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Targeted Delivery of Immunogen to Primate M Cells with Tetragalloyl Lysine Dendrimer

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Effective uptake of Ags by specialized M cells of gut-associated lymphoid tissues is an important step in inducing efficient immune responses after oral vaccination. Although stable nontoxic small molecule mimetics of lectins, such as synthetic multivalent polygalloyl derivatives, may have potential in murine M cell targeting, it remains unclear whether synthetic multivalent polygalloyl derivatives effectively target nonhuman and human M cells. In this study, we evaluated the ability of a tetragalloyl derivative, the tetragalloyl-d-lysine dendrimer (TGDK), to target M cells in both in vivo nonhuman primate and in vitro human M-like cell culture models. TGDK was efficiently transported from the lumen of the intestinal tract into rhesus Peyer’s patches by M cells and then accumulated in germinal centers. Oral administration of rhesus CCR5-derived cyclopeptide conjugated with TGDK in rhesus macaque resulted in a statistically significant increase in stool IgA response against rhesus CCR5-derived cyclopeptide and induced a neutralizing activity against SIV infection. Furthermore, TGDK was specifically bound to human M-like cells and efficiently transcytosed from the apical side to the basolateral side in the M-like cell model. Thus, the TGDK-mediated vaccine delivery system represents a potential approach for enabling M cell-targeted mucosal vaccines in primates. The Journal of Immunology, 2009, 182: 6061–6070.

Human immunodeficiency virus is transmitted primarily via the genital mucosa during sexual intercourse. Elucidating the early events in mucosally transmitted HIV-1 infection plays a critical role in characterizing the virus-host interactions and effective vaccine design and development. Mucosal transmission of HIV-1 infection is mediated by exposure to cell-free viruses and/or cell-associated viruses within mucosal secretions, and established within hours, and can be disseminated to draining lymph nodes within days (1, 2). Recent studies of pathological events in acute infection in nonhuman primates and humans have provided important insights into the disruption of the mucosal immune system. This disruption is evident in the rapid depletion of CD4 T cells within the GALT during acute infection (3, 4), suggesting that once mucosal infection has occurred, immune responses to infection are insufficient to prevent these events. Therefore, a preventive vaccine should effectively target the earliest events in the establishment of HIV infection at the mucosal site.

Conventional vaccines administered from any routes other than the oral route effectively induce protective systemic immune responses, but the level of protective immunity at the major site of HIV mucosal entry is less robust. However, neutralizing Abs administered i.v. at high doses can reach mucosal sites and block genital mucosal transmission of simian/HIV (SHIV) in nonhuman primate models (5), suggesting that sufficient mucosal humoral immune responses induced by mucosal vaccines can prevent HIV infection. Some related studies have shown that mucosal vaccines induce not only secretory IgA at mucosal sites, but also mucosal cell-mediated immunity and systemic Abs against HIV (6–13). These studies suggest that mucosal vaccines have several advantages over conventional systemic vaccines because they can induce multi-immuno responses that prevent HIV infection at the mucosal site.

Current efforts to develop effective mucosal vaccines are mainly directed toward finding more efficient means of delivering appropriate Ags to the mucosal immune system and toward developing effective and safe mucosal adjuvants (14) because it has often proved difficult to stimulate strong mucosal IgA immune responses and protection against pathogens by mucosal administration of Ags without Ag delivery and adjuvant systems. It is generally accepted that M cells in Peyer’s patches (PPs) are instrumental in initiating mucosal immunity against pathogens invading across epithelial barriers (15). The high transcytotic abilities of M cells

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3 Abbreviations used in this paper: SHIV, simian/HIV; DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast; DMF, dimethylformamide; EDS, energy-dispersive x-ray spectroscopy; FAE, follicle-associated epithelium; Fmoc, 9-fluorenylmethoxycarbonyl; GC, germinal center; PEG, polyethylene glycol; PP, Peyer’s patch; PV, poliovirus; rcDDR5, rhesus CCR5-derived cyclopeptide; RT, room temperature; SMPD, synthetic multivalent polygalloyl derivative; TEM, transmission electron microscopy; TGDK, tetragalloyl-d-lysine dendrimer; TRITC, tetramethylrhodamine isothiocyanate; UEA-1, Ulex europaeus agglutinin-1; wpim, weeks postinfection.

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make them an attractive target for mucosally delivered vaccines because mucus secretion may flush away an applied mucosal vaccine at the mucosal site. Some studies showed that mucosal vaccine delivery can be improved using appropriate bioadhesin molecules such as lectins because M cell surface glycoalkyxl differs in carbohydrate composition from that of enterocytes in many species (16–22). *Ulex europaeus agglutinin-1* (UEA-1)-conjugated (23, 24) or α1-protein-conjugated nasal vaccination (13, 25) induces not only strong Ag-specific mucosal IgA and plasma IgG responses, but also CTL immunity. However, lectins such as UEA-1 are of limited value in vaccine delivery because they are toxic and subject to intestinal degradation. Lambkin et al. (26) reported that a stable low m.w. four-copy gallic acid construct is a competitor of UEA-1 and appears to have high affinity for the fucose receptor on murine M cells. Although stable nontoxic small molecular mimetics of UEA-1 have the potential for M cell targeting in mice, it remains unclear whether these mimetics effectively target the nonhuman and human M cells.

