claimed that the frequency of TRIMCyp in Indonesian CMs was higher than that of Indochina and Mauritian populations. However, all these analyses were performed with captive monkeys in breeding and rearing facilities. Therefore, these results may not reflect the natural gene frequencies. For instance, a small number of animals of a certain genotype introduced into facilities may affect the frequency of TRIMCyp via the founder effect. Furthermore, breeding policies may lead to a deviation of specific genotype. Hence, in order to understand the prevalence of TRIMCyp in CM precisely, it is necessary to analyze the frequency of TRIMCyp in wild CM. Therefore, in the present study, we sought to determine the geographic and genetic diversity of the *TRIM5* gene in wild-caught CM.

MATERIALS AND METHODS

SAMPLE COLLECTION

Blood samples from the wild-caught CMs, which had been cryopreserved for veterinary and microbiological examination as quarantine, were used in this study. These animals had been imported in the 1970's from the Philippines, Malaysia, and Indonesia to Japan as the founders of a breeding colony. These animals were directly sent to Japan without breeding in these countries.

DETERMINATION OF *TRIM5* GENOTYPE

The genotyping of *TRIM5* gene was performed as described previously with slight modifications (Saito et al., 2012). Briefly, the genomic DNA was extracted from frozen blood samples of 88 CMs with a QIAamp DNA Blood Mini kit (*Qiagen, Tokyo, Japan*). The genomic DNA was amplified by PCR using Ex Taq HS (TaKaRa, *Otsu, Japan*) with TC forward (5′-TGA CTC TGT GCT CAC CAA GCT CTT G-3′) and TC reverse (5′-ACC CTA CTA TGC AAT AAA ACA TTA G-3′) primers as described by Wilson et al. (2008). After amplification, PCR products were visualized on a 1% agarose gel stained with ethidium bromide.

SEQUENCING OF THE CypA DOMAIN OF TRIMCyp

Amplified products of the CypA domain from 44 TRIMCyp homozygotes and 21 TRIMCyp/TRIM5α heterozygotes were purified using Wizard SV Gel and PCR Clean-Up System (*Promega, Tokyo, Japan*) and then subjected to direct sequencing using primer pairs of MfasCypA_F (5′-CAA CCC TAC CGT GTT CTT CG-3′) and MfasCypA_R (5′-TCG AGT TGT CCA CAG TCA GC-3′). Sequencing products were analyzed on a 3130xl Genetic Analyzer (*Applied Biosystems, Tokyo, Japan*).

RESULTS

HIGHER FREQUENCY OF TRIMCyp IN A WILD PHILIPPINE POPULATION AS COMPARED TO INDONESIAN AND MALAYSIAN POPULATIONS

We first analyzed the frequency of TRIMCyp in these wild-caught animals. The PCR-based assay performed here was designed to differentiate between the presence and absence of the CypA insertion (**Figure 1A**). The electrophoretic pattern of PCR products is shown in **Figure 1B**. The upper bands indicate TRIMCyp, while the lower bands indicate TRIM5α. A heterozygote is expected to possess both bands. As summarized in **Table 1**, we found that the 35 of the 49 Philippine CMs were homozygous for TRIMCyp, 11 were heterozygous, and 3 were homozygous for TRIM5α. In the case of Malaysian CM, 11 of the 29 animals were homozygous for TRIMCyp, 8 were heterozygous, and 10 were homozygous for TRIM5α. Finally, in the case of Indonesian CMs, none of the 10 animals were homozygous for TRIMCyp, 3 were heterozygous, and 7 were homozygous for TRIM5α. The calculated frequency of TRIMCyp in these populations was 82.7%, 48.3%, and 15.0%, respectively. Statistical analysis revealed that the frequency of TRIMCyp in the Philippine population was significantly higher than that in the Indonesian and Malaysian populations.

DIFFERENCE IN THE HAPLOTYPE FREQUENCY OF TRIMCyp BETWEEN WILD AND CAPTIVE PHILIPPINE POPULATIONS

Others and we have recently demonstrated the presence of several haplotypes in TRIMCyp of captive CM (Dietrich et al., 2011; Saito et al., 2012). Specifically, the major haplotype in which amino acid residues at positions 369 (Cyp66) and 446 (Cyp143) are aspartic acid (D) and lysine (K) is abundant in captive-CM TRIMCyp alleles [denoted as TRIMCyp-major (DK)]. In addition, the minor haplotype encoding asparagine (N) and glutamic acid (E) at positions 369 (Cyp66) and 446 (Cyp143) is also present [denoted as TRIMCyp-minor (NE)].

In this study, we further investigated the haplotypes of TRIMCyp in the wild-caught CM and compared the frequency of each haplotype in these animals with those reared in captivity. We found that although both haplotypes were present in wild-caught CM, the frequency of TRIMCyp-minor (NE) in wild Filipino CM was much less than that in captive Filipino CM (1.2% versus 14.3% of TRIMCyp; $p < 0.01$) (**Table 2**). By contrast, the frequency of TRIMCyp-minor (NE) in wild Malaysian CM was comparable to that in captive Malaysian CM (10.7% versus 11.1% of TRIMCyp; $p > 0.05$). In the case of wild Indonesian CM, all animals analyzed here possess TRIMCyp-major (DK), although the size of samples was too small to determine whether this was significant. Thus, the precise frequency of each haplotype in wild Indonesian CM is unclear.

DISCUSSION

In the present study, we analyzed the incidence of TRIMCyp in wild-caught animals and found that its frequency was comparable to that in captive animals (**Table 1**). Although blood samples from other regions were unavailable, it is reasonable to assume that the equivalence in the frequency of TRIMCyp between captive and wild-caught CM in other regions may have a similar tendency. Interestingly, we also found that the frequency of the TRIMCyp-minor (NE) haplotype in wild CM was lower than that in captive CM in the case of the Filipino population, but not in the case of the Malaysian population (**Table 2**). Although the reason for this discrepancy remains to be elucidated, we speculate that it might be hazardous for wild Filipino CM to possess TRIMCyp-minor (NE), as it may render them susceptible to TRIMCyp-minor (NE)-resistant pathogens present in the Philippines, but not in Malaysia. Based on this hypothesis, wild Filipino CM might be forced to expand TRIMCyp-major (DK) in order to counteract invasions from such pathogens. Conversely, weaker attacks, if any, from these pathogens in the breeding and rearing facilities might allow captive Filipino CM to expand TRIMCyp-minor (NE)

