Intradermal and intranasal immunizations with oligomeric middle layer rotavirus VP6 induce Th1, Th2 and Th17 T cell subsets and $CD4^+$ T lymphocytes with cytotoxic potential

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Abstract

Rotavirus (RV) inner capsid VP6 protein is a potential non-live vaccine candidate due to high degree of conservation and immunogenicity, and ability to self-assemble into oligomeric structures, including nanotubes. These VP6 structures induce strong humoral and T cell immunity and protect mice against RV challenge. It has been suggested that intracellular neutralization by IgA antibody and VP6-specific $CD4^+$ T cells mediate protection. We investigated generation of diverse $CD4^+$ T cell subsets by intradermal and intranasal delivery of recombinant VP6 (rVP6) nanotubes in BALB/c mice. Production of antiviral cytokine interferon-γ (IFN-γ), interleukin-4 (IL-4) and pro-inflammatory cytokine IL-17 was analyzed following *in vitro* stimulation of immune cells. Cell surface CD107a expression was measured to determine VP6-specific cytotoxic T cells. Both parenteral and mucosal immunization with oligomeric rVP6 induced VP6-specific Th1, Th2 and Th17 cells. For the first time, cytotoxicity-related degranulation (CD107a surface expression) indicated that RV VP6-specific $CD4^+$ T cells had cytotoxic T lymphocyte (CTL) phenotype. These findings demonstrate an ability of rVP6 nanostructures to induce heterogeneous $CD4^+$ T cells with different effector functions, including CTLs with potential to lyse RV-infected cells, suggesting an additional mechanism of RV VP6-induced protection.

Keywords: rotavirus; VP6; CD4; cytotoxic T lymphocyte; interferon-γ; interleukin-17

1. Introduction

Live attenuated oral rotavirus (RV) vaccines containing species A RVs have been successfully introduced in routine childhood immunization in many parts of the world (1), but RV is still a common cause of acute gastroenteritis in infants and young children worldwide with high mortality in the developing world (2). Alternative second-generation non-live RV vaccine candidates are being considered to alleviate safety concerns and to improve efficacy (1). One of the candidates is RV VP6 protein, which is highly conserved and immunogenic RV protein; it forms the middle layer of the triple-layered RV particle (3, 4). Antibodies directed to this inner capsid protein are the most abundant responses following RV infection (5, 6) or vaccination (7). We have recently developed a combination subunit vaccine against frequent viral causes of childhood gastroenteritis, consisting of RV recombinant VP6 (rVP6) protein and norovirus (NoV) virus-like particles (VLPs) (8, 9). Since protein assemblies with repetitive multivalent expression of antigen are powerful immunogens (10), the ability of VP6 to self-assemble into various oligomeric structures, including nanotubes, (11-13) makes it an attractive new generation RV vaccine candidate. rVP6 subunit vaccine could offer benefits over live attenuated vaccines being safe to administer to immunocompromised people, minimizing the risk of intussusception, preventing shedding of the vaccine virus to the environment and reemergence of new recombinant viruses. As VP6 immunization elicits non-neutralizing antibodies, VP6-induced protection is due to mechanisms different from those of classical neutralization.

We have previously demonstrated that both parenteral and mucosal delivery of the candidate RV – NoV combination vaccine induced broad heterologous and long-lasting immune responses to RV and NoV in mice (8, 9, 14, 15) and protected against live murine RV challenge (14, 16). Furthermore, anti-VP6 mucosal IgA antibodies interfered with RV infection *in vitro* (14, 17), probably by impairing RV replication via intracellular inhibition of viral transcription (18, 19) during transcytosis of the polymeric

IgA (pIgA) antibodies (20, 21). The *in vivo* protective activity of VP6-specific IgA antibodies has been demonstrated by Burns and colleagues (22). rVP6 nanostructures can also induce activation and maturation of antigen presenting cells (APCs) (23), facilitate the APC uptake of co-delivered NoV VLPs, and act as a potent adjuvant for NoV-specific immune responses (24, 25). Others have shown that immunization with rVP6 induces $CD8^+$ cells, which mediate clearance of chronic RV infection in mice (26) and can lyse RV-infected cells *in vitro* (27). In addition, VP6-specific $CD4^+$ T cells have been shown to mediate protection against RV infection in mice (28, 29), either by direct cell-mediated cytotoxic mechanism in mucosa or by antiviral cytokine production. Interferon-γ (IFN-γ) has been identified as the only anti-RV cytokine in stimulated $CD4^+$ T cells from mice immunized with a chimeric VP6 protein (30, 31). Another cytokine suggested to have function in VP6-induced protection is interleukin-17 (IL-17) (32, 33) involved in inflammatory response (34). Although IL-17 possesses no direct antiviral effects on RV replication *in vitro* (30), this pro-inflammatory cytokine, secreted mainly by Th17 cells, plays an important role in recruitment of immune cells to infection sites, especially at the mucosa (34).

$CD4^+$ T cells have been revealed as the principal mediator of protection after mucosal immunization with soluble monomeric VP6 (28, 29, 33). The purpose of this study was to investigate $CD4^+$ T cell effector functions after parenteral immunization with oligomeric rVP6. In the course of this work, VP6-specific $CD4^+$ T cells potentially able to lyse RV–infected cells were identified.

