Mucosal Delivery of Adenovirus-Based Vaccine Protects against Ebola Virus Infection in Mice

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Background. Mucosal vaccination can offer several advantages over conventional intramuscular immunization to protect against Ebola virus (EBOV) infection, such as immune protection at sites of viral entry into susceptible individuals, and can be administered using needle-free devices.

Methods. The present study evaluated oral and nasal vaccination of mice with human adenovirus serotype 5 (Ad) expressing the *Zaire ebolavirus* glycoprotein (Ad-ZGP) in terms of their protection against and underlying immune responses to EBOV.

Results. Similar to intramuscular administration, oral or nasal vaccination of mice with Ad-ZGP fully protected the mice against a lethal challenge with mouse-adapted EBOV. Both T and B cell responses developed in mice receiving oral or nasal vaccination in different body compartments, indicating qualitative improvement of the immune response after mucosal immunization, compared with intramuscular vaccination.

Conclusions. Overall, the breadth of the immune response noted after nasal or oral immunization, including stimulation of $CD8^+$ T cells or effector memory T cells from the gastrointestinal tract or the lungs, was superior to that noted after intramuscular administration of the vaccine. The present study showed that adenovirus-based vaccine is effective against EBOV infection in mice after oral and nasal immunization.

Ebola virus (EBOV) causes hemorrhagic fever, with a survival rate that can be as low as 10% among infected individuals [1, 2]. The precise mode of natural transmission to humans remains elusive, although there are indications that fruit bats could act as a primary source of infection among apes and humans [3]. Filoviruses are particularly infectious, although natural spread of filoviruses among nonhuman primates and secondary transmission to humans normally require direct contact. However, there is experimental and epidemiological evidence indicating that EBOV can be transmitted through mucosal exposure [4, 5]. After entry in susceptible organisms, the virus appears to initially infect monocytes, macrophages, and dendritic cells, leading to deregulated activation of innate immunity and a systemic inflammatory response syndrome. Ultimately, massive destruction of critical organs (e.g., the liver), vascular damage, and hemorrhage can be observed [6, 7]. Outbreaks of EBOV infections have primarily been localized to Central Africa, but, despite their dramatic consequences in affected communities, they have had a relatively low impact on human health worldwide [8]. However, the characteristics of EBOV that make it such a dangerous pathogen also contribute to its utility as a potential agent of biological warfare [9].

Human adenovirus serotype 5 (Ad) was developed as an efficient delivery vehicle for therapeutic transgenes in a wide variety of animal models of genetic disease [10]. However, a significant drawback to the use of recombinant Ad for gene therapy applications originates from possible acute toxicity, transient gene expression in vivo, and limited transgene expression after readministration, arising from the ability of the virus

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to elicit a strong immune response in an immunocompetent host [11]. The inherent ability of recombinant Ad to facilitate strong immune responses against an encoded antigen makes it an attractive vaccine carrier and has compelled many investigators to develop Ad-based vaccines. Adenovirus expressing the *Zaire ebolavirus* (ZEBOV) envelope glycoprotein (Ad-ZGP) has been shown to protect mice, guinea pigs, and nonhuman primates against lethal ZEBOV challenges [12–14]. A phase 1 clinical trial was initiated in healthy adults immunized with adenovirus vector encoding for the ZEBOV GP, with the objective of evaluating the safety of and the immune responses to the vaccine [15].

To date, the primary method of Ad-ZGP vaccine administration is intramuscular injection [12–14], the manner by which most vaccines are given [16]. Although this type of administration ensures that the entire preparation enters the body, contaminated needles may pose significant safety risks to health care providers, patients, and the community. Needle-free–mediated mucosal immunization offers improved safety with respect to disease transmission, and, in many cases, self-vaccination may be possible. This approach may also provide more-appropriate immune protection, because vaccines administered by a mucosal route stimulate efficient mucosal and systemic immune responses, whereas vaccines administered parenterally primarily stimulate systemic immune responses [17, 18]. In small and large animal models, mucosal administration of Ad vaccines has been protective against several infectious agents, including *Mycobacterium tuberculosis,* HIV, rabies, and herpes simplex virus [19, 20]. Nasal immunization of a paramyxovirus-based vaccine expressing the ZEBOV glycoprotein was also shown to protect guinea pigs against lethal challenge [21].

