

A Single Dose Respiratory Recombinant Adenovirus-Based Vaccine Provides Long-Term Protection for Non-Human Primates from Lethal Ebola Infection

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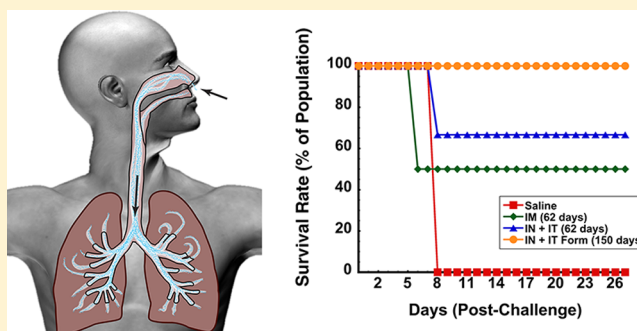
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ABSTRACT: As the Ebola outbreak in West Africa continues and cases appear in the United States and other countries, the need for long-lasting vaccines to preserve global health is imminent. Here, we evaluate the long-term efficacy of a respiratory and sublingual (SL) adenovirus-based vaccine in non-human primates in two phases. In the first, a single respiratory dose of 1.4×10^9 infectious virus particles (ivp)/kg of Ad-CAGoptZGP induced strong Ebola glycoprotein (GP) specific CD8⁺ and CD4⁺ T cell responses and Ebola GP-specific antibodies in systemic and mucosal compartments and was partially (67%) protective from challenge 62 days after immunization. The same dose given by the SL route induced Ebola GP-specific CD8⁺ T cell responses similar to that of intramuscular (IM) injection, however, the Ebola GP-specific antibody response was low. All primates succumbed to infection. Three primates were then given the vaccine in a formulation that improved the immune response to Ebola in rodents. Three primates were immunized with 2.0×10^{10} ivp/kg of vaccine by the SL route. Diverse populations of polyfunctional Ebola GP-specific CD4⁺ and CD8⁺ T cells and significant anti-Ebola GP antibodies were present in samples collected 150 days after respiratory immunization. The formulated vaccine was fully protective against challenge 21 weeks after immunization. While diverse populations of Ebola GP-specific CD4⁺ T cells were produced after SL immunization, antibodies were not neutralizing and the vaccine was unprotective. To our knowledge, this is the first time that durable protection from a single dose respiratory adenovirus-based Ebola vaccine has been demonstrated in primates.

KEYWORDS: adenovirus, Ebola, non-human primate, vaccine, respiratory, sublingual



INTRODUCTION

Since it first appeared as a clinical syndrome in Central Africa in the late 1970s, Ebola hemorrhagic fever, now termed Ebola virus disease, has intrigued infectious disease physicians, virologists, and epidemiologists because of the striking clinical presentation associated with the end stage of the disease, its high case fatality rate, and the ease with which it is transmitted among close contacts, including caregivers.¹ Isolation and identification of the Zaire Ebola virus soon after the first outbreak gave clinicians, scientists, and public health representatives tangible evidence of the pathogen responsible

for severe illness in 318 people which resulted in 280 deaths and fueled four decades of research on the biology of the highly lethal pathogen.² This virus, currently referred to as Ebola virus, is rapidly disseminated to lymph nodes by monocytes, macrophages, and dendritic cells where it quickly spreads to

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the liver and the spleen.³ This process is extremely efficient, making development of a therapeutic regimen to treat Ebola virus disease a race against the clock due to the narrow window between the time when viremia and/or the onset of fever and other clinical symptoms can be detected and death.⁴ Several experimental candidates have shown promise for treating Ebola virus disease in animal models of infection,⁵ however, there are currently no therapeutic or preventative agents approved for human use. Basic supportive care (fluid and electrolyte replacement; administration of antibiotics and antimalarials for concurrent infections and antiemetics for gastrointestinal symptoms) continues to be the cornerstone of therapy for Ebola virus disease and can notably improve outcomes when administered early in the course of the disease.^{6–8}

The current outbreak in West Africa not only emphasizes the important relationship between early detection of infection and supportive treatment, it also highlights a critical need for a well tolerated, highly effective Ebola vaccine that can rapidly elicit protection with a single dose. According to the World Health Organization (WHO) at the time of this writing (October 30, 2014) there have been 13,703 cases of Ebola infection, 4,922 of which have resulted in death.⁹ It is estimated that more than 521 of the reported cases have been healthcare workers, the majority of which (99%) reside in countries with widespread and intense transmission, and that more than half (52%) of these people did not recover from infection.¹⁰ Even though this might be an underestimate of the actual situation due to delays in reporting of data and the rapid evolution of the outbreak, this represents a significant loss to an already understaffed and resource poor healthcare system in a region of extreme poverty and civil unrest.¹¹ Considering that the WHO and other modeling experts have predicted that more than 20,000 new cases of Ebola infection will occur by the end of November 2014 and that in the worst case scenario 1.4 million cases will be seen before the current outbreak ends,^{12,13} an effective needle-free vaccine would bolster the medical response and health care infrastructure of affected nations by allowing a large number of medical personnel to provide aid and immunizations to those under outbreak conditions without concern for their personal health.

The overall goal of these studies was to identify an immunization platform that is easy to administer and capable of eliciting long-term protection from Ebola infection. Using results generated in mouse and guinea pig models,^{14–16} two studies were designed to evaluate the clinical profile of a recombinant adenovirus serotype 5-based vaccine given as a single dose by respiratory or sublingual (SL) administration to non-human primates (NHP). The first study, conducted with 9 male cynomolgus macaques, was designed to identify a subtherapeutic dose for each route of administration to assist in identifying formulations that improved vaccine performance. The second study, conducted with 11 macaques, was designed to evaluate a novel formulation that improved the immunological profile of the vaccine after intranasal administration in rodents and to evaluate the longevity of the immune response conferred by each platform.¹⁷ A comprehensive assessment of the immune response elicited by each vaccine platform after exposure to Ebola virus was performed in this study. Full toxicological profiles were generated for each animal after immunization in both studies. To our knowledge, this is the first evaluation of a needle-free Ebola vaccine in primates and one of the first to demonstrate long-term protection from lethal infection.

■ EXPERIMENTAL SECTION

Adenovirus Production. The E1/E3 deleted recombinant adenovirus serotype 5 vector expressing a codon optimized full-length Ebola glycoprotein sequence under the control of the chicken β -actin promoter (Ad-CAGoptZGP) and a host range mutant adenovirus serotype 5 (AdSMUT) that can replicate in non-human primates were amplified in HEK 293 cells and purified as described.^{18,19} Concentration of each virus preparation was determined by UV spectrophotometric analysis at 260 nm and with the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. Preparations with infectious to physical particle ratios of 1:37 were used in these studies. Buffers and reagents used in the production and purification of each virus preparation were of the highest quality available and were tested for the presence of endotoxin using a QCL-1000 Chromogenic LAL end point assay (Cambrex Bioscience, Walkersville, MD). All reagents contained less than 0.1 E.U./mL, and each virus preparation contained less than 0.2 E.U./mL. Sterility of each preparation was confirmed employing the methods outlined in the United States Pharmacopeia for parenteral products.²⁰

Assay for Detection of Replication Competent Adenovirus (RCA). A two cell line bioassay was performed on each preparation to determine the presence of RCA as described.²¹ Less than one RCA was detected for every 3×10^{12} virus particles tested.

Animal Model. Non-human primate studies were conducted under a contract at Bioqual Inc., Gaithersburg, MD. The animal management program of this institution is accredited by the American Association for the Accreditation of Laboratory Animal Care and meets NIH standards as outlined in the Guide for the Care and Use of Laboratory Animals. This institution also accepts as mandatory PHS policy on Humane Care of Vertebrate Animals used in testing, research, and training. Twenty male cynomolgus macaques (*Macaca fascicularis*) of Chinese origin were allowed to acclimate for 30 days in quarantine prior to immunization. Animals received standard monkey chow, treats, vegetables, and fruits throughout the study. Husbandry enrichment consisted of commercial toys and visual stimulation. Two separate experiments were conducted as summarized in Figures 1 and 7. Specific details about the primates used in each of these studies are summarized in Tables 1 and 2.

Study 1. The first study was conducted with 9 primates. Two animals were given the vaccine by intramuscular injection in a total volume of 1 mL of potassium phosphate buffered saline (KPBS) divided equally between the left and right deltoid muscles. Three animals were given the vaccine by the sublingual route by placing 50 μ L of the preparation under each side of the tongue and waiting for 15 min between doses to allow for absorption. Three animals were given the vaccine in the respiratory tract. This was achieved by slowly dispensing two 250 μ L volumes of the preparation into each nostril and waiting for 15 min between doses to allow for absorption. The remaining dose of the vaccine (5 mL volume) was instilled into the lungs via an endotracheal tube. This route of administration will be referred to as respiratory immunization or as intranasal/intratracheal (IN/IT) throughout the manuscript to illustrate that the vaccine was administered to the respiratory mucosa by two different routes. One primate was given 1 mL of KPBS divided equally between the left and right deltoid muscles. This

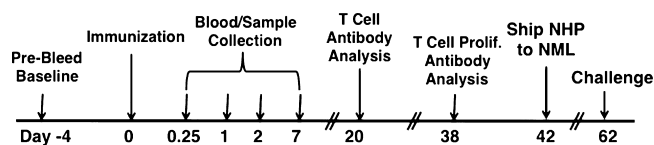


Figure 1. Timeline and sampling schedule for Study 1. Animals were screened for signs of prior exposure to adenovirus (anti-Ad5 NAB, Ad5 DNA, T cell responses) 4 days prior to immunization. Baseline blood chemistry panels were also evaluated at this time. Samples were taken for evaluation of blood chemistry and adenovirus shedding (nasal and oral swabs, urine, feces) 6 h after immunization and on days 1, 2, and 7. On day 20, serum and BAL were collected for assessment of shedding and anti-Ad5 NAB and anti-Ebola GP antibody levels. BAL, PBMCs, and ILNs were also screened for Ebola GP-specific CD8⁺ and CD4⁺ T cells at this time point. On day 38, additional samples were taken for assessment of anti-Ebola GP and anti-Ad5 antibodies and antigen-specific T cell proliferation (Ebola GP and Ad5). 42 days after immunization, NHPs were shipped to the National Microbiology Laboratory in Winnipeg, Canada, for challenge. After an acclimation period, primates were challenged with 1,000 pfu of Ebola (1995, Kikwit) by intramuscular (IM) injection.

Table 1. Study 1: Primate Characteristics and Treatment

animal no.	treatment	wt (kg)	dose (ivp/kg)	route of admin	age (years)
22457	KPBS	8.05		IM	10
22473	Ad-CAGoptZGP	6.36	1.6×10^8	IM	10
40347	Ad-CAGoptZGP	6.16	1.6×10^8	IM	8
50459	Ad-CAGoptZGP	7.31	1.4×10^9	IN/IT	7
52483	Ad-CAGoptZGP	6.98	1.4×10^9	IN/IT	7
52945	Ad-CAGoptZGP	6.84	1.5×10^9	IN/IT	7
52165	Ad-CAGoptZGP	6.30	1.6×10^9	SL	7
62125	Ad-CAGoptZGP	5.59	1.8×10^9	SL	6
62361	Ad-CAGoptZGP	6.38	1.6×10^9	SL	6

animal was the negative control. Blood was collected 6 h after immunization and on days 1, 2, and 7. Full blood chemistry panels and complete blood counts were performed by IDEXX BioResearch (West Sacramento, CA).

Study 2. A second study was conducted with 11 primates. Two animals (negative controls) were given 1 mL each of KPBS divided between the left and right deltoid muscles. The respiratory formulation was prepared at five times the working concentration [10 mg/mL poly(maleic anhydride-*alt*-1-octadecene) substituted with 3-(dimethylamino)propylamine (Anatrace, Maumee, OH)], sterilized by filtration, and diluted with freshly purified virus in KPBS (pH 7.4) prior to use. Three animals were given the vaccine in this formulation in the respiratory tract as described for study 1. Three animals were given an adenovirus serotype 5 host range mutant virus to establish pre-existing immunity (PEI) by IM injection 28 days prior to immunization with the vaccine by the sublingual route as described above. Three animals with no prior exposure to adenovirus were given the vaccine by the sublingual route for comparison.

