JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

Hirai *et al.*, *Journal of General Virology* 2021;102:001590

DOI 10.1099/jgv.0.001590

MICROBIOLOGY SOCIETY

OPEN ACCESS

The infectivity of progeny adenovirus in the presence of neutralizing antibody

Takamasa Hirai[1,2], Anna Sato[1,3], Naoya Koizumi[1,*], Yoh Kurioka[1], Yui Suzuki[1], Junpei Kano[1], Makie Yamakawa[1], Tetsuya Nomura[1], Makiko Fujii[4], Fuminori Sakurai[5], Hiroyuki Mizuguchi[5,6,7], Yoshiteru Watanabe[8] and Naoki Utoguchi[1]

Abstract

Human adenoviruses (Ads), common pathogens that cause upper respiratory and gastrointestinal infections, are blocked by neutralizing antibodies (nAbs). However, Ads are not fully eliminated even in hosts with nAbs. In this study, we assessed the infectivity of progeny Ad serotype 5 (Ad5) in the presence of nAb. The infectivity of Ad5 was evaluated according to the expression of the Ad genome and reporter gene. Infection by wild-type Ad5 and Ad5 vector continued to increase until 3 days after infection even in the presence of nAb. We established an assay for determining the infection levels of progeny Ad5 using a sorting system with magnetic beads and observed little difference in progeny Ad5 counts in the presence and absence of nAb 1 day after infection. Moreover, progeny Ad5 in the presence of nAb more effectively infected coxsackievirus and adenovirus receptor (CAR)-positive cells than CAR-negative cells. We investigated the function of fiber proteins, which are the binding partners of CAR, during secondary infection, observing that fibre proteins spread from infected cells to adjacent cells in a CAR-dependent manner. In conclusion, this study revealed that progeny Ad5 could infect cells even in the presence of nAb, differing from the common features of the Ad5 infection cycle. Our findings may be useful for developing new therapeutic agents against Ad infection.

INTRODUCTION

Human adenovirus (Ad) is a non-enveloped and double-stranded DNA virus with icosahedral symmetry that is categorized into seven subgroups (A–G) based on its genomic and biological properties. Ads cause respiratory, ocular, and gastrointestinal tract infections in immunocompetent individuals, along with severe diseases that are often fatal in immunocompromised patients [1–3]. In the case of Ad serotype 5 (Ad5), the most studied serotype, infection is initiated via binding of the knob region located at the C-terminus of the trimeric fibre protein to the primary receptor, i.e. the coxsackievirus and adenovirus receptor (CAR) [4, 5]. Then, the Ads enter cells via endocytosis mediated by the interaction between the integrins on the cell surface and an arginine–glycine–aspartate motif located in the penton base, followed by escape to the cytosol [6, 7]. After the viral genomes are transported along the microtubules in a dynein-dependent manner, they are translocated to the nucleus [8, 9], where viral DNA replication is activated by the early region proteins, such as E1A and E1B [10, 11]. After the formation of progeny Ad particles in the nucleus, infected cells are lysed by E3-11.6 K protein, followed by particle spread to uninfected cells via cell-free transmission [12, 13].

Although viral infections are suppressed by innate and adaptive immunity, Ads have acquired various strategies to evade host immunity and prolong the duration of infection.

Received 02 October 2020; Accepted 29 March 2021; Published 12 April 2021

Author affiliations: [1]Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Tokyo, Japan; [2]Division of Cell-Based Therapeutic Products, National Institute of Health Sciences, Kanagawa, Japan; [3]Cosmetic Science Laboratory, School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University, Hyougo, Japan; [4]Laboratory of Physical Chemistry, School of Pharmacy, Nihon University, Chiba, Japan; [5]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [6]iPS Cell-Based Research Project on Hepatic Toxicity and Metabolism, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [7]Global Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan; [8]Department of Pharmacy, Tohoku Medical and Pharmaceutical University Hospital, Miyagi, Japan.

***Correspondence:** Naoya Koizumi, koizumi@ac.shoyaku.ac.jp

Keywords: adenovirus; neutralizing antibody; fibre protein; immune evasion.

Abbreviations: Ad5, adenovirus serotype 5; Ad5fiber, fiber protein of adenovirus serotype 5; CAR, coxsackievirus and adenovirus receptor; CTL, cytotoxic T lymphocyte; GFP, green fluorescent protein; IVIG, intravenous immunoglobulin; nAb, neutralizing antibody; TLR, toll-like receptor; VP, viral particle; WT, wild-type.

Five supplementary figures are available with the online version of this article.

One of the aspects of innate immunity is immune responses activated by host pattern recognition receptors such as Toll-like receptors (TLRs). It has been reported that Ad vectors stimulate TLR9 and elicit the production of type I interferon (IFN), a cytokine that induces an antiviral state in host cells, in dendritic cells by virus-associated RNAs [14, 15]. To counteract cytokine production, E1A proteins of Ads suppress the expression of IFN-stimulated genes via the N-terminal/CR1 region [16] and RuvBL1 [17]. In addition, Ad early region four open-reading frame 3, which is critical for efficient viral DNA replication [18, 19], inhibits IFN-induced responses to viral infection [20]. In adaptive immunity, antigen-specific cytotoxic T lymphocytes (CTLs) and antibodies are induced during viral infection. CTLs recognize and kill infected cells, which produce viral proteins and present antigen peptides to major histocompatibility complex class I molecules. Immunodominant Ad-specific CTLs target the hexon protein, which contains conserved sequences of amino acid residues, displaying cross-reactivity with different adenoviral species [21, 22]. In addition, neutralizing antibodies (nAbs) against Ads recognize viral particles including hexon and fibre proteins, which are their major components, outside cells [23, 24]. Anti-Ad5 nAb is carried by more than 40% of people globally and is known to constitute an obstacle to successful gene therapy with Ad vectors [25]. However, it has been reported that patients with nAbs against Ads frequently developed Ad infection after allogeneic stem cell transplantation [26]. In another study, infection by progeny Ad2, which replicates in infected cells in the presence of nAbs, was slightly observed [13]. These reports suggested that a small amount of Ad was not recognized by nAb and remained in the Ad-infected patients, but the infectivity of progeny Ad in the presence of nAb and its mechanism remains unclear.

In this study, we defined the infectivity of progeny Ad5 in the presence of nAb using wild-type (WT) Ad5 and Ad5 vectors containing firefly luciferase and green fluorescent protein (GFP) expression cassettes (Ad5/Luc and Ad5/GFP vectors, respectively). Moreover, we focused on the fibre protein of Ad5 (Ad5fiber), which is critical for cell surface binding, and examined its activity during secondary infection. We found that progeny Ad5 could infect cells adjacent to initially infected cells even in the presence of nAb, which differs from the features of common infection by Ad5.

METHODS

Cells

Cells were maintained in Dulbecco's modified Eagle's medium (Merck, Darmstadt, Germany) containing 10% fetal bovine serum, 1% penicillin–streptomycin solution, and 1% non-essential amino acid solution (Nacalai Tesque, Kyoto, Japan) in an atmosphere of 5% CO_2.

