HCV vaccine.[38] Because HCV infects only humans and chimpanzees, it is difficult to evaluate effective therapeutic vaccine candidates. Recently, as a small animal model for HCV infection study, chimeric humanized mouse harboring a human hepatocyte and hematolymphoid system was established by xenotransplantation technique.[39,40] The xenograft model provides a unique opportunity for HCV vaccine development. However, the generation of this chimeric humanized mouse requires advanced technical skills and the scarcity of adequate human primary material remains a significant logistical challenge.[41,42] Our model showed in the present study is easy to create, and it has Ag-specific T-cell exhaustion and Ag persistent in the liver seen in chronic HCV patients. These features suggest that this system is useful for therapeutic HCV vaccine development.

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Incidence Rate of Needlestick and Sharps Injuries in 67 Japanese Hospitals: A National Surveillance Study

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Abstract

Background: Determining incidence rates of needlestick and sharps injuries (NSIs) using data from multiple hospitals may help hospitals to compare their in-house data with national averages and thereby institute relevant measures to minimize NSIs. We aimed to determine the incidence rate of NSIs using the nationwide EPINet surveillance system.

Methodology/Principal Findings: Data were analyzed from 5,463 cases collected between April 2009 and March 2011 from 67 Japanese HIV/AIDS referral hospitals that participated in EPINet-Japan. The NSI incidence rate was calculated as the annual number of cases with NSIs per 100 occupied beds, according to the demographic characteristics of the injured person, place, timing, device, and the patients' infectious status. The NSI incidence rates according to hospital size were analyzed by a non-parametric test of trend. The mean number of cases with NSIs per 100 occupied beds per year was 4.8 (95% confidence interval, 4.1–5.6) for 25 hospitals with 399 or fewer beds, 6.7 (5.9–7.4) for 24 hospitals with 400–799 beds, and 7.6 (6.7–8.5) for 18 hospitals with 800 or more beds (p-trend<0.01). NSIs frequently occurred in health care workers in their 20 s; the NSI incidence rate for this age group was 2.1 (1.6–2.5) for hospitals having 399 or fewer beds, 3.5 (3.0–4.1) for hospitals with 400–799 beds, and 4.5 (3.9–5.0) for hospitals with 800 or more beds (p-trend<0.01).

Conclusions/Significance: The incidence rate of NSIs tended to be higher for larger hospitals and in workers aged less than 40 years; injury occurrence was more likely to occur in places such as patient rooms and operating rooms. Application of the NSI incidence rates by hospital size, as a benchmark, could allow individual hospitals to compare their NSI incidence rates with those of other institutions, which could facilitate the development of adequate control strategies.

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Introduction

Needlestick and sharps injuries (NSIs) are major risks for blood-borne pathogen exposure in hospitals [1,2]. The risk associated with NSIs varies depending on the devices, sharps waste management practices, degree of experience of health care workers, training opportunities, and the level of universal precaution practices [2,3]. Various measures have been implemented in the United States to minimize the risk of sustaining NSIs, such as the proper use of safety-engineered devices, and the regulatory requirement that these devices be provided; these have reduced the incidence of NSIs [4,5].

Hospitals may monitor NSIs by using adequate surveillance methods, for example EPINet, a tool used in the United States and other countries for monitoring NSIs, in order to develop a strategy for minimizing NSIs [4,6]. EPINet was developed by the International Healthcare Worker Safety Center at the University of Virginia in 1992 [7]. In Japan, nationwide EPINet surveillance projects have used it since 1996 [8,9].

A benchmark for the incidence rate of NSIs would allow hospitals to compare their data with those of other institutions and also assist with the development of measures to minimize NSIs [4,5,10]. There have been few studies focusing on the incidence rate of NSIs across a range of hospitals in Japan [11,12], although some have examined rates in individual hospitals [13–16]. The aim of this study was to determine the incidence rate of NSIs in 67 hospitals located throughout Japan using the nationwide EPINet surveillance system.

Table 1. Number of needle stick injuries and mean incidence rate per 100 beds per year (n = 67 hospitals).

