It has also been suggested that accurate HAV levels in shellfish involved in outbreaks could be of use for the purpose of risk assessment.[15] Although there is no documented incidence rate of HAV infection at revolving sushi bars or sushi shops, HAV infection reportedly happens frequently from sushi bar visits.[16] There are several reports regarding HAV infections at revolving sushi bars and sushi shops.[17–19] In the present outbreak, some of the patients ate only cooked eel, not raw shellfish or oysters. Thus, we could not determine the exact food sources of the HAV. Food-borne outbreaks of HAV may represent an increasing problem in populations not immune to HAV, although it has recently been reported that genetic variants in ABCB1, TGFB1 and XRCC1 appear to be associated with a susceptibility to HAV infection among Mexican Americans.[20]

In Japan, HAV was added to the diseases of Infectious Agents Surveillance in 1987, and HAV infection was listed as one of the reportable diseases on 5 November 2003. At present, all HAV infections have to be reported to a prefectural governor by the physician in charge. In the present cases, the physicians reported the first HAV case to a prefectural governor, and a local public health center intervened in investigating the source of the HAV infection. Their investigation revealed that three shop assistants, one in the present study and two in two other hospitals, were positive for HAV RNA in their stools. Two of them prepared sushi in the revolving sushi bar, one presenting with fever and general fatigue on 19 December 2010, and later he was diagnosed with hepatitis A from another hospital, but he continued to work into January 2011.[21] We could not exclude the possibility that he was the source of the HAV infection because the onsets of the other patients followed his.

Sushi is a Japanese traditional food consisting of rice combined with varieties of raw/cooked fish and shellfish. A revolving sushi shop is a Japanese fast-food sushi restaurant with a revolving conveyor belt that carries plates of sushi. In the present study, 26 of 27 patients went to the same revolving sushi shop. It is unknown whether they ate HAV-contaminated food or not. However, molecular analysis of the HAV infection revealed that a single source might have caused this outbreak.

In Japan, universal vaccination programs against HAV as well as hepatitis B virus are not yet being performed. In recent years, the incidence of hepatitis A in Japan has dramatically decreased,[10] and therefore there might be a decrease in the proportion of persons who have immunity against HAV. Our previous study[5] suggested that hepatitis A cases could increase in the near future. The current outbreak was caused by the HAV subgenotype IA strain, different from HAV subgenotype IIIA that caused the recent Korean outbreak.[22]

In conclusion, we report a hepatitis A outbreak associated with a revolving sushi bar, and that the same HAV subgenotype I strain was detected in 23 of 27 patients. It was again recognized that molecular phylogenetic analysis is useful for detecting the source of HAV infection. In developed countries, because HAV may cause acute hepatitis, particular attention should be paid to hepatitis A.

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Hepatology Research

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Original Article

Possible widespread presence of hepatitis A virus subgenotype IIIA in Japan: Recent trend of hepatitis A causing acute liver failure

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Aim: Recently, the number of acute hepatitis A cases has decreased in Japan. However, six patients with acute liver failure caused by hepatitis A virus (HAV) have been admitted to Chiba University Hospital, Japan, in the last 18 months, between 2010 and June 2011. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

Methods: Hepatitis A virus RNA was detected by strand-specific reverse transcription. Then, HAV VP1/2A regions were amplified by nested polymerase chain reaction (PCR). Sequences were directly determined and phylogenetic trees were constructed for determining HAV subgenotypes.

Results: Analysis of these HAV genomes revealed that 4 and 2 belonged to subgenotypes IA and IIIA, respectively.

Conclusions: Fujiwara *et al.* reported a frequency of HAV subgenotype IIIA of only 2.1% in Japan. We conclude that HAV subgenotype IIIA might be widespread in our country.

Key words: acute liver failure, hepatitis A virus, Japan, subgenotype IIIA

INTRODUCTION

HEPATITIS A VIRUS (HAV) is a member of the genus *Hepatovirus* in the *Picornaviridae* family. HAV is a positive-stranded RNA virus with an approximately 7.5 kb genome, is usually spread via the fecal-oral route, causes acute hepatitis, and occasionally leads to acute liver failure with fatal outcome in unvaccinated individuals.[1,2] There is only one serotype of HAV, but based on sequences of the VP1/2A genomic region, at least six genotypes (I to VI) exist.[3] Three (I, II and III) of the genotypes are of human origin.

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Several studies on HAV genotypes in Japan were reported.[3–6] In 1992, Robertson *et al.*[3] reported the existence of two predominant subgenotypes, IA and IIIB. In 2003, Fujiwara *et al.*[4] determined that 44 of 47 acute hepatitis A cases belonged to subgenotype IA, two to IB, and one to IIIA. In 2006, Takahashi *et al.*[5] also reported that 57 of 58 sequences belonged to IA and only one to IIIA. Toyoda *et al.*[6] reported that all 61 isolates they determined between 1992 and 2003 belonged to subgenotype IA. These reports revealed that the HAV subgenotype IA was endemic to Japan.[4–6]

Recent studies on HAV genotypes from South Korea have shown a distinct pattern change in circulating HAV genotypes over the past 10 years.[7] Until early 2000, almost all isolates tested had been identified as subgenotype IA.[8] A more recent study showed that subgenotype IIIA has been predominant since 2008.[7] In addition, a rise in the frequency of hepatitis A outbreaks has recently been observed in South Korea, our immediate neighbor, although the number of hepatitis A

cases in Japan has been progressively decreasing during the last several years.[9] The two countries have some cultural similarities. There is no universal hepatitis A vaccination program in either country, whereas Korea, but not Japan, has such a program against hepatitis B. We also reported that HAV 5'NTR subgenotype IA from Korea had high homology to Japanese sequences.[9] These circumstances have raised concerns about a possible HAV epidemic in Japan. The aim of this study is to characterize the recent HAV genotypes from an urban hospital in Japan and to compare the clinical differences.

METHODS

Patients

SERA WERE COLLECTED from immunoglobulin M (IgM) antibodies to HAV (IgM-HA) positive patients upon admission to Chiba University Medical School Hospital, Chiba, Japan. HAV infection was defined by positive reactions for IgM-HA and serum HAV RNA by polymerase chain reaction (PCR) with primers from the highly conserved 5' non-translated region (5'NTR).[9] These patients presented with acute liver failure without encephalopathy on admission between 2010 and June 2011 (Table 1). This study was approved by the ethics committee of Chiba University, Japan (permission number 1160), the ethics committee of the National Institute of Infectious Diseases Japan (permission number 305), and complied with the Helsinki Declaration.

RNA extraction and detection of HAV RNA by PCR

RNA was extracted from 100 μL of serum samples according to the guanidium thiocyanate method and subjected to RT-PCR for the VP1/2A region of the HAV genome.[3] Complementary DNA was synthesized with HAV-3273 (5'-CCA AGA AAC CTT CAT TAT TTC ATG-3'), then amplified with HAV-3273 and HAV-2799 (5'-ATT CAG ATT AGA CTG CCT TGG TA-3') for 40 cycles at 94°C, 50°C, and 72°C. Then, the first PCR product was further amplified with inner primer pairs HAV-2907 (5'-GCA AAT TAC AAT CAT TCT GAT GA-3') and HAV-3162 (5'-CTT CYT GAG CAT ACT TKA RTC TTT G-3') in the same manner. Amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Sequencing of the VP1/2A region

Sequences were directly determined as previously described.[9]

Phylogenetic analysis

A phylogenetic tree was constructed by using GENETYX, version 10 (Genetyx, Tokyo, Japan) based on the nucleotide sequences of the amplified VP1/2A region. The GenBank accession numbers for the nucleotide sequences of HAV isolates are AB643799 – AB643804. HAV complete genome sequences were retrieved from the DDBJ/EMBL/GenBank genetic database and used as references in this study.

RESULTS

SIX PATIENTS WITH acute liver failure caused by HAV were admitted during an 18-month period between 2010 and June 2011 (Table 1). All patients had >38.5°C fever on admission. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases) (Fig. 1). Patient no. 2 was a hepatitis B virus carrier. All patients recovered

Table 1 Profiles of six acute liver failure patients infected with hepatitis A virus in Japan

Patient no.	Age (years)/sex/ nationality	Month of onset	Nadir PT (%/INR)	Peak ALT (IU/L)	Peak total bilirubin (mg/dL)	Presumed route of transmission	Isolate name/ subgenotype
1	69/F/JPN	2010 Mar	23/2.88	7731	8.5	Raw scallop	Ch24/IIIA
2	46/M/JPN	2010 Apr	25/2.71	3388	12.6	Unknown	Ch23/IA
3	59/M/JPN	2010 Jun	35/2.01	5693	22.8	Raw oyster	Ch26/IA
4	30/F/KOR	2010 Jul	36/1.98	6958	5.0	Raw oyster	Ch25/IIIA
5	54/M/JPN	2011 Jan	20/3.20	2979	10.1	Sushi	Ch27/IA
6	37/M/JPN	2011 Jan	34/2.11	9826	3.9	Sushi	Ch29/IA

ALT, alanine transaminase; F, female; G, subgenotype; INR, international normalized ratio; JPN, Japan; KOR, South Korea; M, male; PT, prothrombin time.