Cells that expressed GFP, CD90.2, and/or CAR were generated using lentiviral vectors. Briefly, the GFP, mouse CD90.2, and human CAR genes were cloned into the *Bam*HI and *Eco*RI sites of the FG11BSE lentiviral vector. All vesicular stomatitis virus (VSV)-G pseudotyped lentiviral vectors were produced via the calcium phosphate-mediated transient transfection of 293 T cells. Then 293 T cells were co-transfected with appropriate amounts of vector plasmid, the HIV-1 lentiviral packaging constructs pRSVREV [27] and pMDLgpRRE [27], and the VSV-G expression plasmid pHCMVG [28]. The lentiviral vectors were collected from the culture supernatants 3 days after transfection. Cells were incubated with lentiviral vectors for 3 days, followed by the sorting of transduced cells using a cell sorter (SH800, Sony, Tokyo, Japan). GFP, CD90.2, and CAR expression on the surface of sorted cells was confirmed using flow cytometry. Ad5fiber-expressing 293 (Ad5fiber 293) cells, which we generated previously, were used [29].

Virus and nAb against Ad5

WT Ad5 (#VR-5) was obtained from ATCC (Manassas, VA, USA). Ad5 vectors contained a cytomegalovirus promoter-driven firefly luciferase expression cassette and GFP expression cassette. Determination of the viral particle (VP) titres of WT Ad5 and Ad vectors was accomplished using quantitative polymerase chain reaction (PCR) and the method described by Maizel *et al.* [30], respectively.

nAb against Ad5 and control serum were selected from human sera purchased from Cosmo Bio (Tokyo, Japan) using the infectious titres. nAb and control serum were used at 200-fold dilution in this study. Mouse antiserum to Ad5 was prepared by immunizing C57BL/6 mice (female, 10 weeks old) via intraperitoneal injection of the Ad5 vectors exposed to UV radiation 254 nm for 30 min (1.0×10^9 VPs per body) with lipopolysaccharide at 5 µg per body as an adjuvant. PBS acted as the negative control. Eight days after immunization, a second injection was given. Mouse sera obtained 17 days after the first immunization were determined using nAb titres and used at 100-fold dilution in this study. The animal experiment was approved by the institutional ethics committee. Sera were heat-inactivated at 56 °C for 30 min before use.

Ad infection in the presence of nAb

Cells were infected with WT Ad5 at 2.0×10^5 VPs cell^{-1} for 4 h and Ad5/Luc vectors at 200 VPs cell^{-1} for 2 h. After infection, cells were harvested and seeded with non-infected cells at a ratio of 1:10 in six-well plates, followed by incubation in the presence of nAb and control serum. Culture supernatants were collected, and the infection levels in infected cells were determined by quantifying the Ad E4 gene with quantitative PCR (forward primer, 5′-CAC-CAC-CTC-CCG-GTA-CCA-TA-3′; reverse primer, 5′-CCG-CAC-CTG-GTT-TTG-CTT-3′; probe, 5′-[FAM]-AAC-CTG-CCC-GCC-GGC-TAT-ACA-CTG-[MGBEQ]−3′) and a luciferase assay system (PicaGene LT2.0; Toyo Inki, Tokyo, Japan) every 24 h. Cells were treated with the collected culture supernatants for 24 h, followed by measurement of the infection levels. Similarly, 293 T cells were infected with Ad5/GFP vectors at 2000 VPs cell^{-1} for 2 h. After infection, cells were harvested, seeded with 293 cells and mCherry-expressing 293T in a six-well plate, and cultured in the presence of nAb. GFP-positive cells were analysed using a fluorescence microscope (KEYENCE, Osaka, Japan) and a flow cytometer (cytoFLEX S, Beckman Coulter, Brea,

CA, USA). The areas of GFP fluorescence in the images were quantitated using the BZ-X analyser (KEYENCE).

To determine the infection levels of progeny Ad5, 293 T cells infected with Ad5/Luc vectors at 200 VPs cell^{-1} for 2 h were co-cultured with CD90.2-expressing cells at a ratio of 1:10 in the presence of nAb and control serum. At 1–3 days after infection, cells were suspended and treated with magnetic beads against CD90.2 (Becton Dickinson, Franklin Lakes, NJ, USA) at 4 °C for 30 min, after which cell sorting was performed in accordance with the manufacturer's protocol. A luciferase assay system (PicaGene LT2.0) was used to determine luciferase production in sorted cells. To confirm the interference of residual initially infected cells, infected cells were added to CD90.2-expressing cells at a ratio of 1:10 immediately before sorting, followed by sorting at the same time co-cultured cells were sorted. Sorted cells without co-cultivation were used as a sorting control.

Immunostaining

Ad5fiber 293 cells were seeded with GFP-expressing 293 T cells in a 24-well plate on which a cover glass was placed, followed by incubation for 2 days. Cells were fixed in 4% paraformaldehyde for 30 min and incubated in 0.1% Triton X-100 for 15 min. After rinsing with 0.05% Tween-20 in PBS (PBS-T), cells were blocked with 5% bovine serum albumin in PBS-T for 45 min and incubated with anti-fibre Ab (1:1000; Thermo Fisher Scientific, Waltham, MA, USA) for 2 h. After three washes with PBS-T, cells were incubated with conjugated goat anti-mouse IgG Alexa Fluor 680 (1:2000; Thermo Fisher Scientific) for 2 h. After three washes with PBS-T and PBS, cells were carefully mounted onto glass strips. Images of cells were obtained using a confocal laser-scanning microscope (ECLIPS TI-E, Nikon Corp., Tokyo, Japan) equipped with a CCD camera. The fluorescence intensity of the Ad5fiber-positive cells in the images was quantitated using ImageJ.

Flow cytometry

To detect Ad5fiber on the cell surface, 293 T cells infected with Ad5 vectors at 200 VPs cell^{-1} for 2 h and Ad5fiber 293 cells were co-cultured with GFP-expressing cells for 1 day. Cells were treated with anti-fibre Ab (1:500) at 4 °C for 60 min. Subsequently, cells were washed with PBS twice and incubated with allophycocyanin-labelled secondary antibody (1:1000; Thermo Fisher Scientific) at 4 °C for 60 min. After thorough washing, stained cells were analysed using the FACSCalibur (Becton Dickinson) and CellQuest software (Becton Dickinson) or a cytoFLEX S.

Cell ELISA

The 293 T cells infected with Ad5 vectors at 200 VPs cell^{-1} for 2 h were seeded with non-infected cells at a 1:1 ratio in a 24-well plate. After incubation for 40 h, cells were fixed in 4% paraformaldehyde for 30 min and then blocked with 1% Block Ace in PBS for 2 h. Cells were incubated with 0.25 µg ml^{-1} anti-hexon Ab (Millipore, Billerica, MA, USA) and control-Ab (Becton Dickinson) at 4 °C overnight. After four washes with PBS, cells were incubated with horseradish peroxidase-labelled goat anti-mouse IgG (1:20,000; Cosmo Bio) for 2 h. Then, after five washes with PBS, cells were incubated with 3,3′,5,5′-tetramethylbenzidine for 15 min. The enzyme reaction was stopped by the addition of 1 N H_2SO_4, and the absorbance at 450 nm was read using a microplate reader (Varioskan Flash; Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed using SigmaPlot v.12.5 software (Systat Software, San Jose, CA, USA). Statistical significance was analysed using Student's *t*-test and Kruskal–Wallis analysis of variance (ANOVA) on ranks, one-way ANOVA, or two-way ANOVA followed by the Student–Newman–Keuls post-hoc test. $P< 0.05$ was considered significant.

