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**Chopra et al.**

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(54) **METHODS FOR TREATING PLAGUE**

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**Related U.S. Patent Documents**

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**A61K 39/02** (2006.01)  
**A61K 39/00** (2006.01)

(52) **U.S. Cl.**  
 CPC ..... **A61K 39/0291** (2013.01); **A61K 39/025** (2013.01); **A61K 2039/53** (2013.01); **A61K 2039/543** (2013.01); **A61K 2039/545** (2013.01); **A61K 2039/70** (2013.01); **C12N 2710/10043** (2013.01); **Y02A 50/30** (2018.01)

(58) **Field of Classification Search**  
 CPC ..... **A61K 39/0291**; **A61K 39/025**  
 See application file for complete search history.

(56) **References Cited**

**U.S. PATENT DOCUMENTS**

6,261,807 B1 7/2001 Crouzet et al.  
 9,410,129 B2 8/2016 Ranki et al.  
 2010/0209451 A1\* 8/2010 Clarke ..... A61P 31/12  
 435/320.1

**FOREIGN PATENT DOCUMENTS**

WO 2008/045601 A2 4/2008

**OTHER PUBLICATIONS**

Heath et al., *Vaccine* 16(No. 11/12):1131-37, 1998.\*  
 Matson et al., *BMC Microbiol.*, 5:38, pp. 1-10, 2005.\*  
 Agar et al., "Deletion of Braun lipoprotein gene (lpp) and curing of plasmid pPCPI dramatically alter the virulence of *Yersinia pestis* CO92 in a mouse model of pneumonic plague," *Microbiology*, 2009; 155:3247-3259.

Alvarez et al., Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice, *Vaccine*, 2006; 24(44):2447-2490.

Bessis et al., "Immune responses to gene therapy vectors: influence on vector function and effector mechanisms," *Gene Ther*, 2004; 11(Suppl 1):S10-17.

Guyton, "Measurement of the respiratory volumes of laboratory animals," *Am J Physiol*, 1947, 150(1):70-77.

Mizel et al., "Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates," *Clin Vaccine Immunol*, 2009; 16(1):21-28.

Pitt, "Nonhuman Primates as a Model for Pneumonic Plague," Proceedings of the Animal Models and Correlates of Protection for Plague Vaccines Workshop, Food and Drug Administration, National Institute of Allergy and Infectious Disease, and Department of Health and Human Services, Oct. 13-14, 2004; 300 pages.

Powell et al., "Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague," *Biotechnol Prog*, 2005; 21(5): 1490-1510.

Quenee et al., "*Yersinia pestis* *cafI* variants and the limits of plague vaccine protection," *Infect Immun.*, 2008; 76:2025-2036.

Quenee et al., "Prevention of pneumonic plague in mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines," *Vaccine*, 2011, 29:6572-6583.

Rothe, Eric and Chopra, Ashok K. "Evaluation and Production of a Multivalent Adenoviral Plague Vaccine," Grant Abstract, Grant No. AI071634 [online]. National Institute of Allergy and Infectious Diseases, National Institutes of Health, project dates Jul. 1, 2006 to Jun. 30, 2015 [retrieved on May 10, 2018]. Retrieved from the Internet: <URL:http://grantome.com/grant/NIH/R44-AI071634-05>; 3 pgs.

Tao et al., "Mutated and bacteriophage T4 nanoparticle arrayed F1-V immunogens from *Yersinia pestis* as next generation plague vaccines," *PLoS Pathog.*, 2013;9:e1003495.

Welkos et al., "Modified caspase-3 assay indicates correlation of caspase-3 activity with immunity of nonhuman primates to *Yersinia pestis* infection," *Clin Vaccine Immunol*, 15(7):1134-1137.

"African Green monkey (*Chlorocebus aethiops*) animal model development to evaluate treatment of pneumonic plague," Food and Drug Administration (FDA) Anti-Infective Drugs Advisory Committee Meeting, Apr. 3, 2012, Silver Spring, MD; 68 pages.

(Continued)

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(57) **ABSTRACT**

Provided herein are methods for using compositions that include a fusion protein having a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain. In one embodiment the composition is used to confer immunity to plague, such as pneumonic plague, caused by *Yersinia pestis*. In one embodiment, the composition is administered to a mucosal surface, such as by an intranasal route. In one embodiment, the administration to a mucosal surface includes a vector that has a polynucleotide encoding a fusion protein, where the fusion protein includes a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain. The administration is followed by a second administration by a different route, such as an intramuscular route. The second administration includes a fusion protein having the same three domains, and in one embodiment the fusion protein is the same one administered to a mucosal surface.

**19 Claims, 31 Drawing Sheets**

**Specification includes a Sequence Listing.**



(56)

## References Cited

## OTHER PUBLICATIONS

- Agar et al., "Characterization of a mouse model of plague after aerosolization of *Yersinia pestis* CO92," *Microbiology*, Jul. 2008; 154(Pt. 7):1939-1948.
- Agar et al., "Characterization of the rat pneumonic plague model: infection kinetics following aerosolization of *Yersinia pestis* CO92," *Microbes Infect*, 2009; 11:205-214.
- Andrews et al., Protective efficacy of recombinant *Yersinia* outer proteins against bubonic plague caused by encapsulated and nonencapsulated *Yersinia pestis*, *Infect Immun*, 1999; 67(3):1533-1537.
- Anisimov et al., "Amino acid and structural variability of *Yersinia pestis* LcrV protein," *Infect Genet Evol*, 2010; 10(1):137-145.
- Baker et al., "Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*," *J Immunol*, 1952; 68(2):134-145.
- Barouch et al., "Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity," *J Immunol*, 2004; 172(10):6290-6297.
- Barouch et al., "Adenovirus vector-based vaccines for human immunodeficiency virus type 1," *Hum Gene Ther*, 2005; 16(2):149-156.
- Benner et al., "Immune response to *Yersinia* outer proteins and other *Yersinia pestis* antigens after experimental plague infection in mice," *Infect Immun*, 1999; 67(4):1922-1928.
- Bowie et al., "Deciphering the message in protein sequences: tolerance to amino acid substitutions," *Science*, 1990, 247(4948):1306-1310.
- Boyer et al., "Adenovirus-based genetic vaccines for biodefense," *Hum Gene Ther*, 2005; 16(2):157-168.
- Byvalov et al., "Effectiveness of revaccinating hamadryas baboons with NISS live dried plague vaccine and fraction I of the plague microbe," *ZH Mikrobiol Epidermiol Immunobiol*, 1984, 4:74-76. In Russian, with English abstract.
- Cathelyn et al., "RovA, a global regulator of *Yersinia pestis*, specifically required for bubonic plague," *PNAS USA*, 2006; 103(36):13514-13519.
- Chen et al., "Susceptibility of the langur monkey (*Semnopithecus entellus*) to experimental plague: pathology and immunity," *J Infect Dis*, 1965; 115(5):456-464.
- Chen et al., "Immunity in plague: protection induced in *Cercopithecus aethiops* by oral administration of live, attenuated *Yersinia pestis*," *J Infect Dis*, 1976; 133(3):302-309.
- Chen et al., "Fusion protein linkers: property, design and functionality," *Adv Drug Deliv Rev*, 2013; 65(10):1357-1369.
- Chiuchiolo et al., Protective immunity against respiratory tract challenge with *Yersinia pestis* in mice immunized with an adenovirus-based vaccine vector expressing V antigen, *J Infect Dis*, 2006; 194(9):1249-1257.
- Cornelis, "*Yersinia type III* secretion: send in the effectors," *J Cell Biol.*, 2002; 158:401-408.
- Cornelius et al., "Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague," *Infect Immun*, 2008, 76(12):5588-5597.
- Croyle et al., "Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice," *PLoS One*, 2008; 3(10):e3548.
- Cui et al., "Genetic variations of live attenuated plague vaccine strains (*Yersinia pestis* EV76 lineage) during laboratory passages in different countries," *Infect Genet Evol.*, 2014; 26:172-179.
- Danthinne et al., "Production of first generation adenovirus vectors: a review," *Gene Ther*, 2000; 7(20):1707-1714.
- Do et al., "Induction of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor," *Vaccine*, 2012; 30(45):6359-6367.
- Doll et al., "Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona," *Am J Trop Med Hyg*, 1994; 51(1):109-114.
- Fellows et al., "Characterization of a *Cynomolgus Macaque* Model of Pneumonic Plague for Evaluation of Vaccine Efficacy," *Clin Vaccine Immunol.*, 2015; 22:1070-1078.
- Finegold et al., "Studies on the pathogenesis of plague. Blood coagulation and tissue responses of *Macaca mulatta* following exposure to aerosols of *Pasteurella pestis*," *Am J Pathol*, 1968; 53(1):99-114.
- Goujon et al., "A new bioinformatics analysis tools framework at EMBL-EBI," *Nucleic Acids Res*, 2010; 38:W695-9.
- Hackett et al., Antivector and antitransgene host responses in gene therapy, *Curr Opin Mol Ther*, 2000, 2(4):376-382.
- Hallett et al., "Pathogenicity and immunogenic efficacy of a live attenuated plague vaccine in vervet monkeys," *Infect Immun.*, 1973; 8:876-881.
- Hu et al., "Crystal structure of TET2-DNA complex: insight into TET-mediated 5mC oxidation," *Cell*, 2013; 155(7):1545-1555.
- Jones et al., "Prevention of influenza virus shedding and protection from lethal H1N1 challenge using a consensus 2009 H1N1 HA and NA adenovirus vector vaccine," *Vaccine*, 2011; 29(40):7020-7026.
- Koster et al., "Milestones in progression of primary pneumonic plague in cynomolgus macaques," *Infect Immun*, 2010; 78(7):2946-2955.
- Larkin et al., "Clustal W and Clustal X version 2.0," *Bioinformatics*, 2007; 23(21):2947-2948.
- Lathem et al., "Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity," *Proc Natl Acad Sci U S A*, 2005; 102:17786-17791.
- Lathem et al., "A plasminogen-activating protease specifically controls the development of primary pneumonic plague," *Science*, 2007; 315:509-513.
- Lin et al., "IL-17 contributes to cell-mediated defense against pulmonary *Yersinia pestis* infection," *J Immunol.*, 2011; 186:1675-1684.
- Matson et al., "Immunization of mice with YscF provides protection from *Yersinia pestis* infections," *BMC Microbiol*, 2005; 5:38.
- Mett et al., "A plant-produced plague vaccine candidate confers protection to monkeys," *Vaccine*, 2007; 25(16):3014-3017.
- Molinier-Frenkel et al., "Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response," *J Virol*, 2002, 76(1):127-135.
- Motin et al., the difference in the IcrV sequences between *Y. pestis* and *Y. pseudotuberculosis* and its application for characterization of *Y. pseudotuberculosis* strains, *Microb Pathog*, 1992; 12(3):165-175.
- Nanda et al., Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys, *J Virol*, 2005; 79(22):14161-14168.
- Oyston et al., "An *aroA* mutant of *Yersinia pestis* is attenuated in guinea-pigs, but virulent in mice," *Microbiology*, 1996, 142(Pt 7):1847-1853.
- Oyston et al., "The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*," *Infect Immun*, 2000; 68(6):3419-3425.
- Patel et al., "Mucosal delivery of adenovirus-based vaccine protects against Ebola virus infection in mice," *J Infect Dis*, 2007; 196(Suppl 2): S413-20.
- Perry et al., "*Yersinia pestis*—etiologic agent of plague," *Clin Microbiol Rev.*, 1997; 10:35-66.
- Quenee et al., "Plague in Guinea pigs and its prevention by subunit vaccines," *Am J Pathol.*, 2011; 178:1689-1700.
- Ransom et al., "Chronic pneumonic plague in *Macaca mulatta*," *Am J Trop Med Hyg*, 1954, 3(6):1040-1054.
- Rosenzweig et al., "Progress on plague vaccine development," *Appl Microbiol Biotechnol.*, 2011; 91:265-286.
- Russell et al., "A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model," *Vaccine*, 1995; 13:1551-1556.
- Russell, "Adenoviruses: update on structure and function," *J Gen Virol*, 2009; 90(Pt 1):1-20.
- Sambrook et al. (Eds.), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY; 1989. Title page, publisher's page, and table of contents; 30 pgs.