Challenge. Animals were transported to the National Microbiology Laboratory in Winnipeg and, after an acclimation period, transferred to the biosafety level 4 (BSL-4) laboratory

Table 2. Study 2: Primate Characteristics and Treatment

animal no.	treatment	wt (kg)	dose (ivp/kg)	route of admin	age (years)
0810091	KPBS	8.7		IM	6
0805201	KPBS	6.8		IM	6
0802197	Ad-CAGoptZGP	6.2	1.6×10^9	IN/IT	6
0809077	Ad-CAGoptZGP	6.5	1.5×10^9	IN/IT	6
0810003	Ad-CAGoptZGP	5.8	1.7×10^9	IN/IT	6
0805257	Ad-CAGoptZGP	4.9	2.0×10^{10}	SL	6
0804317	Ad-CAGoptZGP	4.8	2.0×10^{10}	SL	6
0808233	Ad-CAGoptZGP	4.8	2.0×10^{10}	SL	6
0809227	Ad5MUT	5.5	1.8×10^{10}	IM	6
	Ad-CAGoptZGP		1.8×10^{10}	SL	
0804819	Ad5MUT	5.2	1.9×10^{10}	IM	6
	Ad-CAGoptZGP		1.9×10^{10}	SL	
0807243	Ad5MUT	4.9	2×10^{10}	IM	6
	Ad-CAGoptZGP		2×10^{10}	SL	

there for challenge. Challenge studies were approved by the Canadian Science Centre for Human and Animal Health (CSCHAH) Animal Care Committee following the Guidelines of the Canadian Council on Animal Care. For challenge, animals were infected by intramuscular injection at two sites with a total volume of 1 mL of freshly prepared Ebola virus (strain Kikwit 95, passage 3 on VeroE6 cells) of an inoculum containing 1,000 times the 50% tissue culture infectious dose (TCID₅₀) in diluent (minimal essential medium containing 0.3% bovine serum albumin). Ebola virus titers were confirmed (1.21×10^3 TCID₅₀/mL) by back-titration of the challenge preparation following administration of the virus. Animals were monitored daily and scored for disease progression using an internal filovirus scoring protocol approved by the CSCHAH Animal Care Committee. The scoring system graded changes from normal in the subject's posture, attitude, activity level, feces/urine output, food/water intake, weight, temperature, and respiration and ranked disease manifestations such as a visible rash, hemorrhage, cyanosis, or flushed skin. Samples were taken for assessment of anti-Ebola GP antibodies and full blood panels on days 3, 7, 14, 21, and 28 postchallenge and upon death. Hematological analysis of samples was performed in the BSL-4 lab with a Horiba ABX Scil ABC Vet Animal Blood Counter, and blood chemistries were analyzed with a VetScan vs1 (Abraxis). Surviving animals were kept until day 28.

ELISpot Assay. IFN- γ ELISpot assays were performed in triplicate according to the manufacturer's protocol (BD Biosciences, San Diego, CA) with 5×10^5 peripheral blood mononuclear cells (PBMCs) per well in cRPMI media (RPMI 1640, 1 mM L-glutamine, 50 μ M β -mercaptoethanol, 10% FBS and 1% penicillin/streptomycin). Cells were stimulated with three peptide pools for the Ebola glycoprotein (2.5 μ g/mL) for 18 h. Spots were visualized with the AEC substrate (BD Biosciences) and quantified with the ELISpot Plate Reader (AID Cell Technology, Strassberg, Germany).

Intracellular Cytokine Staining. PBMCs were isolated from whole blood collected prior to challenge as described.²² The frequency of CD8⁺ and CD4⁺ T cells producing IFN- γ , IL-

2, IL-4, and CD107a were assessed by flow cytometry with the following antibodies: CD3 Alexa Fluor 700 (clone SP34-2) and CD4 Peridinin Chlorophyll Protein (PerCP)-Cy5.5 (clone L200) from BD Biosciences (San Jose, CA); CD8 phycoerythrin (PE)-Cy7 (clone RPA-T8), CD107a Brilliant Violet 421 (clone H4A3), IL-2 Alexa Fluor 488 (clone MQ1-17h12), IL-4 PE (clone 8D4-8), and IFN- γ Allophycocyanin (APC, clone B27) from BioLegend (San Diego, CA). One million PBMCs were stimulated overnight with peptides (5 $\mu\text{g}/\text{mL}$) using GolgiPlug (0.5 $\mu\text{L}/\text{mL}$) and GolgiStop (0.6 $\mu\text{L}/\text{mL}$) in the presence of the anti-CD107a antibody. After surface staining for CD3, CD4, and CD8, samples were incubated two times (30 min each) in Cytofix/Cytoperm (BD Biosciences) for permeabilization. Intracellular staining was performed, and the samples were kept overnight in PBS/1% paraformaldehyde. Approximately 250,000–500,000 events were captured on a BD LSR II flow cytometer and data analyzed with FlowJo vX0.6 software (Tree Star, Ashland, OR).

Measurement of Proliferative Responses by Ki-67 Staining. Blood was collected from each primate in EDTA tubes, shipped same day and PBMCs isolated as described previously.²³ Cells were resuspended in R10 medium (RPMI 1640, 2 mM L-glutamine, 50 μM β -mercaptoethanol, 10% FBS, and 100 IU/mL penicillin and streptomycin) and stimulated using either an Ebola glycoprotein-specific peptide library (2.5 $\mu\text{g}/\text{mL}$), a first generation adenovirus that is genetically identical to the vaccine but does not contain a transgene cassette (AdNull, 1,000 MOI),²² or 5 $\mu\text{g}/\text{mL}$ ConA (Sigma, St. Louis, MO) for 5 days in 5% CO_2 at 37 $^\circ\text{C}$. After 3 days, cells were fed by removing 50 μL of spent medium and replacing it with 100 μL of fresh R10 medium. On day 5, cells were washed with phosphate buffered saline (PBS) for subsequent immunostaining for cell surface markers and for Ki-67, an intracellular marker for proliferation as described.²⁴ Proliferation was calculated by subtraction of values obtained from cells cultured in medium alone.

Anti-Ebola Glycoprotein Antibody ELISA. Flat bottom, Immulon 2HB plates (Fisher Scientific, Pittsburgh, PA) were coated with purified Ebola virus GP_{33–637} Δ TM-HA (3 $\mu\text{g}/\text{well}$) in PBS (pH 7.4) overnight at 4 $^\circ\text{C}$.²⁵ Heat-inactivated serum samples were diluted (1:20) in saline. One hundred microliters of each dilution were added to antigen-coated plates for 2 h at room temperature. Plates were washed 4 times and incubated with a HRP-conjugated goat anti-monkey IgG antibody (1:2,000, KPL, Inc., Gaithersburg, MD) for 1 h at room temperature. Plates were washed and substrate solution added to each well. Optical densities were read at 450 nm on a microplate reader (Tecan USA, Research Triangle Park, NC).

Neutralizing Antibody Assays. *Ebola Virus.* Primate sera were heat inactivated at 56 $^\circ\text{C}$ for 45 min and then serially diluted in 2-fold increments in Dulbecco's modified Eagle's medium (DMEM) in triplicate prior to incubation at 37 $^\circ\text{C}$ for 1 h with an equal volume of medium containing EBOV-eGFP (100 PFU per well) as described.²⁶ Virus-serum mixtures were then added to Vero E6 cells and placed at 37 $^\circ\text{C}$ for 2 days and then fixed in 10% phosphate buffered formalin. GFP levels were quantified by a fluorescent plate reader (AID Cell Technology). These assays were performed under BSL-4 conditions at the National Microbiology Laboratory in Winnipeg.

Adenovirus. Primate sera were heat inactivated and serially diluted as described for the Ebola virus assay. Samples were incubated with a first generation adenovirus serotype 5 expressing beta-galactosidase for 1 h before they were added

to HeLa cell monolayers. An equal volume of medium containing 20% FBS was then added to each well, and infections continued for 24 h. Cells were then histochemically stained for beta-galactosidase expression as described.¹⁴ Positive cells were quantified by visual inspection with a Leica DM LB microscope (Leica Microsystems Inc., Bannockburn, IL). For both assays, the serum dilution that corresponded to a 50% reduction in transgene expression was calculated by the method of Reed and Muench and reported as the reciprocal of this dilution.²⁷

Quantification of Virus Genomes by Real Time PCR. *Ebola Virus.* Total RNA was extracted from whole blood using a QIAmp Viral RNA Mini Kit (Qiagen). Ebola virus RNA was detected by a qRT-PCR assay targeting the RNA polymerase (nucleotides 16472 to 16538, AF086833) and LightCycler 480 RNA Master Hydrolysis Probes (Roche Diagnostics GmbH, Mannheim, Germany). The reaction conditions were as follows: 63 $^\circ\text{C}$ for 3 min, 95 $^\circ\text{C}$ for 30 s, and cycling of 95 $^\circ\text{C}$ for 15 s, 60 $^\circ\text{C}$ for 30 s for 45 cycles with a LightCycler 480 II (Roche). Primer sequences for this assay were as follows: EBOVLF2 CAGCCAGCAATTTCTTCCAT, EBOVLR2 TTTCGGTTGCTGTTTCTGTG, and EBOVLP2FAM FAM-ATCATTGGCGTACTGGAGGAGCAG-BHQ1.

Adenovirus. Urine and BAL fluid were concentrated using Amicon Ultra 100K Centrifugal Filter Devices (Millipore, Billerica, MA). DNA was isolated from blood, concentrated BAL, and oral and nasal swabs using a QIAmp DNA Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). DNA was isolated from rectal swabs using a modified protocol and the QIAmp DNA Mini kit. DNA was extracted from the urine concentrate using a QIAmp Viral RNA mini kit (Qiagen) according to the manufacturer's instructions. DNA was isolated from stool samples using a QIAmp Fast DNA Stool Mini kit (Qiagen). Quantification of viral DNA was determined by real time PCR according to a published protocol.²⁸ DNA amplifications were carried out using a ViiA 7 Real-Time PCR System (Life Technologies, Carlsbad, CA) with the following cycling conditions: 50 $^\circ\text{C}$ for 2 min, 95 $^\circ\text{C}$ for 10 min, 95 $^\circ\text{C}$ for 15 s, and 62 $^\circ\text{C}$ for 1 min for a total of 41 cycles. Primer sequences, used to amplify a region of the adenovirus serotype 5 hexon protein, were 5'-ACT ATA TGG ACA ACG TCA ACC CAT T-3' (forward) and 5'-ACC TTC TGA GGC ACC TGG ATG T-3' (reverse). The internal probe sequence, tagged with 6FAM fluorescence dye at the 5' end and TAMRA quencher at the 3' end, was 5'-ACC ACC GCA ATG CTG GCC TGC-3'. Each sample was run in triplicate in a given PCR assay.

RESULTS

Two primate studies are summarized here. The first, referred to as Study 1, involved 9 male cynomolgus macaques and served to identify suitable doses of vaccine that were semiprotective for further evaluation of test formulations to improve survival in the NHP model. The second study, referred to as Study 2, evaluated a novel formulation for the respiratory platform and involved refinement of the sublingual platform in naive animals and those with prior exposure to adenovirus. The workflow and treatment schedules for each study are depicted in Figures 1 and 7.

Study 1: Clinical Observations. Toxicology and Distribution of Vaccine. Administration of the vaccine at a dose of 1.4×10^9 infectious virus particles (ivp)/kg to the respiratory and the sublingual mucosa was well tolerated with

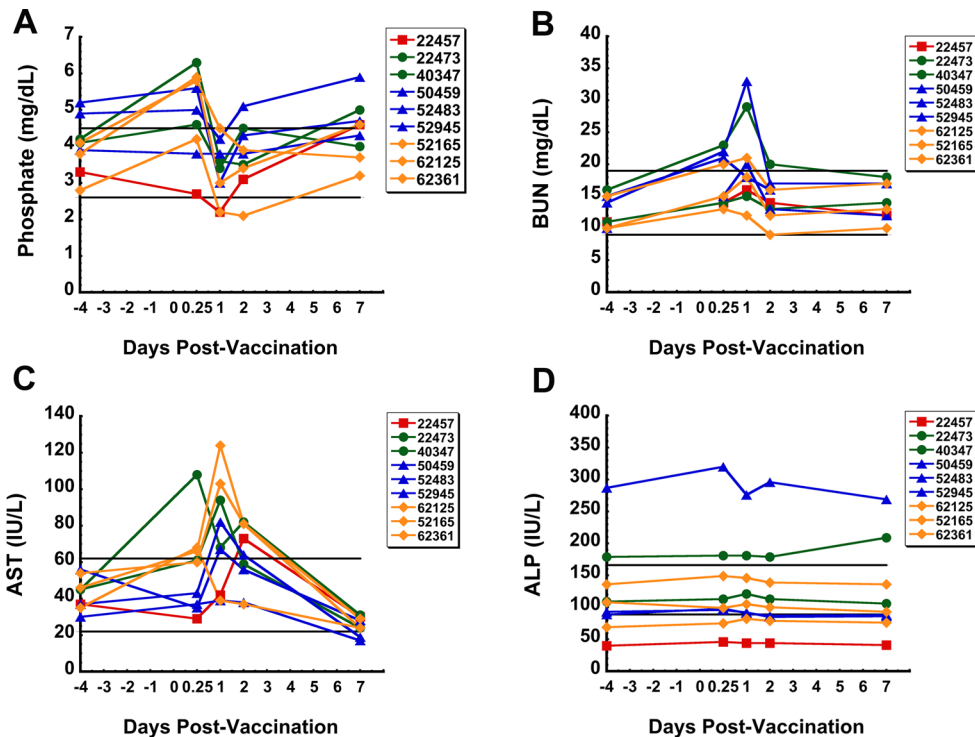


Figure 2. Study 1: Clinical parameters evaluated over time in non-human primates immunized by various routes. *Cynomolgus* macaques were given a single dose of vaccine by IM injection or by the respiratory or the SL route. Each line represents alterations for each parameter during the course of therapy for one primate. In each panel: Red lines/squares: saline control. Green lines/circles: IM injection. Blue lines/triangles: IN/IT immunization. Orange lines/diamonds: SL immunization.

no adverse reactions noted. Of particular note is that all animals experienced a transient increase in serum phosphate levels 6 h after immunization with a primate from each treatment group falling outside normal values (22473, IM, 1.4 times normal, 50459, IN/IT, 1.2 times normal, 62125, SL, 1.3 times normal, Figure 2A). Phosphate levels for all animals reached their nadir at the 24 h time point and were within the normal range for the remainder of the study. Blood urea nitrogen (BUN) levels peaked for all animals 24 h after immunization. Two of these animals, one given the vaccine by IM injection (40347, 29 mg/dL) and another given the vaccine by the IN/IT route (52945, 33 mg/dL), had levels that were notably outside of the normal range (Figure 2B). These values returned to normal by 48 h and remained so throughout the course of the study. Serum aspartate aminotransferase (AST), a standard indicator of adenovirus toxicity,²⁹ was significantly elevated above normal values in all animals 24 h after immunization except for one animal given the vaccine by the SL route (62125) and another given the vaccine by the IM route (40347). AST levels fell 48 h after immunization with only a few animals remaining above normal limits (Figure 2C). AST values for all animals were within normal limits by the 7 day time point. Serum alkaline phosphatase (ALP) of two animals fell outside the normal range during the study. Samples from one animal given the vaccine by IM injection were only mildly over the normal acceptable limit (22473, Figure 2D) while those of an animal immunized by the IN/IT route (52945) were 2 times the normal acceptable limit. In both cases, this parameter was high throughout the study and this elevation was not in response to the vaccine. Other serological parameters evaluated during the first week after immunization (calcium, creatinine, albumin, globulin, total protein, total bilirubin, alanine aminotransferase

(ALT), glucose, sodium, potassium, chloride, and cholesterol) all fell within normal limits during the course of the study.