RESULTS

Infection levels of Ad5 sequentially increased even in the presence of nAb

First, we determined the time course of Ad5 infection levels when infected cells were cultured in the presence of nAb. Human serum that inhibits the transduction of Ad5 vectors by more than 99% at 320-fold dilution and serum with no inhibitory effects were used as nAb and control serum, respectively (Fig. S1a, available in the online version of this article). In addition, at the 200-fold dilution, the nAb could still inhibit the transduction of the Ad5 vectors by more than 99%, at up to 4.3×10^7 infectious units ml^{-1} (Fig. S1b). HeLa cells were co-cultured with cells infected with WT Ad5 in the presence of nAb, followed by determination of the abundance of the Ad genome every 24 h. Then, we also evaluated the infectivity of Ad5 released from infected cells into culture supernatant. The abundance of the Ad genome in cells treated with control serum and serum-free supernatant collected every 24 h increased in a time-dependent manner, whereas that in nAb-treated cells did not detected, indicating that progeny Ad5 released into culture supernatant was completely blocked by nAb (Fig. 1a left). In contrast, the abundance of the Ad genome in the cells, including the initially infected cells, significantly increased at 3 days post-infection under all conditions (Fig. 1a right). Similar results were obtained when Ad5/Luc vectors and 293 cells that allow Ad5 vectors to replicate were used instead of WT Ad5 and HeLa cells (Fig. 1b). To clarify whether the increase of Ad genome and reporter gene expression in the presence of nAb was associated with the increases in the number of infected cells, we evaluated the number of infected cells using Ad5/GFP vectors. The 293 cells were co-cultured with cells infected with Ad5/GFP vectors, and images of cells were obtained using a fluorescence microscope. The images illustrated that the number of GFP-positive cells increased in the presence of nAb without significant cytopathic effects (Fig. 2a). Furthermore, the areas of fluorescence were significantly increased in the presence of the nAb at 3 days post-infection (Fig. 2b). Moreover, to differentiate cells initially infected with Ad5/GFP vectors from those secondarily infected with progeny Ad5/GFP vectors,

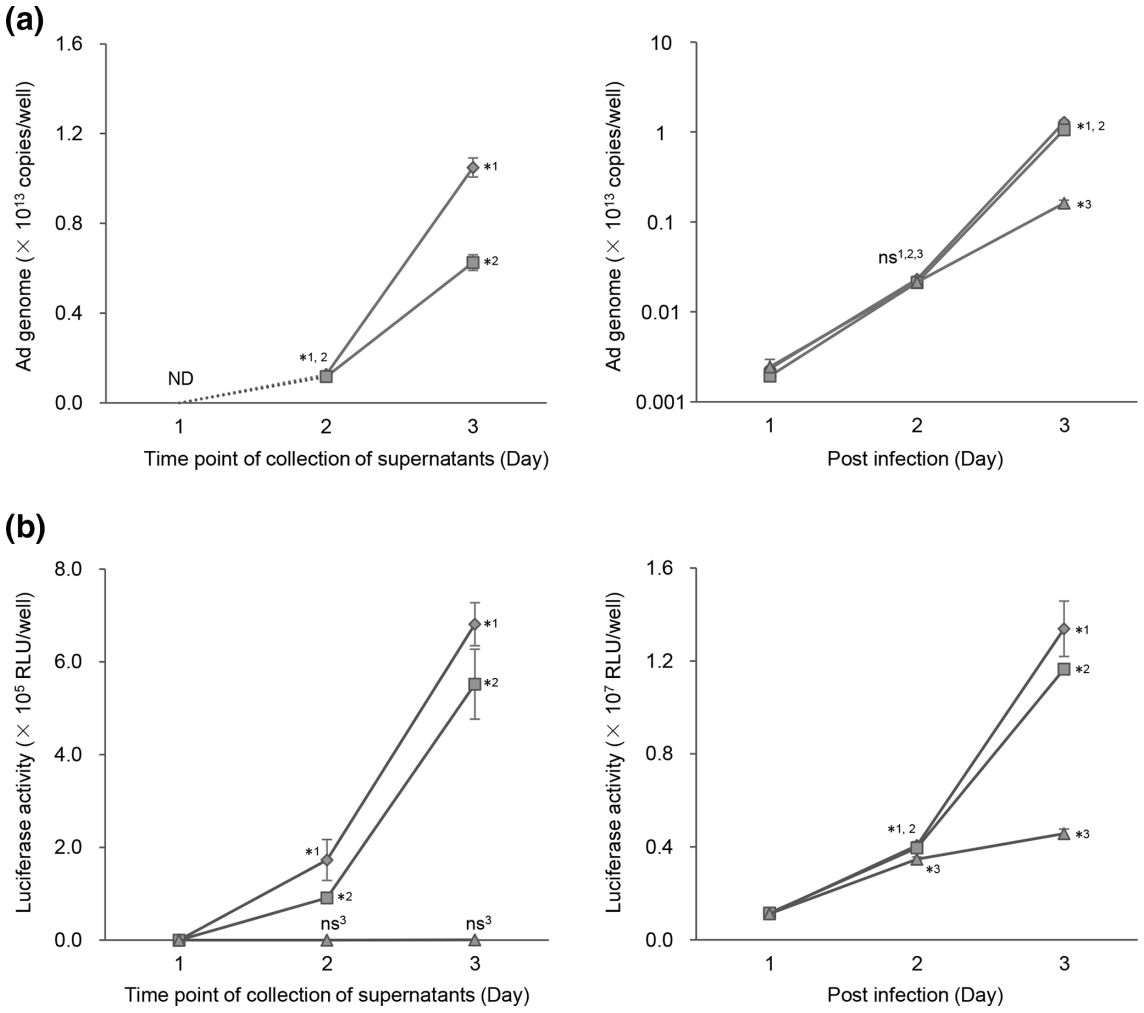

Fig. 1. The time-dependent changes in the infection levels of adenovirus serotype 5 (Ad5) in the presence of neutralizing antibody (nAb). HeLa and 293 T cells that were infected with wild-type (WT) Ad5 (a) and Ad5 vectors containing firefly luciferase expression cassette (b), respectively, were seeded, co-cultured with non-infected cells, and incubated with the control serum and nAb. The levels of infection of cells in the presence of initially infected cells (right) and cells treated with supernatant collected every 24 h (left) were determined. The data are presented as means ± SD (n=3, biological replicates). The infection levels in the cells treated with supernatants collected at 1 day post-infection and those with nAb were below the detection limit when WT Ad5 was used (A, left). Statistical analysis was conducted using the two-way analysis of variance and the Student–Newman–Keuls post-hoc test. ◆ and 1, without nAb; ■ and 2, control serum; ▲ and 3, nAb. *P<0.05, compared with the value on the previous day; ns, not significant; ND, not detected.

we used 293 T cells expressing mCherry, a red fluorescent protein. When 293 T cells infected with Ad5/GFP vectors were co-cultured with mCherry-expressing 293 T cells for 3 days, the number of GFP-positive cells among mCherry-expressing 293 T cells sequentially increased (Fig. 2c, d).