(56)

## References Cited

## OTHER PUBLICATIONS

- Sha et al., "Braun lipoprotein (Lpp) contributes to virulence of yersiniae: potential role of Lpp in inducing bubonic and pneumonic plague," *Infect Immun.*, 2008; 76:1390-1409.
- Sha et al., "Characterization of an F1 deletion mutant of *Yersinia pestis*CO92, pathogenic role of F1 antigen in bubonic and pneumonic plague, and evaluation of sensitivity and specificity of F1 antigen capture-based dipsticks," *J Clin Microbiol.*, 2011; 49:1708-1715.
- Sha et al., "Deletion of the Braun lipoprotein-encoding gene and altering the function of lipopolysaccharide attenuate the plague bacterium," *Infect Immun.*, 2013; 81:815-828.
- Sha et al., "A non-invasive in vivo imaging system to study dissemination of bioluminescent *Yersinia pestis*CO92 in a mouse model of pneumonic plague," *Microb Pathog.*, 2013; 55:39-50.
- Sha et al., "a replication-defective human type 5 adenovirus-based trivalent vaccine confers complete protection against plague in mice and nonhuman primates," *Clinical and Vaccine Immunology*, 2016; 23(7):586-600.
- Sievers et al., "Fast scalable generation of high-quality protein multiple sequence alignments using Clustal Omega," *Mol Syst Biol*, 2011; 7:539.
- Smiley, "Current challenges in the development of vaccines for pneumonic plague," *Expert Rev Vaccines*, 2008; 7:209-221.
- Smiley, "Immune defense against pneumonic plague," *Immunol Rev.*, 2008; 225:256-271.
- Song et al., "Cytotoxic T lymphocyte responses to proteins encoded by heterologous transgenes transferred in vivo by adenoviral vectors," *Hum Gene Ther*, 1997; 8(10):1207-1217.
- Stacy et al., "An age-old paradigm challenged: old baboons generate vigorous humoral immune responses to LcrV, a plague antigen," *J Immunol*, 2008; 181(1):109-115.
- Suarez et al., "Role of Hcp, a type 6 secretion system effector, of *Aeromonas hydrophila* modulating activation of host immune cells," *Microbiology*, 2010; 156:3678-3688.
- Sun et al "Developing live vaccines against plague," *J Infect Dev Ctries*, 2011; 5:614-627.
- Swietnicki et al., "*Yersinia pestis* Yop secretion protein F: purification, characterization, and protective efficacy against bubonic plague," *Protein Expr Purif*, 2005; 42(1):166-172.
- Tatsis et al., "Adenoviruses as vaccine vectors," *Mol Ther*, 2004; 10(4):616-629.
- Tatusova et al., "BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences," *FEMS Microbiol Lett.*, May 1999; 174(2):247-250.
- Tiner et al., "Combinational Deletion of Three Membrane Protein-Encoding Genes Highly Attenuates *Yersinia pestis* while Retaining Immunogenicity in a Mouse Model of Pneumonic Plague," *Infect Immun.*, 2015; 83:1318-1338.
- Tiner et al., "Intramuscular immunization of mice with a live-attenuated triple mutant of *Yersinia pestis*CO92 induces robust humoral and cell-mediated immunity to completely protect animals against pneumonic plague," *Clin Vaccine Immunol*, Dec. 2015; 22(12):1255-1268.
- Titball et al., "Vaccination against bubonic and pneumonic plague," *Vaccine*, 2001; 19(30):4175-4184.
- Titball et al., "*Yersinia pestis*(plague) vaccines," *Expert Opin Biol Ther*, 2004; 4(6):965-973.
- Tripathy et al., "Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors," *Nat Med*, 1996; 2(5):545-550.
- Van Andel et al., "Clinical and pathologic features of cynomolgus macaques (*Macaca fascicularis*) infected with aerosolized *Yersinia pestis*," *Comp Med*, 2008; 58(1):68-75.
- van Lier et al., "Deletion of Braun lipoprotein and plasminogen-activating protease-encoding genes attenuates *Yersinia pestis* mouse models of bubonic and pneumonic plague," *Infect Immun.*, 2014; 82:2485-2503.
- van Lier et al., "Further characterization of a highly attenuated *Yersinia pestis*CO92 mutant deleted for the genes encoding braun lipoprotein and plasminogen activator protease in murine alveolar and primary human macrophages," *Microb Pathog*, 2015, 80:27-38.
- Warren et al., "Cynomolgus macaque model for pneumonic plague," *Microb Pathog*, 2011; 50(1):12-22.
- Williams et al., "Investigation into the role of the serine protease HtrA in *Yersinia pestis* pathogenesis," 2000; 186(2):281-286.
- Williamson et al., "Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates," *Microb Pathog*, 2007; 42(1):11-21.
- Williamson et al., "Recombinant (F1+V) vaccine protects cynomolgus macaques against pneumonic plague," *Vaccine*, 2011; 29:4771-4777.
- Wilson, "Adenoviruses as gene-delivery vehicles," *N. Engl J Med*, 1996; 334(18):1185-1187.
- Xu et al., "An adenoviral vector-based mucosal vaccine is effective in protection against botulism," *Gene Ther*, 2009; 16(3):367-375.
- Yu et al., "Single intranasal immunization with recombinant adenovirus-based vaccine induces protective immunity against respiratory syncytial virus infection," *J Virol*, 2008; 82(5):2350-2357.
- Zhang et al., "An adenovirus-vectored nasal vaccine confers rapid and sustained protection against anthrax in a single-dose regimen," *Clin Vaccine Immunol*, 2013; 20(1):1-8.