	N	(%)	Mean	(95%CI)
Total	5,463		6.2	(5.7–6.7)
Ages of injured health care workers (years)				
20–29	2,946	(53.9)	3.2	(2.9–3.6)
30–39	1,602	(29.3)	1.9	(1.7–2.1)
40–49	583	(10.7)	0.7	(0.7–0.8)
50+	332	(6.1)	0.4	(0.3–0.4)
Occupation of injured health care workers				
Doctors	1,882	(34.4)	2.1	(1.9–2.4)
Nurses	2,838	(51.9)	3.2	(3.0–3.5)
Place injuries occurred				
Patient room	1,728	(31.6)	2.0	(1.8–2.2)
Operating room	1,451	(26.6)	1.7	(1.5–1.9)
Outside of patient room	546	(10.0)	0.6	(0.5–0.7)
Outpatient clinic/office	473	(8.7)	0.5	(0.5–0.6)
Intensive/critical care unit	214	(3.9)	0.2	(0.2–0.3)
Emergency department	207	(3.8)	0.2	(0.2–0.3)
Timing of injury during device use				
Before use of device	246	(4.5)	0.3	(0.2–0.3)
During use of device	1,431	(26.2)	1.7	(1.5–1.8)
Between steps of a multi-step procedure	592	(10.8)	0.7	(0.6–0.8)
Disassembling device or equipment	402	(7.4)	0.4	(0.4–0.5)
In preparation for reuse of a reusable instrument	136	(2.5)	0.2	(0.1–0.2)
While recapping a used needle	476	(8.7)	0.6	(0.5–0.7)
Withdrawing a needle from rubber or another resistant material	170	(3.1)	0.2	(0.1–0.2)
Other after use-before disposal	479	(8.8)	0.5	(0.4–0.6)
While putting the device into a disposal container	409	(7.5)	0.5	(0.4–0.5)
Restraining patient	194	(3.6)	0.2	(0.2–0.3)
Devices causing injuries				
Disposable syringe	1,388	(25.4)	1.6	(1.4–1.7)
Suture needle	910	(16.7)	1.0	(0.9–1.2)
Winged steel needle	629	(11.5)	0.7	(0.6–0.8)
Pre-filled cartridge syringe	424	(7.8)	0.5	(0.4–0.6)
IV catheter stylet	336	(6.2)	0.4	(0.3–0.5)

CI: Confidence interval.

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Materials and Methods

Ethics Statement

The Human Research Committee at the Institute for Science of Labour approved the research methods and processes prior to study commencement (No. 2009-01).

Data Collection

We targeted the HIV/AIDS referral hospitals in Japan since these hospitals are designated as secondary or tertiary care hospitals in their regions and are distributed geographically throughout Japan. These hospitals are also expected to have better precautions for needle stick injuries in place [17]. In 2008, participation agreement forms were sent to the directors of all 364 HIV/AIDS referral hospitals in Japan. Agreement to voluntarily participate in the study was obtained from 117 institutions. The infection control team at each hospital required all workers to report any NSIs and record each case using the EPINet-Japan form. In July 2011, we asked all 117 institutions to provide individualized NSIs data that had occurred between April 2009 and March 2011. We received individualized data from 67 of the 117 institutions.

Statistical Analysis

Participating institutions were asked to report the number of approved beds by their local government and the mean annual bed occupation rate to calculate the actual number of occupied beds. The number of NSIs per year at each hospital was divided by the corresponding number of occupied beds, and the mean NSI incidence rate per 100 occupied beds and 95% confidence interval (CI) were calculated. The incidence rates were also classified according to the characteristics of the injured persons (e.g. age and

job title), place (e.g. operating room), timing (e.g. when recapping the needle), and the devices involved.

The participating hospitals were then classified into three groups according to the number of occupied beds, i.e. those with 399 or fewer beds, those with 400–799 beds, and those with 800 or more beds. This was because of the variance between hospitals of different sizes with regard to their utilization of procedures associated with a high NSI risk, number of patients, number of health care workers, and the infection control system measures used. The NSI incidence rates of each category according to hospital size were analyzed by a non-parametric test of trend to identify any trends among the three hospital sizes. We analyzed the data using Stata version 11 (Stata Corp, College Station, TX).

