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RESEARCH PAPER

Enhancement of Nasal HIV Vaccination with Adenoviral Vector-Based Nanocomplexes Using Mucoadhesive and DC-Targeting Adjuvants

Yuhong Jiang • Man Li • Zhirong Zhang • Tao Gong • Xun Sun

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ABSTRACT

Purpose To investigate the vaccine effect of a replication-defective recombinant adenovirus 5 (rAd5)-based nanocomplex with chitooligosaccharides (Oligo) and mannosylated polyethyleneimine-triethyleneglycol (mPEI) as adjuvants for human immunodeficiency virus (HIV) infection.

Methods Physical characteristics were determined through detecting the size, zeta potential and morphology of Oligo-mPEI-rAd5 nanocomplex, and *in vitro* vaccine uptake and transduction efficiency were estimated. Nanocomplexes were then administered intranasally to Balb/c mice to evaluate *in vivo* rAd5 residence in nasal cavity and HIVgag-specific immune responses using cytotoxic T lymphocyte (CTL), intracellular cytokine staining (ICS) and ELISA assay.

Results The mucoadhesivity of Oligo prolonged nasal residence time, while the dendritic cell (DC) specificity of mPEI improved vaccine uptake. These two adjuvants jointly enhanced transduction efficiency of rAd5. Oligo-mPEI-rAd5 nanocomplex elicited potent HIVgag-specific CTL response and increased IFN-γ positive $CD8^+$T and IL-4 positive $CD4^+$T cells, indicating high cellular immune responses. This vaccine candidate also led to strong humoral immune responses (IgG/IgG1/IgG2a) with balanced Th1/Th2 $CD4^+$T cell activity. Moreover, mice nasally immunized with Oligo-mPEI-rAd5 showed higher levels of SIgA in nasal washes than did mice immunized with rAd5.

Conclusions Intranasal delivery of Oligo-mPEI-rAd5 with a prime-boost regimen is a potential immunization for HIV infection, inducing HIVgag-specific cellular, humoral and mucosal immune responses.

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KEY WORDS adenovirus · chitooligosaccharides · human immunodeficiency virus · intranasal vaccine · mannosylated polyethyleneimine

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BMDCs	Murine bone marrow-derived DCs
BSA	Bovine serum albumin
CFSE	Fluorescent dye carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIV	Human immunodeficiency virus
ICS	Intracellular cytokine staining
mPEI	Mannosylated polyethyleneimine-triethyleneglycol
Oligo	Chitooligosaccharides
PBS	Phosphate buffered saline
rAd5	Replication-defective recombinant adenovirus 5
TEG	Triethyleneglycol
TEM	Transmission electron microscopy
β-gal	β-galactosidase

INTRODUCTION

The human immunodeficiency virus (HIV) epidemic has become one of the most serious global health and development challenges (1). More than 25 million people have died of acquired immunodeficiency syndrome (AIDS) since it was first discovered in 1981, and 35.3 million worldwide were living with HIV in 2012. Recent studies have shown replication-defective recombinant adenovirus (rAd5) was considered as a candidate vector of HIV vaccine for its safety, tolerability, and

immunogenicity (2). In light of the outstanding natural advantages for eliciting immune responses, a rAd5-based vaccine through pharmaceutical modifications, such as the addition of adjuvants or change in administration route, is worthy to be investigated for HIV infection. Additionally, rAd5 encoding simian immunodeficiency virus (SIV)-gag vaccine has been shown to induce potent gag-specific $CD8^+$T responses (3,4). On the other hand, rAd5-based vaccines suffer the major disadvantage that the prevalence of preexisting immunity to Ad5 in the general population, particularly when vaccines are administered intramuscularly (5). A single intramuscular immunization of mice with rAd5 vector failed to generate persistently high antigen-specific $CD8^+$T responses, and performing a second administration impaired the first immune responses due to the preexisting immunity against the rAd5 vector (6). To avoid this problem, a mucosal HIV vaccine has been developed to bypass preexisting anti-Ad5 immunity and improve the HIV vaccine efficacy (7,8).

Intranasal vaccination is a non-invasive approach that can simultaneously induce both systemic and mucosal immunity (9,10). Indeed, the rAd5 vaccine boosted with the same construct by nasal route has been also shown to avoid the preexisting immunity to vector and enhance immune responses (11). These studies suggest that nasal delivery of a rAd5-HIVgag vaccine with a prime-boost regimen may be a more effective alternative for HIV vaccination. However, the mucociliary clearance and low antigen uptake characteristic of the nasal mucosa can reduce the efficacy of intranasal vaccines, rendering them less immunogenic than when administered intramuscularly (12).

Functional materials have been used to overcome barriers to intranasal administration. Chitosan is widely used for mucosal drug administration, and it has been shown to exhibit low toxicity, mucoadhesivity and biodegradability. However, at physiological pH values, chitosan is poorly soluble and tends to aggregate; to avoid this problem, chitosan derivatives known as chitooligosaccharides (Oligo) have been designed. Well-defined chitooligosaccharides (Oligo) have been proposed as an excellent mucoadhesive enhancer for various applications, including drug delivery (13,14). Indeed, chitooligosaccharides (Oligo) have been used as gene delivery vehicles to significantly improve gene transfer efficacy (15,16). Additionally, the cationic polymer polyethyleneimine (PEI), a well-established vehicle, was recently reported to stimulate mixed T cell responses in mucosa and to be a potent adjuvant for eliciting immune responses without also activating proinflammatory pathways (17–19). In our previous study, cross-linked low molecular weight PEI with Ad vectors significantly improved transduction efficiency, which was more pronounced than PEI25K *in vitro* and *in vivo* (20). In addition to its ability to enhance gene transduction efficiency and immune responses, PEI can be modified to target DCs which are professional antigen-presenting cells that express high levels of surface mannose receptors. Therefore, we combined PEI2000 and PEI600 with triethyleneglycol (TEG) to synthesize biodegradable PEI-TEG which was then linked to mannose and investigated as a carrier for DNA vaccine. The resulting mannosylated PEI-TEG (mPEI) could target dendritic cells (DCs) and exhibit a lower cytotoxicity than PEI25K (21).

The present study developed a HIV vaccine candidate for intranasal delivery by combining the latest advances in adjuvant technology to create mucoadhesivity and DC targeting. Chitooligosaccharides (Oligo) and mPEI mannosylated PEI-TEG formed a cationic layer that would coat the negatively charged rAd5 vector, thereby promoting binding and uptake of the complexes to anionic nasal mucosa (22). Subsequent expression of HIVgag encoded by nanocomplexes should then lead to a series of specific immune responses (Fig. 1). In our study, we constructed three kinds of nanocomplexes, *i.e.* Oligo-rAd5, mPEI-rAd5 and Oligo-mPEI-rAd5 nanocomplex to compare Oligo-mPEI-rAd5 with those only one adjuvant, Oligo or mPEI, coating rAd5 in terms of vaccine efficiency. Here, the vaccine uptake by antigen-presenting cells and transduction efficiency in *in vitro* nasal models were analyzed. Then, *in vivo* measurements of rAd5 residence in the nasal cavity and HIVgag-specific immune responses were performed to evaluate the efficiency of nanocomplexes as vaccine candidates.

MATERIALS AND METHODS

Materials

Chitooligosaccharides of 3 kDa molecular weight were purchased from HaiZhiYuan (WeiFan, Shandong, China) and mPEI was synthesized in-house as described (21). The BCA protein assay kit was obtained from KeyGEN (Pierce, USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen (Carlsbad, CA, USA). Alexa Fluor®488 dye (isomeric mixture of carboxylic acid ester), Alexa Fluor®555 dye (isomeric mixture of carboxylic acid ester), FITC-labeled anti-mouse antibodies against CD8a and CD4, PE-labeled anti-mouse antibodies against IFN-γ and IL-4, FITC-labeled anti-mouse antibodies CD80 and PE-Cy5-labeled anti-mouse antibodies CD86, and brefeldin A were all purchased from eBioscience (San Diego, CA, USA). Recombinant mouse GM-CSF was purchased from R&D Systems (Minneapolis, MN, USA). Goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies against IgA, IgG1, and IgG2a were obtained from Santa Cruz (CA, USA), and goat anti-mouse HRP-conjugated antibody against IgG was obtained from ZSGB-BIO (Beijing, China). Mouse ELISA kits to detect IL-4, IFN-γ and IL-2 were obtained from