\* cited by examiner

FIG. 1

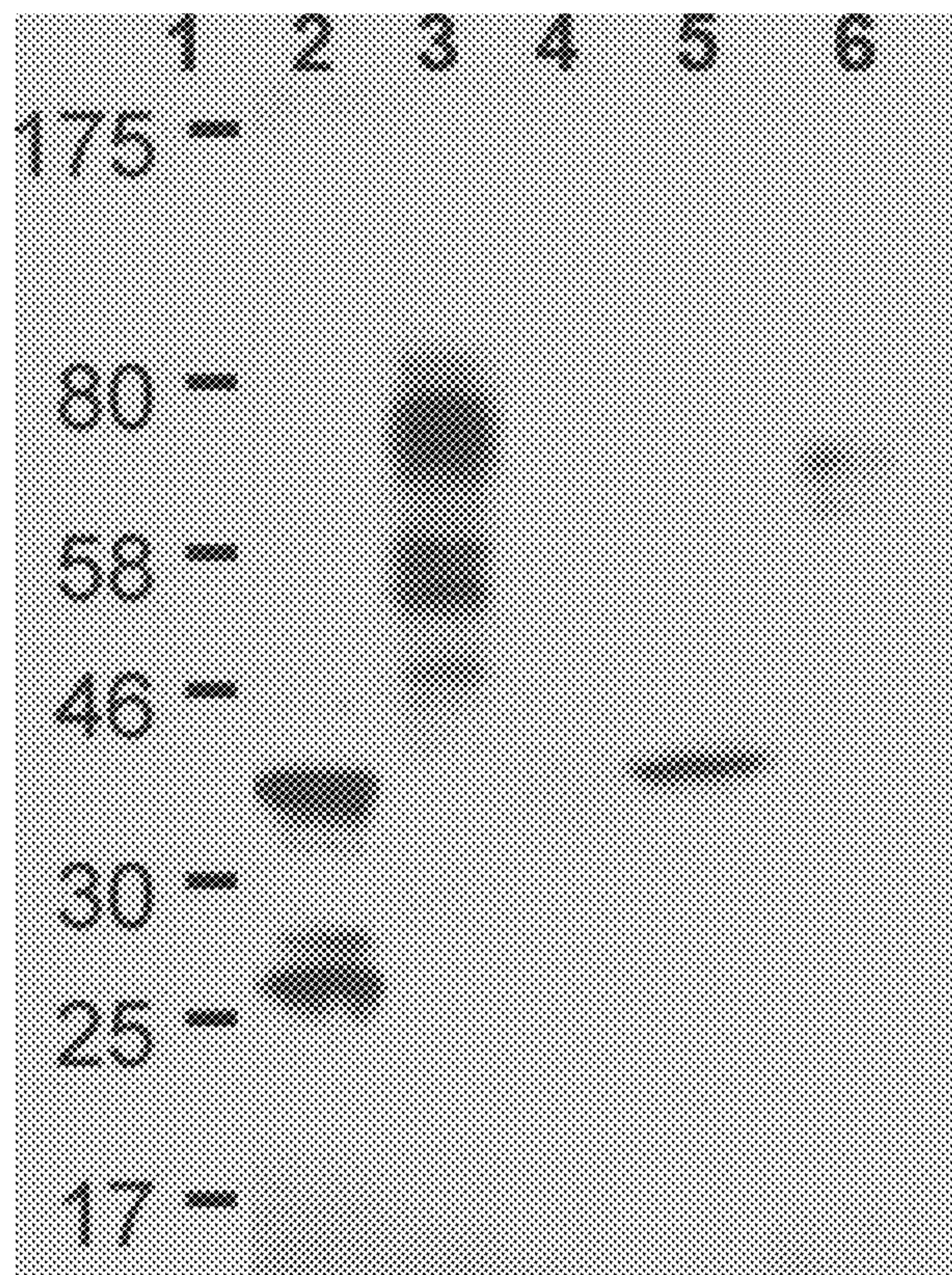




FIG. 2A

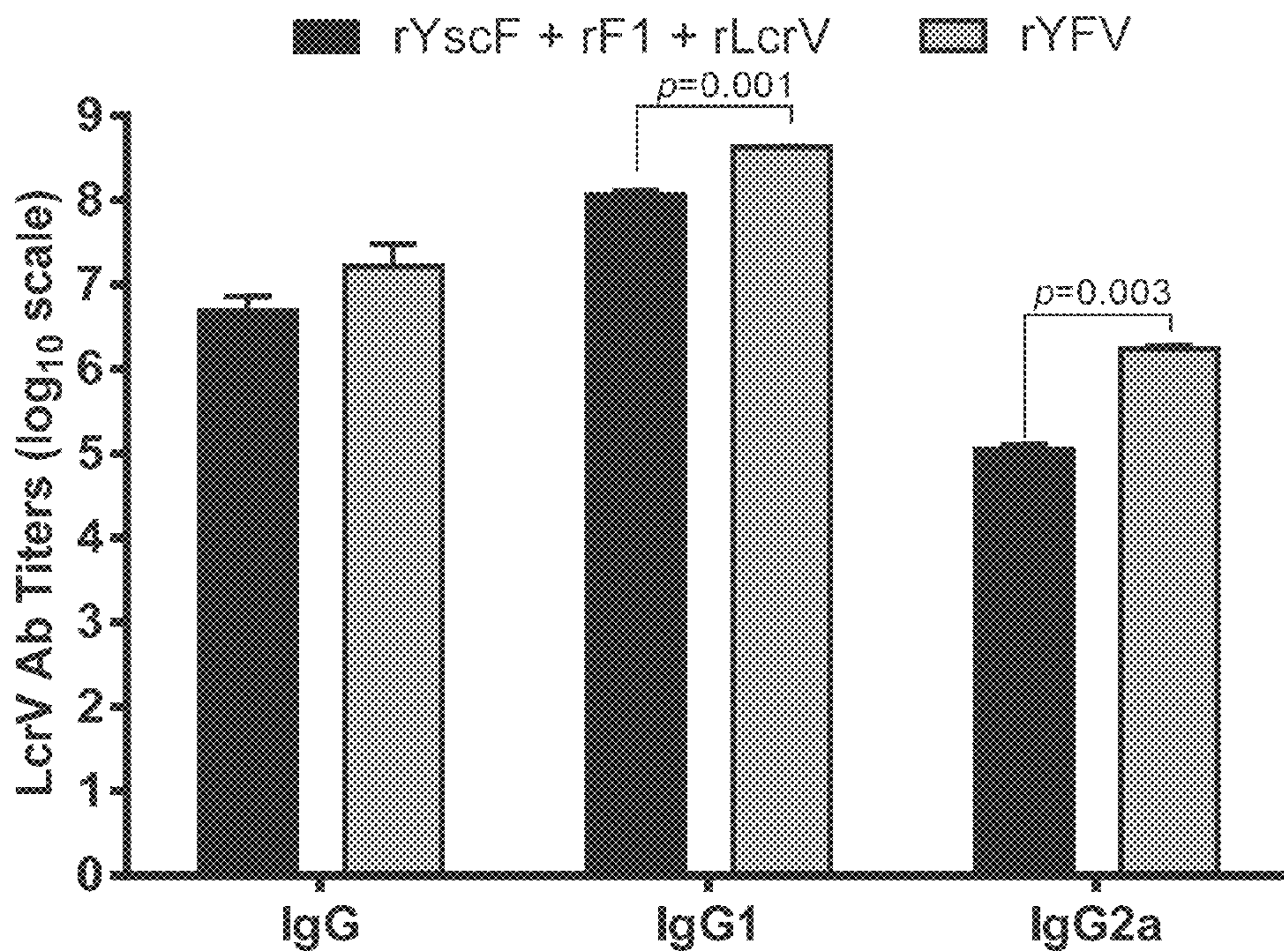


FIG. 2B

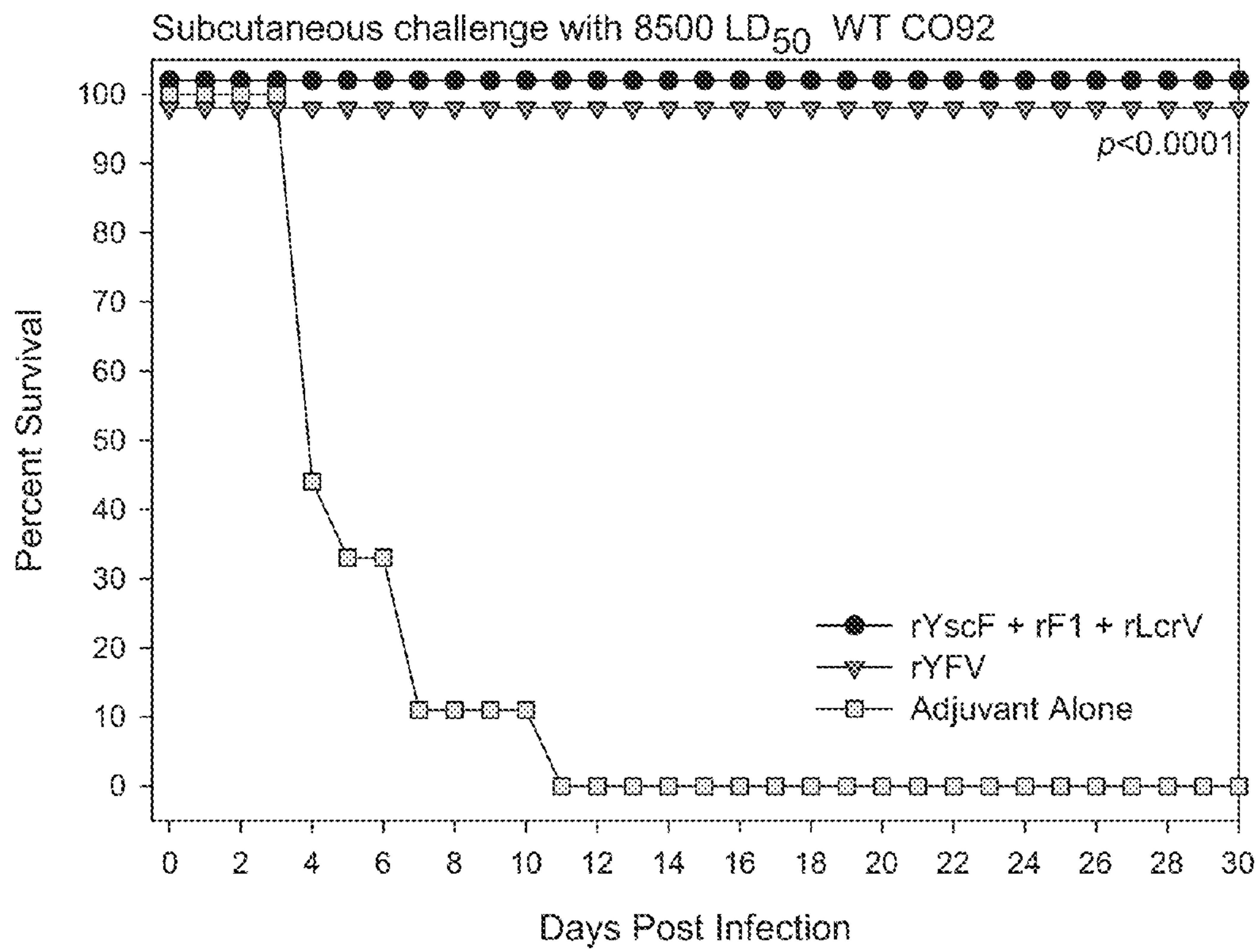


FIG. 2C

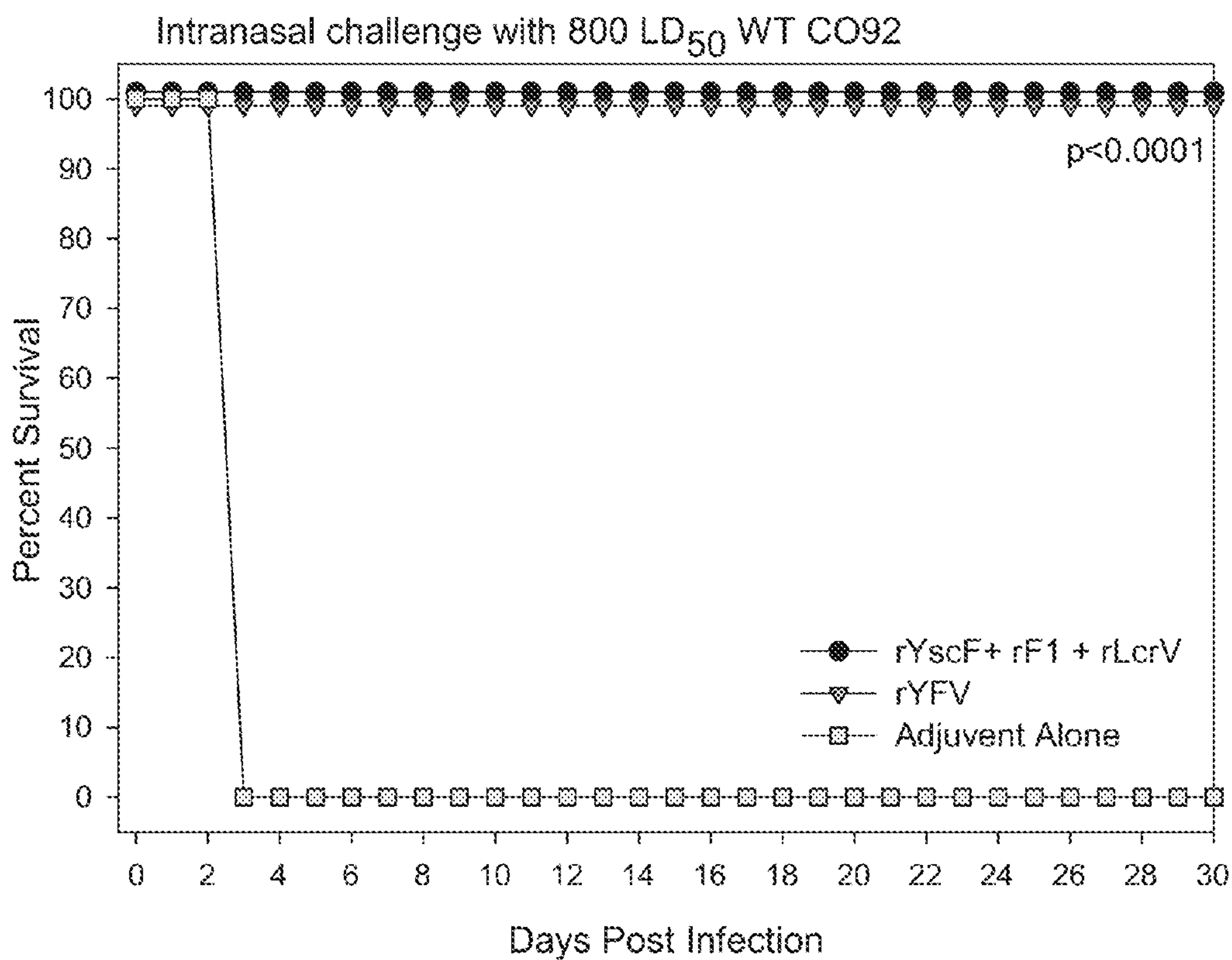