In this study, we synthesized the tetragalloyl-α-lysine dendrimer (TGDK) and demonstrated its M cell targeting potential in both in vivo nonhuman primate and in vitro human M-like cell culture models.

### Materials and Methods

#### TGDK and α-lysine dendrimer

The 9-fluorenylmethoxycarbonyl (Fmoc)-α-MAP4-NH-(CH2)-NH-Trt-resin (in this study referred to as Fmoc-α-lysine dendrimer resin; Watanabe Chemical Industries) was treated with 20% piperidine/dimethylformamide (DMF) for 20 min to remove the Fmoc group. To prepare TGDK, the resin (0.51 mmol) was then washed five times with DMF and reacted with 3,4,5-trimethoxysulfonyl benzoic acid chloride (1 mmol) in triethylamine (7 mmol) at 40°C for 120 min. The resulting resin was washed with 1% trifluoroacetic acid/DMF three times and DMF five times, and then treated with boron tribromide (the amount is 20× mole equivalent of that of DMF in the reaction mixture) at 40°C for 5 min before air drying. TGDK was extracted with Milli-Q water, purified, and lyophilized. The patented synthesis method of TGDK has been obtained (PCT/JP2006/321720). The α-lysine dendrimer was obtained by treating the α-lysine dendrimer resin with acetic acid/trifluoroethanol/dichloromethane (1:1:8). The molecular masses of TGDK and α-lysine dendrimer were determined by MALDI-TOF mass spectrometry (Burker Franzen Analytiik).

#### Animals and tissue samples

Purpose-bred female rhesus macaques (*Macaca mulatta*) obtained from a supplier in China (4–6 years old) were used for this study. This study (permission no. 19-137) was approved and conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

#### Inoculation of TGDK

Rhesus macaques were fasted overnight. They were then inoculated with 1 ml of FITC-labeled TGDK solution (100 nmol) or 0.5 ml of 10 nm gold-labeled TGDK at a site in the ileum (15 cm from the cecum) after ceiliotomy under anesthesia induced by a s.c. injection of urethane (ethyl carbamate, 800 mg/ml; 1.5 ml/kg body weight; Wako Pure Chemical) solution and an i.v. injection of α-chloralose (Wako Pure Chemical; 20 mg/ml; 5.5 ml/kg body weight) into the cecal vein.

#### Inoculation of poliovirus (PV)

Rhesus macaques were fasted overnight. They were then inoculated with PV solution at a site in the ileum (15 cm from the cecum), as described previously (27).

#### Collection of PPs

The rhesus monkeys were euthanized by exsanguination under anesthesia, and the part of the ileum (15 cm from cecum) including the inoculation site was collected. After washing the collected part of the ileum, the blocks of PPs were embedded in the OCT compound (Sakura Finetechical) for immunofluorescence staining or fixed in ice-cold 3% glutaraldehyde/0.1 M sucrose/PBS (pH 7.4) for transmission electron microscopy and energy-dispersive x-ray spectroscopy.

### Histopathological study

Tissue samples were fixed in 10% neutral buffered formalin and were trimmed, embedded in paraffin, sectioned, stained with H&E, and examined by light microscopy.

#### Immunofluorescence staining

To examine the binding and tissue localizations of TGDK, 5 μm frozen sections derived from rhesus macaques inoculated with FITC-labeled TGDK solution were fixed in cold acetone and blocked with 1% nonfat skim milk in PBS*. FITC-labeled TGDK was detected using a rabbit anti-FITC Ab (Zymed Laboratories) for signal amplification and a tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-rabbit IgG Ab or an Alexa488-labeled anti-rabbit IgG Ab as a secondary Ab. To further examine how TGDK is incorporated into the lymphoid organ, the sections were stained with a PE-labeled anti-CD20 Ab (BD Biosciences) or an anti-CD54 Ab (R&D Systems) labeled with Alexa555 using a Zenon mouse IgG labeling kit (Invitrogen). To investigate whether gp2 was expressed in rhesus PP M cells, the sections were pretreated with 0.1% Triton X-100, which is used to solubilize the mucus, and then stained with an anti-PV Ab (II-MAP-01; Japan Poliomyelitis Research Institute) labeled with Alexa488 using a Zenon mouse IgG labeling kit (Invitrogen Corporation) or a rabbit anti-gp2 Ab (IMGENEX) labeled with Alexa555 using a Zenon rabbit IgG labeling kit (Invitrogen). To further examine how FITC-labeled TGDK specifically binds to PP M cells, the sections were also pretreated with 0.1% Triton X-100 and then stained with a rabbit anti-FITC Ab and Alexa488-labeled anti-rabbit IgG Ab as a secondary Ab or a rabbit anti-gp2 Ab (IMGENEX) labeled with Alexa555 using a Zenon rabbit IgG labeling kit (Invitrogen). Some sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to show nuclei.

After the staining, slides were washed and analyzed with a Keyence Biozero BZ-8000 (Keyence) and a Flowview FV3000 (Olympus).