Vaccine-mediated immune protection against EBOV infection in mice seems to require activation of both B and T cell responses [22]. However, the precise role of specific T cell subpopulations in protecting against EBOV infection remains to be determined. $CD8^+$ T memory cells are the crucial component of protective immunity against viral infections. Compared with naive T cells, memory T cells can respond with enhanced kinetics and magnitude to ensure protection against reinfection [23–25]. $CD8^+$ memory T cells are a heterogeneous population that can be broadly segregated into 2 general subsets: central memory and effector memory T cells. Central memory cells are mainly located in lymphoid organs, expressing high levels of CD44 and CD62L ($CD44^{hi}CD62L^{hi}$). They are highly proliferative and require a relatively longer period of activation to express cytolytic function [24]. Effector memory T cells express increased levels of CD44 but markedly reduced levels of CD62L ($CD44^{hi}CD62L^{lo}$). They have less proliferative potential, recirculate preferentially through nonlymphoid tissues, and are immediately cytolytic on antigen reexposure [26].

In the present study, we investigated ZEBOV glycoprotein (GP)–specific cellular and humoral immune responses correlating with protection in mice immunized with a recombinant Ad vaccine by the mucosal route.

MATERIALS AND METHODS

Construction and production of adenoviral vectors. The molecular clone of E1/E3-deleted adenovirus vector expressing the ZEBOV GP was created by cloning of the open reading frame ZEBOV GP in pShuttle (BD Biosciences) for subsequent direct cloning of the expression cassette in the E1 region of Ad (BD Adeno-X Expression System 1; BD Biosciences). Authenticity was confirmed by sequencing, and the recombinant virus was (1) rescued by transfecting the linearized DNA into 293 cells, (2) propagated to large-scale infections (5×10^8 cells), and (3) purified on an affinity column according to the manufacturer's recommendation (Adeno-X Virus Purification Mega Kit; Clontech). Genome structures of vectors were analyzed by restriction digestions of viral DNA in comparison with those of the molecular clones, as described elsewhere [13]. Numbers of particles and infectivity of vectors were determined by standard optical density reading and immunodetection of the hexon protein, respectively, after infection of 293 cells with limiting dilutions of each vector preparation, according to the manufacturer's recommendations (Adeno-X Rapid Titer Kit; Clontech).

Animal models. B10.BR mice (The Jackson Laboratory) were used to evaluate protection against and B and T cell immune responses to ZEBOV, as described elsewhere [13]. The T cell response was analyzed by evaluating the frequency of $CD8^+$ T cells positive for interferon (IFN)–γ production or cell division on peptide stimulation by flow cytometry (FACS) in B10.BR (major histocompatibility complex [MHC] $H\text{-}2^K$), where a dominant CD8 epitope has been mapped.

Immunization of mice with adenoviral vaccine vector and challenge. B10.BR mice were immunized with 1×10^{10} particles/mouse (which is known, from the results of a previous study, to provide protection [13]), by means of intramuscular injection (50 μL) in the right limb or instillation into the nasal cavity (50 μL) or by oral gavage (100 μL) of recombinant adenoviral vector diluted in PBS. Mice were challenged by intraperitoneal injection with 200 $\times$ LD_{50} in 200 μL of mouse-adapted ZEBOV (MA-ZEBOV) [27]. After challenge, the animals were weighed every day for 13 days and were monitored for clinical signs by use of an approved scoring sheet. All procedures and the scoring sheet were approved by the Institutional Animal Care Committee at the National Microbiology Laboratory (NML) of the Public Health Agency of Canada (PHAC), according to the guidelines of the Canadian Council on Animal Care. All infectious work was performed in the biosafety level 4 (BSL4) facility at NML, PHAC.