Adenovirus shedding was also evaluated using a standard real time PCR assay to detect adenovirus genomes²⁸ in serum, nasal swabs, BAL fluid, oral swabs, urine, and feces (Figure 3). A significant number of adenovirus genomes were found in the serum of one animal immunized by the respiratory route 2 days after immunization (50459, 1,452 genomes/mL serum, Figure 3A) and another immunized by IM injection 7 days after treatment (22473, 7,296 genomes/mL serum). As expected, substantial amounts of adenovirus serotype 5 genomes were found in nasal swabs obtained from primates immunized by the IN/IT route (50459, 4.2×10^6 , 52483, 1.4×10^6 , 52945, 7.5×10^5) 24 h after immunization (Figure 3B). Swabs from one primate immunized by the SL route also contained a notable amount of Ad5 genomes (62361, 8,090) at the 24 h time point. Swabs from one animal immunized by the IN/IT route contained a significant amount of adenovirus genomes 2 days after immunization (52945, 6,333). Samples taken at days 7 and 20 fell below detection limits of the assay. Very low amounts of Ad5 genomes were found in the BAL fluid of animals immunized by the IN/IT route 20 days after immunization (Figure 3C). Oral swabs taken 24 h after treatment from one NHP immunized by the IN/IT route (52483, 4.5×10^4 , Figure 3D) and two animals immunized by the SL route (62125, 4.9×10^4 , and 62361, 9.3×10^4) contained significant numbers of adenovirus genomes. Swabs collected from animals at the 2 day time point did not contain any adenovirus genomes. A significant number of virus genomes were detected in the urine of 2 animals within 6 h after treatment (52945, 621 copies/mL, and 62361, 1,228 copies/mL). Adenovirus DNA was also found 24 h after treatment in the urine of 3 animals

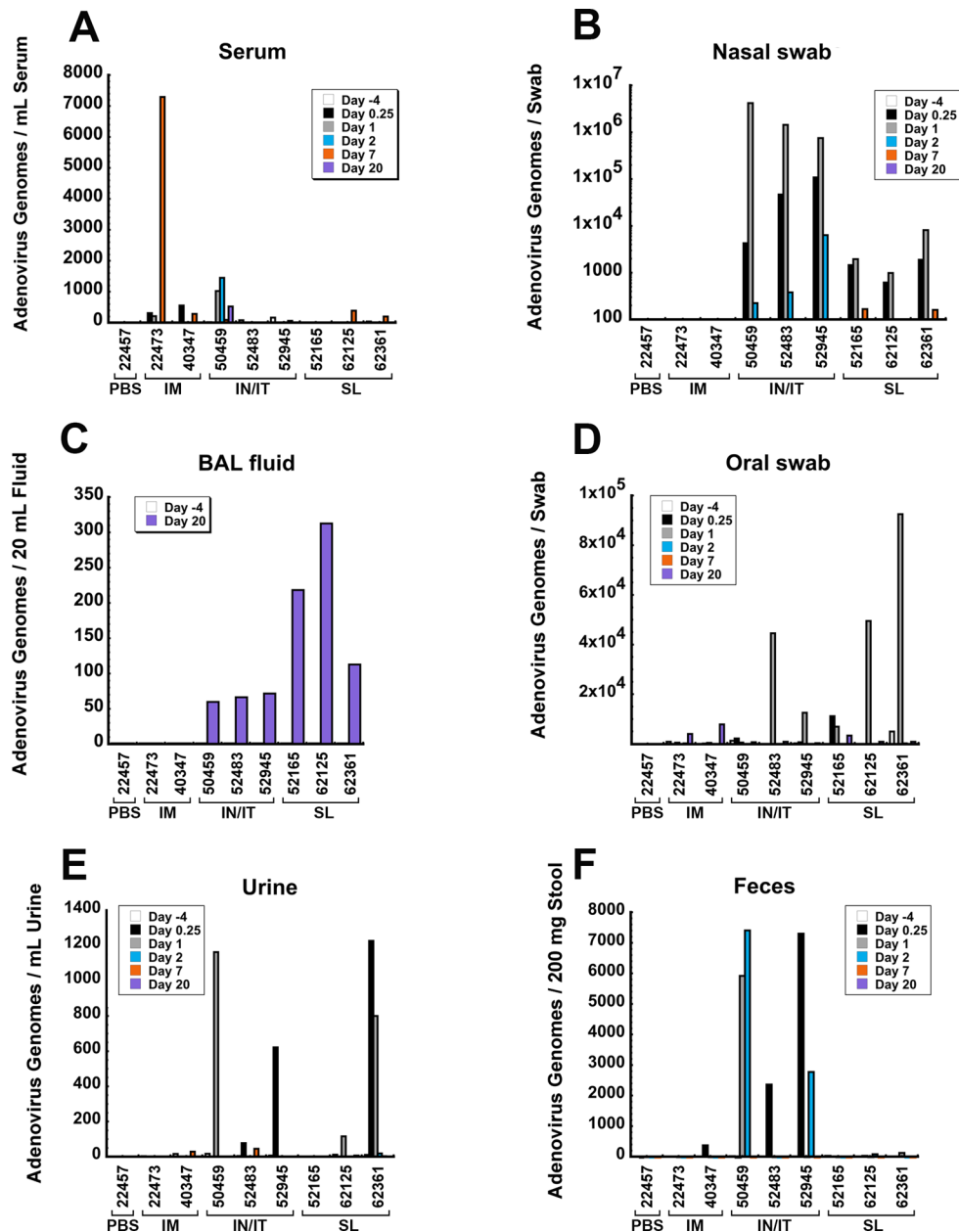


Figure 3. Study 1: Adenovirus genomes are released predominantly in the nasal mucosa and feces after respiratory immunization and in the oral and nasal mucosa after sublingual immunization. Male cynomolgus macaques were given either 1×10^9 ivp by IM injection or 1×10^{10} ivp by the respiratory or the SL route. DNA was isolated from each sample, and viral genomes were determined by real time PCR. Animal numbers and corresponding treatments are outlined in Table 1.

(50459, 1,163, 62361, 801, and 62125, 116 copies/mL, Figure 3E). Samples from all other animals throughout the time course of this study fell below detection limits of the assay. Interestingly, adenovirus genomes were only detected in the feces of animals immunized by the IN/IT route (Figure 3F). As early as 6 h after immunization, 2,362 and 7,302 adenovirus genomes were found in fecal samples from animals 52483 and 52945 respectively. Feces collected from animal 50459 24 h after vaccination contained 5,919 adenovirus genomes. This increased to 7,405 in samples taken from the same animal at the 48 h time point. Samples from animal 52945 also taken 48 h after treatment contained 2,772 virus genomes.

Study 1: The T Cell Response. Twenty days after immunization, PBMCs were isolated from whole blood and incubated with peptides specific for Ebola glycoprotein (GP).

Cells were then subjected to intracellular cytokine staining for CD8⁺ and CD4⁺ surface antigens and IFN- γ and sorted by flow cytometry. At this time point, few cells responsive to Ebola glycoprotein could be detected in PBMCs obtained from any of the animals (data not shown). A similar trend was observed in samples taken from iliac lymph nodes (ILNs) of animals. Profound responses were seen in samples obtained from the BAL fluid of animals given the vaccine by the IN/IT route. The strongest response was seen in CD4⁺ cells with 12.5% of the population obtained from primate 52945 and 3.03% of the population from primate 50459 responding (Figure 4A). Although the response from the third primate in this treatment group (52483) was small in comparison (0.71%), it was significantly higher than that observed in animals given the

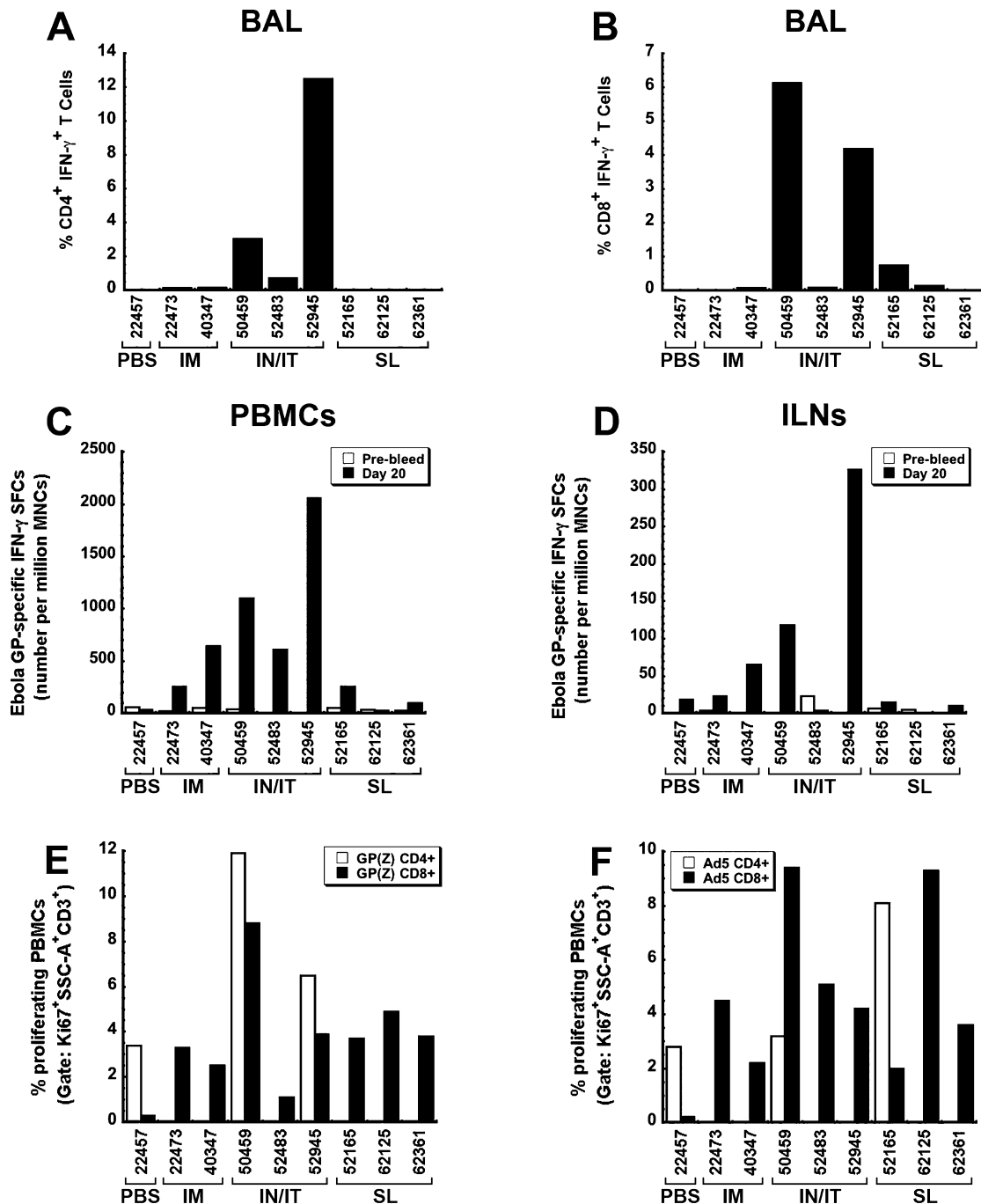


Figure 4. Study 1: Respiratory immunization induces strong antigen-specific T cell responses after administration of a single dose of a formulated adenovirus-based Ebola vaccine. (A) Quantitative analysis of Ebola glycoprotein-specific CD4⁺ T cells in BAL fluid. Cells were isolated from whole blood 20 days after immunization and stimulated with a peptide library for Ebola glycoprotein or peptides specific for the MHC class II associated invariant chain peptide that binds the MHC class II groove of cells (h-Clip, negative control). Positive control cells were stimulated with PMA and ionomycin. Each cell population was stimulated for 5 h, stained for phenotypic markers, and analyzed by flow cytometry. (B) Quantitative analysis of Ebola glycoprotein-specific CD8⁺ T cells in BAL fluid. Cells were treated as described for Panel A. (C) Magnitude of the antigen-specific response of mononuclear cells isolated from whole blood of macaques. PBMCs were isolated 20 days after immunization from whole blood and evaluated for IFN- γ secretion after stimulation with an Ebola GP-specific peptide library by ELISpot. (D) Magnitude of the antigen-specific response in mononuclear cells isolated from iliac lymph nodes (ILNs) of primates. MNCs were isolated 20 days after immunization from ILNs and evaluated for IFN- γ secretion after stimulation with an Ebola GP-specific peptide library by ELISpot. (E) Proliferative capacity of Ebola GP-specific T cells collected 38 days after immunization of naive primates by various routes. The proliferative capacity of CD4⁺ (white bars) and CD8⁺ (black bars) T cells isolated from whole blood was evaluated for each animal by stimulation for 5 days with an Ebola GP-specific peptide library and subsequent staining for Ki-67, an intracellular marker for proliferation.⁵⁹ (F) Proliferative capacity of adenovirus serotype 5-specific T cells after immunization by various routes. Cells were isolated from whole blood 38 days after immunization and stimulated for 5 days with a first generation adenovirus that does not contain a transgene cassette (AdNull, MOI 1:1,000). The proliferative capacity of CD4⁺ (white bars) and CD8⁺ (black bars) T cells was determined by intracellular staining for Ki-67. Animal numbers displayed in each panel and their corresponding treatments are summarized in Table 1.