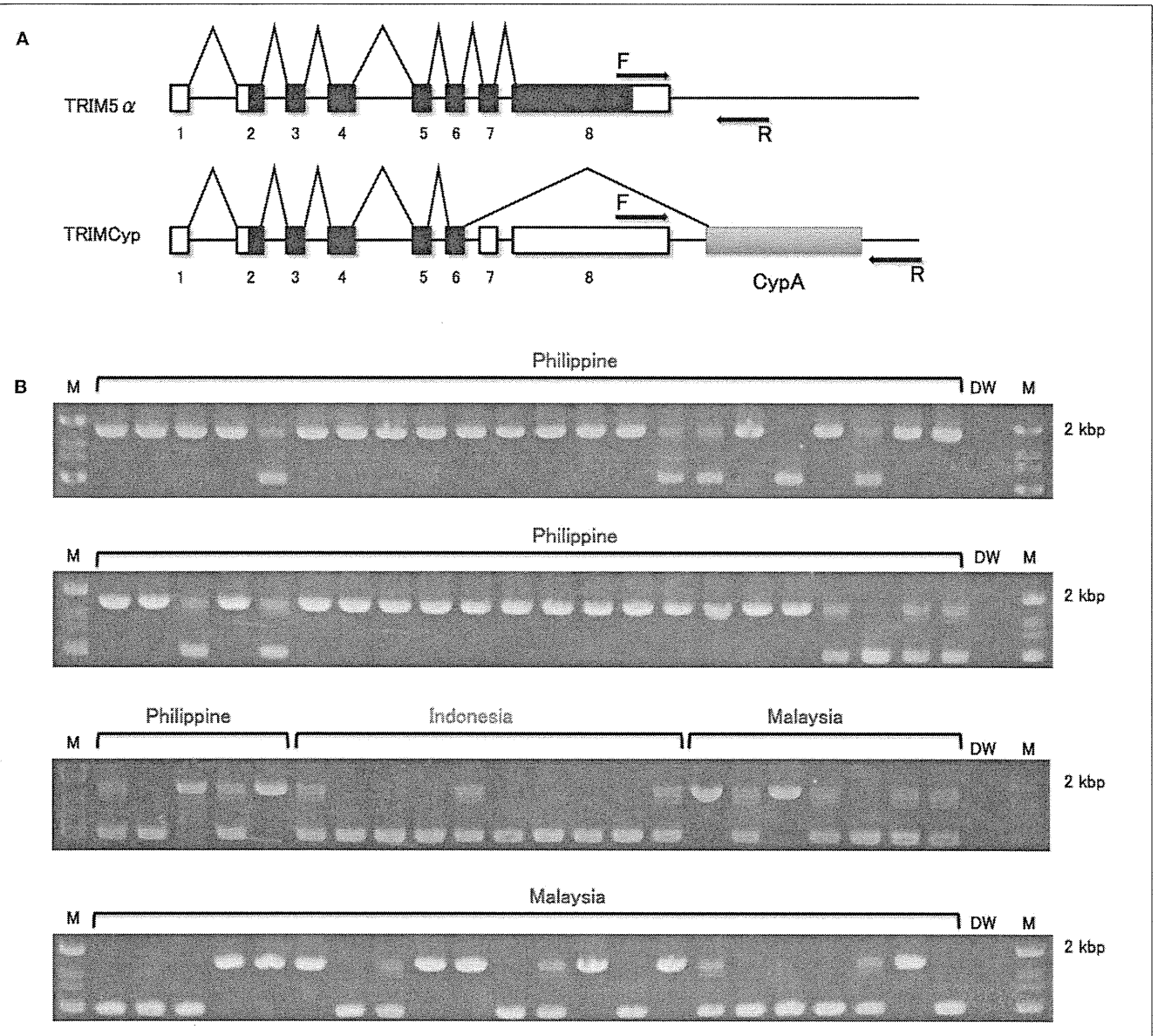

FIGURE 1 | Determination of *CypA* insertion. (A) Diagram indicating splicing of TRIM5α or TRIMCyp. Noncoding and coding exons and CypA sequences are shown in white, black, and shaded white, respectively. F and R denote forward and reverse primers used in this study, respectively. **(B)** The genomic DNA was extracted from whole blood. To test for CypA insertion, the 3′ region of the *TRIM5* gene was amplified by PCR with primers spanning the 3′ UTR and the putative *CypA* insertion. M and DW denote DNA molecular weight standard marker and water control, respectively.

haplotype in their population. Although it might also be hypothesized that the difference in the frequency of these haplotypes between wild-caught and captive animals was a consequence of the founder effect, the fact that more than 100 animals were introduced from wild (wild-caught animals) to breeding and rearing facility (captive animals) by dividing into several times suggests that the difference in the frequency of TRIMCyp haplotype may not be due to founder effect.

Since it is assumed that Filipino CM originated from Indonesian CM stocks (Thierry and Abegg, 2002), the fact that Malaysian and Indonesian CMs also possess TRIMCyp-major (DK) implies that this haplotype arose earlier than the migration of Indonesian CM stocks to the Philippine islands. Probably, TRIMCyp-major (DK) appeared in the ancestor of these CMs for some reason. Since only CM but neither PM nor RM possess TRIMCyp (DK) as one of the TRIMCyp haplotypes, it is reasonable to speculate that some pathogen(s) exerted a strong selection pressure on CM during their evolution. After the appearance of TRIMCyp-major (DK), Malaysian CM continued to maintain TRIMCyp-minor (NE) at a frequency of approximately 10% of total TRIMCyp alleles, while Filipino CM might exclude this haplotype. Alternatively, since Filipino CMs are thought to have originated from a small group of Indonesian CMs (Blancher et al., 2008), the limited prevalence of TRIMCyp-minor (NE) in wild

Table 1 | Frequency of TRIMCyp alleles in wild Philippine, Malaysian, and Indonesian populations.

Country	Origin of sample	#animals	Genotype (# animals)			Allele frequency		Citation
			TRIM5α homozygote	heterozygote	TRIMCyp homozygote	% TRIM5α	% TRIMCyp	
Philippines	Wild-caught	49	3	11	35	17.3	82.7	This study
Philippines	Captive	46	1	10	35	13.0	87.0	Saito et al., 2012
Philippines	Captive	4	0	0	4	0	100	Dietrich et al., 2011
Malaysia	Wild-caught	29	11	8	10	51.7	48.3	This study
Malaysia	Captive	47	11	26	10	51.1	48.9	Saito et al., 2012
Indonesia	Wild-caught	10	7	3	0	85.0	15.0	This study
Indonesia	Captive	33	13	17	3	65.2	34.8	Saito et al., 2012
Indonesia	Captive	18	3	10	5	44.4	55.6	Dietrich et al., 2011

Table 2 | Frequencies of DK and NE haplotypes in TRIMCyps of wild CM.