#### Transmission electron microscopy (TEM) and energy-dispersive x-ray spectroscopy (EDS)

Tissue samples were rinsed in PBS with 0.1 M sucrose (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 2 h. All the samples were rinsed briefly with 50:50, 70:30, 80:20, 90:10, and 95:5 ethanol/water mixtures and 100% ethanol for 10 min each and three times with 100% ethanol for dehydration, and then embedded in epoxy resin (Quatol 812). One-micrometer sections were cut using a glass knife and then stained with toluidine blue. Suitable areas for ultrastructural study were chosen after examining 1-μm sections under a light microscope. Sections of 60–90 nm were cut on a Leica EM UC6 ultramicrotome using a diamond knife, and sections were mounted on a copper grid and stained with 1% uranyl acetate and Reynolds lead citrate. The grids were examined under a JEOL JEM 1200-EX electron microscope. Furthermore, EDS, which was consigned to JOEL Datum, was performed to quantify TGDK by measuring gold concentration within a specimen.

#### Preparation of TGDK-conjugated multiantigens

To examine the in vivo effect of TGDK on M cell targeting, TGDK was conjugated via a Habantigen with rhesus CCR5-derived cyclopeptide (rcDDR5) and BSA. To prepare a Habantigen, an eight-arm functional polyethylene glycol (PEG) with n-nitrophenyl groups, SUNBRIGHT HGE0-200NP (NOF Corporation; 1 equivalent), was mixed with an eight-arm functional PEG with primary amino groups, SUNBRIGHT HGE0-200PA (NOF; 7.2 equivalent), in DMF for 16 h. The resulting Habantigen was dialyzed in Spectrapore dialysis bags (Spectrum Laboratories; molecular weight cut off = 12–14 kDa) against Milli-Q water for 2 days. The dialysate was lyophilized and used as a Habantigen. To prepare rcDDR5, a rhesus CCR5-derived linear dodecapeptide (H,N-KRRQRELQHYTGGCOOH), in which all side-chain groups are protected, was synthesized using an automatic peptide synthesizer, and cyclized, as previously described (28). To bind both TGDK and rcDDR5 to the Habantigen, the amino group of ethylenediamine in TGDK (two equivalents) or of Lys in the deprotected rcDDR5 (two equivalents) was conjugated with a four-arm functional PEG, SUNBRIGHT PTE-100NP (NOF; 1 equivalent). Finally, the Habantigen (168 mg) was coupled to the four-armed PEGylated TGDK (12 μmol) and -rcDDR5 (12 μmol) in DMF for 24 h and then covalently bound to BSA for 6 h. The TGDK-conjugated Ag was dialyzed for 15 h against PBS*, and the dialysate was lyophilized with lactose. The resulting Ag was encapsulated in enteric-coated capsules and included TGDK (56 nmol/capsule), rcDDR5 (90 nmol/capsule), lactose (146 μmol/capsule), and BSA (4.5 nmol/capsule). In contrast, a control Ag also included BSA (4.5 nmol/capsule), but it was not conjugated via a covalent
bond with a Hubtagent. Furthermore, the control Ag did not include TGDK and rcDDR5.

**Immunization schedule**

All of the rhesus macaques were housed in individual cages and maintained in accordance with the rules and guidelines of the National Institute for Infectious Diseases for experimental animal welfare. Five 4- to 6-year-old rhesus macaques (no. 6–10) were orally administered with two enteric-coated capsules containing TGDK-conjugated multiantigens at 0, 2, and 6 wk. Another five rhesus macaques (no. 1–5) were immunized with an enteric-coated capsule including control Ag following the same immunization schedule as that for the controls. Stool samples were obtained at 0, 12, 13, and 14 wk postinitial immunization (wpim), which were then subjected to anti-BSA Ab ELISA and rcDDR5-coupled multipin ELISA in accordance with the method of Misumi et al. (29).

**Sample collection and processing**

Acetone powder was prepared by adding fecal pellets (3 g) with stirring to 3 ml of cold acetone. The powder was then washed three times with cold ether and dried until no trace of ether remained. The acetone powder (100 mg) was resuspended in 400 μl of 1% MPC polymer solution (NOF) and incubated at 37°C for 30 min, and then on ice for 1 h. The suspensions were centrifuged at 13,000 × g for 5 min to remove fecal solids. The processed fecal Ab samples were subjected to anti-BSA Ab ELISA and rcDDR5-coupled multipin ELISA.

**Anti-BSA Ab ELISA**

Abs against BSA in stool samples were detected by ELISA. For evaluation of stool Abs, each well of a flat-bottom 96-well maxisorp microplate (Nunc) was coated with 50 μl of coating buffer (pH 8.0) containing BSA (100 μg/ml) and incubated at room temperature (RT) overnight. The wells were washed with 150 μl of Milli-Q water at 0.1% Tween 80 with complete washing and rinsed with Milli-Q water. Subsequently, 100 μl of blocking buffer, composed of 1% skim milk in Milli-Q water, was added to each well and incubated for 2 h at RT to occupy all unbound sites. Washing was repeated, as described above, followed by the addition of 50 μl of a fecal Ab sample diluted 1/10 in PBS* to each well. Plates were incubated for 2 h at RT and then washed with 0.1% Tween 80, and 50 μl of peroxidase-conjugated goat anti-mouse IgA (diluted 1/5000) was added to each well and incubated for 1 h at RT before the plate was washed. Fifty microliters 3,3`,5,5`-tetramethylbenzidine solution (Wako Pure Chemical) as the substrate was added to each well and incubated at room temperature. Absorbance was measured at 450/630 nm using a microplate reader.