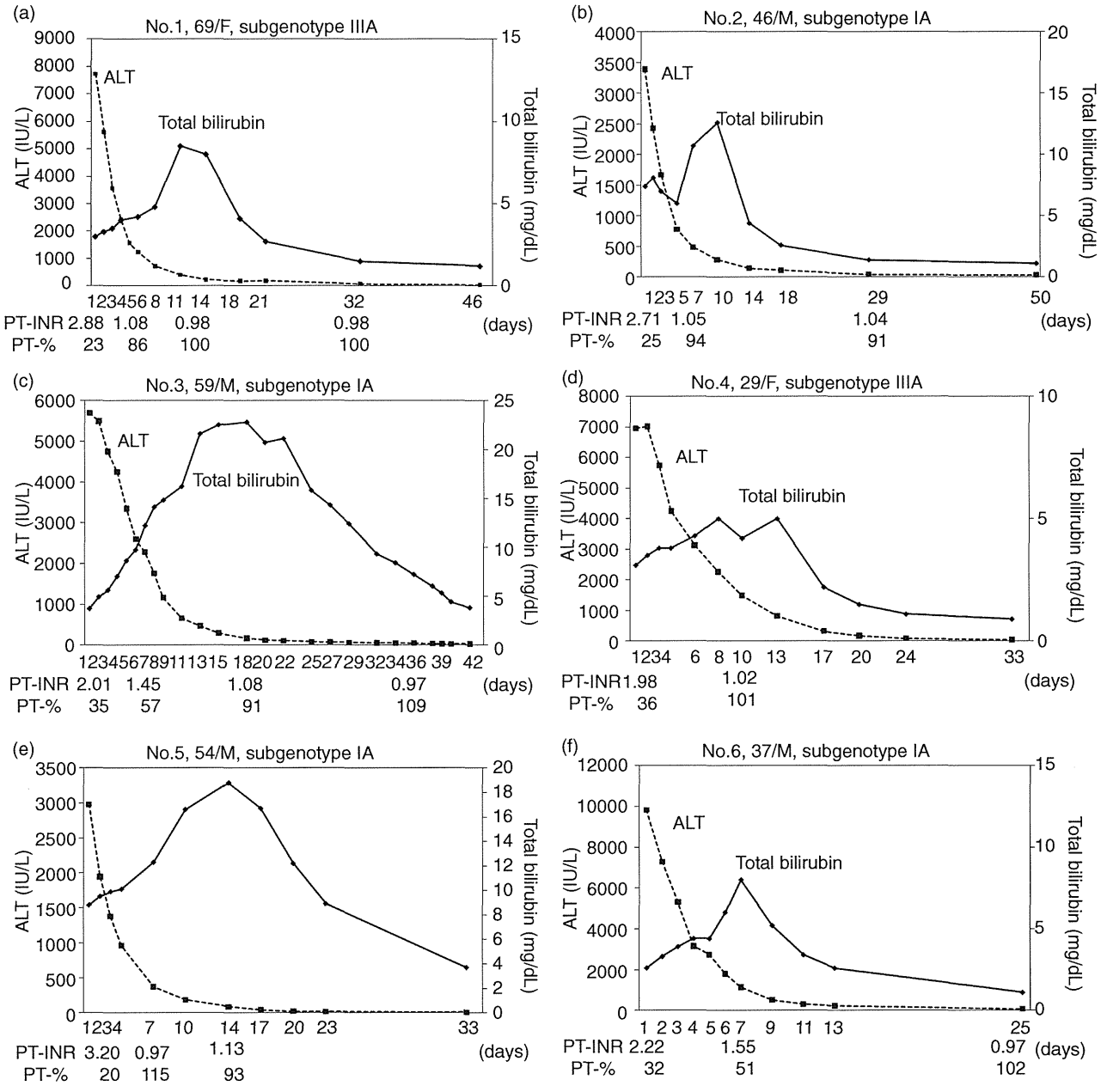

Figure 1 Clinical course of six acute liver failure patients infected with hepatitis A virus (HAV) in Japan. (a), (b), (c), (d), (e) and (f) indicates patient no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6 in Table 1, respectively. All patients presented with acute liver failure with coagulopathy but without encephalopathy (non-fulminant cases). PT, prothrombin time.

without liver transplantation, although patient no. 3 had interstitial pneumonia and was complicated by prolonged cholestasis while hospitalized and bone marrow suppression during the follow-up period, and patient no. 5 was complicated by mild acute kidney injury but recovered.

The nucleotide sequences of the six human HAV isolates in this study were compared with those of 24 published HAV sequences, and the genetic relatedness of the HAV isolates from different genotypes was investigated. Phylogenetic analysis of the nucleotide sequences from the VP1/2A region showed that four isolates (Ch23,

Ch26, Ch27 and Ch29) and two isolates (Ch24 and Ch25) belonged to subgenotype IA and IIIA, respectively (Fig. 2).

The sequences of the four isolates of subgenotype IA closely matched that of one well-characterized subgenotype IA virus: FH1 (GenBank accession no. AB020567) (96–97% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the four isolates of subgenotype IA in this study ranged from 95% to 99%.

The sequences of the two isolates of subgenotype IIIA closely matched that of two well-characterized subgenotype IIIA viruses: A408 (GenBank accession no. AB046904) (99–100% nucleotide identity) and NOR-21 (GenBank accession no. AJ299464) (98% nucleotide identity). Similarity of the nucleotide sequences of the VP1/2A region between the two isolates of subgenotype IIIA in this study was 98%. Our two strains were clustered with A408 (Japan), NOR-21 (Norway), HA-JNG04-90F (Japan), HMH (Germany) and subgenotype IIIA strains reported from Japan in early 2010. Another subgenotype IIIA cluster was formed by two strains, HAJ95-8F (Philippines) and HA-JNG08-92F (Madagascar).

DISCUSSION

IN THE PRESENT study, of six recent patients with HAV-associated acute liver failure, two were caused by subgenotype IIIA. It was reported that almost all acute hepatitis A cases (93.6%) were caused by subgenotype IA and only 2.1% by subgenotype IIIA,[4] and that all acute liver failures were caused by subgenotype IA. Thus, the possibility of a changing pattern in circulating HAV genotypes such as that reported in Korea[7] might need to be entertained in Japan as well.

What about the transmission route? Many high-risk groups such as travelers visiting highly endemic areas, the military, healthcare workers, sewage workers,

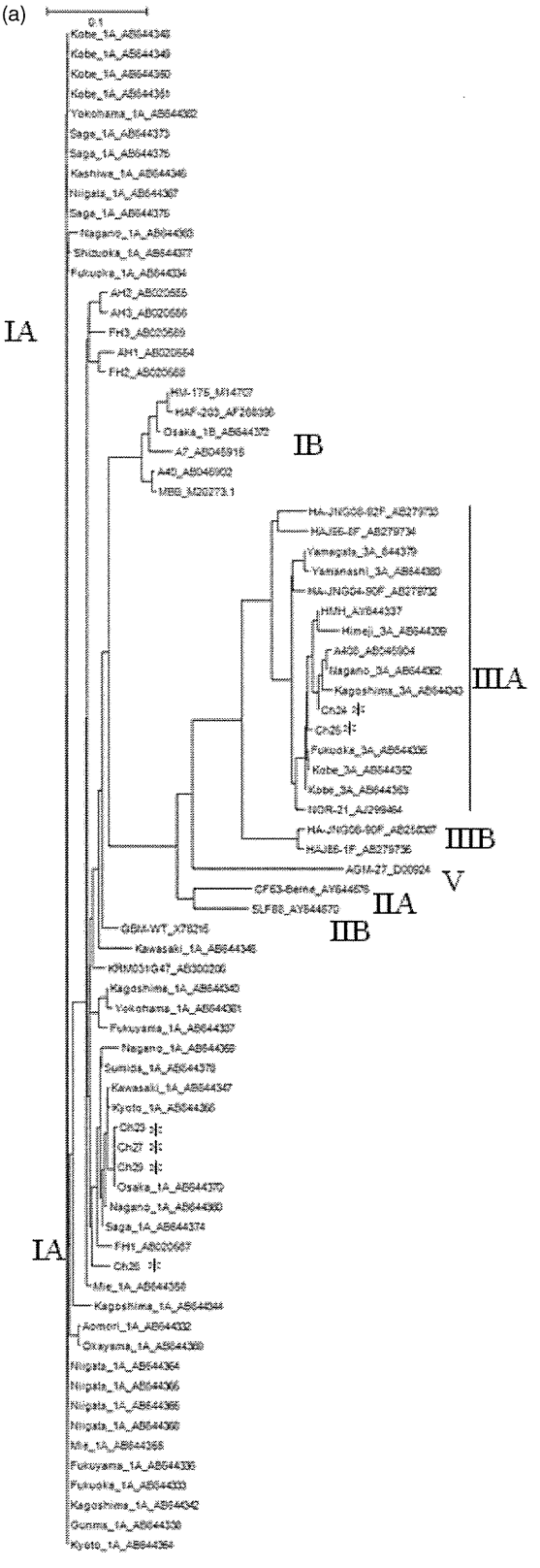

Figure 2 Phylogenetic analysis of hepatitis A virus (HAV) isolates from patients with acute liver failure from Japan. (a), (b) The neighbor joining tree was constructed based on a partial sequence of 451 nt in the VP1/2A region of HAV. Selected reference strains were also included in the phylogenetic analysis to represent the following subtypes: HAV-IA, IB, IIA, IIB, IIIA, IIIB, and V. *Strains sequenced in this study are indicated (Ch23, Ch24, Ch25, Ch26, Ch27 and Ch29), aligned with all the available reference sequences retrieved from data bases (DDBJ/EMBL/Gene Bank).