Neutralization assay. Serum samples collected from im-

munized mice were inactivated at 56°C for 45 min. Serial dilutions of each sample (1:10, 1:20, 1:40, etc., in 50 μL of Dulbecco's modified Eagle medium [DMEM]) were mixed with an equal volume of ZEBOV expressing the enhanced green fluorescent protein (EGFP) reporter gene (ZEBOV-EGFP) [28] (100 transducing units/well, according to EGFP expression) and were incubated at 37°C for 90 min. The mixture was then transferred onto subconfluent Vero E6 cells in 96-well flat-bottomed plates and incubated at room temperature for 5–10 min. Control wells were infected with an equal amount of viral vector without the addition of serum or with the addition of nonimmune serum. A total of 100 μL of DMEM supplemented with 20% fetal bovine serum (FBS) was then added to each well, and plates were incubated at 37°C in 5% CO_2 for 48 h. Cells were subsequently fixed with 10% buffered formalin for 24 h and examined under a fluorescent microscope. The total number of EGFP-positive cells was counted in each well, and sample dilutions that showed a >50% reduction in the number of green cells, compared with that noted in controls, was scored as being positive for neutralizing antibody. That technique has been found to be more accurate and sensitive than traditional plaque assays using immunocytochemical analysis detection methodology (H. Ebihara, J.S., H.F., G.P.K., unpublished data). All infectious in vitro work was performed in the BSL4 laboratory of NML, PHAC.

Frequency of IFN-γ–positive cells. Enzyme-linked immunosorbent spot (ELISPOT) was performed using the ELISPOT Mouse Set (BD PharMingen) according to the manufacturer's recommendation. Splenocytes or mononuclear cells were isolated by grinding tissues of interest (e.g., spleen tissues) in L-15 medium and purifying the cells through filtration and ficoll-hypaque (Amersham Biosciences) density gradient, followed by 3 washes with PBS and resuspension in RPMI 1640. Splenocytes or mononuclear cells isolated from spleen tissues, bronchoalveolar lavage (BAL) fluid, mesenteric lymph nodes (MLNs), and Peyer patches (PPs) were pooled from 4 B10.BR mice per experimental group 45 days after immunization and were added to microwells along with the TELRTFSI [29] peptide that carries the ZEBOV GP immunodominant MHC class I epitope for mice with the H-2^k haplotype (B10.BR). Control cells were incubated either without peptide or with nonspecific stimulator (staphylococcal enterotoxin B; 200 ng/mL). After staining, wells were counted using an ELISPOT reader (AID EliSpot reader system; Cell Technology).

For the evaluation of IFN-γ–positive $CD8^+$ T cells 10 days after immunization, splenocytes (1×10^6 splenocytes/sample) were cultured at 37°C for 5 h in 96-well round-bottom microtiter plate wells in DMEM supplemented with 10% FBS, 10^{-6} mol/L 2-mercapto-ethanol (2-ME; Sigma), and GolgiStop (BD PharMingen) at 1 μL/mL, as described elsewhere [12]. In brief, stimulated cells were stained with a fluorescein isothiocyanate (FITC)–anti–mouse CD8a (BD PharMingen), followed by a phycoerythrin (PE)–anti–mouse IFN-γ antibody (BD PharMingen). Stained cells were run through an LSRII flow cytometer (BD Biosciences), acquiring at least 500,000 events/sample. Final data analyses were performed using the software