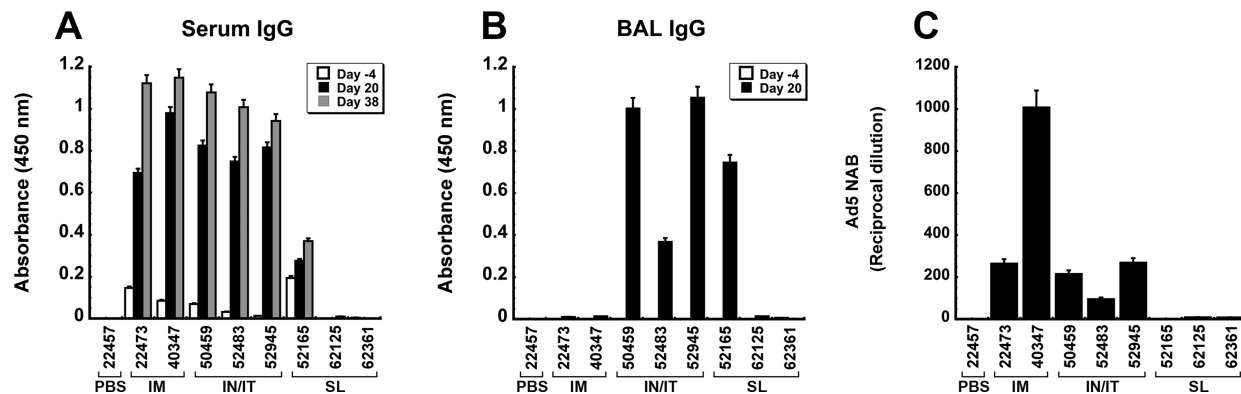


Figure 5. Study 1: Respiratory immunization induces strong anti-Ebola GP and minimal anti-adenovirus antibody responses in serum and BAL fluid. Serum (panel A) was collected 20 and 38 days after immunization. BAL fluid (panel B) was collected 20 days after immunization. These samples were screened for the presence of anti-Ebola GP antibodies by ELISA. Serum collected on day 20 was also screened for anti-adenovirus 5 NABs using an infectious titer assay (panel C). Data in panel C is reported as the dilution at which the infectious titer of a first generation adenovirus expressing the beta-galactosidase transgene was reduced by 50%. In each panel, error bars represent the standard error of samples assayed in triplicate from each primate for each time point.

vaccine by IM injection. The CD8⁺ T cell response followed a similar trend (Figure 4B).

PBMC and ILN populations were further analyzed for IFN- γ production in response to Ebola GP by ELISpot. Samples from animals immunized by the IM route (22473 and 40347) both had significant numbers of IFN- γ producing cells (255 and 642 spot forming cells (SFCs)/million mononuclear cells (MNCs) respectively, Figure 4C). PBMC samples from two NHPs immunized by the SL route (52165, 62361) also had measurable numbers of IFN- γ producing cells (257 and 98 SFCs/million MNCs). Samples from NHPs immunized by the IN/IT route contained the highest numbers of IFN- γ producing cells (1,100, 607, and 2,055 SFCs/million MNCs). Samples from the ILNs of 2 NHPs given the vaccine by the IN/IT route (50459 and 52945) contained approximately 7 and 18 times the number of IFN- γ producing cells found in the saline control (animal 22457) respectively (Figure 4D).

38 days after immunization, the proliferative capacity of CD4⁺ and CD8⁺ cells in response to Ebola GP and adenovirus serotype 5 was assessed by a Ki-67 staining assay.²⁴ Two samples, each obtained from animals immunized by the respiratory route, contained significant numbers of proliferative Ebola GP-specific CD4⁺ T cells (50459, 11.9%, and 52945, 6.5%, white bars, Figure 4E). The sample obtained from NHP 50459 also contained the most Ebola GP-specific CD8⁺ T cells (8.8%, black bars, Figure 4E). The sample from NHP 62125 immunized by the SL route contained the second highest amount of CD8⁺ T cells (4.9%). All remaining samples contained approximately 3–4% CD8⁺ T cells that could proliferate in response to Ebola GP except for that from animal 52483 (1.1%). Only one sample obtained from a primate immunized by the IN/IT route, 52165, contained a significant population of proliferative adenovirus 5-specific CD4⁺ T cells (8.1%, white bars, Figure 4F). One sample from a primate in the IN/IT group (50459) and another from the SL group (62125) contained notable populations of CD8⁺ cells that proliferated in response to Ad5 (9.4 and 9.3% respectively, black bars, Figure 4F). All remaining samples contained approximately 4% CD8⁺ T cells that could proliferate in response to adenovirus except for animal 40347 (2.2%).

Study 1: The Antibody-Mediated Response. Anti-Ebola GP and anti-adenovirus antibody levels were assessed in serum

and BAL fluid 20 and 38 days after immunization (Figure 5). Marked levels of anti-Ebola GP IgG antibodies were found in serum from animals immunized by the IM and the IN/IT routes 20 days after treatment (Figure 5A). These levels increased further 38 days after vaccination. Anti-Ebola GP antibodies were found in the serum of only one of the animals immunized by the SL route (52165). This animal also had Ebola GP-specific IgG antibodies in BAL fluid 20 days after treatment (Figure 5B) that were similar to those found in samples from animals immunized by the respiratory route. BAL from animals immunized by the IM route did not contain any detectable levels of anti-Ebola GP antibodies. One sample from a primate immunized by the IM route (40347) contained a significant amount of circulating anti-adenovirus neutralizing antibodies (NABs, 1,007 reciprocal dilution, Figure 5C). The sample from the remaining animal in the IM group and 2 others from the IN/IT group contained anti-adenovirus NAB titers of ~200 reciprocal dilution. Serum from animals immunized by the SL route did not contain measurable levels of anti-adenovirus 5 NABs.

Study 1: Lethal Challenge with Ebola Virus. 62 days after immunization, NHPs were challenged with 1,000 pfu of Ebola virus (1995, Kikwit). One primate immunized by IM injection (40347) and one animal immunized by the SL route (62125) succumbed to infection 6 days after challenge (Figure 6A). At this time animal 62125 had a clinical score of 23, and substantial petechiae were noted upon necropsy. Primate 40347 had a temperature of 40.3 °C and a clinical score of 25 and experienced notable bleeding. One primate immunized by the IN/IT route (52483) and one primate immunized by the SL route (62361) died the following day. Each of these animals had clinical scores above 25 and significantly decreased food intake the previous day. The remaining primate immunized by the SL route (52165) expired 8 days after challenge. One of the primates vaccinated by IM injection (22473) and two of the animals immunized by the IN/IT route (50459, 52945) survived challenge (50 and 67% survival IM and IN/IT respectively, Figure 6A). Moderate drops in body weight were noted during infection (Figure 6B). A slight increase in weight of one animal immunized by the IN/IT route (50459) was noted during the study period. Changes in body temperature (Figure 6C) and clinical scores (Figure 6D) for each primate

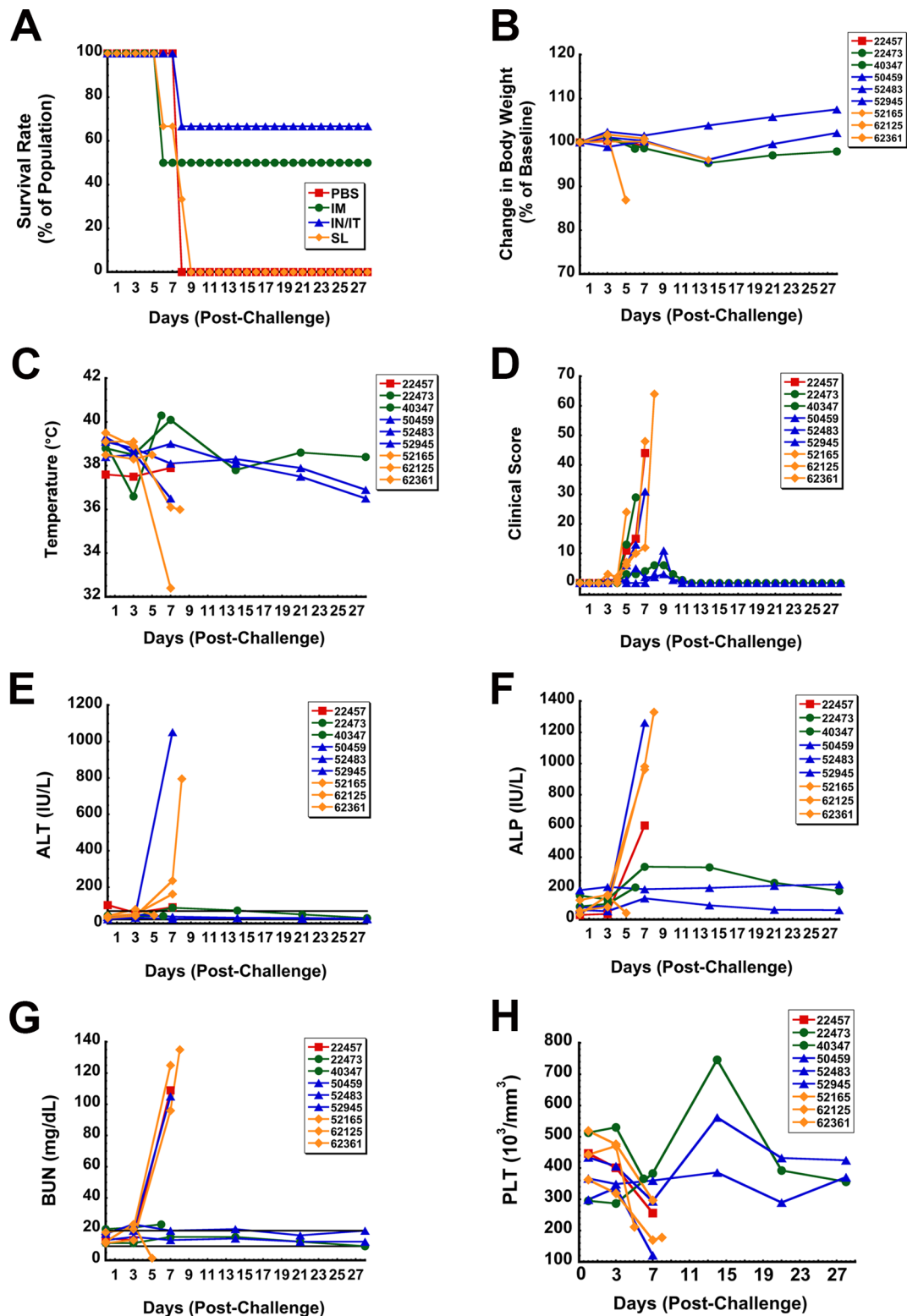


Figure 6. Respiratory immunization confers long-term immunity to Ebola in naive NHPs. Naive male cynomolgus macaques (see Table 1 for characteristics) were challenged 62 days after immunization with a lethal dose of 1,000 pfu (1,000 TCID₅₀) of Ebola virus (1995, Kikwit). (A) Kaplan–Meier survival curve. (B) Body weight profile after challenge. (C) Thermal analysis of animals during challenge. (D) Daily clinical scores for each primate using a standard, approved scoring methodology throughout the challenge. Variations in serum (E) alanine aminotransferase (ALT), (F) alkaline phosphatase (ALP), (G) blood urea nitrogen (BUN), and (H) platelets (PLT) were noted in animals that did not survive challenge. Red line: saline control. Green lines: IM injection. Blue lines: IN/IT immunization. Orange lines: SL immunization.

were in line with survival results. The most striking changes in hematology and blood chemistry values were observed around day 5 postchallenge in the animals that did not survive. These

include significantly elevated liver enzymes with ALT (Figure 6E) and ALP (Figure 6F) values rising to levels 27 and 16 times baseline respectively and blood urea nitrogen levels rising to 7.5

times normal values before the animals expired (Figure 6G). Platelet counts, however, dropped to half the baseline values in these animals (Figure 6H). In contrast, a sharp increase in platelets was noted in samples obtained from animals that survived challenge. Other hematology and blood chemistry values in these animals remained largely unchanged (data not shown).