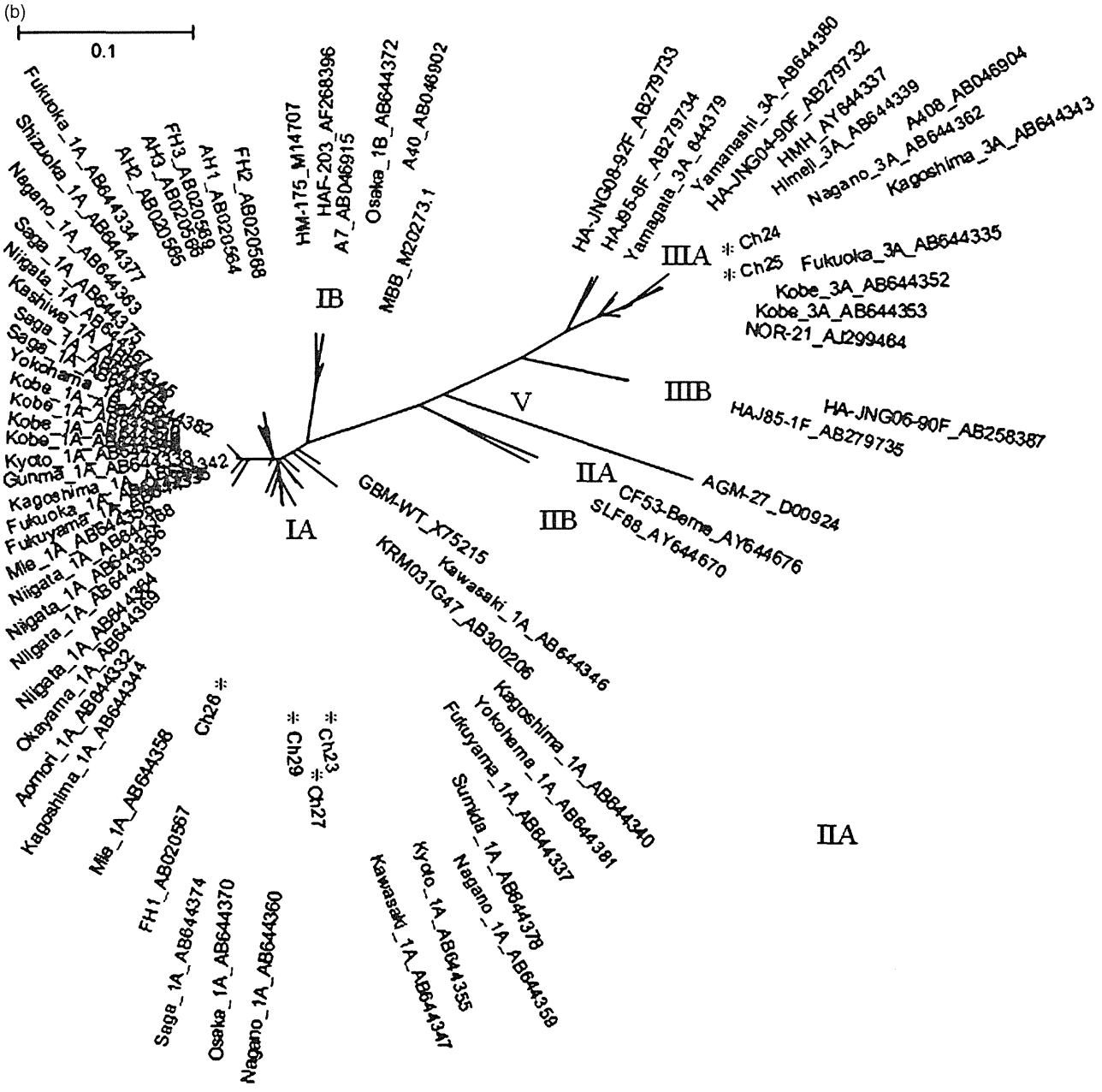

Figure 2 *Continued.*

day-care assistants, drug addicts, and homosexual people have been identified for potential HAV infection.[10] In the present study, four patients with subgenotype IA were male and two with subgenotype IIIA were female. We do not know why there were sex differences between the two subgenotypes. None in the present study was homosexual or HIV-positive. Patients no. 5 and no. 6 were associated with a recent HAV outbreak at a sushi shop in the Chiba area (Table 1).[11] None of the patients had traveled abroad, including to South Korea, during more than one year before admission. That is, all patients were infected with HAV in our country, suggesting that HAV subgenotype IIIA might be widespread in our country. Of interest is that these two patients (no. 1

and no. 4) had eaten raw scallops and raw oysters, respectively (Table 1).

The clinical spectrum of HAV infection ranges from asymptomatic infection to fulminant hepatitis.[12] Clinical presentation of hepatitis A depends on the age of the patient, being more severe in adults than in children.[13] In the present study, the mean age of subgenotype IA and IIIA patients was 49 ± 9.6 and 49.5 ± 27.5 years, respectively. A recent study from Korea reported that HAV genotype influences the severity of liver disease and that a higher ALT level (>1000 IU/L) and longer hospitalization were significantly associated with subgenotype IIIA.[7] All HAV-associated acute liver failure patients in the study of Fujiwara *et al.*[4] belonged to subgenotype IA. In this regard, we also examined whether HAV genotype is directly related to the disease severity of hepatitis A. Two of the six acute liver failure patients in the present study were subgenotype IIIA. It is well-known that viral genotypes occasionally affect disease progression, severity and treatment response in hepatitis B and C.[14,15] Mean ALT levels of subgenotype IA and IIIA patients were 5470 ± 3130 and 7340 ± 546 IU/L, respectively. Further studies will be needed to examine whether there are associations between HAV genotypes and disease severities, as the number of patients was limited and most of the patients in Chiba University Hospital were cases with acute liver failure.

In conclusion, the current study suggested that HAV subgenotype IIIA is also associated with acute liver failure in Japan. We need to make a cautious interpretation of the relation between HAV genotypes and their disease severities.

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Epidemiological and genetic analyses of a diffuse outbreak of hepatitis A in Japan, 2010

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ABSTRACT

Background: Hepatitis A virus (HAV) is still one of the most common causative agents of acute hepatitis in Japan. Although a relatively small number of annual acute hepatitis A cases (approximately 100–150, 0.78–1.17 per million) were recently reported, a larger number of cases (346, 2.71 per million) were reported in 2010.
Objectives: To investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses.
Study design: HAV specimens were obtained from 61 cases from 22 different prefectures. These viral specimens were genotyped by PCR amplification and sequencing of the VP1/2A region of HAV genome.
Results: Phylogenetic analysis revealed that 61 HAV strains could be divided into three genotypes: IA (44 cases), IB (1 case) and IIIA (16 cases). The IA genotype consisted of two genomic sub-lineages. The sequences of one of the two IA sub-lineages (corresponding to 31 cases) were very similar, 26 of these 31 isolates had 100% identity. The other IA sub-lineage corresponded to strains endemic to Japan. The sequences of Japanese IIIA strains were similar to those of strains that caused a large epidemic in the Republic of Korea from 2007 to 2009.
Conclusions: The resurgence of HAV in 2010 can be attributed to importation of two newly emerged HAV genotypes.

1. Background

HAV is a member of the genus *Hepatovirus* within the family *Picornaviridae*, and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kb in length. HAV strains isolated from different parts of the world have been classified into six genotypes (I–VI); genotypes I–III are found in humans, and each of these is further divided into subgenotypes A and B. Most of the human HAV strains belong to genotypes I and III.[1–3] Subgenotypes IA and IB are most often found in North and South America, Europe, China and Japan.[1,4,5] Subgenotype IA appears to be the predominant virus of hepatitis A cases worldwide, whereas subgenotype IB has been prevalent in the European and Mediterranean regions.[3,6–8] Subgenotype IIIA was recovered from various countries in Asia, Europe (especially in Roman ethnic population), Madagascar and the USA,[1,5,9–12] and subgenotype IIIB was responsible for some cases of HAV infection in Denmark and Japan.[1,10,11,13]

HAV infection has been a major public health problem in many countries worldwide. The annual incidence of hepatitis A is 1.5 million cases of clinical disease.[14] HAV is transmitted primarily via the fecal–oral route by contaminated food or water,[15–17] but also has been associated with outbreaks in injecting drug users and men who have sex with men (MSM).[18]

The number of acute hepatitis A patients in Japan has been steadily decreasing since the 1990s. Most of the infections that occurred in Japan were sporadic, with the exceptional occurrence of small-scale outbreaks. In 2007–2009, a relatively low number of annual cases (approximately 100–150, 0.78–1.17 per million) of acute hepatitis A were reported. In 2010, however, 346 cases (2.71 per million) were reported. To investigate the epidemiology of this 2010 HAV resurgence, we collaborated with 28 local institutes of health in Japan to obtain stool and plasma specimens from 98 acute hepatitis A patients. The DNA of these viral isolates was PCR-amplified and sequenced, and the sequences were used to perform phylogenetic analyses.

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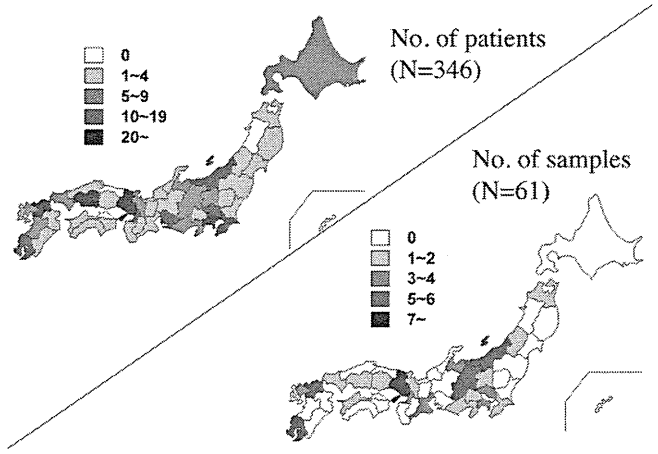

Fig. 1. Geographical distribution of acute hepatitis A patients and collected samples in Japan in 2010.

2. Objectives

The primary objective of this study was to investigate the causes of the 2010 HAV resurgence in Japan by using molecular epidemiological and genetic analyses. This study, performed in collaboration with local institutes of public health, is expected to provide insights useful for setting appropriate public health guidelines for HAV control.

3. Study design

3.1. Data collection

We collected stool and plasma specimens from 98 acute hepatitis A patients in collaboration with 28 local institutes of health in Japan. The collection sites were located at 22 different prefectures (regions in Japan) (Fig. 1).

3.2. RNA extraction, RT-PCR and phylogenetic analysis

A 10% fecal suspension (wt/vol) was prepared with phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 10,000 × *g* for 10 min. Viral RNA was extracted from the fecal suspension or sera by using a QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT) was performed with the SuperScript III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Seven microliters of the purified RNA was added to a reaction mixture (final volume, 20 μl) containing 50 pmol of random hexamer, 25 mM $MgCl_2$ buffer, 10 mM deoxynucleotide triphosphates, 10× RT buffer, 0.1 M dithiothreitol, and 200 U SuperScript III RT. The mixture was incubated at 42 °C for 1 h, after which 10 U of RNase H was added at 37 °C for 20 min.

Four degenerate primers (P1 to P4) were used in PCR to amplify the VP1/2A region of the HAV genome.[1] The sequences of these primers were:

HAV-2799 (5′-ATTCAGATTAGACTGCCTTGGTA-3′)
HAV-2907 (5′-GCAAATTACAATCATTCTGATGA-3′)
HAV-3162 (5′-CTTCYTGAGCATACTTKARTCTTTG-3′)
HAV-3273 (5′-CCAAGAAACCTTCATTATTTCATG-3′)

PCR was carried out using the HAV-2799 and HAV-3273 primer pair, followed by nested PCR with the HAV-2907 and HAV-3162 primer pair. PCR was performed with EX-taq (Takara, Shiga, Japan) according to the manufacturer's instructions. Amplification was performed for 40 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 15 min. Three microliters of the PCR product was used as the template for a second round of PCR amplification under the same conditions. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for direct sequencing.

Phylogenetic trees were constructed with the MEGA software (DNA DATA Bank of Japan) by the neighbor-joining method from a Kimura two-parameter distance matrix, and bootstrap values were determined from 1000 bootstrap re-samplings of the original data.[19–22] All reference sequences used in this study were obtained from GenBank.