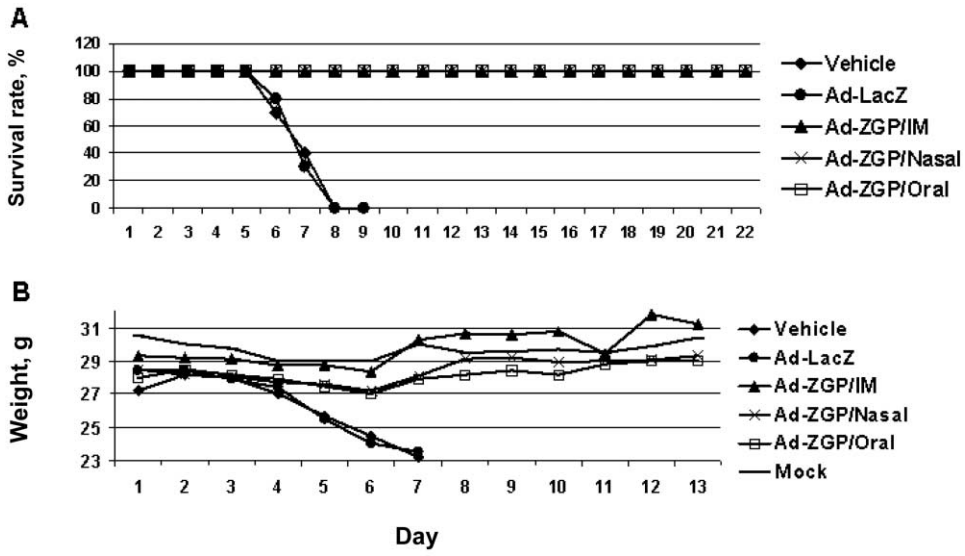

Figure 1. Protective efficacy of human adenovirus serotype 5 expressing the *Zaire ebolavirus* glycoprotein (Ad-ZGP) after intramuscular (IM), nasal, or oral immunization. Groups of 10 B10.BR mice were vaccinated with a single dose of 1×10^{10} particles/mouse in 50 μL, for IM or nasal delivery, or in 100 μL, for oral delivery. At 28 days after vaccination, all mice were challenged with 200 LD_{50} of mouse-adapted *Zaire ebolavirus*. Data are the percentage of mice that survived *(A)* or lost body weight *(B)* over time. Weight is expressed as the mean body weight (in grams) for a group of 10 mice. Ad-LacZ, adenovirus expressing β-galactosidase; mock, age-matched, untreated, unchallenged mice (to indicate normal weight variation over time); vehicle, phosphate-buffered saline.

Flowjo (version 5.7.2; Tree Star). A response was considered to be positive when the frequency from stimulated samples was ⩾3-fold higher than that from nonstimulated samples or control samples stimulated with unrelated peptides. Samples for the ELISPOT assay and the evaluation of IFN-γ–positive $CD8^+$ T cells 10 days after immunization were tested in duplicates, and the experiments were repeated twice.

Splenocytes and mononuclear cells isolated from BAL fluid, MLN, or PP were also analyzed by FACS 45 days after immunization. Cells were cultured in supplemented DMEM (10% FBS and 10^{-6} mol/L 2-ME) and the TELRTFSI peptide at a concentration of 1 μg/mL for 5 days in a 24-well plate (1×10^6 cells/sample per well). Cells were stained with a mix of the anti–mouse antibodies PE-Cy7–anti-CD3, Alexa 700–anti-CD4, perCPCy5.5–anti-CD8, PE–anti-CD44, and APC–anti-CD62L (all from BD PharMingen) at 4°C for 30 min, permeabilized as mentioned above, and stained with an FITC anti–mouse IFN-γ (BD PharMingen). After washing, cells were examined by means of 2- or 6-color FACS performed using an LSRII flow cytometer, and data were analyzed using FACS Diva software (version 5.0.2; BD Biosciences).

Carboxy fluorescein diacetate succinimidyl ester (CFSE) staining. Expansion of T cells was analyzed using the Vybrant CFDA SE Cell Tracer kit (Invitrogen) as described by the vendor's protocol. In brief, 2×10^6 cells were incubated at 37°C for 8 min with 1.5 μmol/L CFDA SE, washed twice with PBS, and cultured for 5 days in supplemented DMEM, as described above, for IFN-γ staining (DMEM supplemented with 10% FBS and 10^{-6} mol/L 2-ME with or without the TELRTFSI peptide). Staining of surface cellular markers was performed after 5 days, as described above, for IFN-γ staining with the same cocktail of antibodies (PE-Cy7–anti-CD3, Alexa 700–anti-CD4, perCPCy5.5–anti-CD8, PE–anti-CD44, and APC–anti-CD62L). Samples were analyzed using 6-color FACS performed using LSRII and FACS Diva software (CFSE and FITC have comparable emission peaks and are detected in the same channel). Samples used in the evaluation of IFN-γ– or CFSE-positive T cells 45 days after immunization were tested in duplicate, and the experiment was repeated once.

Statistical analysis. Data were analyzed for statistical differences by means of unpaired *t* test (with a 2-tailed *P* value and by the method of Kolmogorov and Smirnov), 1-way analysis of variance (ANOVA; Tukey-Kramer multiple comparisons test), or multiple comparisons with the control group (by use of the Holm-Sidak method), when appropriate. The differences in the mean or raw values among treatment groups were considered to be statistically significant when $P < .05$.