Country	Origin of sample	#animals	Genotype (# chromosomes)				Frequency		Citation
			TRIM5α/TRIMCyp heterozygote[a]		TRIMCyp homozygote[b]		% DK	% NE	
			DK	NE	DK	NE			
Philippines	Wild-caught	46	10	1	70	0	98.8	1.2	This study
Philippines	Captive	28	6	1	36	6	85.7	14.3	Saito et al., 2012
Malaysia	Wild-caught	18	7	1	18	2	89.3	10.7	This study
Malaysia	Captive	21	14	1	10	2	88.9	11.1	Saito et al., 2012
Indonesia	Wild-caught	3	3	0	0	0	100	0	This study
Indonesia	Captive	15	12	0	4	2	88.9	11.1	Saito et al., 2012

[a] *Haplotypes were determined by direct sequencing of the PCR products.*

[b] *Haplotypes were inferred by the Maximum-Likelihood estimation using the results of direct sequencing of the PCR products.*

Filipino CMs might be due to a founder effect. Unfortunately, we were unable to place a statistically meaningful value on the prevalence of the TRIMCyp-minor (NE) allele in wild Indonesian CM, since the sample size was too small. In the case of Malaysian CM TRIMCyp, the high frequency of the TRIMCyp-major (DK) allele suggests that it is preferable to posses this haplotype in their habitat. From this point of view, it will be of interest to consider why TRIMCyps of PMs and RMs are NE rather than DK type. In particular, the habitats of PM partially overlap with those of CM, except for the Java and Philippine islands (Thierry and Abegg, 2002). As Dietrich et al. proposed (Dietrich et al., 2010), it is likely that TRIMCyp evolved in the common ancestor of Asian macaques since TRIMCyp is present in both the silenus group, which includes PM, and the fascicularis group, which includes RM and CM. Furthermore, Ylinen et al. speculated that although the CypA sequence that has been retrotransposed into the macaque *TRIM5* locus is expected to be identical to the inherent CypA sequence, an arginine-to-histidine substitution at amino acid 69 may have occurred early in a common ancestor of Asian macaques. This may have been advantageous in that it helped to expand the spectrum of antiviral activity (Ylinen et al., 2010). This group further speculated that TRIMCyp (NE) arose in PMs and RMs independently; however, it is possible that TRIMCyp (NE) arose in the common ancestor of Asian macaques, since TRIMCyp (NE) is also present in CMs (**Table 2**). It is reasonable to imagine that the ancestors of PMs and RMs might fix TRIMCyp (NE) in order to protect themselves from invasion by TRIMCyp (NE)-sensitive pathogens. Specifically, the fact that PMs exclusively possess TRIMCyp (NE) instead of TRIM5α or TRIMCyp (DK) implies the importance of maintaining this *TRIM5* genotype in their habitat. Otherwise, the founder or bottleneck effect might affect the prevalence of TRIMCyp haplotypes in these macaque species. As an alternative hypothesis, TRIMCyp-minor (NE) in CM might be a vestige of an introgression between CMs and RMs with TRIMCyp (NE). In any case, future studies should analyze the prevalence of TRIMCyp in wild CMs by using samples from many regions to verify the correlation of genetic prevalence between wild and captive CMs.

More importantly, these two haplotypes in CM TRIMCyp are reported to show different antiviral activity (Ylinen et al., 2010; Dietrich et al., 2011; Saito et al., 2012). We and other groups reported that TRIMCyp-major (DK) suppresses the replication of HIV-1, but not that of HIV-2. Conversely, it was shown that TRIMCyp-minor (NE) suppresses the replication of HIV-2, but not that of HIV-1. Thus, these haplotypes of TRIMCyp present in CM are expected to show different antiviral activity in nature.

It will be of great interest to investigate the pathogens that acted as a selective pressure to alter the prevalence of TRIMCyp haplotypes.

Taken together, we analyzed the geographic and genetic characteristics of TRIMCyp in wild-caught CM for the first time and found (1) a higher frequency of TRIMCyp in the Philippine population as compared to those in other populations; (2) a similar tendency in the frequency of TRIMCyp between wild-caught and captive CM, and (3) a significant difference in the frequency of TRIMCyp-minor (NE) haplotype between captive and wild Filipino CM. These results provide important insights into the prevalence of CM TRIMCyp and increase our understanding of the evolution of antiretroviral host factors in Asian macaques.

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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

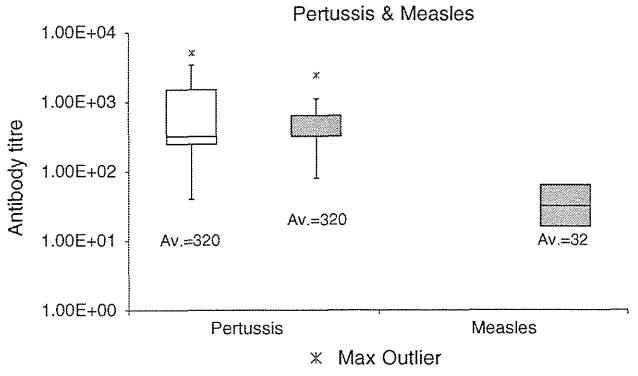

Fig. 1 Seroprevalence and titres of *B. pertussis* and MV antibodies, obtained by use of BAT and HI, respectively, for 14 chimpanzee serum samples. The range of antibody titre against *B. pertussis* was broad (40–5120×), and the average titre for the SB group (chimpanzees reared in KUPRI since birth) 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.)

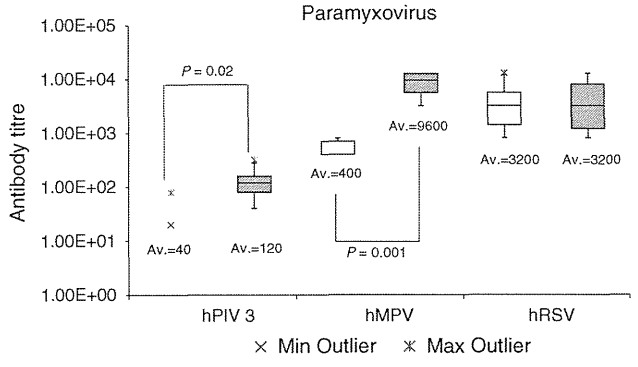

Fig. 2 Seroprevalence and titres of hPIV-3, hMPV, and RSV antibodies obtained by use of HI and ELISA (second two) for 14 chimpanzee serum samples. The titre against hPIV-3 for SB chimpanzees ranged from 20× to 80×, and that for the AB chimpanzees ranged from 80× to 160×. The average titre for the SB 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.)

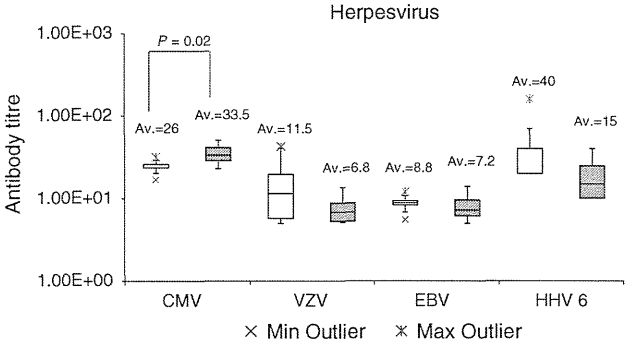

Fig. 3 Seroprevalence and titres of CMV, VZV, EBV, and HHV6 antibodies obtained by use of EIA for 14 chimpanzee serum samples. The antibody titre against CMV ranged from 17× to 32× for SB chimpanzees and from 26× to 51× for AB chimpanzees. The average titres in SB chimpanzees were lower than those in 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

