4. Discussion

In the present study, we investigated the role of BCAA in the HCV life cycle and discovered that these amino acids suppress HCV genome replication but promote viral particle formation. Thus far, many reports have indicated that various cellular factors are involved in the regulation of HCV. In particular, intracellular signaling pathways are important modulators for HCV genome replication [5–10]. BCAA, specifically leucine, were demonstrated to have a role in activating the mTOR pathway, leading to protein synthesis such as upregulation of albumin [4] and HGF production [19]. Recently, mTOR was reported to be involved in IFN-α signaling [17]. IFN-α induced phosphorylation of STAT1 was diminished by rapamycin (but not by LY294002, a PI3 kinase inhibitor). Consequently, rapamycin inhibited the IFN-stimulated regulatory element. Although we demonstrated that BCAA can activate mTOR (Fig. 2A), the inhibition of mTOR revealed that it was not the main pathway for the BCAA suppression of HCV replication. BCAA supplementation did not change the STAT1 phosphorylation status, nor did it induce ISG expression, indicating that the JAK/STAT pathway was not relevant for the suppression of HCV replication. Considering that leucine, the factor required for mTOR activation, did not actually take part in regulation of the HCV replicon (Fig. 1E), it was not surprising that mTOR was shown to not be the responsible molecule.

Very recently, Honda et al. demonstrated that STAT1 phosphorylation was increased by BCAA in a dose-dependent manner [20]. They showed that BCAA increased the phosphorylation levels of STAT1, Foxo3a and p70 S6 kinase leading to downregulation of Socs3 expression and HCV replication. The range of BCAA concentration examined in the present study was between 0 and 2 mM. We ranged the concentration of BCAA between 0 and 2 mM because its concentration in blood is approximately 1.6 mM after oral administration of 5 g of BCAA. However, in the Honda et al. study, the BCAA concentration at which STAT1 was efficiently phosphorylated was more than 4 mM, whereas at 2 mM or less, no obvious increase in phospho-STAT1 was observed. Therefore, we may have detected no change in phospho-STAT1 due to BCAA levels used in this study. Thus, BCAA may be capable of suppressing HCV genome replication at a low concentration by inhibiting HCV IRES activity while decreasing virus replication by augmentation of IFN signaling at high concentrations.

Although BCAA suppressed replication of HCV replicon, they increased HCVcc production in infected cells. The life cycle of HCV has many steps that are required to achieve infection, such as attachment to the cell surface, endocytosis of the virus, uncoating and releasing genome RNA, RNA replication, polyprotein synthesis and processing, viral assembly, and release of progeny virus. Among these, the HCV replicon system only represents the steps of genome RNA replication and non-structural protein synthesis in the cells, and BCAA affects these by impairing protein synthesis via suppression of HCV IRES activity. However, HCVcc replication requires all of these steps. We assumed that the increase of HCVcc due to BCAA indicated that some step(s) must be upregulated by BCAA to the extent of overcoming the decreased genome replication. The study of particle formation-deficient viruses suggested that virus assembly or some downstream step in the virus life cycle was critical for the augmentation of HCVcc by BCAA. A single-cycle virus production assay indicated that the production of an infectious virus was prominent in the presence of BCAA, while virus secretion was not strongly affected. Although HCV genome replication was suppressed by BCAA, more infectious virus particles were secreted into the media, and they could have re-infected the Huh7 cells. We suggest that the abundant infectious HCV in BCAA-supplemented medium causes amplification of the virus during repetition of such re-infection, which leads to an accumulation of HCV in the cells, and thus, the abundance of HCV RNA in the cells with BCAA medium overcomes that without BCAA. Further investigation is needed on the detailed mechanisms defining how BCAA regulates HCV particle formation. Clarification of this process could contribute to new insights into HCV replication and could also suggest a basis for treatment of HCV patients.

Acknowledgments and disclosures

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.051.

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1(OH) Vitamin D3 Supplementation Improves the Sensitivity of the Immune-Response during Peg-IFN/RBV Therapy in Chronic Hepatitis C Patients-Case Controlled Trial

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Abstract

Objective: 1,25(OH)$_2$ vitamin D3 can affect immune cells. However, the mechanism responsible for the favorable effects of 1(OH) vitamin D3, which becomes 1,25(OH)$_2$ vitamin D3 in the liver, is not clear. The aim of this study is to analyze the immunological response of 1(OH) vitamin D3 supplementation in CH-C patients.

Design: Forty-two CH-C patients were treated with 1(OH) vitamin D3/Peg-IFNα/RBV. Forty-two case-matched controls were treated with Peg-IFNα/RBV. The expression of Interferon-stimulated genes (ISGs)-mRNA in the liver biopsy samples and JFH-1 replicating Huh-7 cells were quantified by real-time PCR. Ten kinds of cytokines in the plasma were quantified during treatment by using a suspension beads array. A trans-well co-culture system with peripheral blood mononuclear cells (PBMCs) and Huh-7 cells was used to analyze the effect of 1(OH) vitamin D3. The activities of the Th1 response were compared between subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV and those treated with Peg-IFN/RBV therapy alone.

Results: 1(OH) vitamin D3/Peg-IFN/RBV treatment could induce rapid viral reduction, especially in *IL28B T/T* polymorphism. Several kinds of cytokines including IP-10 were significantly decreased after 4 weeks of 1(OH) vitamin D3 treatment ($p<0.05$). Th1 responses in the subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV were significantly higher than those treated with Peg-IFN/RBV at 12 weeks after Peg-IFN/RBV therapy ($p<0.05$). The expression of ISGs in the patient's liver biopsy samples was significantly lower than in those treated without 1(OH) vitamin D3 ($p<0.05$).

Conclusion: 1(OH) vitamin D3 could improve the sensitivity of Peg-IFN/RBV therapy on HCV-infected hepatocytes by reducing the IP-10 production from PBMCs and ISGs expression in the liver.

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Introduction

Hepatitis C Virus (HCV) is a non-cytopathic virus that causes chronic inflammation, fibrosis and hepatocellular carcinoma (HCC) [1]. Recently, it has been reported that vitamin D3 supplementation could improve the SVR in chronic hepatitis C (CH-C) patients [2,3]. Moreover, the amount of 25-hydoxyvitamin D3 (25(OH) vitamin D3) in the serum could affect the treatment response to pegylated interferon α (Peg-IFN-α)/ribavirin (RBV) therapy and is complementary to interleukin 28B (*IL-28B*) rs1297860 C/T polymorphism in enhancing the correct prediction of the SVR in CH-C [4]. Another group reported that, in patients with genotype 1 HCV persistent infection, the 25(OH) vitamin D serum levels and *IL28B* polymorphism were independently associated with the likelihood of achieving a rapid viral response and SVR after treatment with Peg-IFN/RBV [5].

Although several kinds of mechanisms for the favorable effects of vitamin D3 supplementation were reported, the total effect of vitamin D3 supplementation remains unclear [6,7]. One group reported that 25(OH) vitamin D3, but not vitamin D3 or 1, 25 dihydoxyvitamin D3 (1, $25(OH)_2$ vitamin D3), appeared to inhibit the viral life cycle at the level of infectious HCV assembly [7]. Another group reported that vitamin D3 or $1,25(OH)_2$ vitamin D3 and IFN-α could synergistically inhibit HCV production by enhancing the IFN signaling pathway [6]. However, the effect of vitamin D3 on the adaptive immune system in CH-C patients has not been reported yet.

It has been reported that vitamin D3, as synthesized in the skin by photolysis from 7-dehydrocholesterol or ingested with food, is transported in the blood to the liver where it is hydoxylated at the C-25-position [8]. Then, it is hydoxylated at the C-1α-position to form the active metabolite 1,25 $(OH)_2$ vitamin D3 in the kidney [9,10]. In this study, we selected 1(OH) vitamin D3, since the local concentration in the liver should be higher than other metabolites of vitamin D3. Moreover, 1 (OH) vitamin D3 is safe and commonly used in worldwide. 1,25 $(OH)_2$ vitamin D3 is known to regulate calcium and phosphorus metabolism in skeletal homeostasis [11]. It has been reported that $1,25(OH)_2$ vitamin D3 plays an important role as an immune-modulator targeting various immune cells [12–15]. Various kinds of immune cells express not only vitamin D receptors (VDRs) but also vitamin D-activating enzymes, allowing local conversion of inactive vitamin D into 1,25 $(OH)_2$ vitamin D3 within the immune system [16,17]. The active metabolite$1,25(OH)_2$ vitamin D3 could enhance the anti-mycobacterial activity in monocytes by enhancing the chemotactic and phagocytic capacity of macrophages [18]. Moreover, $1,25(OH)_2$ vitamin D3 might play an important role in the binding and capturing of antigens by dendritic cells (DCs) at the initiation of the immune response [19]. On the other hand, some groups reported that 1, $25(OH)_2$ vitamin D3 could inhibit the differentiation and maturation of DCs [19,20]. In addition to monocyte-derived cells, $CD3^+$ T cells, $CD19^+$ B cells, natural killer cells (NK cells) could be directly and/or indirectly affected by 1, $25(OH)_2$ vitamin D3[12,17,21–24]. It has been reported that 1, $25(OH)_2$ vitamin D3 could contribute to the suppression of the immune response in autoimmune diseases [14,15,25]. More Recently, the expression of specific VDRs in liver cells and reduced expression of VDRs in CH-C patients have been reported [26]. In addition, an inverse relationship between the liver VDR expression and inflammation severity has been found [26]. However, the effects of 1, $25(OH)_2$ vitamin D3 for the adaptive immune system in the condition of CH-C patients and during treatment with peg-interferon α and ribavirin (Peg-IFN/RBV) are still unclear. Therefore, it is urgent to analyze the effect of 1, $25(OH)_2$ vitamin D3 on the adaptive immune responses that could contribute to the outcome of Peg-IFN/RBV therapy.