The infectivity of progeny Ad5 in the presence of nAb

To evaluate the infectivity of progeny Ad5 in the presence of nAb, we established an assay that determined the infection levels in cells infected with progeny Ad5. This assay was constructed using a sorting system featuring cells that constitutively expressed mouse CD90.2 as a marker and anti-mouse CD90.2 magnetic beads. After co-cultivating CD90.2-expressing cells with infected cells, CD90.2-expressing cells were sorted using anti-mouse CD90.2 magnetic beads. The infection levels in sorted cells indicate the infectivity of progeny Ad5 when initially infected cells were fully eliminated by the sorting system (Fig. 3a). In preliminary experiments, CD90.2-negative cells were not detected among sorted cells via flow cytometry. Moreover, to confirm the interference by initially infected cells (CD90.2-negative) after sorting, we determined the infection levels in a control in which CD90.2-expressing cells were temporarily co-cultured with initially infected cells in the tube immediately before sorting using anti-CD90.2 magnetic beads. Expectedly, the infection levels in the sorting control were at background in all experiments, implying that the sorting system eliminated

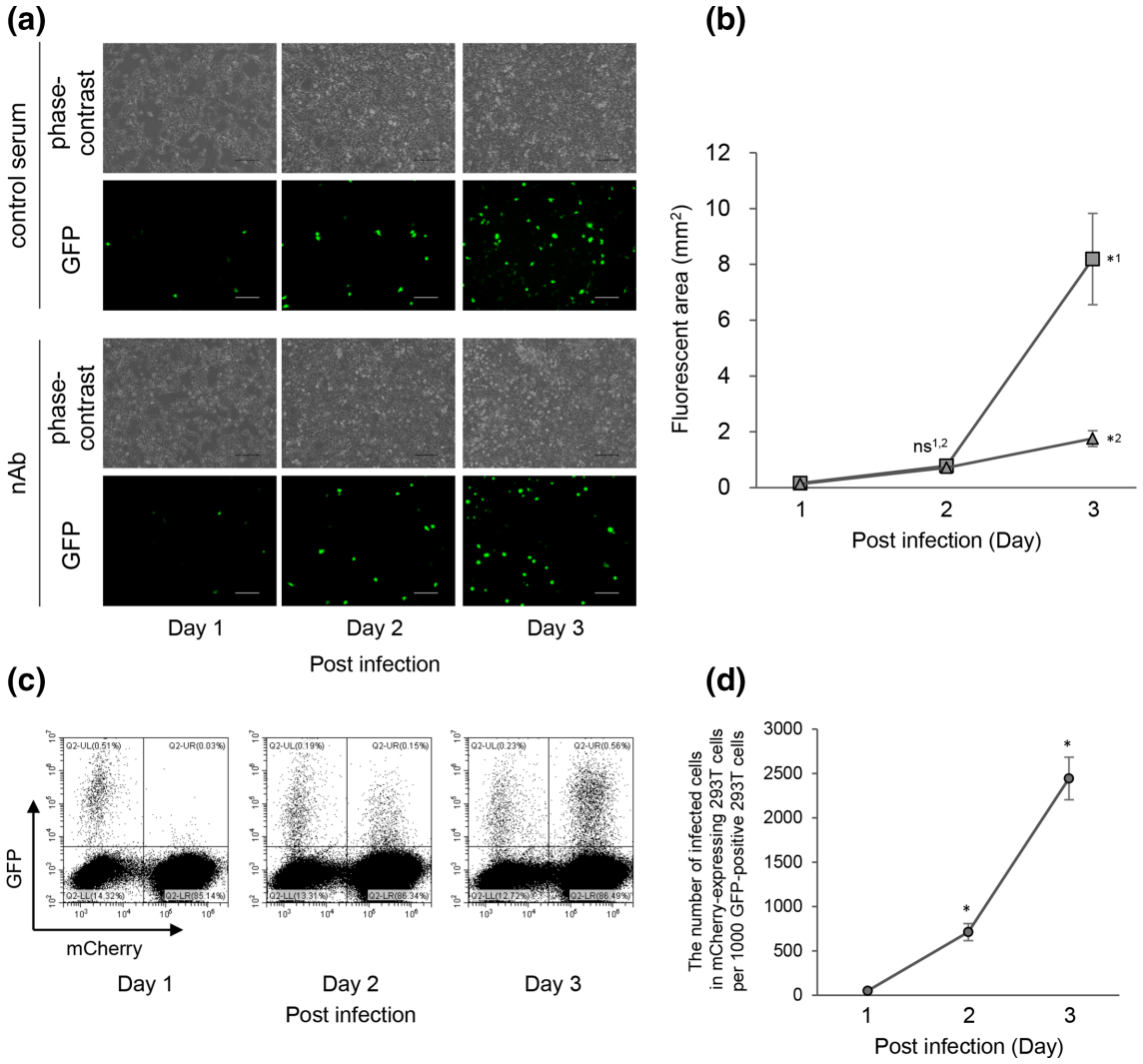

Fig. 2. Infected cell counts were increased even in the presence of neutralizing antibody (nAb). (a, b) The 293 T cells infected with adenovirus serotype 5 vectors containing green fluorescent protein expression cassettes (Ad5/GFP) were seeded, co-cultured with 293 cells, and incubated with the control serum and nAb. A fluorescence microscope was used to acquire the images. The bar represents 100 µm. The images are representative of three independent experiments. The GFP-positive areas were analysed using the BZ-X analyser. The data are presented as the mean±SD (error bars; *n*=3, biological replicates). The *P* values (one-tailed) were analysed using two-way analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) post-hoc test. ■ and 1, control serum; ▲ and 2, nAb. **P* < 0.05; compared to the value on the previous day; ns, not significant. (c, d) The 293 T cells infected with Ad5/GFP were seeded, co-cultured with mCherry-expressing 293 T cells, and incubated with the nAb. GFP-positive cells were identified by flow cytometry. The data are presented in representative dot plots as the mean±SD (error bars) number of GFP-positive cells in mCherry-expressing 293 T cells per 1000 GFP-positive 293 T cells (mCherry-negative; *n*=3, biological replicates). Statistical analysis was conducted using one-way ANOVA and the SNK post-hoc test (**P*<0.05, compared to the value on the previous day).

initially infected cells below the level that could cause interference (Fig. 3). In addition, luciferase activity in the sorted cells was significantly higher than that in the sorting control when the initially infected cells were 293 T cells, but not HepG2 cells, in which the Ad5 vectors were not replicated. These data indicated that the progeny Ad5 vectors replicated in the initially infected 293 T cells infected the CD90.2-expressing cells (Fig. S2).