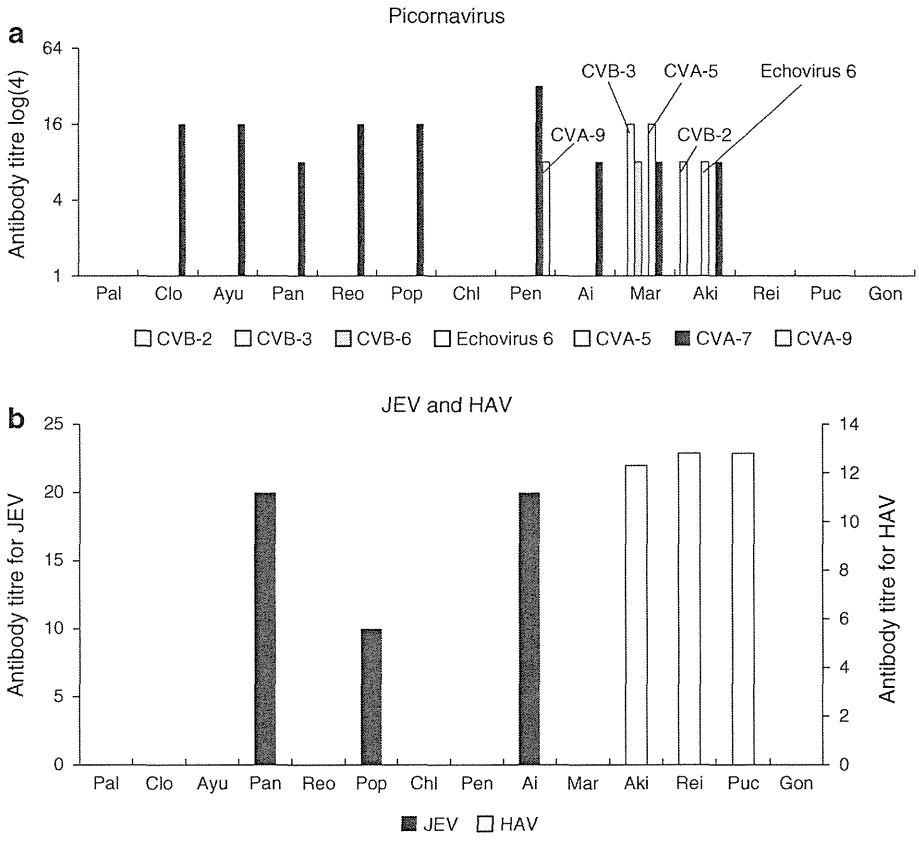

Fig. 4 **a** Seroprevalence of antibodies against picornaviruses, CVB-2, CVB-3, CVB-6, echovirus-3, echovirus-6, CVA-5, CVA-6, CVA-7, CVA-9, and PV-1 for each chimpanzee. The antibodies in serum were detected by use of NT. The AB female Mari had antibodies against four picornaviruses (CVB-3, CVB-6, CVA-5, 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

zoonotic and originate from wildlife via direct or indirect contact. For example, a case of Ebola virus infection resulted from contact with an infected wild chimpanzee (Morell 1994; Le Guenno et al. 1995; Taylor et al. 2001). Chimpanzee caretakers and researchers are at risk of exposure to unknown chimpanzee infectious pathogens. In addition to basic hygiene, keepers and researchers need appropriate vaccinations and should wear masks, gloves, and protective clothing during quarantine periods.

Disease prevention management for wild chimpanzees

Our study provides important information for hygiene management in wild chimpanzee conservation by adding information about possible human–chimpanzee zoonotic diseases. Infectious agents newly identified by their antibodies as agents possibly transmittable to chimpanzees (ADV-1, 2, 3, 5, 6, 19, CVA-5, 7, and JEV) or previously detected pathogens might cause the next outbreak in wild chimpanzees, not only in primate institutes, but also zoological gardens. Furthermore, we should consider agents that were not seropositive in the chimpanzees because the chimpanzees do not possess immunity against them, or at least not antibodies.

For chimpanzees at KUPRI, unlike some groups of wild chimpanzees, a previous infection of RSV or hMPV did not cause a severe respiratory disease. Captive chimpanzees might have more opportunity to be exposed to human pathogens compared with wild populations, but their environment and diet might not be as harsh as in the wild, which could alter the incidence and effects of the same pathogens in captive and wild populations. In addition, captive chimpanzees are at risk of new infectious diseases to which they have never been exposed and against which they have not established immunity. Consequently, monitoring results should be analysed carefully at each research institute or zoo, and care should be taken with all pathogens, including those to which chimpanzees are highly susceptible and those for which only a few or no chimpanzees were seropositive. Ultimately, without direct surveillance of wild populations, we cannot elucidate the prevalence of human-borne infectious diseases in the wild, but our data may still be used as a model. Our data suggest that a means of detecting antibodies in faeces should be developed to facilitate further studies in the wild.

In recent years, a vaccination program to protect wild chimpanzees against Ebola virus has been planned and is in preparation (Walsh 2009) after vaccine challenge against polio virus in wild chimpanzees at Gombe and against measles virus in gorillas at Virunga (Whittier et al. 2001). Furthermore, its effectiveness has been reported in one case of intervention (Robbins et al. 2011). This study shows that groups of chimpanzees under captive conditions produced specific antibodies against human diseases and that the chimpanzees were probably protected by their acquired immunity. Therefore, pre-immunity probably effectively protects wild chimpanzees from the human infectious diseases that tourists or researchers unknowingly transmit. However, the vaccination campaign needs careful consideration in terms of negative side effects for wild chimpanzees and nature. Recently, Ryan and Walsh (2011) reviewed the positives and negatives of the intervention and described the available vaccines against human pathogens.

Conclusion

We conducted serological surveillance for human-borne zoonoses in chimpanzees, and revealed the possibility of disease transmission between humans and chimpanzees. To reduce the chance of transmitting disease to captive chimpanzees in research institutes and zoos and to prevent disease transmission among researchers, animal caretakers, and chimpanzees, it is necessary to evaluate the risk of disease transmission. The serology of captive chimpanzees provides important information for hygiene management in ecotourism involving wild chimpanzees and other great apes.

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EXPERT OPINION

Targeting TLR3 with no RIG-I/MDA5 activation is effective in immunotherapy for cancer

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Introduction: Many forms of RNA duplexes with agonistic activity for pattern-recognition receptors have been reported, some of which are candidates for adjuvant immunotherapy for cancer. These RNA duplexes induce cytokines, interferons (IFNs) and cellular effectors mainly via two distinct pathways, TLR3/TICAM-1 and MDA5/MAVS.
Areas covered: We determined which pathway of innate immunity predominantly participates in evoking tumor immunity in response to RNA adjuvants.
Expert opinion: In knockout (KO) mouse studies, robust cytokine or IFN production is dependent on systemic activation of the MAVS pathway, whereas maturation of dendritic cells (DCs) to drive cellular effectors (i.e., NK and CTL) depends on the TICAM-1 pathway in DCs. MAVS activation often causes endotoxin-like cytokinemia, while the TICAM-1 activation does not. Unlike the TLR/MyD88 pathway, this TICAM-1 pathway barely accelerates tumor progression. Although the therapeutic effect in human patients of MAVS-activating or TICAM-1-activating RNA duplexes remains undetermined, the design of a TLR3 agonist with optimized toxicity and dose is an important goal for human immunotherapy. Here we summarize current knowledge on available RNA duplex formulations, and offer a possible approach to developing a promising RNA duplex for clinical tests.