**rcDDR5-coupled multipin ELISA**

A rhesus CCR5-derived linear dodecapeptide (H,N-ERSQREGHLHTY-GOOGOH) in which all side-chain groups are protected was synthesized using an automatic peptide synthesizer and was cyclized by bond formation between the α-carboxyl group of Gly and the α-amino group of Glu after removal of the protecting group of the resin. The γ-carboxyl group of Glu in the protected cyclic dodecapeptide was conjugated to MultiPin block (Mimotopes). The block was used for detecting anti-CCR5 Abs in stool samples in accordance with the method of Misumi et al. (28).

**Determination of total number of SIV DNA copies**

The total number of SIVmac239 DNA copies was determined to monitor SIV infection and estimate the neutralizing activity of antisera. The relative change in the number of SIV DNA copies indicates the degree of neutralization activity. Percentage of copies in HSC-F infected with SIVmac239 (50 ng, measured using p27 master mix containing modified DyNAamo hot start DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM MgCl₂, a dNTP mix including dUTP (Finnzymes), 2 μl of each primer, and 8 μl of cDNA. PCR was conducted as follows: initial activation of hot start DNA polymerase at 95°C for 15 min; 40 cycles of four steps of 95°C for 10 s, 57°C for 20 s, 72°C for 20 s, and 76°C for 2 s. At the end of the amplification cycle, melting temperature analysis was conducted by gradually increasing temperature (0.5°C/s) to 95°C. Amplification, data acquisition, and analysis were conducted with the DNA Engine Opticon 2 System (Bio-Rad) using Opticon Monitor version 3.0 software.

**In vitro human M cell model**

The human M cell model was constructed using cocultures of Caco-2 cells and Raji cells in accordance with the method of Kernerés et al. (32) with slight modification. We seeded Caco-2 cells by adding 1 × 10⁶ cells on the lower face of 3-μm-pore Transwell filters and culturing them overnight. The filters were then transferred to the Transwell device with the epithelial cells facing the lower chamber of the cluster plates. Caco-2 cells were cultured until they were fully differentiated (21 days). Raji B cells (10⁵) were added to the upper chamber facing the basolateral side of Caco-2 cells. The cultures were maintained for 3 days. Caco-2 cell monolayers were washed with PBS and incubated with FITC-labeled TGDK or FITC-labeled γ-linseye dendrimer with or without PV (type II, 10⁷–10⁸ cell culture infective doses 50%) for 30 min. To examine the localization of PV and M cell makers (i.e., gp2, CD54, and integrin β₁), the monolayers were stained with an anti-PV Ab (II-MAP-01; Japan Poliomyelitis Research Institute), an anti-gp2 polyclonal Ab (IMGENEX) labeled with
FIGURE 2. Association of TGDK with rhesus PP FAE and accumulation of TGDK within the GCs. A. Schematic diagram of rhesus ileum. B and C, H&E staining of rhesus PPs. D–F, FITC-labeled TGDK was inoculated into the lumen of the ileum at 15 cm from the ileocecal valve. One hour after TGDK inoculation, the portion between the injection site of TGDK and the ileocecal valve was excised and subjected to immunofluorescence analysis. Frozen sections of rhesus macaque PPs were labeled with mAbs (anti-CD20 Ab (I) and anti-CD54 Ab (M); red), and TGDK was stained, as described in Materials and Methods (red or green) (D–F, H, and L). Differential interference contrast (DIC) (G and K), merged (J and N), and control (O, Alexa488-labeled anti-rabbit IgG, or P, TRITC-labeled anti-rabbit IgG) images are shown.
FIGURE 3. TGDK can efficiently penetrate into rhesus PP M cells. PV, FITC-labeled TGDK, or 10-nm gold-labeled TGDK was inoculated into the lumen of the ileum, as described in Materials and Methods. After the inoculation, the portion between the inoculation site and the ileocecal valve was excised and subjected to electron microscopies (A, B, I, and J), immunofluorescence analysis (C–H), or EDS (K and L). A, TEM image of typical M cells in the PP. B, TEM image of B shown in A. Inset (the magnified TEM image of dotted square in B), PV is transcytosed through rhesus PP M cells. C–H, Frozen sections of rhesus macaque PPs were labeled with mAbs (anti-PV Ab (C) and anti-gp2 Ab (D and G)), and TGDK were stained, as described in Materials and Methods (F). Merged images are shown (E and H). I, TEM view of rhesus PP M cells, which had short (arrowhead) and irregular microvilli and pocket structures containing lymphocytes (indicated by L). J, Depicts a higher-magnification image of I. K and L, Graphs show EDS of spots C and D in J, confirming the presence of gold-labeled TGDK. The a signals come from Cu (8.904 keV), which is attributed to the sample holder, and the b signal contains the signal of OsLβ (10.354 keV).
Alexa555 using a Zenon mouse IgG labeling kit (Invitrogen), a mouse anti-human CD54 Ab (R&D Systems), or a mouse anti-human integrin α5β1 Ab (Chemicon International), and then incubated with or without TRITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). At the end of the staining, slides were washed and incubated with DAPI for nuclear staining. Finally, the monolayers were washed and analyzed with a Keyence Biozero BZ-8000. To investigate whether TGDK was specifically transported in human M cell model, the assay was conducted in the presence of 100 μM FITC-labeled TGDK or FITC placed in the lower chamber. The monolayers including M cells or Caco-2 control monolayers were incubated for 2 h at 37°C. FITC-labeled TGDK or FITC, transported from the lower chamber to the upper chamber, was quantified using a CORONA Multi Microplate Reader.