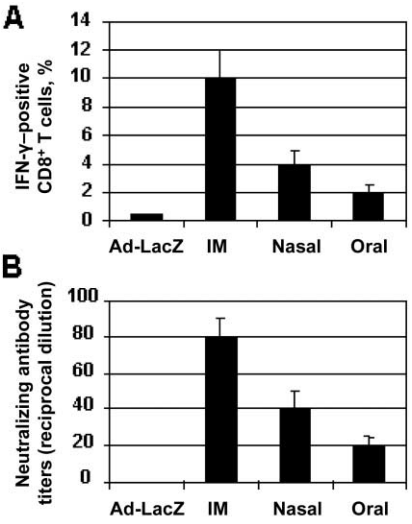

Figure 2. T and B cell responses after immunization. B10.BR mice were vaccinated intramuscularly (IM), nasally, or orally with 1×10^{10} particles/animal, and splenocytes were harvested 10 days later for interferon (IFN)–γ $CD8^+$ T cell frequency analysis *(A)* or determination of neutralizing antibody (NAb) titers *(B)*. The specific TELRTFSI peptide to *Zaire ebolavirus* (ZEBOV) glycoprotein (GP) (0.4 μg/well) was incubated with 10^6 splenocytes in the presence of Brefeldin A (GolgiStop; BD Biosciences), and the cells were subsequently analyzed by flow cytometry for expression of CD8 and IFN-γ. Four mice were analyzed per group. The levels of NAb to ZEBOV expressing the enhanced green fluorescent protein (EGFP) reporter gene (ZEBOV-EGFP) were evaluated 25 days after vaccination ($n = 10$ mice/group). Error bars denote the SD of the data. Ad-LacZ, adenovirus expressing β-galactosidase.

RESULTS

Mucosal vaccination and protection against lethal EBOV challenge. To investigate the relevance of mucosal vaccination in protecting against EBOV, the protective efficacy of adenovirus-based ZEBOV vaccine was evaluated after intramuscular, nasal, or oral administration. Mice were vaccinated with phosphate-buffered saline (PBS; vehicle) or adenovirus expressing β-galactosidase (Ad-LacZ) given in the same dose that was administered to experimental groups. Challenge was performed 28 days after immunization with 200 LD_{50} of MA-ZEBOV. All control mice (those given vehicle and those given AdHu5-LacZ) died between 5 and 8 days after challenge, whereas all mice vaccinated with Ad-ZGP administered intramuscularly, nasally, or orally survived the challenge (figure 1*A*). Significant weight loss was observed only in both control groups (i.e., the vehicle and AdHu5-LacZ groups) (figure 1*B*) ($P < .05$). These results indicate that mucosal vaccination with Ad-ZGP can protect mice against lethal MA-ZEBOV infection.

Induction of both cellular and humoral immunity by mucosal vaccination. T and B cell–specific immune responses were analyzed with respect to the different routes of immunization. Peptide-specific activation of $CD8^+$ T cells, as measured by the production of IFN-γ as determined by FACS, was detected in the spleens of Ad-ZGP–vaccinated animals immunized by the intramuscular, nasal, or oral route at a mean

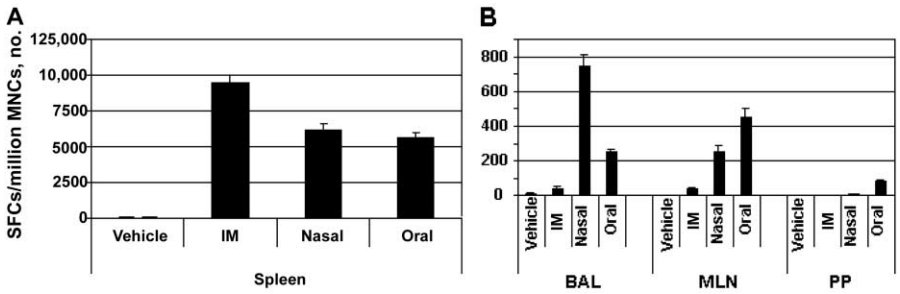

Figure 3. Frequency of interferon (IFN)–γ–positive mononuclear cells (MNCs) in different body compartments after vaccination. The frequency of IFN-γ–positive mononuclear cells was analyzed, by enzyme-linked immunosorbent spot (ELISPOT) assay, in spleen tissues *(A),* bronchoalveolar lavage (BAL) fluid, mesenteric lymph nodes (MLN), or Peyer patches (PP) samples *(B)* obtained 45 days after immunization. Mononuclear cells isolated from different body compartments were plated at 1×10^5 or 1×10^4 cells/well and stimulated with the TELRTFSI peptide. Expression of IFN-γ was evaluated using an anti–mouse IFN-γ antibody. Cells isolated from BAL fluid, MLNs, or PP samples obtained from 4 mice were pooled, and the 2 cell concentrations were tested in duplicate. The number of spot-forming cells per million mononuclear cells is denoted on the *y*-axis. Error bars denote the SD of the data. SFCs, spot-forming cells.