Keywords: double-stranded RNA, MAVS (IPS-1, Cardif, VISA), immunotherapy, TICAM-1 (TRIF), TLR3

Expert Opin. Ther. Targets [Early Online]

1. Introduction

Toll-like receptor 3 (TLR3) was first identified in 2001 as a membrane-associated dsRNA sensor in TLR3-/- mice in which polyI:C-mediated NF-κB activation was severely hampered [1]. While it was suggested that signaling via TLR3, like TLR4, induced type I IFN [2], no conclusive data as to how this was achieved by dsRNA recognition were offered in that report [1]. We have established a mAb, TLR3.7, against human TLR3, that blocks dsRNA (polyI:C)-mediated type I IFN production in the human fibroblast line MRC5, and that demonstrates TLR3 localization on the cell surface membrane of these fibroblasts [3]. Moreover, immunoprecipitation analysis demonstrated that polyI:C-stimulated TLR3 formed a molecular complex with cytoplasmic proteins [3]. Ultimately, TICAM-1 (TRIF) was identified as the TLR3 adaptor [4]. By demonstrating that TLR3 recognizes dsRNA on the cell membrane and delivers an intracellular signal for the induction of type I IFN, these reports collectively link TLR3 to the type I IFN production pathways in fibroblasts in both human and mouse (**Figure 1A**).

RIG-I and MDA5 were soon discovered as cytoplasmic sensors for dsRNA that induced type I IFN [5,6]. It is reasonable that virus dsRNA replicating in cytoplasm is recognized by RIG-I/MDA5 of infected cells. What is the role of the

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Article highlights.

- dsRNA or polyI:C induces cytokines/IFNs through recognition by RNA sensors, TLR3 or RIG-I/MDA5.
- The cytoplasmic sensors RIG-I/MDA5 cause systemic cytokinemia in response to *in vivo* administration of polyI:C while the endosomal dsRNA sensor TLR3 does not.
- The TLR3 pathway resides in myeloid cells and mainly involved in DC-driven effector cells induction.
- TLR3-specific dsRNA stimulation with no involvement of RIG-I/MDA5 generates antitumor adjuvant activity without robust cytokine induction.
- TLR/MyD88 is a main pathway for NF-κB activation, which harbors protumor activity: yet, the TLR3 pathway does not involve MyD88 activation.
- Although polyI:C is too toxic to culminate driving cellular immunity against cancer, developing nontoxic antitumor adjuvants from derivatives of dsRNA would be feasible by redesigning dsRNA.

This box summarizes key points contained in the article.

TLR3 pathway for IFN induction in viral infection should have been reconsidered. PolyI:C has been historically used as an IFN inducer, and has been shown to engage both TLR3 and MDA5 [7,8]. Gene disruption studies in mice suggest that serum IFN levels in polyI:C-injected mice are increased primarily by the RIG-I/MDA5 pathway [7]. TLR3 is involved in local secretion of type I IFN limitedly in nearby tissues and organs (**Figure 1B**). Current understanding of this issue is that the fundamental function of TLR3 is not to induce robust IFNs/cytokines to alarm infection over whole body but to evoke cellular immunity, as mentioned below.

An alternative function of the TLR3 pathway has been clarified through studies of dsRNA (polyI:C)-stimulated or virus-infected tumor cells (**Figure 1A**). Cell death or growth is promoted in response to dsRNA, since TLR3 links to the RIP1 pathway to induce NF-κB activation and RIP1/RIP3-mediated cytolysis in tumor cells [9,10]. In addition, tumor-associated macrophages (Mf) (TAM) switches from a tumor-supporting to a tumoricidal phenotype in response to dsRNA [11]. Hence, the physiological role of the TLR3 pathway in tumor cells appears to provide dead cell-derived tumor Ag to DC and promote tumor immunity. Ultimately, these results suggest that if Ag and TLR3 agonist are provided for DC maturation, tumor cells expressing the Ag will be targeted by effector cells induced via the DC-derived immune response (**Figure 2**). Here, we focus on the role of dsRNA in evoking cellular immunity for cancer.

2. TLR3: distribution and localization

The gene encoding TLR3 is evolutionarily conserved across humans, mice, chickens and teleost fish [12-14] and TLR3 in all these species induces IFN production in response to polyI:C [13], implicating TLR3 as a potential viral dsRNA sensor in vertebrates [14]. However, viruses usually replicate in cytoplasm where no TLR3 is distributed. Furthermore, cell types expressing TLR3 are limited, at least in humans and mice, therefore TLR3 is unlikely to systemically protect tissues or organs from virus infection.

A human mAb against TLR3, TLR3.7, blocks dsRNA (polyI:C)-mediated type I IFN production in the human fibroblast lines where TLR3 localized on the cell surface membrane [4]. In contrast, the TLR3.7 mAb does not block the production of IFN by TLR3 in human monocyte-derived dendritic cells (MoDC) [15]. Electron or confocal microscopic analysis in these cells suggested that TLR3 is localized to endosomal compartment [15,16], which were later identified as early endosomes. Hence, human TLR3 is localized in a cell type-specific fashion, such that epithelial cells and fibroblasts, including MRC5 cells, express TLR3 on the cell surface, while in myeloid cells, TLR3 localizes to the endosome (**Figure 1A**). The surface-expressed type of TLR3 is positioned next to sample dsRNA in the extracellular milieu to transmit IFN-inducing signaling, whereas endosomal TLR3 engages phagocytosed dsRNA for signaling. Both types of endogenous TLR3 could be detected by TLR3.7 mAb by imaging analysis [15].

Since myeloid cells, including DC and Mf, take up external dsRNA into the endosome via phagocytosis [15-17], our current hypothesis is that endosomal TLR3 plays a role in sampling viral materials for augmenting antigen (Ag) presentation (**Figure 1B**). This model is gaining approval as the authentic adjuvant function of TLR3.

Human endosomal TLR3 is primarily expressed by myeloid cells, including dendritic cells (DCs) (**Table 1**) [18]. There are many subsets of DCs in humans, of which human CD141 $(BDCA3)^+$ DCs express high levels of TLR3 [19]. Human epithelial and endothelial cells express detectable amounts of TLR3 on their cell surfaces [3,20,21]. In contrast, tumor cells and malignantly transformed cells express TLR3 in endosomes rather than on their cell surfaces [18]. Tumor cells, therefore, unlike normal epithelial and endothelial cells, cannot directly sample environmental RNAs.