FIG. 3A

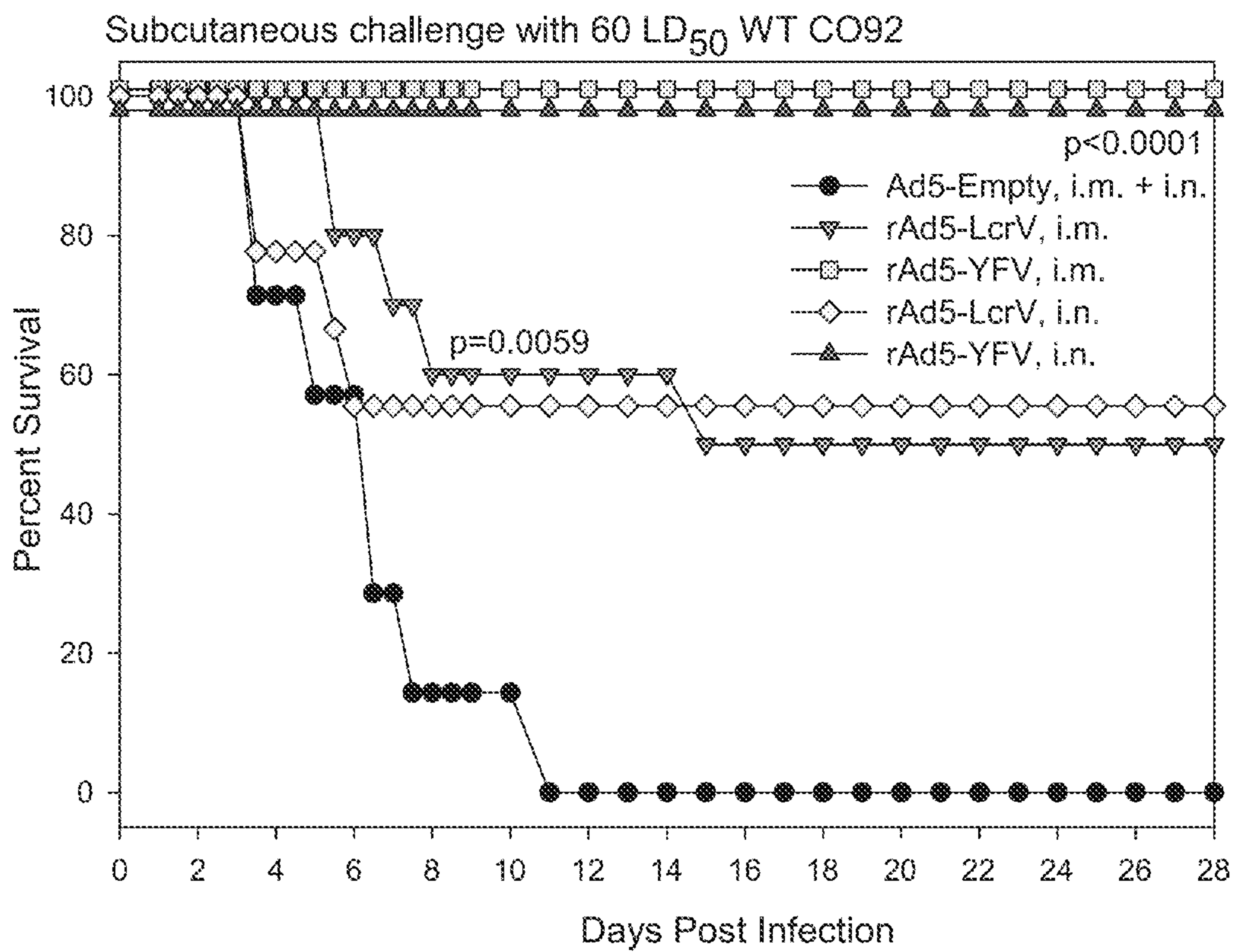




FIG. 3B

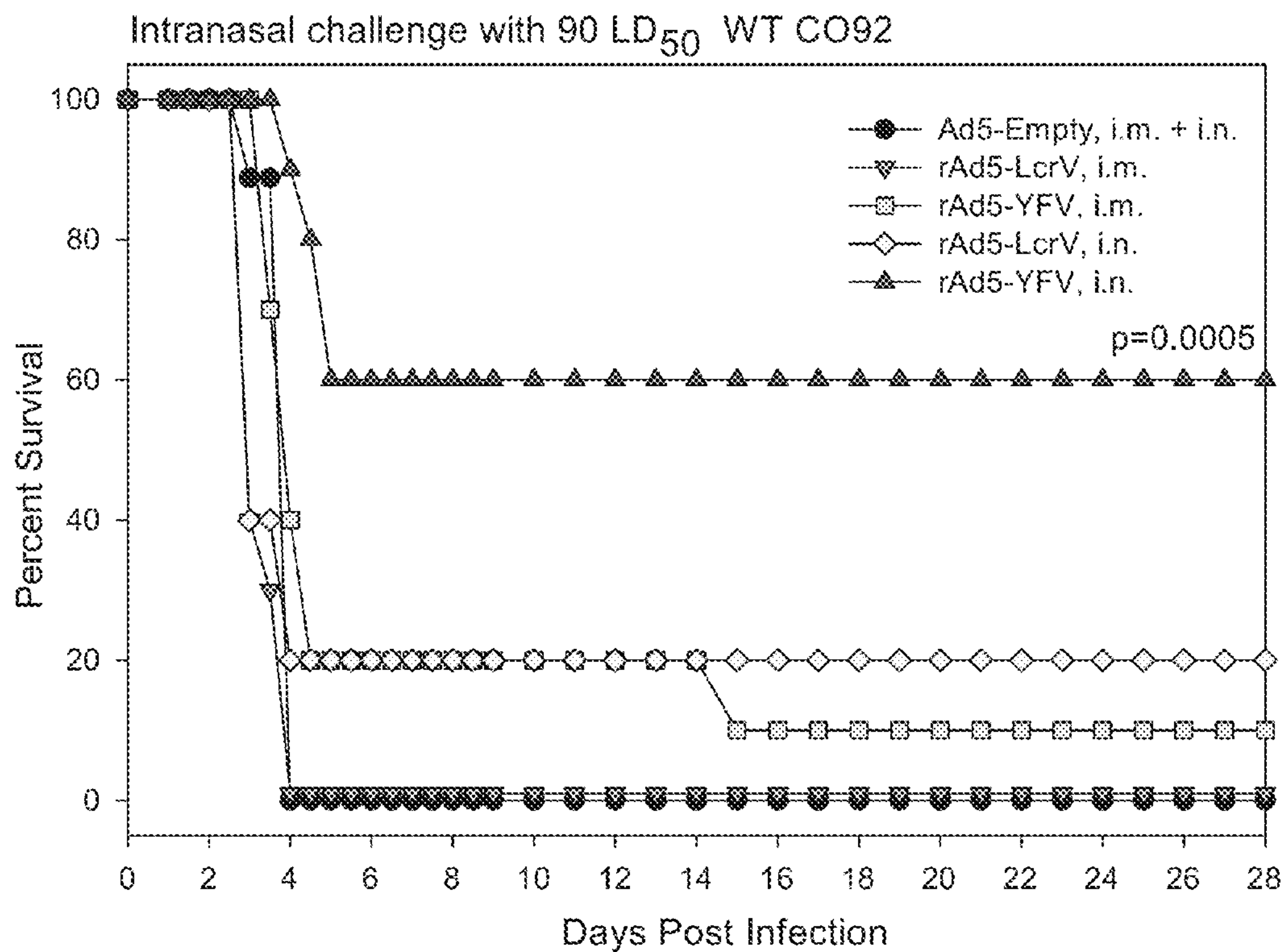


FIG. 3C

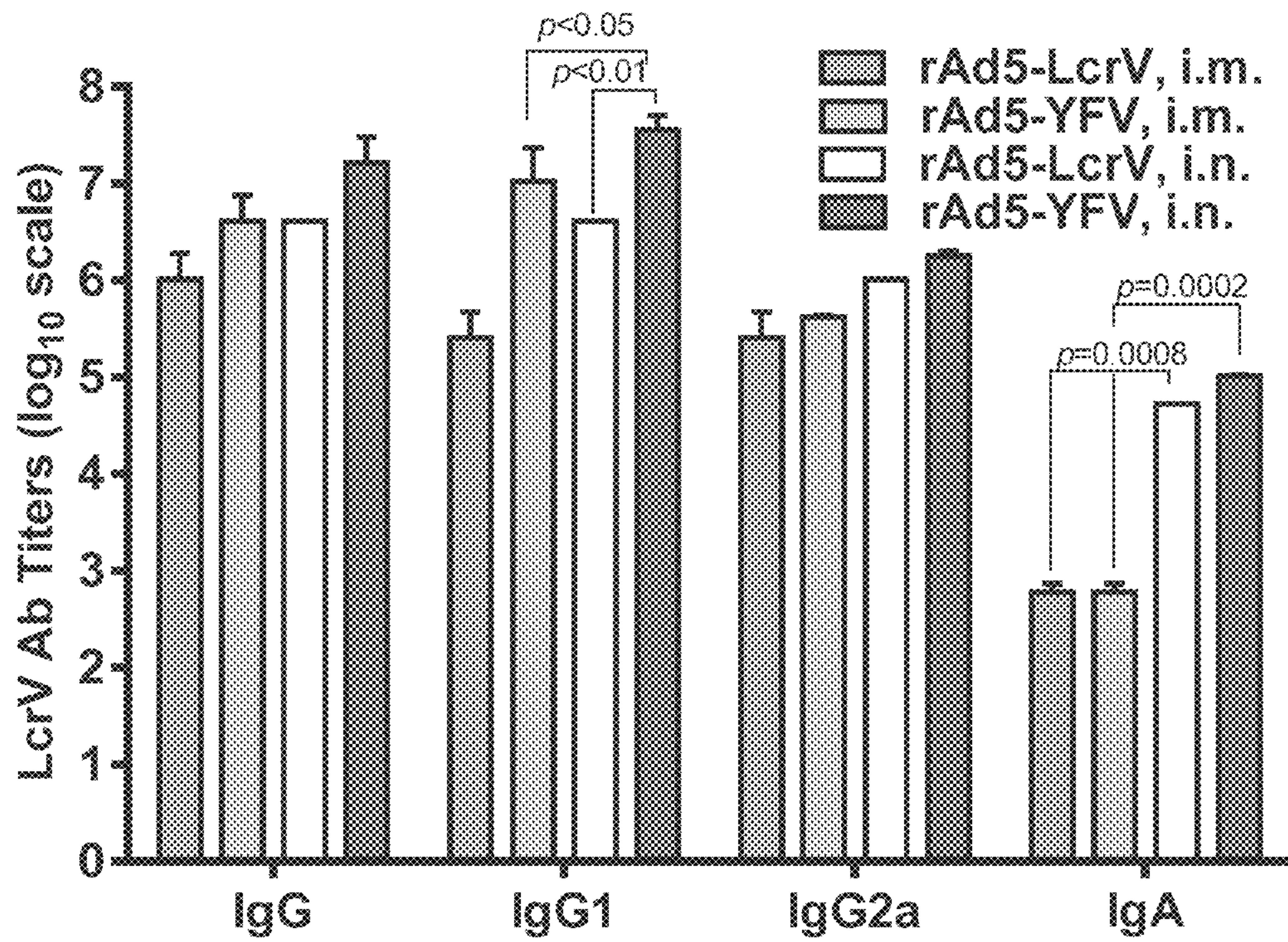




FIG. 4A

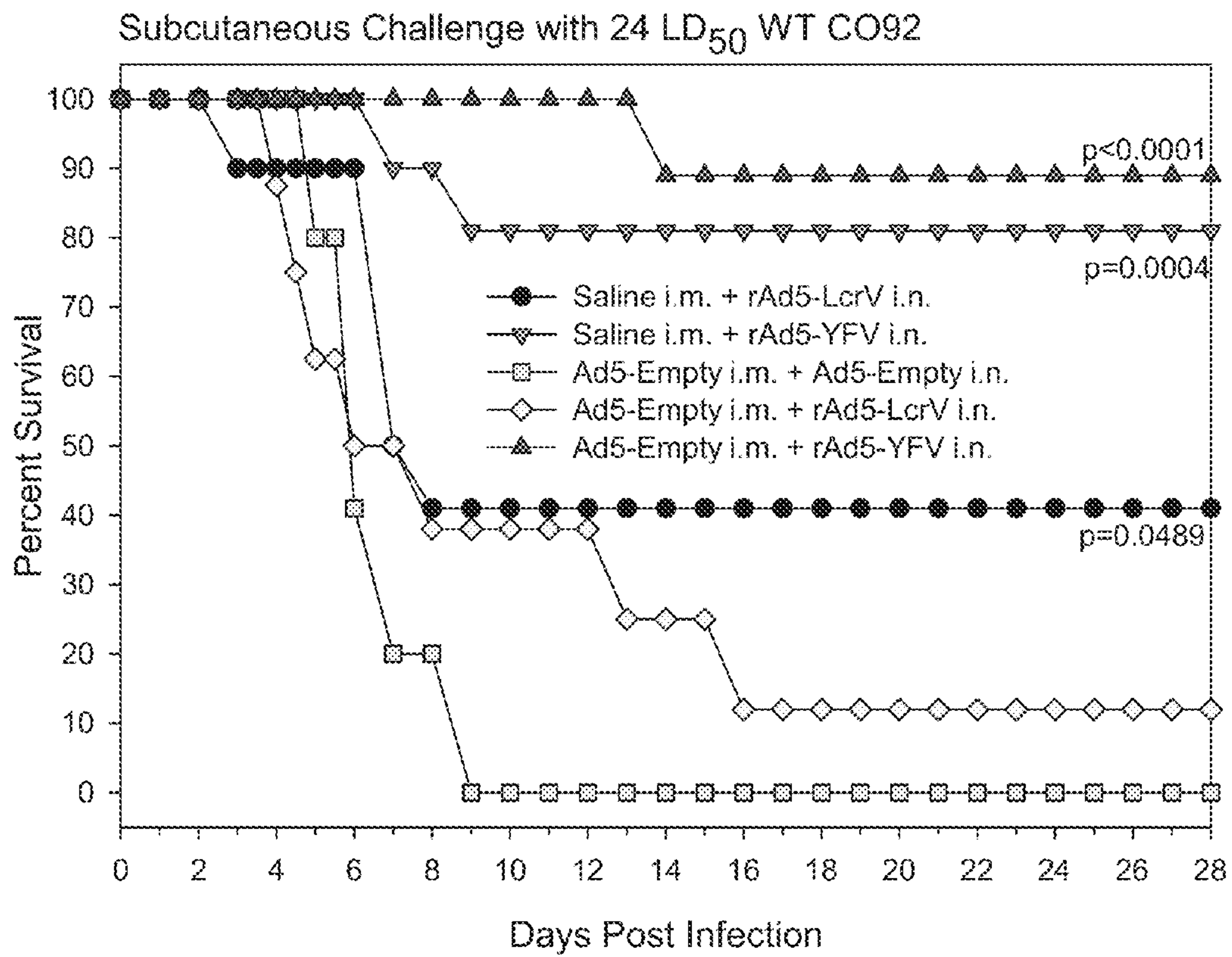


