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ORIGINAL ARTICLE

Affinity maturation of an anti-V antigen IgG expressed in situ *through adenovirus gene delivery confers enhanced protection against* Yersinia pestis *challenge*

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Genetic transfer of neutralizing antibodies (Abs) has been shown to confer strong and persistent protection against bacterial and viral infectious agents. Although it is well established that for many exogenous neutralizing Abs increased antigen affinity correlates with protection, the effect of antigen affinity on Abs produced in situ *after adenoviral gene transfer has not been examined. The mouse IgG2b monoclonal Ab, 2C12.4, recognizes the* Yersinia pestis *type III secretion apparatus protein, LcrV (V antigen), and confers protection in mice when administered as an IgG intraperitoneally or after genetic immunization with engineered, replication-defective serotype 5 human adenovirus (Ad). The 2C12.4 Ab was expressed as a single-chain variable fragment (scFv) in* Escherichia coli *and was shown to display an equilibrium dissociation constant (K_D) = 3.5 nM by surface plasmon resonance analysis. The 2C12.4 scFv was subjected to random mutagenesis, and variants with increased affinity were isolated by flow cytometry using the anchored periplasmic expression bacterial display system. After a single round of mutagenesis, variants displaying up to 35-fold lower K_D values (H8, K_D = 100 pM) were isolated. The variable domains of the H8 scFv were used to replace those of the parental 2C12.4 IgG encoded in the Ad vector, AdαV, giving rise to AdαV.H8. The two adenoviral vectors resulted in similar titers of anti-V antigen Abs 3 days after immunization, with 10^9, 10^{10} or 10^{11} particle units (pu). After intranasal challenge with 363 LD_{50} (lethal dose, 50%) of* Y. pestis *CO92, 54% of the mice immunized with 10^{10} pu of AdαV.H8 survived through the 14 day end point compared with only 15% survivors for the group immunized with AdαV expressing the lower-affinity 2C12.4 ($P < 0.04$; AdαV versus AdαV.H8). These results indicate that affinity maturation of a neutralizing Ab delivered by genetic transfer may confer increased protection not only for* Y. pestis *challenge but also possibly for other pathogens.*
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Introduction

Yersinia pestis is the etiological agent of plague and has been responsible for pandemic outbreaks occurring throughout the course of history[1]. Although advances in our current living conditions, public health practices and antibiotic therapies make future pandemics unlikely, outbreaks of plague resulting from biological warfare are a real threat. The features of *Y. pestis* that make it an attractive option for use as a biological weapon include availability of the organism, capacity for aerosol dissemination, potential for spread of secondary cases and the high fatality rate of the pneumonic form of plague. In endemic regions of the world, the bacterium survives by causing chronic disease in animal reservoirs. It is spread among these animals and occasionally to humans predominantly through a flea vector, such as *Xenopsylla cheopis*.[2,3] Without prompt antibiotic therapy, ~50% of bubonic plague infections are fatal and can progress to the more dangerous pneumonic plague.[2] Respiratory droplets from a pneumonic plague-infected individual promote rapid spread through a susceptible population. Symptoms develop in 1–6 days after infection and the disease progresses rapidly from a flu-like illness to severe pneumonia with cough, chest pain and bloody sputum. To be effective, antibiotic therapy must be administered early. If treatment is delayed more than 24 h following the onset of symptoms, the fatality rate is high.[4] In addition, the presence of antibiotic-resistant strains of *Y. pestis* renders antibiotic therapy unreliable. For these reasons, *Y. pestis* is a likely agent to be used as a biological weapon as aerosolized bacteria can confer widespread pneumonic plague.[2]

Of the 11 *Yersinia* species, only *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* are human pathogens. *Y. pestis* is

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a gram-negative, nonmotile, non-spore-forming bacterium that replicates intracellularly during the early stages of infection and grows predominantly extracellularly at later stages of the infectious cycle.[2] At present, no plague vaccine has been approved for use in the United States. Passive immunization with antibodies (Abs) specific for the LcrV protein (V antigen) is an attractive alternative to vaccines, and has been shown to be effective against lethal challenges with *Y. pestis*.[1,5–7] The V antigen has a central role in plague pathogenesis. It activates the type III secretion system and thus mediates translocation of effector proteins (Yops) into host macrophages. It is also released from the bacteria and has immunosuppressive functions manifested by increasing levels of the anti-inflammatory cytokine interleukin-10 and decreasing levels of tumor necrosis factor-α (TNF-α).[2] A recently developed anti-V antigen monoclonal antibody (mAb) 2C12.4 has been shown to confer protection against lethal challenge with intranasally administered *Y. pestis* in a dose-dependent manner.[1]

For several neutralizing Abs, the degree of protection against challenge with pathogen correlates with antigen-binding affinity.[8–11] For example, although mAbs and Ab fragments to the protective antigen (PA) of *Bacillus anthracis* with a equilibrium dissociation constant (K_D) = 11 nM fail to confer protection against challenge with the holotoxin or with intranasally administered spores, engineered Ab variants displaying 40- to 200-fold higher affinities were protective in different animal models.[8,12] Notably, protection appeared to be mediated by blocking the ability of PA to bind to its receptor with PEGylated Ab fragments exhibiting a $K_D = 35$ pM, but lacking an Fc domain, and hence incapable of engaging innate immunity mechanisms of pathogen clearance, were protective.[12] Engineering Abs with high affinity has been shown to improve protection against other protein toxins and viruses including Botulism, human immunodeficiency virus and human respiratory syncytial virus, and have increased efficacy when targeting inflammatory cytokines such as TNF-α.[8–11,13,14]

