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Modeling Pre-Existing Immunity to Adenovirus in Rodents: Immunological Requirements for Successful Development of a Recombinant Adenovirus Serotype 5-based Ebola Vaccine

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Abstract

Pre-existing immunity (PEI) to human adenovirus serotype 5 (Ad5) worldwide is the primary limitation to routine clinical use of Ad5-based vectors in immunization platforms. Using systemic and mucosal PEI induction models in rodents (mice and guinea pigs), we assessed the influence of PEI on the type of adaptive immune response elicited by an Ad5-based vaccine for Ebola with respect to immunization route. Splenocytes isolated from vaccinated animals revealed that immunization by the same route in which PEI was induced significantly compromised Ebola Zaire glycoprotein (ZGP)-specific IFN- γ ⁺ CD8⁺ T cells and ZGP-specific multifunctional CD8⁺ T cell populations. ZGP-specific IgG1 antibody levels were also significantly reduced and a sharp increase in serum anti-Ad5 neutralizing antibody (NAB) titers noted following immunization. These immune parameters correlated with poor survival after lethal challenge with rodent-adapted Ebola Zaire virus (ZEBOV). Although the number of IFN- γ ⁺ CD8⁺ T cells was reduced in animals given the vaccine by a different route from that used for PEI induction, the multifunctional CD8⁺ T cell response was not compromised. Survival rates in these groups were higher than when PEI was induced by the same route as immunization. These results suggest that antigen-specific multi-functional CD8⁺ T cell and Th2 type antibody responses compromised by PEI to Ad5 are required for protection from Ebola. They also illustrate that methods for induction of PEI used in pre-clinical studies must be carefully evaluated for successful development of novel Ad5-based vaccines.

Keywords

Pre-existing immunity; Adenovirus serotype 5; Zaire Ebola virus; Vaccine; Mouse; Guinea pig; Multifunctional CD8⁺ T cell response

Introduction

Recombinant human adenovirus serotype 5 (Ad5) is currently one of the most potent vaccine platforms that can induce strong adaptive immune responses against encoded antigens^{1–3}. Therefore, Ad5 vectors have been actively developed as vaccines for many pathogenic bacteria and viruses (anthrax, hepatitis C, malaria, botulism, rabies and

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Ebola) ⁴⁻⁹. Although pre-clinical results obtained with adenovirus-based vaccines are quite encouraging, pre-existing immunity (PEI) to Ad5 in the general population has prevented progression to clinical use^{10, 11}. Approximately 45% of the healthy adult population in the United States and India, 72% in China and 80% in South Africa, Gambia and Thailand have serological evidence of PEI to Ad5 in the context of measurable anti-Ad5 neutralizing antibody (NAB) titers in the circulation ¹²⁻¹⁵. This may manifest as high levels of pro-inflammatory cytokines at the time the vaccine is given, a humoral antibody response that neutralizes the adenovirus, or a cellular immune response that targets and destroys cells expressing adenoviral antigens ^{11, 16}.

Several different approaches have been taken to resolve issues associated with PEI to Ad5. Increasing the vaccine dose to override vector neutralization by NAB was the first and most obvious tactic ^{17, 18}. Even though this can incrementally improve the potency of Ad5-based vaccines in those with PEI, the risk of severe side effects due to the large dose of virus required to achieve this effect outweighed the utility of this approach. Sophisticated methods to improve antigen expression through optimization of promoter and codon sequences and incorporation of protein adaptor sequences in antigen expression cassettes has allowed for notable antigen expression from the portion of the vaccine which is not neutralized in those with PEI ^{19, 20}. Use of rare human adenovirus serotypes (Ad11, Ad12, Ad35) or those that infect other species (chimpanzee, porcine, bovine) as vaccine vectors alone or in combination with other Ad serotypes in prime-boost regimens represents another rational approach taken to improve vaccine efficacy in those with PEI to Ad5 ²¹⁻²⁵. However, many of these viruses are difficult to produce on a large scale and are less immunogenic than Ad5. In addition, timelines needed for prime-boost protocols are often not amenable to effective, widespread immunization campaigns in regions where transportation and access to medical care are limited.

Modification of Ad5 capsid proteins at the genetic level to create hexon-chimeric adenoviruses and chemical modification virus proteins with biocompatible polymers represent some of the most highly sophisticated efforts to protect vectors from recognition by the immune system in those with PEI to Ad5 ^{26, 27}. Unexpected changes in the natural *in vivo* transduction efficiency of the modified viruses and additional modifications required to maintain potency of these vaccines currently limit the feasibility of these platforms in a clinical setting ²⁸. More recent studies have focused on eliminating the necessity of virus transduction for effective immunization through fusion of antigen to capsid proteins on the outer surface of intact virions or generation of hapten-conjugates with antigen and disrupted virus capsids ^{29, 30}.

After careful review of the literature, it is evident that intramuscular (IM) injection currently remains the primary route of administration of many vaccines ³¹. This is not surprising since it is the most direct way to induce strong, systemic immune responses to antigens. Thus, early studies designed to test novel immunization platforms that circumvent PEI to Ad5 established PEI by IM injection of an unrelated Ad5 vector prior to immunization ^{10, 11, 16}. Considering that Ad5 naturally infects humans through the respiratory tract ³², direct injection of virus into the systemic circulation of a given animal model in this manner does not resemble what naturally occurs in the human population. Even though there is a general consensus within the scientific community that PEI to vectors developed from viruses commonly found in the environment is a significant issue that must be addressed when developing a clinical therapeutic containing these agents ¹⁰, a standardized method for establishing PEI that closely reflects natural infection currently does not exist. In addition, very little is known about this particular immunological state in the context of how it influences the immune response to an antigen and other microbial pathogens.

One of the primary goals of our laboratory is to develop a potent, long-acting Ad5-based vaccine for Ebola Zaire that is highly effective in those with prior-exposure to Ad5. In order to meet this objective, we conducted the study summarized here to determine how PEI to Ad, when induced by either the systemic or mucosal route, influences the immune response to a model antigen, Ebola Zaire glycoprotein (ZGP). A secondary aim was to identify a method for induction of PEI to Ad5 which could be viewed as the most stringent test under which to evaluate novel formulation candidates designed to improve vaccine potency in those with PEI. Assays to evaluate quantitative and qualitative antigen-specific CD8+ T cell responses were performed on samples obtained from naïve animals and those with PEI established by different routes. Antibody responses to ZGP and to the Ad5 vector were also assessed. Survival after lethal challenge served as a final indicator of the stringency of each model of PEI. Data obtained from these studies assisted us in identifying specific types of immune responses that must be reconstituted through formulation or other modification of the vaccine in order to promote survival from Ebola in those with prior-exposure to Ad5.

Experimental Section

Adenovirus Production

The codon optimized full-length Zaire Ebola glycoprotein sequence (Genbank/NCBI; Mayinga strain 76, Gene accession number: AF086833) was cloned in an E1/E3-deleted adenovirus serotype 5 vector under the control of chicken- β -actin promoter (Ad-CAGoptZGP) and further amplified in HEK 293 cells (ATCC, CRL-1573) as previously described^{19,33}. Virus was purified from cell lysates by two rounds of cesium chloride gradient ultracentrifugation. Virus bands were desalted by dialysis overnight in 100 mM potassium phosphate buffer (pH 7.4) and infectious titer was determined using the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Preparations with infectious to physical particle ratios below 1:200 were used in this study.

Animal Studies

All procedures were approved by the Institutional Animal Care and Use Committees at The University of Texas at Austin and the University of Texas Medical Branch in Galveston and are in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals. The time course for establishment of PEI, immunization, sample collection and analysis is illustrated in Figure 1.

Immunization—Six-week-old male B10.Br mice (MHC H-2k) and male Hartley guinea pigs (body weight: 400 g) were obtained from the Jackson Laboratory (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA), respectively. Animals were housed in a temperature-controlled, 12-hour light-cycled facility at the Animal Research Center of The University of Texas at Austin and were given free access to standard rodent chow (Harlan Teklad, Indianapolis, IN) and tap water. Animals were anesthetized by a single intra-peritoneal injection of a 3.9:1 (mice) or 7.5:1 (guinea pigs) mixture of ketamine (100 mg/ml, Wyeth, Fort Dodge, Animal Health, Overland Park, KS) and xylazine (100 mg/ml, Sigma Aldrich, St. Louis, MO). Once deep plane anesthesia was achieved, animals were immunized with 1×10^8 (mice) or $1 \times 10^7 - 1 \times 10^9$ (guinea pigs) infectious virus particles (ivp) of Ad-CAGoptZGP by various routes. Intramuscular injection involved direct injection of the preparation into each gastrocnemius muscle located on the hind limb (mice: 50 μ l/muscle, guinea pigs: 100 μ l/muscle). Nasal immunization was performed by slowly dripping the preparation into each nostril (mice: 10 μ l/nostril, guinea pigs: 50 μ l/nostril) using a standard micropipette (Gilson, Middleton, WI). For sublingual immunization, sterile forceps were placed under the tongue of the animal and 10 μ l of the preparation slowly dispensed

with a standard micropipette as described previously³³. Animals given the vaccine by the nasal and sublingual routes were maintained in an upright position for 30 minutes after immunization to minimize choking and accidental swallowing of the vaccine.