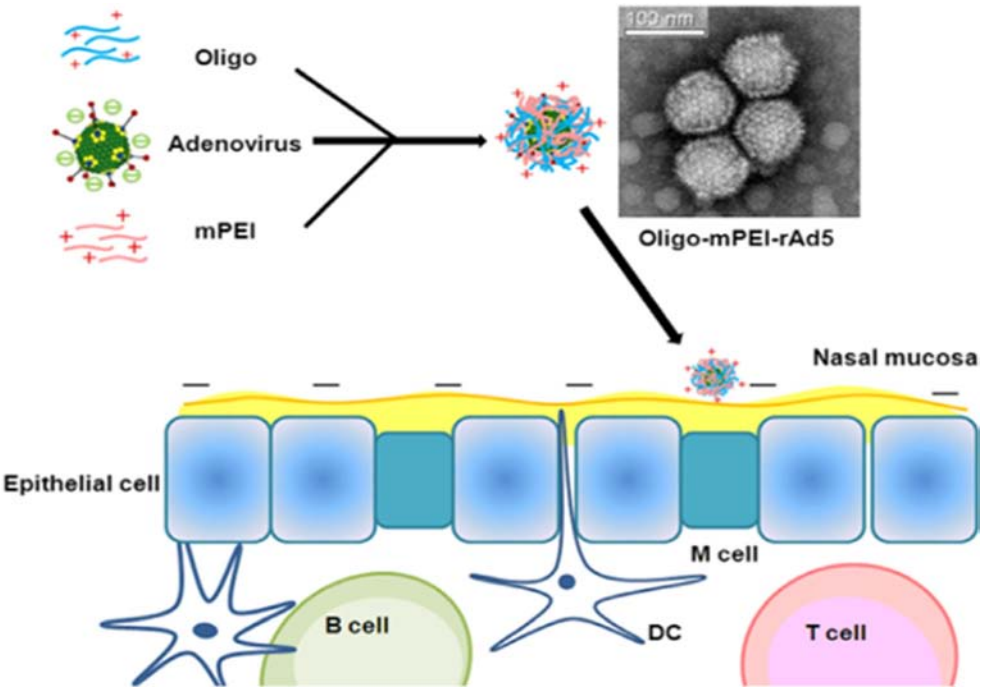

Fig. 1 Model for how the Oligo-mPEI-rAd5 vaccine candidate may initiate HIVgag-specific immune responses in nasal mucosa. Chitooligosaccharides (Oligo) and mannosylated polyethyleneimine-triethyleneglycol (mPEI) form a cationic layer, coating the adenovirus. The nanocomplex can tightly bind the anionic nasal mucosa, and subsequently contact dendritic cells (DCs) through the relatively loose nasal epithelium or *via* the transcytosis of M cells. Alternatively, rAd5 can infect nasal epithelial cells and express the encoded antigen, which will be presented to DCs after the lysis of the infected cells by natural killer (NK) cells.

R&D (USA). HIVgag peptide (The H-2 Kd-restricted immunodominant CTL epitope AMQMLKETI) was purchased from GenScript (Piscataway, NJ, USA) and recombinant human HIV-1 gag p24 was purchased from USBiological (Swampscott, MA, USA).

Cell Culture and Animals

The following cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China): Calu-3 human lung carcinoma cells, Madin-Darby cane kidney (MDCK) cells, DC2.4 dendritic cells and 293 T human kidney epithelial cells. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) or 1640 medium (Hyclone, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 units/mlpenicillin and 100 μg/mL streptomycin. Murine bone marrow-derived DCs (BMDCs) were generated as described (23). Cells were suspended in R5 medium (RPMI-1640 supplemented with 10% FBS, 10 mM HEPES, 50 μM β-mercaptoethanol, 20 ng/mL GM-CSF, antibiotic) at 37°C in a humidified atmosphere with 5% CO_2.

Balb/c and C57BL/6 mice between 6 and 8 weeks old were obtained from West China Experimental Animal Center of Sichuan University (Chengdu, China) and housed in a specific pathogen-free, light-cycled and temperature-controlled facility. All experiments were carried out according to China's Animal Welfare Legislation and the institutional animal care and use guidelines of Sichuan University.

Recombinant Adenovirus Vectors

The E1/E3-deleted adenovirus serotype 5 vector encoding HIV-1 gag p24 protein was kindly provided by Prof. Hildegund C.J. Ertl (Wistar Institute of Anatomy and Biology, Philadelphia, PA, USA). The rAd5-HIVgag vector was amplified in 293 T cells and purified through two rounds of CsCl density gradient centrifugation as the established adenovirus vector construction manual. The rAd5-lacz vector was rAd5 contain the lacz gene insteading of the HIVgag gene. The virus particles number of purified adenoviral vector was determined by measuring the optical density at 260 nm. Infectious titer was determined by serially diluting the recombinant adenovirus, infecting 293 T cells and observing them by inverted microscopy according to the TCID50 method.

Preparation of Oligo-mPEI-rAd5 Nanocomplex

Oligo-mPEI-rAd5 nanocomplex was assembled through electrostatic interaction by mixing particles of rAd5-HIVgag or rAd5-lacz with chitooligosaccharides and mPEI. The rAd5 particles were diluted in 5% glucose solution to a final dosage of 2×10^9 or 2×10^{10} viral particles (vp). Then, the same volume of Oligo and mPEI in 5% glucose solution were mixed by vortex for 30 s before dropped into the adenovirus solution, followed by vortex for 30 s and incubation for 30 min at room temperature. Similarly, Oligo and mPEI was also formulated with rAd5, respectively (Oligo-rAd5, mPEI-rAd5).

Characterization of rAd5-HIVgag and Nanocomplexes

Mean particle size, zeta potential and polydispersity index were determined for rAd5-HIVgag, Oligo-rAd5, mPEI-rAd5 and Oligo-mPEI-rAd5 nanocomplex at 25°C using photon correlation spectroscopy (Zetasizer Nano ZS90, Malvern Instruments Ltd, Worcestershire, UK). Before measurement, 0.2 mL of each sample was diluted to a final volume of 1 mL in 5% glucose solution. Measurements were taken at a fixed angle of 90° with a 1-min equilibrium time and automatic measurement cycling. Measurements were performed in triplicate over at least 20 runs in each experiment.

The morphology of rAd5-HIVgag and nanocomplexes were observed at an accelerating voltage of 200 kV using a transmission electron microscopy (TecnaiG2F-20, FEI, Holland). Each sample was processed by negative stain method. The samples were freshly prepared, loaded on a copper grid and incubated for 5 min. Then the samples were stained with 1% (w/v) aqueous uranyl acetate for 2 min, the samples were air-dried after removing the dye.

In Vitro Transduction Efficiency Assay

Calu-3 or MDCK cells were seeded at a density of 1.5×10^5 cells/well in 24-well plates and infected at 37°C with nanocomplexes and rAd5-lacZ dissolved in DMEM medium without FBS. Preliminary experiments (supplementary Figure 1) indicated that the optimal reagent ratios were 2×10^6 pfu (a multiplicity of infection (MOI) of 20) rAd5 per well, and 500 ng per well of Oligo or mPEI. After 4 h of infection, adenovirus complexes were replaced with complete DMEM medium and cells incubated a further 48 h. The transgene expression of rAd5-lacz in cells was quantitated using the β-galactosidase enzyme assay system according to the manufacturer's instructions (Beyotime, China). The total protein content per well was determined using the BCA assay. The transduction efficiency was defined as the units of β-gal activity per mg total protein.

In Vitro Uptake Assay

BMDCs or DC2.4 cells were seeded in 6-well plates at a density of 6×10^5/well. The rAd5-HIVgag was labeled with Alexa Fluor®488 dye and purified by dialysis. These nanocomplexes were formed with Alexa Fluor®488-labeled rAd5-HIVgag, respectively (the same optimal reagent ratios as transduction experiment). Cells were incubated 1 h at 37°C with labeled rAd5-HIVgag (negative control) or nanocomplexes. Cells were then digested and analyzed by flow cytometry using a FC500 flow cytometer (Beckman Coulter, Brea, CA, USA).

In Vivo Fluorescence Imaging Assay

Mice were administered with Alexa Fluor®555-labeled rAd5-HIVgag, Oligo-rAd5, mPEI-rAd5 or Oligo-mPEI-rAd5 nanocomplex intranasally (2×10^9 viral particles in 20 μl) under anesthesia. Then mice were scanned at 0 min, 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, 180 min with an IVIS® Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA, USA). Absolute fluorescence in the nasal cavity was quantified over time using an excitation wavelength of 555 nm and an emission wavelength of 565 nm, and the absolute fluorescence intensity was not captured when the fluorescence disappeared in nasal cavity. The spectral unmixing technology in Living Image software (version 4.2; Caliper Life Sciences) was applied in order to distinguish different fluorescent reporters and reduce background. To facilitate comparison of nasal residence times between groups, absolute fluorescence was converted to relative fluorescence (% of the maximum/initial fluorescence in the nasal cavity).