In this study, we evaluated whether adenovirus (Ad)-mediated delivery of an engineered 2C12.4 IgG, exhibiting markedly increased affinity directed toward the V antigen can improve protection against *Y. pestis* challenge in mice.[1] Improvement would be shown by conferring a higher level of protection through immunization with an equivalent dose of a recombinant Ad encoding the higher-affinity neutralizing Ab. However, the mutations needed to confer higher affinity could have unexpected effects on protein expression from the Ad-delivered IgG DNA or biodistribution of the IgG, thus making it difficult to predict how protection to pathogen challenge might be affected. A single-chain variable fragment (scFv) incorporating the variable heavy chain (V_H) and variable light chain (V_L) domains of the 2C12.4 IgG was constructed and subjected to affinity maturation by screening a library generated by random mutagenesis. The latter was performed using the *Escherichia coli* display technique, anchored periplasmic expression (APEx), coupled with fluorescent-activated cell sorting (FACS).[15] In APEx, the protein library is tethered to the periplasmic side of the inner membrane of the bacterium through fusion to the six N-terminal amino acids of the native lipoprotein NlpA. The resulting NlpA-scFv fusion protein is lipidated *in vivo* and thus becomes attached to the inner membrane. After permeabilization of the *E. coli* outer membrane by chemical and enzymatic means, the cells are labeled with fluorescent antigen and the most fluorescent cells are isolated by FACS. A single round of random mutagenesis and APEx screening resulted in the isolation of a clone, H8, displaying a $K_D = 100$ pM that has a 35-fold higher affinity than the parental 2C12.4 Ab. The H8 V_H and V_L domains were used to exchange with those of 2C12.4 IgG in the adenoviral vector AdαV, giving rise to AdαV.H8. We show that delivery of the two vectors *in vivo* resulted in approximately the same Ab serum titers, and that mice immunized with 10^{10} particle units (pu) of AdαV.H8 conferred a statistically significant increase in protection relative to the parental AdαV.

Results

Engineering high-affinity anti-V antigen Abs

Genes encoding the V_H and kappa light chain (V_K) domains of the *Y. pestis*-neutralizing mAb 2C12.4 were used to create scFv Ab. This scFv gene was cloned into pMoPac16 for soluble expression of the Ab fragment in the single-chain Ab (scAb) format.[16] The scAb format is comprised of an scFv in which the C-terminus of the V_K domain is fused to the human κ-constant domain, resulting in improved expression in *E. coli* without altering the Ab-binding affinity.[17] Lysates from cells expressing 2C12.4 scAb were confirmed to express full-length protein by western blotting and displayed binding activity, as determined by enzyme-linked immunosorbent assay (ELISA) using purified *Y. pestis* V antigen.

The gene encoding the scFv variant of 2C12.4 was subjected to one round of random mutagenesis by error-prone PCR. Amplified DNA was cloned into pFLAG-APEx and the ligation product was transformed into *E. coli* Jude-1 cells yielding $>2 \times 10^6$ independent transformants. DNA sequencing of 10 clones selected at random revealed an average of 1.75% nucleotide substitutions with a standard deviation of 0.75%. Cells were grown in liquid culture, protein expression was induced and 4 h later, cells were harvested and converted into spheroplasts. The spheroplasted cells were then labeled with anti-FLAG R-phycoerythrin (PE) and V antigen-BODIPY (dipyrromethene boron difluoride) to determine full-length scFv expression levels and antigen binding, respectively (Figure 1a). A total of 8×10^7 cells (40-fold library coverage) were subjected to high-throughput FACS, and the top 2% events in terms of PE and BODIPY fluorescence emissions were collected (Figure 1b). The collected spheroplasts were immediately resorted as above without additional labeling to maximize the isolation of Abs with slow dissociation rate constants toward V antigen.[15] The scFv genes in the spheroplasts collected during the resort were amplified by PCR, and the DNA was subcloned back into the pFLAG-APEx vector. After transformation, the cells were subjected to two additional rounds of sorting using increasingly stringent collection criteria, namely, a higher fluorescence threshold. After the third round of sorting, the number of events falling within the window used for the first round increased from 2 to 23% (Figure 1b).