2. Materials and Methods

2.1. Recombinant VP6 production

An oligomeric human-derived RV rVP6 (Subgroup (SG) II; GenBank reference strain accession no. **GQ477131**) was produced in Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA) in Sf9 insect cells and purified by ultracentrifugation in sucrose gradients according to the previously published procedures (8, 17). The assembly of rVP6 (resuspended in PBS pH 7.3-7.5) into nanotubes was confirmed by electron microscopy (8, 9).

2.2. Viruses and synthetic peptides

RV cell culture antigens were prepared from RV strains Wa (G1P1A[8], SGII), BrB (G4P2[6], SGII) and WC3 (G6P7[6], SGI) by propagation in a fetal rhesus monkey kidney (MA104) cell line as described elsewhere (14). The amount of VP6 protein in the cultures was quantified by the Ridascreen® Rotavirus test (R-Biopharm AG, Darmstadt, Germany) with rVP6 as an internal standard (17). An 18-mer VP6-derived R6-2 peptide (^{242}DGATTWYFNPVILRPNNV^{259}, $AA_{242-259}$), originally identified as an VP6-specific H-2^d $CD4^+$ T cell epitope (35), was synthesized by Proimmune LtD. (Oxford, UK). A 17-mer peptide (^{323}ISQAVHAAHAEINEAGR^{339}, $AA_{323-339}$, InvivoGen, San Diego, CA) derived from chicken ovalbumin (OVA) was used as an irrelevant negative control in the assays.

2.3. Mouse immunization and sample preparation

Female 6-week-old BALB/c OlaHsd mice were purchased from Envigo (Horst, the Netherlands) and acclimatized prior to the start of the experiment. Five mice/experimental group were immunized twice (at study weeks 0 and 3) with a combination vaccine formulation containing rVP6 nanotubes (8, 9), via ID or IN delivery at a dose of 10 µg rVP6/immunization point. No external adjuvants were included in

the vaccine formulation. Mice administered with a carrier (sterile PBS only) served as control groups. Immunizations were conducted under general anesthesia induced with a formulation of Hypnorm® (VetaPharma Limited, Leeds, UK) and Dormicum® (Roche Pharma AG, Grenzach-Wyhlen, Germany). Whole blood and spleens were collected at the time of sacrifice (week 5) and prepared according to the previously published procedures (36). All of the experimental procedures were conducted according to the regulations and guidelines of the Finnish National Experiment Board.

2.4. RV VP6-specific ELISA

Two-fold serial dilutions from 1:200 diluted sera of individual mice were tested in ELISA for the presence of RV VP6-specific IgG and IgG subtype antibodies as described elsewhere (8, 17). Shortly, 96-well half-area polystyrene plates (Corning Inc, Corning, NY) were coated with 50 ng of rVP6 per well. Serum anti-VP6 antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), IgG1 (Invitrogen), or IgG2a (Invitrogen) and SIGMA FAST OPD substrate (Sigma-Aldrich). Optical density values at 490 nm (OD_{490}) were measured by Victor^2 1420 microplate reader (PerkinElmer, Waltham, MA). A result was interpreted as a positive when the OD_{490} was above the cut-off value (mean OD_{490} + 3 × SD of the control mice and OD_{490} >0.1). The end-point titer was expressed as Log_{10} of the reciprocal of the highest dilution with an OD_{490} above the cut-off value.

2.5. ELISPOT IFN-γ, IL-4 and IL-17

Quantification of IFN-γ, IL-4 and IL-17 production from liquid nitrogen frozen splenocytes of immunized and control mice was performed according to the previously published ELISPOT IFN-γ and IL-4 assays (9, 17, 37). Briefly, Multiscreen 96-well HTS-IP filter plates (Millipore, Billerica, MA) were coated with 5 µg/mL of anti-mouse monoclonal IFN-γ antibody AN18 or IL-4 antibody 11B11 or 10

μg/mL of anti-mouse monoclonal IL-17A antibody IL17-I (all from Mabtech AB, Nacka Strand, Sweden). Group-wise pooled splenocytes (0.2×10^6 cells/well for IFN-γ; 0.4×10^6 cells/well for IL-4 and IL-17) from experimental or control mice were stimulated in duplicates with rVP6 protein (5 μg/mL), R6-2 peptide (5 μg/mL) or RV (Wa, BrB or WC3) cell culture antigens (0.5 μg VP6/mL). OVA-peptide and MA104 mock cell culture were used as negative controls. Background (culture medium (CM) only) and cell viability controls (10 μg/mL of T cell mitogen Concanavalin A; Sigma-Aldrich) were included in each assay. Following incubation of 20h (for IFN-γ) or 45h (for IL-4 and IL-17) at 37^{o}C, cytokine secretion was detected with 0.5 μg/mL of biotinylated anti-mouse IFN-γ (R4-6A2), IL-4 (BVD6-24G2) or IL-17A (MT2270) monoclonal antibody and 1:1000 diluted streptavidin-alkaline phosphatase conjugate (all from Mabtech AB). The spots developed with BCIP/NBT substrate (Mabtech AB) were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany). The results were expressed as mean spot forming cells (SFC)/10^6 splenocytes of duplicate wells.

2.5.1. Blocking of T cell activation

In order to define cell type responsible for the IFN-γ production, splenocytes were pre-incubated prior to *in vitro* stimulation with the antigens for 1 h at 37^{o}C with 30 μg/mL of functional blocking antibodies rat anti-mouse CD4, rat anti-mouse CD8a or rat IgG2a isotype control (all from eBioscience) (17). The blocking efficiency of the anti-CD4 and anti-CD8 antibodies to block IFN-γ production in the ELISPOT assay has been previously confirmed (38).