Materials and Methods

Study Design and Patients

Multi-centers that belong to the Tohoku-liver-study-group (TLG) were involved in this study. Dr. Abu-Mouch et al. reported that the SVR rate of Peg-IFN/RBV plus Vitamin D treatment group was 86% in the AASLD 2009 annual meeting [27]. On the

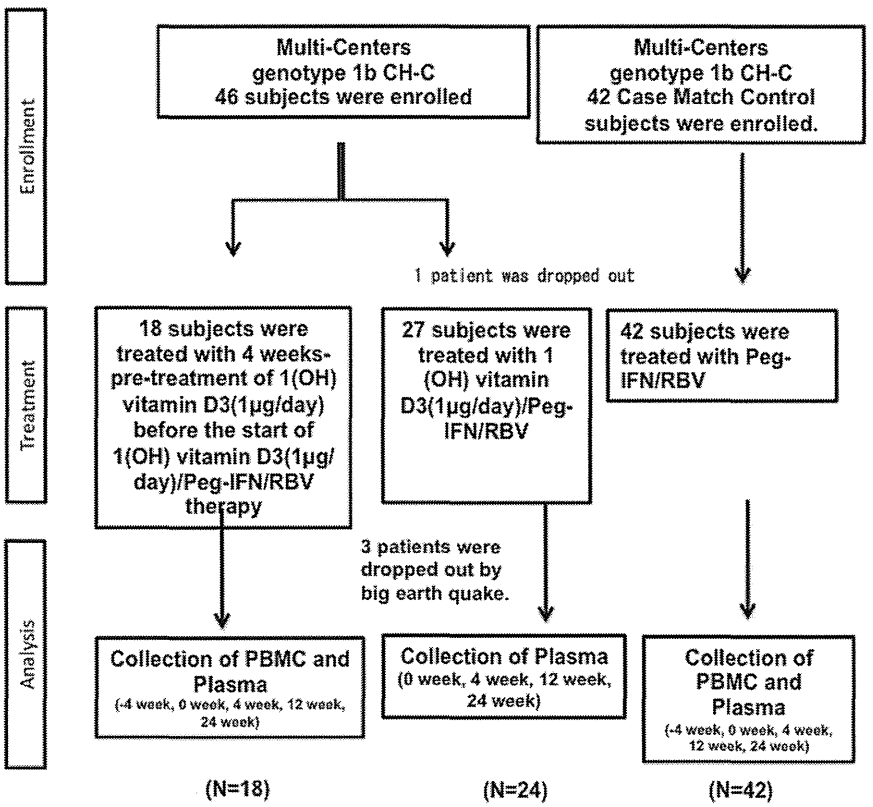

Figure 1. Enrollment of CH-C patients. 46 patients with genotype 1b and high viral loads were enrolled in this study. In total, 4 patients were dropped from this study.
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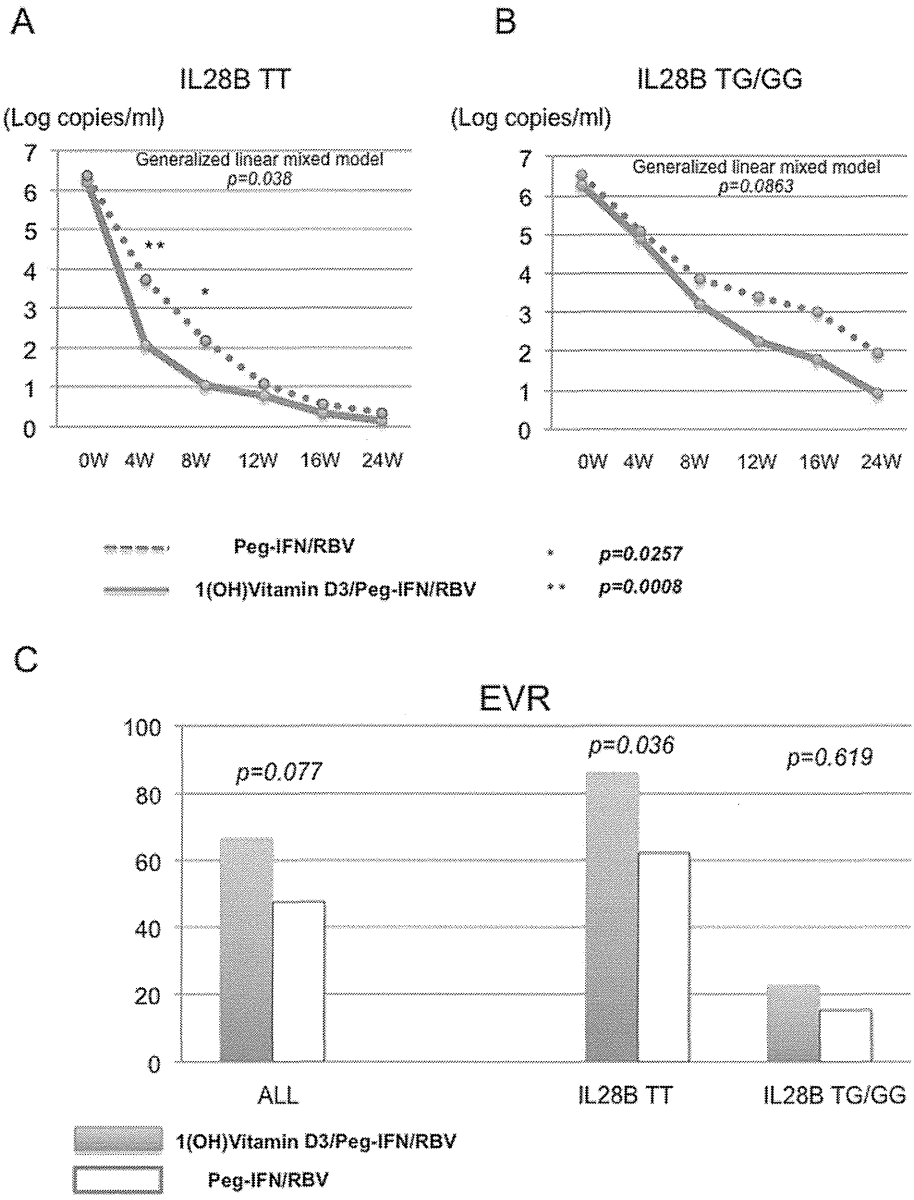

Figure 2. Comparison of viral dynamics and treatment response. Viral dynamics of subjects with IL28B T/T major homo polymorphism are shown (A). Viral dynamics of subjects with IL28B T/G or G/G minor polymorphism are shown (B). Blue lines indicate viral dynamics of subjects treated with 1(OH) Vitamin D3/Peg-IFN/RBV. Dotted lines indicate viral dynamics of subjects treated with Peg-IFN/RBV. *p<0.05 **p<0.01 The rates of early virological response in the patients treated with 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV are shown (C).
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other hand, the SVR rate of Peg-IFN/RBV treatment group was 42%. Considering the uncertainty, we speculated that the EVR rate might be 90% of the EVR rate in the Peg-IFN/RBV plus Vitamin D treatment group because the reported EVR rate in this study was remarkably high. Based on the results of this study, we enrolled about 80 patients including control patients: there was 10% loss in the proportion of patients during the 48 weeks therapy ($\alpha = 0.05$, statistical power 90%) (EVR rate 77% vs 42%). The alfa level was two-sided. Forty-six CH-C (Genotype 1b) patients were enrolled in this study (Fig. 1). Forty-two matched historical controls treated with Peg-IFN-α/RBV therapy were analyzed. The inclusion criteria were as follows: age between 20 and 75 years, high viral load (>5.0 log copies/mL) by real time PCR analysis of HCV-RNA, absolute white blood cell count >2,000/ml, neutrophil count >1,000/ml, platelet count >90,000/ml, and hemoglobin concentration >11 g/dL in laboratory tests. The exclusion criteria were as follows: other liver diseases, including autoimmune hepatitis and alcoholic hepatitis, decompensated liver cirrhosis, liver failure, severe renal disorders, abnormal thyroid function, poorly controlled diabetes, poorly controlled hypertension, medication with immune-modulators, interstitial pneumonia and severe depression. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2010-114) (UMIN000003694). The date of the protocol fixation was 10th June 2010. The anticipated trial start date was 11th June 2010. Patients in the 1(OH) vitamin D3/Peg-IFN/RBV group were treated from June 2010 to June 2012. Patients in the Peg-IFN/RBV group were treated from March

2009 to June 2012. Liver biopsy samples of the historical control were from previous studies (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent of the control subjects treated with Peg-IFN/RBV treatment was obtained in the previous study and in the present study (Permission no. 2009-166) (UMIN000002326), (Permission no. 2009-209), and (Permission no. 2010-404). Written informed consent was obtained from all the participants enrolled in the 1(OH) vitamin D3/Peg-IFN/RBV treatment group. Participants were monitored for a year. At each assessment, patients were evaluated by hematological test, biochemical laboratory tests, immunological test and virological tests. Liver histology was analyzed at the start of Peg-IFN/RBV therapy using the METAVIR score.

Detection of IL-28B Polymorphism

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using an automated DNA isolation kit. Then, the polymorphism of *IL28B* (rs8099917) was analyzed using real-time polymerase chain reaction (PCR) (TaqMan SNP Genotyping Assay, Applied Biosystems, CA, USA). Detection of the *IL28B* polymorphism was approved by the Ethics Committee at Tohoku University Graduated School of Medicine (permission no. 2010-323).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs), $CD4^+$ Cells and Cell Culture

PBMCs were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation (Amersham Bioscience, Uppsala, Sweden). Primary $CD4^+$ cells were isolated using magnetic beads (Dynal). PBMCs were used to analyze the effect of the metabolite of α-calcidol(1(OH) vitamin D3) without direct cell to cell contact in an Huh-7 cells-transwell system. PBMCs and Huh-7 cells were cultured with serum-free complete medium that were previously made by our group [28]. A thousand times higher amount of 1(OH) vitamin D3 was used to analyze the effect of 1,25 $(OH)_2$ vitamin D3, which comes from the lower part of chamber, since the Huh-7 cells have several enzymes that could convert 1(OH) vitamin D3 to 1,25 $(OH)_2$ vitamin D3. The supernatant was harvested at 48 hours after the addition of 1(OH) vitamin D3 or 1,25 $(OH)_2$ vitamin D3.

Flow Cytometry Analysis

PBMCs were stained with CD3-pacific-blue, CD4-PE/Cy7, CD25-PE, CD127-APC, CD183 (CXCR3)-APC/Cy7, CD195 (CCR5)-FITC, Viaplobe and isotype control antibodies (BD pharmingen, San Jose, CA, USA) for 15 min on ice to analyze the frequency of CD3+CD4+CXCR3+CCR5+ cells (Th1) and CD3+CD4+CD25+CD127− (Tregs) by FACSCanto-II (BD). The FCS files 3.0 were analyzed by Flowjo 7.60 software.

Multiplex Beads Suspension Array

The culture supernatant of PBMCs treated with the active vitamin D3 metabolite (1,25 $(OH)_2$ vitamin D3) and the plasma obtained from CH-C patients treated with or without alfa-calcidol (1(OH) vitamin D3) were sequentially analyzed by suspension beads array (BIO-RAD Laboratories, Tokyo, Japan). Suspension beads array was performed following the manufacturer's instruction. Briefly, the supernatant was incubated with first-antibody binding magnetic beads. Then, the detection antibody and PE

Table 1. Clinical characteristics of subjects enrolled in this study.

	PEG-IFNα/RBV	PEG-IFNα/RBV+VD3	PEG-IFNα/RBV+VD3	
	(n = 42)	(n = 42)	With Pre-VD3	Without Pre-VD3
			(n = 18)	(n = 24)
Gender(M/F)	19/23	15/27	6/12	9/15
Age	58.3(35–72)	59.1(29–71)	58.6(29–71)	58.5(43–71)
Body Weight	58.4	58.1(41.2–81)	56.4(41.2–81)	59.4(43–78)
History of IFN(+/−)	13/29	13/29	7/11	6/18
IL-28B(TT/TG,GG)	29/13	29/13	10/8	19/5
Sampling Point (week)	0W	All 0W	−4W	0W
HCV-RNA	6.3(5.1–7.2)	6.3(5.2–7.4)	6.3(5.2–7.1)	6.4(5.3–7.4)
ALT	68.5 (15–234)	66.4(16–242)	47.9(22–108)	78(16–242)
AST	55.2(16–161)	58.1(21–251)	45.3(22–112)	66.1(21–251)
WBC	5045(3050–7800)	5165(2400–9300)	5055(3100–9300)	5530(2400–8130)
RBC	441.3(355–522)	441.5(375–567)	450(375–567)	446(383–515)
PLT	16.6(9.4–29.4)	16.7(9.3–27.6)	16.6(9.3–27.6)	16.7(9.3–23.9)
Nue	2845(1750–5020)	2911(1190–7160)	2792(1190–7160)	3476(1533–5070)
Hb	13.8(11.8–15.9)	13.6(12–16.3)	13.7(12–15.2)	14.1(12.6–16.3)
Serum Ca	9.3(8.5–9.8)	9.2(8.6–10.1)	9.4(8.9–10.1)	9.2(8.6–10)
Insulin	9.4(6.8–20.2)	9.5(1.6–25.5)	9(4.76–20.8)	9.6(1.6–25.5)
T-cho	170.6(118–214)	172.4(116–227)	168.2(116–226)	173.7(119–227)
TG	108.5(55.6–210)	106.4(37–427)	118.9(37–259)	103.2(51–427)

HCV-RNA(log copies/ml), ALT(U/l), AST(U/l), WBC(counts/μl), RBC(x10^3counts/μl), PLT(x10^4counts/μl), Neut(counts/μl), Hb (g/dl), Serum Ca (mg/dl), Insulin (μU/ml), T-cho (mg/dl), TG (mg/dl).