frequency (±SD) of 10% ± 2%, 4% ± 1%, and 2% ± 0.5%, respectively (figure 2*A*). In contrast, splenocytes harvested from control mice immunized with Ad-LacZ contained 0.5% of $CD8^+$ T cells positive for IFN-γ. The B cell response to ZEBOV GP in serum samples obtained from immunized mice was analyzed for neutralization of ZEBOV-EGFP [28]. ZEBOV-EGFP–specific neutralizing antibodies (NAbs) were detected in mice 25 days after vaccination with Ad-ZGP administered intramuscularly, nasally, or orally at mean levels (±SD) equal to 80 ± 10, 40 ± 10, and 20 ± 5 (reciprocal dilutions), respectively (figure 2*B*).

Induction of immune responses in different body compartments by means of mucosal vaccination. Local mucosal immunity would be important in providing protection against different exposures to ZEBOV. Consequently, ELISPOT was used to measure T cell immune responses in different body compartments 45 days after intramuscular, nasal, or oral vaccination of mice with Ad-ZGP. The number of activated, IFN-γ–positive T cells harvested from the spleen was 35%–45% less in mice vaccinated nasally or orally than in mice that received Ad-ZGP intramuscularly (figure 3*A*). In contrast, a mean (±SD) of 740 ± 55 IFN-γ–positive cells (spot-forming cells/million mononuclear cells) was detected in BAL fluid after nasal immunization, whereas a mean (±SD) of 240 ± 10 and 30 ± 5 IFN-γ–positive cells was found in animals after oral and intramuscular vaccination, respectively (figure 3*B*). Oral vaccination induced the development of more IFN-γ–positive cells in MLNs and PPs (mean ± SD, 445 ± 50 and 92 ± 1, respectively) than did nasal immunization (mean ± SD, 255 ± 25 and 8 ± 0, respectively) or intramuscular vaccination (47 ± 1 and 0, respectively) (figure

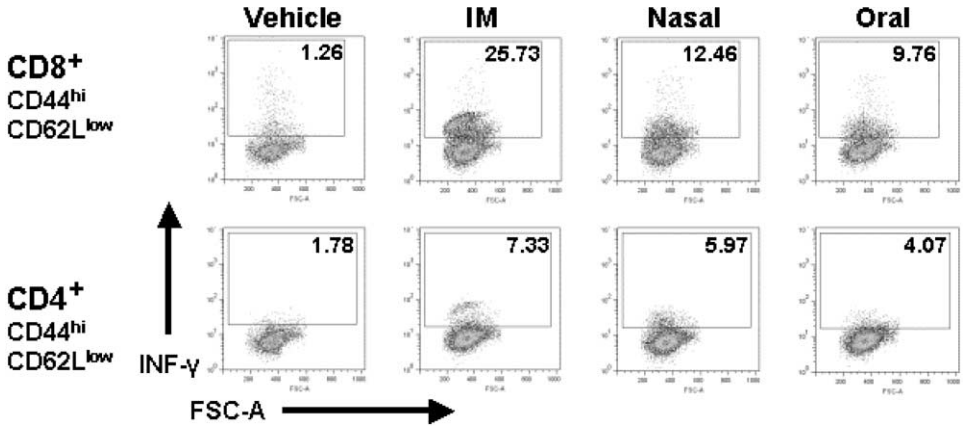

Figure 4. Activation of effector/effector memory ($CD44^{hi}CD62L^{lo}$) $CD4^+$ and $CD8^+$ T cells after intramuscular or mucosal immunization. Splenocytes pooled from 4 mice/experimental group 45 days after vaccination were stimulated in the presence of the TELRTFSI peptide for 5 days. Six-color flow cytometry was then performed using antibody against mouse CD3, CD4, CD8, CD44, and CD62L T cells and interferon (IFN)–γ. Gated population positive for CD8, CD3, $CD44^{hi}$, and $CD62L^{lo}$ T cells or CD4, CD3, $CD44^{hi}$, and $CD62L^{lo}$ T cells was analyzed for expression of IFN-γ (*y*-axis). The number in each dot plot denotes the percentage of gated IFN-γ–positive cells for each subpopulation, as indicated. Pictures are from the second of 3 experiments and are representative of the mean to higher response from each group (from the same experiment).