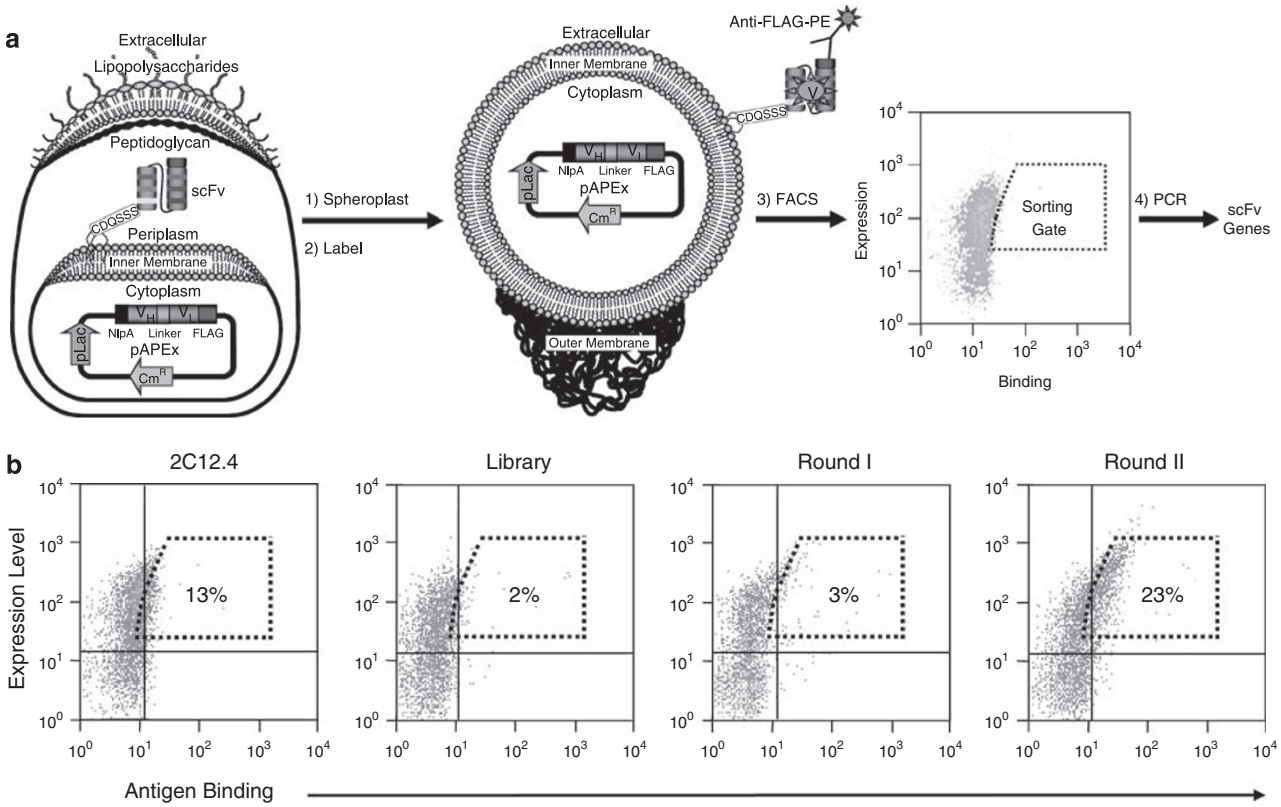

Figure 1 Affinity maturation of the anti-V antigen scFv 2C12.4 using anchored periplasmic expression (APEx). (**a**) Schematic diagram of APEx (**b**) FACS analysis of *E. coli* spheroplasts expressing 2C12.4 scFv, the library of random mutants and the populations recovered after two rounds of FACS. Spheroplasts were labeled with 500 nM V antigen-BODIPY and 5 μg ml^{-1} Anti-FLAG-PE. The dashed box represents the gate used to isolate cells by FACS from the original library. The percentage of the spheroplast in this gate for each population is indicated.

Table 1 Kinetic analysis of anti-V antigen antibody fragments

Antibody	k_{on} $(M^{-1}s^{-1})$[a]	k_{off} (s^{-1})[a]	K_D *(pM)*	*Affinity improvement*[b]	*Expression improvement*[c]
2C12.4	$9.2\ (0.005) \times 10^4$	$3.2\ (0.005) \times 10^{-4}$	3500	1 ×	1 ×
H8	$1.2\ (0.0009) \times 10^5$	$1.2\ (0.08) \times 10^{-5}$	100	35 ×	4 ×
E4	$1.0\ (0.0007) \times 10^5$	$1.8\ (0.07) \times 10^{-5}$	180	20 ×	5 ×
F4	$1.2\ (0.0007) \times 10^5$	$2.6\ (0.06) \times 10^{-5}$	220	16 ×	3 ×
G8	$9.8\ (0.007) \times 10^4$	$2.4\ (0.07) \times 10^{-5}$	250	14 ×	2 ×

[a]Global fit (and standard error for the fit) of triplicate injections at four different antibody concentrations.
[b]Affinity improvement relative to the parental antibody 2C12.4.
[c]Yield of purified scAb (mg l^{-1}; A_{600}) compared with 2C12.4.

The scFv gene pool from the third round of sorting was amplified by PCR and cloned into pMoPac16 for expression in the scAb format. Surface plasmon resonance (SPR) was used to rank order the dissociation rate constants of individual clones (Supplementary Figure 1). Of the 72 colonies tested, 41 (57%) expressed scAbs that displayed significant binding toward V antigen immobilized on the Biacore chip (GE Healthcare, Uppsala, Sweden). Of these 41 clones, four clones exhibited dissociation rate constants at least 10-fold slower than those expressing the parental Ab (Supplementary Figure 1e and f).

These four scAbs, as well as the parental 2C12.4 and the digoxin specific 26.10 scAbs, were expressed at the 500 ml scale in shake flasks, the periplasmic fraction was isolated by osmotic shock and monomeric scAb proteins were purified by immobilized metal affinity chromatography and size-exclusion fast protein liquid chromatography. Consistent with the isolation of the respective clones on the basis of increased PE fluorescence, the protein yields for all four scAbs was higher than that of the parental 2C12.4 scAb (Table 1). All Abs exhibited at least 10-fold lower K_D relative to the parental 2C12.4 scAb (Table 1), which is consistent with the measurements obtained using crude cell lysates (Supplementary Figure 1f). The highest-affinity clone, H8, exhibited an affinity of 100 pM that translates to a 35-fold improvement compared with the parental Ab 2C12.4. The improved affinity for all clones was almost exclusively the result of a decrease in the dissociation rate constant, whereas the association rate constant remained essentially unchanged. The four variants, H8,

Table 2 Sequence analysis of anti-V antigen antibody fragments

	Variable heavy chain[a]									*Variable light chain*[a]								
	FW1		*CDR1*	*FW2*		*CDR2*		*FW3*		*FW1*		*CDR1*		*FW2*	*CDR2*	*FW3*		*CDR3*
Clone	*3*	*11*	*32*	*45*	*49*	*51*	*65*	*67*	*82*	*1*	*5*	*24*	*32*	*39*	*53*	*72*	*84*	*97*
2C12.4	*T*	*I*	*S*	*L*	*L*	*I*	*S*	*L*	*I*	*D*	*T*	*R*	*Y*	*K*	*N*	*T*	*A*	*T*
H8					S	V				N	S	K	F					R
E4		V	P		S								F	R	S		T	
F4	A			P	S			V		N	S	K	F					R
G8					S		G		T	N	S	K	F			A		R

Abbreviations: CDR, complementary determining region; FW, framework.
[a]Amino acid substitutions based on Kabat numbering.