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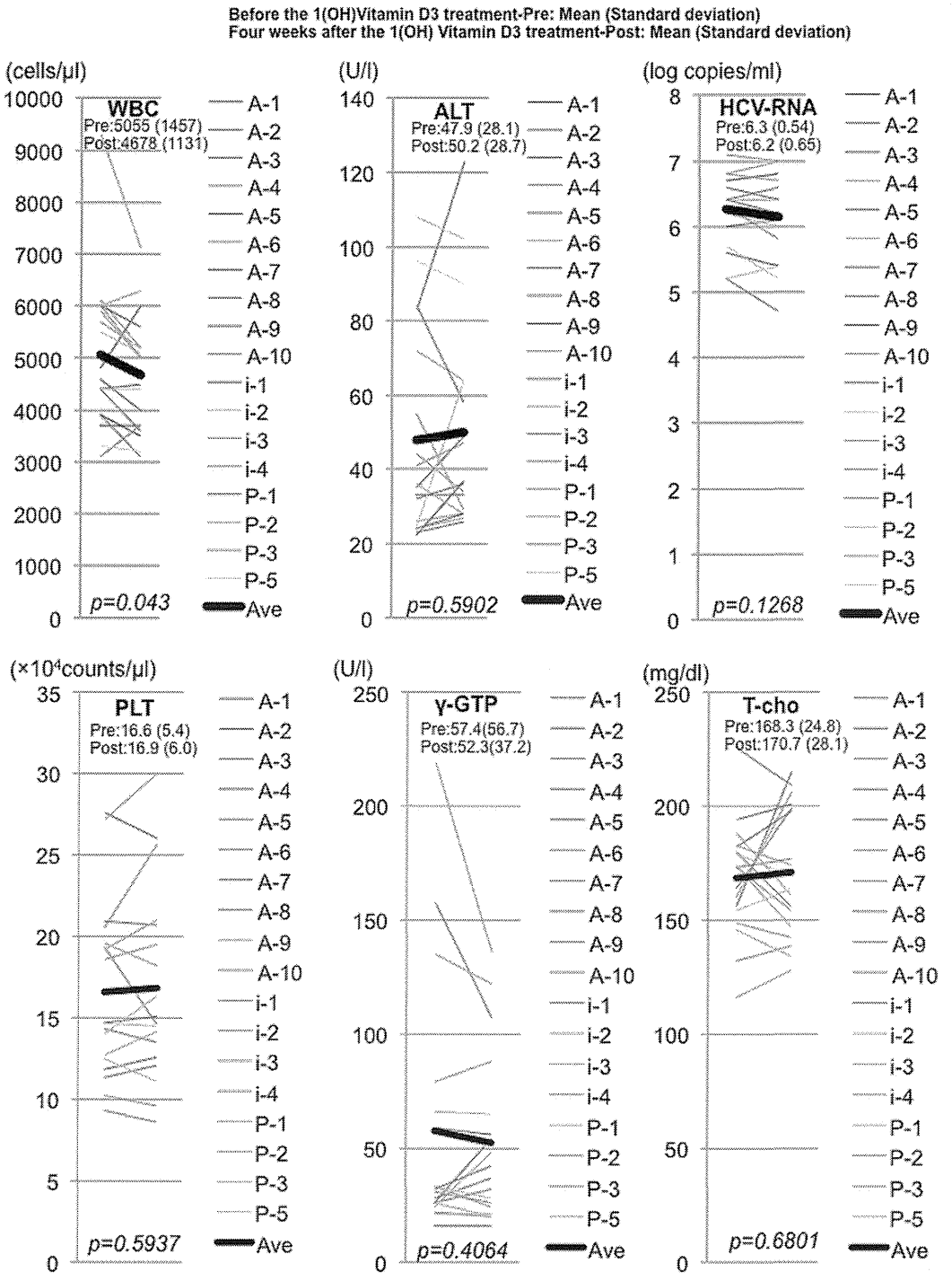

Figure 3. Comparison of hematological and biochemical analysis between before and after 4-week 1(OH) vitamin D3 treatment. Representative hematological, biochemical and virological data are shown. WBC indicates white blood cell count. ALT indicates alanine transaminase. HCV-RNA indicates titer of hepatits C virus RNA. PLT indicates platelet count. γ-GTP indicates gamma-glutamyl traspeptidase. T-cho indicates total cholesterol. The data at pre- and post-4weeks administration of 1(OH) vitamin D3 without Peg-IFN/RBV are shown. Black lines indicate the average of each analysis.

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conjugated streptavidin were reacted after the appropriate washing steps. Finally, the reaction plates were analyzed by Bio-plex 200 system.

Real-time Polymerase Chain Reaction

RNA was isolated using a Qiagen RNeasy mini kit (Valencia, CA) and the yields were determined by absorption spectroscopy using a Nano-Drop (NanoDrop Products, Wilmington, DE). After the extraction of total RNA and the reverse transcription (RT)

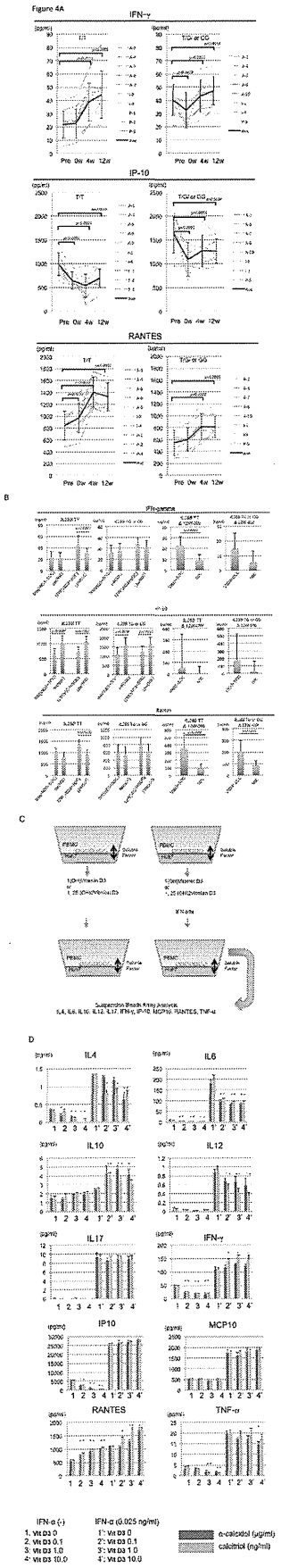

Figure 4. Cytokine profiles in the *ex vivo* and *in vitro* samples treated with vitamin D3. Sequential data of quantification of 3 cytokines (IFN-γ, IP-10 and RANTES) during 1(OH) vitamin D3 pre-treatment (pre vs 0w), 1(OH) vitamin D3/Peg-IFN/RBV therapy are shown (A). Dotted lines indicate the data of each subject. Black lines indicate the averaged data. Error bars indicate standard deviation. The data from IL28B (T/T) subjects or IL28B (T/G or G/G) subjects are shown in the independent graphs (A). Comparisons of the amounts of 3 cytokines (IFN-γ, IP-10 and RANTES) between the 1(OH) vitamin D3/PEG-IFN/RBV group (VitD3+standard of care (SOC)) and Peg-IFN/RBV group (SOC) at 0 weeks and 12 weeks after the start of Peg-IFN/RBV treatment are shown (B). Analysis of the changes in the amounts of the 3 cytokines (IFNγ, IP-10 and RANTES) during 12 weeks treatment of Peg-IFN/RBV is shown. Schema of *in vitro*-analysis of co-culture is shown (B). alfa-calcidol: 1(OH)vitamin D3 and calcitriol: 1,25(OH)vitamin D3 were used to analyze the cytokine production *in vitro*. Black bars indicate the data from samples treated with alfa-calcidol. Gray bars indicate the data from samples treated with calcitriol. *$p<0.05$.
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procedure, real-time polymerase chain reaction (PCR) using a TaqMan Chemistry System was carried out. The ready-made set of primers and probe for the amplification of IFN-γ, T-bet, Mx1 (ID Hs00895608), IFI44 (ID Hs00197427), IFIT1 (ID Hs01911452) and glyceraldehyde 3-phsphate-dehydrogenase (GAPDH) were purchased from Perkin-Elmer Applied Biosystems (Carlsbad, CA, USA). The relative amount of target mRNA was obtained by using the comparative threshold (CT) cycle method.

The Quantification of ISGs mRNA in Hepatocyte Cell Culture

Huh-7 cells were treated with ethanol (control), 1(OH) vitamin D3 (1.0 μM) or 1,25(OH)2 vitamin D3 (1.0 μM) after transfection of poly IC (Sigma-Aldrich, St. Louis, MO) or in vitro transcribed JFH-1 full-length RNA. Cells were harvested 30 hour after transfection, and the expression levels of Mx, IFI44 and IFIT1 mRNA were assessed by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) in accordance with the manufacturer's instructions. The expression levels of genes with or without vitamin D3 treatment were expressed by the log fold increase of untreated Huh-7 cells.

Statistical Analysis

The data in Fig. 2A and B were analyzed using a generalized linear mixed model (Treatment group of 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV were fixed-effect. Duration of treatment was random-effect.) and Student's *t* test. The data in Fig. 2C were analyzed by χ^2 test. The data in Fig. 3, Fig. 4A and Fig. 5B were analyzed by paired *t* test. The data in Fig. 4C were analyzed by Dunnett's test. The data in Fig. 5C were analyzed by Tukey's test. The data in Fig. 4B, Fig. 5D and Fig. 6 were analyzed by Student's *t* test. The cut-off of acceptance of test's results was $p<0.05$ with a confidence interval of 95%. All statistical analyses were carried out using JMP Pro version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Efficacy and Tolerability of 1(OH) Vitamin D3 Combined with Peg-IFN/RBV Therapy

The characteristics of 42 patients treated with 1(OH) vitamin D3 (1 μg/day)/Peg-IFN/RBV therapy are shown in Table 1. The subjects enrolled in this study were 29 to 71 years old. 13 patients