3. TLR3: structure and function

TLR3 is a type I membrane protein consisting of extracellular leucine-rich repeats and a cytoplasmic Toll-IL-1 receptor homology domain (TIR). The acidic environment in the endosomal lumen allows TLR3 to more tightly bind dsRNA than neutral cell-surface irrespective of the sequence. Forty to fifty base pairs of non-mismatched dsRNA interacts with homodimeric TLR3 almost exclusively with the sugar phosphate backbone rather than through the base moieties [22-24]. Intriguingly, the interaction of dsRNA with TLR3 does not trigger conformational changes, but rather facilitates homodimerization, which brings the intracellular TIRs of TLR3s in close proximity of each other [23]. TLR3 homodimerization recruits the adaptor TICAM-1 (TRIF) to the TIR domains [3],

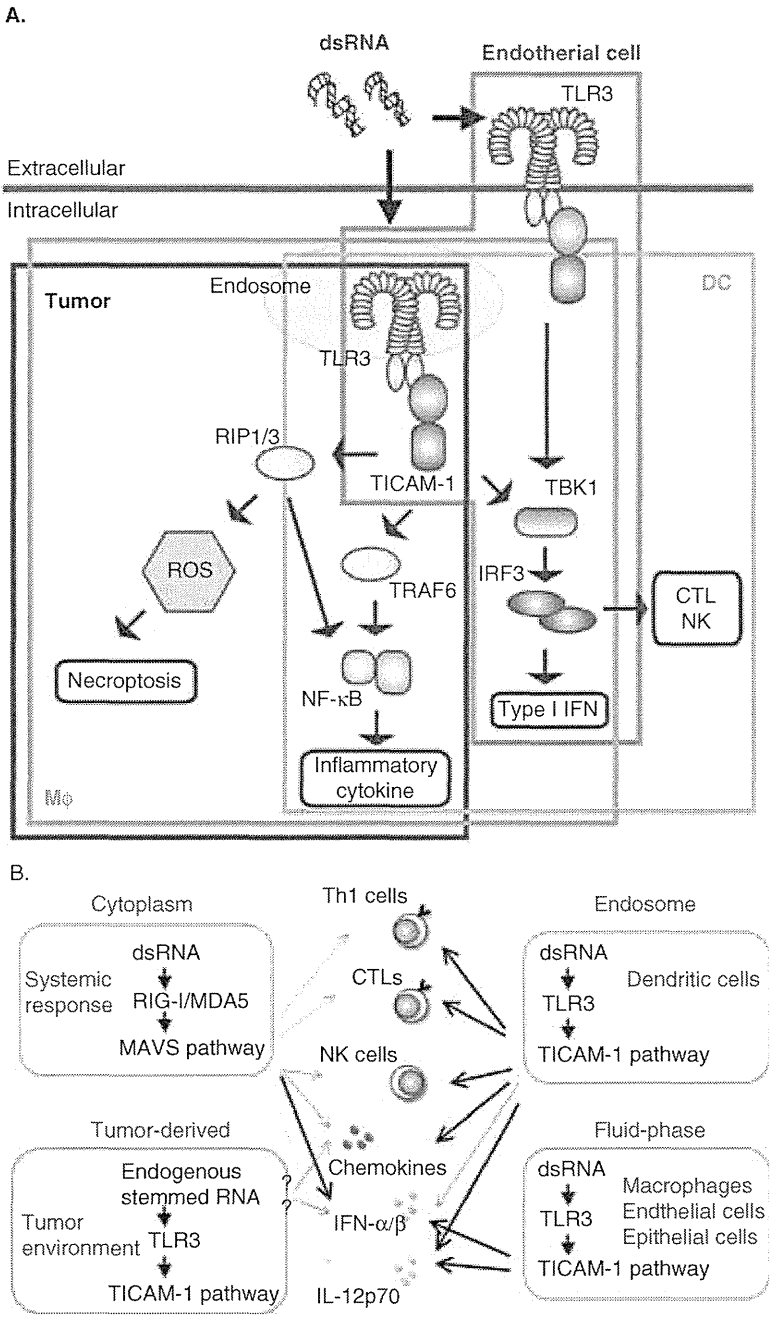

Figure 1. A. Cell type-specific activation of the TLR3/TICAM-1 pathway. Endothelial cells, epithelial cells and some macrophage subsets of human and mouse express TLR3 on the surface of the membrane and extracellularly sample naked double-stranded or stem/bulged RNA (red box). Tumor cells usually express TLR3 in the endosome and some cells activate the RIP1/3 pathway in response to dsRNA (black box). Most myeloid cells express TLR3 in the early endosome and take up debris-encapsulated dsRNA (blue and brown boxes). Cell death is induced in some tumor cells through the RIP1/3 pathway, which causes liberation of RNA-containing debris. Macrophages have unique properties of RIP1/3 and release DAMP. See the text for the functional properties of dendritic cell TLR3. **B.** A variety of output induced by dsRNA. Viruses produce dsRNA in the cytoplasm of infected cells during replication, and the cytoplasmic dsRNA is sensed by RNA sensors, RIG-I/MDA5 (left top panel). The cytoplasmic sensors contribute to production of robust type I IFN, leading to systemic cytokinemia, while they only weakly trigger other effectors without participation of the IFNAR pathway. On the other hand, dsRNA, either naked or encapsulated, can be incorporated into the endosomes of dendritic cells to induce cellular and soluble effectors (right top). Roles of surface-expressed TLR3 and endogenous stemmed RNA in this context still remain poorly characterized (bottom panels).

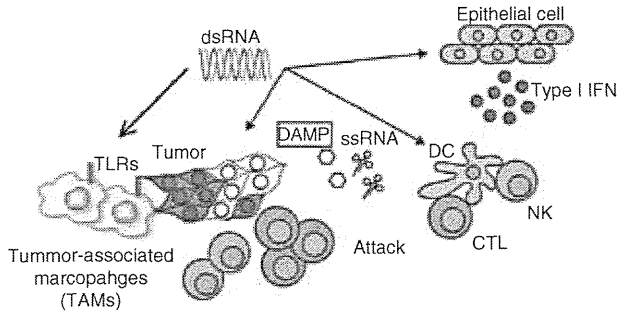

Figure 2. dsRNA-mediated inflammation modulates tumor microenvironment. A dsRNA or stemmed ssRNA affects tumor environment by acting on RNA sensors in epithelial cells, dendritic cells, tumor-infiltrating macrophages and tumor cells. Immune cells infiltrating the tumor mass may cause necroptosis of tumor cells. Tumor cells undergoing necrosis liberate DAMPs and debris containing Ag and nucleic acids with modified structural signatures of stem and bulge. These signatures can activate endosomal TLR3 in dendritic cells to promote the inflammatory response. Tumors in some cases benefit from the inflammatory response and in other cases regress in response to inflammation, and the mechanism determining this switch remains to be clarified.

which in turn recruits kinases TBK1 and IKK to the N-terminal domain of TICAM-1 [25]. The TICAM-1 complex thereafter dissociates from TLR3 and forms cytoplasmic speckles which are distinct from endosomes [26]. The kinase substrates IRF-3 and NF-κB are activated within the speckles, suggesting that the latter contain kinases and their substrates along with TICAM-1 [26,27]. After phosphorylation, IRF-3 migrates from the speckled region to the nucleus and acts as a transcription factor to induce type I IFN [27]. Without activation of the MAVS pathway, production of type I IFN is therefore a primary endpoint of dsRNA-mediated TLR3 activation in most cell types.