Results

The distribution of the hospitals that participated by location (n = 67) was evenly distributed throughout the country. The number of hospitals was highest in the Chubu area. The number of hospitals with a daily average of 399 or fewer occupied beds, 400–799 beds, or 800 or more beds, was 25, 24 and 18 hospitals, respectively.

The total number of reported NSI incidents was 2,680 from April 2009 to March 2010, and 2,783 from April 2010 to March 2011, equating to 5,463 incidents for the study period. Table 1 shows the annual NSI incidence rate per 100 occupied beds in relation to the demographic characteristics of the injured persons, place, timing, device, and the patients' infectious status. The mean annual NSI incidence rate per 100 occupied beds with 95% CI was 6.2 (5.7–6.7). The injured health care workers were most frequently aged in their 20 s (3.2 [2.9–3.6]); nursing was the most frequently reported occupation (3.2 [3.0–3.5]). NSIs occurred predominantly in patient rooms (2.0 [1.8–2.2]) and operating rooms (1.7 [1.5–1.9]). NSIs most often occurred during the use of medical devices (1.7 [1.5–1.8]), and involved mainly disposable syringes (1.6 [1.4–1.7]) and suture needles (1.0 [0.9–1.2]).

Table 2 shows the annual NSI incidence rates for three different hospital sizes in relation to the demographic characteristics of the injured persons, place, timing, and devices involved. Among injured persons 39 years of age or younger, there was a significant trend for the incidence rate to rise as the hospital size increased (p-trend<0.01). Among injured persons in their 40 s and older, there was no significant trend to suggest a relative increase in incidence rates within larger hospitals. When we focused on the injured staff member's occupation, there was a similar increase in the incidence rate according to larger hospital size. Among the various places the injuries occurred, no hospital size-related trend was observed for emergency departments. NSIs occurred most frequently during the use of medical devices, regardless of hospital size. There was no significant trend indicating a rise in recapping injuries with increasing hospital size.

Discussion

We determined annual NSI incidence rates in 67 HIV/AIDS referral hospitals in Japan, and clarified incidence trends in hospitals of three sizes, based on the number of occupied beds. The number of NSIs tended to be higher for larger hospital sizes and in workers aged less than 40 years; injury occurrence was more likely to occur in places such as patient rooms and operating rooms. Application of the NSI incidence rates by hospital size, as a benchmark, could allow individual hospitals to compare their NSI incidence rates to those of other institutions, which could facilitate the development of adequate control strategies.

The mean NSI incidence rate in Japan calculated per 100 beds per year was 6.2, which is lower than the corresponding rates in the United States, Taiwan, and South Korea [18–20]. The place of injury associated with the highest rate in the United States is the operating room (9.9 per 100 beds), followed by patient room (6.7 per 100 beds) [10]. In contrast, the corresponding figures in this study were 1.7 and 2.0 per 100 beds, respectively, showing relatively fewer injury occurrences compared with the United States. The lower number of NSIs per 100 beds in Japan may be explained by the fact that fewer sharps devices are handled per unit bed, because the mean hospital stay of patients is longer in Japan. The mean hospital stay of patients in 2009 was 18.8 days in Japan, much longer than 4.9 days in the United States [21]. Thus, the number of devices used in Japan per bed on a daily basis, may also be lower, and this could have possibly reduced the overall NSI incidence rates per hospital bed. However, the level of precautions among hospitals may be varied; for example, the studied hospitals might take more precautions since they are conscious of the risks of NSI as they are HIV/AIDS referral hospitals.

Previous studies have shown that NSIs occur more frequently among nurses than doctors [14–16]. However, our study suggests there is a chance that doctors are at greater risk of NSIs than nurses. Although, there are (on average) 3.5 times more nurses than doctors within medical institutions in Japan [22], the mean number of NSIs was 1.5-fold higher in nurses than doctors in our study. Since denominator data were lacking in this study (i.e. the number of persons engaged in a particular occupation), precise calculations were not possible. However, previous studies have shown that injuries are more common in surgeons [23] and medical residents [24] in particular, and that the number of NSIs varies among different specialties [25]. It is important to determine, more precisely, the risks for various medical specialists and to provide efficient interventions.