Results
Synthesis of TGDK
UEA-1, an α-1-fucose-specific lectin, has been of particular interest owing to its M cell specificity in the mouse model and its applicability to proof-of-concept studies of vaccine delivery to APCs (33). However, lectins are susceptible to proteolytic degradation in the gastrointestinal tract, and their cytotoxic effects also limit their use as targeting agents to deliver vaccines to M cells. One approach to overcoming these limitations is to synthesize small organic molecules that are able to mimic the function of lectins. Lambkin et al. (26, 34) reported that a synthetic multivalent polygalloyl derivative (SMPD) is a competitor of UEA-1 and appears to have high affinity for the fucose receptor on murine M cells. Its advantages include its stability and suitability for incorporation into delivery systems using routine chemical procedures. In this study, we also chose a versatile scaffold with branched α-lysine (Fig. 1A). An aminomethyl group was introduced into the lysine dendrimeric scaffold to allow its linkage with a candidate immunogen. Furthermore, gallic acid was selected as a polyphenolic functional group and coupled with α- and ε-amino groups of lysine residues in the lysine dendrimer (Fig. 1B). As shown in Fig. 1C, the spectra of purified (3,4,5-trimethoxybenzoyl)α-lysine dendrimer and TGDK exhibited major peaks at m/z 1222.0 (Fig. 1C, upper spectrum) and 1054.2 (Fig. 1C, lower spectrum), respectively. The difference in molecular mass indicates the complete deprotection of the methyl group by boron tribromide.

TGDK transport through follicle-associated epithelium (FAE) of rhesus macaque PPs
To examine whether TGDK was effectively transported through FAE of PPs in vivo, FITC-labeled TGDK was inoculated into the rhesus macaque ileum (Fig. 2A). One hour after the injection, the tissue was subjected to immunofluorescence analysis. As shown in Fig. 2B and C, the large PP is found in the lumen of the terminal ileum in rhesus macaques. Light microscopy revealed the typical structure of a mucosal lymphoid follicle, composed of germinal centers (GCs) and a dome area bulging into the lumen (Fig. 2B and C). The closer the section to the injection site, the larger the amount of TGDK reaching GCs through FAE of PPs (Fig. 2D–F). When PPs from different sections (~7.5 cm from the injection site) were further observed, TGDK was clearly detected in the GCs of PPs (Fig. 2H and L). GC cells can be stained with anti-CD20 and anti-CD54 Abs, the staining patterns reflecting reactivity with B cell lymphocytes and the follicular dendritic reticulum in nonhuman primates and humans, respectively (35–37). As shown in Fig. 2, I and M, CD20 and CD54 were expressed within the GCs of rhesus macaques. Fig. 2J and N, shows the patterns of double fluorostaining for TGDK/CD20 or TGDK/CD54 in the GCs of PPs. These results indicate that TGDK efficiently enters into GCs through FAE of PPs in rhesus macaques.

FIGURE 4. Oral immunization schedule and detection of anti-BSA or anti-rcDDR5 Abs in rhesus macaques. A. Immunization schedule for rhesus macaques. Five rhesus macaques (no. 6–10) were orally immunized at 0, 2, and 6 wk with TGDK-conjugated multiantigens. Another five macaques (no. 1–5) were immunized with the control Ag. Stool sampling was performed at 12, 13, and 14 wpim. Stool samples (1/10 dilution) obtained after immunization with TGDK-conjugated multiantigen (B; macaque no. 6 (○), 7 (△), 8 (■), 9 (▲), and 10 (×)) or control Ag (C; macaque no. 1 (○), 2 (△), 3 (■), 4 (▲), and 5 (×)) were examined to investigate whether the anti-BSA mucosal IgA (B and C) or anti-rcDDR5 mucosal IgA (D and E) can be raised in rhesus macaques by anti-BSA ELISA and the rcDDR5-coupled multipin ELISA, as described in Materials and Methods. ○, Show the average OD values. F. Furthermore, the inhibitory effect of the stool samples from vaccinated (no. 6–10) and control groups (no. 1–5) on in vitro SIV infection was also investigated, as described in Materials and Methods. Percentage of copies in HSC-F infected with SIVmac239 in the presence of the 14-wpim stool sample (■) is expressed relative to that in the presence of the 0-wpim stool sample (○), which is considered 100%.