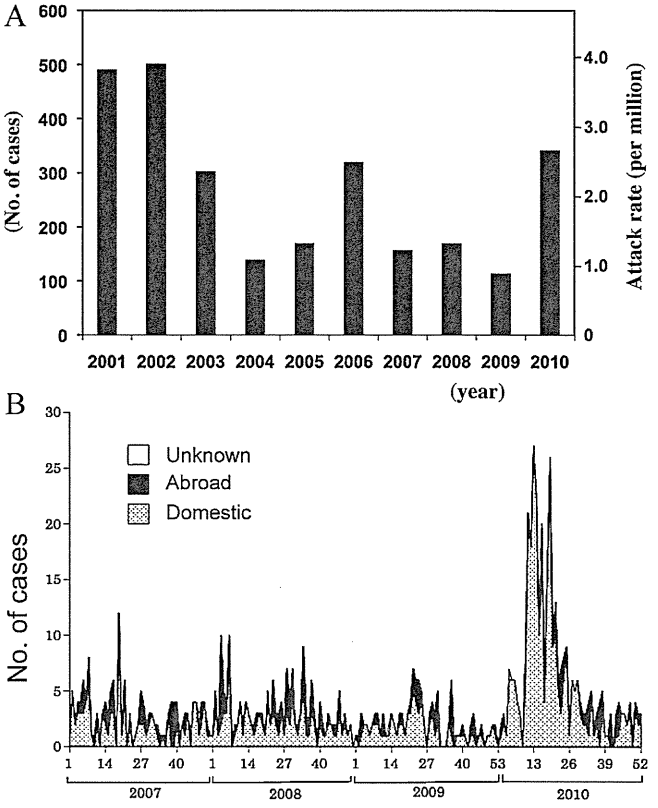

Fig. 2. (A) Reported number of acute hepatitis A patients in Japan from 2001 through 2010. The increase of the number in 2010 was statistically significant compared with the number in 2007 ($t = 5.4 \times 10^{-}$), 2008 ($t = 5.6 \times 10^{-5}$) and 2009 ($t = 1.8 \times 10^{-5}$). (B) Weekly acute hepatitis A cases from week 1 of 2007 to week 52 of 2010.

4. Results

In 2010, the number of acute hepatitis A cases increased to 346 (2.71 per million) (Fig. 2A) because of a diffuse outbreak that occurred from March through May (Fig. 2B). Most of the patients in this outbreak reflected domestic infection events (Fig. 2B). Clinical descriptions of these patients are summarized in Table 1.

Sera and fecal samples from 98 patients were available for PCR. Of these, 61 yielded a PCR product that could be used for sequencing. Among these 61 isolates, 44 were of genotype IA, one was of genotype IB and 16 were of genotype IIIA by phylogenetic analysis (Fig. 3). The genotype IA isolates could be sorted into two sub-lineages. One sub-lineage (referred to as IA-1 in this paper) grouped with several isolates found in 2006,[23–25] suggesting that the isolates in this lineage were endemic to Japan. In contrast, the sequences of most of the genotype IA isolates belonged to a second sub-lineage (referred to as IA-2 in this paper) with sequences almost identical to one another. Among the IA-2-infected patients, two had developed acute hepatitis shortly after returning from Philippines,

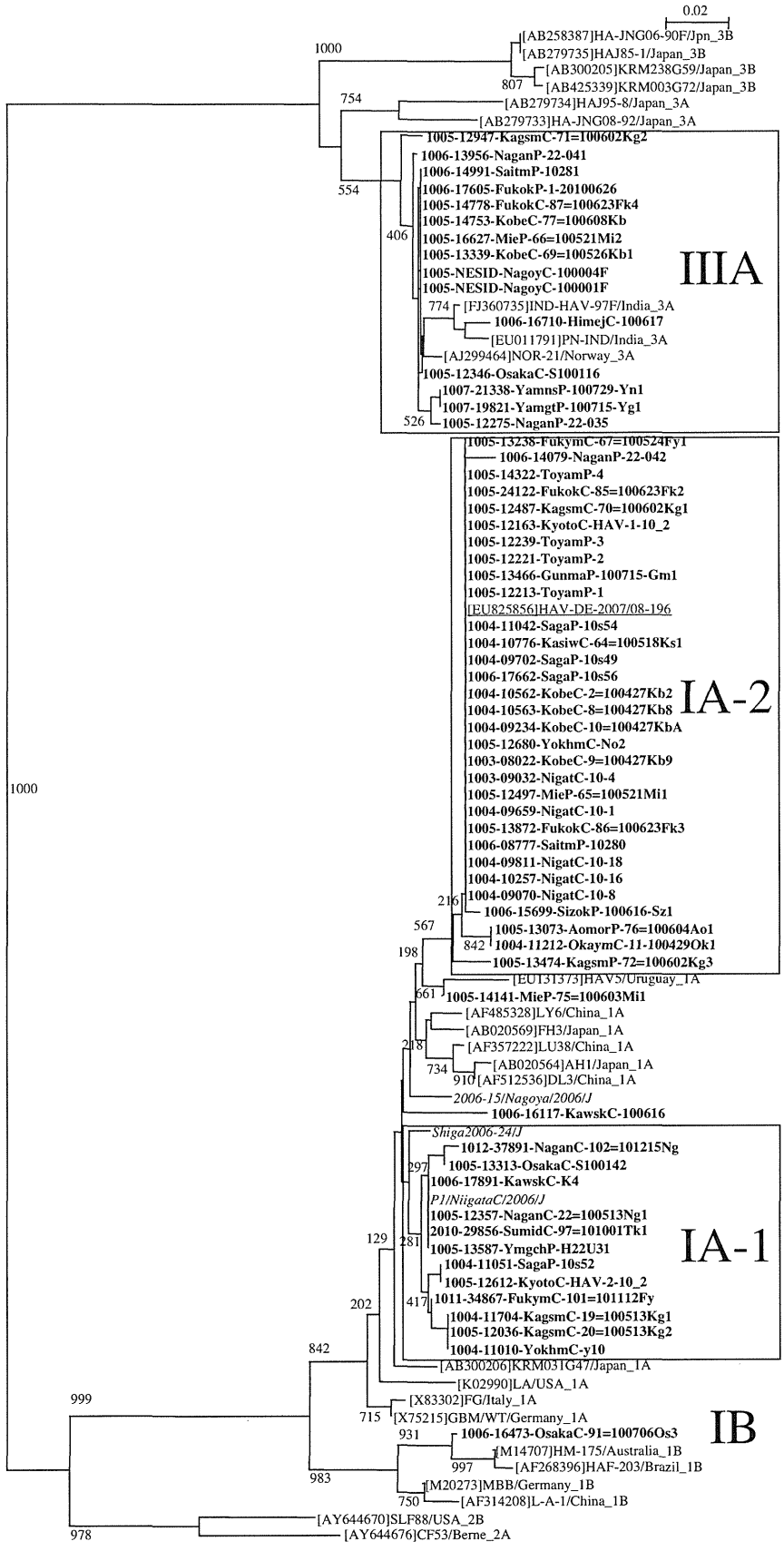

Fig. 3. Neighbor-joining phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus isolates. Reference strains are used in this study and indicated as GenBank accession numbers. Sequences of 61 isolates from this study are shown as YYMM-NESID-KKKKKKKKK (YYMM represents the reported year (YY) and month (MM); NESID (National Epidemiological Surveillance of Infectious Diseases) is the ID number of the patient; KKKKKKKKK is the name of the isolate given by local institute). The scale bar at the bottom indicates nucleotide distance. Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.

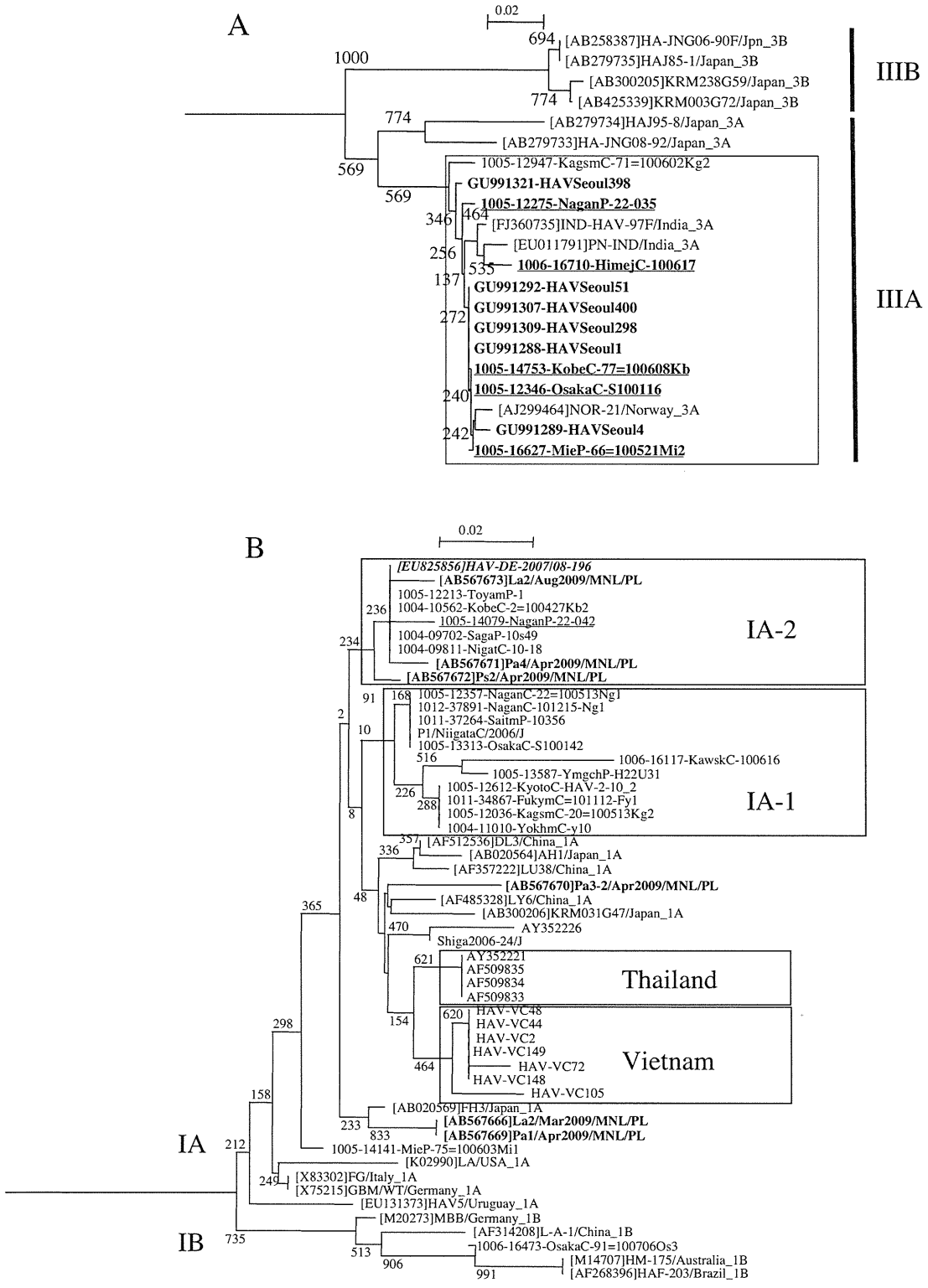

Fig. 4. (A) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from HAV strains (genotype IIIA) isolated from Japan (bold underline) and Korea (bold). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling. (B) Phylogenetic tree of the nucleotide sequences of the VP1/2A junction region from hepatitis A virus strains (genotype IA) isolated from Japan, Thailand, Vietnam and river and sewage from Philippines (shown in bold). HAV sequences of Japanese patients who developed acute hepatitis shortly after travel to Philippines are underlined. HAV-DE-2007/08-196 is shown in italics. In IA-2 sub-lineage, 26 identical sequences are represented by four sequences (1005-12213, 1004-10562, 1004-09702, 1004-09811). Numbers at the branches show boostrap percentages obtained after 1000 replications of bootstrap sampling.

suggesting the relationship of this lineage with HAV viruses from that geographical source.