Figure 5

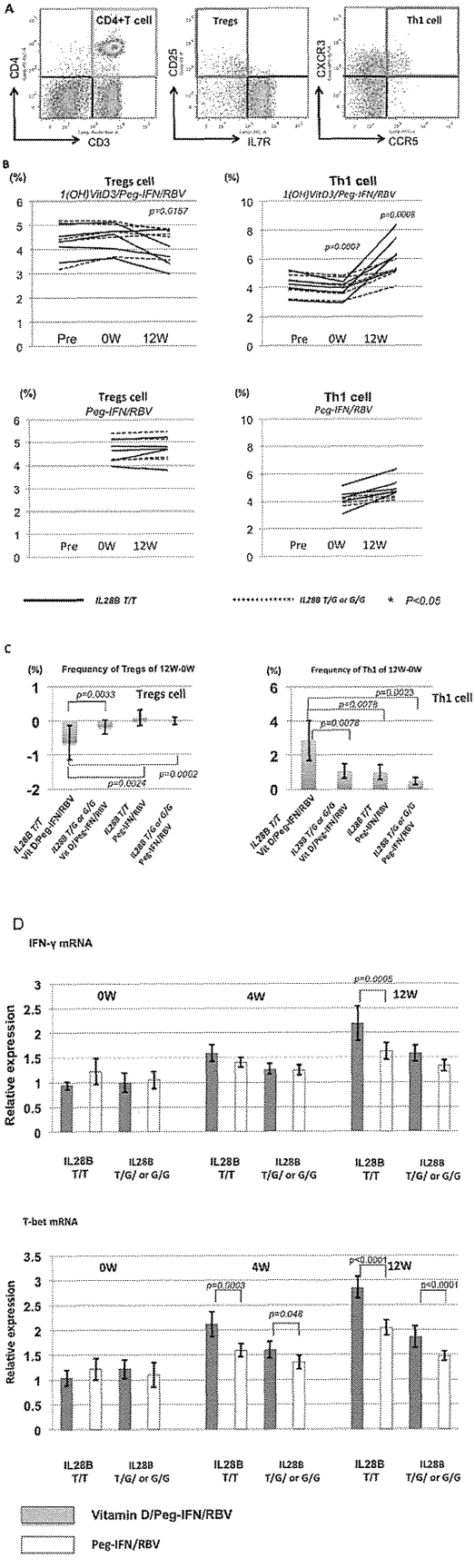

Figure 5. Comparison of Th1 and Tregs between 1(OH) vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV. Representative dot plots of $CD3^+CD4^+CD25^+IL7R^-$ (Tregs) and $CD3^+CD4^+CXCR3^+CCR5^+$ (Th1 cells) are shown. (A) Frequencies of Th1 and Tregs among the 4 groups (IL28B T/T vitamin D3/Peg-IFN/RBV, IL28B T/G or G/G vitamin D3/Peg-IFN/RBV, IL28B T/T Peg-IFN/RBV, and IL28B T/G or G/G Peg-IFN/RBV) are shown. (B) Comparison of the T-bet and IFN-γ mRNA expression between subjects treated with vitamin D3/Peg-IFN/RBV therapy and those treated with Peg-IFN/RBV therapy. Each group included 5 patients. Total mRNA was extracted from isolated $CD4^+$ T cells. The relative expression levels are shown in bar graphs. The statistical analysis was carried out by independent student *t*-test.
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were previously treated with IFN-based therapy and failed to achieve SVR. Another 29 patients were treatment naïve. Case match control subjects treated with Peg-IFN/RBV therapy were enrolled in this study (Fig. 1) (Table 1). All of the enrolled patients had over 5 log copies/ml HCV-RNA and genotype 1b HCV RNA. Thirteen patients had the hetero/minor *IL28B* allele (T/G) (rs8099917) that was reported to be a marker of patients difficult-to-treat with Peg-IFN/RBV therapy [29]. Twenty-nine patients had the major homo *IL28B* allele (T/T) that was reported to be favorable for achieving SVR [29]. Therefore, we compared the viral dynamics between subjects treated with the 1(OH) vitamin D3/Peg-IFN/RBV and subjects receiving the Peg-IFN/RBV with the same IL28B polymorphism (Fig. 2A and B). The titers of HCV-RNA in the *IL28B* (T/T)-HCV patients treated with 1(OH) vitamin D3/Peg-IFN/RBV therapy were significantly lower than those treated with Peg-IFN/RBV at 4 weeks after the start of Peg-IFN/RBV therapy ($p<0.01$). The rate of early virological response in the *IL28B* (T/T) patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was significantly higher than that in those treated with Peg-IFN/RBV alone (Fig. 2C). None of the patients showed side effects from 1(OH) vitamin D3 administration such as hypercalcemia or renal dysfunction, etc. The rate of the sustained virological response (SVR) in the overall patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was 59.45% (45.24% in the overall patients treated with Peg-IFN/RBV) ($p=0.2059$) The rate of SVR in the *IL28B* (T/T) patients treated with 1(OH) vitamin D3/Peg-IFN/RBV was 73.07% (55.17% in *IL28B* (T/T) patients treated with Peg-IFN/RBV) ($p=0.1657$). However, this study was conducted to analyze the immunological response during the early phase of Peg-IFN/RBV. The sample size might not be large enough to analyze the SVR rate.

Biological Effect of 1(OH) Vitamin D3 Treatment during Peg-IFN/RBV Therapy

The biochemical and hematological analysis was carried out at 4 weeks before the start of Peg-IFN/RBV therapy and at the start of Peg-IFN/RBV therapy. Of those data, only the absolute counts of white blood cells were significantly decreased after 4 weeks-1(OH) vitamin D3-treatment ($p<0.05$) (Fig. 3). The titers of HCV-RNA were not significantly changed after the 4-week administration of 1(OH) vitamin D3 without Peg-IFN/RBV therapy. Therefore, we examined the immunological effects of 1(OH) vitamin D3. At first, we quantitated 10 cytokines (IL 4, IL 6, IL10, IL12, IL17, IFN-γ, IP-10, MCP-1, RANTES, TNF-α) in the peripheral blood samples during 1(OH) vitamin D3/Peg-IFN/RBV therapy using multiple beads suspension array (Fig. 4A and Fig. S1). Among the *IL28B* T/T polymorphism patients, the amounts of IL4, IP-10 and MCP1 in the peripheral blood serum were significantly reduced after 4-week-1(OH) vitamin D3-treatment. On the other hand, the amounts of IL6, RANTES and TNF-α in the serum were significantly increased after 4-week 1(OH)vitamin D3 treatment. In the *IL28B* T/G or G/G

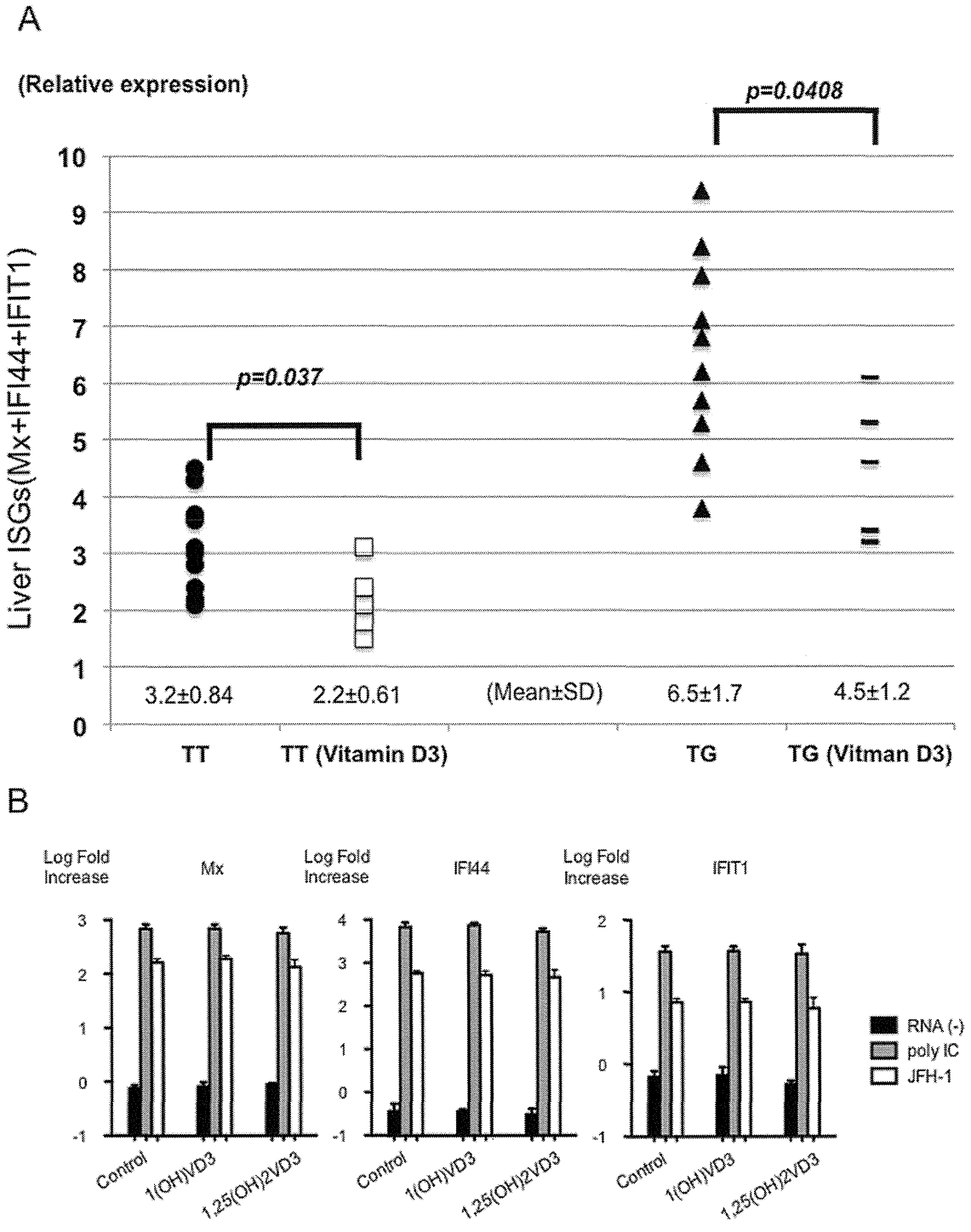

Figure 6. The effect of vitamin D3 on the expression of ISGs mRNA in the liver. The relative amount of target mRNA was obtained by using a comparative threshold cycle (CT) method. The expression levels of Mx, IFI44 or IFIT1 mRNA in an *IL28B T/T* patient treated without 1(OH) vitamin D3 are represented as 1.0 and the relative amounts of target mRNA in the other patients were calculated by the comparative Ct method [42]. Therefore, the standard amount of 3 ISGs (Mx, IFI44 and IFIT1) is 3. The relative amounts of the 3 kinds of ISGs were added and shown in the graph (A). Black circles indicate the data from IL28B (T/T) subjects treated without 1(OH) vitamin D3. White boxes indicate the data from IL28B (T/T) subjects treated with 1(OH) vitamin D3. Black triangles indicate the data from IL28B (T/G or G/G) subjects treated without 1(OH) vitamin D3. Black lines indicate the data from the subjects treated with 1(OH) vitamin D3 (A). The effect of vitamin D3 on the expression of ISGs mRNA in the hepatocyte cell culture are shown (B). Huh-7 cells were treated with ethanol (control), 1(OH) vitamin D3 (1.0 μM) or 1,25(OH)$_2$ vitamin D3 (1.0 μM) after transfection of poly IC (Sigma-Aldrich, St. Louis, MO) or in vitro transcribed JFH-1 full-length RNA. Cells were harvested 30 h after transfection, and the expression levels of Mx, IFI44 and IFIT1 mRNA were assessed by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) in accordance with the manufacturer's instructions. The expression levels of genes with or without vitamin D3 treatment were expressed by log fold increase of untreated Huh-7 cells.
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polymorphism patients, the amount of RANTES in the serum was significantly increased after 4-week 1(OH) vitamin D3-treatment. The amounts of IL4, IFN-γ, IP-10, MCP-1 in the serum were significantly decreased after 4-week 1(OH) vitamin D3-treatment. The administration of 1 (OH) vitamin D3 could reduce the high IP-10 status that is reported to be difficult-to-treat. Then, we compared the amounts of 10 cytokines between 1(OH) vitamin D3/Peg-IFN/RBV group and Peg-IFN/RBV group at 0 week and 12 weeks after the Peg-IFN/RBV treatment. The amounts of cytokines in the patients treated with 1(OH) vitamin D3/Peg-IFN/RBV at 0 week were affected by 4 weeks 1(OH) vitamin D3 pre-treatment. The amounts of IP-10 in the patients treated with 4 weeks-1(OH) vitamin D3 were significantly lower than those in the group treated without 1(OH) vitamin D3. However, the amounts of IFN-gamma and RANTES in the *IL28B* TT patients treated with 1(OH) vitamin D3/Peg-IFN/RBV were significantly higher than those in the *IL28B* TT patients treated with Peg-IFN/RBV without 1(OH) vitamin D3 at 12 weeks after the start of Peg-IFN/RBV treatment (Fig. 4B). In addition to the absolute amounts of several cytokines, the changes in the amounts after the 12 weeks Peg-IFN/RBV treatment were analyzed (Fig. 4B and Fig. S2). Changes in the amounts of IL4, IL-12, IFN-gamma and RANTES during the 12 weeks-treatment of Peg-IFN/RBV were significantly different between the 1(OH) vitamin D3/Peg-IFN/RBV group and Peg-IFN/RBV group ($p<0.05$) (Fig. 4B and Fig. S2).