Notably, in some tumor cells and macrophages, ligand-stimulated TLR3 can facilitate RIP1 activation via the C-terminal TICAM-1 pathway, resulting in apoptosis or necroptosis (Figure 2) [28,29]. Furthermore, the TICAM-1 pathway triggers a chemokine/cytokine cascade via NF-κB activation that facilitates tumor progression in concert with the tumor microenvironment [30,31]. On the other hand, antigen-presenting cells (DC and Mf) express high amounts of TLR3, together with MHC and co-stimulatory molecules, and interact positively or negatively with lymphocytes. The primary function of myeloid cell TLR3 is to drive activation of NK cells by up-regulating surface-expressed NK-activating ligands (cell cell contact-mediated activation) [32] or induction of IL-12 and IL-15 (cytokine-mediated activation) [33]. Another function of myeloid cell TLR3 is to induce cross-presentation in DC and cross-prime antitumor CD8 T lymphocytes (CTL) [34]. Together, these functions of TLR3 are crucial for induction of antitumor cellular immunity (Figure 2).

4. Therapeutic TLR3 agonists

Three synthetic dsRNAs which harbor therapeutic potential as TLR3 agonists have been developed from polyriboinosinic polyribocytidylic acid (polyI:C), originally synthesized in the late 1960s to mimic viral responses [35]. IPH-3102, a dsRNA of unknown structure, appears to have a similar function to polyI:C [36]. In addition to these classical dsRNAs, single-stranded RNAs with nuclease-resistant stems are potential TLR3 agonists (Table 2) [37,38].

Ampligen (also known as rintatolimod) is a synthetic dsRNA consisting of polyI:C with one mismatch every 12 C, designating poly(I:C12U). It acts on DCs to induce tumoricidal effects, resulting in tumor growth retardation *in vivo* [39]. Ampligen, though has not been immunobiologically well-characterized as a specific agonist for TLR3, appears to operate in a multimodal fashion, encompassing the activation of natural killer (NK) cells, the proliferation of CTL, as well as direct cytostatic/cytotoxic effects on cancer cells. Hence, Ampligen targets a putative dsRNA sensor, most likely TLR3 across various cell types. Ampligen may not be a ligand for RIG-I/MDA5 [39].

Hiltonol (polyI:CLC) is a particular formulation of polyI:C that contains carboxymethylcellulose and poly-L-lysine as stabilizing agents [40]. Hiltonol is less vulnerable to degradation by serum nucleases or high temperature than Ampligen [41]. Hiltonol significantly elevates the levels of circulating IFNs in monkeys under conditions where an equivalent dose of Ampligen did not [40]. This has been attributed to the ability of Hiltonol to act as a ligand for both TLR3 and MDA5 [7,8] to exert potent immunostimulatory effects. Robust increases of serum type I IFN have been observed in multiple preclinical models, including mice and monkeys, although these are accompanied by an increased risk of side-effects [40-44]. Phase I/II clinical trials have been designed with patients having multifarious malignancies to assess the safety and efficacy of Hiltonol [45]. In general, these trials have concluded that low doses of Hiltonol are not particularly toxic in terms of cytokine induction and are moderately efficient in boosting antitumor immune responses. To date, ~ 20 Phase I/II clinical trials have suggested that Hiltonol is adaptable to immunotherapy for cancer in most cases, including brain tumor, malignant melanoma, breast cancer, and colorectal cancer [45].

Polyadenylic polyuridylic acid (polyA:U) is a synthetic dsRNA with immune-enhancing function *in vivo* on 1967 [46]. polyA:U was later found to stimulate TLR3 in Flt3-derived conventional DCs to generate IL-12 [47]. RIG-I/MDA5 recognizes only high amounts of polyA:U [46,48]. When combined with anticancer vaccines, poly(A:U) promotes Th1 responses that control tumor growth and are associated with the establishment of immunological memory [49]. On the other hand, poly(A:U) has protumor functions [50], because it stimulates TLR3 expressed on tumor cells to induce tumor cell proliferation [28,51-53]. There are currently no

Table 1. TLR repertoire in human dendritic cells.

Human TLRs	Monocyte	Freshly isolated mDC (BDCA1+)	Freshly isolated mDC (BDCA3+)	*In vitro*-differentiated cells pDC (BDCA4+)	*In vitro*-differentiated cells Monocyte-derived DCs
mAb					
TLR1 (1.136)	++	+	++	-	+
TLR2 (2.45)	++	++	+	-	++
TLR3 (3.7)	-	++	+++	-	++
TLR4	++	+	-	-	+
TLR6 (6.127)	++	+	++	-	+
TLR7		-	-	+	-
TLR8	+	+	+/-	-	+
TLR9	-	-	-	+	-

+: Protein or mRNA expression; Nucleotide-recognizing TLRs, TLR3, 7, 8 and 9 reside in intracellular compartments. MAVS pathway is ubiquitous while TICAM-1 pathway limitedly works in myeloid, epithelial and endothelial cells.

Table 2. Host response to RNAs and other DAMPs.

PAMP/DAMP receptors	
Microbial nucleic acids (PAMP)	
Cytosolic long dsRNA	MDA5
Cytosolic 5'-PPP-RNA	RIG-I
Endosomal > 140 bp dsRNA	TLR3
Fluid-phase dsRNA	TLR3
Encapsulated virus RNA	TLR3
Bulged or stemmed RNA	TLR3
Self nucleic acids (DAMP?)	
Modified self mRNA	TLR3
Some miRNA	TLR3
Denatured ssRNA	TLR3
Self molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14,TLR2/4
HSPs	CD14,TLR2/4
S100 proteins	RAGE

clinical trials evaluating its efficacy for either oncological or cancer-unrelated indications.

More exact information about clinical trials and oncological indications of these TLR3 agonists has been published by Galluzzi *et al.* [45].

5. Specific ligands for TLR3 without activation of the MAVS pathway

dsRNA and its synthetic analog, polyI:C, have long been known to be potent type I inducers [54] and modulators for cellular immunity [55]. Indeed, mouse and human versions of TLR3 recognize dsRNA and transduce TICAM-1 signals for NF-κB and IRF-3 activation. Type I IFN/cytokine and cellular immunity induced by the cytoplasmic dsRNA receptors RIG-I/MDA5 and NLRP3 have been identified more recently [45,48,56]. The type I IFN production induced by dsRNA is largely attributable to the MAVS pathway rather than the TICAM-1 pathway [7]. Furthermore, KO mice studies with *in vivo* administration of polyI:C have suggested that dsRNA contributes to NK cell activation and CTL proliferation even in MAVS-/- and IFNAR-/- mice, the initial response to which is independent of MAVS- or IFNAR-mediated type I IFN production [34,57]: i.e., tumor-specific NK cell and CTL can be induced without increasing serum type I IFN level in mice. Hence, the activation of cellular immunity occurs irrespective of the serum level of IFN in tumor-bearing host, although IRF-3 is essential for cellular immune activation [57].