Establishment of Pre-Existing Immunity to Adenovirus—First generation adenovirus expressing β -galactosidase under the control of the cytomegalovirus promoter (AdlacZ) was used to establish PEI in mice and guinea pigs^{33, 34}. Twenty-eight days prior to immunization, systemic PEI (IM PEI) or mucosal PEI (IN PEI) was induced by administration of 5×10^{10} (mice) or 1×10^{12} (guinea pigs) virus particles (vp) to the muscle of each hind limb or in the nasal cavity as described above. Blood was collected via the saphenous (mice) or cephalic (guinea pigs) vein and serum screened for anti-Ad5 neutralizing antibodies (NAB). At the time of vaccination, animals had an average anti-Ad5 circulating NAB titer of $1:171 \pm 48$ (IM PEI) or $1:103 \pm 26$ (IN PEI), which falls within the lower range of average values reported in humans after natural infection^{12–15}.

Challenge with Ebola Zaire—All challenge experiments were performed under biosafety level 4 (BSL-4) conditions in an AAALAC accredited animal facility at the Robert E. Shope BSL-4 Laboratory at The University of Texas Medical Branch in Galveston, Texas. Twenty-four days post-immunization, vaccinated mice were transferred to the BSL-4 lab where they were challenged on day 28 by intraperitoneal injection with 1,000 pfu of mouse (MA-ZEBOV; $30,000 \times \text{L.D.}_{50}$)³⁵ or guinea pig adapted Ebola Zaire (GPA-ZEBOV; $100 \times \text{L.D.}_{50}$)³⁶. Animals were monitored for clinical signs of disease and weighed daily. At the end of the study, survivors were bled and serum samples gamma-irradiated (5 Mrad) prior to removal from the BSL-4 lab for cytokine and transaminase analysis.

ELISPOT

IFN- γ ELISPOT assays were performed using the ELISpot Mouse Set (BD Pharmingen) according to the manufacturer's instructions. Briefly, a 96-well ELISPOT plate was pre-coated with 5 $\mu\text{g}/\text{ml}$ anti-mouse IFN- γ capture antibody overnight at 4 °C. Coated wells were blocked with complete Dulbecco's Modified Eagle's Medium (DMEM, Mediatech) for 2 hours at room temperature. Cells were isolated from the spleen, lung, mesenteric lymph nodes (MLNs) and submandibular lymph nodes (SMLNs) as described previously³³, washed twice with complete DMEM and added to each well (5×10^5 cells/well) with the TELRTFSI peptide (0.5 $\mu\text{g}/\text{well}$, New England Peptide, Gardner, MA) that bears the ZGP immunodominant MHC class I epitope for mice with the H-2k haplotype (B10.Br)³⁷. Plates were placed at 37°C for 20 hours with 5% CO₂. Wells were washed twice with deionized water, washed with buffer (0.05% Tween-20 in PBS, pH 7.4) and subsequently incubated with biotinylated anti-mouse IFN- γ antibody at a concentration of 2 $\mu\text{g}/\text{ml}$ for 2 hours at room temperature. Following three consecutive washes with buffer, wells were incubated with 5 $\mu\text{g}/\text{ml}$ of horseradish peroxidase (HRP)-conjugated streptavidin antibody for an additional hour. Wells were then washed and plates developed with AEC substrate (Sigma). Spots were counted using an automated ELISpot reader (CTL-ImmunoSpot® S5 Micro Analyzer, Cellular Technology Ltd., Shaker Heights, OH).

Intracellular cytokine staining

Splenocytes isolated from mice were washed three times with complete DMEM containing 50 μM β -mercaptoethanol (Sigma), penicillin (10,000 I.U./ml)/streptomycin (10,000 $\mu\text{g}/\text{ml}$) (Gibco, Invitrogen, Grand Island, NY), L-glutamine (1mM, Hyclone, Salt Lake City, UT), 1 mM sodium pyruvate (Lonza, Walkersville, MD), 1 mM non-essential amino acids (Lonza) and stimulated with TELRTFSI peptide (0.5 $\mu\text{g}/\text{well}$) in the presence of Brefeldin A at 37°C for 6 hours. Following stimulation, cells were surface stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD8 α antibody (1:150 in PBS, BD Pharmingen,

San Diego, CA) for 30 minutes at 4 °C and treated with Cyto-fix/Cytoperm (BD Pharmingen) for 20 minutes at 4 °C. For intracellular cytokine staining, cells were labeled with PE-labeled anti-mouse IFN- γ , PerCP-Cy5.5-labeled anti-mouse TNF- α and APC-labeled anti-mouse IL-2 antibodies (1:150 in Permash, BD Pharmingen) for 30 minutes at 4 °C. Cells were washed and positive cells counted using four-color flow cytometry (FACS Fortessa, BD Biosciences, Palo Alto, CA) in The University of Texas at Austin Flow Cytometry Core Facility. Over 500,000 events were captured per sample. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Characterization of Ebola Zaire glycoprotein-specific antibodies

Full length Ebola Zaire glycoprotein was produced using the Zaire GP₃₃₋₆₃₇ Δ TM-HA construct according to published methods³⁸. Sub-confluent HEK 293T cells were transfected with the plasmid using a standard calcium phosphate transfection protocol. Four days later, supernatant was collected, concentrated by centrifugation (Centricon Plus-100, Millipore, Billerica, MA) and purified by immunoaffinity chromatography (Anti-HA Affinity Matrix, Roche, Mannheim, Germany). To assess anti-ZGP immunoglobulin levels in serum, Immulon 2HB plates (Fisher Scientific, Pittsburgh, PA) were coated with purified Zaire GP₃₃₋₆₃₇ Δ TM-HA (3 μ g/well) in PBS (pH 7.4) overnight at 4°C. Plates were washed 4 times with PBS containing 0.05% Tween 20 and blocked in PBS containing 1% BSA (Sigma) for 1 hour at room temperature. Heat-inactivated serum samples were diluted 1:20 in sterile PBS. One hundred microliters of each dilution was added to the antigen-coated plates for 2 hours at room temperature. Plates were washed 4 times with PBS containing 0.05% Tween 20 and incubated with HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgM (1:2,000, Southern Biotechnology Associates, Birmingham, AL) or HRP-conjugated goat anti-guinea pig IgG, IgG1, IgG2, IgM antibodies (1:2,000, Bethyl Laboratories, Inc., Montgomery, TX) in separate wells for 1 hour at room temperature. Plates were washed and 200 μ l of substrate solution (0.4 mg/ml o-phenylenediamine (Sigma) in 50 mM phosphate-citrate buffer, pH 5.0 with 0.03 % (v/v) hydrogen peroxide) were added to each well. The plate was incubated at room temperature for 10 minutes and optical densities read at 450 nm on a microplate reader (GloMax-Multi+ Detection System, Promega, Madison, WI).

Neutralizing antibody assay

Anti-Ad5 neutralizing antibody (NAB) titers were assessed in serum samples from mice and guinea pigs prior to and after immunization according to established methods^{33, 34, 39}. Heat-inactivated serum was diluted in DMEM in twofold increments starting from a 1:20 dilution. Each dilution was mixed with AdlacZ for 1 hour at 37 °C and added to HeLa cells grown on 96-well plates. Two hours later, 100 microliters of DMEM supplemented with 20% FBS were added to each well. Cells were incubated additional 24 hours and β -galactosidase expression visualized by histochemical staining. For each sample, the serum dilution that corresponded to a 50% reduction in transgene expression was obtained by the method of Reed and Muench as described previously³⁹. The absence of neutralization in samples containing medium only (negative control) and FBS (serum control) and an average titer of 1:1,280 \pm 210 read from an internally generated positive control stock serum were criteria for qualification of each assay.