Study 2: Effect of Formulation on Establishing Long-Lasting Immunity to Ebola and Refinement of Dose for Sublingual Immunization. The most exciting finding extracted from Study 1 was that the combined IN/IT administration of the vaccine was able to confer long-term immunity to Ebola. Since it was not known if immunity induced by adenovirus-based vaccines for Ebola is persistent over time,^{30,31} we decided to extend the length of time between respiratory administration of a formulated version of the vaccine and challenge. A secondary goal was to increase the dose of vaccine given by the sublingual route and to evaluate the ability of the sublingual vaccine to confer protection in animals with prior exposure to adenovirus since improved responses in this population were observed in studies with rodents.¹⁵ The long-term immune response of surviving animals postchallenge was also a major point of interest in this study especially in animals receiving vaccine containing a novel formulation¹⁷ and in those given the sublingual vaccine to identify parameters to target during additional refinement of each immunization platform.

Three male cynomolgus macaques were given the vaccine in a potassium phosphate buffer (pH 7.4) containing an amphiphilic polymer (formula weight (FW) ~39,000) formulation that improved the antigen-specific immune response in rodent models of infection.¹⁷ The goal was to immunize this group as early in the study as possible so that there would be a significant amount of time between immunization and challenge (Figure 7). 42 days after these animals were immunized, 3 macaques were given 1×10^{11} particles of a host range mutant adenovirus serotype 5 that can replicate in non-human primates^{19,32} by intramuscular injection to establish pre-existing immunity. 42 days later, animals were then given the vaccine by the sublingual route. At this time the animals had an average circulating anti-adenovirus antibody titer of 320 ± 160 reciprocal dilution. Three naive animals were also given the same dose of vaccine by the sublingual route at the same time for comparison.

Study 2: Toxicology and Vaccine Shedding. In contrast to the first study, a notable spike in creatine phosphokinase (CPK) was detected in the serum of all animals 24 h after immunization (Figure 8A). This enzyme increased to 8 times baseline in one animal immunized by the IN/IT route (810003, 8,209 IU/L) and to 10 times baseline in a primate with pre-existing immunity to adenovirus immunized by the sublingual route (804819, 4,483). A notable spike in serum lactate dehydrogenase (LDH) was also noted at the 24 h time point. This was not as sharp as that seen with CPK with the highest elevations found to be approximately 3 times baseline (804317, 849 IU/L, Figure 8B). Both parameters returned to normal within 3 days after treatment. As seen in the first study, serum AST increased in all primates after immunization. This occurred at the 24 h time point for animals immunized by the respiratory and sublingual routes but was not observed in primates with pre-existing immunity to adenovirus until 48 h (Figure 8C). As in the first study, serum alkaline phosphatase (ALP) levels varied between primates, however, in this trial a

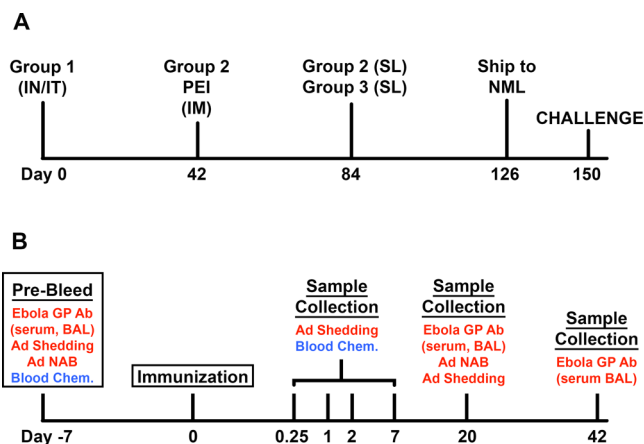


Figure 7. Study 2. (A) Immunization schedule. Eleven male cynomolgus macaques of Chinese origin were immunized according to the schedule depicted in the figure. Animals were shipped to the National Microbiology Laboratory (NML) in Winnipeg 126 days after immunization for challenge on day 150 of the study. (B) Sample collection scheme for Study 2. Animals were screened for signs of prior exposure to adenovirus and Ebola (anti-Ad5 NAB, Ad5 DNA, anti-Ebola GP antibodies) 1 week prior to the initiation of the study. Baseline blood chemistry panels were also evaluated. Samples were taken for evaluation of blood chemistry and adenovirus shedding (nasal, oral, rectal swabs, urine, feces) 6 h after immunization as well as on days 1, 2, and 7. On day 20, serum and BAL were collected for assessment of shedding, anti-Ad5 NABs, and anti-Ebola GP antibodies. On day 42, additional samples were taken for assessment of anti-Ebola GP and anti-Ad5 antibodies and antigen-specific T cell proliferation (Ebola GP and Ad5). 150 days after immunization, NHPs were shipped to the National Microbiology Laboratory in Winnipeg for challenge. IN/IT: intranasal/intratracheal. IM: intramuscular. SL: sublingual. PEI, pre-existing immunity.

distinct drop in this parameter was noted in samples collected from most animals between the 6 and 24 h time points, after which values remained constant (Figure 8D). Other serological parameters evaluated during the first week after immunization (calcium, creatinine, albumin, globulin, total protein, gamma glutamyl transferase (GGT), total bilirubin, alanine aminotransferase (ALT), BUN, glucose, sodium, potassium, phosphate, chloride, and cholesterol) all fell within normal limits throughout the course of the study (data not shown).

Adenovirus genomes were only found in serum samples collected from animals immunized by the respiratory route (Figure 9A). The most significant numbers of virus genomes detected in any of the biological samples collected throughout the second study were found in nasal swabs collected from primates 6 h after IN/IT immunization [810003 (8.18×10^6 genome copies (GC)), 809077 (1.44×10^7 GC), and 802197 (1.36×10^7 GC, Figure 9B)] and in oral swabs collected from primates 6 h after sublingual immunization: [804317 (9.06×10^6 GC), 805257 (1.74×10^5 GC), and 808233 (7.92×10^6 GC, Figure 9D)]. As seen in the first study, adenovirus genomes were only found in the BAL fluid of animals immunized by the IN/IT route (Figure 9C). Urine collected from one naive animal immunized by the SL route and another with pre-existing immunity also immunized by the SL route 6 h after treatment contained notable amounts of adenovirus (808233, 9,821 GC; 807243, 2,363 GC, Figure 9E). Adenovirus genomes were found in feces collected from one primate with pre-existing immunity to adenovirus 24 h after immunization by the SL route (804819, 2.71×10^6 GC) and in another primate

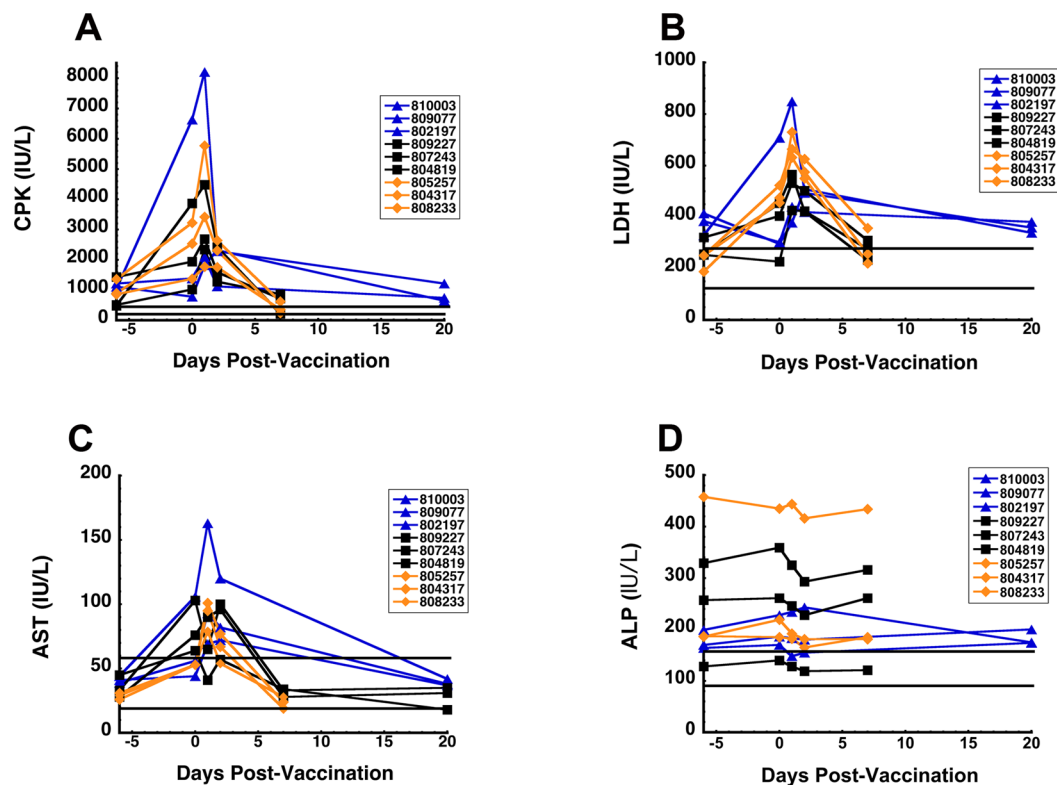


Figure 8. Study 2: Clinical parameters demonstrating transient changes after immunization of naive non-human primates and those with pre-existing immunity to adenovirus immunized by various routes. Naive cynomolgus macaques were given a single dose of vaccine by the respiratory (IN/IT) or the SL routes. A separate group of animals first received a dose of an adenovirus serotype 5 host range mutant virus 42 days prior to immunization. Each line represents alterations for each parameter after immunization for one individual primate. Blue lines/triangles: IN/IT immunization. Black lines/squares: SL immunization (primates with pre-existing immunity to adenovirus). Orange lines/diamonds: SL immunization (naive primates).

2 days after it was immunized by the IN/IT route (802197, 6.51×10^6 GC, Figure 9F). Virus continued to be shed in feces of this animal 1 week after immunization (802197, 2.52×10^6 GC). Adenovirus DNA was found on rectal swabs collected from each animal throughout the course of the study (Table 3).

Study 2: The Long-Term T Cell Response. The Ebola virus glycoprotein-specific T cell response was examined in PBMCs isolated from whole blood immediately prior to challenge, 150 days postimmunization. Multiparameter flow cytometry provided a comprehensive analysis of the types of antigen-specific T cells elicited by each treatment (Figure 10). The CD4⁺ T cell population present in animals immunized by the IN/IT route was much more diverse than the CD8⁺ T cell population (Figure 10A,B). Six specific CD4⁺ T cell subpopulations were found in animal 802197 with the most predominate phenotype being CD4⁺ CD107a⁺ IL-2⁺ (39% of the CD4⁺ population, Figure 10A). This animal also had the most diverse antigen-specific CD8⁺ T cell population with 4 different subpopulations detected by intracellular staining (Figure 10B). Samples from NHP 809077 contained four different CD4⁺ subpopulations. Cells that were CD4⁺ IL-2⁺ were most prevalent (45%) in this primate. The CD8⁺ population in this animal was composed of 3 specific subtypes with relatively equal distribution (CD8⁺ CD107a⁺ IL-2⁺, CD8⁺ IFN- γ ⁺, and CD8⁺ IL-2⁺). The CD4⁺ T cell population was less diverse in primate 810003 with the majority of antigen-specific cells also having the CD4⁺ IL-2⁺ phenotype (85%). The CD8⁺ IL-2⁺ subpopulation was the most prominent of two types of antigen-specific CD8⁺ T cells found in this primate.

CD4⁺ and CD8⁺ T cell populations were noticeably less diverse in animals immunized by the SL route (Figure 10C). Antigen-specific CD4⁺ T cells were not detected in samples collected from primate 808233. CD4⁺ IFN- γ ⁺ IL-2⁺ cells were present to a lesser degree than CD4⁺ IFN- γ ⁺ cells in samples collected from animal 805257 (25% and 75% of the population respectively). The most diverse CD4⁺ population elicited by SL immunization was found in primate 804317 with CD4⁺ IL-2⁺ cells being the most prominent of 5 different subtypes identified in this population. Antigen-specific CD8⁺ T cells were only found in samples collected from this animal with the majority being of the CD8⁺ CD107a⁺ phenotype (92.6%) and the remaining cells of the CD8⁺ IL-2⁺ phenotype (7.4%, data not shown).

Pre-existing immunity to adenovirus did not noticeably alter the diversity of T cells elicited by sublingual immunization (Figure 10D). Five distinct subpopulations of CD4⁺ T cells were found in primate 809227 with those of the CD4⁺ IL-2⁺ being the most prominent (63.1%). A single population of CD8⁺ CD107a⁺ cells was also found in samples collected from this animal (data not shown). CD4⁺ IL-4⁺ cells were the most prominent of the two antigen-specific CD4⁺ T cell populations found in samples collected from primate 807243. Antigen-specific CD8⁺ T cells were not detected in samples collected from this animal. SL immunization induced a single population of CD4⁺ IL-2⁺ cells and a single population of CD8⁺ CD107a⁺ cells in primate 804819.