HepG2 cells that expressed mouse CD90.2 (CD90.2 HepG2 cells) were co-cultured with 293 T cells infected with Ad5/Luc vectors in the presence of nAb, followed by measurements of luciferase activity in cells sorted 1 and 3 days after infection. We then confirmed that nAb completely blocked the infection of progeny Ad5 released into culture supernatant (Figs S3 and S4). The luciferase activity in cells sorted 1 and 3 days after co-cultivation with infected cells in the presence of nAb was considerably higher than that of the sorting control (Fig. 3b). Notably, the ratio of evasion of nAb responses (nAb/control serum) was higher on day 1 than on day 3. Similar results were obtained using serum collected

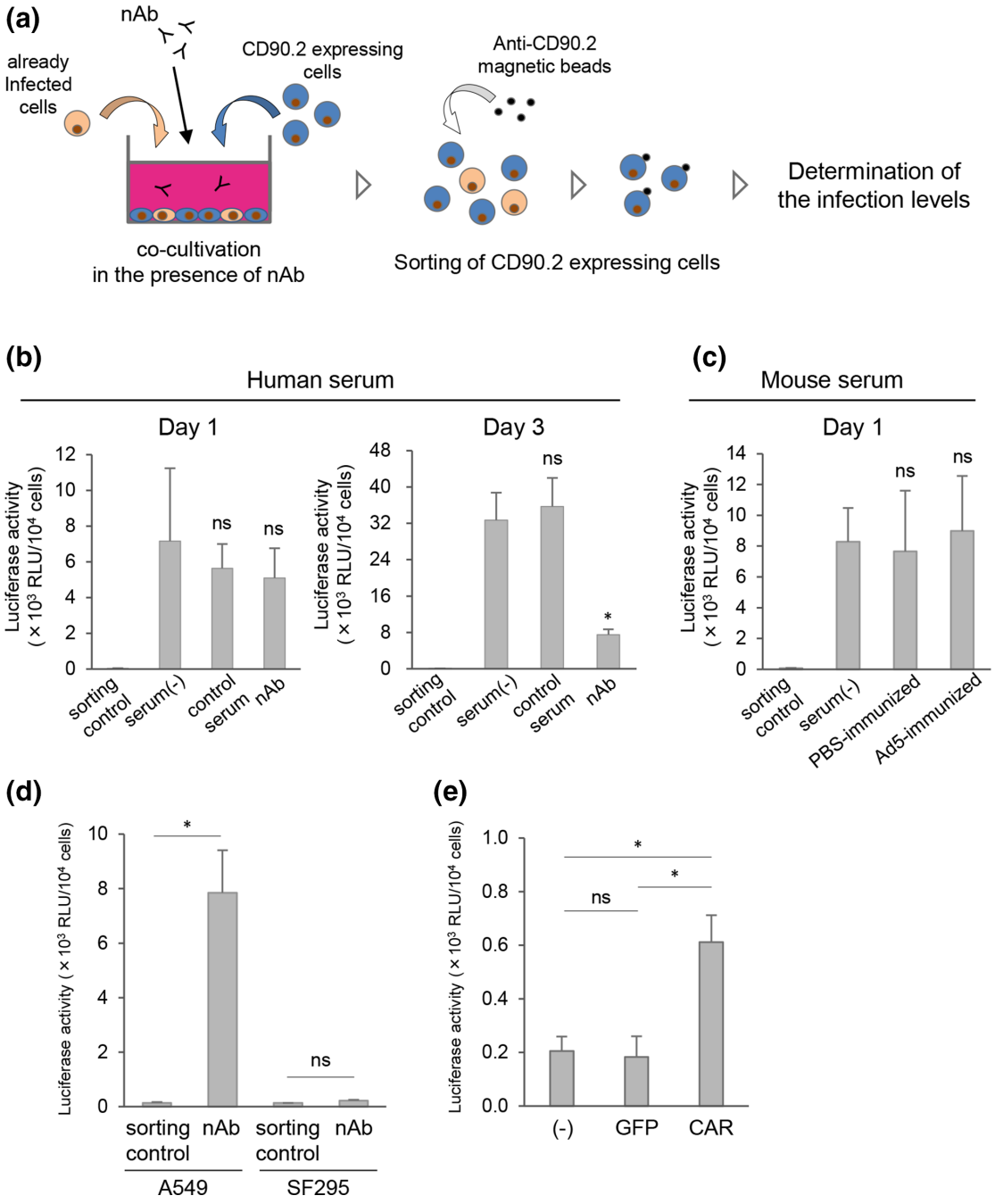

Fig. 3. The infection levels of progeny adenovirus serotype 5 (Ad5) in the presence of neutralizing antibody (nAb). (a) Scheme of the assay using magnetic sorting to determine the infection levels of progeny Ad5. (b, c) The 293T cells infected with Ad5 vectors containing the firefly luciferase expression cassettes (Ad5/Luc) were seeded, co-cultured with CD90.2-expressing HepG2 cells, and incubated with the nAb and serum obtained from Ad5-immunized mice. After 1 or 3 days of co-culturing, the CD90.2-expressing cells were sorted using magnetic beads against CD90.2. Luciferase activities in the sorted cells were determined. Data are presented as the mean±SD (error bars; *n*=3, biological replicates). Statistical analysis was conducted using two-way analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) post-hoc test. Significant differences were observed in all groups as compared to the sorting control. **P*<0.05, compared to without serum; ns, not significant. (d, e) The 293T cells infected with Ad5/Luc vectors were seeded, co-cultured with A549, SF295, and coxsackievirus and adenovirus receptor-expressing SF295 cells expressing CD90.2, and incubated with the nAb. After co-culturing in the presence of the nAb for 2 days, the CD90.2-expressing cells were sorted using magnetic beads against CD90.2. Luciferase activity in the sorted cells was determined. Data are presented as the mean±SD (error bars; *n*=3, biological replicates). Statistical analysis was conducted using two-way ANOVA and SNK post-hoc test (d) or Kruskal–Wallis ANOVA on ranks and SNK post-hoc test (e) (**P*<0.05; ns, not significant).

from Ad5-immunized mice with equal inhibitory potency as nAb (Figs 3c and S5).

To evaluate the infectivity of progeny Ad5 in the presence of nAb in other cell lines, we prepared A549 and SF295 cells that expressed CD90.2. The luciferase activity in SF295 cells, which do not express CAR, in the presence of nAb was comparable to that of the sorting control, whereas that of A549 cells, which express CAR, was significantly higher than that of the sorting control (Fig. 3d). To uncover the contribution of CAR, the infectivity of progeny Ad5 in the presence of nAb in SF295 cells that constitutively expressed CAR was evaluated. The luciferase activity in CAR-expressing SF295 cells was significantly increased compared with that in GFP-expressing SF295 cells as a negative control, demonstrating that CAR was also important for infection by progeny Ad5 in the presence of nAb (Fig. 3e).