FIG. 4B

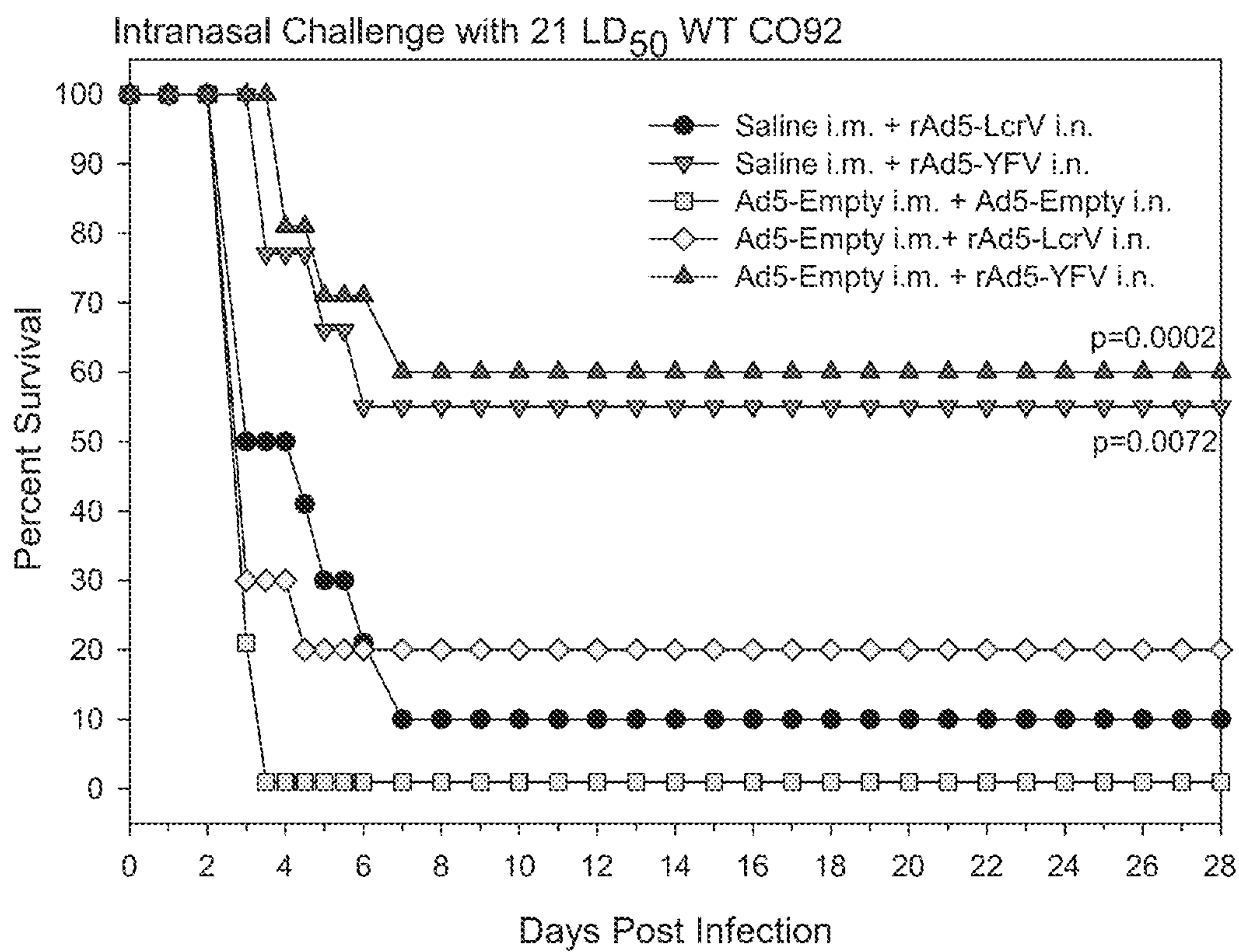




FIG. 4C

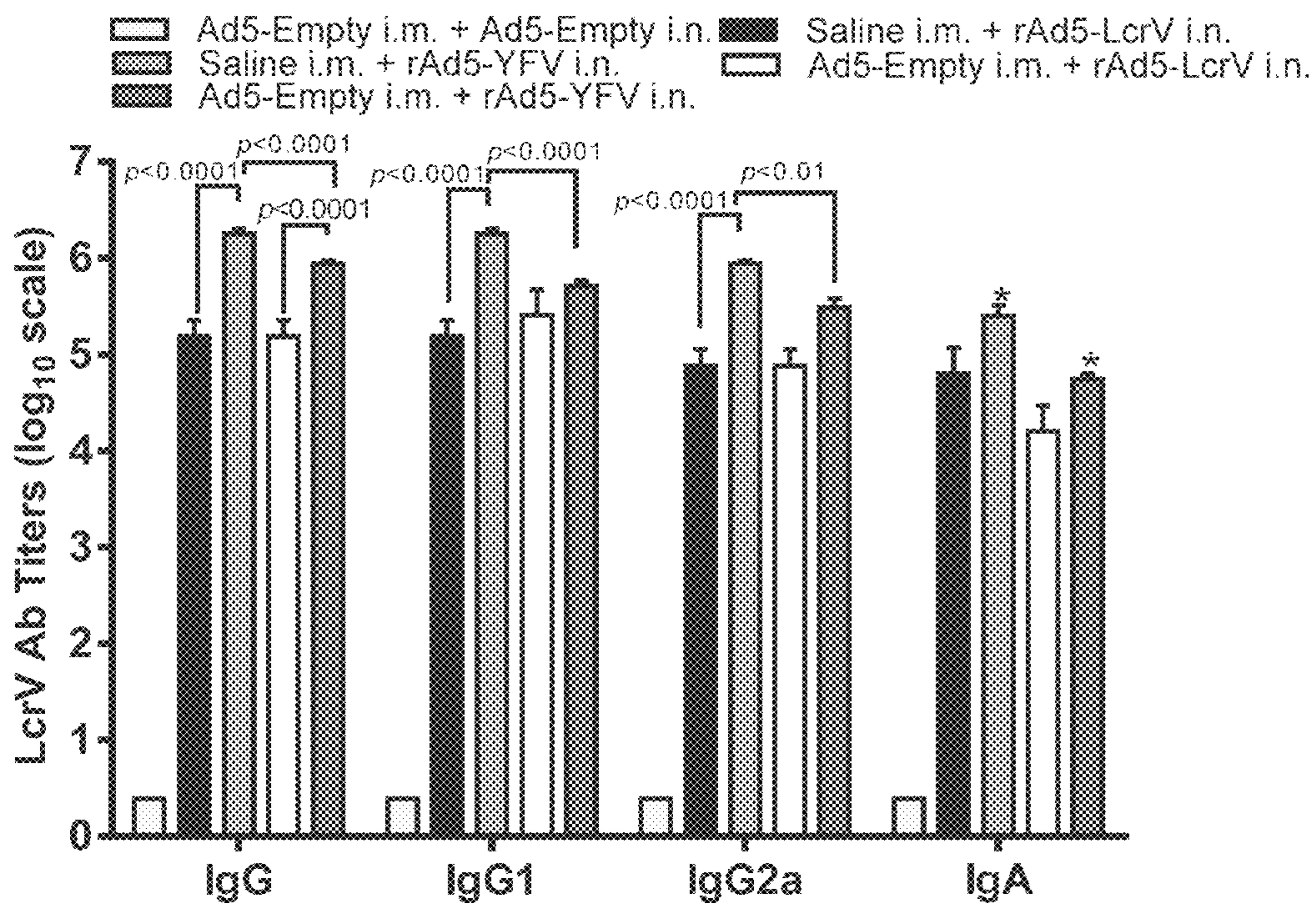


FIG. 5

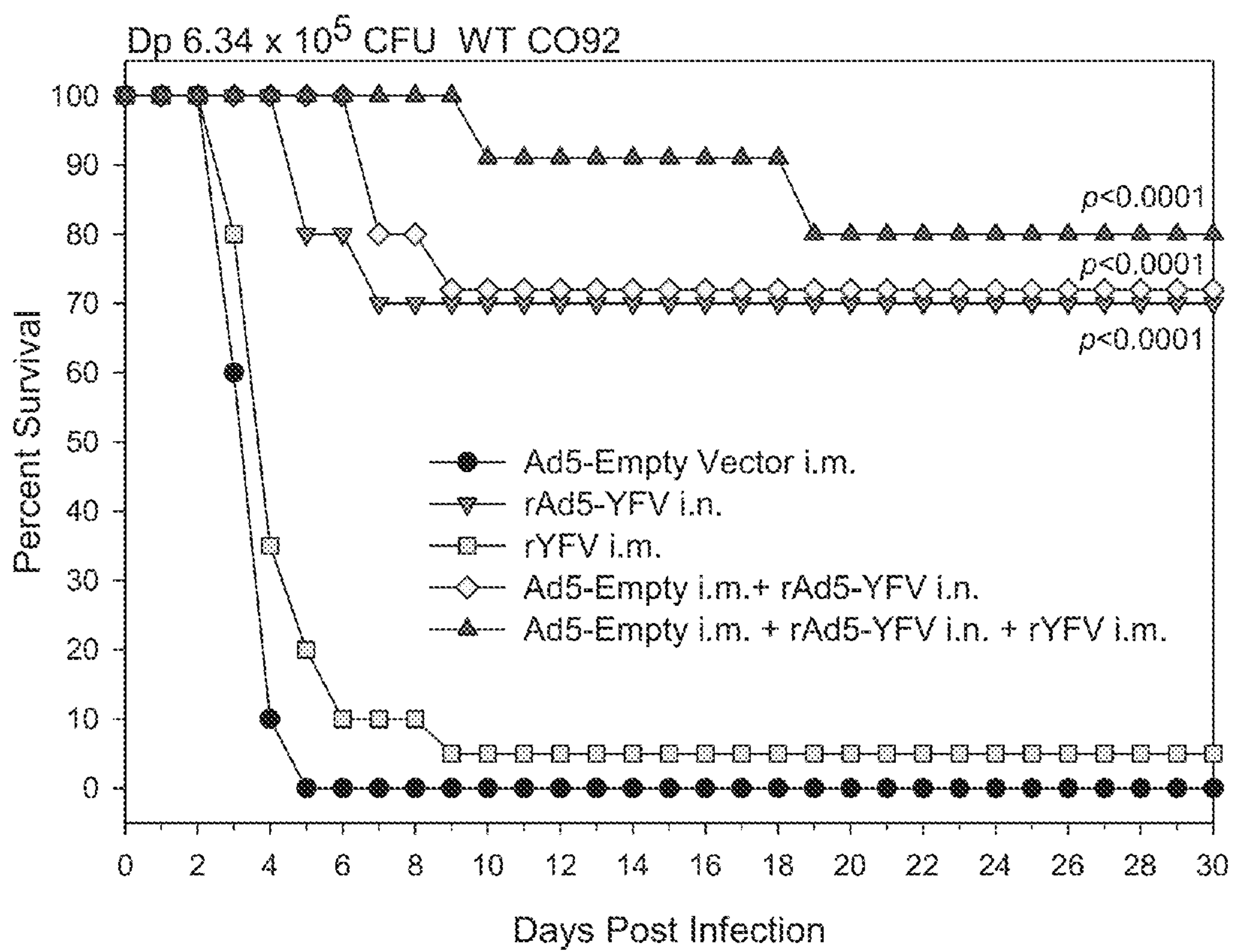




FIG. 6A

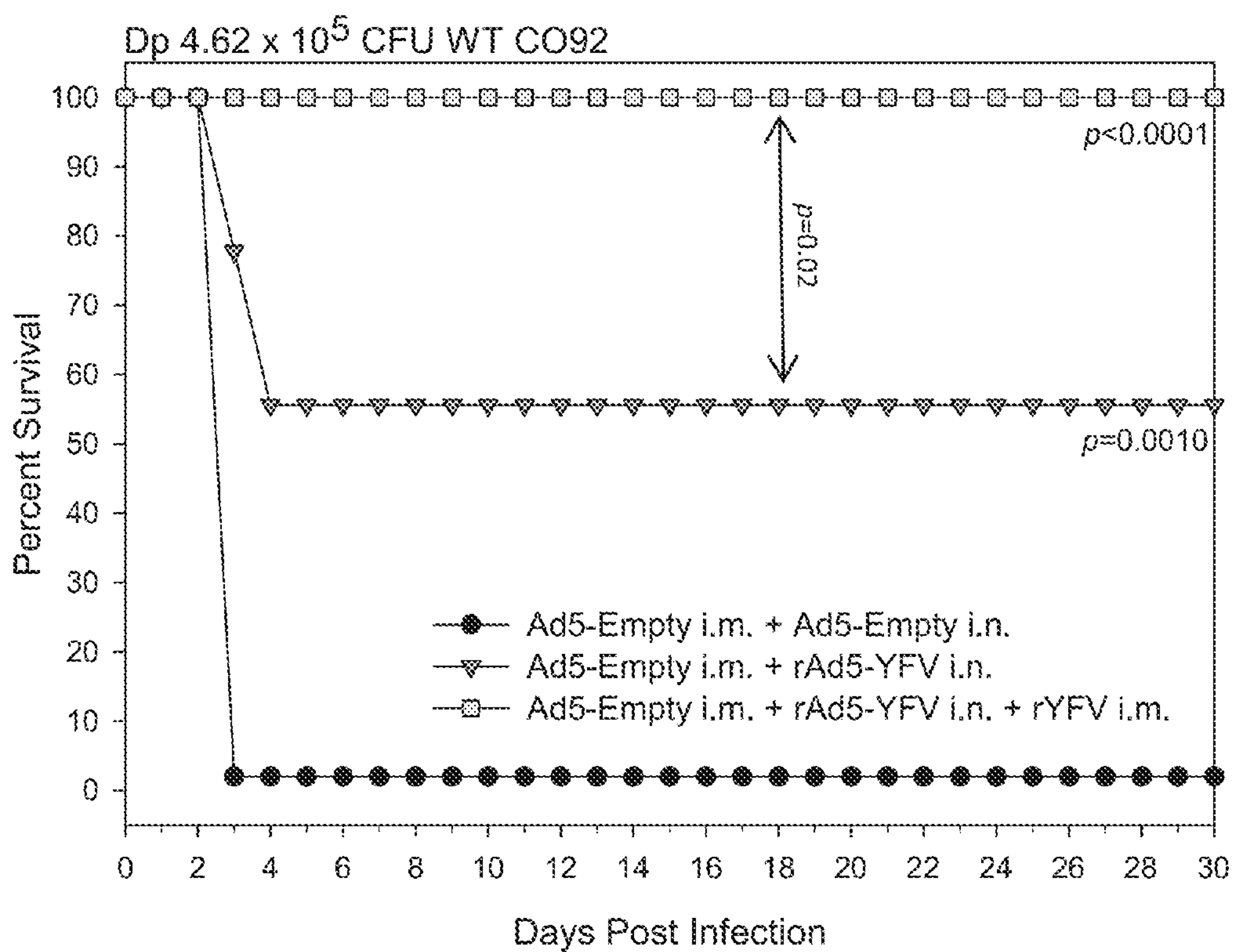