Analysis of NSI incidence rates by age showed that NSI rates in health care workers less than 40 years of age increased with hospital size. In contrast, there was no significant upward trend identified in the NSI rate in personnel over 40 years of age, relative to hospital size. This might be because middle-aged workers at hospitals deal less frequently with blood collection or other tasks that are associated with a high risk of NSI, and are focused on more administrative tasks. It is noteworthy that 51.6% of cases with NSIs were 20–29 years old, while 30.6% were 30–39 years old. Smith et al. reported that nurses 25 years of age and younger at a university affiliated hospital had 2.2 times the risk of NSIs compared with nurses over 25 years old [14]. Young health care workers are clearly a priority when providing educational training concerning NSI prevention [26].

Whether a particular work location carries a high risk of NSIs depends on the types of medical procedures carried out in that place. Procedures with direct access to blood vessels, such as collection of blood samples and placement of intravenous catheters, are frequently undertaken in patient rooms [27,28]. As a consequence, NSIs occurring in patient rooms account for a high proportion of total NSI incidents [29,30].

NSIs during recapping might be prevented by the placement of sharps containers in convenient places to help facilitate effective and safe disposal [31]. In this study, NSIs that occurred during recapping accounted for 9.7% of all NSIs, a lower incidence than the corresponding figure in Taiwan (16.5%) [32], but higher than 2006/07 data from the United States (about 4%) [10]. We observed no discernible trend in the number of NSIs due to recapping according to hospital size during this study. The Ministry of Health, Labour and Welfare, Japan has requested prohibiting re-capping in their guideline of infection control [33].

Table 2. Annual incidence rates of needle stick injuries per 100 beds in hospitals of three different sizes.

	399 or fewer beds (n = 25 hospitals)		400–799 beds (n = 24 hospitals)		800 or more beds (n = 18 hospitals)		p-value
	Mean	(95%CI)	Mean	(95%CI)	Mean	(95%CI)	(Trend)
Total	4.8	(4.1–5.6)	6.7	(5.9–7.4)	7.6	(6.7–8.5)	<0.01
Ages of injured health care workers (years)							
20–29	2.1	(1.6–2.5)	3.5	(3.0–4.1)	4.5	(3.9–5.0)	<0.01
30–39	1.6	(1.3–1.9)	1.9	(1.6–2.2)	2.2	(1.8–2.5)	<0.01
40–49	0.8	(0.6–1.0)	0.8	(0.7–0.9)	0.6	(0.4–0.7)	0.15
50+	0.4	(0.3–0.4)	0.4	(0.3–0.5)	0.4	(0.3–0.5)	0.2
Occupation of injured health care workers							
Nurses	2.8	(2.5–3.2)	3.3	(2.9–3.8)	3.9	(3.4–4.3)	<0.01
Doctors	1.4	(1.1–1.8)	2.5	(2.1–3.0)	2.6	(2.1–3.1)	<0.01
Place injuries occurred							
Patient room	1.6	(1.3–1.9)	2.0	(1.7–2.3)	2.5	(2.1–2.8)	<0.01
Operating room	1.2	(0.9–1.6)	1.9	(1.6–2.3)	2.0	(1.7–2.3)	<0.01
Outside patient room	0.5	(0.4–0.6)	0.6	(0.5–0.7)	0.8	(0.7–0.9)	<0.01
Outpatient clinic/office	0.4	(0.3–0.5)	0.6	(0.4–0.7)	0.7	(0.6–0.8)	<0.01
Intensive/critical care unit	0.2	(0.1–0.2)	0.2	(0.1–0.3)	0.3	(0.2–0.4)	<0.01
Emergency department	0.2	(0.1–0.3)	0.3	(0.2–0.4)	0.2	(0.2–0.3)	0.2
Timing of injury during device use							
Before use of device	0.2	(0.1–0.3)	0.3	(0.2–0.4)	0.4	(0.3–0.5)	<0.01
During use of device	1.2	(1.0–1.5)	2.1	(1.8–2.4)	1.8	(1.6–2.0)	<0.01
Between steps of a multi-step procedure	0.5	(0.4–0.7)	0.7	(0.5–0.8)	0.9	(0.7–1.1)	<0.01
Disassembling device or equipment	0.2	(0.1–0.3)	0.6	(0.5–0.7)	0.6	(0.4–0.7)	<0.01
In preparation for reuse of a reusable instrument	0.1	(0.1–0.2)	0.2	(0.1–0.2)	0.2	(0.1–0.3)	<0.01
While recapping a used needle	0.6	(0.4–0.7)	0.6	(0.5–0.7)	0.6	(0.5–0.7)	0.76
Withdrawing a needle from rubber or another resistant material	0.1	(0.1–0.2)	0.2	(0.1–0.2)	0.3	(0.1–0.4)	0.01
Other after use-before disposal	0.4	(0.3–0.5)	0.5	(0.4–0.6)	0.8	(0.6–1.0)	<0.01
While putting the device into disposal container	0.4	(0.3–0.6)	0.5	(0.4–0.6)	0.5	(0.5–0.6)	0.03
Restraining patient	0.2	(0.1–0.3)	0.2	(0.1–0.2)	0.3	(0.2–0.4)	<0.01
Devices causing injuries							
Disposable syringe	1.2	(1.0–1.5)	1.7	(1.4–1.9)	1.9	(1.7–2.1)	<0.01
Suture needle	0.7	(0.5–1.0)	1.2	(1.0–1.4)	1.2	(1.1–1.4)	<0.01
Winged steel needle	0.5	(0.3–0.6)	0.8	(0.6–1.0)	0.9	(0.7–1.1)	<0.01
Pre-filled cartridge syringe	0.4	(0.3–0.5)	0.4	(0.3–0.6)	0.7	(0.5–0.8)	<0.01
IV catheter stylet	0.4	(0.3–0.5)	0.4	(0.3–0.5)	0.4	(0.3–0.6)	0.34