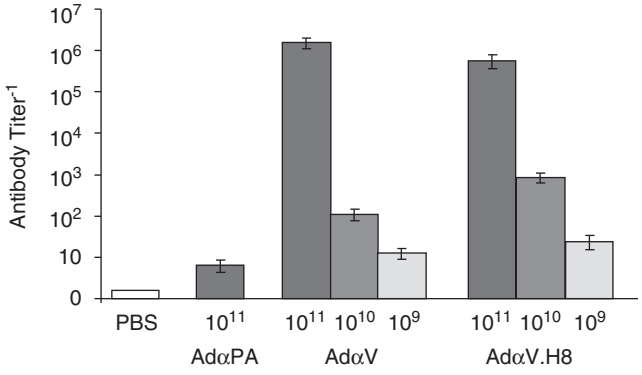

Figure 2 Anti-V antigen IgG titers in mice after immunization with Ad vectors. C57BL/6 mice ($n = 13$ per group for PBS, AdαPA, AdαV and AdαV.H8) were intravenously administered with the indicated Ad vectors or PBS. Serum anti-V antigen antibody titers were measured 3 days after immunization by ELISA. PBS, negative control; AdαPA, vector encoding an IgG specific for the *B. anthracis* PA; AdαV, vector encoding the 2C12.4 IgG; AdαV.H8, vector encoding the 2C12.4 IgG mutant H8.

E4, F4 and G8, contained either 7 or 9 amino acid mutations compared with the parental Ab (Table 2), of which two were common: L49S in framework 2 of the V_H, which is immediately before complementary determining region 2, and Y32F in complementary determining region 1 of the light chain.[18] Further, three of the variants contained an additional four common mutations (D1N, T5S, R24K and T97R) in the light chain.

Genetic immunization using IgG-encoding Ad vectors and challenge with Y. pestis

The V_H and V_K domains of the 2C12.4 mouse IgG2b Ab were replaced with those from the highest-affinity Ab variant, H8, and engineered into a replication-defective Ad serotype 5 E1–E3 gene transfer vector to create AdαV.H8.[1] Mice were immunized with varying doses of AdαV and AdαV.H8 (10^9–10^{11} pu) or, as controls, the highest dose (10^{11} pu) of AdαPA, an Ad vector encoding a mouse IgG mAb specific for the *B. anthracis* PA, or phosphate-buffered saline (PBS). Serum anti-V antigen Ab titers were measured by ELISA 3 days after immunizations (Figure 2). A similar dose-dependent response was observed for both AdαV and AdαV.H8. The titers for equivalent immunization levels were within 10-fold for both AdαV and AdαV.H8. Both AdαPA and PBS controls did not result in any measurable anti-V antigen Ab titers. At 3 days after immunization, the mice were challenged intranasally with 363 LD_{50} (lethal dose, 50%) of the fully virulent *Y. pestis* CO92 and survival was monitored for 2 weeks (Figure 3). All mice immunized with the highest dosage (10^{11} pu) of AdαV and AdαV.H8 survived (Figure 4). However, at a 10-fold lower dose (10^{10} pu), only 15% of the mice immunized with AdαV survived, whereas 54% of the mice immunized with this dose of AdαV.H8 survived ($P < 0.04$; AdαV versus AdαV.H8). In addition, 15% of the mice immunized with an additional 10-fold lower dose (10^9 pu) of AdαV.H8 survived, but none survived with the same dosage level of AdαV ($P < 0.1$; AdαV versus AdαV.H8).

Discussion

Passive immunization with engineered Abs displaying enhanced antigen affinity has been shown to increase the neutralization efficacy of a number of bacterial or viral pathogens and toxins.[8–11,13,14] However, to the best of our knowledge, neither the effect of antigen affinity for Abs expressed *in situ* after viral transfer of the Ab gene nor the relationship between V antigen affinity and protection to the potential biowarfare agent *Y. pestis*, have been examined. Here, we found that increasing the affinity of the *Y. pestis*-neutralizing Ab 2C12.4 from 3.5 nM to 100 pM (35-fold) could confer significant protection against a lethal challenge with *Y. pestis* at a 10-fold lower dose of a recombinant Ad encoding the neutralizing Ab.

The affinities of Abs generated by the natural immune system are constrained by the kinetics of *in vivo* selection.[19] Most high-affinity mouse mAbs display nanomolar affinities and this is the case for the anti-V antigen Ab 2C12.4, which displays a $K_D = 3.5$ nM by SPR. Combinatorial mutagenesis and library screening by phage display or other high-throughput techniques have been used for affinity maturation of the variable domains of mAbs.[8–11,13,14] In earlier studies, we used random mutagenesis and screening of *E. coli* displayed libraries by APEx to isolate picomolar affinity Abs to the *B. anthracis* PA, which in turn proved to have markedly improved neutralization potency *in vitro* and in various animal models.[8,12,20] Key advantages of this strategy are the ease with which libraries of random mutants can be constructed in *E. coli* and screened by FACS. FACS is