Regarding the question as to through which target receptor, MDA5 or TLR3, polyI:C induces antitumor cellular immunity, evidence suggests that it is TLR3 [32,34]. Previous studies demonstrated that both MDA5 and TLR3 were equally associated with initiation of cellular immunity in response to i.p. injection of polyI:C + Alum in mice [58]. However, initial IRF-3 activation in myeloid cells is closely linked to NK cell activation but not robust IFN induction [57-59]. In syngenic mouse tumor-implant models, TLR3-TICAM-1 is more important than MDA5-MAVS for CD8α$^+$ DC to evoke antitumor cellular immunity below the protumorigenic polyI:C dose [32,34].

The optimal *in vivo* doses for induction by polyI:C of type I IFN, NK activation or CTL induction (cross-priming) are as yet unknown. It is likely that the optimal dose for inducing type I IFN, largely attributable to the RIG-I/MDA5 pathway, differs from those for facilitating NK activation and/or cross-priming induced by human DC [19], particularly a subset of a human counterpart of mouse CD8α$^+$ DC, namely CD141$^+$ DC. In both human and mouse DCs, 10 μg of polyI:C activates NK cells to kill tumor cells *in vitro* [60]. However, the dose discrepancy appears to cause different immune responses between human and mouse with respect to *in vivo* polyI:C administration. In the C57BL/6 mouse, 1 μg of i.p. injection of polyI:C per mouse is sufficient to induce type I IFN and IFN-inducible genes in spleen cells, but is insufficient

for causative NK/CTL activation to effect regression of implant tumors [32,34,61]. Likewise, in human volunteers, 1.6 mg of s.c. polyI:C has been shown to induce type I IFN in whole blood [62]. It is currently unknown whether this dose is sufficient for activation of NK/CTL in humans. The reported doses were restricted by the toxicity of polyI:CLC, and may have been sufficient for RIG-I/MDA5 activation followed by the feedback activation of the amplifiable IFNAR pathway in mice and humans, however, the dose has not been determined to optimize IRF-3-dependent NK/CTL activation by Ag-presenting DC.

NK cell activation requires > 10 µg of polyI:C per mouse, although the quality of polyI:C, including the average length of the duplex region, varies and critically affects the optimal dose for induction of cellular immunity [62,63]. For induction of cross-priming in mice, > 50 µg/20 g is actually required by i.v. or i.p injection. With regard to s.c. injection of dsRNA, several shots in different areas would be ideal for administration of the dsRNA reagent. If high-dose administration of dsRNA is also mandatory for induction of cellular immunity in humans, the dose 1.2 1.6 mg/volunteer would be a short dose in humans. If the dose limitation of polyI:C in human trials is mostly due to side-effects such as cytokinemia and protumor activity, the development of less toxic RNA reagents is indispensable for facilitating human immunotherapy.

There are several points of concern in the context of high-dose polyI:C therapy. Firstly, the likelihood of a cytokine storm is increased in healthy volunteers receiving > 1.6 mg polyI:C due to systemic activation of the MAVS pathway. Erythema, arthralgia and general malaise have been reported and may be secondary to elevated type I IFN [64]. The other point concerns the protumor activity of the TICAM-1-RIP1 pathway. Appropriate doses that neither activate the RIP1 pathway in tumor cells nor induce tumor growth should be chosen for antitumor therapy. Moreover, the duration of the effects is currently also unknown, although a single-shot dsRNA has only a short duration over ED_{50}. IFN-α/β levels may be kept high, being sustained by the IFNAR pathway [65]. TLR3 is endosomally expressed in myeloid DCs as well as in tumor cells. The types of cell involved in the immune response against high-dose polyI:C remain undetermined in humans, and the role of RNA-sensing receptors in other cell types therefore warrants further exploration.

6. Other RNA derivatives in tumor environment

Recent reports have suggested that single-stranded (ss)RNAs with incomplete stems serve as ligands for TLR3 [37,38]. ssRNA with a ~ 200 bp duplex may act as a TLR3 agonist without activation of MDA5 [24,63]. As mentioned above, the capacity of ssRNA to activate TICAM-1, but not MAVS, makes it suitable for antitumor immunotherapy, since it has only marginal cell-proliferative activity but fully activates NK cells and CTL in relevant tissues with induction of only low levels of IFN. The results are promising in the context of the synthesis of TLR3-specific ligands which do not participate in the MAVS pathway, and which can be applicable to humans without marked toxicity.

It has been believed that viral dsRNA is liberated from virus-infected cells through cell death events, apoptosis or necrosis. Oncogenic viruses may trigger death signals by activating cytoplasmic RNA sensors in transformed cells. An EB virus RNA with an incomplete stem, named EBER, also activates TLR3 [66] and, together with RIG-I, induces live signals and sometimes accelerates tumorigenesis in infected hosts [67]. Alternatively, transformed cells release live signals in the form of type I IFN and proinflammatory cytokines (IL-6, IL-12, TNF-α, etc.), which are liberated through IRF-3/7 and NF-κB activation as the output from living virus-infected cells. TLR, NLR and other cytosolic nucleic acids sensors are closely associated with RNA recognition (**Table 2**), and inflammation states are therefore fundamentally variable and individually modified by these factors [56,68]. It is notable that type III IFN (IFN-λ) is also generated via the TICAM-1 pathway in $CD8\alpha^+$ DC in mice and human $CD141^+$ DC in response to polyI:C [69]. Yet, in other cell types such as hepatocytes, the MAVS pathway participates in IFN-λ production [70].

In addition, tumor cells may liberate self mRNA, miRNA and other endogenous noncoding RNAs (**Table 2**), which become TLR3 ligands through conformational alterations which result in the formation of incomplete stems [71,72]. These self RNAs allow TLR3-positive host cells to induce IFNs and chemokines (**Figure 1B**). Once type I IFN and IFN-γ are robustly produced, the synergistic function of these IFNs results in the induction of IFN-stimulated genes (ISG) in the tumor and surrounding cells, including CXCL10 (IP-10) and CCL5 (RANTES) [72,73]. CXCR3 ligands (CXCL9, 10 and 11) are also expressed by these cells [73]. Since CXCR3 is mainly expressed on activated T and NK cells, these cytotoxic effectors converge upon the inflammatory nest, which includes the tumor microenvironment as well as secondary-affected organs. The tumor microenvironment is likely to be modified by these mediators in conjunction with cellular immune response.

These immunological aberrations may coincide with ecological environmental factors besides viral infection. Indeed, in mouse models, UV-B irradiation effects conformational changes in dermal mRNA to convert nonstimulatory mRNA to active TLR3 ligands by forming with incomplete stems [37], which then activate the TLR3 pathway, similar to virus-derived RNA [38], resulting in inflammatory sunburn. In any case, RNAs with bulged stems are functional as TLR3 agonists to induce IFN-α/β and possibly cellular immunity [37,38,66].

Whether endogenous TLR3 ligands are tumorigenic or tumoricidal remains to be determined. Necrosis-like cell death occurs in a cell type-specific manner as a result of death