2.6. Degranulation assay

The cytotoxic phenotype of RV VP6-specific mouse T cells was tested by degranulation assay determining the percentages of T cells expressing CD107a on the surface, according to previously

published method with few modifications (39). In brief, splenocytes (1×10^6 cells/mL) were pre-incubated for an hour at 37^{o}C in complete CM with fluorescein isothiocyanate (FITC)-conjugated anti-CD107a antibody (clone 1D4B) (40) in the presence of the VP6-specific R6-2 peptide (4 µg/mL) or RV Wa cell culture antigens (0.5 µg VP6/mL), and incubated overnight in the presence of brefeldin A and monensin. MA104 mock cell culture was used as negative control. Background control (CM only) was included in each assay for determining spontaneous expression of CD107a. The cells were treated with mouse Fc Block (Clone 2.4G2) and Horizon Fixable Viability stain 780 (live/dead discrimination) before staining the cell surface antigens CD3 (PE-Cy7 Rat Anti-Mouse CD3) and CD8 (PerCP-Cy5.5 Rat Anti-Mouse CD8a, Clone 53-6.7). All reagents were purchased from BD Biosciences. The cells were acquired on BD FACSCanto II flow cytometer and analyzed using FlowJo v.10 software (ThreeStar Inc., San Carlos, CA). Live $CD3^+$ lymphocytes were gated and analyzed for CD8a and CD107a expression.

2.8. Statistical analyses

Fisher's exact test was employed to assess the intergroup differences in the IgG endpoint titers. The Mann-Whitney U-test and Kruskal-Wallis test were used to compare differences between the non-parametric observations of two or more independent groups. Analyses were conducted by IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY), Version 23.0. The statistically significant difference was defined as $p \leq 0.05$.

3. Results

3.1. VP6-specific serum IgG antibodies and Th1/Th2 dichotomy

Immunization of the mice with a vaccine formulation containing 10 µg of rVP6 nanotubes elicited robust VP6-specific IgG antibody levels (geometric mean titers, GMTs >4.9 $\log_{10}$) irrespective of the delivery route (**Figs. 1A and B**). Although ID immunization resulted in slightly lower antibody response, no statistically significant difference (p=0.143, Fisher's exact test) was observed in the magnitude of the responses induced by rVP6 via ID or IN route (**Fig. 1B**). No VP6-specific antibodies were detected in the sera of control mice (**Figs. 1A and B**).

Determination of IgG subtype IgG1 and IgG2a titers, representing Th2- and Th1-type responses, revealed induction of strong Th2-type (GMTs >5.1 $\log_{10}$) and Th1-type (GMTs >5.5 $\log_{10}$) responses by both administration routes (**Figs. 1C and D**). Similar to IgG responses, no significant difference was observed in the magnitude of the IgG1 (p=0.143, Fisher's exact test) or IgG2a (p=1.0, Fisher's exact test) responses generated by 10 µg dose of rVP6 via ID or IN route. No anti-VP6 IgG or IgG subtype antibodies were detected in sera of control mice (**Figs. 1A**–**D**).

3.3. Both ID and IN route induce VP6-specific IFN-γ secreting $CD4^+$ T cells

Induction of T cell responses via ID and IN routes was characterized by analyzing Th1-type cytokine IFN-γ production from the splenocytes of immunized and control mice. Regardless of the administration route, cells from the mice receiving 10 µg dose of oligomeric rVP6 responded with considerable IFN-γ release to stimulation *ex vivo* with Wa, BrB, and WC3 RV cell cultures (**Figs. 2A and B**). T cells were highly reactive to Wa and BrB RV strains belonging to SGII, homologous to the rVP6 used for immunizations, and somewhat lower cross-reactive responses to heterologous WC3 RV belonging to SGI were detected. Moreover, both experimental groups developed significant T cell responses to rVP6 protein as well as the R6-2 peptide (**Figs. 2A and B)**, a previously identified VP6-specific $CD4^+$ T cell epitope (35). Similar quantities of IFN-γ secreting cells were detected in both experimental groups

(p=0.841, Mann-Whitney U-test). No VP6-specific IFN-γ response was detected by the cells of negative control mice (**Fig. 2C**). MA104 mock cell culture, CM alone or negative control OVA peptide stimulated no IFN-γ production by the cells from any of the groups (**Figs. 2A**–**C**).

To determine the T cell type responsible for IFN-γ production, anti-CD4, anti-CD8, and control IgG antibodies were used to block the interaction of the antigen and T cell receptor and thereby T cell activation. When the splenocytes of mice immunized IN with rVP6 were stimulated with Wa RV cell culture, rVP6 or R6-2 peptide in the presence or absence of the functional blocking antibodies, 68-90% of IFN-γ production was significantly blocked only with anti-CD4 antibodies (p=0.005, Kruskal-Wallis test), indicating $CD4^+$ but not $CD8^+$ T cells as the main effectors producing IFN-γ (**Fig. 2D**). Some unspecific blocking of RV Wa with IgG control antibody was detected, similarly to our previously published observation (17).