Serum Cytokines and Transaminases

Cytokines (IL-6, IL-12, TNF- α , IL-2 and IL-10) were quantitated with commercially available ELISA kits (Invitrogen, BioSource International, Camarillo CA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using Vitros AST/SGOT and ALT/SGPT DT slides on a Vitros DTSC autoanalyzer (Ortho-Clinical Diagnostics, Rochester, NY).

Statistical Analysis

Data were analyzed for statistical significance using SigmaStat (Systat Software Inc., San Jose, CA) by performing a one-way analysis of variance (ANOVA) between control and experimental groups, followed by a Bonferroni/Dunn post-hoc test when appropriate. Differences in the raw values among treatment groups were considered statistically significant when $p < 0.05$.

Results

Pre-Existing Immunity and the Magnitude of the T Cell Response in Mice

In order to determine how establishment of PEI by either the systemic (IM) or mucosal (IN) route influenced the antigen-specific T cell response, mononuclear cells were collected 10 days after immunization. Ad-CAGoptZGP induced the strongest responses in the systemic and mucosal compartments of naïve animals than in those with PEI, regardless of immunization route (Figure 2). Prior exposure to the virus through IM injection followed by vaccination by the same route reduced the number of activated cells in the spleen to a much greater degree than when PEI was established by the nasal route (IM PEI/IM: 44.0% reduction with respect to naïve animals vs. 25.8% reduction IN PEI/IM, $p < 0.001$, Figure 2A). Establishment of PEI by instillation of virus in the nasal cavity had the most profound effect on ZGP-specific IFN- γ secreting mononuclear cells (MNCs) in the spleen of animals also immunized by the IN route (IN PEI/IN, 55.3% reduction). Establishment of PEI by the nasal route had a similar effect on the systemic antigen-specific T cell response after sublingual immunization. Prior exposure to Ad by the IN route suppressed production of these cells by 56.5% while establishment of PEI by the IM route reduced cell numbers by 19.5% (Figure 2A).

Mucosal PEI significantly reduced the average number of ZGP-specific IFN- γ^+ MNCs in BAL fluid of mice immunized by the IN route to a much greater degree than when PEI was established by IM injection (IN PEI/IN: 62.5% reduction with respect to naïve animals vs. 33.5% reduction IM PEI/IN, $p < 0.001$, Figure 2B). A similar trend was noted in BAL of mice immunized by the SL route (IN PEI/SL: 58.8% reduction; IM PEI/SL: 47.9% reduction, $p < 0.001$). In sharp contrast, prior exposure to Ad5 significantly enhanced the number of activated cells in MLNs and SMLNs with respect to naïve animals when the vaccine was given by either the IN or SL route (Figures 2C and 2D). The most prominent increases were found in samples from SMLNs of mice immunized by IN route in the presence of systemic PEI (85 ± 27.83 SFCs/million MNCs: Naïve/IN vs. 122.5 ± 19.6 SFCs/million MNCs: IM PEI/IN, $p < 0.05$) and mice immunized by the SL route in the presence of mucosal PEI (Naïve/SL: 65.5 ± 23.46 SFCs/million MNCs, IN PEI/SL: 294 ± 15.64 SFC/million MNCs; $p < 0.001$; Figure 2D).

Pre-Existing Immunity and Multifunctional CD8+ T cell Responses in Mice

Previously, we have shown that, even though a single dose of vaccine given by the IN route to naïve animals induced significantly less ZGP-specific IFN- γ^+ CD8+ T cells than immunization by IM injection, it could afford full protection from lethal challenge with Ebola Zaire^{33, 34}. When PEI was established by IM injection, animals immunized by the IN route survived while those immunized by the IM route succumbed to infection. Further analysis revealed that the amount of ZGP-specific IFN- γ^+ CD8+T cells produced in animals with systemic PEI given the vaccine nasally was substantially lower than that found in naïve mice immunized by IM injection, yet similar to that seen in naïve animals vaccinated by the IN route. Collectively, these data suggest that the magnitude of the antigen-specific IFN- γ response is not fully predictive of vaccine-elicited protection from ZEBOV challenge in mice with PEI to the vaccine carrier.

To further characterize the influence of PEI induced by systemic or nasal route on antigen-specific CD8⁺ T cell responses, we performed a comprehensive functional analysis using multiparameter flow cytometry^{40,41}. With this strategy, we were able to delineate seven distinct cytokine-producing cell populations and characterize them at the single-cell level based on IFN- γ , IL-2 and TNF- α secretion patterns. The relative frequency of these distinct populations defines the quality of the vaccine-induced CD8⁺ response. Complete analysis of IFN- γ producing cells identified four distinct populations: IFN- γ ⁺, IFN- γ ⁺IL-2⁺, IFN- γ ⁺TNF- α ⁺ and IFN- γ ⁺IL-2⁺TNF- α ⁺. This analysis further revealed a correlation between the frequency of robust, multifunctional CD8⁺ T cells (IFN- γ ⁺IL-2⁺TNF- α ⁺), which are most effective at responding to a given antigen, and the manner by which PEI was induced in mice immunized by IM, IN or SL routes. Ten days after immunization, systemic PEI not only suppressed the total number of Zaire GP-specific IFN- γ producing CD8⁺ T cells in the spleen of mice given the vaccine by intramuscular injection (5.30%: Naïve/IM vs. 1.82%: IM PEI/IM; Figure 3A), but also reduced ZGP-specific multifunctional CD8⁺ T cells (triple producers: IFN- γ ⁺IL-2⁺TNF- α ⁺; 35.4 \pm 0.37 %: Naive/IM vs. 24.29 \pm 2.01 %: IM PEI/IM, p <0.001; red arcs Figure 3G) with a concurrent increase in single cytokine producers (blue arcs Figure 3G). When PEI was established by the nasal route, the number of IFN- γ ⁺ CD8⁺ T cells was reduced to a lesser degree (5.30%: Naïve/IM vs. 3.18%: IN PEI/IM; Figure 3A) and the multifunctional CD8⁺ T cell population was not significantly compromised (35.4 \pm 0.37 %, Naive/IM vs. 37.88 \pm 0.83 %, IN PEI/IM, Figure 3G).

The next series of experiments was designed primarily to determine how nasal administration of our Ad5-based Ebola vaccine was able to bypass PEI induced by IM injection³⁴. In this context, mucosal PEI suppressed the number of IFN- γ ⁺ CD8⁺ T cells to a larger degree than systemic PEI (2.58%: Naïve/IN vs. 0.89%: IM PEI/IN vs. 0.77%: IN PEI/IN, Figure 3B). A similar trend was also noted in the multifunctional CD8⁺ T cell response (triple producers: 41.11 \pm 3.59 %: Naive/IN vs. 30.74 \pm 1.30 %, IM PEI/IN, p <0.05; 41.11 \pm 3.59 %: Naive/IN vs. 23.52 \pm 4.22 %, IM PEI/IN, p <0.01; red arcs; Figure 3H). These parameters were also evaluated after SL immunization. The total frequency of IFN- γ ⁺ CD8⁺ T cells was reduced to a larger degree in mice with PEI induced by the mucosal route than in mice with systemic PEI (0.21%: IN PEI/SL and 0.66%: IM PEI/SL, respectively; Figure 3C). Despite this notable reduction in the number of IFN- γ ⁺ CD8⁺ T cells, a significant increase in multifunctional CD8⁺ T cells was observed (triple producers: 24.20 \pm 0.91 %: Naive/SL vs. 37.04 \pm 1.91 %: IM PEI/SL, p <0.01; red arcs; Figure 3I). The multifunctional CD8⁺ T cell population was not affected by mucosal PEI (24.20 \pm 0.91 %: Naive/SL vs. 20.93 \pm 4.92 %: IN PEI/SL, p >0.05; red arcs; Figure 3I).