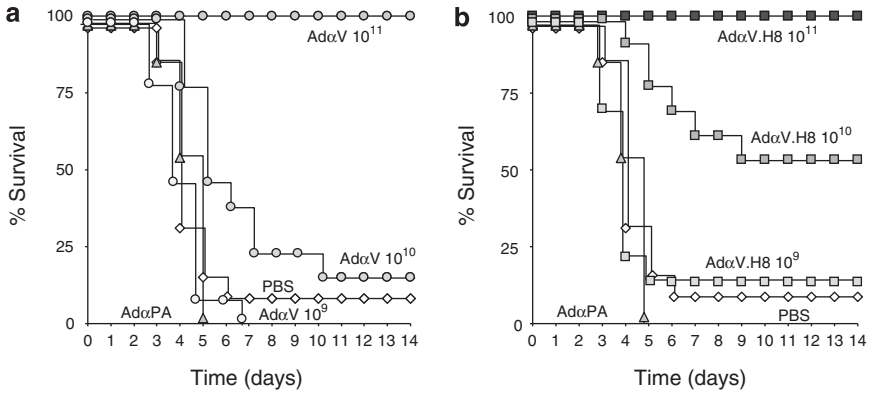

Figure 3 Survival of mice challenged with *Y. pestis* after prophylactic administration of Ad vectors. At 3 days after intravenous administration of Ad vectors or PBS, C57BL/6 mice ($n = 13$ per group for PBS, AdαPA, AdαV and AdαV.H8) were challenged with a lethal dose of *Y. pestis* intranasally. Survival of the mice was monitored for 14 days after challenge. (**a**) Immunization with AdαV compared with AdαPA and PBS. (**b**) Immunization with AdαV.H8 compared with AdαPA and PBS.

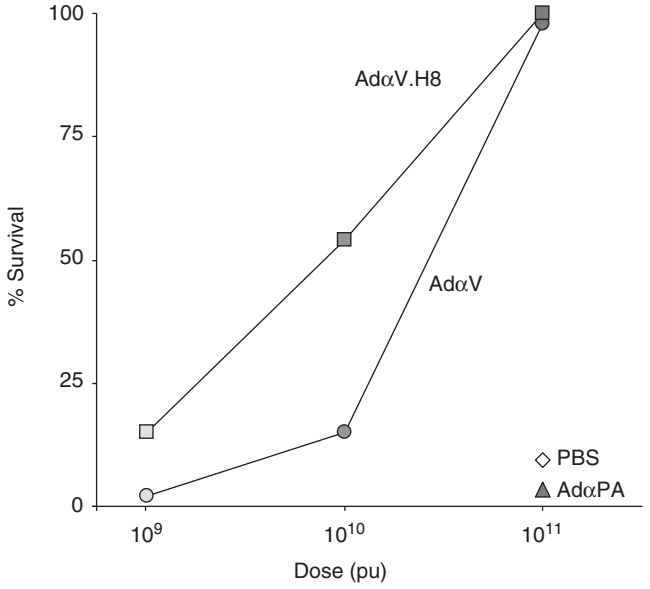

Figure 4 Net survival of mice challenged with *Y. pestis* after prophylactic administration of Ad vectors. Percentage of C57BL/6 mice ($n = 13$ per group for PBS, AdαPA, AdαV and AdαV.H8) surviving 14 days after intranasal challenge with a lethal dose of *Y. pestis*.

a high-throughput screening technique that enables real-time quantitative multi-parameter analysis on a cell by cell basis. Therefore, multiple properties can be monitored simultaneously to specifically isolate Abs with desired characteristics. In this work, Ab fragments were isolated from a library on the basis of both antigen affinity and expression level (Figure 1). Subsequently, clones isolated from the last round of FACS were rank-ordered by comparing the dissociation rate constants in crude lysates (Supplementary Figure 1). This approach significantly expedited the analysis of the clones obtained after screening, a process that often represents the rate-limiting step in Ab affinity maturation. Thus, a single round of mutagenesis followed by three rounds of FACS screening resulted in the isolation of Abs displaying affinities in the 100 pM range and also higher expression yields in *E. coli* (Figure 1b, Table 1). In addition, the conversion of these monovalent Ab fragments to full-length Abs can further enhance their potency owing to the avidity associated with these bivalent molecules, leading to an even higher apparent affinity.

Genetic delivery of full-length Abs *in vivo* has been carried out using Ad, adeno-associated virus and vaccinia virus vectors,[21–23] and it is an attractive alternative to the administration of Ab protein, which requires production, purification and formulation before injection into the patient.[22,24] The administration of Abs as injectable therapeutics is complicated by bioavailability issues and by the potential for increased immunogenicity resulting from large dosages required for therapeutic purposes and the ensuing aggregation issues.[25] A common side effect is infusion reactions with symptoms ranging from mild to life-threatening and typically occur near the time of administration.[26] After infusions, complicated pharmacokinetics result in a continual decrease in serum concentrations that effects the bioavailability and the pharmacodynamic properties of the therapeutic Ab.[25,27] However, genetic delivery can provide immediate and sustained serum levels for several months from a single treatment with both Ad and adeno-associated virus vectors encoding the same mAb.[28] This approach has been shown using a neutralizing Ab, and it offered almost immediate protection against anthrax toxin challenge *in vivo*, which was sustained for 6 months.[29] This approach circumvents many of the issues associated with conventional methods and has the additional benefits of increasing patient quality of life and protecting soldiers entering combat for extended periods.

Here, we show that the affinity of Abs produced *in situ* after adenoviral gene transfer correlates with protection. Although human Ad serotype 5 has been effectively used in a variety of animal models for genetic delivery, it has limited use because ~35–50% of humans have preexisting neutralizing Abs against it.[30] However, several nonhuman primate Ad vectors for which no natural immunity exists in humans have been described and can be deployed to circumvent the neutralization or clearance of the vector.[28] There are many pathogens to which an effective vaccine has yet to be produced, and

neutralizing Abs remain the only means for prophylaxis and therapy. The potency of neutralizing Abs can benefit from engineering their affinity, stability and neutralization potency (for instance, through mutations that increase Ab-dependent cell-mediated cytotoxicity[31]). The *in situ* synthesis of optimized neutralizing Abs by low-immunogenicity genetic delivery vectors could represent a promising alternative for the treatment of a number of infectious diseases.