Animal Immunization

Balb/c mice 6–8 weeks old were immunized intranasally through prime-boost regime. In the prime immunization, the mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (50 mg/ml) and inoculated intranasally with rAd5, Oligo-rAd5, mPEI-rAd5 or Oligo-mPEI-rAd5 nanocomplex (2×10^9 viral particles of rAd5-HIVgag in 20 μl). Two weeks later, mice were boosted intranasally with the same construct as the prime administration (rAd5, Oligo-rAd5, mPEI-rAd5 or Oligo-mPEI-rAd5, respectively) but high-dose rAd5-HIVgag (2×10^{10}vp) in each sample. All intranasal formulations were administered to the left and right nostril in a total volume of 10 μl, respectively. Mice were maintained in an upright position on a hook for 30 min to ensure uniform dosing. Given that neutralizing antibody produced after boost vaccination impairs the strong immunity induced by intramuscular immunization, positive control mice were immunized with a single intramuscular dose (2×10^9vp) of rAd5-HIVgag. Mice were sacrificed 2 weeks after the boost immunization.

CTL Assay

The cytotoxicity of antigen-specific $CD8^+$ T cells was examined in an *in vivo* CTL killing assay (24). Briefly, splenocytes were isolated from naïve mice, red blood cells were lysed with ACK buffer (0.15 M NH_4Cl, 10.0 mM $KHCO_3$,0.1 mM EDTA, pH 7.4), and the cell suspension was divided equally into two aliquots. One aliquot was incubated with 2 μM synthetic HIV-gag peptide and the other with medium for 2 h at 37°C. The peptide-pulsed cells were stained with a high CFSE concentration (4 μM), while the medium-treated (non-

pulsed) cells were stained with a low CFSE concentration (0.4 μM) for 5 min at 37°C. CFSE labeling (Invitrogen, Carlsbad, CA, USA) was then quenched by addition of FBS to a final concentration of 20% (v/v). The two aliquots (10^7/100 μl) were mixed together, and given to naïve (control) or immunized mice *via* tail vein injection. After 18 h, spleen mononuclear cells from recipient mice were prepared and then analyzed by flow cytometry. The percentage of specific lysis was calculated as follows: specific lysis (%) = 100× [1 − (ratio of $CFSE^{low}/CFSE^{high}$ cells recovered from naive mice/ratio of $CFSE^{low}/CFSE^{high}$ cells recovered from immunized mice)].

Intracellular Cytokine Staining (ICS)

Splenocytes were harvested from immunized mice on day 28 and red blood cells were lysed using ACK buffer. Splenocytes were then incubated in the presence of 2 μl/ml HIVgag peptide and 1 μl/ml brefeldin A for 5 h at 37°C. As a negative control, cells were also incubated without peptide. The splenocytes were resuspended in staining buffer and incubated in the dark for 30 min at 4°C with FITC-labeled anti-mouse antibody against CD8a or CD4. Then cells were fixed in the dark for 20 min with IC fixation buffer and permeated with 1× permeabilization buffer. After resuspension in 1× permeabilization buffer, splenocytes were incubated in the dark for 20 min with PE-labeled anti-mouse antibody against IFN-γ or IL-4. Finally, the cells were examined using two-color flow cytometry (Beckman Coulter, Inc, Brea, CA, USA), and data were analyzed using the Kaluza software (Beckman Coulter Inc).

Antigen-Specific Cytokine Assays (IFN-γ, IL-4, IL-2)

Levels of HIVgag-specific cytokines were assayed in serum and spleen cells to evaluate immune responses induced by rAd5-HIVgag vaccines. Splenocytes (5×10^6/ml) from immunized mice were incubated for 72 h at 37°C with or without HIVgag peptide in 96-well plates. The culture supernatant was collected and concentrations (pg/ml) determined by cytokine ELISA kit or using the paired monoclonal antibodies and purified commercial preparations of recombinant mouse IFN-γ, IL-4, and IL-2.

Blood was collected from the retro-orbital plexus of immunized or control mice with a capillary tube. After centrifugation, serum was obtained. Serum concentrations of IFN-γ, IL-4, and IL-2 were quantitated using the paired ELISA kit.

Antigen-Specific Serum Antibody Responses

Levels of anti-HIV-gag antibodies (IgG, IgG1, IgG2a) were determined in serum by ELISA. ELISA plates (Corning, USA) were coated overnight at 4°C with recombinant HIV-1gag/p24 protein, and blocked with PBS containing 1% BSA for 2 h at 37°C. Then, two-fold serial dilutions of serum (from 1:16 to 1:2,048) were added to the plates and incubated for 2 h at 37°C. Plates were washed and incubated with HRP-conjugated anti-mouse antibodies against IgG (1:20,000), IgG1 or IgG2a (1:10,000) (Santa Cruz, CA, USA) for 1 h at 37°C. The TMB substrate was added, and the reaction was stopped 30 min later by addition of 2 M H_2SO4. Absorbance of the wells was determined at 450 nm, and endpoint titers were expressed as reciprocal $\log_2$ titers of the last dilution (OD value of samples/OD value of background is more than or equal to 2.1).

Antibody Secretion in the Nasal Cavity

The amount of secretory immunogobulin A (SIgA) secreted by the mucosa has been used as an index of mucosal immune response (25). Therefore, to analyze the mucosal antibody response induced by our vaccination regimen, the nostrils of mice were flushed three times with PBS containing 1% BSA and the secretory IgA level was determined by ELISA. The level of HIVgag-specific SIgA antibody in the nasal washes was expressed as OD_{450} value (26).

Statistical Analysis

Quantitative data were depicted in graphs as mean ± SD of at least three independent experiments. Statistical significance of intergroup differences was checked using one-way analysis of variance (ANOVA) and Student's *t*-test test; $*p<0.05$ was defined as the threshold of significance.

RESULTS

Physical Characteristics of rAd5-HIVgag and Nanocomplexes

Some physical characteristics (*i.e.* mean particle size, zeta potential and polydispersity index) were determined for rAd5, Oligo-rAd5, mPEI-rAd5, and Oligo-mPEI-rAd5 nanocomplex Nanocomplexes of Oligo-rAd5 (mean size, 178.1 ± 2.2 nm), mPEI-rAd5 (175.8 ± 1.7 nm), and Oligo-mPEI-rAd5 (180.5 ± 2.3 nm) were much larger than rAd5 (103.8 ± 0.5 nm) (Fig. 2a). This presumably reflects the fact that chitooligosaccharides and mPEI formed a cationic layer that coated the adenovirus. The addition of cationic polymers also converted the net negative charge of rAd5 particles to a net positive charge in the complexes (Fig. 2b). The zeta potential of Oligo-mPEI-rAd5 nanocomplex was 24.1 ± 0.9 mV, while that of rAd5 was −9.2 ± 0.5 mV. Indeed, the cationic nanocomplexes can effectively bind anionic nasal epithelium and increase the chance of entering cells.