Study 2: The Antibody-Mediated Response. Anti-Ebola GP and anti-adenovirus antibody levels were assessed in serum and BAL fluid at various time points after immunization

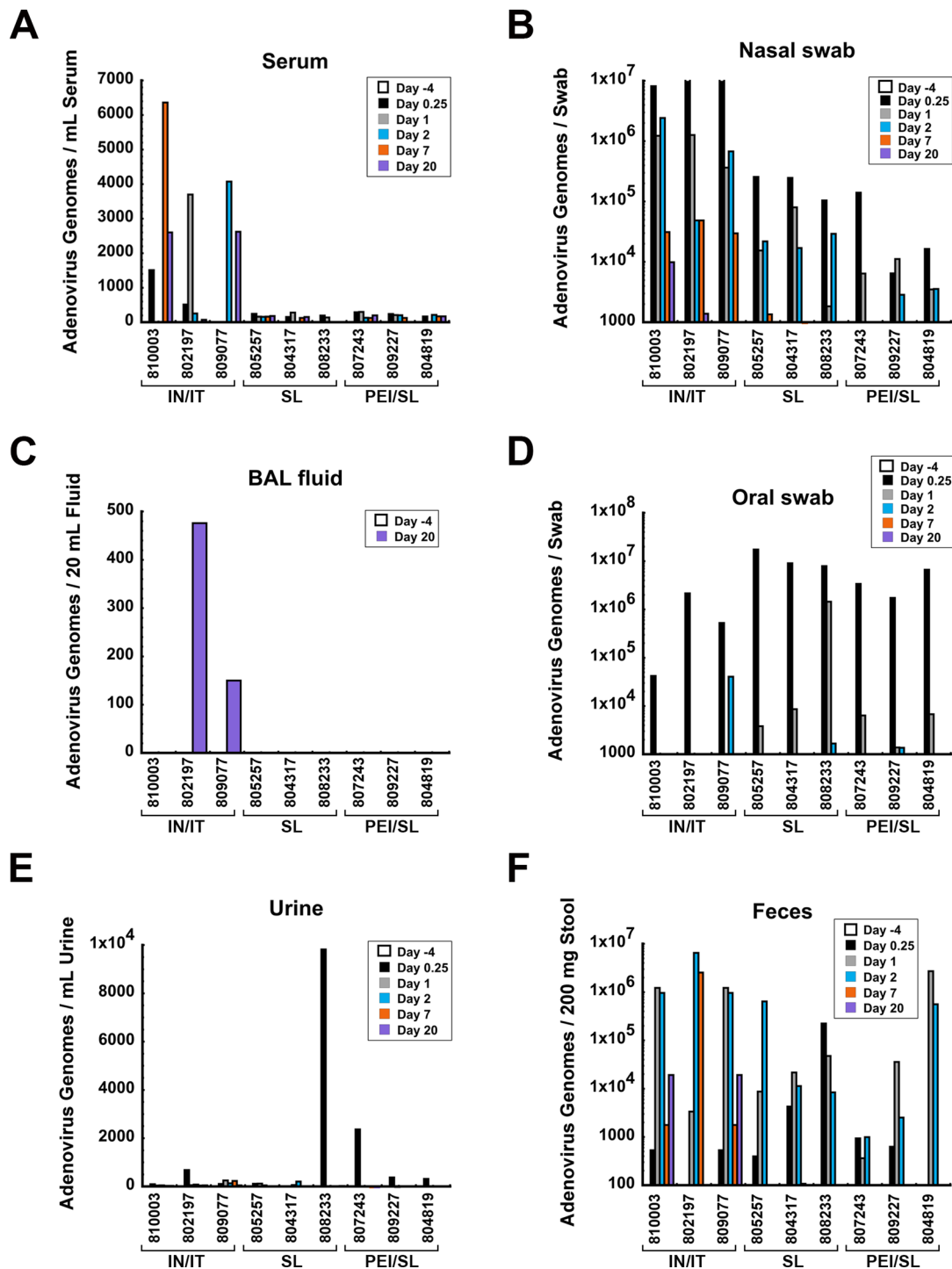


Figure 9. Study 2: Adenovirus genomes are released in the serum and nasal mucosa after IN/IT administration of formulated vaccine and in the oral mucosa after sublingual immunization. Male cynomolgus macaques were given either 1.6×10^9 ivp/kg of vaccine in a formulation of 10 mg/mL poly(maleic anhydride-*alt*-1-octadecene) substituted with 3-(dimethylamino)propylamine by the respiratory route or 2×10^{10} ivp/kg of vaccine in potassium phosphate buffered saline by the SL route. DNA was isolated from each sample, and viral genomes were determined by real time PCR. Animal numbers and corresponding treatments are outlined in Table 2

(Figure 11). Antigen-specific antibody levels mildly increased between day 20 and day 104 in serum collected from two animals immunized by the IN/IT route (0810003, 1.5-fold increase, 0809077, 1.3-fold increase, 0802197, no change, Figure 11A). Antibody levels remained high at the 142 day time point and were comparable to those found in animals immunized by the respiratory route in the first primate study. Significant anti-Ebola GP antibody levels were detected in the

BAL fluid of only one primate immunized by the IN/IT route (0802197, Figure 11B). Samples obtained from one of the animals immunized by the sublingual route (0808233) contained the highest level of anti-Ebola GP antibodies than any of the other animals given a single dose of vaccine (Figure 11C). It is also important to note that a significant change in anti-Ebola GP antibody levels between day 20 and day 57 postimmunization was detected in samples obtained from only

Table 3. Study 2: Shedding Patterns of Adenovirus DNA from the Rectal Mucosa of Non-Human Primates after a Single Dose of AdCAGoptZGP^a

route of immunization	animal #	pre	0.25 d	1 d	2 d	7 d	20 d
IN/IT	0810003	— ^b	1,500 ^c	1.0×10^5	3.7×10^4	2,000	2,100
	0802197	—	380	7.5×10^5	2.6×10^5	420	540
	0809077	—	—	3.1×10^4	9.0×10^4	620	2,600
SL	0805257	—	83	6.0×10^6	3.6×10^5	780	79
	0804317	—	1,400	3.5×10^4	1.5×10^4	640	58
	0808233	—	200	5,600	1,600	5,600	24
	0807243	—	1,100	1.2×10^5	1,100	190	58
PEI-SL	0809227	—	920	440	1.1×10^4	130	—
	0804819	—	2,000	1.4×10^6	1,900	30	—

^aData were obtained by real-time TaqMan PCR on DNA isolated from samples as described. ^bNone detected. Sample fell below the detection limit of the assay (10 viral genomes/100 ng of DNA). ^cUnits are genome copies per swab.

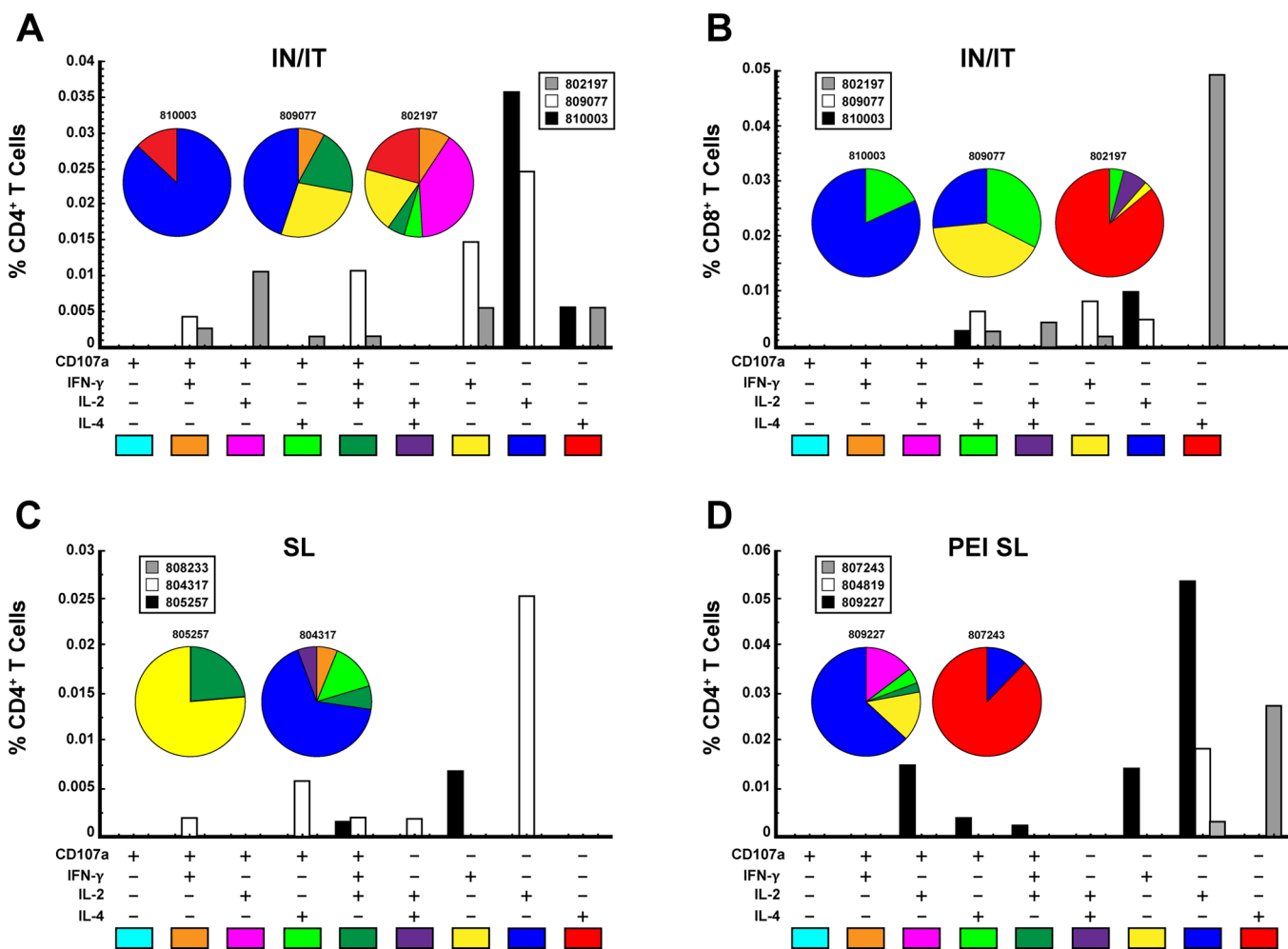


Figure 10. Study 2: Mucosal immunization elicits diverse populations of T cells capable of responding to Ebola glycoprotein 150 days after treatment. Quantitative analysis of CD4⁺ T cell populations secreting individual and combinations of cytokines in response to antigen stimulation after IN/IT administration (panel A), SL administration to naive animals (panel C), and SL administration to those with pre-existing immunity to adenovirus (panel D). Panel B reflects the quantitative analysis of CD8⁺ T cell populations after immunization by the IN/IT route. Each positively responding cell was assigned to one of 8 possible categories reflecting the production of IFN- γ , IL-2, and IL-4 alone or in combination. Pie charts depict the variety of T cell populations found in each individual animal. CD4⁺ T cells were not found in samples obtained from primate 808233 (SL immunization). A single CD8⁺ IL-2⁺ population was detected in samples from primate 804819 (PEI-SL) and is not illustrated as a pie chart.

one animal in this treatment group (0805257, 2.4-fold increase). Samples from only one of the animals with prior exposure to adenovirus immunized by the sublingual route contained anti-Ebola GP antibodies above the detection limit of the assay (809227, Figure 11D). While a notable amount of

anti-adenovirus neutralizing antibody (NAB) was detected in the serum of one primate 20 days after immunization by the IN/IT route (802197, 1:640 reciprocal dilution), circulating anti-adenovirus NABs were low in samples obtained from other primates immunized in the same manner (Figure 11E). Anti-

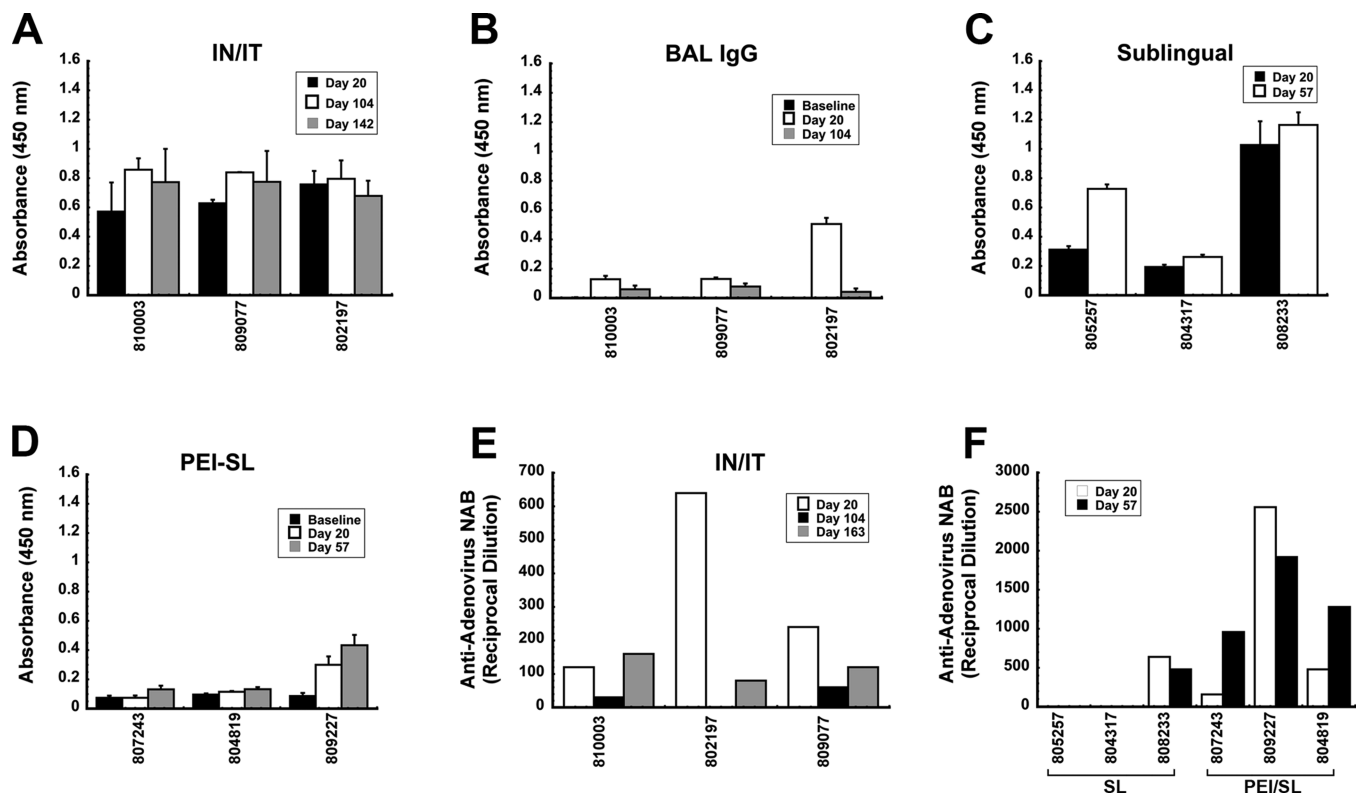


Figure 11. Study 2: Respiratory immunization induces production of antigen-specific antibodies that are sustained over time. Serum was collected from cynomolgus macaques immunized by the IN route (panel A) on days 20, 104, and 142 after immunization and analyzed for anti-Ebola GP IgG by ELISA as described.¹⁵ Serum was also collected from naive primates (panel C) and those with pre-existing immunity to adenovirus (panel D) on days 20 and 57 after immunization. These samples along with BAL fluid (panel B) collected from all primates were screened for anti-Ebola GP antibodies in the same manner. Serum from animals immunized by the IN/IT route (panel E) and from animals immunized by the SL route (panel F) was also screened for anti-adenovirus neutralizing antibodies. In each panel, error bars represent the standard error of samples assayed in triplicate from each primate for each time point.