Fiber protein on the cell surface could spread to nearby cells in a CAR-dependent manner

Progeny Ad5 must spread from infected cells to nearby cells to facilitate infection, whereas infection by progeny Ad5 released into culture supernatant was inhibited by nAb. We previously demonstrated that Ad5fiber localized to the cell surface without release into the culture supernatant [31]. We focused on Ad5fiber and examined its behaviour on the cell surface. Flow cytometry analysis illustrated the presence of Ad5fiber on the cell surface of both infected cells and GFP-expressing HepG2 cells after co-cultivation (Fig. 4a). Similar results were obtained when Ad5fiber 293 cells were used instead of infected cells (Fig. 4b), demonstrating that Ad5fiber spread from infected cells to nearby cells. Moreover, we investigated whether the spread of Ad5fiber occurs in a CAR-dependent manner. As presented in Fig. 4c, Ad5fiber spread from infected cells to the surface of A549 cells, but not SF295 cells. In contrast to the findings for SF295 cells, Ad5fiber spread to the surface of CAR-expressing SF295 cells similarly as observed for HepG2 and A549 cells (Fig. 4c). In addition, immunostaining for Ad5fiber revealed its localization in the intercellular space in which CAR resides on cells distant from Ad5fiber 293 cells (Fig. 4d).

Finally, we investigated whether progeny Ad spreads on the cell surface in the presence of nAb. In cells that were not permeabilized with surfactant, the levels of hexon, a major component of Ad, were determined using anti-hexon antibody. The absorbance in cells detected using anti-hexon antibody was significantly higher when infected cells were co-cultured with CAR-expressing SF295 cells than with SF295 cells. This indicated that progeny Ad5 was efficiently transferred to CAR-positive cells but not CAR-negative cells, similarly as noted for Ad5fiber (Fig. 4e).

DISCUSSION

Host immunity is important for controlling viral infection and preventing infectious diseases. However, viruses have developed various strategies for evading host immunity. In this study, we found that progeny Ad5 can infect cells even in the presence of nAb.

Ads cause acute infection as well as persistent or latent infection [32–34], implying that they are not completely cleared by host immunity. We found that nAb did not completely inhibit infection by progeny Ad5 despite being able to inhibit infection by the virus when released into culture supernatant. It has been reported that Ad2, which similarly belongs to subgroup C as Ad5, requires approximately 50 h to disrupt the cell membrane, followed by its release from infected cells for secondary infection [13]. In accordance, our results illustrated that culture supernatants contained infectious progeny Ad5 starting 2 days after infection. Interestingly, infection by progeny Ad5 occurred until 1 day after infection, and there was no difference in transgene expression levels at this time. These findings suggested the existence of a novel mechanism by which progeny Ad5 infects cells while evading nAb responses, especially in the early phase of infection.

There are two major models of viral spread: cell-free transmission and cell-to-cell transmission. Other groups have reported that Ad2 and influenza A virus move on the cell surface via receptors [35, 36]. We found that progeny Ad5 spread to CAR-positive cells, but not to CAR-negative cells, in the presence of nAb. Moreover, Ad5fiber itself can spread between cells in a CAR-dependent manner, and it localizes to intercellular space. Fiber protein is a homotrimer with three binding sites for CAR [37], implying that it can simultaneously bind multiple CARs. CAR, a 46 kDa transmembrane glycoprotein, is composed of two extracellular immunoglobulin-like domains (D1 and D2), one transmembrane domain, and one cytoplasmic tail containing a PDZ domain. CAR localizes in tight junctions in cells and homodimerizes through intracellular and intercellular interactions [38, 39]. Therefore, it is possible that progeny Ad5 spreads via cell-to-cell transmission through binding between fiber proteins and CARs, thereby sustaining the capacity of infection. Cell-to-cell transmission of viruses such as HIV is known to be effective for evading nAb responses [40] because one of the main roles of nAb is inhibiting the binding of viruses to the cell surface. Although further investigation is needed to uncover the mechanism by which progeny Ad5 infects cell in the presence of nAb, it is considered that the binding of progeny Ad5 to the cell surface before being recognized by nAb and its spread to nearby cells without detachment from the cell surface permitted its evasion of nAb.

Ad can cause severe disseminated infections in immunocompromised patients such as bone marrow or solid organ transplant recipients. Controlling complications, including infections, is important for successful transplantation. However, there is no approved therapeutic agent for Ad infections. To date, it has been reported that ribavirin and cidofovir treatment [41–44] and adoptive transfer of Ad-specific T cells [45, 46] effectively prevented Ad infections in patients following transplantation. Intravenous immunoglobulin (IVIG) has been used to treat severe Ad infection in combination with ribavirin and cidofovir [47–49], but the therapeutic