The Biological Effects of 1(OH)vitamin D3 and 1,25(OH)$_2$ Vitamin D3 on the Production of Cytokines from PBMCs

Then, we examined whether the administration of 1(OH) vitamin D3 could affect the production of various kinds of cytokines from PBMCs. We used trans-well systems to analyze the effects of hepatocytes with various kinds of enzymes that affect the metabolism of 1(OH) vitamin D3 (Fig. 4C). We used a ng/ml order of calcitriol(1,25(OH)$_2$ vitamin D3) as the active form of vitamin D3 and a μg/ml order of 1(OH) vitamin D3 as the pre-active form of vitamin D3 with or without IFN-α (0.025 ng/ml). The amounts of IL4, IL6, IFN-γ, IP-10 and TNF-α were significantly decreased by the active and pre-active form of vitamin D3 without IFN-α (Fig. 4D). Among them, the amount of IP-10 was dose-dependently decreased by 1(OH)vitamin D3 and 1,25(OH)$_2$vitamin D3 without IFN-α. On the other hand, the amount of RANTES was dose-dependently increased by 1(OH)-vitamin D3 and 1,25 (OH)$_2$ vitamin D3 with or without IFN-α. The amounts of IL10 and IFN-γ were significantly increased by 1(OH)vitamin D3 and 1,25(OH)$_2$vitamin D3 with IFN-α (Fig. 4D). These data indicated that 1(OH)vitamin D3 and 1,25(OH)$_2$vitamin D3 could modulate the immunological status of PBMCs, especially the down-regulation of IP-10 production.

Comparison of the Frequency of Th1 and Tregs between 1(OH) Vitamin D3/Peg-IFN/RBV and Peg-IFN/RBV

Sequential analyses of $CD3^+CD4^+CXCR3^+CCR5^+$(Th1 cells) and $CD3^+CD4^+CD25^+CD127^-$ (Tregs) were carried out during 1(OH) vitamin D3/Peg-IFN/RBV or Peg-IFN/RBV treatment. Representative dot plots indicating Th1 and Tregs are shown (Fig. 5A). The subsets of these cells could be clearly recognized by flow cytometry. Four-week treatment of 1(OH) vitamin D3 could significantly decrease the frequency of Th1 cells but not Tregs ($p<0.05$) (Fig. 5B). However, the frequency of Th1 cells was rapidly increased after the start of Peg-IFN/RBV therapy, especially in the *IL28B* T/T subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV therapy (Fig. 5B and C). The frequency of Th1 cells in the subjects treated with 1(OH) vitamin D3 was significantly higher than in those treated with Peg-IFN/RBV at 12 weeks after the Peg-IFN/RBV therapy, especially in the *IL28B* T/T patients (Fig. 5C). Moreover, the expression of IFN-γ and T-bet mRNA in the isolated $CD4^+$ cells of subjects treated with 1(OH) vitamin D3/Peg-IFN/RBV therapy was significantly higher than in those treated with Peg-IFN/RBV therapy at 4 weeks and 12 weeks after Peg-IFN/RBV therapy (Fig. 5D).

Changes in ISG mRNA Expression in Liver with 1(OH) Vitamin D3 Treatment

The administration of 1(OH) vitamin D3 could reduce various kinds of cytokines in the serum. Therefore, we carried out quantification of ISG mRNA in samples from liver biopsies (Fig. 6A). We selected the Mx, IFI44, IFIT1 genes among the various kinds of ISGs, since another group previously reported that these ISGs could clearly recognize patients as difficult-to treat or easy-to-treat with IFN-based therapy [30]. The expression level of ISGs in the *IL28B* TT polymorphism was significantly lower than in the *IL28B* TG or GG polymorphism. Moreover, the expression levels of liver ISGs in the CH-C patients receiving 4 week-administration of 1(OH) vitamin D3 were significantly lower than those in the CHC patients without administration of 1(OH) vitamin D3.

Direct Effect of Vitamin D on the Expression of ISGs in Hepatocyte without Immune Cells

We used Huh-7 cells with a JFH-1 system that mimicks the acute phase of ISG induction in HCV infection, since we wanted to determine whether 1 (OH) vitamin D3 and 1, 25 (OH)2 vitamin D3 could affect the ISG expression directly. Three representative ISGs (MxA, IFI44 and IFIT1) were analyzed by real-time PCR. JFH-1 replication could induce these ISGs in Huh-7 cells (Fig.6 B). We used 1(OH) vitamin D3 and 1,25(OH)$_2$ vitamin D3 to analyze the ISG expression after JFH-1 inoculation. These ISGs were not affected by 1(OH) vitamin D3, and 1, 25(OH)$_2$ vitamin D3 in vitro.

Discussion

Recently, it has been reported that supplementation of vitamin D3, a potent immunomodulator, could improve the HCV response to antiviral therapy [2,3,31]. We used 1(OH) vitamin D3, since hepatocytes have various kinds of enzymes to convert 1(OH) vitamin D3 to the active metabolite 1,25(OH)$_2$ vitamin D3. Therefore, we speculated that the administration of 1(OH) vitamin D3 could affect the liver adaptive immune cells since the local concentration of 1,25(OH)$_2$ vitamin D3 might be higher than the systemic concentration of this active metabolite. Another group reported that 25(OH) vitamin D3, but not vitamin D3 or 1,25(OH)$_2$ vitamin D3, could have direct-antiviral activity at the level of infectious virus assembly [7]. However, the antiviral activity of 25(OH) vitamin D3 is not so remarkable. Moreover, the system of HCV replication in that study did not include the immune cells that are important for the control of HCV replication [32–35].

In this study, we first reported that administration of 1(OH) vitamin D3 could affect the cytokine production from PBMCs and suppress the ISGs mRNA expression in the liver samples. Among the various kinds of cytokines, IP-10, which was reported to be an important biomarker for the treatment response, could be significantly decreased after 1(OH) vitamin D3 treatment in vivo [36,37]. It has been reported that a high amount of IP-10 is a promising biomarker for difficult-to-treat patients regardless of the *IL28B* polymorphism [36,37]. IP-10 can be produced from various kinds of immune cells including monocytes. In this study, we found

that calcitriol could reduce the production of IP-10 from PBMCs dose-dependently *in vitro*. In addition to the production of IP-10, the expression of ISG mRNA in the liver biopsy samples with 1(OH) vitamin D3 treatment was significantly lower than in those without 1(OH) vitamin D3 treatment regardless of the *IL28B* polymorphism. The excessive expression of ISG mRNA before the Peg-IFN/RBV therapy might induce a poor response to IFN administration [38,39]. In addition to these results, we confirmed that the amounts of IFN-gamma and RANTES induced by 12-weeks 1 (OH) vitamin D3/Peg-IFN/RBV treatment was significantly higher than those induced by 12 weeks Peg-IFN/RBV treatment without 1 (OH) vitamin D3. 1 (OH) vitamin D3 could suppress the basal levels of the immune response in the CH-C patients. However, the subsequent response of the adaptive immune system after the start of Peg-IFN/RBV treatment could have been augmented by 1(OH) vitamin D3. These data indicated that calcitriol might be able to stabilize the adaptive immune systems that were out of control in CH-C patients instead of inducing their activation. In this study, we could not detect a significantly higher rate of SVR in the 1(OH) vitamin D3/Peg-IFN/RBV group in comparison with those in the Peg-IFN/RBV group. However, the addition of 1(OH) vitamin D3 could improve the adaptive immune response. Therefore, the SVR rate in the 1(OH) vitamin D3/Peg-IFN/RBV group might have been significantly higher than in the Peg-IFN/RBV group, if the sample size had been large enough to analyze the SVR.

In addition to previous reports, our data indicated that calcitriol could affect the production of cytokines from PBMCs [25,40]. However, we could not exclude the possibility of affecting cytokines other than the 10 cytokines we analyzed in this study. Moreover, other groups reported that vitamin D3 might modulate the expression of TLRs and/or their signaling, which are important in the immunopathogenesis of hepatitis C virus persistent infection [6,14,41]. This study was not a randomized control trial and did not have a large number of patients, since it focused on the effect of 1,25 $(OH)_2$ Vitamin D3 on the immune cells. For this purpose, the number of included patients was sufficient for the analysis. Moreover, we are conducting a randomized control trial that includes a large number of chronic hepatitis C patients with sever fibrosis and low vitamin D3 concentrations (ongoing study) (UMIN000007400).

In conclusion, the active metabolite of vitamin D3, calcitriol, could improve the response to Peg-IFN/RBV therapy. Supplementation of 1(OH) vitamin D3 or 1,25$(OH)_2$ vitamin D3 should be reasonable for the conditioning of IFN-based treatment including Direct Acting Antiviral (DAA)/Peg-IFN/RBV, DAA/Peg-IFN, Peg-IFN/RBV and Peg-IFN monotherapy.

Supporting Information

Figure S1 Cytokine profiles in the *ex vivo* treated with 1(OH) vitamin D3/Peg-IFN/RBV. Sequential data of quantification of 7 cytokines (IL4, IL6, IL10, IL12, IL17, MCP-1 and TNF-α) during 1(OH) vitamin D3 pre-treatment (pre vs 0w), 1(OH) vitamin D3/Peg-IFN/RBV therapy are shown. Dotted lines indicate the data of each subject. Black lines indicate the averaged data. Error bars indicate standard deviation. The data from IL28B (T/T) subjects or IL28B (T/G or G/G) subjects are shown in the separate graphs.
(TIFF)

Figure S2 Comparison of the cytokine profiles between 1(OH) vitamin D3 plus SOC and SOC. Comparisons in the amounts of 7 cytokines (IL4, IL6, IL10, IL12, IL17, MCP-1 and TNF-α) between 1(OH) Vitamin D3/PEG-IFN/RBV group (VitD3+standard of care (SOC)) and Peg-IFN/RBV group (SOC) at 0 weeks and 12 weeks after the start of Peg-IFN/RBV treatment are shown. Analysis of the changes in the amounts of 7 cytokines (IL4, IL6, IL10, IL12, IL17, MCP-1 and TNF-α) during 12 weeks treatment of Peg-IFN/RBV is shown.
(TIFF)

Author Contributions

Conceived and designed the experiments: YK T. Kato OK TI MN EK MM TA YM T. Kobayashi MI NK KS HN TI NO YU TM TS. Performed the experiments: YK T. Kato OK TI MN EK MM TA YM T. Kobayashi MI NK KS HN TI NO YU TM TS. Analyzed the data: YK T. Kato OK TI MN EK MM TA YM T. Kobayashi MI NK KS HN TI NO YU TM TS. Wrote the paper: YK T. Kato OK TI MN EK MM TA YM T. Kobayashi MI NK KS HN TI NO YU TM TS. Immunological analysis: YK OK MM TA. Virological analysis: YK T. Kato MN EK.