3.4 Both ID and IN route induce VP6-specific IL-4 producing T cells

Production of IL-4 by the splenocytes of experimental mice was analyzed as a marker of a true Th2-type response. Cells of mice immunized ID or IN with oligomeric rVP6 produced significant levels of IL-4 when stimulated *ex vivo* with Wa RV cell culture antigen, R6-2 peptide and rVP6 (**Figs. 3A and B**). Similar quantities of IL-4 secreting cells were induced via both delivery routes (p=1.0, Mann-Whitney U-test). No VP6-specific IL-4 response was observed in control mice (**Fig. 3C**). MA104 mock cell culture, CM alone or negative control OVA peptide did not stimulate IL-4 secretion by the splenocytes from any of the groups (**Figs. 3A**–**C**).

3.5 Both ID and IN route induce VP6-specific IL-17 producing T cells

Both ID and IN administration routes resulted in considerable quantities of VP6-specific IL-17 secreting cells in response to *ex vivo* stimulation with Wa and BrB RV cell cultures (**Figs. 4A and B**). Immunization of mice via either delivery routes elicited an IL-17 response, when splenocytes were stimulated with WC3 RV culture, the 18-mer R6-2 peptide or with rVP6 (**Figs. 4A and B**). However, substantially lower levels of IL-17 were induced via ID delivery compared with the responses induced via IN route (p=0.002, Mann-Whitney U-test). MA104 mock cell culture, CM alone or negative control OVA-peptide stimulated no IL-17 production by the cells of the experimental groups (**Figs. 4A and B**). Cells of the control mice did not produce IL-17 in response to any of the stimulants (**Fig. 4C**).

3.6. Cell surface expression of degranulation marker on $CD4^+$ T cells

VP6-specific T cells generated by rVP6 immunization were further analyzed for cytotoxic effector phenotype ($CD107a^+$) and therefore the ability to potentially lyse RV infected cells (41). CD107a cell surface expression was detected on $CD3^+CD8^-$ T cells of mice immunized with the rVP6 (**Fig. 5**). Following VP6 derived R6-2 peptide (a VP6-specific H-2^d $CD4^+$ T cell restricted epitope (35)) stimulation, up to 2.0% of $CD4^+$ T cells expressed CD107a (**Figs. 5A and C**), while 3.5% of RV Wa stimulated T cells were positive for CD107a expression (**Fig. 5A**). No VP6-specific degranulation was observed in the cells of negative control mice stimulated with R6-2 peptide (0.2% $CD107a^+$) or RV Wa (0.3% $CD107a^+$) (**Fig. 5B**).

4. Discussion

We have proposed that polymeric forms of RV VP6 are potentially useful for a combined RV – NoV vaccine candidate to combat many cases of acute gastroenteritis in children (8, 9). rVP6 oligomers, including nanotubes and nanospheres, are potent inducers of antibodies and T cell responses and protect immunized mice against RV challenge (14, 16, 17, 25). The mechanisms by which subunit RV VP6 vaccines exert protection are distinct from mechanisms suggested for replicating live RV vaccines, protection being independent from traditional neutralizing antibodies or $CD8^+$ T cells (29, 42, 43). VP6-specific secretory IgA, but not IgG, was shown to mediate intracellular neutralization of RV (20, 21). Others have shown that $CD4^+$ T cells are the only effector cells required for VP6-triggered protection after mucosal immunization with a monomeric soluble VP6 formulated with the adjuvant LT (R192G) (29). Subsequently, protection has been associated with the production of the Th1 and Th17 cytokines, IFN-γ and IL-17 by intestinal $CD4^+$ T cells (28, 32). In order to elucidate possible mechanisms of protection mediated by oligomeric VP6, the present study investigated induction of different $CD4^+$ T cell subsets by rVP6 nanotubes via parenteral and mucosal delivery, primarily Th1-type, Th2-type and Th17-type responses, but most importantly, previously unexplored VP6-specific $CD4^+$ cytotoxic T cells.

High levels of anti-VP6 serum IgG antibodies were induced by ID and IN delivery routes, demonstrating the immunogenicity of rVP6 nanotubes and successful immunizations. Despite the robust serum IgG levels, VP6-specific IgG response has not directly been indicted in protection from RV infection in mice (28, 33, 44, 45). Both deliveries also induced considerable levels of IgG2a and IFN-γ (markers of a Th1-type response), as well as IgG1 and IL-4 (markers of a Th2-type response). Although BALB/c mice preferentially develop Th2-type responses to certain antigens (46, 47), our results show that rVP6 induces balanced Th1/Th2-type responses by both deliveries. IFN-γ and IL-4 are the key cytokines of Th1 and

Th2 cells, which drive B cell switching to IgG2a and IgG1, respectively. Further, the IgG2a isotype antibodies have been shown to be particularly potent in viral clearance due to their capacity to activate the complement (48, 49) and induce antibody-dependent cell-mediated cytotoxicity (50).

rVP6 induced Th1 response based on IFN-γ production when stimulated with rVP6, different RV cell culture antigens and a VP6-derived immunodominant $CD4^+$ T cell epitope (35). These cells reacted with RVs belonging to SGI and SGII, indicating broad reactivity due to the highly conserved nature of VP6 among all group A RVs (3). This cross-reactive immunity shows potential of rVP6 to induce protection against different circulating RV strains. Blocking experiments confirmed $CD4^+$ T cells as the main effectors producing IFN-γ after IN immunization, which is in concordance with our previous results observed after parenteral immunization (17). Although the protective role of $CD4^+$ T cells is generally restricted to their traditional helper functions, these cells can also act through other mechanisms to prevent and resolve viral infections, including antiviral activity of the cytokines they produce and direct cytotoxicity. According to a previous report, IFN-γ can block RV replication *in vitro* (51). Association of IFN-γ with VP6-induced protection against murine RV infection (28, 32) support antiviral activity of IFN-γ as a possible mechanism by which VP6 mediates protection. Instead, induction of VP6-specific Th2 cells may stimulate humoral immunity by promoting proliferation and differentiation of antigen-primed B lymphocytes into antibody secreting cells as well as memory B cells through secretion of IL-4.