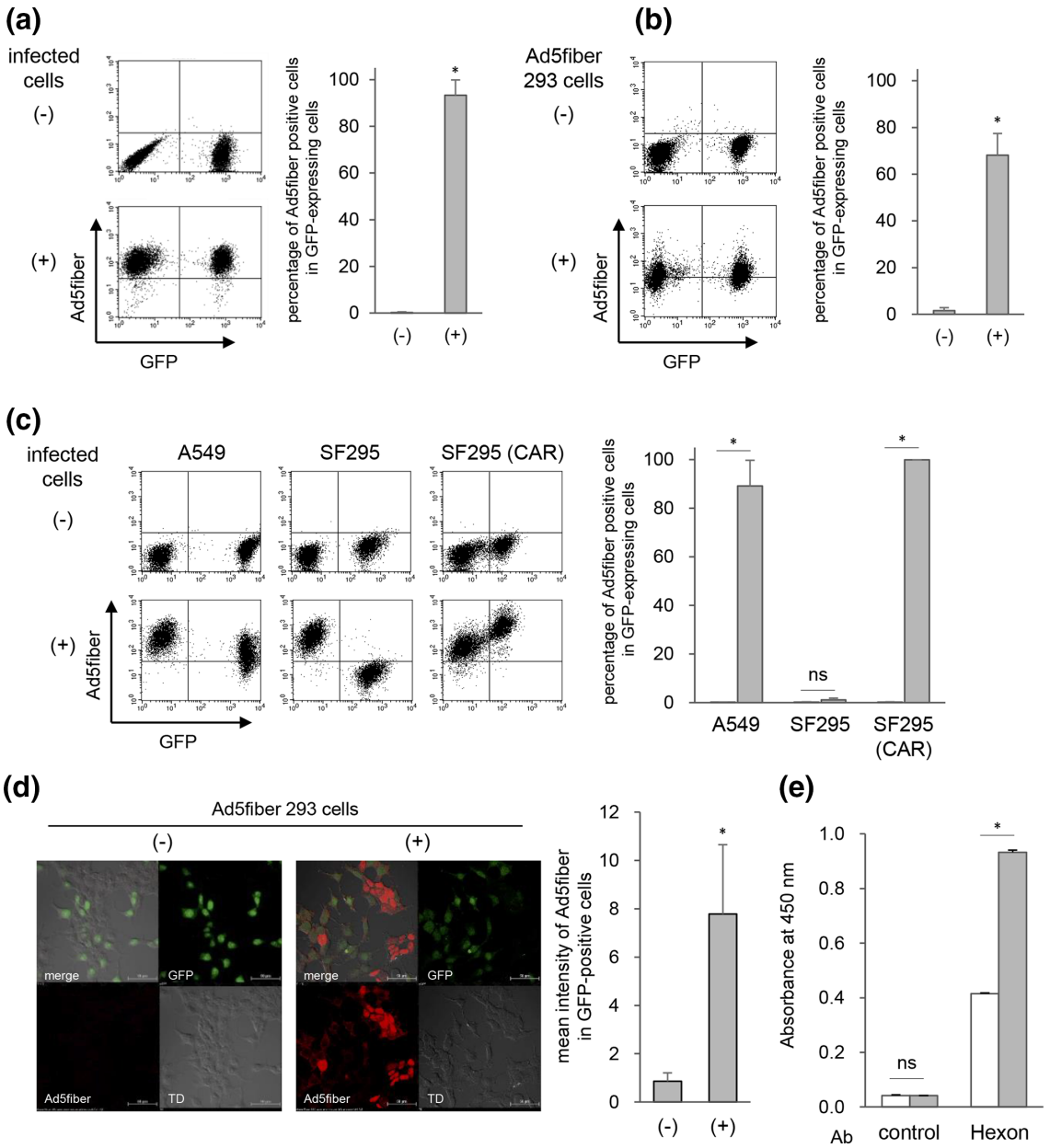

Fig. 4. The spread of the fiber protein of adenovirus serotype 5 (Ad5fiber) between cells in a coxsackievirus and adenovirus receptor (CAR)-dependent manner. (a–c) GFP-expressing cells [a and b, HepG2; c, A549 (left), SF295 (middle), and CAR-expressing SF295 (right)] were co-cultured with the 293T cells infected with adenovirus serotype 5 (Ad5) vectors or the Ad5fiber-expressing 293 cells for 1 day. The levels of Ad5fiber were determined via flow cytometry. The data from three independent experiments were presented as representative dot plots. The percentages of Ad5fiber-positive cells among the GFP-expressing cells were shown as means ± SD (error bars) of three independent experiments (c, open columns, without infected cells; filled columns, with infected cells). (d) GFP-expressing 293T cells were co-cultured with Ad5fiber-expressing 293 cells for 2 days. After treatment with fluorescently labelled antibodies, the representative images of the cells were obtained using a confocal laser-scanning microscope equipped with a CCD camera. The experiments were performed at least twice and obtained similar results. The mean fluorescence intensity of the Ad5fiber-positive cells among the GFP-positive cells was quantified using ImageJ. The data are shown as means ± SD (error bars) of three independent images. The bar represents 50 µm. (e) The SF295 (open columns) and CAR-expressing SF295 cells (filled columns) were co-cultured with the 293T cells infected with Ad5 vectors for 40 h in the presence of nAb and fixed with 4% paraformaldehyde. The levels of hexon proteins were determined using Cell ELISA. The data are presented as the mean±SD (error bars; *n*=3, biological replicates). Statistical analysis was conducted using the Student's t-test (a, b, and d) or two-way analysis of variance and the Student–Newman–Keuls post-hoc test (c, e) (*P<0.05; ns, not significant).

effects of IVIG itself are unclear [50]. Our results suggest that the effect of IVIG is attenuated by progeny Ad in the presence of nAb and that inhibition of this infection is an attractive strategy for patients treated with IVIG. Therefore, understanding the mechanisms of infection by progeny Ad and the evasion of nAb responses may both provide a new therapeutic target and enhance the therapeutic effects of IVIG.

In summary, we revealed that progeny Ad5 can infect cells in the presence of nAb, differing from the common features of the Ad5 infection cycle. Furthermore, we found that Ad5fiber on the cell surface spread to nearby cells in a CAR-dependent manner, suggesting its involvement in the evasion of nAb responses and delivery of Ad particles. Our findings may be useful for developing treatments against Ad infection and identifying novel strategies for evading host immunity.

Funding information
This work was supported by a Grant-in-Aid for Young Scientists of Showa Pharmaceutical University and Scholarship Fund for Young Researchers of The Promotion and Mutual Aid Corporation for Private Schools of Japan.

Author contributions
T. H., Methodology; Investigation; Formal Analysis; Writing – Original Draft Preparation. A. S., Investigation. N. K., Conceptualization; Writing – Review and Editing; Project Administration. Y. K., Validation. Y. S., Validation. J. K., Investigation. M. Y., Investigation.T. N., Writing – Review and Editing. M. F., Writing – Review and Editing. F. S., Resources; Writing – Review and Editing. H. M., Resources; Writing – Review and Editing. Y. W., Writing – Review and Editing. N. U., Supervision.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The animal experiment was approved by the institutional ethics committee of Showa Pharmaceutical University.