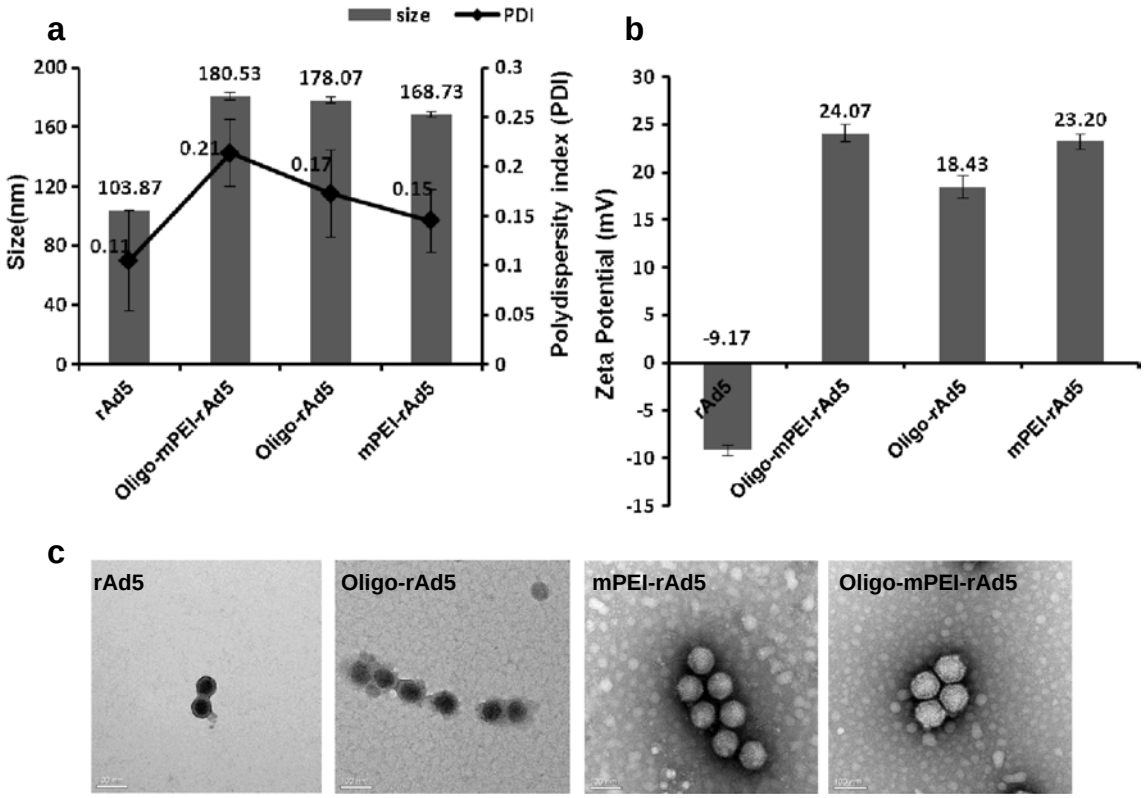

Fig. 2 Physical characteristics (size distribution, zeta potential and morphology) of rAd5-HIVgag and nanocomplexes. (**a**) Mean size and polydispersity index (PDI) of nanocomplexes. The *histogram and lines* depict data for mean size (*left axis*) and PDI (*right axis*), respectively. (**b**) Zeta potential of nanocomplexes and rAd5. Measurements were carried out in triplicate with at least 20 runs. (**c**) Transmission electron micrograph images of rAd5, Oligo-rAd5, mPEI-rAd5 and Oligo-mPEI-rAd5 nanocomplex. Note: *Bar* = 100 nm.

The photographs of transmission electron microscopy presented the morphology of the polymers wrapping adenovirus. As shown in Fig. 2c, in the TEM images of each nanocomplex group, rAd5 particles were surrounded by thin membranes indicated that cationic polymers had been ionically anchored onto the surface protein of rAd5, forming coated rAd5. Meanwhile, as rAd5 and polymers had different electron cloud densities, mPEI was observed to form white membranes coating rAd5 in the photographs of mPEI-rAd5 and Oligo-mPEI-rAd5 nanocomplex.

Transduction Efficiency of the Nanocomplexes in *In Vitro* Nasal Models

The efficiency of gene transduction is also an indicator of the potency of genetic vaccines. The transduction efficiency of nanocomplexes was investigated in both Calu-3 and MDCK cells. The Calu-3 cell line was used as an *in vitro* nasal platform, because its properties are similar to those of the serous cells of the upper airway (27). MDCK cells are widely used as a model of epithelium featuring tight junctions and apico-basolateral polarity (28). Transduction efficiency was significantly higher for the Oligo-mPEI-rAd5 nanocomplex than for Oligo-rAd5 nanocomplex, mPEI-rAd5 nanocomplex and rAd5-lacZ (*$p<0.05$, **$p<0.01$, Fig. 3a). These results showed that chitooligosaccharides and mPEI together increased the efficiency of adenovirus transduction to a greater extent than did either chitooligosaccharides or mPEI on their own.

In Vitro Antigen Uptake by Antigen-Presenting Cells

Since vaccines inducing immune responses would firstly undergo antigen recognition and presenting processes, it is necessary to investigate antigen uptake by the professional antigen presenting cells, dendritic cells (DCs). Uptake of rAd5-HIVgag and nanocomplexes by murine bone marrow-derived DCs (BMDCs) and DC2.4 cells was determined. The rAd5 and Oligo-rAd5 showed similarly low uptake, while both mPEI-rAd5 and Oligo-mPEI-rAd5 nanocomplex showed significantly greater uptake, presumably due to the presence of mannose on mPEI. The uptake of the Oligo-mPEI-rAd5 nanocomplex was 17- and 4-fold higher than that of rAd5 in BMDCs and DC2.4 cells, respectively (***$p<0.001$, Fig. 3b). Therefore, Oligo-mPEI-rAd5 and mPEI-rAd5 nanocomplex both have been showed a potent capability to induce high cellular uptake.

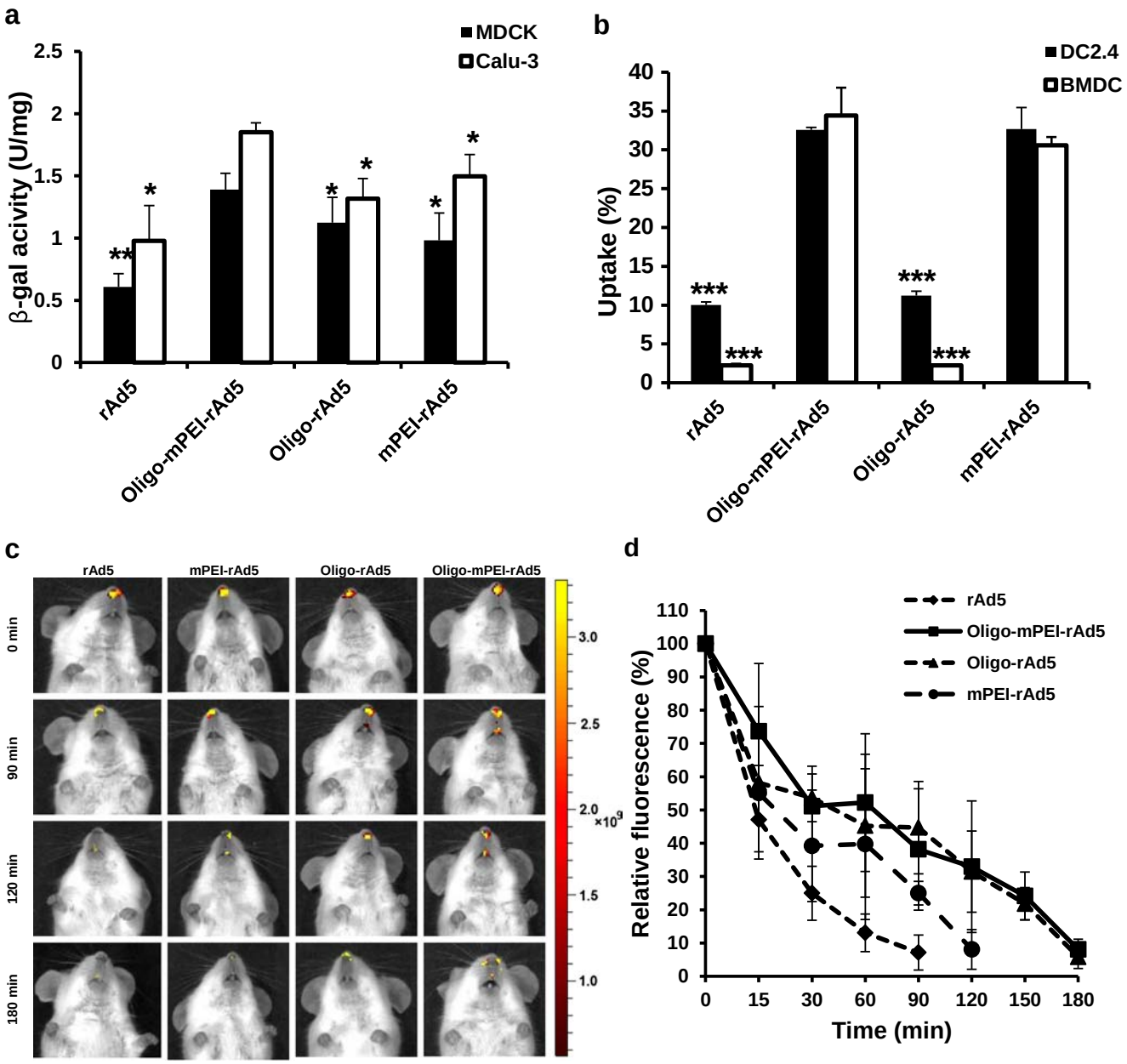

Fig. 3 *In vitro* transduction, uptake efficiency and nasal residence time of rAd5-lacZ and nanocomplexes. (**a**) Transduction efficiency of rAd5 and nanocomplexes in an *in vitro* nasal model were detected. Calu-3 and MDCK cells were infected with rAd5-lacZ or nanocomplexes. 2×10^6 pfu (MOI = 20) rAd5-lacZ with 500 ng Oligo and/or 500 ng mPEI per well. $^*p < 0.05$ and $^{**}p < 0.01$ vs Oligo-mPEI-rAd5 in Calu-3 or MDCK cell, respectively ($n = 4$). (**b**) Celluar uptake of rAd5-HIVgag and nanocomplexes by BMDCs or DC2.4 were determined by flow cytometry. The internalization of the virus was quantitated as percent uptake, defined as the percentage of total cells that were positive for intracellular virus. $^{***}p < 0.001$ vs Oligo-mPEI-rAd5 and mPEI-rAd5 in BMDCs or DC2.4 cell, respectively ($n = 4$). (**c**) Nasal residence time analyzed by *in vivo* fluorescence imaging. Balb/c mice were inoculated intranasally with Alexa Fluor®555-labeled rAd5 and nanocomplexes, and the fluorescence in the nasal cavity was monitored over time. (**d**) Relative fluorescence intensity, defined as the percent of the initial fluorescence in the nasal cavity. The absolute fluorescence intensity was not captured when the fluorescence disappeared in nasal cavity ($n = 4$).