Recent studies have revealed the role of Th17 cells and IL-17 production in infection and vaccine-induced immunity against some pathogens, including *S. pneumoniae*, *M. tuberculosis*, and influenza (52, 53). Increased expression levels of IL-17 have also been detected in the peripheral blood cells of children with RV infection (54). Chimeric VP6 monomer administered mucosally in combination with an adjuvant generates both IL-17 and IFN-γ producing $CD4^+$ T cells, reduces fecal shedding, and protects

against live RV challenge (28, 30, 32, 33). In the present study, we observed that immunization with oligomeric rVP6 induces IL-17 secreting T cells, not only by using the mucosal (IN) but also by the parenteral (ID) route. Although we did not directly show a T cell subset responsible for IL-17 production, Smiley and co-workers (32) have demonstrated that IL-17 induced after VP6 immunization is solely produced by $CD4^+$ T cells and not by $CD8^+$ T cells. In contrast to direct antiviral function of IFN-γ, IL-17 is a pro-inflammatory cytokine that may act more indirectly, probably by affecting immune cell recruitment via upregulation of chemokines (52, 53). In addition, IL-17 is critical in generating mucosal IgA responses (55) and therefore plays protective role in immunity against infectious disease at the mucosa. Although we showed that both administration routes induced IL-17 production, IN delivery of rVP6 generated a better Th17 response. Concordantly, we have previously shown that mucosal immunization with rVP6 induces higher level of mucosal IgA antibodies compared with parenteral immunization (14, 17). These VP6-specific secretory pIgA antibodies confer protection from RV infection via pIgR-mediated intracellular neutralization (20, 21). Since pIgR is considered a key factor in mucosal immunity, mediating the delivery of pIgA and pIgM to the apical surface of epithelial cells via transcytosis, IL-17 may have a role in VP6-induced protective immunity by up-regulating pIgR expression in mucosal epithelia and thus enhancing secretory IgA levels (55).

Although earlier studies have indicated that VP6-specific $CD4^+$ T cells may protect from RV infection, probably by direct cytotoxic effect (29, 30), to the best of our knowledge, this is the first study showing the cytotoxic phenotype of VP6-specific $CD4^+$ T cells. Even if $CD8^+$ CTLs are the classical effectors of protection against intracellular pathogens, $CD4^+$ CTLs have been identified in humans with chronic viral infections, such as with human cytomegalovirus, HIV-1 and different hepatitis viruses (56-58), as well as in the lungs of mice infected with influenza virus A (59). CTLs mediate killing of target cells in an antigen-specific fashion via secretion of cytotoxic granules containing perforin and granzymes (60). In

order to identify CTLs induced by rVP6, we employed degranulation assay detecting cell surface CD107a expression. As CD107a/b is not normally found on the surface of T cells, an increased cell surface expression of degranulation molecule CD107a on T cells of rVP6-immunized mice can be associated with the loss of intracellular perforin (41), indicating the ability of these cells to lyse RV-infected cells. In the degranulation assay, we used R6-2 peptide (a VP6-specific H-2^d $CD4^+$ T cell restricted epitope) as the stimulating antigen, as immunization with this peptide ($AA_{242–259}$) alone has been sufficient for protection against RV challenge, but the mechanism was not investigated (35). Our results suggest that $CD4^+$ CTLs are able to recognize the epitope on the cell surface and lyse the infected cells. In addition, RV Wa was selected as the stimulating antigen due to the importance of demonstrating cytotoxic potential of VP6-specific T cells to viable virus. However, further studies to prove the $CD4^+$ T cell cytotoxicity, either by *in vivo* depletion or *ex vivo* cell killing assay, are warranted.

Most of the studies exploring effectors or mechanisms of VP6-induced protection have been accomplished by mucosal immunizations with soluble *E.coli*-expressed chimeric VP6 monomers in combination with a strong adjuvant, where generation of strong immune responses and protection has depended strictly on co-administration of an adjuvant (30, 32, 33, 44). Although protective immunity generated by VP6 may vary with the type of immunogen, immunization route, and use of adjuvants (28, 42, 44), oligomeric rVP6 used in here induced T cell immunity similar to that induced by a chimeric VP6 with the external adjuvant. This is probably due to superior immunogenicity of protein assemblies over soluble proteins, which is strongly associated with the multivalent antigen expression (10, 61). In addition, due to the size range (0.2—1.5μm) and morphology (17, 62, 63), rVP6 nanotubes are efficiently internalized by macrophages and dendritic cells (23), resulting in activation and maturation of these APCs and efficient presentation of the antigen to T cells in the lymphoid organs (61).

In conclusion, the current study demonstrated that oligomeric rVP6 induces IFN-γ, IL-4 and IL-17 producing $CD4^+$ T lymphocytes, independently from the delivery route and in the absence of an adjuvant. Most importantly, rVP6 generated $CD4^+$ CTLs with the potential to lyse infected cells. These findings provide new insights into the mechanisms of protective immunity to RV induced by the rVP6 protein and support the general notion of using rVP6 nanostructures as a non-live RV vaccine.