A slightly different region of VP1-2A (nt: 2975–3364) was used for phylogenetic analysis in South Korea (Yoo et al., unpublished, available on GenBank) compared to the region of VP1-2A (nt: 2930–3161) used in the present study. Unfortunately, the overlap between these sequences was not long enough for comparison between the two studies. To permit such a comparison, we

Table 1
Clinical descriptions of hepatitis A cases during diffuse outbreak period (from 10th to 28th week of 2010, 236 cases).

Items	Data
Age (median)	5–88 yr (48 yr)
Sex	Male 138 (58%), female 98 (42%)
Suspected infection route	Fecal–oral 199 (84%), others/unknown 37 (16%)
Suspected food vehicle	Oyster 58 (29%), fishery product 27 (14%), well water/tap water in foreign country 4 (2%), others/unknown 46 (23%), unnoted 64 (32%)
Icteric	171 (72%)
Fulminant (severe) hepatitis	6 (3%)
Diagnosed by	IgM 223 (94%), PCR 2 (1%), IgM and PCR 11 (5%)

sequenced VP1-2A fragments (nt: 2822–3272) generated by the first PCR reaction on some of the Japanese genotype IIIA strains. These sequences were compared with Korean genotype IIIA strains. Phylogenetic analysis revealed that the Japanese and Korean genotype IIIA isolates could be classified into a single cluster (Fig. 4A). This observation suggests a close relationship between the Japanese genotype IIIA strains and those derived from the recent Korean outbreak.

5. Discussion

In recent years, the incidence of hepatitis A in developed countries has decreased dramatically. Changes in the genotypes or subtypes of HAV strains, including the emergence of HAV strains that are new to the area, have been observed in patients with acute hepatitis A in developed countries,[26] probably due to the transport of HAV strains via international transport of foods and agricultural products. HAV strains also could be imported by unvaccinated human carriers who have traveled to endemic countries. National surveillance of HAV in Japan has shown that more than 90% of people over 65 years of age, but fewer than 10% of people under 34 years of age, are seropositive for HAV.[27] Most of the infections that have occurred in Japan represent sporadic events, with exceptional occurrences of small-scale outbreaks. In 2010, however, there was a spike of hepatitis A infections in Japan, with 346 cases reported by the Infectious Disease Surveillance Center, NIID.

One of the genotype IA sub-lineages (referred to as IA-1 in this paper) was related to an isolate found in small outbreaks in Shiga and Niigata prefectures in 2006.[23,24] The isolates belonging to this sub-lineage have been detected in Japan since at least 2001 (Tamada and Yano, personal communication), suggesting that the isolates of this sub-lineage were locally endemic strains of Japan. On the other hand, more than half of genotype IA isolates displayed identical or virtually identical sequences across a 230-nt interval of the VP1-2A segment of the genome. Among the isolates in this sub-lineage (IA-2 in this paper), two (Fig. 4B, underlined) were from patients who had recently visited the Philippines, suggesting a relationship between IA-2 sub-lineage and this geographical site. This sequence also was found to be identical to HAV-DE-2007/08-196 (Fig. 4B, italics), which was identified in Germany in 2007.[28] The patient of HAV-DE-2007/08-196 was an 11-year old female who developed acute hepatitis shortly after traveling to the Philippines (Faber et al., personal communication). To assess this proposal, we also obtained sequence data for HAV derived from river and sewage of Manila and included these sequences in our phylogenetic analysis (Fig. 4B; HAV from river and sewage of Manila are shown in bold). Some sequences classified with the IA-2 sub-lineage, supporting the hypothesized Philippine connection. Genotype IA isolates of HAV from other Southeast Asian countries, such as Vietnam[29] and Thailand,[30] formed distinct clusters (Fig. 4B). However, caution is necessary with this result, because

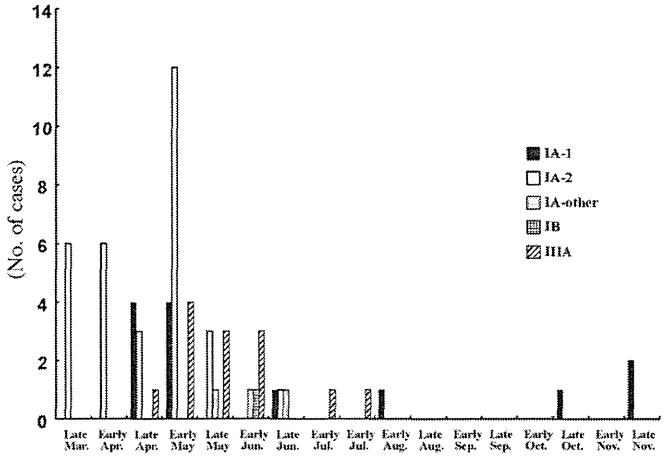

Fig. 5. Temporal distribution of HAV genotypes from late March to late November in 2010.

the sequences of HAV from these countries were determined 4–5 years before the Japanese diffuse outbreak in 2010, and a shorter 168-bp fragment (nt: 3024–3191, corresponding to the sequence data of Thai isolates) was used for the analysis. The isolates belonging to the IA-2 sub-lineage were detected mainly from late March through May, and could not be detected after June (Fig. 5). On the other hand, a regional imbalance of hepatitis A cases associated with this strain was not observed. Together with the uniformity of this cluster, we propose that this strain expanded from a single infection source (possibly an imported food product) that caused diffuse outbreak without a secondary expansion. Unfortunately the source(s) of HAV isolates belonging to the IA-2 remain unidentified.

Until recently, Japanese isolates of genotype IIIA were detected only on rare occasion, with the exception of some imported cases.[31–33] However, in 2010, approximately 26% of HAV isolates were classified as genotype IIIA. In South Korea, the incidence of reported HAV cases were increased dramatically since 2005, and most of the HAV isolates from this period clustered within genotype IIIA lineage. These results suggest genotype IIIA as the major epidemic strain for this outbreak, despite the fact that the predominant genotype in Korea, until 2005, was genotype IA.[12,34] Since the VP1-2A region of HAV genome amplified by nested RT-PCR for phylogenetic analysis in Korea differed from that in our study, we could compare only those Japanese IIIA isolates for which we obtained sequences of the region amplified by the first PCR reaction. Phylogenetic analysis revealed that the Japanese and Korean IIIA isolates clustered together (Fig. 4A), suggesting a correlation between the Japanese IIIA strain in 2010 and the recent Korean outbreak.

In conclusion, our data revealed that the diffuse outbreak of hepatitis A in Japan in the spring of 2010 was derived not only from locally circulating strains, but also from two other newly emerged HAV strains, possibly imported from the Philippines (IA-2) and Korea (IIIA). More detailed and extensive epidemiological analyses, ideally in collaboration with these countries, are needed to determine the source of the imported strains. However, in order to provide a better phylogeny, the use of a longer fragment, such as the entire VP1 gene and/or VP3 gene, is highly desirable. Together with the changing epidemiology of HAV infection, our findings may help the authorities in formulating public guidelines, including HAV vaccination policies targeted at susceptible populations.

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Competing interests

None.

Ethical approval

Not required.

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PLOS ONE

Dendritic Cells from Oral Cavity Induce Foxp3$^+$ Regulatory T Cells upon Antigen Stimulation

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Abstract

Evidence is accumulating that dendritic cells (DCs) from the intestines have the capacity to induce Foxp3$^+$CD4$^+$ regulatory T cells (T-regs) and regulate immunity versus tolerance in the intestines. However, the contribution of DCs to controlling immunity versus tolerance in the oral cavity has not been addressed. Here, we report that DCs from the oral cavity induce Foxp3$^+$ T-regs as well as DCs from intestine. We found that oral-cavity-draining cervical lymph nodes contained higher frequencies of Foxp3$^+$ T-regs and ROR-γt$^+$ CD4$^+$T cells than other lymph nodes. The high frequency of Foxp3$^+$ T-regs in the oral-cavity-draining cervical lymph nodes was not dependent on the Toll like receptor (TLR) adaptor molecules, Myd88 and TICAM-1 (TRIF). In contrast, the high frequency of ROR-γt$^+$ CD4$^+$T cells relies on Myd88 and TICAM-1. *In vitro* data showed that CD11c$^+$ DCs from oral-cavity-draining cervical lymph nodes have the capacity to induce Foxp3$^+$ T-regs in the presence of antigen. These data suggest that, as well as in the intestinal environment, antigen-presenting DCs may play a vital role in maintaining tolerance by inducing Foxp3$^+$ T-regs in the oral cavity.

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Introduction

Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells (T-regs), constitute about 5–10% of peripheral CD4$^+$T cells and control immunological self-tolerance in rodents and human [1,2,3,4]. The expansion and induction of CD25$^+$Foxp3$^+$ T-regs in the periphery are controlled by professional antigen-presenting cells, dendritic cells (DCs) [5,6]. DCs can expand thymic-derived natural occurring T-regs [7,8,9]. DCs are the most efficient antigen presenting cells to induce Foxp3$^+$T-regs from Foxp3$^-$ precursors in the periphery [10,11]. Peripheral DCs directly control the numbers and homeostasis of Foxp3$^+$T-regs *in vivo* [12].