Pre-Existing Immunity and Antibody responses to Zaire GP and Ad5 in Mice

To characterize the impact of systemic and mucosal PEI on B cell-mediated antibody responses, anti-ZGP-specific immunoglobulin isotypes and adenovirus serotype 5-specific neutralizing antibody (NAB) levels were assessed in samples collected 42 days after immunization. Induction of PEI by the IM route reduced serum anti-ZGP-specific total IgG levels by 63.6% with respect to levels of naïve mice vaccinated by IM injection (Figure 4A). Of the serotypes tested in this group, IgG1 was the most profoundly affected by PEI (90.8% reduction). IgG2a and IgG2b were reduced by 78.8 and 78.4%, respectively. When PEI was established by the mucosal route, a significant decrease in the production of IgG1 was observed with respect to naïve animals immunized by instillation of the vaccine in the nasal cavity (IgG1: 88.99% reduction; p <0.05; Figure 4B). Aside from this, none of the other anti-ZGP-specific isotypes were affected by PEI induced by either route in animals immunized by the IN route. Mucosal PEI also did not significantly affect anti-ZGP-specific IgG isotype levels in mice given the vaccine by the SL route (Figure 4C). Systemic induction of PEI,

however, did significantly reduce ZGP-specific total IgG (66.3%), IgG1 (77.5%), IgG2a (80.6%) and IgM (80.5%) levels with respect to those seen in naïve mice (Figure 4C).

In order to understand how PEI affects the B cell-mediated immune response against the adenoviral vector itself, circulating anti-Ad5 NAB levels were evaluated in serum samples collected 42 days after immunization. Systemic PEI greatly increased the amount of circulating anti-Ad5 NABs after IM immunization as levels rose by a factor of 5 from an average of $1:171 \pm 48$ prior to vaccination to $1:925 \pm 213$ after treatment ($p < 0.05$, Figure 4D). However, the anti-Ad5 NAB titers found in mice with IM PEI after IN or SL immunization were not significantly higher than levels measured prior to vaccination ($1:171 \pm 40$ (IM PEI only), $1:184 \pm 40$ (IM PEI/IN), $1:314 \pm 186$ (IM PEI/SL); $p > 0.05$; Figure 4D). A similar trend was found in samples from mice immunized by IM or IN route in the presence of mucosal PEI.

Pre-Existing Immunity and Survival of Mice after Lethal Challenge

The most direct way of evaluating the impact of PEI on vaccine potency is to assess protection from a lethal dose of pathogen. We did this by monitoring survival rate, weight loss and toxicity after exposure to mouse-adapted ZEBOV (MA-ZEBOV). The virus was uniformly lethal in control mice given saline (PBS, Figure 5A). In contrast, all naïve mice immunized by IM injection survived without notable loss of body weight (Figure 5A, D). As seen previously^{33, 34}, systemic PEI significantly compromised the efficacy of the vaccine when it was given by the IM route with only 20% survival observed (IM PEI/IM; $p < 0.01$; Figure 5A). Chemical analysis of samples taken from mice in this treatment group (IM PEI/IM) post-challenge revealed serum ALT levels that were 47.5 times that of naïve, immunized animals (IM) and AST levels that were increased by a factor of 27, indicative of severe liver damage from Zaire Ebola infection ($p < 0.001$; Figure 5G). As is commonly seen in samples taken from non-survivors of Ebola infection⁴²⁻⁴⁴, samples from these animals also contained elevated levels of cytokines (IL-6: 597.72 ± 577.32 pg/ml, IL-10: 1383.8 ± 143.96 pg/ml, IFN- γ : 635.5 ± 294.65 pg/ml; Figure 5J). As routinely found in survivors of Ebola hemorrhagic fever, samples from the Naïve/IM group contained only trace amounts of each compound (IL-6: not detected, IL-10: 17.49 ± 3.02 pg/ml, IFN- γ : not detected, Figure 5J).

Interestingly, 90 % of the mice given the vaccine by the IM route in the presence of mucosal PEI survived (IN PEI/IM, Figure 5A) without a notable increase in Ebola-induced liver toxicity (Figure 5G). To fully define how PEI affects protective efficacy following nasal immunization, naïve mice and those with systemic or mucosal PEI were also given a lethal dose of MA-ZEBOV. Consistent with previous studies³⁴, naïve mice (Naïve/IN) and those with systemic PEI (IM PEI/IN) survived without notable changes in body weight (Figures 5B and 5E). However, 60% of the animals with mucosal PEI survived challenge (IN PEI/IN, Figure 5B). As seen with animals immunized by IM injection, samples taken from this group also revealed sharp elevations in serum ALT (768.75 ± 337.48 U/L) and AST (IN PEI/IN: 1086 ± 584.97 U/L, $p < 0.001$, Figure 5H) with respect to naïve animals immunized by the same route that survived challenge (Naïve/IN: 144 ± 30.19 (ALT) and 330 ± 102.94 U/L (AST) respectively)). They also contained significant amounts of IL-10 and IFN- γ with respect to those from naïve immunized mice ($p < 0.05$, Figure 5K).

Naïve mice and those with systemic or mucosal PEI vaccinated by the SL route were also challenged with MA-ZEBOV 28 days after vaccination. Eighty percent of naïve mice and 100% of those with systemic PEI to Ad5 survived (Figure 5C). Interestingly, mucosal PEI did not significantly compromise the efficacy of the vaccine when given by the SL route with 87.5% survival observed in this treatment group (IN PEI/SL, Figure 5C). Serum samples taken from this group post-challenge revealed moderate increases in ALT ($118.75 \pm$

24.09 U/L ($p < 0.05$) and IFN- γ ($1,384.4 \pm 222.15$ pg/ml ($p < 0.001$)) with respect to naïve, immunized animals (Figures 5I and 5L).

Pre-Existing Immunity and the Zaire GP-specific Antibody Response in Guinea Pigs

Several animal models have been developed to study the pathogenesis of Ebola virus infection, assess the safety and efficacy of vaccine candidates and identify the immune mechanisms necessary for optimal protection against lethal challenge. Currently, the progression and pathogenesis of infection in guinea pigs and non-human primates most closely resembles that of the human disease^{36, 45}. Thus, in order to determine if our observations in mice would translate to that which occurs in humans, systemic or mucosal PEI was established in male Hartley guinea pigs 28 days prior to immunization with doses of vaccine ranging from 1×10^7 to 1×10^9 ivp as outlined in Figure 1B. Due to the lack of commercially available reagents to perform T cell assays in guinea pigs, we were not able to characterize the ZGP-specific T cell responses as was done in mice.

Systemic PEI reduced serum anti-ZGP-specific total IgG levels by 67.4% in all animals immunized by IM injection ($p < 0.05$, Figure 6A). IgG1 was also suppressed (~ 95.2% reduction) without regard to vaccine dose. At the highest vaccine dose tested (1×10^9 ivp), IgG1 was the only isotype affected by mucosal PEI in animals given the vaccine nasally (82.2% reduction, $p < 0.05$, Figure 6B). Although antibody production was significantly blocked in animals given the lowest dose of vaccine nasally regardless of the method by which PEI was induced, mucosal PEI held the most prominent effect (Total IgG: 92.1%, IgG1: 97.3%, IgG2: 84.7 and IgM: 99.2% reduction $p < 0.01$, Figure 6B). There was no significant difference in the amount of each ZGP-specific IgG isotype produced after SL immunization of naïve animals and those with mucosal or systemic PEI ($p > 0.05$, Figure 6C).

Anti-Ad5 NAB levels were evaluated in serum collected 24 days after immunization. Systemic PEI greatly increased the amount of circulating anti-Ad5 NABs after IM immunization by a factor of 10 from an average of $1:212 \pm 79$ prior to vaccination to $\sim 1:1,985 \pm 363$ after treatment for all vaccine doses tested ($p < 0.01$, Figure 6D). Mucosal PEI also boosted circulating anti-Ad5 NABs after IN administration of 1×10^8 and 1×10^9 ivp of vaccine by a factor of 4 ($p < 0.01$) while there was no significant difference between anti-Ad5 NAB levels of naïve guinea pigs given 1×10^7 ivp of vaccine and those with mucosal PEI immunized in the same manner ($p = 0.08$). Systemic PEI increased anti-Ad5 NAB levels in guinea pigs immunized by the SL route fourfold while mucosal PEI increased NAB levels fivefold ($p < 0.01$).

Pre-Existing Immunity and Survival of Guinea Pigs After Challenge

In a final effort to determine how changes in antibody production in response to PEI impacts survival, guinea pigs were challenged with 1,000 pfu ($\sim 100 \times LD_{50}$) of guinea pig-adapted Ebola Zaire (GPA-ZEBOV). Unimmunized guinea pigs (negative control, PBS) experienced significant weight loss starting from day 5 post-challenge that progressed until death on days 6 through 9 (Figure 7A). Naïve guinea pigs given the vaccine by the IM route (dose: 1×10^9 ivp) were fully protected. Consistent with mouse challenge results, PEI established by the IM route significantly decreased protective efficacy in a dose dependent manner (IM PEI/IM (1×10^9), 80% survival; IM PEI/IM (1×10^8), 40% survival, $p < 0.01$, Figure 7A). Survival was reduced by 60% when PEI was established by the mucosal route and the vaccine given by IM injection (IN PEI/IM (1×10^8)). Samples taken after challenge revealed that serum AST levels were significantly elevated in guinea pigs with PEI established by either route with respect to naïve, immunized animals (Naïve/IM, 104 ± 37.1 U/L) by a factor of 2.4 (IM PEI/IM: 1×10^9), 6.2 (IM PEI/IM: 1×10^8) and 5.8 (IN PEI/IM: 1×10^8 , Figure 7G).