Materials and methods

Bacterial strains and plasmid vectors

E. coli Jude-1 (*E. coli* DH10B F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ* ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL endA1 nupG* harboring an F′ from *E. coli* XL1-blue introduced by conjugation) was used for all Ab engineering experiments. Plasmid pMoPac16 was used for soluble scAb fragment expression and has been previously described.[16] Plasmids pAPEx1 was used for *E. coli* display of soluble scFv Abs.[15] pFLAG-APEx was constructed by replacing the polyhistidine and c-myc sequences in pAPEx1 through *Bam*HI and *Not*I restriction sites with the FLAG peptide epitope sequence amplified by PCR using primers TVB100 (5′-GTCGCT GCGGCCGCAGATTACAAAGACGACGATGACAAGTA GTGATATCGCAAGCTTGACC-3′) and TVB101 (5′-CAG CGAGGATCCGTGACGCAGTAGCGGTAAACGGC-3′).

Protein expression and purification

Recombinant V antigen was expressed and purified as previously described.[1,32] Monomeric scAbs were expressed and purified according to the published procedures.[15,16] Briefly, *E. coli* Jude-1 containing pMoPac16 derivatives encoding the desired scAb genes were streaked from frozen stocks onto agar plates containing Luria–Burtani Miller (LB) medium (Becton Dickinson Difco, Sparks, MD, USA) supplemented with 2% (wt/vol) glucose (2% glc) and 200 μg ml^{-1} ampicillin (Amp200) (20 h, 25 °C). Individual colonies were cultured in 2 ml of Terrific Broth (TB) medium (Becton Dickinson Difco)+2% glc+Amp200 (8 h, 250 r.p.m., 30 °C) then 1 ml was used to inoculate 500 ml of TB medium+2% glc+Amp200 (250 r.p.m., 30 °C). After overnight growth, cells were pelleted by centrifugation (15 min, 4400 × *g*, 4 °C) and resuspended in 500 ml of TB medium+Amp200+1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) to induce protein expression. After 4 h of incubation at 25 °C, the cells were pelleted by centrifugation (15 min, 4400 × *g*, 4 °C) and the periplasmic fraction from the cell pellet was isolated by osmotic shock according to the published procedures.[16] scAb proteins were purified from the shockate by immobilized metal affinity chromatography using Ni-NTA agarose on the basis of the manufacturer's protocol (Qiagen, Hilden, Germany), and monomeric scAbs were further isolated by size-exclusion fast protein liquid chromatography on a Superdex 200 column (GE Healthcare) using HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered saline with surfactant P20 (HBS-P) (GE Healthcare). Proteins were quantified on the basis of the absorbance (A_{280}) measured using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and the appropriate extinction coefficients calculated using Protein Calculator (http://www.scripps.edu/~cdputnam/protcalc.html, Putnam Lab at The Scripps Research Institute, La Jolla, CA, USA). Relative concentrations were verified and purity determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 4–20% gel (NuSep, Lawrenceville, GA, USA) stained with GelCode Blue (Thermo Fisher Scientific, Rockford, IL, USA). All scAbs used in this work were at least 90% pure.

Anti-V antigen Ab fragment cloning, expression and analysis

Genes encoding the V_H and the V_K domains of anti-V antigen 2C12.4 IgG were amplified by PCR from the previously described Ad vector plasmid DNA[1] using primers TVB102 (5′-GCAGCGAGGCCCAGCCGGCCATG GCGCAGGTAACTCTGAAAGAGTCTG-3′) and TVB103 (5′-AGAGCCGCCGCCGCCGCTACCACCACCACCAGAA CCACCACCACCTGAGGAGACTGTGAGAGTGGTG-3′) for V_H and TVB104 (5′-GGTGGTGGTGGTAGCGGCGGCG GCGGCTCTGGCGGCGGCGGCTCCGACATTGTGCTGAC ACAGTCG-3′) and TVB105 (5′-CGAATTCGGCCCCCGAG GCCCGTTTTACTTCCAGCTTGGTC-3′) for V_K.[1] The resulting PCR products were combined by overlap extension PCR and cloned into pMoPac16 through the non-compatible *Sfi*I restriction sites for soluble expression as a scAb. The resulting ligation product was transformed into *E. coli* Jude-1, and scAb expression was performed as previously described.[16,33] Briefly, individual colonies were cultured overnight in 2 ml of TB medium+2% glc+Amp200 at 30 °C. After overnight growth, cells were pelleted by centrifugation (10 min at 4600 × *g*, 4 °C) and resuspended in 2 ml of TB medium+Amp200+1 mM IPTG to induce protein expression. After 4 h incubation at 25 °C, 1 ml of cells were pelleted by centrifugation (10 min at 4600 × *g*, 4 °C), and the cell pellet was resuspended in 1 ml of lysis buffer that consisted of BugBuster HT Protein Extraction Reagent (Novagen, Madison, WI, USA) diluted in the ratio 1:4 in PBS.[34] The cells were incubated in lysis buffer while rotating on an inverter (30 min, 60 r.p.m., 25 °C) and then the insoluble fraction was removed by centrifugation (1 min, 10 000 × *g*, 4 °C).

The soluble fraction was analyzed for the presence of full-length scAb specific for the V antigen by western blot analysis and ELISA. Western blot analysis with anti-polyhistidine (anti-His) peroxidase (HRP) conjugate (Sigma-Aldrich) was performed as previously described.[35] ELISA was performed by coating Costar high-binding 96-well EIA/RIA plates (Corning, Corning, NY, USA) overnight at 4 °C with 50 μl of 5 μg ml^{-1} recombinant V antigen or bovine serum albumin in PBS. The plates were washed three times with PBS, followed by blocking with 400 μl PBS supplemented with 2% milk for 4 h at room temperature. Samples were diluted in the ratio 1:1 in PBS with 2% milk and incubated for 1 h at room temperature. The plates were washed three times with PBS and an additional three times with PBS containing 0.1% Tween-20. Immunocomplexes were detected using an anti-human κ-light chain (anti-HuCκ) polyclonal serum HRP conjugate (Sigma-Aldrich) applied at a 1:10 000 dilution and incubated for 30 min at room temperature. The plates were washed as described above and developed using the chromogenic HRP substrate TMB+ (Dako, Glostrup, Denmark), as

described by the manufacturer. The A_{450} was measured with a mircroplate reader (BioTek, Winooski, VT, USA).