Foxp3$^+$T-regs induced by DCs in the intestine control the balance between inflammation and tolerance in the gut [13,14,15]. CD103$^+$DCs in the intestine use the retinoic acid-metabolizing enzyme retinaldehyde dehydrogenase and induce Foxp3$^+$T-regs to maintain oral tolerance [16,17]. Intestinal DCs use signaling through ß-catenin to induce Foxp3$^+$T-regs, which suppress Th17 and Th1 responses in the intestine [18]. Specific pathogens or Toll-like receptor (TLR) signals have been shown to induce Foxp3$^+$T-regs in the intestine [19,20]. Moreover, Foxp3$^+$T-regs control Th17 cells using interleukin (IL)-10 in the intestine [21,22]. Thus, Foxp3$^+$T-regs in the intestine are important in maintaining mucosal tolerance where there are vast numbers of commensal microbes and food antigens.

As in the intestine, many commensal microbes and food antigens also exist in the oral cavity [23,24,25,26]. Oral cavity is often involved with systemic immunological diseases such as graft versus host diseases, Stevens-Johnson syndrome, Behçet diseases, pemphigus vulgaris and Sjögren's syndrome. In addition, oral cavity is the place where many viruses, including influenza, herpes, common cold etc., start to infect. Therefore, it is important to identify how immune response is regulated in the oral cavity. Here we found that the DCs from oral cavity have the capacity to induce Foxp3$^+$T-regs. To our knowledge, this is the first report showing that DCs from the oral cavity induce Foxp3$^+$T-regs to maintain tolerance.

Results

The Frequency of Foxp3$^+$T-regs is Increased in Cervical Lymph Nodes (CLNs) in a Myd88/TICAM-1- Independent Manner

We considered whether Foxp3$^+$T-regs played an important role in the skin or oral cavity because the skin and oral cavity are exposed to many commensal microbes and antigens, like the intestine. First, we investigated the frequencies of Foxp3$^+$T-regs in lymph nodes (LNs) at different anatomical locations, which included skin- and oral-cavity-draining LNs. We found that

cervical LNs (CLNs) contained a higher frequency of Foxp3$^+$ T-regs than other skin-draining LNs, such as axillary LNs (ALNs) and inguinal LNs (ILNs; paired t-test: $p<0.005$; Fig.1A arrow, Fig.1B and Fig.S1). CLNs contained a slightly, but significantly, higher frequency of Foxp3$^+$ T-regs than mesenteric LNs (MLNs; paired t-test $p<0.05$; Fig.1B). In MLNs, Foxp3$^+$T-regs are actively induced by CD103$^+$ DCs [16,17]. These data suggest that Foxp3$^+$T-regs may be also induced in CLNs, as in MLNs.

To investigate if the frequency of DCs correlates with the frequency of Foxp3$^+$T-regs, the frequency of CD11c$^+$DCs in total cells was compared between CLNs and ALNs (Fig.S2). The frequency of DCs was similar between CLNs and ALNs.

Recent reports showed that signals though TLR-2 induce Foxp3$^+$T-regs [20,27,28]. To examine whether signals from TLRs are required for the high frequency of Foxp3$^+$ T-regs in CLNs, we took advantage of Myd88 and TICAM-1 (TRIF) double knockout mice (Myd88/TICAM1 DKO), which lack all TLR signaling [29,30]. In Myd88/TICAM1 DKO mice, CLNs still contained a significantly higher frequency of Foxp3$^+$T-regs than inguinal LNs (ILNs; paired t-test: $p<0.05$; Fig.1B). The frequency of Foxp3$^+$T-regs in CLNs did not differ between Myd88/TICAM1 DKO and wild-type (WT) mice (t-test: $p=0.09$; Fig.1B, 1C). The frequency of Foxp3$^+$T-regs in MLNs did not differ between Myd88/TICAM1 DKO and WT mice also (t-test: $p=0.4$; Fig.1B).

Thus, Foxp3$^+$T-regs are increased in CLNs in a Myd88/TICAM1-independent manner, suggesting that TLR signals are not involved in the increase in Foxp3$^+$ T-regs in CLNs.

The Frequency of ROR-γt$^+$ CD4$^+$T Cells is Increased in CLNs in a Myd88/TICAM1- Dependent Manner

The induction of Foxp3$^+$T-regs in the intestine is reciprocally controlled by Th17 [18,31]. To examine the balance between Th17 and Foxp3$^+$T-regs, we next compared the frequencies of ROR-γt$^+$ CD4$^+$T cells in different anatomical locations. ROR-γt is a transcription factor expressed by Th17 cells [32]. We found that CLNs had a significantly higher frequency of ROR-γt$^+$ CD4$^+$T cells than other skin-draining LNs and spleen (paired t-test: $p<0.05$; Fig.2A closed arrows and Fig.2B). As expected, MLNs contained a higher frequency of ROR-γt$^+$ CD4$^+$T cells than other LNs (Fig.2A gray arrows and Fig.2B).

To assess whether signals from microbes through TLRs are required for the induction of ROR-γt$^+$ CD4$^+$T cells in CLNs, we investigated Myd88/TICAM1 DKO mice. In Myd88/TICAM1 DKO mice, frequencies of ROR-γt$^+$ CD4$^+$T cells did not differ between CLNs and ILNs (paired t-test: $p=0.05$; Fig.2B). Moreover, the frequency of ROR-γt$^+$ CD4$^+$T cells in CLNs was significantly reduced in Myd88/TICAM-1 DKO mice (t-test: $p<0.05$; Fig.2B). The frequency of ROR-γt$^+$ CD4$^+$T cells in MLNs did not differ between Myd88/TICAM1 DKO and WT mice also (t-test: $p=0.1$; Fig.2B).

Thus, both Foxp3$^+$T-regs and Th17 may be induced in CLNs. However, Myd88/TICAM1 signaling is important for the development of ROR-γt$^+$CD4$^+$-Th17 T cells in CLNs (Fig.2B), but not for the induction of Foxp3$^+$T-regs (Fig.1B).

CLNs are Oral-cavity-draining Lymph Nodes

We considered that the higher frequency of Foxp3$^+$T-regs in CLNs may reflect their response to antigens in the oral cavity. To confirm whether CLNs were draining LNs from the oral cavity, we investigated the proliferation of transferred OT-II CD4$^+$-T cells in CLNs after sublingual (s.l.) administration of ovalbumin (OVA; Fig.3). OT-II mice are commonly used OVA-specific CD4$^+$ T-cell receptor transgenic mice [7,10,28]. In the absence of OVA s.l. administration, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II T cells did not divide, in CLNs or ALNs (Fig.3, top). With OVA s.l. administration, CFSE-labeled OT II T cells divided well in CLNs (Fig.3, bottom arrow), but not in ALNs. Thus, we confirmed that CLNs were draining LNs of the oral cavity because s.l.-administered OVA antigen was presented to OT II CD4$^+$-T cells.

DCs from Oral-cavity-draining CLNs Locate Close to Foxp3$^+$T-regs and have the Capacity to Induce Foxp3$^+$ T-regs on Antigen Stimulation

Next, to investigate the interaction between DCs and Foxp3$^+$T-regs in CLNs, we microscopically examined CLNs. We found that Foxp3$^+$T-regs and CD11c$^+$ DCs were closely located, as reported previously in MLNs [33] (Fig.4A). This suggests that DCs from CLNs may induce Foxp3$^+$T-regs as DCs do in MLNs.

To determine whether DCs from the oral cavity can in fact induce Foxp3$^+$T-regs, we compared the capacity to induce Foxp3$^+$T-regs *in vitro* using DCs from ALNs, MLNs, and oral-cavity-draining CLNs. Purified CD11c$^+$ DCs from CLNs, ALNs, or MLNs were cultured with OT II CD4$^+$T cells with or without antigen for 5 days. In the presence of antigen, CLN DCs induced a higher frequency of Foxp3$^+$T-regs compared with ALN DCs (paired t-test: $p<0.005$; Fig.4B, 4C). The frequency of Foxp3$^+$T-regs induced by antigen plus DCs did not differ between the culture with CLN DCs and that with MLN DCs (paired t-test: $p=0.878$; Fig.4C).

These results indicated that DCs from the oral-cavity-draining CLNs had the capacity to induce Foxp3$^+$T-regs with antigen, as DCs from MLNs do.

CD103$^+$DCs may not be Involved in Inducing Foxp3$^+$ T-regs in Oral-cavity-draining CLNs

To determine whether DCs from the oral cavity contain a specific DC subset to induce Foxp3$^+$T-regs as in the intestine, we first performed real-time PCR. When we investigated the mRNA expression of retinal dehydrogenase 2 (RALDH2), transforming growth factor (TGF)-ß, and IL-10, there was no difference between DCs from CLNs and ALNs (Fig.4A). DCs from MLNs had higher mRNA expression of RALDH2 as previously reported (Fig.5A). We also measured the protein production of TGF-ß1 and IL-10 in the culture supernatant. TGF-ß1 was not detected in the culture supernatants of CLN DCs with or without latent TGF-ß activation (data not shown). We did not detect IL-10 in the culture supernatants from CLN DCs and OT II CD4$^+$T cells without peptide in Fig.4B and 4C (data not shown). Theses results indicate that TGF-ß1, IL-10 and RALDH2 may not involve in the induction of Foxp3$^+$T-regs by CLN DCs.

To investigate whether CD103$^+$DCs play a role in inducing Foxp3$^+$T-regs in CLNs, we compared the frequency of CD103$^+$DCs in each location. However, oral cavity-draining CLNs had a lower frequency of CD103$^+$ DCs than MLNs (Fig.5B).

Plasmacytoid DCs have a capacity to induce Foxp3$^+$T-regs [34,35,36,37]. Epidermal Langerhans cells and migratory dermal DCs have also been reported to induce Foxp3$^+$T-regs [38,39,40]. However, the frequencies of plasmacytoid DCs and migratory class II^{high} DCs did not differ between CLNs and ALNs (Fig.5C). Next, we investigated the classical CD8$^+$ and CD8$^-$ DC subsets in CLNs and ALNs. The frequency of CD8$^+$ DCs was similar between CLNs and ALNs (paired t test: $p=0.065$) (Fig.5D). However, CLNs had a significantly higher frequency of CD8$^-$ DCs than ALNs (paired t test: $p<0.0005$) (Fig.5D).