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Figure legends

Fig. 1. RV VP6-specific serum IgG (**A, B**) and IgG subtype (**C, D**) antibody responses induced by ID or IN immunization with 10 µg of RV rVP6 nanotubes. Control (Ctrl) mice receiving carrier only (PBS) served as controls. (**A**) OD_{490} values of anti-VP6 IgG antibodies in 1:200 diluted sera of individual mice. The horizontal lines represent the mean $OD_{490\ nm}$ values of the experimental groups. End-point titration curves of anti-VP6 IgG (**B**), IgG1 (**C**), and IgG2a (**D**) antibodies in sera of the experimental groups. Mean titration curves with standard errors of the mean of the experimental groups (n=5) are shown. The dashed lines indicate maximum background level (cut-off limit).

Fig. 2. RV VP6-specific IFN-γ production by T cells. RV Wa, BrB, and WC3 cell cultures, rVP6 protein and R6-2 peptide were used to stimulate IFN-γ production from the group-wise pooled splenocytes of mice (n=5) immunized ID (**A**) or IN (**B**) with rVP6 or carrier only (**C**). Mean IFN-γ spot-forming cells (SFC)/10^6 splenocytes of duplicate wells of 2–4 independent experiments with standard errors of the means are shown. Results of negative control mice receiving PBS via ID and IN were combined. The dashed line indicates maximum background level (cut-off limit) originating from the cells incubated in a culture medium (CM) only (mean SFC + 3×SD). MA104 mock culture and an OVA peptide were used as negative control stimulants. (**D**) Blocking of IFN-γ production by stimulating the cells of IN immunized mice in the presence and absence of anti-CD4 and anti-CD8 or control antibodies. Results are expressed as the mean % inhibition of two independent experiments. Statistical significance was determined using Mann-Whitney U-test or Kruskal-Wallis test.

Fig. 3. RV VP6-specific IL-4 production by T cells. Group-wise pooled splenocytes of mice (n=5) immunized ID (**A**) or IN (**B**) with rVP6 nanotubes or carrier only (**C**) were stimulated with RV Wa cell culture antigen, rVP6 protein and R6-2 peptide and analyzed for IL-4 production. Mean IL-4 spot-

forming cells (SFC)/10^6 splenocytes of duplicate wells of 2 independent experiments with standard errors of the means are shown. Results of negative control mice receiving PBS via ID and IN were combined. The dashed line indicates maximum background level (cut-off limit) originating from the cells incubated in a culture medium (CM) only (mean SFC + 3×SD).

Fig. 4. RV VP6-specific IL-17 production by T cells. Group-wise pooled splenocytes of mice (n=5) immunized ID (**A**) or IN (**B**) with rVP6 nanotubes or carrier only (**C**) were stimulated with RV Wa, BrB, and WC3 cell cultures, rVP6 protein and R6-2 peptide and analyzed for IL-17 production. Mean IL-17 spot-forming cells (SFC)/10^6 splenocytes of duplicate wells with standard errors of the means are shown. Results of negative control mice receiving PBS via ID and IN were combined. The dashed line indicates maximum background level (cut-off limit) originating from the cells incubated in a culture medium (CM) only (mean SFC + 3×SD).

Fig. 5. Expression of CD107a in RV VP6-specific $CD4^+$ T cells. The group-wise pooled splenocytes of mice (n=5) immunized with rVP6 nanotubes via ID delivery (**A**) or the control mice (**B**) were stimulated with R6-2 peptide (a VP6-specific H-2^d $CD4^+$ T cell restricted epitope (35)) or RV Wa or negative control antigens (culture medium (CM) or mock culture) in the presence of CD107a FITC before flow cytometry analysis. Due to the shortage of the cells, the splenocytes of mice immunized with rVP6 nanotubes via IN delivery (**C**) were stimulated only with R6-2 peptide or CM. Events (%) shown are gated on live $CD3^+$ T lymphocytes. Percentages indicated in the upper left quadrant denote $CD3^+CD8^-$ $CD107^+$ T cells.