Isolation of high-affinity variants of the 2C12.4 scFv

The anti-V antigen 2C12.4 scFv and the anti-digoxin 26.10 scFv were cloned into pAPEx1 and pFLAG-APEx through the non-compatible *Sfi*I restriction sites.[15] Purified recombinant V antigen was conjugated to BODIPY using 6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino) hexanoic acid succinimidyl ester (Invitrogen, Carlsbad, CA, USA) at a molar ratio of 1:5, as described by the manufacturer. Free BODIPY FL-X SE was removed using a NAP-10 gel filtration column, as described by the manufacturer (GE Healthcare). The extent of conjugation was determined on the basis of the ratio of A_{280} and A_{504} measured using a NanoDrop 1000 and the appropriate extinction coefficients and correction factors as recommended by the manufacturer (http://www.scripps.edu/~cdputnam/protcalc.html, Putnam Lab at The Scripps Research Institute; Invitrogen).

The anti-V antigen 2C12.4 scFv was subjected to random mutagenesis by error-prone PCR,[36] the amplified DNA was cloned into pFLAG-APEx through the non-compatible *Sfi*I restriction sites, and the ligation product was transformed into electrocompetent *E. coli* Jude-1.[15] Cells were cultured on agar plates containing LB medium+2% glc+30 μg ml^{-1} chloramphenicol (Cm30) (22 h, 25 °C). Cells were transferred to LB medium+2% glc+15% glycerol (v/v), to $A_{600} \sim 10$ and then stored at −80 °C. Frozen cell stocks were thawed on ice and subcultured to $A_{600} = 0.1$ in TB medium+2% glc+Cm30 grown up at 37 °C, to $A_{600} \sim 0.8$–1.2, cooled for 30 min to 25 °C and protein expression was induced with the addition of 1 mM IPTG. After 4 h induction at 25 °C, 1 ml of cells at $A_{600} = 5.0$ were converted to spheroplasts using Tris/sucrose/EDTA/lysozyme as previously described.[15,34] The spheroplasted cells were first labeled with 5 μg ml^{-1} PhycoLink anti-FLAG R-phycoerythrin (Prozyme, San Leandro, CA, USA) in PBS supplemented with 1% bovine serum albumin (PBSB) (30 min, 150 r.p.m., 25 °C). The spheroplasts were washed with PBSB and incubated with 500 nM V antigen conjugated to BODIPY (V antigen-BODIPY) in PBSB (1 h, 150 r.p.m., 25 °C). Labeled spheroplasts were washed with PBSB and analyzed on a FACSAria (Becton Dickinson Biosciences, San Jose, CA, USA) droplet deflection flow cytometer by exciting with a 488-nm laser and measuring the fluorescence emission spectrum of BODIPY and PE with 530/40 and 570/40 band-pass filters, respectively. Fluorescence compensation was performed using the APEx controls as previously described.[34] The library population was gated to avoid aggregates, as determined by the forward scatter and side scatter parameters, and the brightest 1–2% of the population based on both the BODIPY and PE emission spectrums was collected.[15,34] The collected spheroplasts were immediately resorted, and the scFv genes in the resort solution were amplified by PCR using primers BRH06 (5′-GCGGATAACAATT TCACACAGG-3′) and AHX89 (5′-CGCAGTAGCGGT AAACGGC-3′).[15] The amplified DNA was cloned into pFLAG-APEx through the non-compatible *Sfi*I restriction sites, and the ligation mixture was transformed into electrocompetent *E. coli* Jude-1. At least 10-fold excess of colonies relative to the number of events in the resort were obtained. Colonies were scraped from the agar plates into liquid media as explained above and subjected to an additional two rounds of sorting, exactly as described above.

The scFv genes from the third round of sorting were subcloned into pMoPac16, and the ligation product was transformed into *E. coli* Jude-1 for expression of soluble scAb similar to above.[15] In addition, *E. coli* Jude-1 containing pMoPac16 encoding 2C12.4 scAb was streaked from a frozen stock onto an agar plate containing LB medium+2% glc+Amp200. Further, the anti-digoxin 26.10 scFv was cloned into pMoPac16 through the non-compatible *Sfi*I restriction sites, and the ligation product was transformed into *E. coli* Jude-1. A total of 72 individual colonies from round III, 8 colonies of 2C12.4 scAb and 4 colonies of 26.10 scAb were picked and cultured overnight in sterile Costar round-bottomed 96-well microtiter plates (Corning) containing 200 μl of TB medium+2% glc+Amp200 on a microtiter plate shaker. After overnight growth (150 r.p.m., 30 °C), 4 μl aliquots were transferred to a master microtiter plate well containing 156 μl of TB medium+2% glc+Amp200, cultured on a plate shaker (8 h, 150 r.p.m., 30 °C) and stored at 4 °C for up to 2 weeks. The remaining cells were pelleted by centrifugation (10 min at 4600 × *g*, 4 °C) and resuspended in 200 μl of TB medium+Amp200+1 mM IPTG to induce protein expression. After 4 h induction at 25 °C, cells were pelleted by centrifugation (10 min at 4600 × *g*, 4 °C), the cell pellet was resuspended in 1 ml of the lysis buffer described above for 30 min at 25 °C and the insoluble fraction was removed by centrifugation (20 min at 4600 × *g*, 4 °C). The soluble fraction was further clarified using 96-well MultiScreen HTS NA clearing filter plates (Millipore, Billerica, MA, USA), as described by the manufacturer. Filtered lysates were transferred to Costar non-binding 96-well plates (Corning) and covered with microplate foil (GE Healthcare). Relative expression levels of scAb proteins in filtered lysate were obtained using a Minifold I Dot Blot System (Whatman, GE Healthcare) by transferring 10 μl of filtered lysate diluted with 90 μl of PBS to a nitrocellulose membrane following the manufacturer guidelines. Immunological detection of the polyhistidine-tagged scAb proteins immobilized on the nitrocellulose membrane was performed with anti-His HRP conjugate, as previously described.[35] The scAbs in filtered lysate were analyzed by SPR, as described below.