FIG. 6B

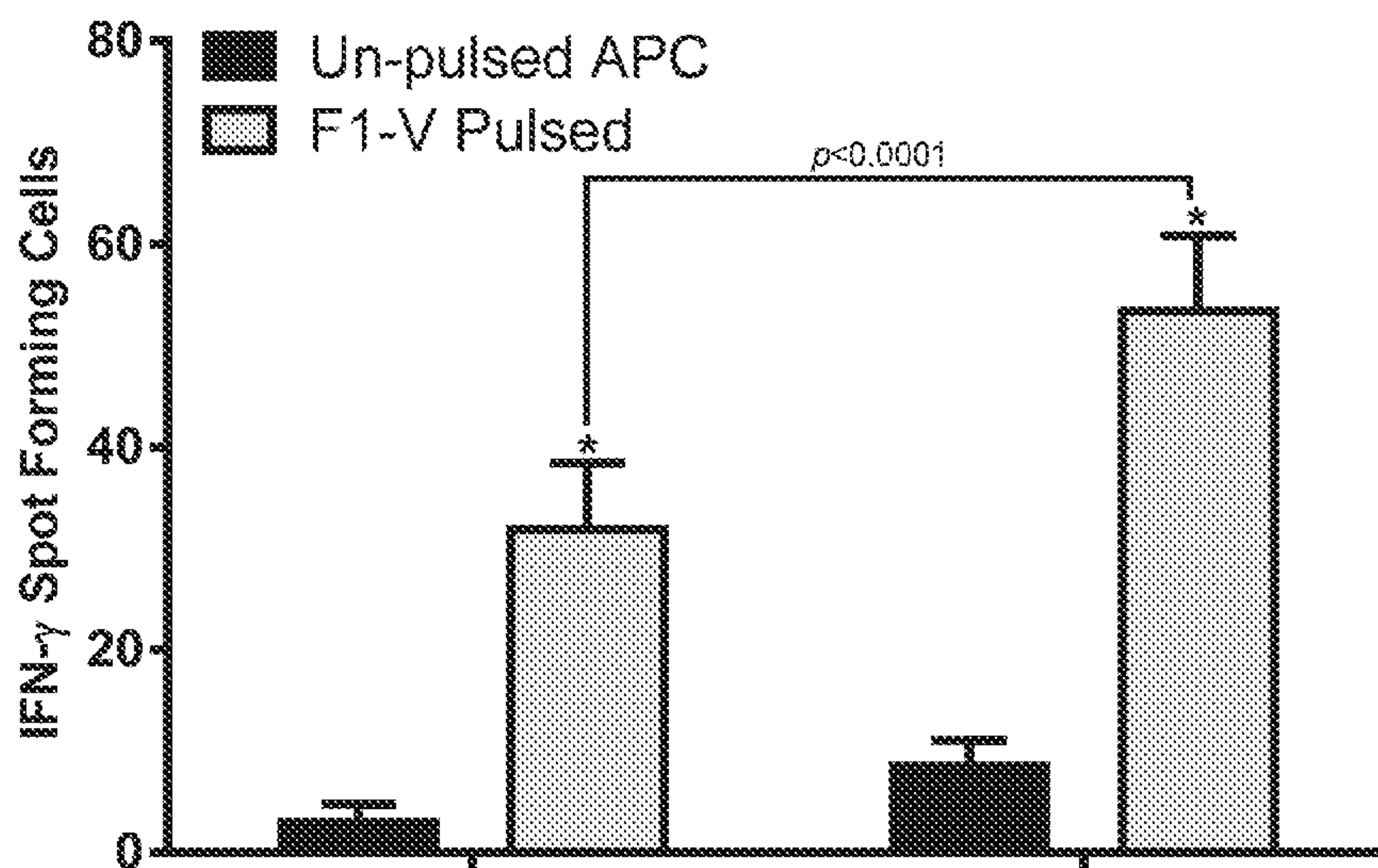


FIG. 6C

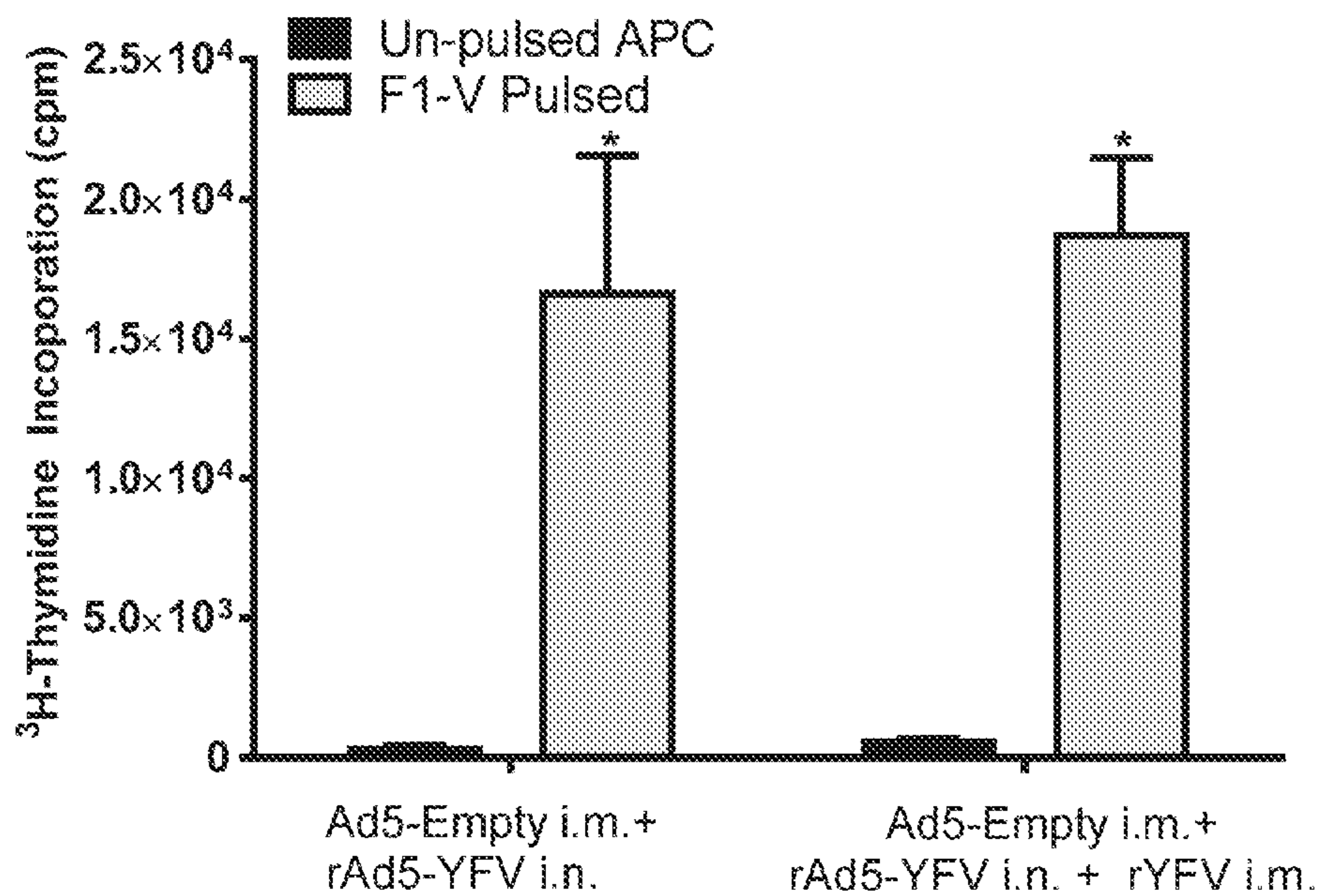




FIG. 7A

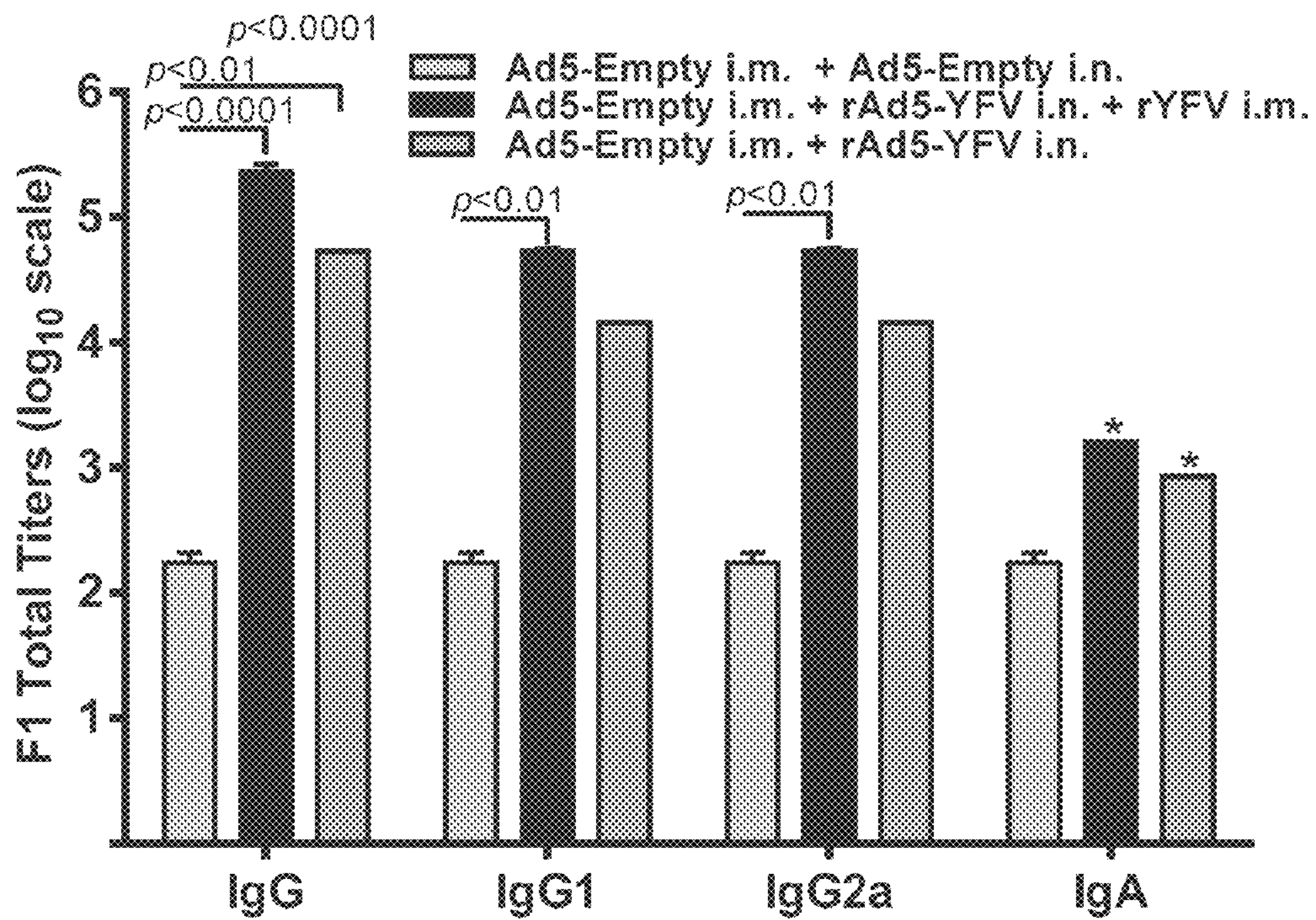


FIG. 7B

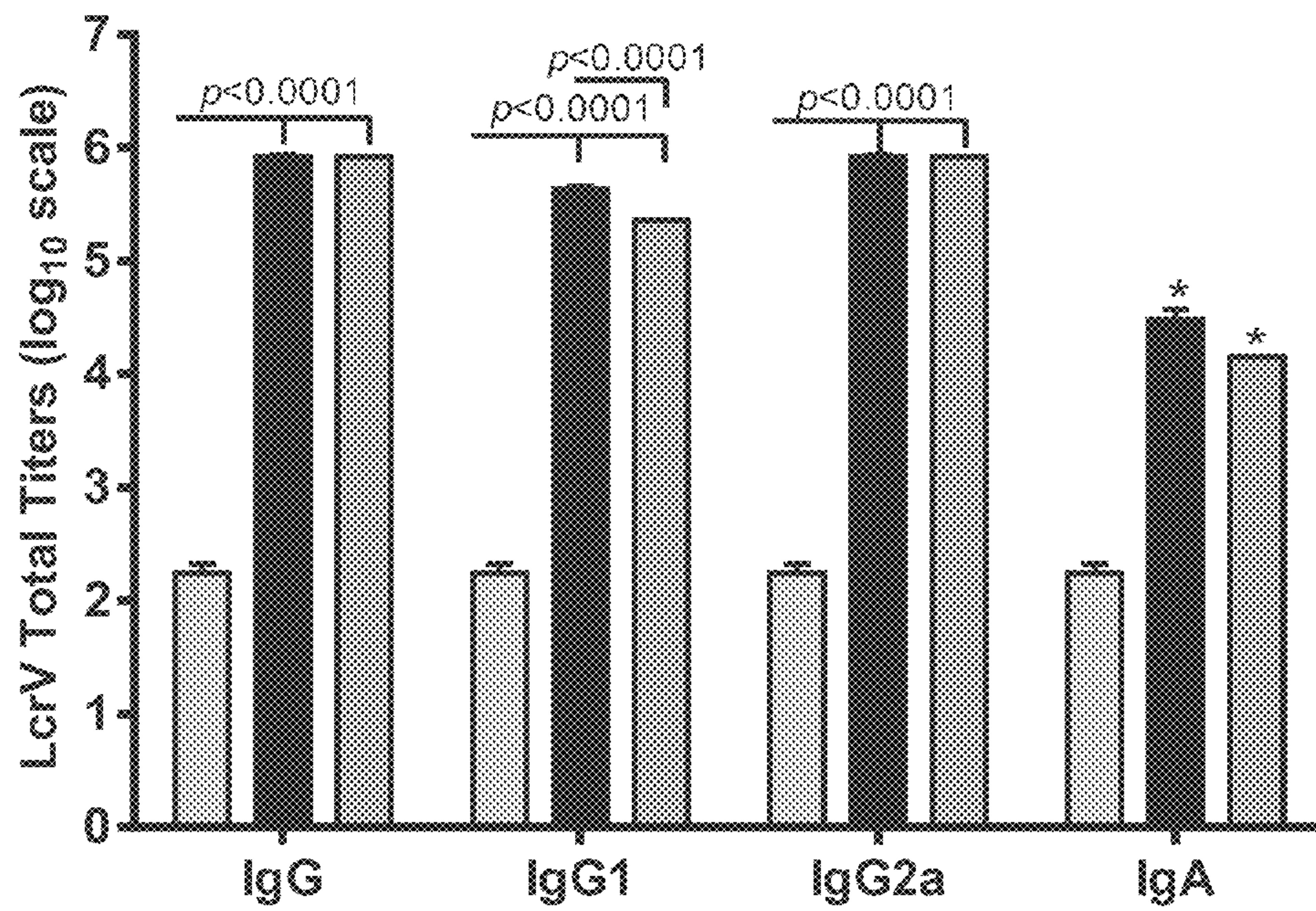