adenovirus NABs were not found in the BAL of any of the primates immunized by the IN/IT route during the course of the study (data not shown). While anti-adenovirus NABs were quite high in the serum of one animal with pre-existing immunity 20 days after immunization by the SL route (809227, 1:2,560 reciprocal dilution), they were not detected in samples collected from two naive primates immunized in the same manner (805257, 804317, Figure 11F).

Study 2: Lethal Challenge with Ebola Virus. 150 days after immunization, animals were challenged with 1,000 pfu of Ebola virus (1995, Kikwit). Six days after challenge, both primates given saline, two animals immunized by the SL route (804317, 808233), and one animal with pre-existing immunity to adenovirus immunized by the SL route (809227) expired from infection (Figure 12A). The remaining primates with pre-existing immunity succumbed to infection on days 7 (804819) and 8 (807243) respectively. The remaining animal given the vaccine by the SL route (805257) expired on day 9. Each animal immunized by the respiratory route survived challenge. These animals experienced minimal changes in body weight (Figure 12B) and temperature (Figure 12C) during the course of infection with their clinical scores peaking at about 4–7 days after challenge (Figure 12D).

A notable drop in lymphocyte levels of all animals was observed 3 days after challenge (Figure 12E). Lymphocytes abruptly spiked in one animal immunized by the SL route (808233) and another with pre-existing immunity to adenovirus (804819) 6 days after challenge. Lymphocyte levels

of primates immunized by the IN/IT route slowly increased to day 14 where they remained constant. Lymphocytes of all other animals remained low until the time of death. ELISpot analysis revealed that a significant amount of MNCs capable of producing IFN- γ in response to stimulation with Ebola GP peptides were present in PBMCs isolated from whole blood of surviving animals 14 days after challenge (Figure 12F). A sharp drop in platelet counts was noted in all animals that did not survive challenge (Figure 12G). Mild drops in platelet counts were observed in animals immunized by the IN/IT route 3 days after challenge. These values continued to drop through day 28. ALT (Figure 12H) and BUN (Figure 12I) sharply rose to values as high as 24 and 6 times baseline respectively in animals that succumbed to Ebola infection. These values remained unchanged throughout Ebola infection in surviving animals.

Assessment of sera taken during challenge revealed that primates immunized by the IN/IT route had very high levels of circulating anti-Ebola GP antibodies (Figure 13A). These were neutralizing since very low levels of infectious Ebola were found in samples taken from two primates 3 days postchallenge (Figure 13B). Infectious Ebola virus was not detected in any samples collected from the third animal in this treatment group (809077). Ebola virus genomes were also not detected in samples taken from any of the animals immunized by the respiratory route (Table 4). Although samples from two animals immunized by the sublingual route also contained high levels of anti-Ebola neutralizing antibody (804317, 808233, 1,280 reciprocal dilution, Figure 13C), they were only partially

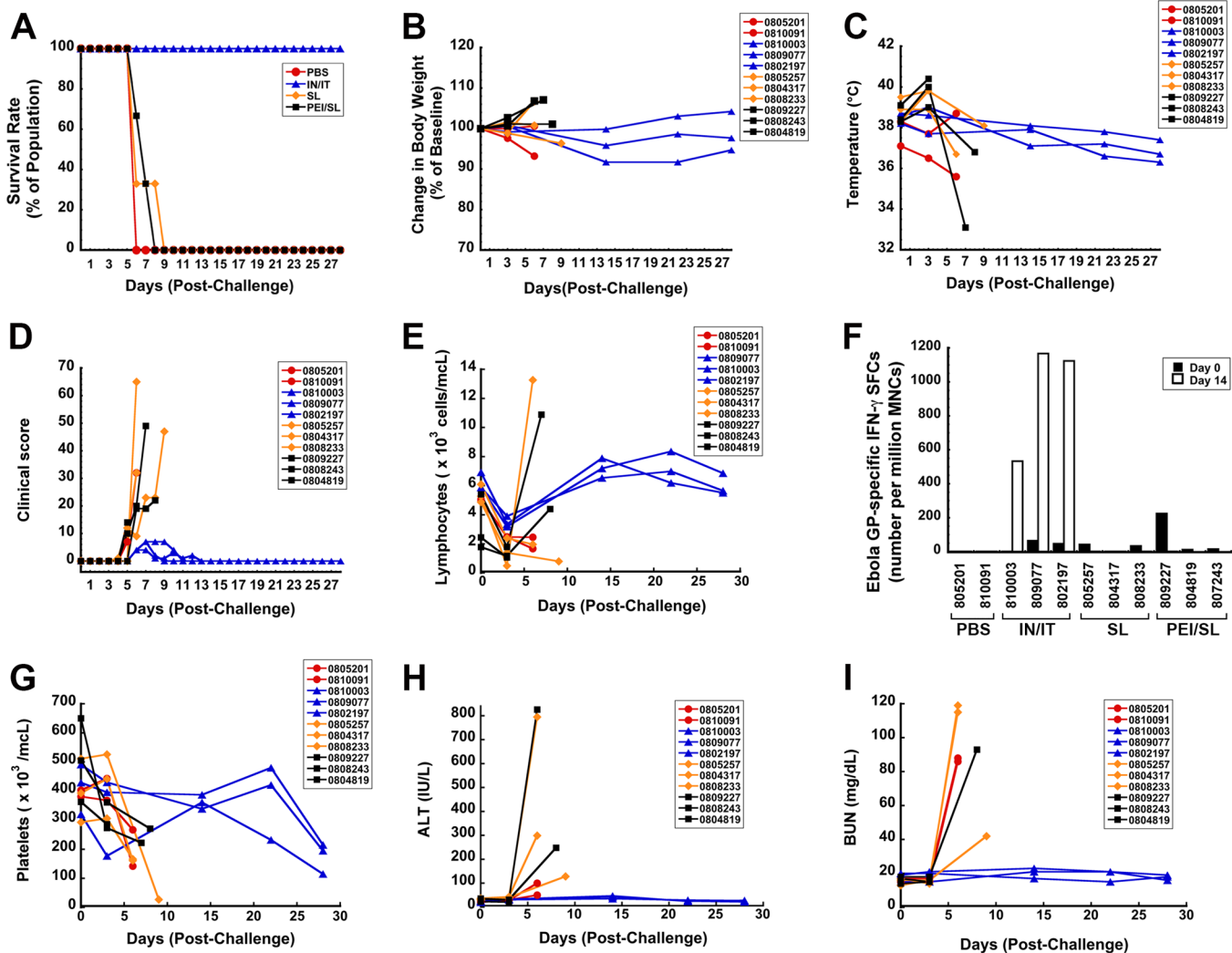


Figure 12. Study 2: A single dose of a formulated adenovirus-based vaccine protects from lethal challenge 150 days after immunization. (A) Kaplan–Meier survival curve. Cynomolgus macaques given a single dose of 1.4×10^9 ivp of Ad-CAGoptZGP formulated with 10 mg/mL poly(maleic anhydride-*alt*-1-octadecene) substituted with 3-(dimethylamino)propylamine in phosphate buffered saline survived lethal challenge. (B) Body weight profiles of immunized animals challenged with Ebola. Animals succumbing to infection experienced a change of $\pm 10\%$ of body weight during the active infection period. (C) Body temperature of primates during challenge. Body temperature declined in each animal during challenge with the most dramatic drops observed in animals that were not protected from infection. (D) Clinical scores. Primates were observed on a daily basis during the challenge period. Clinical scores were recorded for each primate by a blinded technician using a standard, approved scoring methodology. (E) Lymphocyte profiles. Lymphocytes of surviving animals recovered from an initial drop 3 days after challenge and remained stable throughout the remainder of the study. (F) ELISpot analysis of the cellular immune response in surviving animals 14 days after challenge. PBMCs were isolated from whole blood and stimulated with a peptide pool spanning the Ebola glycoprotein. (G) Platelet counts of primates during challenge. A notable drop in platelets was observed in all animals during challenge. (H) Serum alanine aminotransferase (ALT) levels during challenge. Samples were collected from animals on day 3 and day 14 and at the time of death. (I) Blood urea nitrogen (BUN) profile of immunized animals during challenge. This parameter remained unchanged in immunized animals that survived challenge. Red lines/circles: saline controls. Blue lines/triangles: IN/IT immunization. Orange lines/diamonds: SL immunization. Black lines/squares: animals with pre-existing immunity to adenovirus immunized by the SL route.

neutralizing since a concentration of 316 TCID₅₀/mL was found in samples collected from both primates at the 3 day time point that escalated to 1.47×10^8 and 6.81×10^8 TCID₅₀/mL respectively by the 6 day time point (Figure 13D). The number of circulating virus genomes in these animals followed a similar trend (Table 4). One animal that was exposed to the adenovirus serotype 5 host range mutant prior to immunization by the SL route (804819) also had high levels of anti-Ebola GP circulating antibodies (1,280 reciprocal dilution, Figure 13E), however, Ebola virus RNA was detected in samples collected from this animal at a concentration of 8.19×10^6 genome

copies/mL (Table 4). This animal expired before any infectious virus could be detected in its serum (Figure 13F).

DISCUSSION

The ongoing epidemic in West Africa is the largest Ebola outbreak ever recorded and is rapidly crossing borders. In response to this public health crisis, the WHO has supported a movement to initiate small phase I clinical trials of vaccine candidates that have successfully prevented non-human primates from developing Ebola virus disease after exposure to a lethal dose of the virus.³³ One candidate, an attenuated