CI: Confidence interval.

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Larger hospitals might take this stance in the prevention from NSIs by prohibiting recapping or installing a needle with automatic needle retraction system resulting in the observed decrease of recapping-related injuries.

The introduction of devices with safety equipment is known to be effective for preventing device-related NSIs [34,35]. In this study, injuries relating to winged steel needles, IV catheter stylets, and suture needles accounted for 33.9% of incidents; the devices responsible for these injuries were equipped with safety mechanisms, which are available in Japan. However, disposable syringes with safety features have not, to date, been widely used in Japan [36]. Therefore, the introduction of disposable syringes in Japan should be promoted, employing an approach suited to the local health care service.

This study has limitations. An underreporting of injuries may have resulted in an underestimation of true NSI incidence rates. In addition, it should also be noted that staff in larger hospitals may be encouraged to report NSIs and this may account for the higher incidence of NSIs observed in these institutions. Further studies are needed to more accurately estimate the incidence of NSIs. This could be achieved by combining different study designs, such as including an anonymized questionnaire survey. Second, HIV/AIDS referral hospitals are facilities designated by the Ministry of Health, Labour and Welfare. These hospitals must implement a higher than usual standard of occupational infection control and exceed routine levels of NSI control, a potential confounder. Generalizability of our results to other types of hospitals still needs to be considered, especially if the parameters within our work are

set as a goal to strive for. Finally, we also need to further elaborate underlying factors, such as number of rooms or patients per staff member and the number of treatments requiring more invasive practices for each institute, in order to validate our findings of the increased incidence rate of NSIs in larger hospitals.

In conclusion, the incidence rate of NSIs tended to be higher for larger hospitals and in workers aged less than 40 years; injury occurrence was more likely to occur in places such as patient rooms and operating rooms. Application of the NSI incidence rates by hospital size, as a benchmark, could allow individual hospitals to compare their NSI incidence rates with those of other institutions, which could facilitate the development of adequate control strategies.

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Author Contributions

Conceived and designed the experiments: TY KW JL KK TO SK KM. Performed the experiments: TY JL TM HK YM MA. Analyzed the data: TY KW. Contributed reagents/materials/analysis tools: TY KW KK SK. Wrote the paper: TY KW.

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PLOS ONE

New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

* Resolved individuals were HBsAg negative and HBcAb positive.

** 419 of 467 healthy controls were de-identified, without information on age.

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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: logit(p) = −3.905+0.083*age+(−0.929)*sex+(−0.684)**DPB1*02:01*+1.814*c1+(−0.478)*c2+0.782*c3. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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