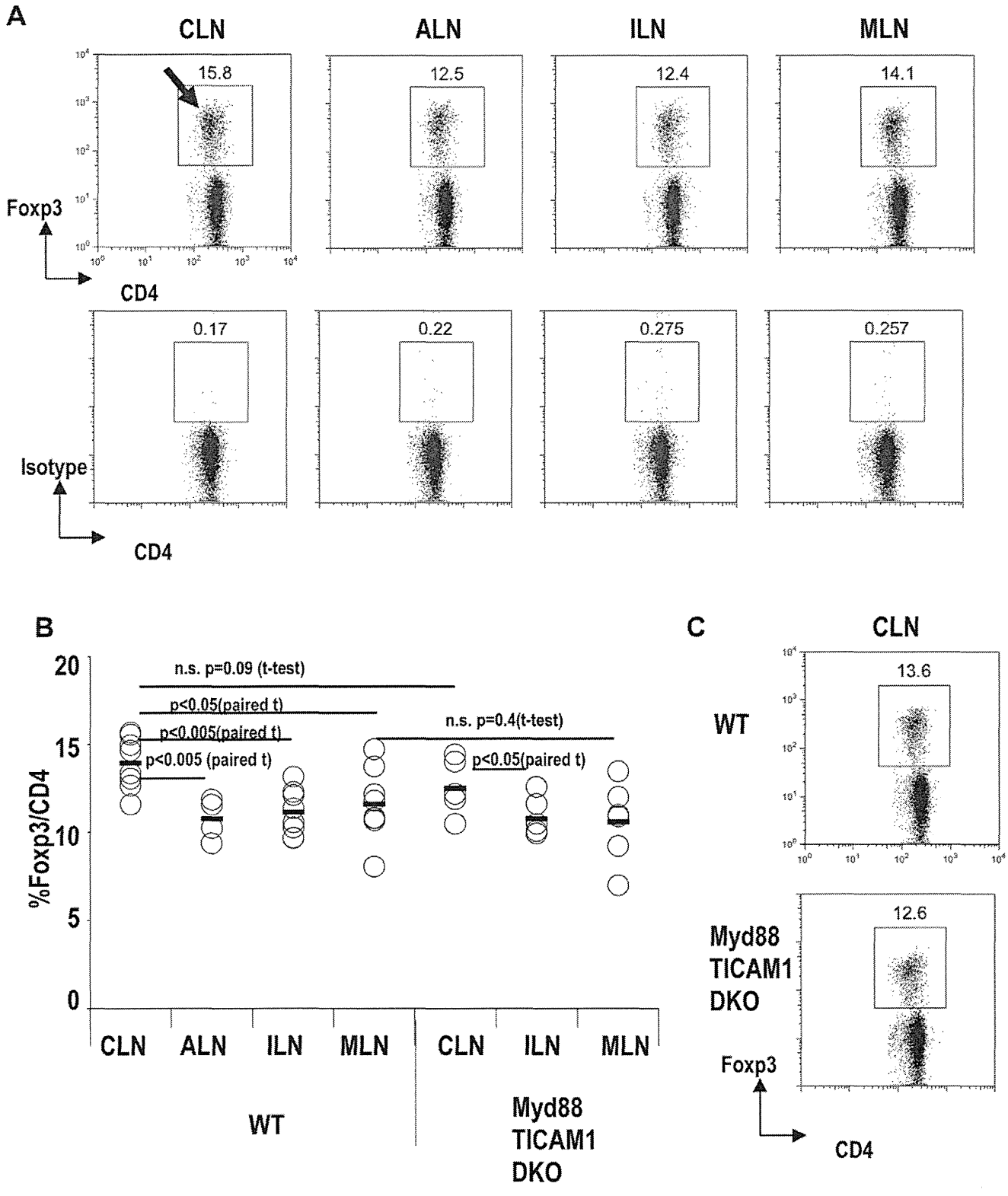

Figure 1. Frequency of Foxp3$^+$ T-regs in cervical lymph node is increased in a Myd88/TICAM-1 independent manner. (A) Cervical lymph nodes (CLN), axillary lymph nodes (ALN), inguinal lymph nodes (ILN), and mesenteric lymph nodes (MLN) from wild type B6 mice were analyzed for the expression of Foxp3. The isotype control is shown at the bottom. Plots were gated on CD4$^+$ T cells. Representative of seven separate experiments. (B) As in (A), but CLN, ALN, ILN and MLN from wild-type B6 mice (WT) or Myd88/TICAM1 double knockout mice (Myd88/TICAM1 DKO) were analyzed for the expression of Foxp3. Summary from seven separate experiments. P value provided is by paired t-test or t-test. "n.s." = "not significant". (C) As in (B), but a representative of CLN is shown.
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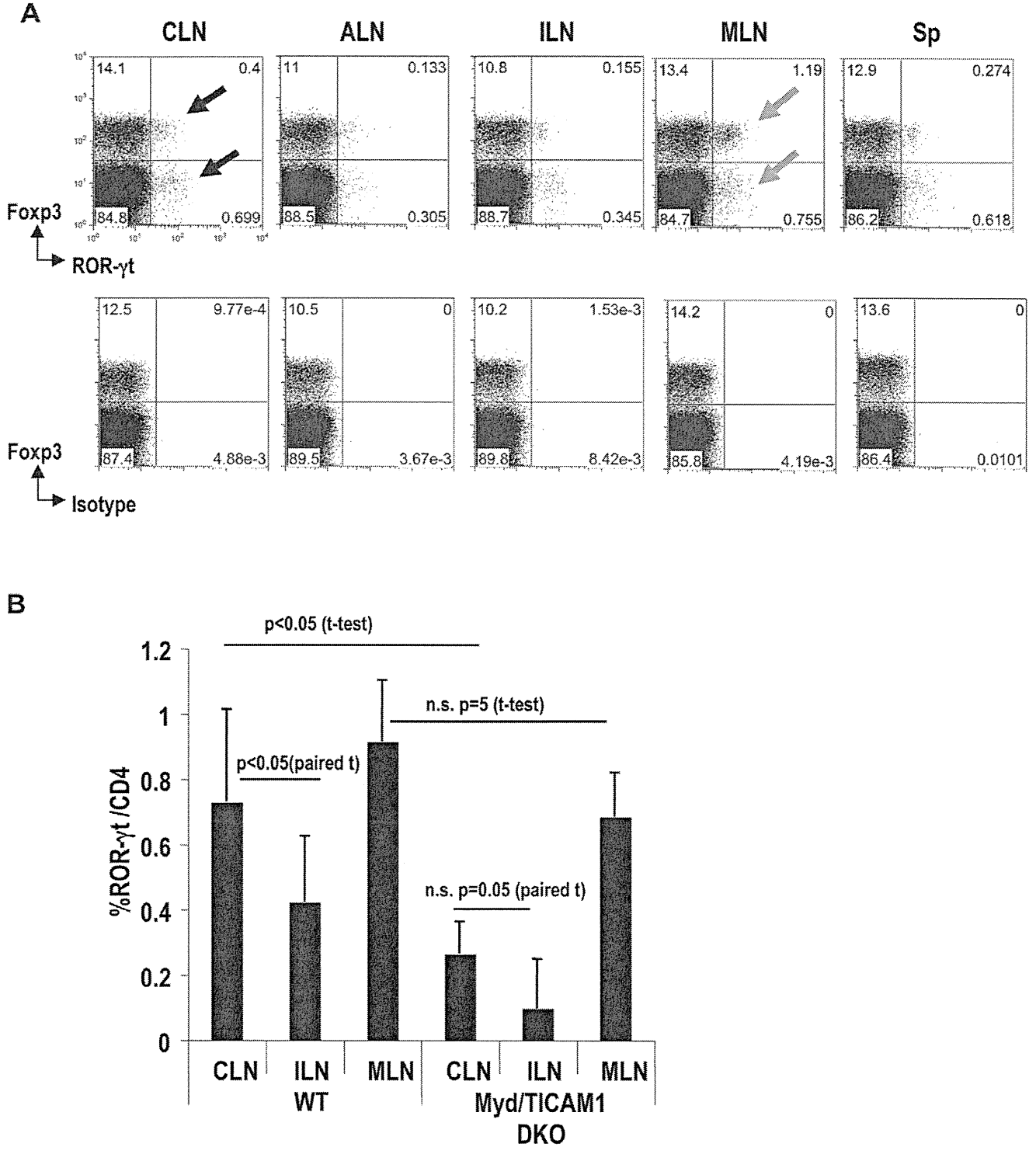

Figure 2. Frequency of ROR-γt$^+$CD4$^+$ T cell in cervical lymph node is increased in a Myd88/TICAM-1 dependent manner. (A) CLN, ALN, ILN, MLN, and spleen (Sp) from WT B6 mice were analyzed for the expression of Foxp3 and ROR-γt. The plots were gated on CD4$^+$ T cells. Isotype staining for ROR-γt is shown at the bottom. Representative of three separate experiments. (B) As in (A), but cells from WT mice or Myd88/TICAM-1 DKO mice were analyzed for the expression of ROR-γt and CD4. The graphic shows a summary from two separate experiments. P value provided is by paired-t test or t-test. "n.s." = "not significant".
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These results suggest that CD103$^+$ DCs and retinoic acid may not contribute to inducing Foxp3$^+$T-regs in CLNs. It is possible that the classical CD8$^-$ DC subset in CLNs may participate in the induction of Foxp3$^+$T-regs. Further studies are required.