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ORIGINAL ARTICLE

Epidemiological study of zoonoses derived from humans in captive chimpanzees

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Abstract Emerging infectious diseases (EIDs) in wildlife are major threats both to human health and to biodiversity conservation. An estimated 71.8 % of zoonotic EID events are caused by pathogens in wildlife and the incidence of such diseases is increasing significantly in humans. In addition, human diseases are starting to infect wildlife, especially non-human primates. The chimpanzee is an endangered species that is threatened by human activity such as deforestation, poaching, and human disease transmission. Recently, several respiratory disease outbreaks that are suspected of having been transmitted by humans have been reported in wild chimpanzees. Therefore, we need to study zoonotic pathogens that can threaten captive chimpanzees in primate research institutes. Serological surveillance is one of several methods used to reveal infection history. We examined serum from 14 captive chimpanzees in Japanese primate research institutes for antibodies against 62 human pathogens and 1 chimpanzee-borne infectious disease. Antibodies tested positive against 29 pathogens at high or low prevalence in the chimpanzees. These results suggest that the proportions of human-borne infections may reflect the chimpanzee's history, management system in the institute, or regional epidemics. Furthermore, captive chimpanzees are highly susceptible to human pathogens, and their induced antibodies reveal not only their history of infection, but also the possibility of protection against human pathogens.

Keywords Chimpanzee · Serology · Captive · Human-borne infection

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Introduction

Emerging infectious diseases (EIDs) in wildlife, which may arise as a result of complex relationships between social and environmental factors, are a major threat both to human health and biodiversity conservation (Daszak et al. 2000, 2001; Jones et al. 2008; McMichael 2004; Morens et al. 2004). Such diseases often reduce wildlife populations in isolated communities (e.g., an Ebola outbreak in gorillas and chimpanzees in Gabon and Congo), increasing their probability of extinction, especially in frequency-dependent outbreaks (De Castro and Bolker 2005; Gerber et al. 2005; Bermejo et al. 2006; Nunn et al. 2008). Most EID events have been caused by zoonotic pathogens from a non-human animal source (Taylor et al. 2001; Woolhouse and Gowtage-Sequeria 2005; Jones et al. 2008). Moreover, it is estimated that 71.8 % of such zoonotic events are

caused by pathogens that originate in wildlife, for example the emergence of Nipah virus in Perak, Malaysia, and severe acute respiratory syndrome (SARS) in Guangdong Province, China. Pathogens originating from wildlife have also increased significantly with time (Jones et al. 2008). This supports the suggestion that zoonotic EIDs are an increasing, very significant, threat to global health (Morens et al. 2004; Weiss and McMichael 2004; King et al. 2006). It also emphasizes the importance of understanding the factors that increase contact between wildlife and humans for developing predictive approaches to disease emergence (Daszak et al. 2000; Patz et al. 2004).

Human infectious diseases are also being increasingly transmitted to wildlife, especially non-human primates. Chimpanzees and humans are closely related species evolutionarily and genetically, not only in their anatomical and physiological characteristics but also in their immunological features, which are much more similar than those between other animals (Brack 1987; Woodford et al. 2002; Clark et al. 2003). Wild chimpanzees may be susceptible to human infectious diseases because their immune system is naïve to them, so they may not be protected against human pathogens. According to recent reports, in addition to poaching and habitat fragmentation by deforestation, human-borne disease epidemics spread by ecotourism have contributed to the decline in wild chimpanzee populations (Kaiser 2003; Whitfield 2003; Kondgen et al. 2008). Unknown respiratory diseases have also been reported in other chimpanzees residing in protected areas, including Bossou, Guinea, and Gombe and Mahale, Tanzania; these have resulted in chimpanzee deaths and are suspected of having been transmitted by humans (Goodall 1986; Hosaka 1995; Ferber 2000; Nishida et al. 2003; Matsuzawa et al. 2004; Hanamura et al. 2008). Infectious disease transmission is possible without close contact via sneezing, coughing, etc. Muehlenbein and Ancrenaz (2009) reported that 39 species of pathogens were recovered from throat swabs of tourists viewing orang-utans, revealing the possibility that many human-borne agents may be transmitted to apes by several modes of infection. Therefore, continuous health monitoring and investigation of human-borne infectious diseases in apes is needed for risk management.

In this study we sought serological evidence of zoonoses in captive chimpanzees at the Kyoto University Primate Research Institute (KUPRI) in Japan to obtain basic epidemiological information on zoonoses affecting wild chimpanzees in Africa and to prevent pandemic outbreaks. The chimpanzees examined in this study had been reared for 10–30 years since birth at KUPRI or approximately 40 years after introduction from other zoos or western Africa. They had not been in individual cages, but rather in social groups, as in the wild; they were, therefore, a good model for wild chimpanzee studies.

Methods

Animals

This study was conducted under the guidelines of KUPRI. After obtaining the approval of the Institutional Animal Welfare and Care Committee, serological surveillance was conducted on 14 chimpanzees between 10 and 44 years old kept in an indoor–outdoor enclosure at KUPRI (see Matsuzawa 2003, 2006 for further information about the animals). Information about the chimpanzees is summarised in Table 1. The chimpanzees had been subjects for behavioural, psychological, and evolutionary studies. As summarised in Table 1, Pendesa suffered from allergic dermatitis, Mari and Reiko had severe colds in 1984 and 1980, respectively, and Reo developed tetraparesis resembling acute transverse myelitis in 2006 (Miyabe-Nishiwaki et al. 2010). Pal, Cleo, Ayumu, Pan, Reo, and Popo were born at KUPRI; Pendesa was born in another institution in Japan and transferred to KUPRI when she was 2 years old. Mari and Akira were born in Africa and reared at other institutes in Japan, then transferred to KUPRI; Chloe was born in a French Zoo; Puchi and Gon were born in Africa and reared as pets in Japan; Ai and Reiko were transported directly from Africa. The health of each animal was monitored daily by their keepers, and each individual underwent a periodic health examination every 1–2 years. None of these chimpanzees had been vaccinated against any pathogens.

Sample collection

Samples were collected between April 2007 and February 2010, when each chimpanzee was anaesthetised for research purposes or for a periodic health examination. The chimpanzees were anaesthetised with a combination of 3.5 mg/kg ketamine hydrochloride (Ketalar; Sankyo Parke Davis, Japan) and 0.035 mg/kg medetomidine hydrochloride (Domitor; Meiji Seika Kaisha, Tokyo, Japan) with or without premedication with oral midazolam (1 mg/kg) or droperidol (0.2 mg/kg). Anaesthesia was maintained with isoflurane (Isoflu; Dainippon Sumitomo Pharma, Osaka, Japan) when necessary. Blood samples were collected in plain tubes with a coagulant, and the serum or plasma was separated by centrifugation at 3000*g* for 20 min and then analysed within 1 day or stored at −80 °C until serological tests were performed.

Human infectious microbiological agent tests

Human respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) serological analyses were conducted at the Virus Research Centre of Sendai Medical

Table 1 Information on each chimpanzee

Name (abbr.)	Sex	Age[a]	History[b]	Introduction[c]	Medical record
Pal (Pal)	F	10	D: Pendesa, S: Akira	2000	
Cleo (Cle)	F	10	D: Chloe, S: Reo	2000	
Ayumu (Ayu)	M	10	D: Ai, S: Akira	2000	
Pan (Pan)	F	27	D: Puchi, S: Gon	1983	
Reo (Reo)	M	28	D: Reiko, S: Gon	1982	Total paralysis from 2006[d]
Popo (Pop)	F	28	D: Puchi, S: Gon	1982	
Chloe (Chl)	F	30	Paris Zoo-KUPRI	1985	
Pendesa (Pen)	F	33	JMC[e]-KUPRI	1979	Atopic dermatitis
Ai (Ai)	F	34	Africa-KUPRI	1977	
Mari (Mar)	F	34	Africa-JMC-KUPRI	1978	Severe cold in 1984[f]
Akira (Aki)	M	34	Africa-KUPRI	1978	
Reiko (Rei)	F	44	Africa-KUPRI	1968	Severe cold in 1980[f]
Puchi (Puc)	F	44	Africa-Pet in JPN-KUPRI	1979	
Gon (Gon)	M	44	Africa-Pet in JPN-KUPRI	1979	

F female, *M* male

[a] Age in December 2009

[b] The parents are shown for the chimpanzees born at KUPRI (D, dam; S, sire); relocation history is shown for transferred chimpanzees

[c] The year transferred or the years Pal, Cleo, Ayumu, Pan, Reo, and Popo were born at KUPRI

[d] Reo contracted tetraparesis resembling acute transverse myelitis in 2006

[e] Japan Monkey Centre

[f] These chimpanzees were separated from the others and hospitalized for therapy

Centre, Sendai, Japan (Okamoto et al. 2010). The other analyses were outsourced to the Tokai Chuo Laboratory (ISO15189: 2003) at Falco Biosystems, Kyoto, Japan, and The Corporation for Production and Research of Laboratory Primates, Tsukuba, Japan. The HBV test was conducted with Espline HBs-N (Fujirebio Diagnostics, Tokyo, Japan), an immunochromatographic test that uses serum and gives a visible result. These tests were repeated, and positive and negative controls were prepared to reduce non-specific reactions.

The antibodies analysed targeted causative agents of respiratory diseases and hepatitis in humans in addition to retroviruses, encephalitis virus, and chimpanzee foamy virus (CFV). The specific antibodies examined reacted against *Mycoplasma pneumoniae*; *Chlamydia pneumoniae*; *Bordetella pertussis* (Japanese Higashihama or Yamaguchi strains); influenza A virus; influenza B virus; human parainfluenza virus types 1–4 (hPIV-1–4); hMPV; RSV; mumps virus; measles virus (MV); adenovirus (ADV)-1 through 8 and 11, 19, and 37; coxsackievirus types A5–7, 9, 10, and 16 (CVA-5–7, 9, 10, and 16) and B1–6 (CVB-1–6); echovirus types 3, 6, 7, and 13; enterovirus 71; poliovirus types 1–3 (PV-1–3); herpes simplex virus-1 and 2 (HSV-1 and 2); cytomegalovirus (CMV); varicella zoster virus (VZV); Epstein–Barr virus (EBV); human herpesvirus 6 (HHV-6); hepatitis A virus (HAV); hepatitis B virus (HBV); hepatitis C virus (HCV); rubella virus; reovirus; rotavirus; human parvovirus B19 (HPV-B19); Japanese encephalitis virus (JEV); human immunodeficiency virus type I (HIV-1); human T cell lymphotropic virus type I (HTLV-1); CFV; filovirus; and *Entamoeba histolytica*.

For statistical analysis, Student's *t* test was used to compare the average antibody titres between chimpanzees reared since birth (SB) and chimpanzees reared after birth (AB). A P value of <0.05 was considered to indicate statistical significance of the results (Figs. 2, 3).