Nasal Residence Time of rAd5-HIVgag and Nanocomplexes

An *in vivo* fluorescence imaging assay was used to study the nasal residence time of each group. The nasal residence time of Oligo-mPEI-rAd5 group was at least 180 min, whereas that of rAd5-HIVgag and mPEI-rAd5 was 60–90 min and 90–120 min, respectively (Fig. 3c and d). Analysis of relative fluorescence decay showed that at 90 min, the relative fluorescence of Oligo-rAd5 and Oligo-mPEI-rAd5 nanocomplex was still more than 40%, whereas the relative fluorescence of rAd5-HIVgag was less than 8%. By 120 min, relative fluorescence for Oligo-rAd5 and Oligo-mPEI-rAd5 nanocomplex was nearly 30%, compared to less than 8% for mPEI-rAd5

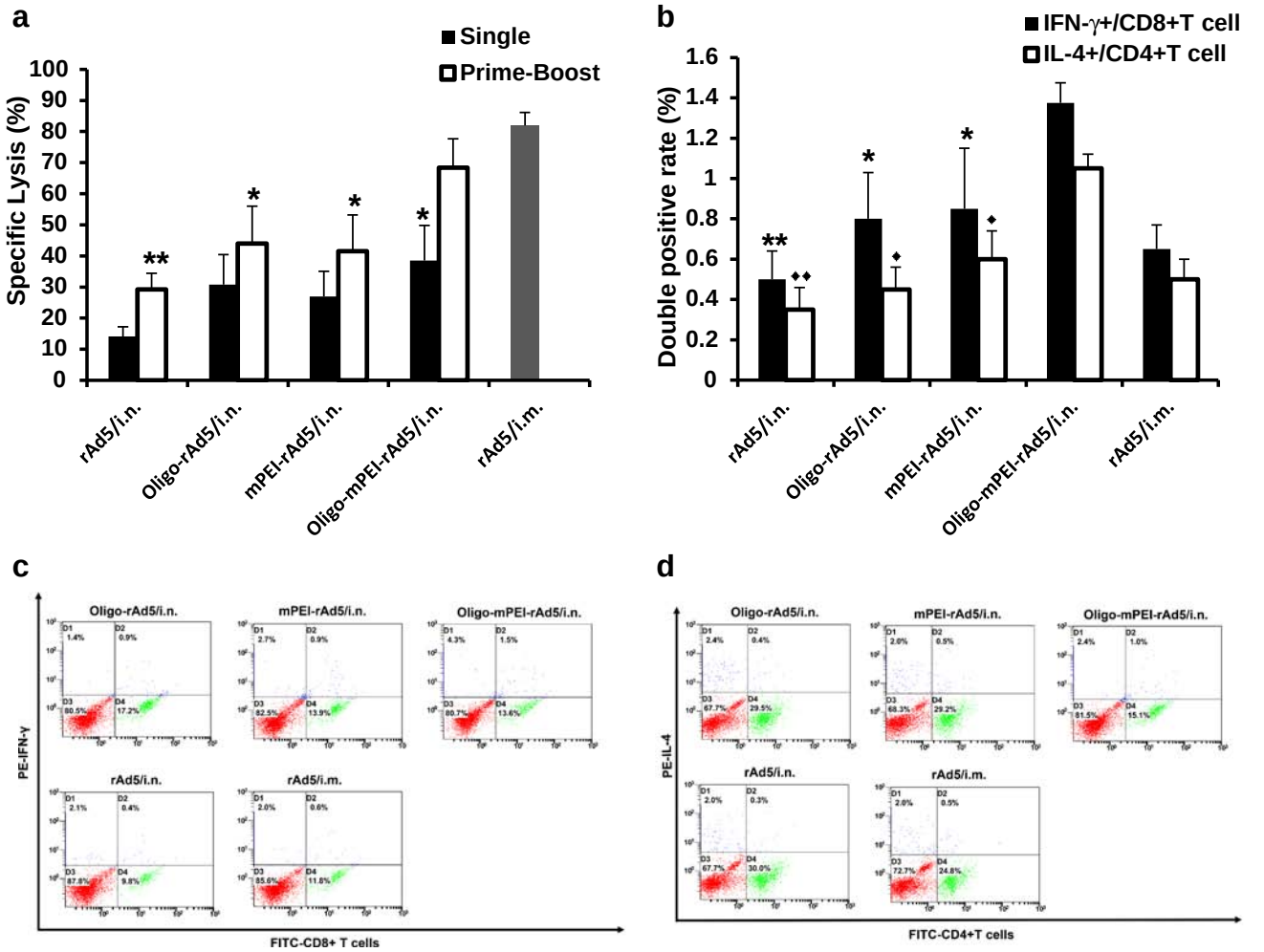

Fig. 4 HIVgag-specific cytolytic Tcell responses and cytokines (IFN-γ/IL-4) production by $CD8^+$Tor $CD4^+$Tcells induced by intranasal immunization with rAd5 and nanocomplexes and intramuscular rAd5 vaccination (mice immunized with a single dose (2×10^9vp) of rAd5-HIVgag). (**a**) The percentage of HIVgag-specific cytotoxic $CD8^+$Tcell lysis was calculated shown and the mean values obtained from five mice per treatment were statistically analyzed. *$p<0.05$, **$p<0.01$ vs intranasal prime-boost immunization with Oligo-mPEI-rAd5 ($n=5$). (**b**) The presence of double positive cells (*i.e.* $CD8^+$ Tcells producing IFN-γ or $CD4^+$Tcells producing IL-4) were analyzed using an intracellular cytokine staining assay. The mean percentages of double positive cells were calculated by the percentages of cytokines positive specific Tcells to total Tcells. *$p<0.05$ and **$p<0.01$ vs Oligo-mPEI-rAd5 in IFN-γ^+/$CD8^+$Tcell; $^{\blacklozenge}p<0.05$ and $^{\blacklozenge\blacklozenge}p<0.01$ vs Oligot-mPEI-rAd5 in IL-4^+/$CD4^+$Tcell ($n=4$). (**c**~**d**) One representative dot plot of each sample in the intracellular cytokine staining assay is shown. The *numbers in the upper right quadrant* of each graph shows frequencies of the cytokines positive specific Tcells (IFN-γ^+/$CD8^+$Tcells (**c**) and IL-4^+/$CD4^+$Tcells (**d**)).

(Fig. 4d). These results indicate that chitooligosaccharides has mucoadhesive properties that helped nanocomplexes significantly reduce the rate of nasal mucociliary clearance, which subsequently prolonged nasal residence.

In Vivo CTL Activity

To study the antigen-specific $CD8^+$ CTL response induced by intranasal vaccination with rAd5-HIVgag, we measured the percentage of specific cytotoxic lysis in vaccinated and control mice. In this assay, the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) was used to assess CTL-mediated lysis by monitoring injected HIVgag peptide-pulsed $CFSE^{high}$-labeled splenocyte populations relative to non-pulsed $CFSE^{low}$-labeled population (29). Since the potent antigen-specific T cell response peaks at 4 weeks after intramuscular administration (1), a single intramuscular immunization with rAd5-HIVgag was used as the positive control. For control mice, $CFSE^{high}$ and $CFSE^{low}$ peak heights were similar, and a single intranasal dose (2×10^9vp) of rAd5 or nanocomplexes did not elicit strong CTL responses (Fig. 4a). After boosted with an increased dose (2×10^{10}vp) of the same construct, Oligo-mPEI-rAd5 nanocomplex significantly enhanced antigen-specific $CD8^+$ T cell responses (*$p<0.05$; Fig. 4a), whereas rAd5-HIVgag group showed relatively low CTL respones (prime 14.12%, and boost 29.23%). Meanwhile, the percentages of specific cytotoxic lysis in Oligo-rAd5 (30.81%, 43.95%) and mPEI-rAd5 group

(26.96%, 41.49%) were also higher than rAd5, but the two nanocomplexes groups both exhibited less effective specific lysis of T cells than Oligo-mPEI-rAd5 nanocomplex (38.56%, 68.43%). Indeed, intranasal immunization with Oligo-mPEI-rAd5 induced significantly greater HIVgag-specific cytotoxic lysis than did rAd5-HIVgag (**$p<0.01$), Oligo-rAd5 (*$p<0.05$) and mPEI-rAd5 (*$p<0.05$).