FIG. 7C

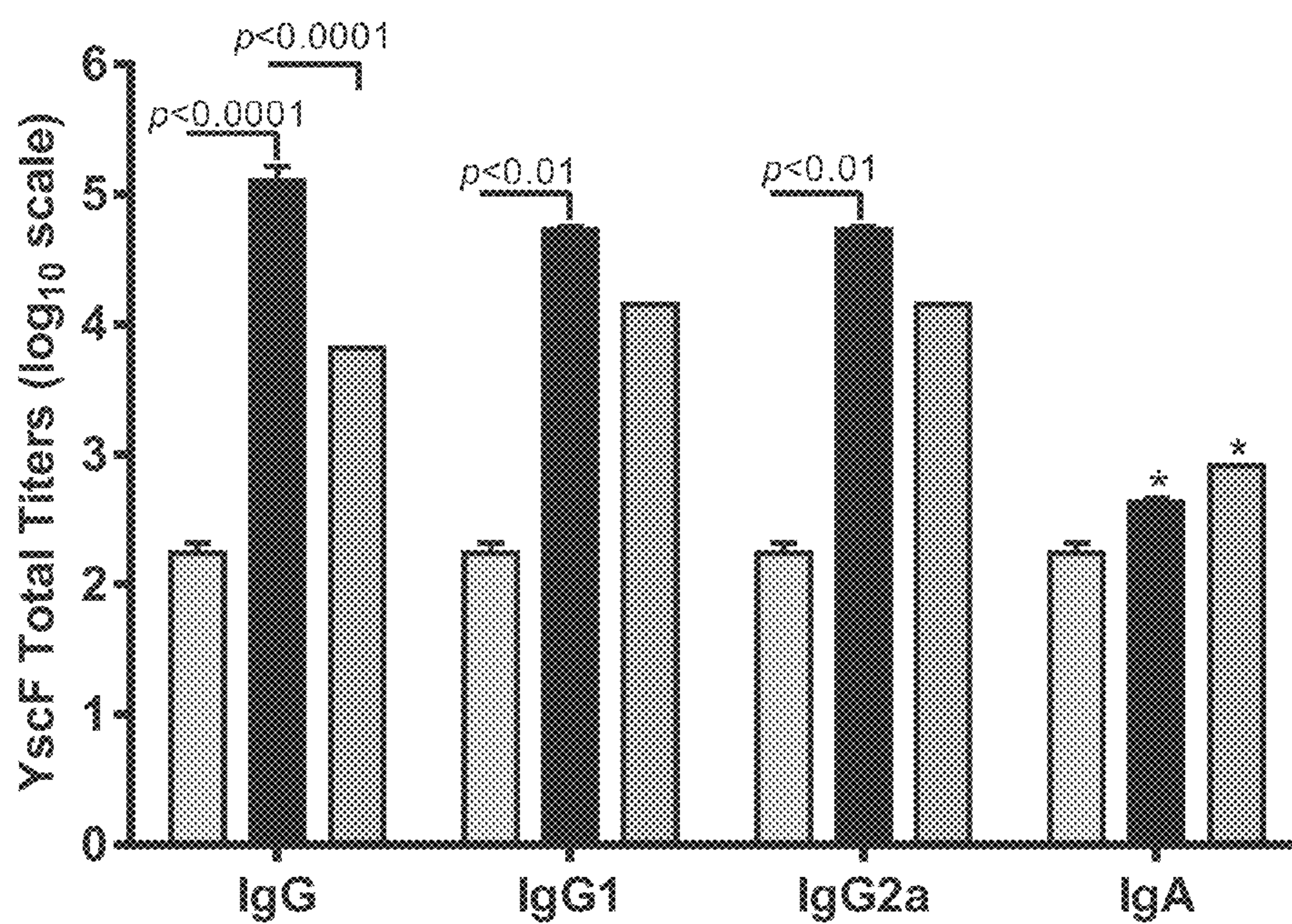
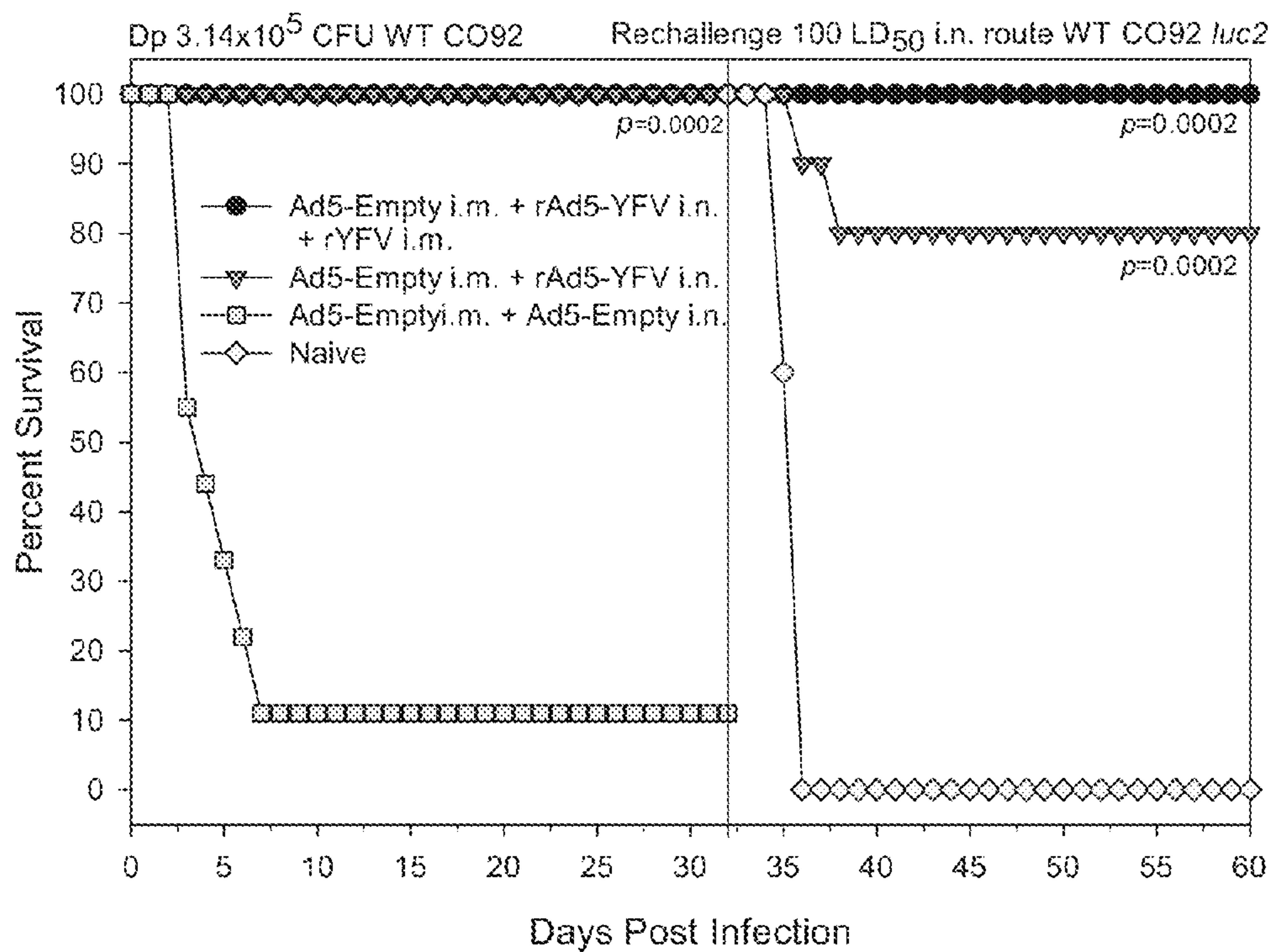


FIG. 8A





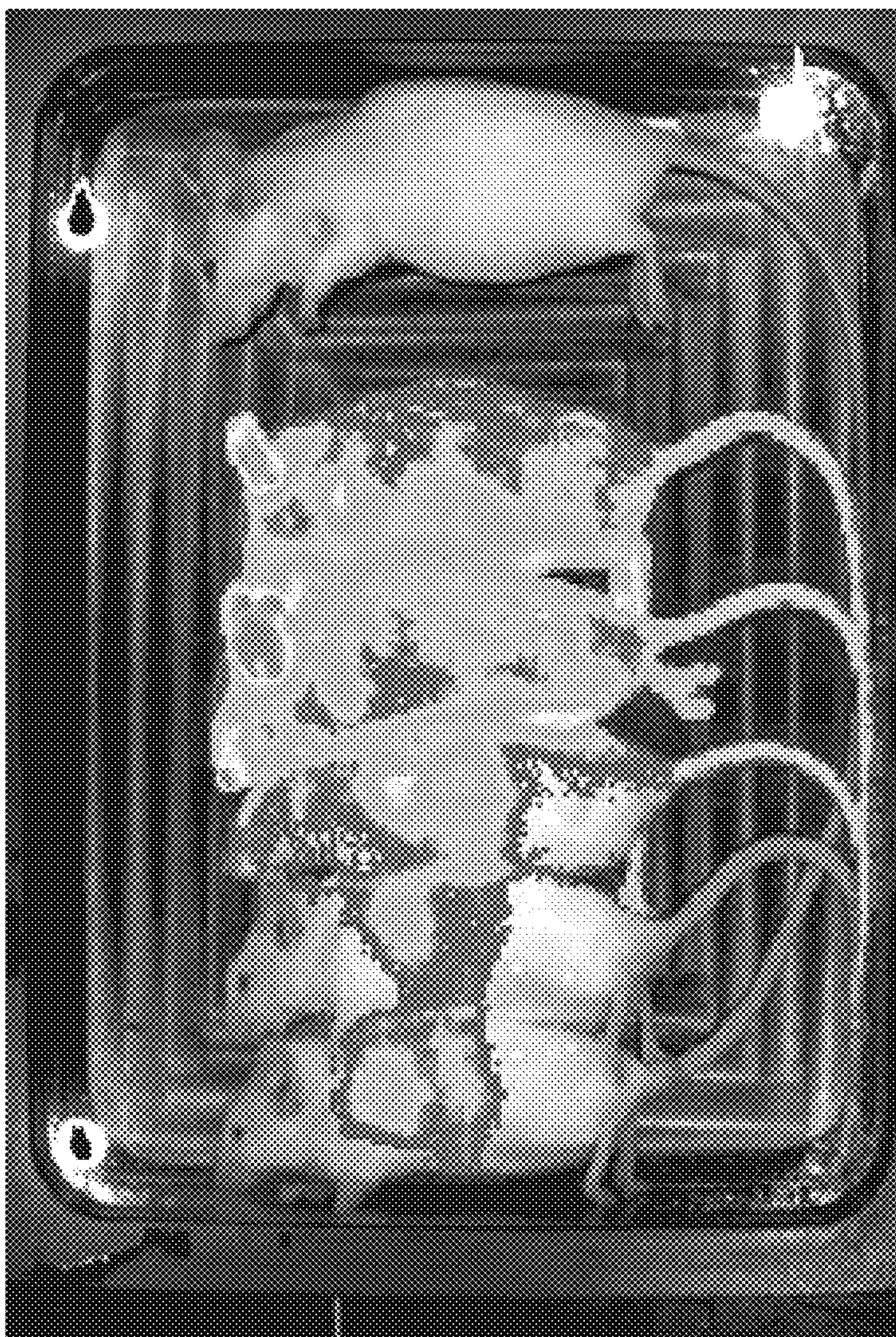
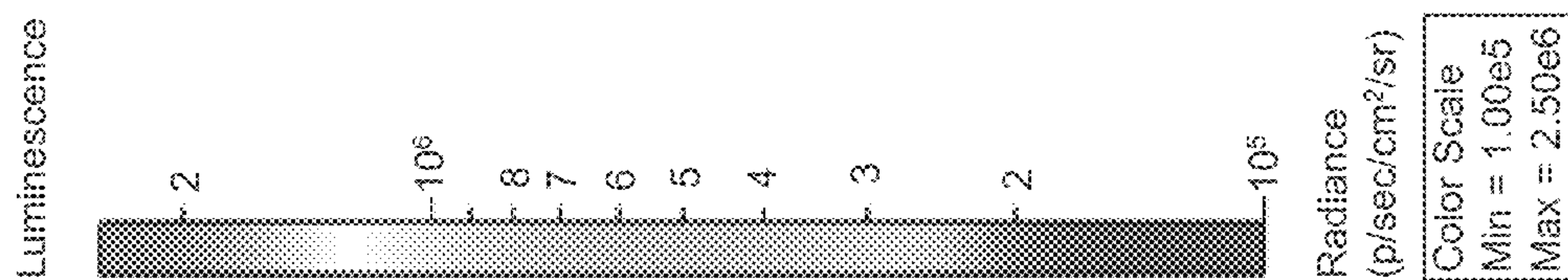