Results

We investigated the prevalence of antibodies against human-borne pathogens in chimpanzees at Japanese primate institutes using standard procedures for human serological analysis.

Table 1 summarises the name, sex, age, history, year of arrival at KUPRI, and medical notes for each chimpanzee. Six chimpanzees were born at KUPRI: Pal, Cleo, Ayumu, Pan, Reo, and Popo. Eight chimpanzees were born in Europe or Africa: Chloe, Pendesa, Ai, Mari, Akira, Reiko, Puchi, and Gon.

The seroprevalence of human and chimpanzee-originating pathogens in the chimpanzees are listed in Supplementary Table S1. Antibodies against 29 of 62 human pathogens were detected by use of standard procedures for

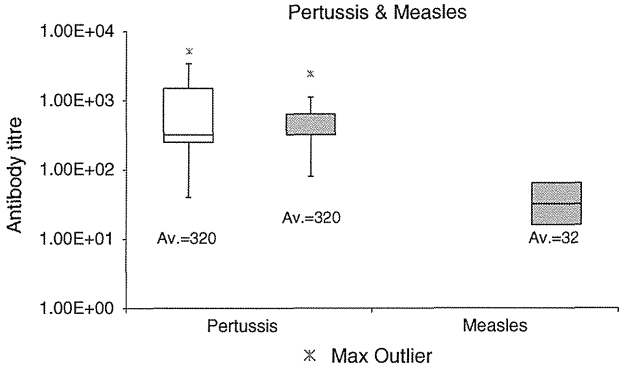

Fig. 1 Seroprevalence and titres of *B. pertussis* and MV antibodies, obtained by use of BAT and HI, respectively, for 14 chimpanzee serum samples. The range of antibody titre against *B. pertussis* was broad (40–5120×), and the average titre for the SB group (chimpanzees reared in KUPRI since birth) was higher than that for the AB group (chimpanzees reared in KUPRI after birth). MV antibody titre ranged from 16× to 64×, and most positive chimpanzees were in the AB group (in the SB group only one chimpanzee was positive: 64×). The *hollow box* indicates SB, and the *solid box* indicates AB. The *bars* indicate the average (Av.)

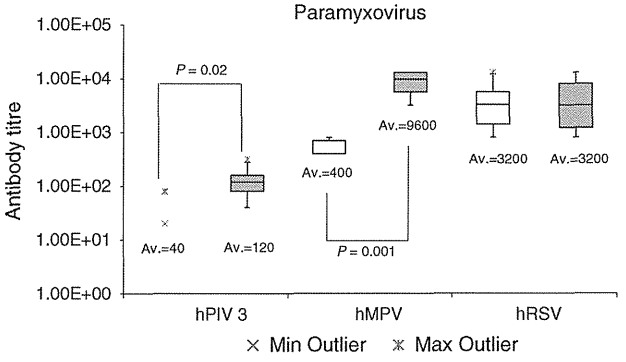

Fig. 2 Seroprevalence and titres of hPIV-3, hMPV, and RSV antibodies obtained by use of HI and ELISA (second two) for 14 chimpanzee serum samples. The titre against hPIV-3 for SB chimpanzees ranged from 20× to 80×, and that for the AB chimpanzees ranged from 80× to 160×. The average titre for the SB group was lower than that for the AB group. The titre against hMPV for SB chimpanzees ranged from 400× to 800×, which was lower than that for AB chimpanzees (3200–12800×). The average titre for the SB group was also lower than that for the AB group. The titre against hRSV ranged broadly from 800× to 12800× for both SB and AB chimpanzees. Average titres for SB chimpanzees were not much higher than those for AB chimpanzees. The *hollow box* indicates SB, and the *solid box* indicates AB. The *bars* indicate the average (Av.)

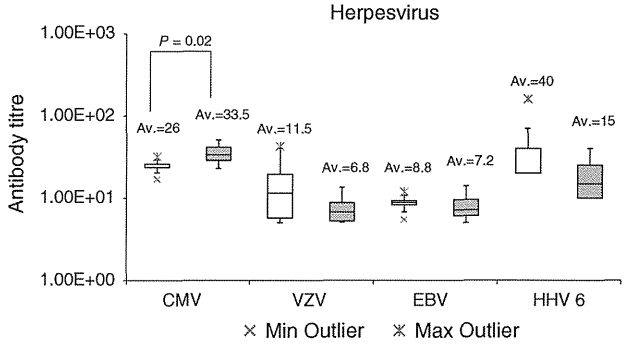

Fig. 3 Seroprevalence and titres of CMV, VZV, EBV, and HHV6 antibodies obtained by use of EIA for 14 chimpanzee serum samples. The antibody titre against CMV ranged from 17× to 32× for SB chimpanzees and from 26× to 51× for AB chimpanzees. The average titres in SB chimpanzees were lower than those in AB chimpanzees. The antibody titre against VZV in SB chimpanzees ranged from 5.0× to 42.7×; that in AB chimpanzees ranged from 5.1× to 13.6×. The antibody titre against EBV ranged from 5.5× to 12× for the SB group, which was not much higher than that for the AB group (5×–14×). The antibody titre against HHV6 ranged from 20× to 160× for SB chimpanzees and from 20× to 40× for AB chimpanzees. Differences in the average titres of VZV, EBV, and HHV6 were not statistically significant between SB and AB chimpanzees. The *hollow box* indicates SB, and the *solid box* indicates AB. The *bars* indicate the average (Av.)

serological analysis of humans. Briefly, more than 50 % of the chimpanzees were positive (high prevalence) for 14 human pathogens: pertussis, hPIV-3, hMPV, RSV, ADV-1, ADV-2, ADV-4, ADV-5, ADV-6, CVA-7, CMV, VZV, EBV, and HHV-6. In addition, 15 human pathogens were found in some chimpanzees (low prevalence): influenza A (H3N2), MV, ADV-3, ADV-19, CVB-2, CVB-3, CVB-6, echovirus-6, CVA-5, CVA-9, HAV, reovirus, rotavirus, HPV-B19, and JEV. CFV was also detected in chimpanzees. Antibodies for the following 26 pathogens were not detected in any chimpanzee: *M. pneumonia*, *C. pneumonia*, influenza A (H1N1), influenza B, hPIV-1, hPIV-2, mumps virus, ADV-8, ADV-11, CVB-1, CVB-4, CVB-5, echovirus-7, echovirus-13, CVA-10, CVA-16, enterovirus 71, PV-2, PV-3, HSV, HBV, HCV, rubella virus, HIV-1, HTLV-1, and filovirus. In addition, hPIV-4, ADV-7, ADV-37, echovirus-3, CVA-6, PV-1, and *E. histolytica* were positive, but their positive sample antibody titres were equal to the cut-off titre (asterisk in Supplementary Table S1).

Figures 1a, b, 2a–c, 3a–d, and 4a, b show the antibody titres against pertussis, MV, hPIV-3, hMPV, RSV, CMV, VZV, EBV, and HHV-6 grouped by the birthplace of the chimpanzee. SB indicates that chimpanzees were reared in KUPRI since birth; AB indicates that chimpanzees were introduced and reared in KUPRI after birth. The AB group was older than the SB group. Their birthplaces are listed in Table 1.

Pertussis is caused by *B. pertussis* infection. The antibody titres against pertussis varied from 40 to 5120×, and the average titre in SB chimpanzees (320×) was equal to that in AB chimpanzees (320×) (Fig. 1). Antibody titres against MV were 16–64×, and the average titre in AB chimpanzees was 32× (Fig. 1). Only one chimpanzee was

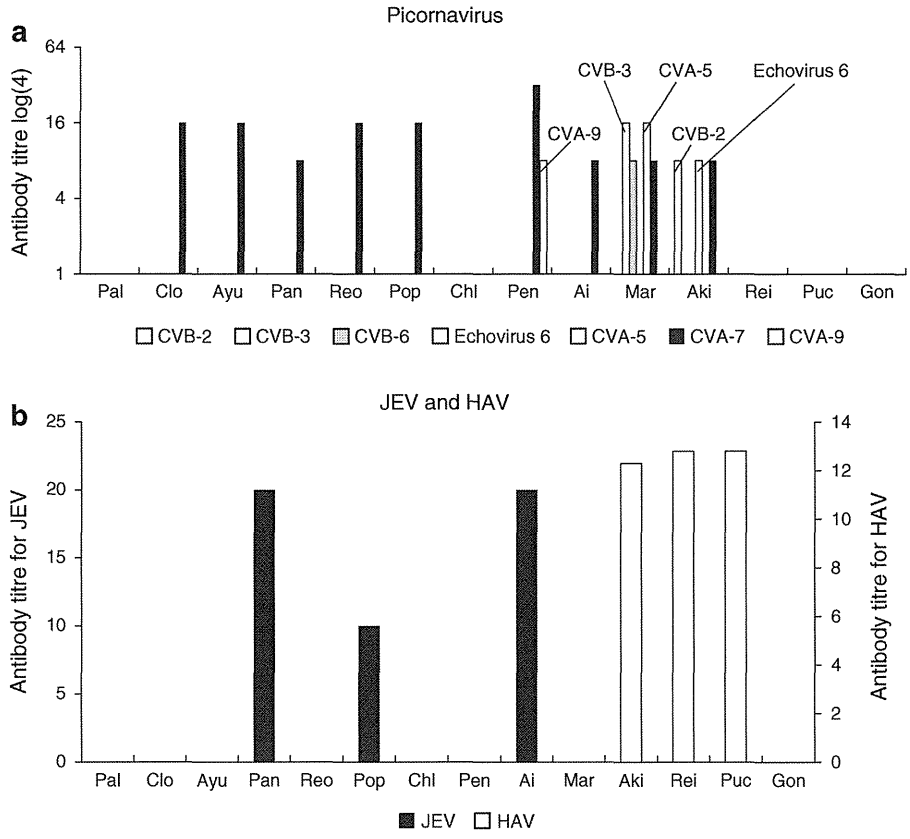

Fig. 4 **a** Seroprevalence of antibodies against picornaviruses, CVB-2, CVB-3, CVB-6, echovirus-3, echovirus-6, CVA-5, CVA-6, CVA-7, CVA-9, and PV-1 for each chimpanzee. The antibodies in serum were detected by use of NT. The AB female Mari had antibodies against four picornaviruses (CVB-3, CVB-6, CVA-5, and CVA-7); the AB male Akira had antibodies against CVB-2, echovirus-6, and CVA-7. Abbreviations of each chimpanzee name are listed in Table 1. **b** The seroprevalence of antibodies against HAV and JEV for each chimpanzee detected by use of CLIA for HAV and CF for JEV. Antibody against JEV was detected in both SB and AB chimpanzees, but HAV was detected in AB chimpanzees only

positive against MV in the SB group. The average antibody titre against hPIV-3 in SB chimpanzees (40×) was lower than that in AB chimpanzees (120×) (Fig. 2). The range of titre against RSV was broad, from 800–12800×, and the average titre for SB chimpanzees (3200×) was equal to that for AB chimpanzees (3200×) (Fig. 2). The titre against hMPV in SB chimpanzees was 800–12800×, and the SB animals (400–800×; average: 400×) had lower levels than the AB chimpanzees (3200–12800×; average: 9600×) (Fig. 2). Titres of PIV3 and hMPV increased with age ($P < 0.05$).