Antigen-Specific T Cells Responses Monitored by ICS

Levels of HIVgag-specific $CD8^+$ and $CD4^+$T cell responses were determined by ICS assay. In this approach, antigen-specific $CD8^+$T responses are evaluated by measuring IFN-γ production, and IL-4 is an index of $CD4^+$ Th2 immune response. After splenocytes from immunized or control mice were incubated for 5 h with HIVgag peptide, IFN-γ secretion by $CD8^+$ T cells and IL-4 production by $CD4^+$ T cells were analyzed by flow cytometry. Oligo-mPEI-rAd5 immunization induced IFN-γ production by antigen-specific $CD8^+$T cells (1.38%) and IL-4 production by antigen-specific $CD4^+$T cells (1.05%) to a significantly greater extent than did rAd5-HIVgag (0.50% and 0.35%, respectively; **$p<0.01$, Fig. 4b, c and d). Additionally, Oligo-rAd5 (0.80% and 0.45%) or mPEI-rAd5 (0.85% and 0.60%, *p< 0.05) induced far less IFN-γ secreting $CD8^+$T cells and IL-4 secreting $CD4^+$T cells than Oligo-mPEI-rAd5 nanocomplex. More importantly, induction by Oligo-mPEI-rAd5 nanocomplex was even greater than that caused by intramuscular immunization (0.65% and 0.50%, respectively). According to the kinetics of the IFN-γ positive $CD8^+$ T cells reported previously, the strong, specific $CD8^+$T responses induced by intramuscular immunization with rAd5-HIVgag peaked at day 10 and declined thereafter (30). The Oligo-mPEI-rAd5 immunization with a prime-boost regimen led to significantly better $CD4^+$Th2 and cellular immune responses, both of which were notably higher than using either adjuvant alone or no adjuvant at all. The results were also consistent with the above detection of pronounced antigen-specific CTL responses, indicating the vaccine efficiency of Oligo-mPEI-rAd5 nanocomplex for cellular immune responses.

Antigen-Specific Cytokine Production After Prime-Boost Vaccination

Cytokine levels are considered to play an important role in controlling HIV infection and disease progression. To examine antigen-specific IFN-γ, IL-4, and IL-2 in response to vaccination, we used a special ELISA technique to measure cytokine secretion from spleen cells after 72 h of stimulation with HIVgag peptide. Indeed, the antigen-specific cytokine productions of Oligo-rAd5 and mPEI-rAd5 group were higher than rAd5 but lower than Oligo-mPEI-rAd5 group. Although there was no markedly significant difference between Oligo-mPEI-rAd5 nanocomplex and Oligo-rAd5/mPEI-rAd5 alone in stimulating the secretion of antigen-specific cytokines, the results have shown that Oligo-mPEI-rAd5 nanocomplex evoked the highest level of cytokines in the four experimental groups. Meanwhile, the IFN-γ concentration in culture supernatants was significantly higher from in cells treated with Oligo-mPEI-rAd5 (1521.21 pg/ml) than in cells treated with rAd5 (870.77 pg/ml; *$p<0.05$, Fig. 5a), consistent with the ICCS results.

Since Th1 immune responses are associated not only with IFN-γ secretion by activated NK and T cells but also with serum IFN-γ, we measured the concentrations of serum IFN-γ and another Th1 cytokine, IL-2, and also assayed levels of the Th2 cytokine IL-4. Prime immunization with Oligo-mPEI-rAd5 vaccine was sufficient to induce a serum IFN-γ concentration (1147.32 pg/ml) significantly higher than that observed with rAd5-HIVgag (813.30 pg/ml; *$p<0.05$, Fig. 5b). Furthermore, the serological level of IFN-γ was significantly elevated after boost immunization with the same construct, which was far higher than rAd5-HIVgag group (Fig. 5b, **$p<0.01$). Meanwhile, the serological profile of IL-4 and IL-2 by Oligo-mPEI-rAd5 vaccine was similar with IFN-γ. Oligo-mPEI-rAd5 caused much higher serum IL-4 production (73.01 pg/ml) than did rAd5-HIVgag (30.95 pg/ml) with a prime-boost immunization (Fig. 5c); the analogous results were observed for IL-2 secretion (Fig. 5d). All the results have shown that Oligo-mPEI-rAd5 could also stimulate a potent production of antigen-specific cytokines.

Antibody Production in Serum and Nasal Cavity

After serum was obtained from the retro-orbital plexus of immunized mice, the levels of antigen-specific IgG, IgG1 and IgG2a antibodies in serum were measured to evaluate humoral immune response. The rAd5-HIVgag group induced the lowest IgG and its subtypes (IgG1and IgG2a) responses, all of which were notably increased by adding two polymer adjuvants to coat rAd5. Moreover, HIVgag-specific antibody responses generated by Oligo-mPEI-rAd5 were stronger than other intranasal groups after prime vaccination, and these responses in all the groups significantly improved after boost vaccination. The mice immunized with pime-boost Oligo-mPEI-rAd5 nanocomplex produced significantly higher IgG/IgG1/IgG2a titers than rAd5-HIVgag as well as rAd5 with one adjuvant (Oligo-rAd5 and mPEI-rAd5). More importantly, these antibody titers were similar in intranasal Oligo-mPEI-rAd5 and the intramuscular group that had been considered as a potent route for vaccination (*$p<0.05$, ** $p<0.01$***$p<0.001$, Fig. 6a, c and d). Since the ratio of IgG1 to IgG2a isotype is generally taken to indicate the relative strength of Th1 or Th2 responses, we compared the levels of IgG1 and IgG2a. It was generally accepted that IgG1/IgG2a ratio≤0.5 indicated a Th1-dominant profile, while IgG1/IgG2a≥2.0 suggested a Th2-dominant profile. The ratios between 0.5 and 2.0 implied a mixed or balanced