References

1. Baldwin A, Kingman H, Darville M, Foot AB, Grier D *et al*. Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. *Bone Marrow Transplant* 2000;26:1333–1338.
2. Pham TTN, Burchette JL, Hale LP. Fatal disseminated adenovirus infections in immunocompromised patients. *Am J Clin Pathol* 2003;120:575–583.
3. Cederwall S, Påhlman LI. Respiratory adenovirus infections in immunocompetent and immunocompromised adult patients. *Epidemiol Infect* 2020;147:e328.
4. Henry LJ, Xia D, Wilke ME, Deisenhofer J, Gerard RD. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in Escherichia coli. *J Virol* 1994;68:5239–5246.
5. Roelvink PW, Lizonova A, Lee JG, Li Y, Bergelson JM *et al*. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol* 1998;72:7909–7915.
6. Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol* 1994;127:257–264.
7. Chiu CY, Mathias P, Nemerow GR, Stewart PL. Structure of adenovirus complexed with its internalization receptor, alphavbeta5 integrin. *J Virol* 1999;73:6759–6768.
8. Suomalainen M, Nakano MY, Keller S, Boucke K, Stidwill RP *et al*. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. *J Cell Biol* 1999;144:657–672.
9. Leopold PL, Kreitzer G, Miyazawa N, Rempel S, Pfister KK *et al*. Dynein- and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. *Hum Gene Ther* 2000;11:151–165.
10. Spindler KR, Eng CY, Berk AJ. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. *J Virol* 1985;53:742–750.
11. Hu MC, Hsu MT. Adenovirus E1B 19K protein is required for efficient DNA replication in U937 cells. *Virology* 1997;227:295–304.
12. Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ *et al*. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* 1996;70:2296–2306.
13. Yakimovich A, Gumpert H, Burckhardt CJ, Lütschg VA, Jurgeit A *et al*. Cell-Free transmission of human adenovirus by passive mass transfer in cell culture simulated in a computer model. *J Virol* 2012;86:10123–10137.
14. Basner-Tschakarjan E, Gaffal E, O'Keeffe M, Tormo D, Limmer A *et al*. Adenovirus efficiently transduces plasmacytoid dendritic cells resulting in TLR9-dependent maturation and IFN-alpha production. *J Gene Med* 2006;8:1300–1306.
15. Yamaguchi T, Kawabata K, Kouyama E, Ishii KJ, Katayama K *et al*. Induction of type I interferon by adenovirus-encoded small RNAs. *Proc Natl Acad Sci U S A* 2010;107:17286–17291.
16. Ackrill AM, Foster GR, Laxton CD, Flavell DM, Stark GR *et al*. Inhibition of the cellular response to interferons by products of the adenovirus type 5 E1A oncogene. *Nucleic Acids Res* 1991;19:4387–4393.
17. Olanubi O, Frost JR, Radko S, Pelka P. Suppression of type I interferon signaling by E1A via RuvBL1/Pontin. *J Virol* 2017;91:pii: e02484–16.
18. Bridge E, Ketner G. Redundant control of adenovirus late gene expression by early region 4. *J Virol* 1989;63:631–638.
19. Huang MM, Hearing P. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J Virol* 1989;63:2605–2615.
20. Ullman AJ, Reich NC, Hearing P. Adenovirus E4 ORF3 protein inhibits the interferon-mediated antiviral response. *J Virol* 2007;81:4744–4752.
21. Tang J, Olive M, Pulmanausahakul R, Schnell M, Flomenberg N *et al*. Human CD8+ cytotoxic T cell responses to adenovirus capsid proteins. *Virology* 2006;350:312–322.
22. Leen AM, Christin A, Khalil M, Weiss H, Gee AP *et al*. Identification of hexon-specific CD4 and CD8 T-cell epitopes for vaccine and immunotherapy. *J Virol* 2008;82:546–554.
23. Roberts DM, Nanda A, Havenga MJE, Abbink P, Lynch DM *et al*. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 2006;441:239–243.
24. Bradley RR, Lynch DM, Iampietro MJ, Borducchi EN, Barouch DH. Adenovirus serotype 5 neutralizing antibodies target both hexon and fiber following vaccination and natural infection. *J Virol* 2012;86:625–629.
25. Seshidhar Reddy P, Ganesh S, Limbach MP, Brann T, Pinkstaff A *et al*. Development of adenovirus serotype 35 as a gene transfer vector. *Virology* 2003;311:384–393.
26. Veltrop-Duits LA, van Vreeswijk T, Heemskerk B, Thijssen JCP, El Seady R *et al*. High titers of pre-existing adenovirus serotype-specific neutralizing antibodies in the host predict viral reactivation after allogeneic stem cell transplantation in children. *Clin Infect Dis* 2011;52:1405–1413.
27. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M *et al*. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72:8463–8471.
28. Yee JK, Friedmann T, Burns JC. Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol* 1994;43 Pt A:99–112.
29. Koizumi N, Mizuguchi H, Sakurai F, Yamaguchi T, Watanabe Y *et al*. Reduction of natural adenovirus tropism to mouse liver by

fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J Virol* 2003;77:13062–13072.

30. Maizel JV, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7a, and 12. *Virology* 1968;36:115–125.
31. Sato A, Hirai T, Koizumi N, Hatakeyama S, Watanabe A *et al*. Adenovirus fiber can distribute itself to the cell surface without membrane damage. *BPB Reports* 2019;2:113–118.
32. Garnett CT, Erdman D, Xu W, Gooding LR. Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. *J Virol* 2002;76:10608–10616.
33. Lichtenstein DL, Wold WSM. Experimental infections of humans with wild-type adenoviruses and with replication-competent adenovirus vectors: replication, safety, and transmission. *Cancer Gene Ther* 2004;11:819–829.
34. Alkhalaf MA, Guiver M, Cooper RJ. Prevalence and quantitation of adenovirus DNA from human tonsil and adenoid tissues. *J Med Virol* 2013;85:1947–1954.
35. Burckhardt CJ, Suomalainen M, Schoenenberger P, Boucke K, Hemmi S *et al*. Drifting motions of the adenovirus receptor CAR and immobile integrins initiate virus uncoating and membrane lytic protein exposure. *Cell Host Microbe* 2011;10:105–117.
36. Sakai T, Nishimura SI, Naito T, Saito M. Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. *Sci Rep* 2017;7:45043.
37. Bewley MC, Springer K, Zhang YB, Freimuth P, Flanagan JM. Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science* 1999;286:1579–1583.
38. Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT *et al*. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci U S A* 2001;98:15191–15196.
39. Patzke C, Max KEA, Behlke J, Schreiber J, Schmidt H *et al*. The coxsackievirus-adenovirus receptor reveals complex homophilic and heterophilic interactions on neural cells. *J Neurosci* 2010;30:2897–2910.
40. Gupta P, Balachandran R, Ho M, Enrico A, Rinaldo C. Cell-to-cell transmission of human immunodeficiency virus type 1 in the presence of azidothymidine and neutralizing antibody. *J Virol* 1989;63:2361–2365.
41. Liles WC, Cushing H, Holt S, Bryan C, Hackman RC. Severe adenoviral nephritis following bone marrow transplantation: successful treatment with intravenous ribavirin. *Bone Marrow Transplant* 1993;12:409–412.
42. Miyamura K, Hamaguchi M, Taji H, Kanie T, Kohno A *et al*. Successful ribavirin therapy for severe adenovirus hemorrhagic cystitis after allogeneic marrow transplant from close HLA donors rather than distant donors. *Bone Marrow Transplant* 2000;25:545–548.
43. Legrand F, Berrebi D, Houhou N, Freymuth F, Faye A *et al*. Early diagnosis of adenovirus infection and treatment with cidofovir after bone marrow transplantation in children. *Bone Marrow Transplant* 2001;27:621–626.
44. Yusuf U, Hale GA, Carr J, Gu Z, Benaim E *et al*. Cidofovir for the treatment of adenoviral infection in pediatric hematopoietic stem cell transplant patients. *Transplantation* 2006;81:1398–1404.
45. Feucht J, Opherk K, Lang P, Kayser S, Hartl L *et al*. Adoptive T-cell therapy with hexon-specific Th1 cells as a treatment of refractory adenovirus infection after HSCT. *Blood* 2015;125:1986–1994.
46. Feuchtinger T, Matthes-Martin S, Richard C, Lion T, Fuhrer M *et al*. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. *Br J Haematol* 2006;134:64–76.
47. Permpalung N, Mahoney MV, Alonso CD. Adjunctive use of cidofovir and intravenous immunoglobulin to treat invasive adenoviral disease in solid organ transplant recipients. *Pharmacotherapy* 2018;38:1260–1266.
48. Serrano RM, Darragh RK, Parent JJ. Successful treatment of disseminated adenovirus infection with cidofovir and intravenous immunoglobulin in an infant following heart transplant. *Cardiol Young* 2018;28:888–889.
49. Park UJ, Hyun SK, Kim HT, Cho WH, Han SY. Successful treatment of disseminated adenovirus infection with ribavirin and intravenous immunoglobulin in an adult renal transplant recipient: a case report. *Transplant Proc* 2015;47:791–793.
50. Piedra PA, Kasel JA, Norton HJ, Gruber WC, Garcia-Prats JA *et al*. Evaluation of an intravenous immunoglobulin preparation for the prevention of viral infection among hospitalized low birth weight infants. *Pediatr Infect Dis J* 1990;9:470–475.