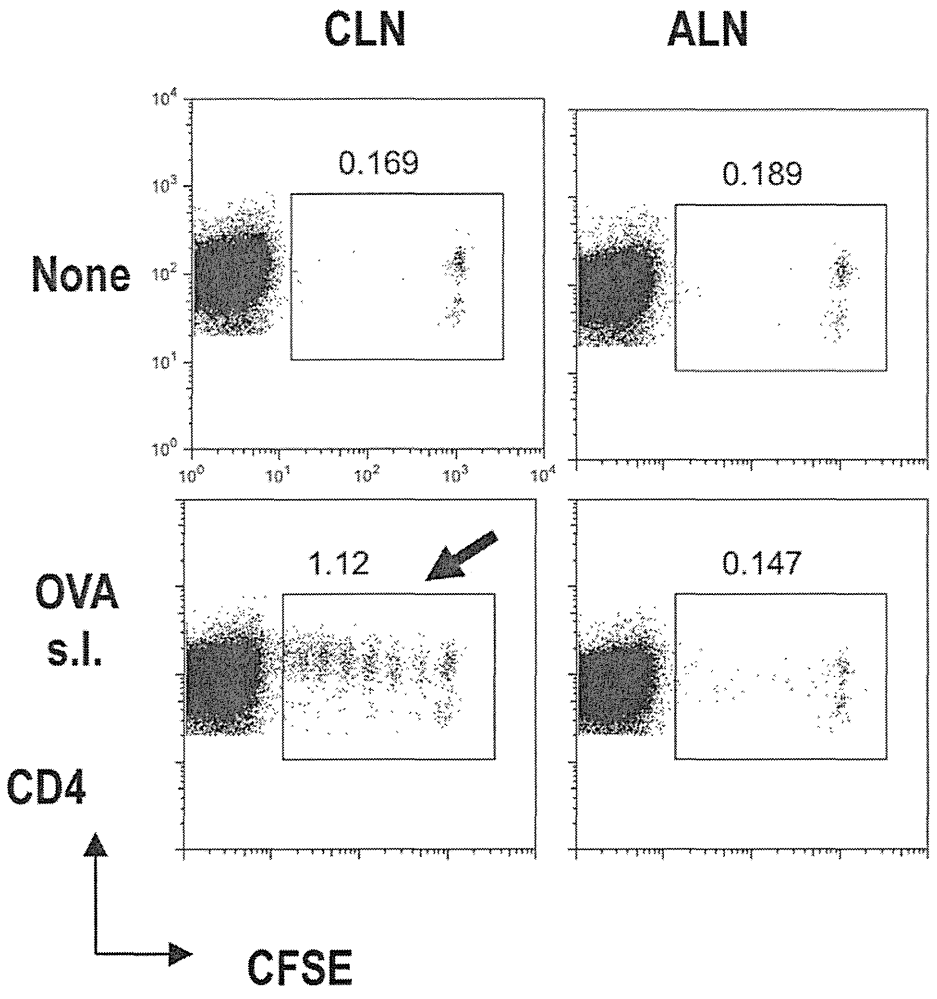

Figure 3. Cervical lymph nodes are draining lymph nodes from the oral cavity. CFSE-labeled OTII CD4$^+$T cells were adoptively transferred into B6 mice on day −1. On day 0, 500 μg of OVA was administered sublingually (s.l.). CLN or ALN was analyzed for CFSE dilution at day 3. One of two similar experiments is shown for the FACS plots. Plots were gated on CD4$^+$T cells.
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Discussion

The oral cavity is exposed to many antigens and commensal organisms every day [23,24,25,26]. The oral cavity is frequently associated with systemic immunological disorders, such as graft versus host diseases, Stevens-Johnson syndrome and Behçet diseases. However, it is unknown how tolerance in the oral cavity is maintained. Here, we showed that the balance between Th17 and Foxp3$^+$T-regs may play a role in maintaining tolerance in the oral cavity. We found that the frequencies of Foxp3$^+$T-regs and ROR-γt $^+$CD4$^+$T cells were increased in oral-cavity-draining CLNs, compared with skin-draining LNs and mesenteric LNs. DCs from oral-cavity-draining CLNs have the capacity to induce Foxp3$^+$ T-regs *in vitro* on antigen stimulation, as much as DCs from mesenteric LNs do. These data suggest that the induced Foxp3$^+$T-regs in oral-cavity-draining CLNs may be important in maintaining mucosal tolerance in response to microbes and food antigens in the oral cavity.

Although some TLR signaling is involved in inducing Foxp3$^+$ T-regs [20,27,28,41,42], the high frequency of Foxp3$^+$ T-regs in CLNs was not dependent on Myd88/TICAM1 (Fig. 1). Thus, TLR signaling is apparently not involved in the induction of Foxp3$^+$T-regs in the oral cavity. However, we cannot exclude the possibility that some specific microbes may be involved in the inducing Foxp3$^+$T-regs in the oral cavity in a Myd88/TICAM1 independent manner. For example, Atarashi et al recently showed that *Clostridum* induces Foxp3$^+$T-regs in the colon in a Myd88-independent manner [19]. It might be interesting to investigate if there are specific microbes that contribute to the induction of Foxp3$^+$T-regs in the oral cavity, especially as the bacterial community varies between the oral cavity and gut [26].

Our results showed that the high frequency of ROR-γt$^+$CD4$^+$T cells was dependent on Myd88/TICAM1 in the oral-cavity-draining CLNs (Fig.2). This is consistent with recent findings in skin [43]. Th17 cells in skin are reduced in Myd88/TICAM1 knockout mice and skin-resident commensal bacteria induce Th17 cells in a Myd88- and IL-1 receptor-dependent manner [43]. It is also known that some microbes induce Th17 using TLR signals; for example, Th17 cells induced by *Chlamydia* infection are reduced in Myd88 KO mice [44]. Thus, it is possible that TLR signals through some oral microbes are responsible for the increase of ROR-γt $^+$CD4$^+$T cells in oral-cavity-draining CLNs. Interestingly, the intestine may use a different mechanism from the oral cavity and skin to maintain Th17 cells, because Th17 cells are not reduced in the intestine in Myd88/TICAM1(TRIF)-knock out mice [45,46].

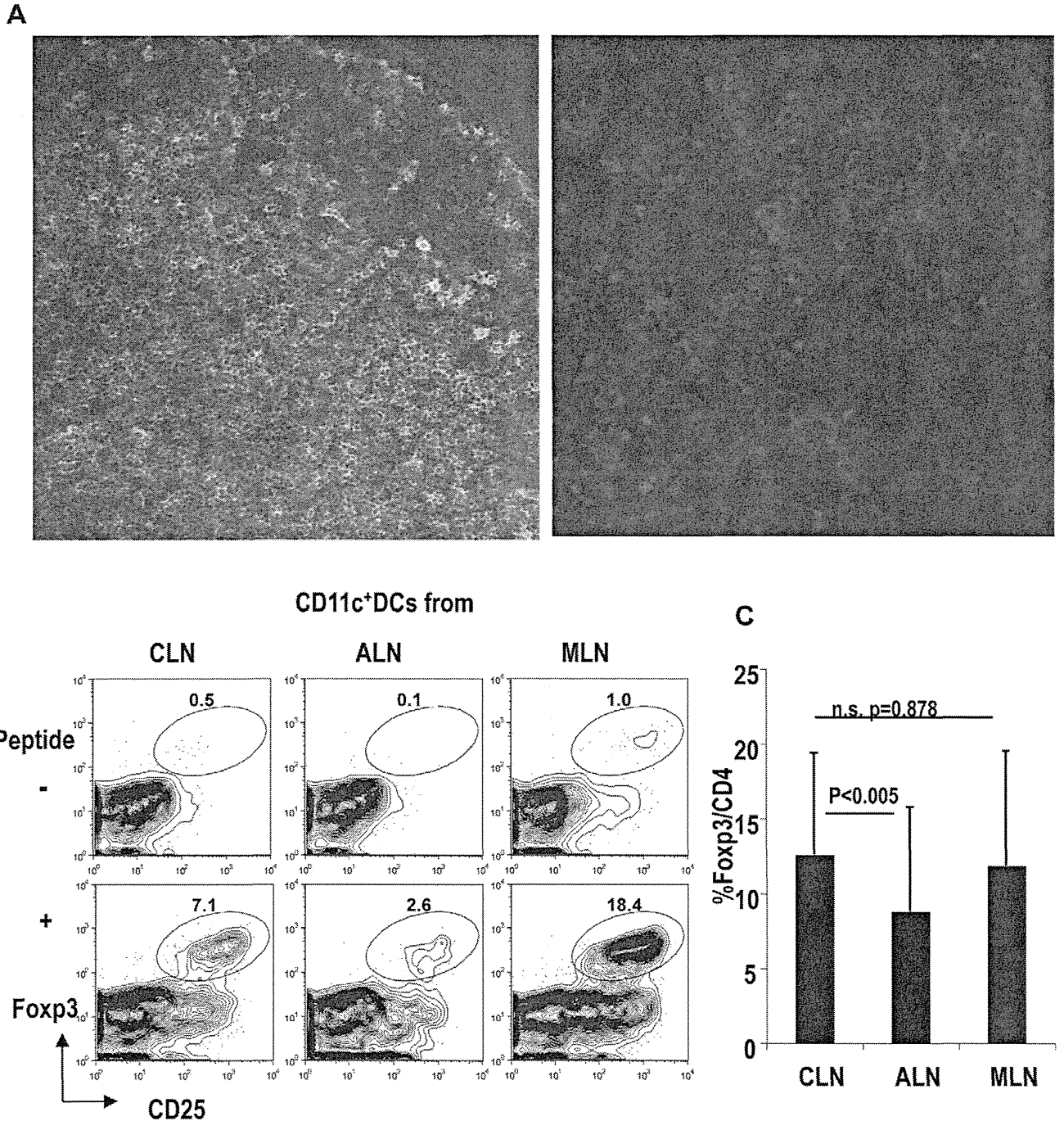

Figure 4. Dendritic cells from oral-cavity-draining cervical lymph nodes induce Foxp3$^+$ T-regs. (A) CLNs were stained with Foxp3 (red), CD4 (green) and CD11c (blue). Representative of three similar separate experiments. (B) OT II CD4$^+$ T cells (5×10^4) were cultured with dendritic cells (DCs) from CLN, ALN, or MLN (5×10^4) with or without OVA peptide. After 5 days, cells were stained with Foxp3, CD25 and CD4. The plots were gated on CD4$^+$ T cells. Representative of four separate experiments. (C) As in (A), but the graphic shows a summary of four separate experiments. P value provided is by paired t-test. "n.s." = "not significant".
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Furthermore, we found that DCs from oral-cavity-draining CLNs induce Foxp3$^+$T-regs in the presence of antigen, as do DCs from MLNs (Fig.4B). It has been reported that cutaneous CD103$^+$DCs induce Foxp3$^+$T-regs using RALDH2, as intestinal CD103$^+$DCs do [47]. Here, we would like to propose that DCs in the oral cavity use a different mechanism(s) to induce Foxp3$^+$Tregs from DCs in the intestine. First, CLNs have few CD103$^+$ DCs compared with MLNs (Fig. 5B). Second, DCs from CLNs do not express RALDH2 at the mRNA level (Fig. 5A). We have not yet found any specific DC subset in the oral-cavity-draining CLNs. However, CD8$^-$ classical DCs are increased in CLNs versus ALNs. Our previous report showed that CD8$^+$DEC205$^+$DCs induce Foxp3$^+$T-regs from Foxp3$^-$ cells and that CD8$^-$33D1$^+$DCs expand natural occurring Foxp3$^+$T-regs