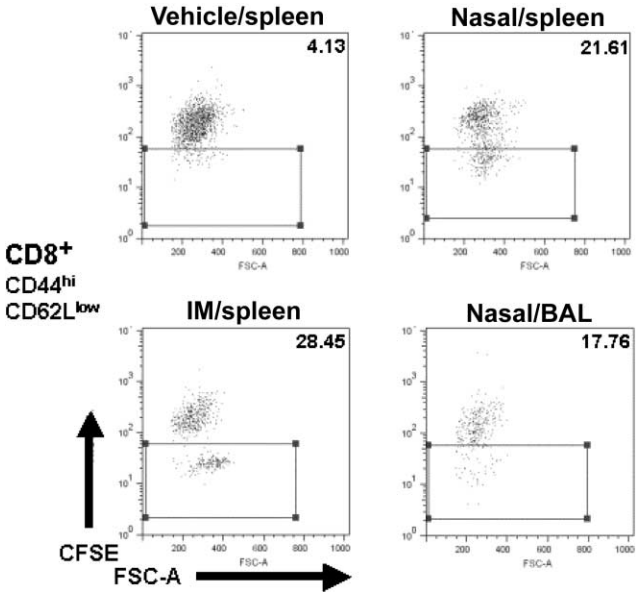

Figure 5. Expansion of $CD8^+$ effector/effector memory cells ($CD44^{hi}CD62L^{lo}$) isolated from the spleen or bronchoalveolar (BAL) fluid. Mononuclear cells from the spleen or BAL fluid were isolated 45 days after intramuscular (IM) or nasal immunization, stained with carboxy fluorescein diacetate succinimidyl ester (CFSE), and stimulated with the TELRTFSI peptide for 5 days. Cells positive for $CD8^+$, $CD44^{hi}$, and $CD62L^{lo}$ were then evaluated for CFSE staining by 6-color flow cytometry. A decrease in CFSE staining denotes cell division/expansion. CFSE-labeled cells expressing $CD8^+$, $CD44^{hi}$, and $CD62L^{lo}$ isolated from the spleen of 4 mice immunized intramuscularly or nasally are shown in the lower left and upper right panel, respectively. The effector memory $CD8^+$ CFSE-labeled cell population originating from the BAL fluid obtained from 4 nasally immunized mice is shown in the right lower panel. The number in each dot plot denotes the percentage of gated CFSE-negative cells for each subpopulation, as indicated. Pictures are from the second of 3 experiments and are representative of the higher response in each group (from the same experiment). FSC-A, forward scatter.

3*B*). BAL fluid obtained from mice immunized by the nasal route contained 3 and 15 times more IFN-γ–positive cells than did BAL fluid obtained from animals vaccinated by the oral or intramuscular route, respectively (figure 3*B*). The lower but significant response in the MLNs of nasally vaccinated mice and in BAL fluid from orally vaccinated mice ($P < .05$) most likely results from an overspill of the vaccine during the immunization procedure. Animals immunized nasally were seen to swallow at least part of the vaccine dose, and bubbles were observed to be coming out of the nostrils of some orally immunized mice. The NAb response was also monitored in BAL fluid samples obtained 45 days after vaccination. NAb to ZEBOV-EGFP was undetectable in samples from control or intramuscularly immunized mice, whereas mean levels ($\pm$SD) of 40 $\pm$ 10 and 10 $\pm$ 5 (reciprocal dilutions) were detected in the BAL fluid samples obtained from nasally and orally vaccinated animals, respectively (data not shown). These results suggest that mucosal delivery induces substantial local immunity, compared with intramuscular vaccination.