PEI induced by the nasal route also compromised the protective efficacy of the vaccine after IN immunization in a dose dependent manner (Figure 7B). A high vaccine dose (1×10^9 ivp) fully protected immunized animals with mucosal PEI (IN PEI/IN (1×10^9)). However, the challenge was uniformly lethal when the vaccine dose was reduced to 1×10^7 ivp regardless of the manner by which PEI was induced (IM PEI/IN (1×10^7), IN PEI/IN (1×10^7): no survivors, Figure 7B). Samples taken from guinea pigs with systemic or mucosal PEI after challenge revealed that serum ALT (IM PEI/IN (1×10^7): 229 ± 6.78 U/L, IN PEI/IN (1×10^7): 323 ± 157.72 U/L) and AST (IM PEI/IN (1×10^7): $1,525 \pm 123.64$ U/L, IN PEI/IN (1×10^7): $2,192 \pm 726.29$ U/L) levels were significantly elevated in those with PEI established by either route with respect to naïve, immunized animals (naïve/IN: ALT: 37 ± 4.85 U/L, AST: 74.4 ± 13.78 U/L, $p < 0.001$, Figure 7H).

The protective efficacy of sublingual (SL) immunization in guinea pigs in the presence of systemic or mucosal PEI was also evaluated only with the highest dose of the vaccine (1×10^9 ivp, Figure 7C). Consistent with the mouse data, 80% of naïve guinea pigs and those with systemic PEI vaccinated by the SL route survived without notable loss of body weight (Figure 7C, 7F). However, when PEI was induced by the IN route, the efficacy of the SL vaccine was significantly compromised with only 40% survival observed in this treatment group (IN PEI/SL, $p < 0.05$, Figure 7C). None of the samples taken post-challenge from guinea pigs immunized by the SL route contained significantly elevated serum ALT and AST from baseline values (Figure 7I).

Discussion

Ebola hemorrhagic fever is a severe, often-fatal disease caused by a single stranded RNA virus of the *Filoviridae* family⁴⁶. Four of the five known species of Ebola are infectious to humans with case fatality rates up to 90%⁴⁷. Lack of remedies, unpredictability of outbreaks and its potential use as a bioweapon highlight the necessity for an effective strategy to prevent Ebola infection. Although there are currently no licensed vaccines or post-exposure treatments available for preventing or managing Ebola infection, several promising vaccine platforms that employ recombinant viruses to deliver genetic sequences for Ebola proteins have been developed^{48–53}. Among these, recombinant Ad5 and human parainfluenza virus type 3 (HPIV3) vectors, generated from common respiratory tract viruses are currently the most efficacious in animal models. It is not clear, however, how either of these platforms will perform in those with prior exposure by natural infection.

Natural transmission of Ebola involves either direct contact with body fluids of infected individuals or aerosol transmission of virus particles to the respiratory mucosa^{54–56}. Therefore, fully protective vaccines against the virus require strong systemic and mucosal antigen-specific adaptive immune responses to prevent or substantially limit infection^{57, 58}. Administration of a vaccine by different routes can have profoundly different effects on the type and strength of the immune response generated against a given antigen^{59, 60}. This is illustrated by the fact that IM injection of Ad5-based vaccines induces strong, systemic compartment-restricted immune responses while mucosal immunization (nasal, sublingual) induces both systemic and mucosal immune responses. Even though mucosal immunization often generates low-grade antigen-specific CD8+ T cell responses in comparison to those generated by IM injection, it fully protects naïve animals and those with prior exposure to Ad5 from lethal Ebola infection^{33, 34, 61}. In this regard, mucosal administration of adenovirus-based vaccines has long been thought to be a promising strategy to bypass PEI. However, specific immunological parameters altered by PEI to Ad5 and the mechanisms to explain poor survival following systemic injection of an Ad5-based Ebola vaccine in animals with PEI are not fully delineated.

The initial paradigm to explain how PEI to Ad dampens the adaptive immune response to an encoded antigen identified neutralization of virus particles to block transduction of target cells by anti-Ad antibodies and killing of Ad infected cells by cytotoxic T cells as primary events that limit the utility of Ad-based vaccines^{62–65}. We believe that this supports our observation that establishment of PEI by the same route significantly compromises the antigen-specific multifunctional CD8+ T cell response. It does not, however, fully explain why establishing PEI by a route different from that of immunization has little to no effect on this facet of the immune response. Although a few early reports suggested that Ad5 might transduce and activate dendritic cells (DCs), the role of transduced DCs in priming T cell responses was not clear^{66, 67}. The recent observation that neutralizing antibodies in the systemic circulation block uptake of Ad and subsequent transgene expression in CD11c+ DCs with a corresponding reduction in the antigen-specific CD8+ T cell response *in vivo* represents an important shift in the PEI model⁴¹. Given this information, we believe that there is a minimal threshold and/or requirement for the number and types of DCs and other antigen presenting cells (APCs) needed to induce Th1 and CD8+ T cell responses that varies according to the route of exposure (for PEI) or administration (for vaccination). This minimum is clearly not met when PEI is established by the same route as that of immunization while differing routes stimulate a diverse array of APCs to support production of antigen-specific multifunctional T cells sufficient to protect animals from lethal challenge⁶⁸. This may also explain why PEI appeared to strengthen the antigen-specific CD8+ T cell response in SMLNs and MLNs after IN or SL immunization as different types of local, resident DCs and other migratory APCs recruited to the area in response to local inflammation processed antigen within these regions to magnify the immune response⁶⁹. Additional studies to identify and characterize the types of APCs recruited to immunization sites and their distribution patterns are necessary to validate this model. Another very important role of DCs, cross-presentation of antigen to promote inhibitory CD8+ T cell responses was not addressed in the studies outlined here^{70, 71}. Additional studies to evaluate the activation status of CD4+CD25+ regulatory T cells (T_{reg}) in naïve animals and those with PEI are currently underway in our laboratories to further characterize the influence of PEI on the immunogenicity of our Ad5-based vaccine.

A controversy exists regarding which specific immune parameters correlate with protection against Ebola virus infection. A recent report showed that the antigen-specific CD8+ T cell response is unequivocally required for protection in non-human primates⁵⁷. Others have provided evidence suggesting that amount of antigen-specific IgG is the immune parameter that correlates with survival^{58, 72, 73}. This is the first report in which multi-parameter analysis of T cell isolates has been performed in rodents infected with and immunized against Ebola. Identification of changes in cell populations that have enhanced antigen-specific CTL activity (IFN- γ + and TNF- α +) as well as those that foster memory T cell differentiation (IFN- γ +, TNF- α +, IL-2+) allowed us to determine that the quality of the CD8+ T cell response more closely predicts survival rather than the quantitative measure of this response^{74–77}. We also found that the antigen-specific antibody response is highly dependent on the manner by which PEI is induced and the immunization route. Since T cells play a key role in B cell activation for antibody production, we believe that the suboptimal activation of the multifunctional CD8+ T cell response in conjunction with reduced level of serum ZGP-specific IgG isotypes, especially IgG1, were collectively responsible for poor survival. Even though we could not characterize the T cell response elicited by the vaccine in the guinea pig model, it was clear that the antigen-specific antibody response alone was not sufficient for protection from lethal challenge. Studies in naïve primates and those with prior exposure to Ad5 in which multifunctional analysis of both antigen-specific T and B cell subsets produced by our vaccine are evaluated are currently underway and will provide additional information about requirements for protection from Ebola.

One critical issue not addressed by this report and many others evaluating the role of PEI to Ad5 on *in vivo* performance of vaccine candidates is that the viruses used to establish PEI do not replicate as the wild type virus does during the natural course of human infection. In fact, replication of wild-type Ad5 obtained from human isolates is restricted in most rodent animal models and non-human primates⁷⁸. Thus, we realize that our PEI model may not fully reflect the immunological state induced by replication competent Ad5 in humans after natural exposure. Very recently, a host range mutant adenovirus, capable of replicating in non-human primates has been developed and used in studies where PEI to Ad5 is required^{79, 80}. Several pilot studies using this virus in primates are currently planned to determine how virus replication alters the immune response to our vaccine and parameters required for protection from lethal Ebola challenge.