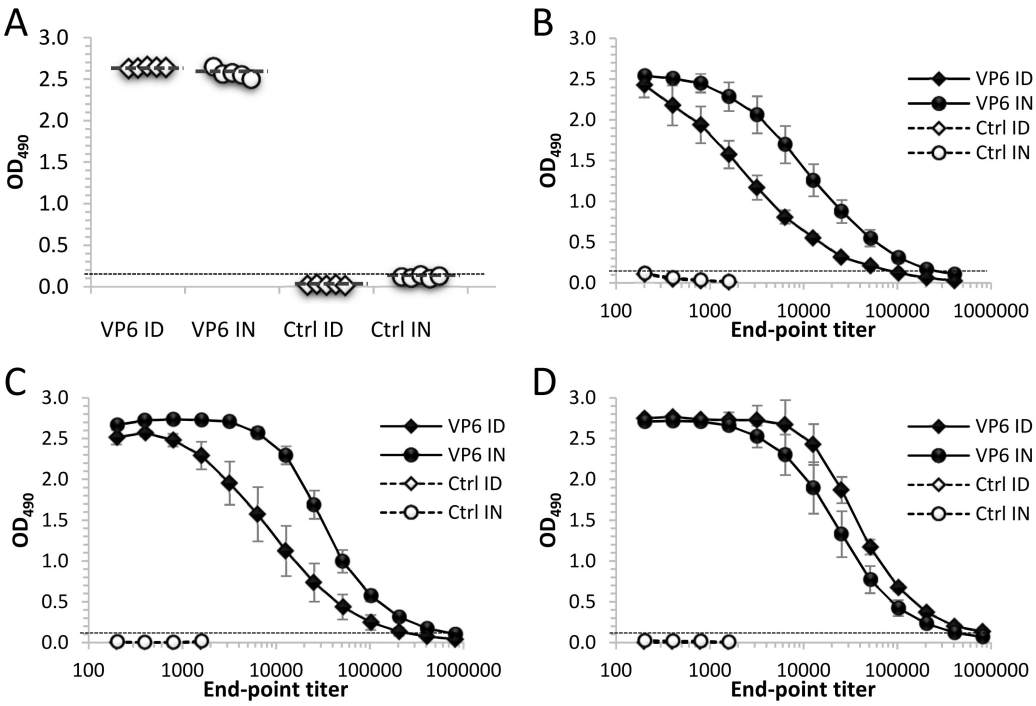


Figure 1

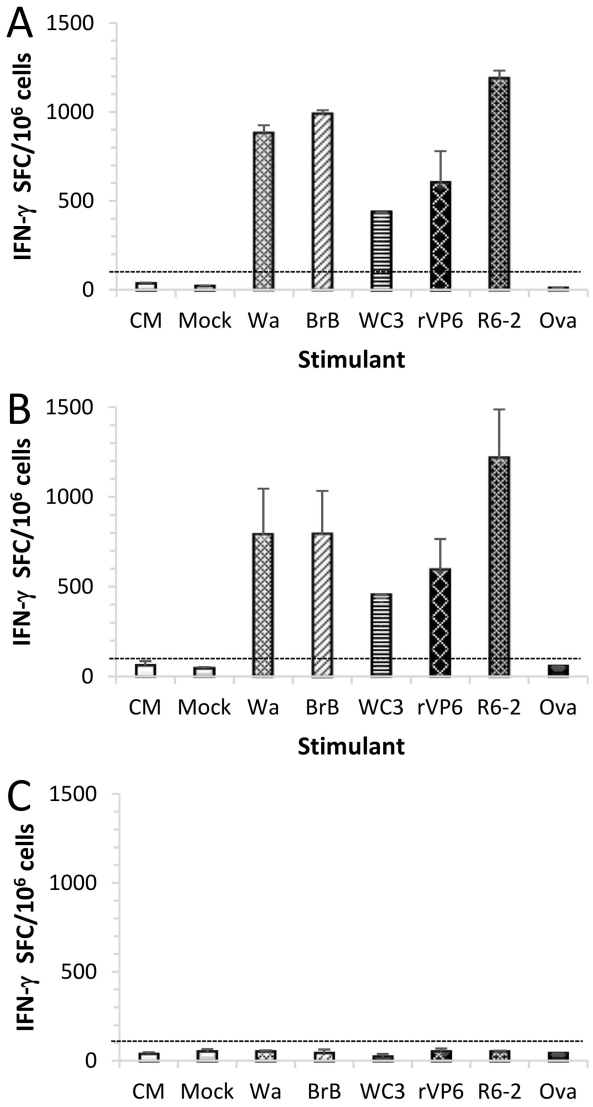
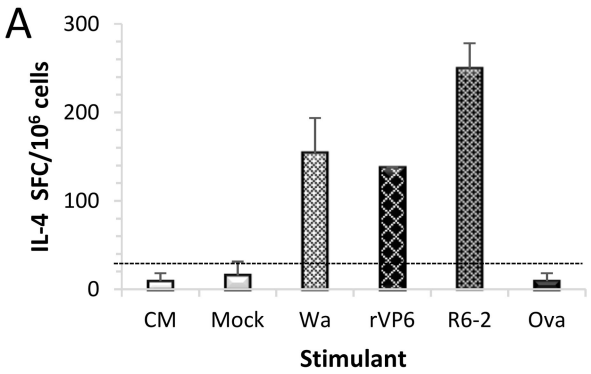
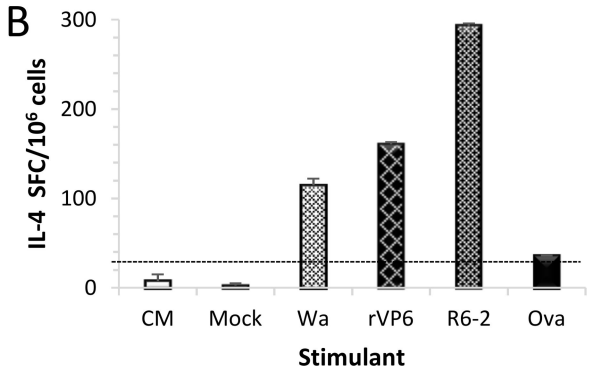