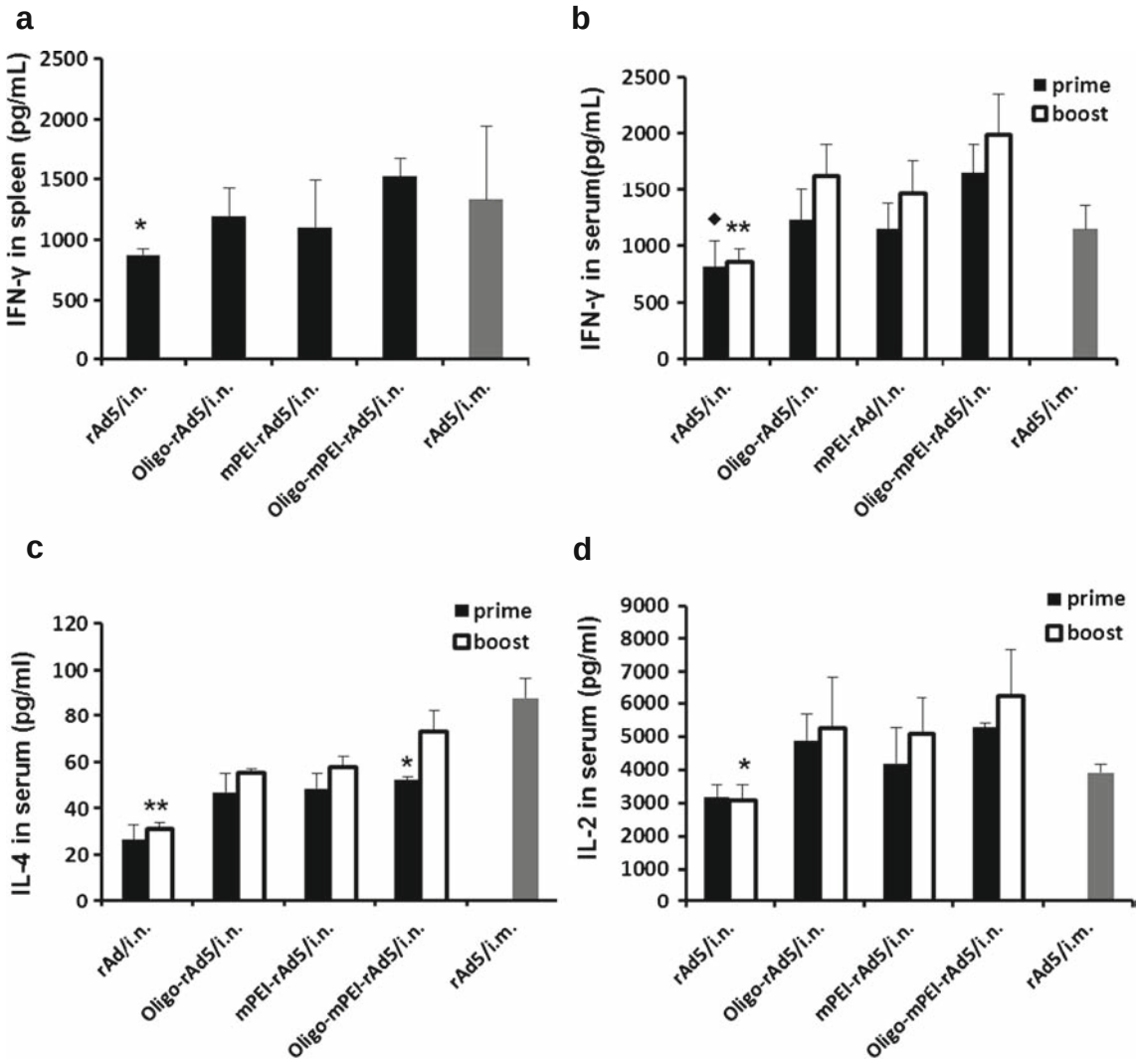

Fig. 5 Intranasal immunization with Oligo-mPEI-rAd5 strongly induced IFN-γ secretion by splenocytes and increased serum levels of IFN-γ, IL-4 and IL-2. (**a**) IFN-γ levels in splenocyte supernatants.*$p < 0.05$ vs intranasal immunization of Oligo-mPEI-rAd5. (**b**–**d**) Secretion of IFN-γ (**b**), IL-4 (**c**), and IL-2 (**d**) in serum. The intramuscular group was mice immunized with a single dose (2×10^9vp) of rAd5-HIVgag vaccine. The mean value of cytokine levels (IFN-γ, IL-4 and IL-2) was also determined by ELISA for each treatment. $^{\blacklozenge}p < 0.05$ vs Oligo-mPEI-rAd5 at day 14 after prime immunization, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs Oligo-mPEI-rAd5 at day 14 after boost immunization ($n = 4$; $^{*}p < 0.05$, $^{**}p < 0.01$).

response (25,31,32). As shown in Fig. 6e, the IgG1/IgG2a ratios of all groups were within 0.5–2.0. Following boost immunization with Oligo-mPEI-rAd5, the IgG1/IgG2a ratio was 1.07, indicating a balanced Th1 and Th2 immune response.

The secretory immunoglobulin A (SIgA) is the predominant immunological defense system at the mucosal surface, where it blocks microbial infections; it is considered an important indicator of mucosal immunity (33). Since the nasal cavity features a large surface area of mucous membrane, the local secretion of antibody was usually determined. The nasal cavities of the mice were washed with three successive PBS containing 1% BSA and SIgA secretion in nasal washes was detected by ELISA. Oligo-mPEI-rAd5 immunization induced a significantly higher HIVgag-specific mucosal SIgA level (OD=0.74) than did rAd5 (OD=0.25) and mPEI-rAd5 immunization (OD=0.41, $^{*}p<0.05$, Fig. 6b). Indeed, Oligo-rAd5 nanocomplex stimulated nasal mucosa producing SIgA antibodies as much as Oligo-mPEI-rAd5, partly for chitooligosaccharides enhanced the adhesion to the nasal cavity and may be easier to evoke the local immune response.

DISCUSSION

Acquired immunodeficiency syndrome (AIDS), a life-threatening infectious disease with no effective treatment, is transmitted mainly by sexual contact, through the blood and from mother-to-child. The highest concentrations of HIV

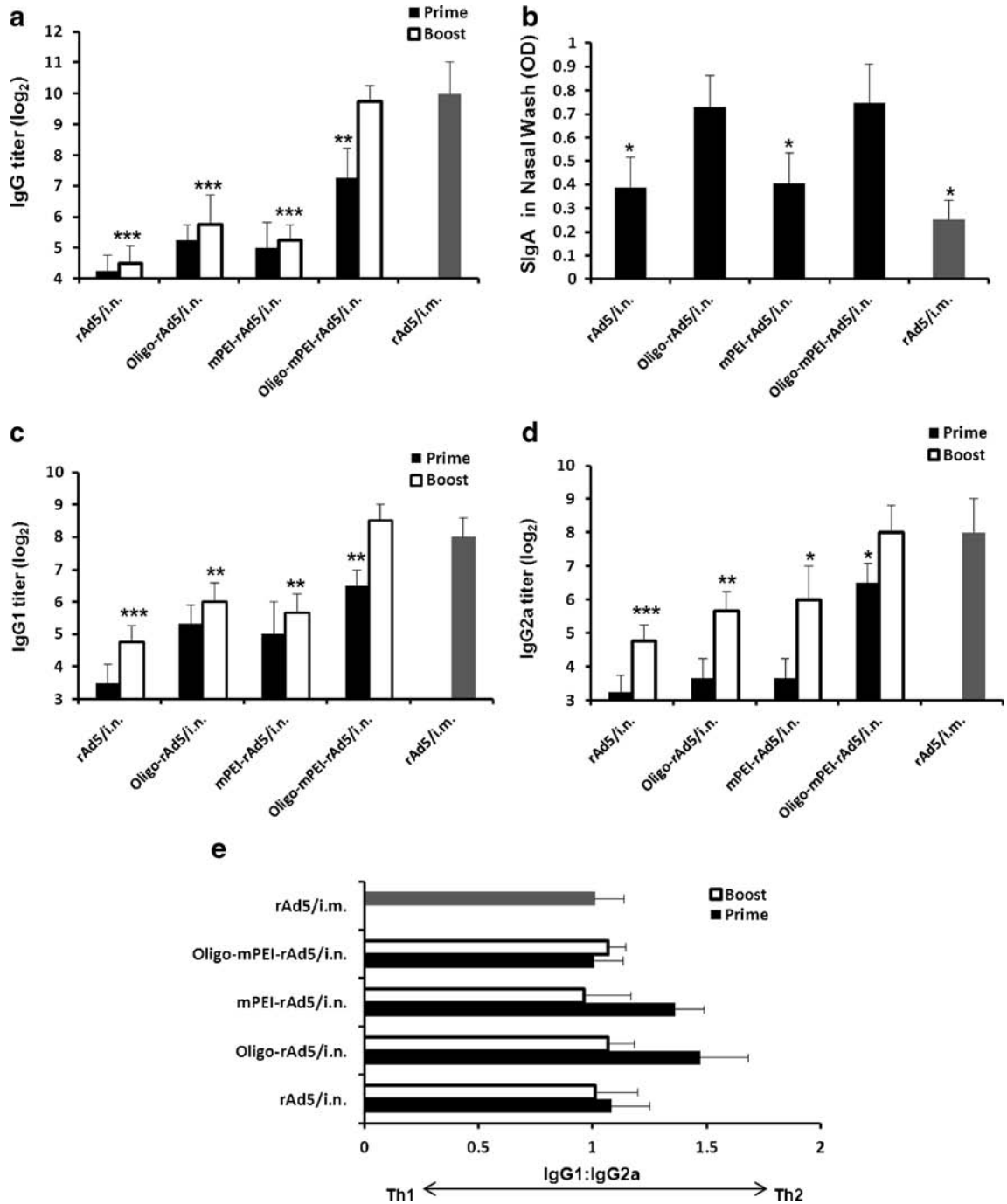

Fig. 6 Serum levels of IgG, IgG1 and IgG2a and antigen-specific SIgA secretion in nasal mucosa following vaccination. (**a**) The serum was obtained from the retro-orbital plexus of mice, and IgG titers in serum were assayed. ***$p < 0.001$ vs intranasal prime-boost immunization with Oligo-mPEI-rAd5. (**b**) Antigen-specific sIgA levels in nasal washes were determined by ELISA. *$p < 0.05$ vs Oligo-mPEI-rAd5 and Oligo-rAd5, respectively ($n = 4$). (**c**~**d**) IgG isotypes (IgG1/IgG2a) titers in serum were detected by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs intranasal prime-boost immunization with Oligo-mPEI-rAd5, respectively. (**e**) The IgG1/IgG2a ratio was calculated as an indicator of Th1/Th2 immune responses ($n = 4$). The intramuscular group in all the experiments was mice immunized with a single dose (2×10^9vp) of rAd5-HIVgag.

virus have been detected in the blood, semen and vaginal secretions. Mucosal immunization such as nasal vaccination can induce robust IgA secretion in various mucosas, including the vaginal and nasal layers, which may prevent HIV at disseminate site (34,35). Moreover, nasal administration has been shown to allow induction of immune responses at distal mucosal tissues, such as the gut that is the main site where a profound depletion of $CD4^+$ T cells occurs after parenteral or mucosal HIV-1 infection (36,37). Therefore, intranasal immunization may be an effective route for HIV vaccination.