One of the most important findings described in this manuscript is that the ZGP-specific multifunctional T cell response induced by our vaccine is more predictive of survival from lethal Ebola challenge than the quantitative T cell response in rodents. This cannot be considered alone but in tandem with the strength of the antibody response in larger animal models of Ebola infection. We also found that induction of pre-existing immunity to Ad5 through the nasal mucosa, which closely models natural exposure, may be limiting for nasal vaccine platforms using adenoviral vectors but not for those that plan to administer the vaccine by other routes. Thus, establishing PEI in this manner during pre-clinical study of nasal vaccine candidates will provide the most stringent test for evaluating novel formulations or other strategies designed to bypass PEI. Lastly, we believe that formulations that can restore and strengthen components of the adaptive immune response impaired by PEI to Ad5 and which are necessary for protection from Ebola will greatly improve vaccine potency in naïve individuals and those with PEI.

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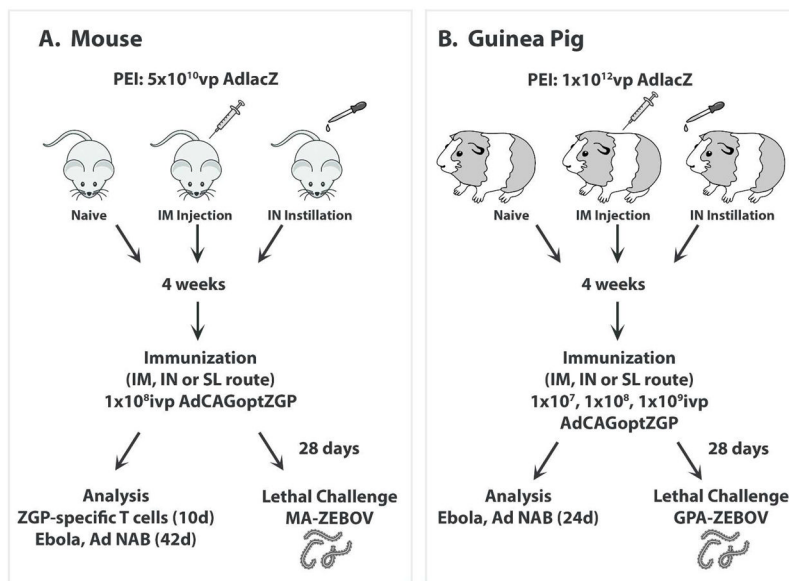


Figure 1. Timeline for Establishment of Pre-Existing Immunity to Adenovirus, Immunization, Sample Collection and Challenge for (A) Mice and (B) Guinea Pigs

PEI was established by administration of either 5×10^{10} (mice: $n=12$) or 1×10^{12} (guinea pigs: $n=5$) particles of adenovirus containing the β -galactosidase transgene by the intramuscular (IM) or intranasal (IN) route 28 days prior to vaccination. These groups are denoted as IM/systemic PEI or IN/mucosal PEI, respectively throughout the manuscript. Naïve animals and those with PEI to Ad5 were then given either 1×10^8 ivp (mice) or $1 \times 10^7 \sim 1 \times 10^9$ ivp (guinea pigs) of our vaccine (Ad-CAGoptZGP) by various routes. Control, naïve mice were given saline (PBS). Mononuclear cells were collected from the spleen, bronchioalveolar lavage (BAL) fluid, mesenteric lymph nodes (MLNs) and submandibular lymph nodes (SMLNs) of mice 10 days post-immunization for characterization of the ZGP-specific T cell response. Serum was collected 24 (guinea pigs) and 42 (mice) days after immunization for assessment of circulating anti-ZGP antibodies. Animals were challenged with 1,000 pfu of either MA-ZEBOV ($30,000 \times \text{L.D.}_{50}$) or GPA-ZEBOV ($100 \times \text{L.D.}_{50}$) 28 days after immunization.

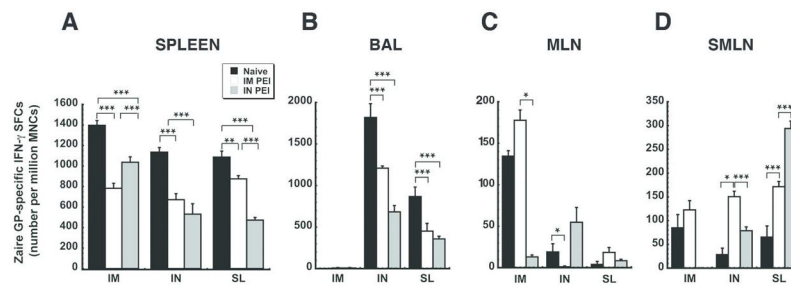


Figure 2. The Manner by which PEI is Induced Significantly Affects the Magnitude of the CD8+ T Cell Response Against Ebola Zaire Glycoprotein in the Systemic and Mucosal Compartments of Mice Immunized by Various Routes

The number of IFN- γ secreting mononuclear cells was quantitated in isolates taken from the spleen (Panel A), bronchioalveolar lavage (BAL) fluid (Panel B), mesenteric lymph nodes (MLN, Panel C) and submandibular lymph nodes (SMLN, Panel D) from naïve mice and those with PEI to Ad5 10 days after immunization by ELISPOT. Results are expressed as average values \pm the standard error of the mean and are representative of data collected over the course of three separate experiments. * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.