Internalization of TGDK by PP M cells
To investigate whether rhesus macaque PP M cells can specifically take up TGDK, the rhesus macaque ileum was inoculated with FITC-labeled TGDK and subjected to immunofluorescence...
fluorostaining for TGDK/DAPI. gp2/DAPI, CD54/DAPI, and integrin β2/DAPI, Abs (G, J, and M). Show the patterns of double fluorostaining for the FITC-labeled D-lysine dendrimer (A, C, F, I, and L, green) or the FITC-labeled n-lysine dendrimer (B, green), as described in Materials and Methods. Because it is known that PV can bind to M-like cells, the Caco-2/Raji B monolayer was labeled with an anti-PV Ab (D, red). The monolayer was further labeled with mAbs (anti-gp2 (G), anti-CD54 (J), and anti-integrin β2 Abs (M), red). A and C. Show the merge image of DIC and a fluorescence image of FITC-labeled TGDK. B. Shows the merge image of DIC and a fluorescence image of FITC-labeled n-lysine dendrimer. D. Shows the merge image of DIC and a fluorescence image of monolayer labeled with an anti-PV Ab. F. I, and L. Show the patterns of double fluorostaining for TGDK/D-labeled TGDK was colocalized with gp2. These results indicated that gp2 was expressed in rhesus PP M cells in FAE of PPs. Taken together, these results indicate that inoculation of TGDK into the rhesus macaque ileum containing PPs shows the clear targeting of M cells and transcytosis of TGDK, and rhesus macaque PP M cells have the ability to take up TGDK from the lumen.

Immunization of rhesus macaques with TGDK-conjugated multiantigens and induction of BSA- and rcDDR5-specific Abs

To examine the in vivo effect of TGDK on M cell targeting in nonhuman primates, five rhesus macaques were immunized with TGDK-conjugated multiantigens containing BSA and rcDDR5 by oral administration according to the time schedule shown in Fig. 4A. Another five rhesus macaques were immunized with the control Ag. Although BSA-specific IgA in stool samples was significantly induced in the group immunized with TGDK-conjugated multiantigens at 14 wpim (Fig. 4B; p < 0.05 in repeated measures ANOVA), the immunization with the TGDK-conjugated multiantigen unfortunately induced only weak anti-BSA Ab responses. The result suggests that the induction of a stronger anti-BSA Ab response may be required for a higher content of BSA in the TGDK-conjugated multiantigen. In contrast, the immunization of rhesus macaques with the control Ag did not induce BSA-specific IgA in stool samples (Fig. 4C).

Our previous studies demonstrated that the rhesus macaque antiseras raised against cDDR5 mimicking the conformation-specific domain of human CCR5 reacted with both human and macaque CCR5s, and potently suppressed infection by the R5 HIV-1 laboratory isolate (HIVJRFL). R5 HIV-1 primary isolates (clade analysis (Fig. 3). Recently, Terahara et al. (38) reported that gp2 is specifically expressed at a high level in mouse PP M cells. However, the expression of gp2 in rhesus PP M cells remains to be clarified in rhesus macaque. Sicinski et al. (39) reported that PV is found specifically adhering to the surface projections of M cells and in vesicles in M cells. Fig. 3A shows a typical TEM image of PP M cells. As expected, PV was transcytosed through typical intestinal rhesus PP M cells (Fig. 3B). Therefore, we confirmed whether gp2 was expressed in rhesus PP M cells, in which PV was transcytosed. Immunofluorescence analysis demonstrated that gp2 was expressed in rhesus PP M cells (Fig. 3D), which were recognized by the anti-PV Ab (Fig. 3C). These results indicated that gp2 was expressed in rhesus PP M cells. To further investigate whether TGDK specifically binds to PP M cells, the localization of TGDK was confirmed by counterstaining with the anti-gp2 Ab. As shown in Fig. 3, F and G, TGDK was colocalized with gp2. These results indicated that TGDK specifically binds to rhesus M cells.