Induction of generation of specific memory T cells by mucosal vaccination. The presence of activated cells expressing IFN-γ on peptide-specific restimulation 45 days after immunization indicates that T cells of the memory compartment were stimulated at the time of vaccination. These activated T cells are also likely to be important in providing protection against EBOV infection, because they were found to play a critical role in the establishment of protective immune responses to different viruses, including HIV [30]. FACS showed that ZEBOV peptide-specific $CD44^{hi}CD62L^{lo}$ effector/effector memory CD4 and CD8 T cells were detected in mice 45 days after vaccination. IFN-γ production was detected in a mean of 25.7% of $CD44^{hi}CD62L^{lo}$ effector/effector memory $CD8^+$ T cells isolated from the spleen of intramuscularly immunized mice, compared with a mean of 12.5% and 9.8% of such cells obtained from nasally and orally vaccinated animals, respectively (figure 4, *top row*). In control mice immunized with vehicle, 1.3% of effector $CD8^+$ T cells were found to be positive for IFN-γ under identical conditions. Effector memory $CD4^+$ T cells ($CD44^{hi}CD62L^{lo}$) also demonstrated specific expression of IFN-γ in response to peptide restimulation, with a trend comparable to the effector memory $CD8^+$ T cells subpopulation, although with lower overall mean levels: 7.3%, 6.0%, and 4.1% of cells from intramuscularly, nasally, and orally immunized mice, respectively (figure 4, *bottom row*), were found to be positive for IFN-γ. Under the same conditions, <2% of control effector memory $CD4^+$ T cells were found to be positive for IFN-γ.

ZEBOV-specific T cells were also tested for their ability to rapidly proliferate when reencountering the cognate antigen. Cells from the spleen and BAL fluid were labeled with CFSE and restimulated with the ZEBOV immunodominant peptide. CFSE is a fluorescent dye that is evenly diluted during cell division. A decrease in CFSE staining is indicative of cell division and expansion. Five days after restimulation, a mean of 28.5% of effector memory $CD8^+$ T cells obtained from the spleens of intramuscularly immunized mice had undergone proliferation, compared with 21.6% of cells derived from the spleens of nasally immunized animals (figure 5). Interestingly, a mean of 17.8% of $CD44^{hi}CD62L^{lo}$ effector memory $CD8^+$ T cells isolated from the BAL fluid of mice vaccinated nasally could also proliferate in response to the antigen (figure 5, *lower right panel*). These results suggest that, despite being associated with a quantitatively lower induction of the immune response with respect to that achieved by intramuscular immunization ($P < .05$), adenovirus-mediated mucosal immunization can stimulate a broad, prolonged T cell immune response in mice.

DISCUSSION

The present study evaluated the protective efficacy of recombinant adenovirus-based Ebola vaccine vector against ZEBOV in mice and the potency of the systemic and mucosal immune responses after intramuscular, nasal, or oral immunization. The evaluation of immune responses focused on effector memory cells rather than central memory cells, because the former cells have a significant presence in the periphery, which was the main interest here. Overall, mucosal vaccination could stimulate a systemic response of every subpopulation monitored that also responded to intramuscular immunization, indicating that the extent of the systemic immune response after mucosal vaccination is comparable to the response stimulated from intramuscular immunization. These results indicate that mucosal vaccination substantially improved the overall quality of the systemic and mucosal immune responses by involving more body compartments, including the lungs and the gastrointestinal tract. It is possible that this significant increase in the diversity of the immune response can compensate for the lower response in such lymphoid organs as the spleen.

Developing a vaccine strategy that can generate both mucosal and systemic protective immunity against EBOV infection is important for any individual who has potentially been exposed to EBOV. Whether exposure results from contact with infected animals, infectious body fluids, or contaminated biological samples or occurs after an intentional release of the virus, the mucosa is likely to be the primary site of exposure in all cases and should participate in the host defense of the individual after appropriate vaccination. In addition, self-administered needle-free vaccination may have advantages in remote places, and the lower toxicity associated with mucosal vaccination may facilitate quantitative improvement in the immune response after safe immunization with higher doses of Ad vaccine. It may also be more suitable for efficient protection of a population at risk, including great apes, which could be immunized using bait vaccines.

Mucosal vaccination with Ad-ZGP protected mice against lethal ZEBOV challenge with concomitant B and T cell–mediated immune responses. Immunization by each route was also capable of establishing antigen-specific memory responses, which is indicative of long-term protection. These results support further evaluation of adenovirus-based mucosal immunization regimens against EBOV in other relevant animal models of EBOV infection, such as guinea pigs and nonhuman primates.

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