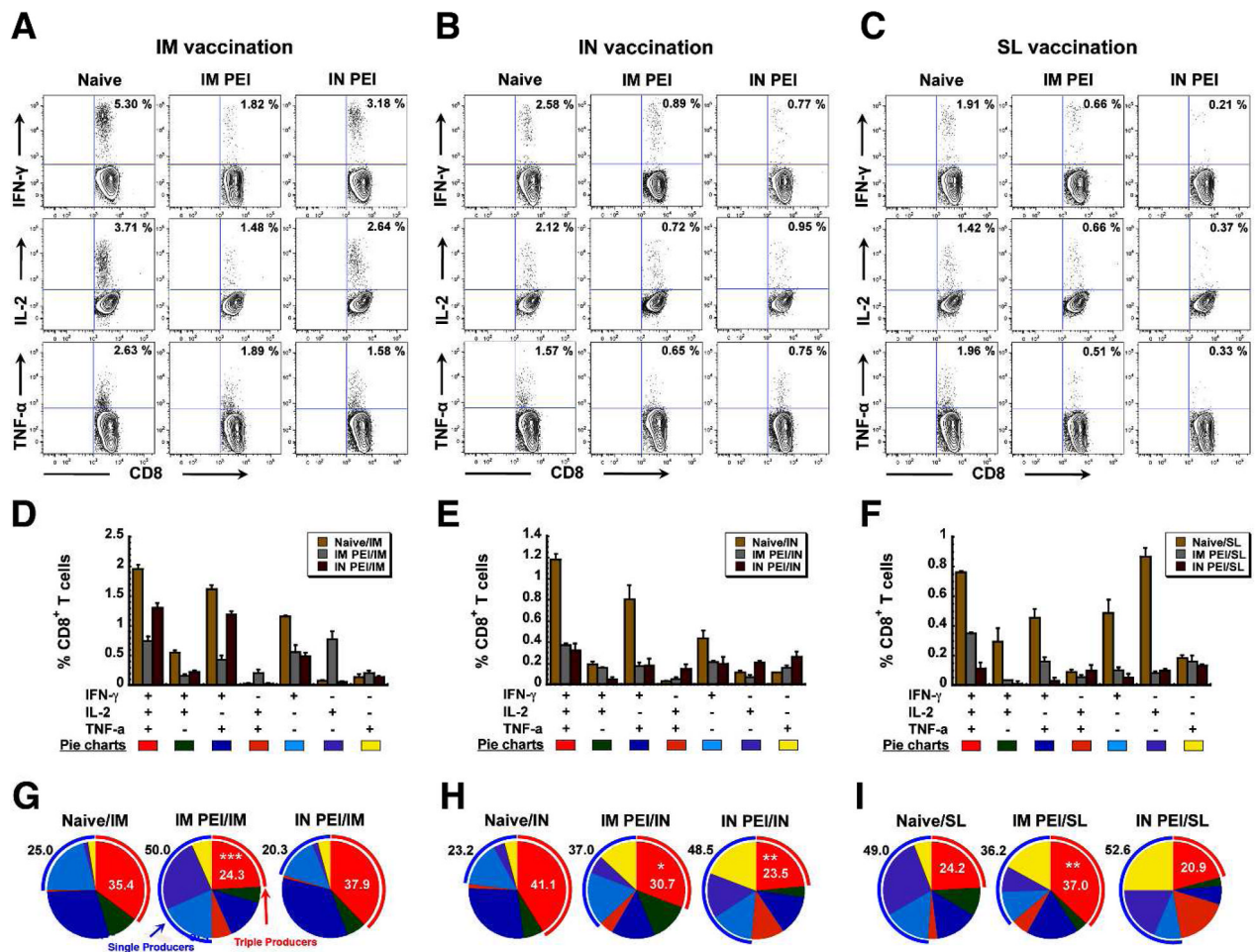


Figure 3. Prior Exposure to Adenovirus Profoundly Affects the Ebola Zaire Glycoprotein-Specific Multifunctional CD8⁺ T cell Response Elicited After Immunization by Various Routes Naïve B10.Br mice and those with PEI established by the IM (systemic) or IN (mucosal) route (10/group) were given 1×10^8 ivp of Ad-CAGoptZGP by the IM, IN or sublingual (SL) route. Mononuclear cells were harvested from the spleen 10 days later and the multifunctional T cell response assessed by FACS. **Panels A–C. Representative FACS analysis of cell populations secreting individual cytokines.** Numbers written in the upper right corner of each scatter plot represent the percentage of CD8⁺ T cells secreting the cytokine listed at the far left of each row after TELRTFSI peptide stimulation. **Panels D–F. Quantitative analysis of cell populations secreting individual and combinations of cytokines in response to antigen stimulation.** Each positively responding cell was assigned to one of 7 possible categories reflecting the production of IFN- γ , IL-2 and TNF- α alone or in combination. Average values and standard errors were calculated for each treatment group for statistical evaluation. **Panels G–I. Depiction of Zaire glycoprotein-specific multifunctional CD8⁺ T cells in pie chart format.** The relative frequency of triple producers defines the quality of the vaccine-induced CD8⁺ T cell response. Triple (IFN- γ^+ IL-2⁺TNF- α^+) and single cytokine producers are highlighted in the red and blue arcs, respectively. Numbers in the pie chart denote the percentage of triple (red) or single (black) cytokine producers in a given population. Results are reported as the mean \pm the standard error of the mean. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.

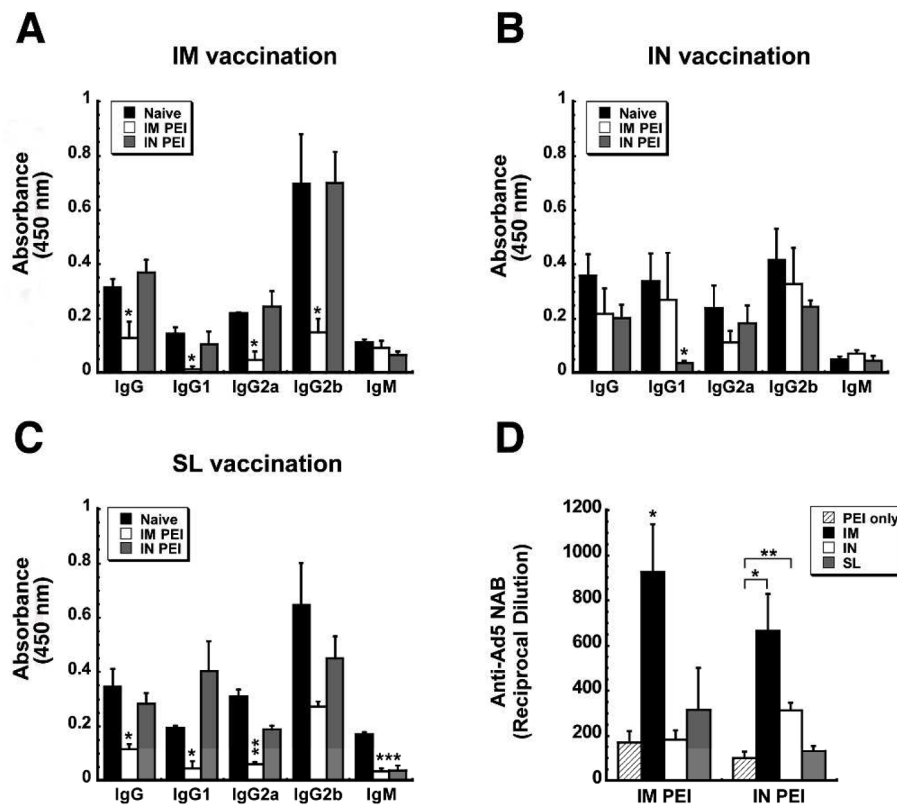


Figure 4. Establishment of Pre-Existing Immunity to Adenovirus by the Same Route as that used for Immunization Significantly Impairs Ebola Zaire Glycoprotein-Specific Antibody Production and Exacerbates the Anti-Adenovirus Antibody Response

Serum was collected from naïve B10.Br mice and those with PEI established by the IM (systemic) or IN (mucosal) route 4 days prior to and 42 days after immunization. **Panels A–C. Characterization of the Antigen-Specific Response.** Individual samples were evaluated for ZGP-specific IgM and IgG isotypes by ELISA. The average optical density read from samples obtained from each treatment group are presented to serve as a measure of relative antibody concentration. **Panel D. Characterization of Anti-Adenovirus Antibody Production After Induction of PEI (PEI only) and Post-Immunization.** Neutralization was assessed by serial dilution of each sample and incubation with a fixed concentration of adenovirus expressing beta-galactosidase (AdlacZ) prior to infection of HeLa cells. The reciprocal dilution plotted for each treatment group reflects the dilution at which the ability of the AdlacZ vector to infect target cells was reduced by 50%. In each panel, results are expressed as average values \pm the standard error of the mean and are representative of three separate experiments each containing 12 mice per immunization route. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.

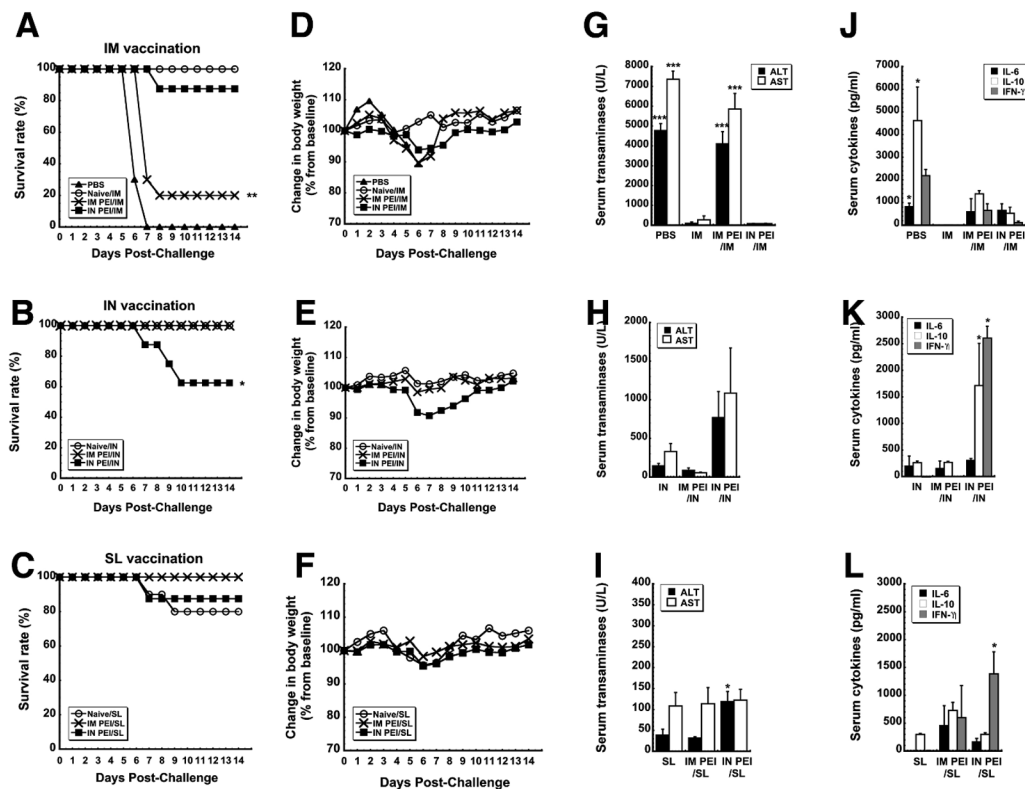


Figure 5. Prior Exposure to Adenovirus by a Route Different from that of Immunization Affords Better Protection from Lethal Challenge than when the Same Route is Used for Both Procedures in Mice