We examined specific antibodies against herpesviruses in the captive chimpanzees. Surprisingly, the chimpanzees had high prevalence of antibodies against the four herpesviruses CMV (100 %), VZV (100 %), EBV (100 %), and HHV-6 (75 %), but not against HSV-1 or 2 (Supplementary Table S1). The titres against CMV, VZV, EBV, and HHV-6 are shown in Fig. 3. Titres of antibodies against CMV, only, increased with age ($P < 0.05$).

In humans, ADVs are common causes of respiratory, eye, urologic, and gastrointestinal diseases. ADV-8, 19, and 37 (species D ADV) are the causative pathogens of epidemic keratoconjunctivitis in humans; in this study, however, antibodies against ADV-19 and 37 were detected in three chimpanzees and one chimpanzee, respectively, without specific symptoms. ADV-1 to 7 (mainly ADV3) cause pharyngoconjunctival fever in humans; in this study, ADV-1, 2, 4, 5, and 6 were detected with comparatively high seroprevalence (57.1, 78.6, 100, 100, and 85.7 %, respectively), whereas ADV-3 was detected in one chimpanzee only (Supplementary Table S1). There were no significant differences in prevalence between SB and AB chimpanzees.

Figure 4a shows antibody titres and chimpanzee seroprevalence against picornaviruses; CVB-2, 3, and 6; echovirus-6; and CVA-5, 7, and 9. The titres of antibodies against CVA-7 varied; no other picornavirus antibodies were found in SB chimpanzees. Mari, an AB female, had antibodies against four picornaviruses: CVB-3 and 6 and CVA-5 and 7. Akira, an AB male, had antibodies against CVB-2, echovirus-6, and CVA-7, although he had relatively low titres.

Figure 4b shows antibody titres and chimpanzee seroprevalence for mosquito-borne encephalitis virus (JEV) and HAV. The antibody against JEV was detected in SB and AB chimpanzees. Antibody against HAV was detected in AB chimpanzees only.

Discussion

We surveyed whether captive chimpanzees have specific antibodies against human-borne infectious pathogens. We tested for antibodies against 62 infectious diseases in serum from 14 captive chimpanzees and found high or low

prevalence for 29 antibodies. Therefore, standard procedures for human serological analysis may be very useful for detecting specific antibodies against human-borne infectious pathogens in captive chimpanzees.

Data for older and younger chimpanzees can be compared to assess hygiene conditions at an institute. This study found fewer positive pathogens than in a study of US primate centres conducted by Kalter and Heberling (1971) more than 30 years ago. This may be because the infectious agents had been removed from the chimpanzee environment with improved knowledge about infectious diseases, and antibody titres of chimpanzees had been reduced to undetectable levels over time.

Serological tests against human-borne pathogens

The serological tests selected were conducted at commercial laboratories. The methods used for each test varied, and the types of detectable immunoglobulin (Ig), and test sensitivity, differed. Hence, the ideal serological screening against several diseases is an assay that can detect as many Igs as the chimpanzees can produce (i.e., IgM, IgA, IgG, and IgE). However, monitoring these human-borne pathogens should not be complicated. Easily available test methods are required for public zoological gardens and primate research institutes.

We could determine most of the history of infection by use of conventional serological analyses, but we could not determine when the antibody titre had decreased to a barely detectable level. Other serum sample-related factors affected the tests, for example cross-reaction of antibodies between chimpanzee viruses and human viruses, effect of haemolytic samples, and contamination. In this study, seven pathogens were detected, and all of their titres were equal to the standard cut-off titre. Among the pathogens, ADV-7, ADV-37, hPIV-4, echovirus-3, and CVA-6 are viruses of common human diseases, and thus it is highly possible they may infect chimpanzees. On the other hand, the West Pacific area has been free from PV-1 (poliomyelitis) since October 2000 (WHO/WPRO Kyoto conference, Kyoto, Japan), and a false-positive case of *E. histolytica* infection was reported despite the fact that titres were equal to the cut-off level (Tachibana et al. 2000). Thus, we conservatively assumed that PV-1 and *E. histolytica* are indeterminate, although their titres are normally deemed positive.

Implications of seroprevalence against human-borne infections

Chimpanzee foamy virus is a common virus in chimpanzees that is transmitted by a variety of routes. The 100 % prevalence of CFV indicates that the rearing conditions in KUPRI provide an environment for spread among chimpanzees. Therefore, human pathogens may not only be directly transmitted to chimpanzees from humans, but may also spread among chimpanzees.

Pertussis infection of chimpanzees was reported in a zoological garden in Sweden, and the affected chimpanzees had typical clinical signs of *B. pertussis* infection (Gustavssona et al. 1990). However, no clinical signs have been detected in the KUPRI chimpanzees despite their higher antibody titres. On the basis of their high antibody titres the chimpanzees at KUPRI may have been infected recently and may have developed immunity against pertussis. Pertussis in humans is common in children and the efficacy of the vaccine is proved, but antibody titre decreases after 2–5 years. Hence, permanent immunity is not established, and adults may be reinfected as antibody levels decrease. For example, some previously vaccinated adults in Japan were recently re-infected with pertussis (NIH 2008).

Measles virus antibody was found in six chimpanzees in this study. Interestingly, only one of the chimpanzees born in Japan (reared since birth in KUPRI: SB) had specific antibodies against MV, compared with five chimpanzees that were positive among those born in Europe or Africa (reared after birth in KUPRI: AB). More than 80 % of people have an antibody titre against MV, and antibody production is believed to indicate that the person has acquired immunity against MV (Taya et al. 2011). Chimpanzees with lower antibody titres are still at risk of MV infection.

PIV3, RSV, and hMPV antibody prevalence in humans increases with age. The prevalence of PIV-3 (HI test) antibody in humans peaks at 64–128× (Kishi et al. 1978), whereas that of hMPV (ELISA) peaks at 800–1600× (Okamoto et al. 2010). Antibody titres against RSV in chimpanzees did not vary with age, in contrast with humans, who normally acquire immunity by adulthood (Bhattarakosol et al. 2003). In humans, PIV-3 and the paramyxoviruses hMPV and RSV cause severe diseases of childhood and mild diseases of adults, who have acquired immunity after repeated infection in the process of growing. Therefore, the high prevalence of these viruses in chimpanzees may indicate that KUPRI chimpanzees have developed, or are developing, immunity against PIV-3, hMPV, and RSV. Increasing antibody prevalence with age was similar to that in humans with PIV-3 and hMPV. RSV and hMPV outbreaks in wild chimpanzees have, however, been causes of death or severe diseases (Kaur et al. 2008; Kondgen et al. 2008). RSV was identified as the chimpanzee coryza agent when it was isolated for the first time at a primate institute in the USA; hence, chimpanzees are highly susceptible to RSV (Morris et al. 1956).

Adenoviruses are common in humans, but not all infections lead to disease, and people develop adequate

immunity against reinfection by the same serotype. In this study, 9 of 11 ADV serotypes were found in chimpanzee serum, and the seroprevalence of ADV-1, 2, and 4 to 6 were high (Supplementary Table S1). Chimpanzee adenoviruses have also been identified, and the chimpanzee ADV Pan 9 neutralises human ADV-4 (Willimzik et al. 1981). More recently, 30 novel great ape ADVs from chimpanzees, bonobos, and gorillas were detected in captive non-human primates held in facilities and zoological gardens in North America (Roy et al. 2009). Typically, each ADV has a narrow host range that is restricted to one animal species or to closely related host species (Wold 2007).

Herpesviridae is highly infectious in its host animals, persistently or latently. EBV, CMV, HHV-6, and HSV-2, like viruses in chimpanzees, have been reported previously, so a neutralising antibody test is needed to distinguish among viruses of human and chimpanzee origin (Gerber et al. 1976; Swinkels et al. 1984; Lacoste et al. 2005; Luebcke et al. 2006). In this study 100 % of the chimpanzees were positive for EBV, CMV, and VZV and 75 % were positive for HHV-6, but no antibodies against HSV-1 or 2 were found (Supplementary Table S1). In humans, antibody prevalence against herpesviruses increase with age, with acquisition of immunity; hence, antibody prevalence against CMV is similar to that in humans. Repeat reactivation of the other herpesviruses, EBV, VZV, and HHV-6, may occur in the host animals, or human herpesviruses may be transmitted by chimpanzees in KUPRI. Human VZV infection has been reported in chimpanzees; the affected chimpanzees had a mild skin rash over the entire body (Cohen et al. 1996). In humans, VZV infection sometimes causes herpes zoster, which is thought to be triggered by stress or weak immunity. A case of severe haemorrhagic symptoms as a result of reactivation of its original VZV (simian varicella virus) has been reported in a cynomolgus monkey (Takasaka et al. 1990). However, although all of the KUPRI chimpanzees had the antibodies, the chimpanzees never developed symptoms. The original great ape VZV has not been identified, but the high incidence of infection may indicate the existence of a VZV-related virus. HSV is a common virus not only in humans but also in other primates. However, no transmission has been observed in KUPRI chimpanzees.

JEV is an arbovirus transmitted by the mosquito *Culex tritaeniorhynchus*, which feeds on the blood of host pigs. Its seroprevalence in a pig population in a prefecture, Aichi, neighbouring that where KUPRI is located, suggests it is not rare, and there is even a report of a human resident suffering from JEV infection there (NIID 2008; Sato et al. 2009). Pan and Popo, who were born in KUPRI, were infected with JEV, suggesting that JEV occurs in the region around KUPRI and that chimpanzees might be bitten by mosquitoes carrying the virus. However, this is the first report of detection of an antibody against JEV in chimpanzees.

HAV is transmitted via the faecal–oral route. Outbreaks of human HAV spreading from chimpanzee to human and chimpanzee to chimpanzee have been reported elsewhere (Dienstag et al. 1976). The human is the only host for HAV, and normally humans in developing countries are infected during childhood and acquire immunity against it. The three seropositive chimpanzees were born in Africa, and might have been infected in their childhood (Fig. 4b). HAV infection is now rare in Japan, and most cases are imported; therefore, no transmission might ever have occurred in the other chimpanzees at KUPRI.

Among the picornaviridae infections, poliomyelitis by poliovirus is the most clinically important disease among enteroviruses. In this study, the chimpanzees were positive, with high incidence, for CoxA7 virus only. This causes herpangina in human children. Reiko and Akira were seropositive for several picornaviruses, indicating they may have been infected by different persons without chimpanzee-to-chimpanzee transmission.

Influenza virus is prevalent in humans of all ages, but only Ayumu had antibody against H3N2. The severe symptoms of influenza infection may force workers not to enter the rearing zone, and then the chances of transmission may decrease.

Managing captive chimpanzees and zoonotic risks to humans

An important issue to examine in future studies is whether the caretakers have specific antibodies against the same human-borne infectious pathogens as our captive chimpanzees.

One of the tasks of zoos and research institutes is to protect and maintain endangered species, including chimpanzees. Therefore, it is important to keep the chimpanzees healthy under conditions that are quite different from their native habitats. It may be difficult for some small zoos or research institutes to establish their own examination systems. We successfully detected chimpanzee antibodies against human pathogens by use of commercial tests developed for humans. This should facilitate routine monitoring and surveillance of captive chimpanzees. We will continue serological examinations to analyse antibody levels and other respiratory pathogens. Furthermore, our data are limited to one institute, and more information should be gathered that will be useful for great ape conservation. In this study, we performed one-point serological surveillance, which provides only the history of each chimpanzee. Therefore, a phase examination should be performed to analyse disease prevalence in the future.

This study focussed on the transmission of human-borne infections to chimpanzees, but the reverse should also be considered. Of human emerging diseases, 75 % are