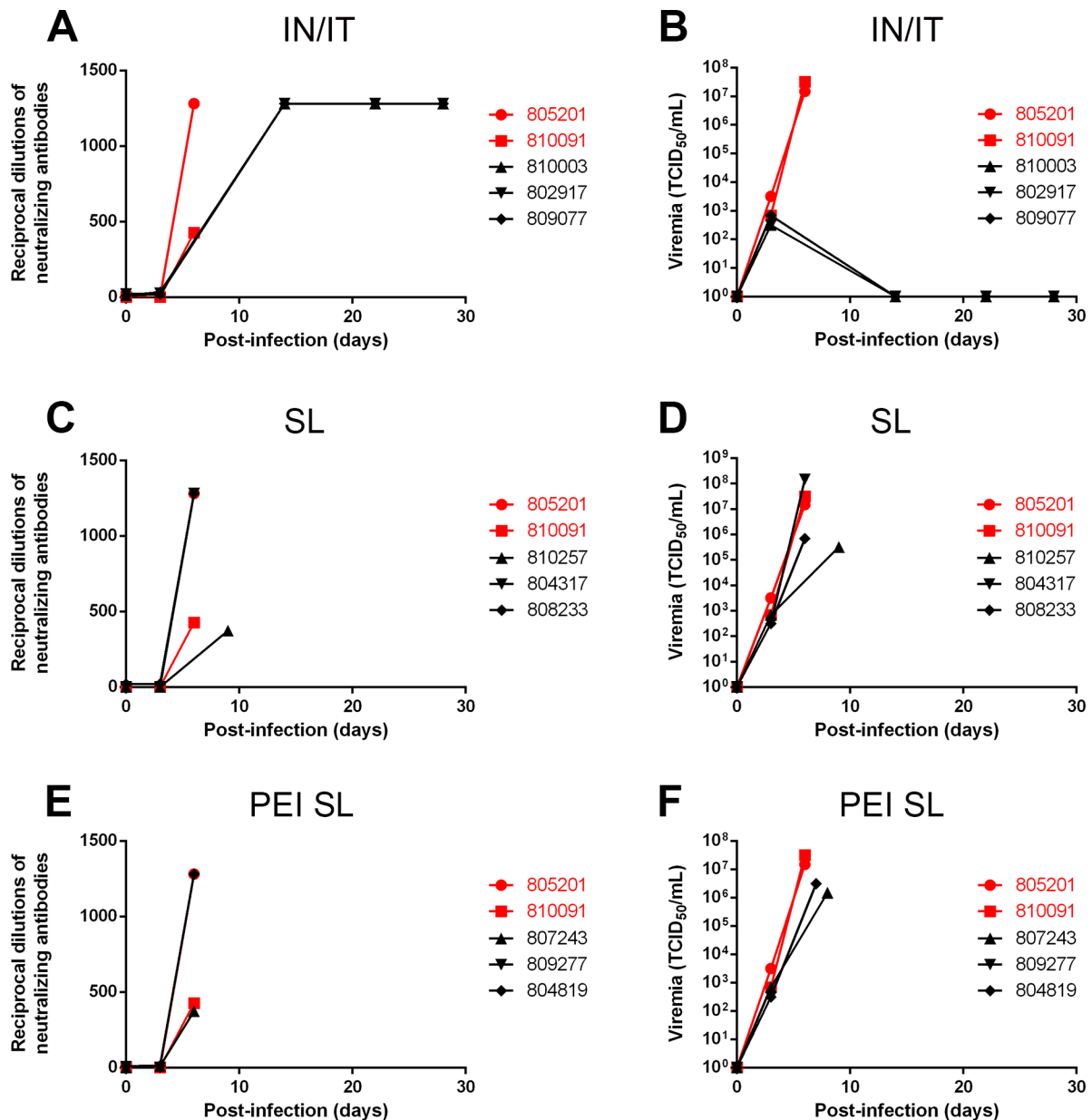


Figure 13. Anti-Ebola GP antibodies generated by a formulated adenovirus-based respiratory vaccine are neutralizing while those produced by an unformulated sublingual vaccine are partially neutralizing. The neutralizing capacity of antibodies in serum collected from each primate was assessed using a fluorescence neutralization assay (panels A, C, and E). The amount of Ebola virus present in the serum of animals during challenge was determined using a standard infectious titer assay (panels B, D, and F). In each panel, data obtained from animals given saline prior to challenge with Ebola are included as red symbols and lines for reference. TCID₅₀ = median tissue culture infectious dose 50 or the amount of virus that will produce pathological change in 50% of cells that are infected in culture. These assays were performed under BSL-4 conditions at the National Microbiology Laboratory in Winnipeg.

vesicular stomatitis virus (VSV) that expresses the Ebola glycoprotein in place of its own envelope protein, has shown efficacy in inducing both prophylactic and postexposure protection from infection.³⁴ While this fast-acting platform clearly holds promise for people in high-risk settings who may have already been exposed to Ebola, the longevity of the immune response elicited by the vaccine has only just been evaluated in rodent models of infection.³⁵ A second vaccine candidate, a bivalent recombinant chimpanzee adenovirus serotype 3 virus expressing the glycoproteins of both Ebola and Sudan species, the most lethal of the known Ebola viruses,³ is also undergoing clinical testing.^{36,37} This platform, which does not generate a protective immune response as rapidly as

the VSV-based vaccine, requires supplementation with a modified vaccinia Ankara virus also expressing the Ebola and Sudan glycoproteins to generate long-term protective immunity.³⁸ Both of these vaccine candidates have been developed and are entering the clinic as injectable products.

The ideal characteristics for an effective Ebola vaccine should be greatly influenced by the population where infections are endemic. Long-lasting protection from Ebola is necessary for at-risk populations (medical personnel) and for rural villagers where repeated prime-boost regimens are not feasible. Development of easy to administer, noninvasive immunization platforms eliminates the potential for transmission and spread of other blood-borne pathogens like hepatitis and HIV due to

Table 4. Study 2: Circulating Ebola Virus Genomes in Primates Challenged with Ebola Virus 150 Days after Immunization with a Single Dose of AdCAGoptZGP^a

animal no.	treatment/route	day 0	day 3	day 3.8	day 14	day 21	day 28
0810091	KPBS	— ^b	880 ^c	1.84 × 10 ⁵	<i>d</i>	N.A. ^e	N.A.
0805201	KPBS	—	—	7.79 × 10 ⁵	<i>d</i>	N.A.	N.A.
0802197	IN/IT	—	—	N.A.	—	—	—
0809077	IN/IT	—	—	N.A.	—	—	—
0810003	IN/IT	—	—	N.A.	—	—	—
0805257	SL	—	—	N.A.	<i>d</i>	N.A.	N.A.
0804317	SL	—	1.74 × 10 ⁴	9.84 × 10 ⁶	<i>d</i>	N.A.	N.A.
0808233	SL	—	1.01 × 10 ³	1.14 × 10 ⁶	<i>d</i>	N.A.	N.A.
0809227	PEI-SL	—	2.08 × 10 ⁴	1.57 × 10 ⁴	<i>d</i>	N.A.	N.A.
0804819	PEI-SL	—	—	8.19 × 10 ⁶	<i>d</i>	N.A.	N.A.
0807243	PEI-SL	—	3.33 × 10 ⁴	3.3 × 10 ⁵	<i>d</i>	N.A.	N.A.

^aData were obtained by quantitative RT-PCR on RNA isolated from whole blood as described. ^bNone detected. Sample fell below the detection limit of the assay (86 viral genomes/mL). ^cUnits are genome copies per milliliter of whole blood (GC/mL). ^dAnimal expired prior to sample collection at this time point. ^eNot assayed at this time point.

needle stick injuries that occur from unsafe practices during large immunization campaigns and from improper handling of biomedical waste.^{39,40} Establishment of mucosal as well as systemic immunity to Ebola is also important since transmission of the virus occurs through direct contact of mucosal areas with body fluids of infected individuals.⁴¹ While the manner by which we delivered the vaccine to the respiratory mucosa may seem complex by traditional standards, the IN/IT dual route method was previously found to stimulate a significant IgG response as early as 14 days after immunization, which is much earlier than that observed after intramuscular injection.⁴² Thus, we envision this approach to be quite attractive when rapid immune protection is desired especially in the case of first responders to an outbreak. Another important consideration is that of the prevalence of pre-existing immunity (PEI) to adenovirus serotype 5 in the global population and the negative impact it might have on the potency of our vaccine.^{43,44} We have previously found that the performance of the unformulated vaccine when given by IN/IT administration to primates at a dose equivalent to that used in the studies outlined in this manuscript was not affected by pre-existing immunity to adenovirus 5.⁴² However, survival was not complete as 25% of the naive animals and those with pre-existing immunity to adenovirus succumbed to infection. It is also important to realize that, in this case, the vaccine was not given alone but in combination with another adenovirus vector expressing interferon alpha, which may play a significant role in development of the antigen-specific immune response necessary for survival from Ebola infection in animals with prior exposure to adenovirus. In contrast, respiratory administration of our formulated vaccine alone afforded full protection to all animals challenged at a much later time after immunization (Figure 12). While this improvement in the absence of an immunostimulatory cytokine may suggest that our formulated vaccine may improve the potency of the vaccine in those with prior exposure to adenovirus, additional dose ranging studies in animals with PEI to adenovirus are greatly warranted.

Both the unformulated and the formulated vaccines given by the respiratory route were well tolerated by each primate with only mild, transient changes in a few blood chemistry parameters noted (Figures 2 and 8). Shedding of the vaccine was also minimal as the adenovirus was fully cleared from the nasal passages, urine, and feces within 48 h after immunization (Figures 3 and 9). Administration of the unformulated vaccine

by the respiratory route induced notable systemic and mucosal Ebola GP-specific T cell responses (Figure 4) as well as anti-Ebola GP-specific antibodies in the circulation and BAL fluid (Figure 5). To date, a considerable body of evidence indicates that, regardless of vaccine platform, a robust antibody response to Ebola glycoprotein is essential for protection from lethal infection.^{4,34,45} Additional studies have shown that a strong antigen-specific T cell response is required to prevent the dysregulation of host protective immune responses during Ebola infection⁴⁶ and is supportive when the antibody-mediated response is suboptimal.^{4,45} These principles were illustrated by the primate given the respiratory vaccine that did not survive challenge in Study 1 as the T cell response and the antibody mediated response were significantly lower in samples collected from this animal than in samples collected from other primates immunized in the same manner.

For the past 20 years, there has been solid evidence in the scientific literature that heterologous prime-boost regimens are required for induction of long-lived protective CD8⁺ T cells against a variety of microbial infections.³⁰ As a result, there are very little if any data delineating the durability of the immune response generated by a single dose of a recombinant adenovirus-based vaccine since most of these platforms involve a priming dose of a recombinant DNA plasmid containing an antigenic sequence similar to that encoded in a recombinant adenovirus used as a boost or the use of two different adenovirus serotypes expressing the same antigen.^{31,38,47} Detailed study of prime-boost regimens revealed that this approach elicited a diverse CD8⁺ T cell population with a variety of immunological functions as defined by the production of two or more cytokines at one time and cell surface mobilization of the degranulation marker CD107a.⁴⁴ While the presence of polyfunctional T cells has been shown to be important for protection in some infectious disease models,^{48,49} the role of polyfunctional T cells is not yet clear in the context of Ebola infection. Previous studies in rodent models of Ebola infection revealed that pre-existing immunity to adenovirus compromised the production of polyfunctional CD8⁺ T cells,¹⁵ which was indicative of poor survival upon challenge. In the non-human primate model, it has recently been found that the presence of CD8⁺ IFN- γ ⁺TNF- α ⁺ T cells correlated with survival³⁷ while other studies suggest that polyfunctional CD4⁺ T cells, especially those producing IFN- γ and IL-2 in response to stimulation with Ebola GP peptides, are

important for survival.^{22,26} Data generated in our study best correlate with these latter studies as samples obtained from two of the primates receiving the respiratory vaccine that survived challenge contained highly diverse CD4⁺ T cell populations and the most diverse CD8⁺ T cell populations of all immunized animals (Figure 10). However, one common cell surface marker/cytokine profile could not be detected among the surviving animals. To our knowledge, this is the first time that durable protection from a single dose respiratory recombinant adenovirus-based Ebola vaccine has been demonstrated in non-human primates.

The STEP and HVTN studies, which utilized an adenovirus serotype 5-based HIV vaccine, and some non-human primate data generated with recombinant adenoviruses have raised concerns that induced antigen-specific, vector-specific, or total CD4⁺ lymphocytes at mucosal surfaces may lead to enhanced HIV-1 infection.^{50–53} Here, we show that respiratory administration of a recombinant adenovirus 5-based vaccine does induce strong CD4⁺ T cell responses in BAL fluid and in the periphery that are long lasting. In contrast, SL immunization did not foster production of antigen-specific CD4⁺ T cells in BAL but did support antigen-specific T cell responses in the periphery, which were not hampered by prior exposure to adenovirus. While it is not clear if these observations would have adverse impact in populations where there is currently a heightened need for an Ebola vaccine and where HIV is quite prevalent, the fact that adenovirus genomes were detected from rectal swabs taken from animals at 7 and 20 days should be further evaluated. Because these samples were contaminated with residual fecal matter, screening of rectal biopsies taken 104 days after immunization for signs of inflammation and cellular activation are currently underway to further address this important question.

One very important point illustrated in the second primate study is that the antibody response generated by a given vaccine platform may not be fully neutralizing. This was somewhat evident in the first primate study as two animals with what appeared to be strong Ebola GP-specific antibody responses as determined by an ELISA assay did not survive challenge (Figure 5 and 6). TCID₅₀ and quantitative RT-PCR assays performed in the second study provided solid evidence that antibodies generated after immunization by the SL route could not effectively neutralize the virus (Figure 13). The highly diverse Ebola GP-specific CD4⁺ T cell populations found in one naive animal immunized by the SL route and another with pre-existing immunity could not compensate for the poor antibody-mediated immune response generated by this immunization strategy. While these results were disappointing, it is important to realize that the primary goal of this project was to develop an adenovirus-based vaccine that could be given by either the respiratory or the oral route. During development of the oral platform, we discovered that sublingual administration of the vaccine could elicit protective responses to rodent-adapted Ebola.^{14,15} Recently, studies evaluating this route of immunization have gained presence in the scientific literature with promising results for a variety of pathogens.^{54–56} The respiratory platform is attractive for use in areas where there is a dire need for Ebola vaccines and therapeutics because it allows for self-immunization in a needle free capacity. However, devices required for instillation into the nose and lung can be bulky, require some skill for proper use, and may require refrigeration for storage.⁵⁷ Many of the dosage forms used for sublingual delivery are compact and can stabilize

compounds at ambient temperature.⁵⁸ Thus, refinement of this platform for adenovirus-based vaccines and other Ebola vaccine candidates is warranted.

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Author Contributions

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Notes

The authors declare the following competing financial interest(s): X.Q., J.S., J.A., L.F., G.W., A.B., and G.P.K. have participated in the development of a variety of vaccines and therapeutics for Ebola virus disease including the vaccine described in this manuscript. D.W. and his laboratory have several commercial relationships with companies in the area of vaccines. These include him receiving consulting fees or stock ownership for Advisory/Review Board service, speaking support, or research support from commercial entities including Inovio Pharmaceuticals, Bristol-Myers Squibb, VGXI, Pfizer, Virxsys Co., Johnson & Johnson, Merck & Co., Sanofi Pasteur, Althea, Novo Nordisk, Statens Serum Institut, Aldevron, Novartis, Incyte, and possibly others. The work described in this manuscript was not supported by any of these entities. J.H.C., K.J.-S., D.J.S., J.X.X., K.L.M., M.J.M., I.B., L.Q.T., S.C.S., and M.A.C. have no competing interests to declare.

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