Surface plasmon resonance

Antigen-binding kinetics of scAb proteins in filtered lysate were analyzed by SPR using a Biacore 3000 (GE Healthcare). Approximately 200 response units of V antigen in 10 mM sodium acetate (pH 5.0) were coupled to a CM5 chip (GE Healthcare) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxy succinimide chemistry. Bovine serum albumin was similarly coupled to the chip and used for in-line subtraction. Kinetic analysis was performed in HBS-P (GE Healthcare) at a flow rate of 25 μl min^{-1} at 25 °C unless indicated. Samples were injected for 3 min and dissociation was monitored for 5 min. The surface was regenerated with two 12 s injections of 50 mM phosphoric acid. The antigen-binding kinetics of purified monomeric scAb were determined in HBS-P at a flow rate of 50 μl min^{-1}. Samples were injected for 2 min and dissociation was monitored for 10 min. The surface was

regenerated with two 12-s injections of 50 mM phosphoric acid. Four two-fold dilutions of each Ab beginning at 160 nM were performed in triplicate, double referenced with a blank and globally fit to a 1:1 Langmuir binding model for the calculation of both k_{on} and k_{off} using BIAevaluation software (GE Healthcare).[37]

Ad vectors

The AdαV is a replication-defective human Ad serotype 5, $E1^-E3^-$ gene transfer vector constructed to direct the expression of the heavy and light chains of the anti-V antigen mAb 2C12.4 from a single promoter.[1] The expression cassette contains (5′ to 3′) the cytomegalovirus immediate early promoter/enhancer, the Ab IgG2b heavy chain coding sequence, a four amino acid furin cleavage site, the 24 amino acid self-cleaving 2A peptide, the κ-light chain coding sequence and the simian virus 40 polyadenylation signal. AdαV.H8 was constructed by assembling the genes encoding the V_H and V_K domains of the high-affinity anti-V antigen 2C12.4 variant, H8, by overlap PCR using the AdαV IgG2b expression cassette as a template and the resulting fragment was used to replace the 2C12.4 coding sequence in AdαV. AdαPA is a similarly constructed gene transfer vector encoding an unrelated Ab (14B7–1H) against *B. anthracis* PA and was used as a negative control.[29] AdαV, AdαV.H8 and AdαPA were produced in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA, USA) and purified by centrifugation twice through a CsCl gradient, as described.[38] The titer of each recombinant Ad preparation was determined spectrophotometrically and expressed as pu, as described.[39]

Assessment of AdαV.H8 in vivo

Male C57BL/6 mice ($n=13$ per group) (The Jackson Laboratory, Bar Harbor, ME, USA) were housed under specific-pathogen-free conditions and used at 6–8 weeks of age. Mice were administered AdαV or AdαV.H8 (10^9, 10^{10} or 10^{11} pu) through the intravenous route. Naïve mice or mice injected with AdαPA (10^{11} pu) were used as negative controls. Ad vectors were diluted with saline to the specified dose. Serum was collected through the tail vein 3 days after vector administration, centrifuged at $8000\times g$ for 20 min and stored at −20 °C. Anti-V antigen serum titers were assessed by ELISA using flat-bottomed 96-well EIA/RIA plates (Corning) coated with 100 µl of 5 µg ml^{-1} recombinant V antigen in 0.05 M carbonate buffer (pH 7.4) overnight at 4 °C. The plates were washed three times with PBS and blocked with 200 µl PBS supplemented with 5% milk for 1 h at 23 °C. Serial serum dilutions were added to each well and incubated for 1 h at 23 °C. The plates were washed three times with PBS containing 0.05% Tween-20. Immunocomplexes were detected using a sheep anti-mouse IgG HRP conjugate (Sigma-Aldrich) applied at a 1:10 000 dilution in PBS supplemented with 1% milk incubated for 1 h at 23 °C. The plates were washed four times with PBS containing 0.05% Tween-20 and once with PBS, developed using 100 µl of peroxidase substrate (Bio-Rad, Hercules, CA, USA) incubated for 15 min at 23 °C and the reaction quenched by the addition of 100 µl of 2% oxalic acid. The A_{415} was measured using a microplate reader (Bio-Rad). Ab titers were calculated based on log(optical density)−log(dilution) normalized with purified anti-V antigen Abs to account for affinity differences. Mice challenge studies were conducted at The Public Health Research Institute (PHRI) at the International Center for Public Health (Newark, NJ, USA) under biosafety level 3 conditions. *Y. pestis* CO92 was grown aerobically in heart infusion broth (Becton Dickinson Difco) at 30 °C and diluted in saline solution for a challenge dose of 2×10^4 colony-forming units, which corresponds to 363 LD_{50}. A total of 25 µl of bacterial suspension was used for intranasal infection of mice; bacterial dose was controlled by plating on *Yersinia* selective agar (Oxoid, Hampshire, UK) and counting colonies for colony-forming unit determination. Survival was monitored daily for 14 days.

Statistical analyses

The data are presented as mean ± standard error of the mean. Statistical analyses were performed using the non-paired two-tailed Student's *t*-test, assuming equal variance. Survival evaluation was carried out using Kaplan–Meier analysis. Statistical significance was determined at $P<0.1$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)