Figure 2





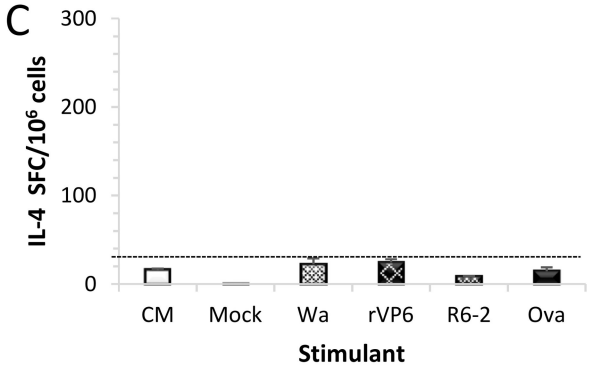
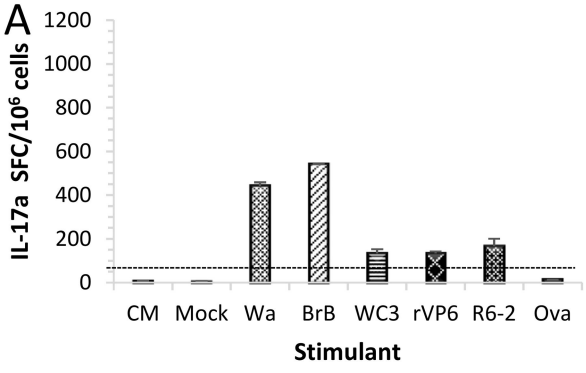


Figure 3



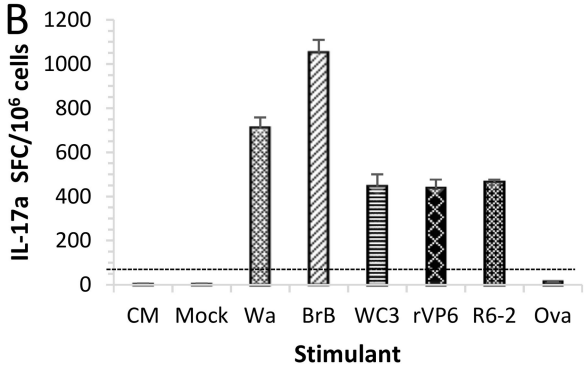


Figure 4

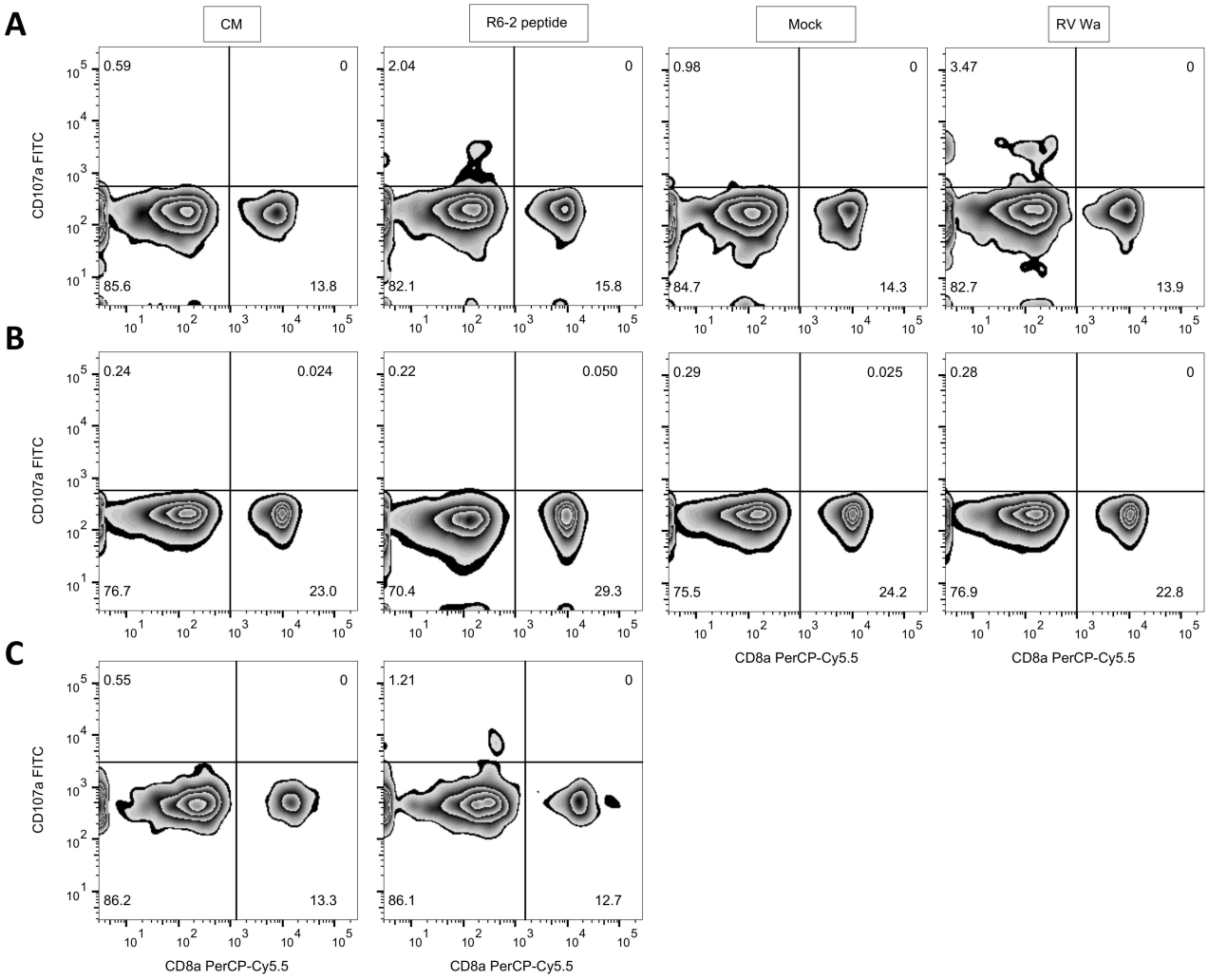

A
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Figure 5