To investigate whether TGDK is transcytosed through PP M cells, the rhesus macaque ileum was inoculated with gold-labeled TGDK and subjected to EDS (Fig. 3). The advantage of EDS is that gold-labeled TGDK can be directly detected when TGDK is completely embedded in an ultrathin section. The characteristic x-ray peak from gold (AuL-α: ∼9.712 keV) is used to confirm the presence of nano-gold particles in the cytoplasm of a PP M cell within a section (Fig. 3F). EDS demonstrated the presence of gold-labeled TGDK in the cytoplasm of a PP M cell (spot C, Fig. 3, J and K), but not in the cytoplasm of a lymphocyte within a PP M cell (spot D; Fig. 3, J and L). In contrast, gold-labeled TGDK was not detected in intestinal epithelial cells in FAE of PPs. Taken together, these results indicate that inoculation of TGDK into the rhesus macaque ileum containing PPs shows the clear targeting of M cells and transcytosis of TGDK, and rhesus macaque PP M cells have the ability to take up TGDK from the lumen.
SHIVSF162P3 bulk isolate in vitro (29). In addition, our recent data demonstrate that mouse anti-recDDR5-specific IgG can inhibit in vitro SIV infection (40). Therefore, the stool samples from the group immunized with TGDK-conjugated multiantigens were also examined by recDDR5-coupled multipin ELISA to determine whether recDDR5-specific mucosal IgA was induced. As shown in Fig. 4D, recDDR5-specific IgA in stool samples was significantly induced in the group immunized with TGDK-conjugated multiantigens. In contrast, the immunization of rhesus macaques with the control Ag did not induce recDDR5-specific IgA in stool samples (Fig. 4E). Although we investigated whether recDDR5-specific mucosal IgM or IgG was also induced, the recDDR5-specific IgM or IgG in stool samples was not significantly induced (data not shown). To further assess neutralizing activity, we performed an in vitro neutralization assay using SIVmac239. Interestingly, neutralizing activity tended to increase with the titer of the anti-recDDR5 IgA in stool samples (Fig. 4F). These data suggest that TGDK-mediated vaccine delivery system represents a potential approach to develop M cell-targeted mucosal vaccines.

Selective binding and transcytosis of TGDK in human in vitro M cell model

Although TGDK promises to be an M cell targeting molecule in nonhuman primates, it still remains unclear whether TGDK effectively targets human M cells. Kernéis et al. (32) developed an in vitro human M cell model that is useful for facilitating the design of oral vaccines and efficient mucosal drug delivery systems. Therefore, we evaluated whether TGDK selectively binds to human M-like cells and is capable of transcytosis. As shown in Fig. 5, A and B, the spotlike staining of the apical surface of epithelial cells in the model with the FITC-labeled TGDK was observed, but hardly in the model with the FITC-labeled d-lysine dendrimer. Because Sicinski et al. (39) demonstrated that PV enters the human host through intestinal M cells, we examined whether TGDK can colocalize with PV on the apical surface of the model (Fig. 5, C–E). Immunofluorescence analysis demonstrated that M cells in the model showed colocalization of TGDK and PV. Furthermore, because human M cells express gp2, CD54, and α5β1 integrin on their surface, the apical surface of epithelial cells in the model was also stained with the anti-gp2, anti-CD54, or anti-α5β1 integrin Ab (Fig. 5, G, J, and M). Fig. 5, G, J, and M, shows that gp2, CD54, and α5β1 integrin are expressed on the apical surface. Fig. 5, H, K, and N, shows the patterns of triple fluorostaining for TGDK/gp2/DAPI, TGDK/CD54/DAPI, or TGDK/α5β1 integrin/DAPI in this model.

Transcytotic activity was also monitored for 120 min at 37°C. TGDK was effectively transported at 37°C through the monolayers containing M cells, but not through control monolayers (Fig. 5O; p < 0.05 in Mann-Whitney U test). To further confirm TGDK-mediated Ag transport, we investigated whether FITC-labeled TGDK is more efficiently transported than FITC, which is postulated as an Ag. As shown in Fig. 5P, FITC-labeled TGDK is more efficiently transported than FITC (p < 0.05 in Mann-Whitney U test). These results indicate that TGDK can significantly bind to human M cells and is capable of transcytosis through M cells in inductive sites, such as PPs.

Discussion

The sexual route is the most important route of HIV transmission in heterosexuals, in which the genital tract provides the virus access to lymphoid cells. In the majority of patients, the initial acquisition of HIV involves passage of the virus across a mucosal surface. Thus, blocking HIV mucosal transmission is key to prevent HIV infection.

Some studies demonstrated that mucosal Ab responses may contribute to the apparent resistance to HIV-1 infection. The studies, in which humoral and cellular responses against HIV-1 in the vaginal secretions of women who remain uninected despite frequent unprotected sex with HIV-1-infected partners were analyzed, indicated the presence of mucosal IgA Abs to HIV-1 (44–46). Furthermore, the second type of natural resistance is found in persons with CCR5-specific mucosal autoantibodies (47). To attempt to reproduce some of the functional aspects of this natural resistance to HIV infection, many researchers have examined various types of mucosal vaccine candidate against SIV/SHIV infection because they are capable of inducing not only the immunity at the mucosal sites of transmission, which prevents the virus from gaining entry into immune cells, but also the immunity in the systemic circulation.

Could CCR5 be an attractive target for the development of mucosal vaccines? Persons with the homozygous 322 CCR5 mutation, a 32-bp deletion of the CCR5 gene that results in a lack of cell surface expression of CCR5, have strongly reduced susceptibility to CCR5-dependent HIV-1 infection (48–50). Furthermore, Pastori et al. (51) reported that long-lasting CCR5 internalization by anti-CCR5 Abs in a subset of long-term nonprogressors is associated with a possible protective effect against disease progression, suggesting that induction of anti-CCR5 Abs by a vaccine can reproduce the immune status in long-term nonprogressors. Our previous study demonstrated that the high induction of the anti-CCR5 Ab can suppress viral propagation during acute HIV-1 i.v. transmission in cynomolgus macaques i.p. and s.c. immunized with cDDR5 mimicking the conformation-specific domain of human CCR5 (29). In this study, recDDR5 was synthesized to induce more specific anti-rhesus CCR5 Abs. Our recent study demonstrates that the immunization of recDDR5-conjugated KLH induces mouse anti-recDDR5-specific IgG that specifically binds to rhesus CCR5 and inhibits in vitro SIV infection (40). These observations suggest that CCR5 can be an attractive target for the development of mucosal vaccines. Hence, to reproduce the functional aspects found in long-term nonprogressors with CCR5-specific mucosal autoantibodies, we sought new types of vaccine delivery system for the effective delivery of recDDR5 to mucosa-associated lymphoid tissues such as PPs, the inductive site for the induction of the Ag-specific immune response.