Naïve B10.Br mice and those with prior exposure to adenovirus 5 were challenged with a lethal dose of MA-ZEBOV 28 days after immunization by the IM, IN or SL route. Control, naïve mice were given saline (PBS). **Panels A–C. Kaplan–Meier survival curves.** In each panel, asterisks indicate a significant difference with respect to naïve, immunized animals. **Panels D–F. Body Weight Profiles after Challenge.** No significant change in body weight was noted in animals that survived challenge. **Panels G–I. Post-challenge serum alanine (ALT) and aspartate (AST) aminotransferase levels.** Samples were taken from survivors at the termination of the study and from non-survivors at time of death. Asterisks indicate a significant difference with respect to naïve, immunized animals. **Panels J–L. Serum Cytokines Levels After Challenge.** Samples were taken from survivors 14 days after challenge and from non-survivors at time of death. In all panels, data reflect average values \pm the standard error of the mean for 9–10 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.

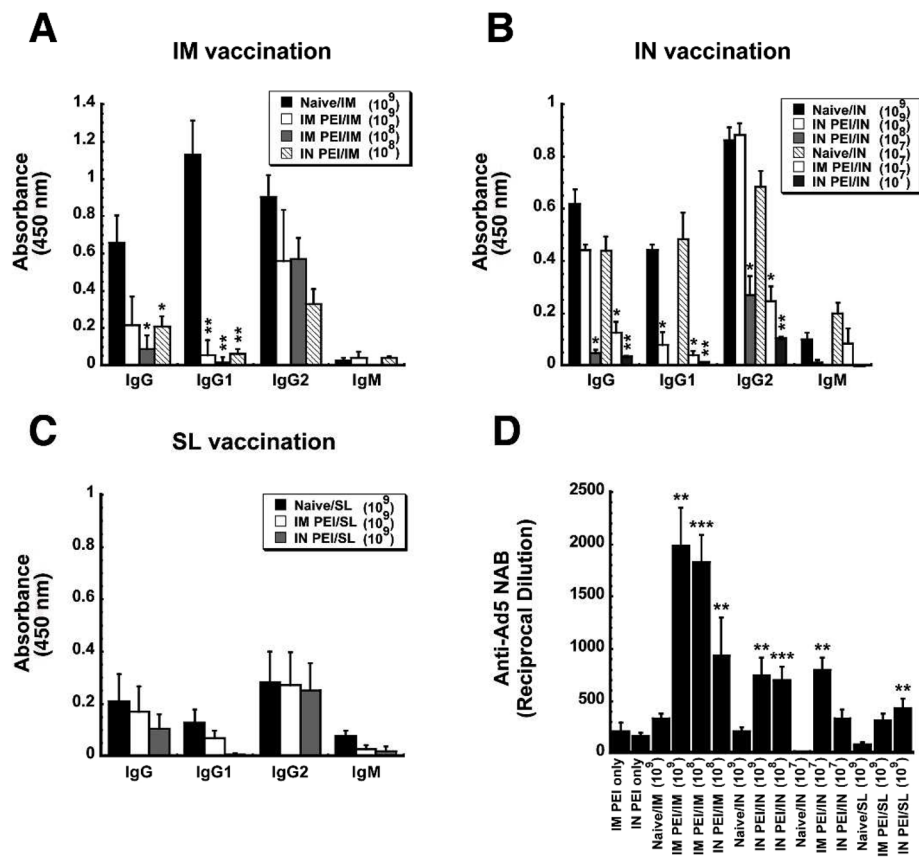


Figure 6. Pre-Existing Immunity to Adenovirus Significantly Reduces Ebola Zaire Glycoprotein-Specific Antibody Response and Exacerbates the Anti-Adenovirus Antibody Response in Guinea Pigs in a Dose-Dependent Manner

Serum was collected from naïve guinea pigs and those with PEI established by the IM (systemic) or IN (mucosal) route 4 days prior to and 24 days after immunization. **Panels A–C. Characterization of the Ebola-Zaire Glycoprotein-Specific Response.**

Characterization of the Antigen-Specific Response. Individual samples were evaluated for ZGP-specific IgM and IgG isotypes by ELISA. **D. The Anti-Adenovirus Response.** The value plotted for each treatment group reflects the dilution at which the ability of an AdlacZ vector to infect target cells was reduced by 50%. In each panel, results are expressed as average values \pm the standard error of the mean and are representative of three separate experiments each containing 5 guinea pigs per treatment. *In each panel, indicates a significant difference with respect to naïve, immunized animals. * $p < 0.05$, ** $p < 0.01$, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.

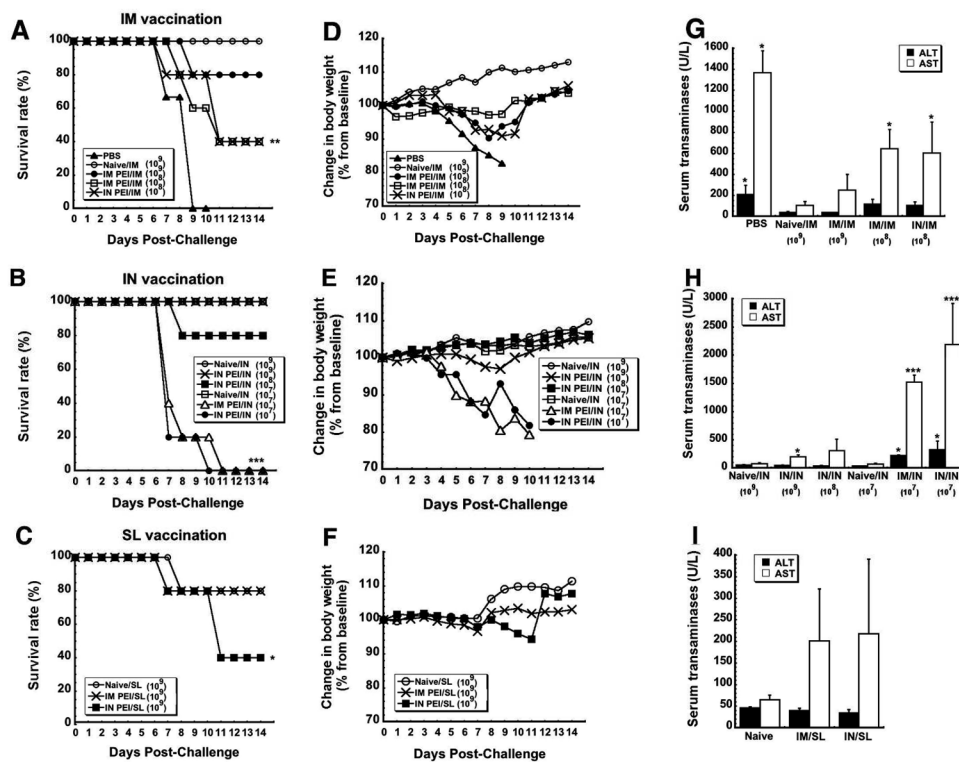


Figure 7. Protective Efficacy of Various Doses of an Ad-Based Ebola Vaccine in Guinea Pigs with Pre-Existing Immunity to Ad5

Naïve guinea pigs and those prior exposure to adenovirus were challenged with a lethal dose of GPA-ZEBOV 28 days after immunization with $1 \times 10^7 \sim 1 \times 10^9$ i.p.v. of Ad-CAGoptZGP by the IM, IN or SL route. **Panels A–C. Survival curves.** In each panel, asterisks indicate a significant difference with respect to naïve, immunized animals. **Panels D–F. Body Weight Profiles after Challenge.** Significant changes in body weight were only noted in animals that did not survive challenge. **Panels G–I. Serum alanine (ALT) and aspartate (AST) aminotransferase levels.** Samples were taken from survivors at the termination of the study and from non-survivors at time of death. Asterisks indicate a significant difference with respect to naïve, immunized animals. In all panels, data reflect average values \pm the standard error of the mean for 5 guinea pigs per treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA, Bonferroni/Dunn post-hoc analysis.