Although the rAd5 vector has already been used as a viral vector in vaccines, it was still necessary to explore a versatile adenovirus carrier for vaccination. Main barriers of rAd5 vector-based vaccines were discussed in our studies, such as preexisting immunity to Ad5 and relatively low immunogenicity of intranasal administration. Here, we describe and evaluate an intranasal HIV vaccine candidate based on rAd5 and two cationic polymers, chitooligosaccharides and mPEI, which have been shown to slow nasal mucociliary clearance and improve immune efficacy (38). We found that, *in vitro* and *in vivo*, the Oligo-mPEI-rAd5 complex indeed worked better than the rAd5 vector or the vector complexed with either one of the cationic polymers on its own.

Chitooligosaccharides and mPEI formed cationic layer around rAd5, shielding their negative charge and increasing affinity for binding nasal epithelial cell layer. Chitooligosaccharides with mucoadhesive properties first helped nanocomplexes resist mucociliary clearance and prolong residence in the nasal cavity for Oligo-rAd5 and Oligo-mPEI-rAd5 nanocomplex, thereby increasing the likelihood that the nanocomplexes would enter cells. These findings are similar to those reported for vaccine (15,39). As DC-targeting adjuvant, mPEI helped anchor rAd5 on DCs that express high levels of mannose receptors, and indeed played an important role in increasing the celluar uptake of nanocomplexes with mPEI. Meanwhile, chitooligosaccharides and mPEI have also been shown to co-operate in enhancing transduction efficiency in nasal epithelial cells, but not chitooligosaccharides or mPEI alone.

To evaluate the immune efficiency of Oligo-mPEI-rAd5 complex, we immunized Balb/c mice intranasally. The previous studies showed that the HIVgag-specific $CD8^+$T cells appear to play a dominant role in the initial state of viral replication during acute HIV infection (40,41). The *in vivo* CTL assay has been shown to be a sensitive and specific method for quantitating the HIVgag-specific $CD8^+$ CTL response and evaluating vaccine efficacy (42). Accordingly, the results showed that Oligo-mPEI-rAd5 complexes enhanced the ability of the specific effector cells to kill the HIVgag peptide-pulsed target cells significantly. The Oligo-mPEI-rAd5 nanocomplex elicited a stronger antigen-specific CTL response in this assay than did rAd5-HIVgag, Oligo-rAd5 and mPEI-rAd5.

To complement the CTL results, we used ICSS to measure the cytokine secretion status of antigen-specific $CD4^+$ and $CD8^+$ T cells. Antigen-specific $CD8^+$ T cells produce abundant IFN-γ and induce cytotoxicity to kill antigen-pulsed cells. Thus, the cellular immune response can be assessed by measuring the frequencies of the effector $CD8^+$ T positive for IFN-γ detected to evaluate cellular immune responses. Antigen-specific $CD4^+$T cells can also induce cytokine secretion, supporting specific $CD8^+$T and antibody responses to prevent HIV infection (43–45). The $CD4^+$T helper cells have been known to differentiate in Th1 and Th2 subtypes. Th1 immune responses are primarily cellular and Th2 immune responses are mostly associated with antibody production. IL-4 and IL-10 are measured as Th2 markers, while IFN-γ and IL-2 are Th1 markers (46). The vigorous HIV gag-specific $CD4^+$Th1 cell responses might provide help to promote specific $CD8^+$CTL activities. Meanwhile, the HIVgag-specific $CD4^+$Th2 cell response was assessed by measuring secretion of the main Th2 cytokine IL-4, which was a main Th2 cytokine to partly reflect humoral immune responses. The Oligo-mPEI-rAd5 nanocomplex was found to activate significantly not only $CD8^+$ T cells positive for IFN-γ but also $CD4^+$ T cells positive for IL-4 that indicates a Th2 response. This activation was even superior to that obtained at 28 days after intramuscular immunization. It is worth mentioning that the peak time of the antigen-specific CTL responses and levels of $CD8^+$ T cells positive for IFN-γ induced by intramuscular immunization has been demonstrated to be the fourth week and tenth day, respectively. Indeed, the CTL and ICS assays both showed that although Oligo-rAd5 and mPEI-rAd5 nanocomplex had a moderate increase compared with adenovirus vector, Oligo-mPEI-rAd5 elicit more robust antigen-specific immune responses than Oligo or mPEI alone with rAd5, which were in line with the results of *in vitro* experiments. For antigen-specific cytokine profiles, we also found that prime-boost intranasal immunization with Oligo-mPEI-rAd5 significantly provoked to generate numerous antigen-specific cytokines much more (IFN-γ, IL-4, IL-2) in serum or spleen than did rAd5.

Meanwhile, rAd5 with one adjuvant, Oligo or mPEI, failed to evoke high IgG/IgG1/IgG2a titers despite an increase in antibody responses after boost vaccination. Oligo-mPEI-rAd5 nanocomplex after nasal vaccination promoted the comparable production of total IgG and IgG isotype, and these elicited antibody responses were nearly as strong as intramuscular immunization, implying these two adjuvants could augment antibody-mediated immune responses together. Th2/Th1 immune response of the intranasal HIV vaccine candidate was also confirmed to be a mixed humoral/cellular immune response by calculating the ratio of serum IgG1/IgG2a antibodies. Additionally, intranasal vaccination offers the advantage of inducing strong local and mucosal responses, mediated to a large extent by SIgA (34). To determine whether this was also the case with our vaccine candidate, we measured SIgA levels in nasal washes. Oligo-mPEI-rAd5 and Oligo-rAd5 both evoked higher SIgA levels than mPEI-rAd5 nanocomplex and rAd5 group, partly because the mucoadhesive properties of Oligo enhanced the residence of nasal cavity and then evoked stronger local immune response.

Although the exact mechanism of the immune response evoked by nasal rAd5 vaccine is unclear, recent studies have shown some possible processes after rAd5 vaccination.

Particulate antigens such as rAd5 could be transported by M cells in the nasal-associated lymphoid tissue (NALT) and presented to underlying antigen presenting cells (APCs) to prompt local or distant mucosal responses (47). Alternatively, rAd5 vectors could infect epithelial cells, which later would be lysed by natural killer (NK) cells. DCs could successively take up the released antigens priming antigen-specific T responses and evoking Ig class switching and production of SIgA. Additionally, compared to other epithelial surfaces, the nasal epithelium is thin and porous (48). Therefore, some DCs can be partially exposed to nasal mucosa through the epithelial tissue space and directly take up the rAd5 vaccine to trigger immune responses (Fig. 1) (47,49). Based on the present results, we preliminary deduced that Oligo-mPEI-rAd5 nanocomplex elicited HIVgag-specific through these multiple routes, including first uptake by DCs or infected by epithelial cells.

Taken together, our results indicate that Oligo and mPEI, these two adjuvants can jointly improve the potency of rAd5-based nasal vaccines. Intranasal prime-boost immunization with the Oligo-mPEI-rAd5 nanocomplex elicited the strongest cellular and humoral responses as well as local mucosal immune responses as a nasal vaccine candidate in these nanocomplexes and rAd5 groups, involving a balance of Th2 and Th1 $CD4^+$ T cell activity.

CONCLUSION

In our study, an adenoviral vector-based nasal vaccine was formulated and investigated in order to develop an effective nasal immunization for HIV prevention. Chitooligosaccharides (Oligo) and mannosylated polyethyleneimine-triethyleneglycol (mPEI) formed a cationic layer coating rAd5 vector, which could enhance the binding affinity with the anionic nasal mucosa. As a mucosal adhering adjuvant, chitooligosaccharides significantly improved the residence of nasal cavity and SIgA production while mPEI was a DC-targeting adjuvant increasing DC uptake. Moreover, Oligo and mPEI improved the transduction efficiency in nasal epithelial cells together. Our results also showed that the prime-boost immunization of Oligo-mPEI-rAd5 nanocomplex by nasal route induced a robust HIVgag-specific CTL responses and high cytokine secretion levels of HIVgag-specific $CD8^+$/$CD4^+$T cells as well as potent antibody-mediated (humoral) immunity. This intranasal delivery of Oligo-mPEI-rAd5 nanocomplex also elicited a potent mucosal antibody response (SIgA) with a balance of Th1 (IFN-γ, IL-2, IgG2a) and Th2 (IL-4, IgG1) $CD4^+$ helper T-cell response. Indeed, the prime-boost intranasal immunization with the Oligo-mPEI-rAd5 nanocomplex is a potential vaccine candidate for HIV infection. Further studies are needed for exploring the exact mechanism of this vaccination and developing an even more versatile HIV mucosal vaccine system.

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