Lectins have been investigated for targeted Ag delivery to mucosa-associated lymphoid tissues. UEA-1 and Aleuria aurantia lectin have high specificity for the carbohydrate moiety α-L-fucose located on the apical membranes of mouse M cells (19, 21, 52). There have been successful efforts made in vivo targeting in mouse M cells by conjugating UEA-1 to polymerized liposomes (33) and latex particles (53), or by coating poly(D, L-lactide-co-glycolide) particles with the A. aurantia lectin (52). However, lectins are of limited value in vaccine delivery owing to their toxicity to humans or sensitivity to intestinal degradation. To overcome these limitations, SMPDs that appear to have high affinity for the fucose receptor on murine M cells were identified from a high-throughput screening of mixture-based compound libraries in a competitive UEA-1-binding assay (26). Although SMPDs may have the potential in oral vaccine targeting in mouse model, it remained unclear whether SMPDs effectively target nonhuman and human M cells.

The macaque model serves several important purposes in current HIV vaccine research. It allows analysis of vaccine safety and proof of immunogenicity in a species more closely related to humans. Furthermore, there are a few interesting options for testing
the effect of a vaccine on a mucosal pathogenic challenge system, such as SIV or SHIV challenges. Therefore, we investigated the M cell targeting potential of a tetragalloyl derivative, TGDK, in an in vivo nonhuman primate model. Our findings suggest that TGDK can serve as a useful targeting molecule for nonhuman primate M cells (Fig. 3). Interestingly, TGDK accumulated in GCs after it transcytosed through M cells from the gut lumen (Fig. 2, D–F). Although the mechanism underlying the behavior of TGDK in PP still remains to be elucidated, the ability of TGDK to accumulate in GCs may increase the possibility of interaction of an immunogen, in the form of an immune complex trapped on follicular dendritic cells, with GC B cells.

To assess the efficiency of TGDK as a mucosal delivery system, it is important to examine whether rcDDR5-specific Abs in mucosal secretions are induced in nonhuman primate models. It is generally difficult to induce a long-lasting anti-CCR5 Ab response because CCR5 is continuously exposed to the immune system. Our previous study showed that immunization with cDDR5-MAP induces anti-CCR5 serum production for ~15 wk after the third immunization, although the titer of anti-CCR5 sera declined over time until 21 wpim (29), suggesting that cDDR5-MAP is not suitable as a model Ag for the estimation of TGDK owing to its weak immunogenicity. Therefore, we introduced BSA as a standard model Ag into TGDK-conjugated multiantigens with rcDDR5 via the Hubantigen to estimate the efficiency of TGDK. Furthermore, our recent data indicate that anti-rcDDR5 serum is produced for more than 56 wk when rhesus macaques were immunized with the TGDK-conjugated multiantigens containing rcDDR5 and BSA without a specific adjuvant (our unpublished data), suggesting that the immunogenicity of TGDK-conjugated multiantigens used to evaluate the efficiency of TGDK was improved. Five animals were orally immunized with the TGDK-conjugated multiantigens (vaccinated group), and another five were immunized with the Hubantigen and BSA only (control group). Although the immunization with the TGDK-conjugated multiantigens induced only weak anti-BSA Ab responses in the vaccinated group (Fig. 4B; p < 0.05), rcDDR5-specific IgA in stool samples was significantly induced in the vaccinated group, as shown in Fig. 4D. Furthermore, the neutralizing activity tended to increase with the titer of the anti-rcDDR5 Ab in the stool samples (Fig. 4F). Taken together, these results show that TGDK is useful for inducing rcDDR5-specific mucosal IgA responses with neutralizing activity, although it is necessary to re-examine the dose of the orally administered TGDK-conjugated multiantigens for the perfect reproduction of the functional aspects found in long-term nonprogressors with CCR5-specific mucosal autoantibodies.

Finally, we examined whether TGDK can be available for human use by using the human in vitro M cell model. Giammasca et al. (54) reported that the UEA-1 receptor is not expressed in human faeces. Because it further remained unclear whether the binding receptor of UEA-1 is completely the same as that of TGDK, we examined whether TGDK was directly capable of binding to human M-like cells. As shown in Fig. 5, TGDK can specifically bind to human M-like cells and transcytose through M-like cells. These results suggest that the TGDK-mediated vaccine delivery system can be available for mucosal vaccine delivery in humans.

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Disclosures
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