FIG. 8B-01



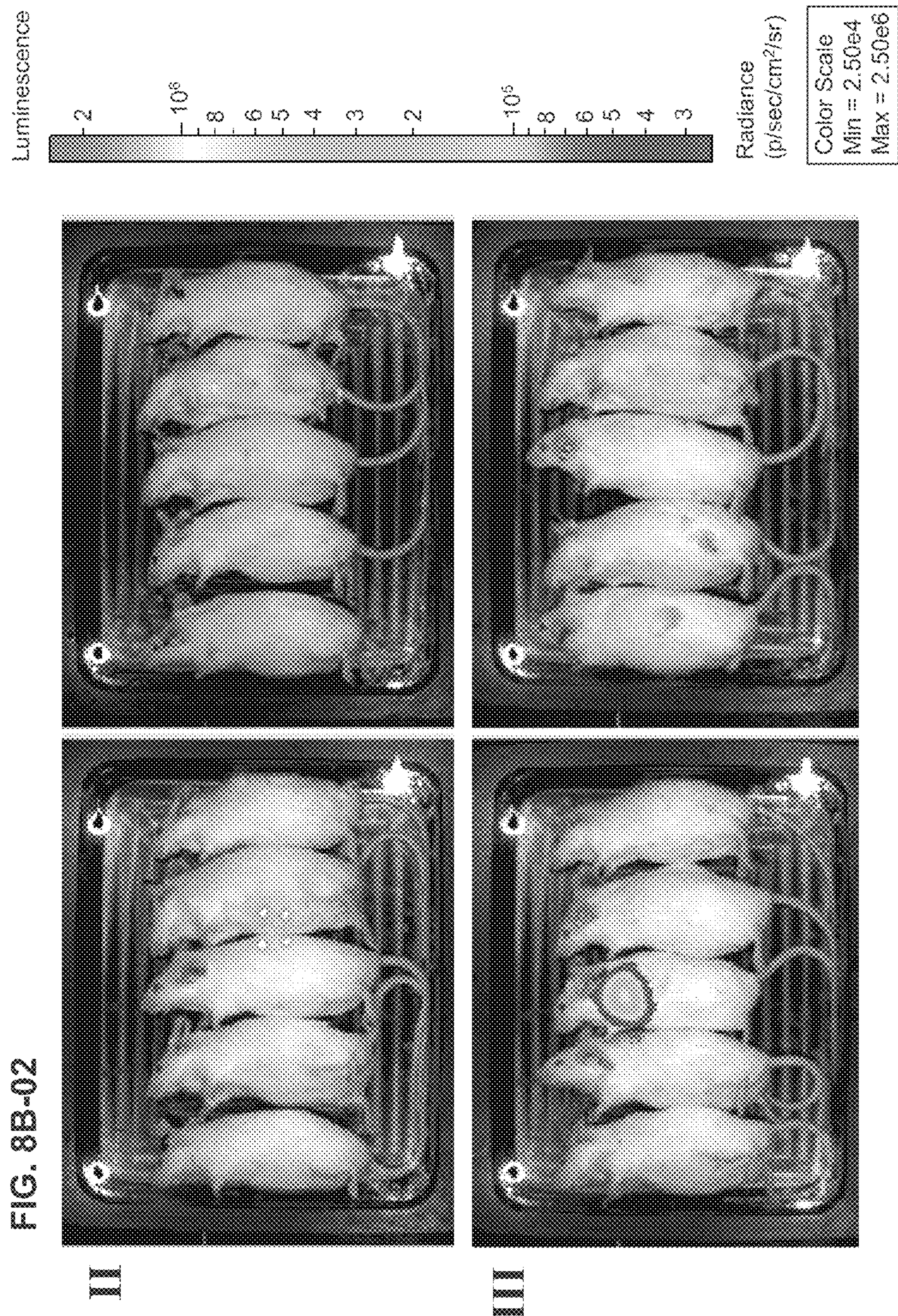


FIG. 8B-02



FIG. 9

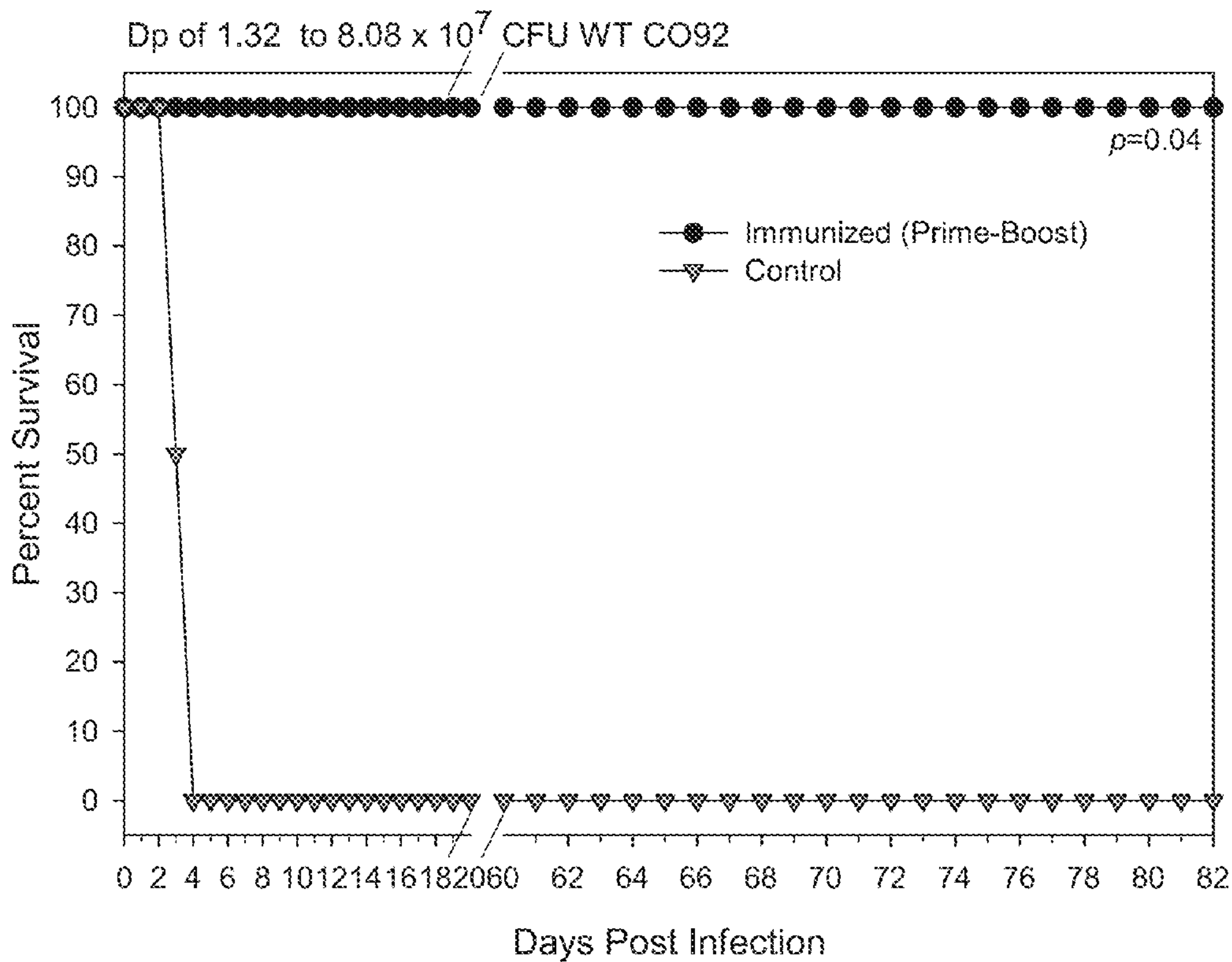




FIG. 10C

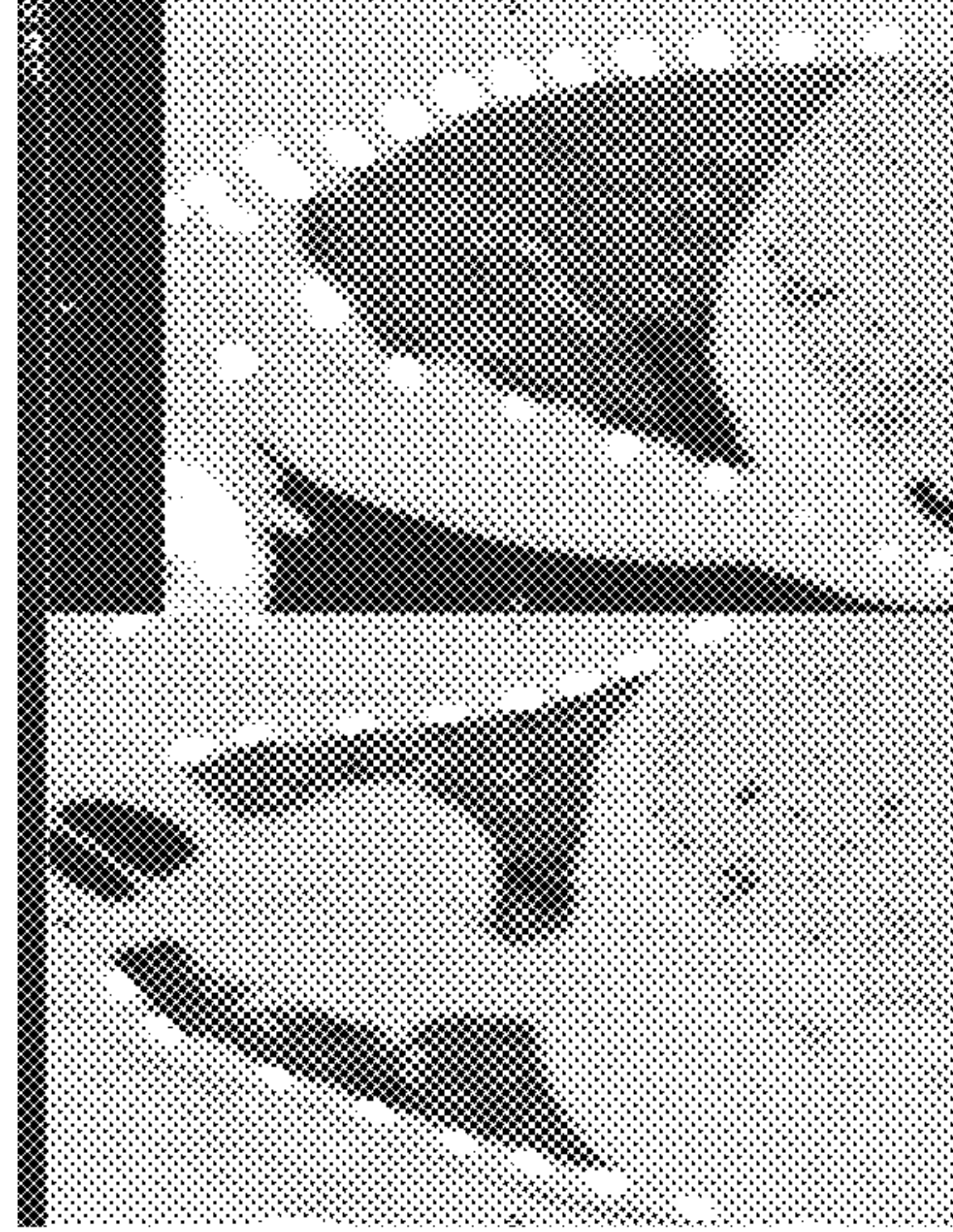


FIG. 10B

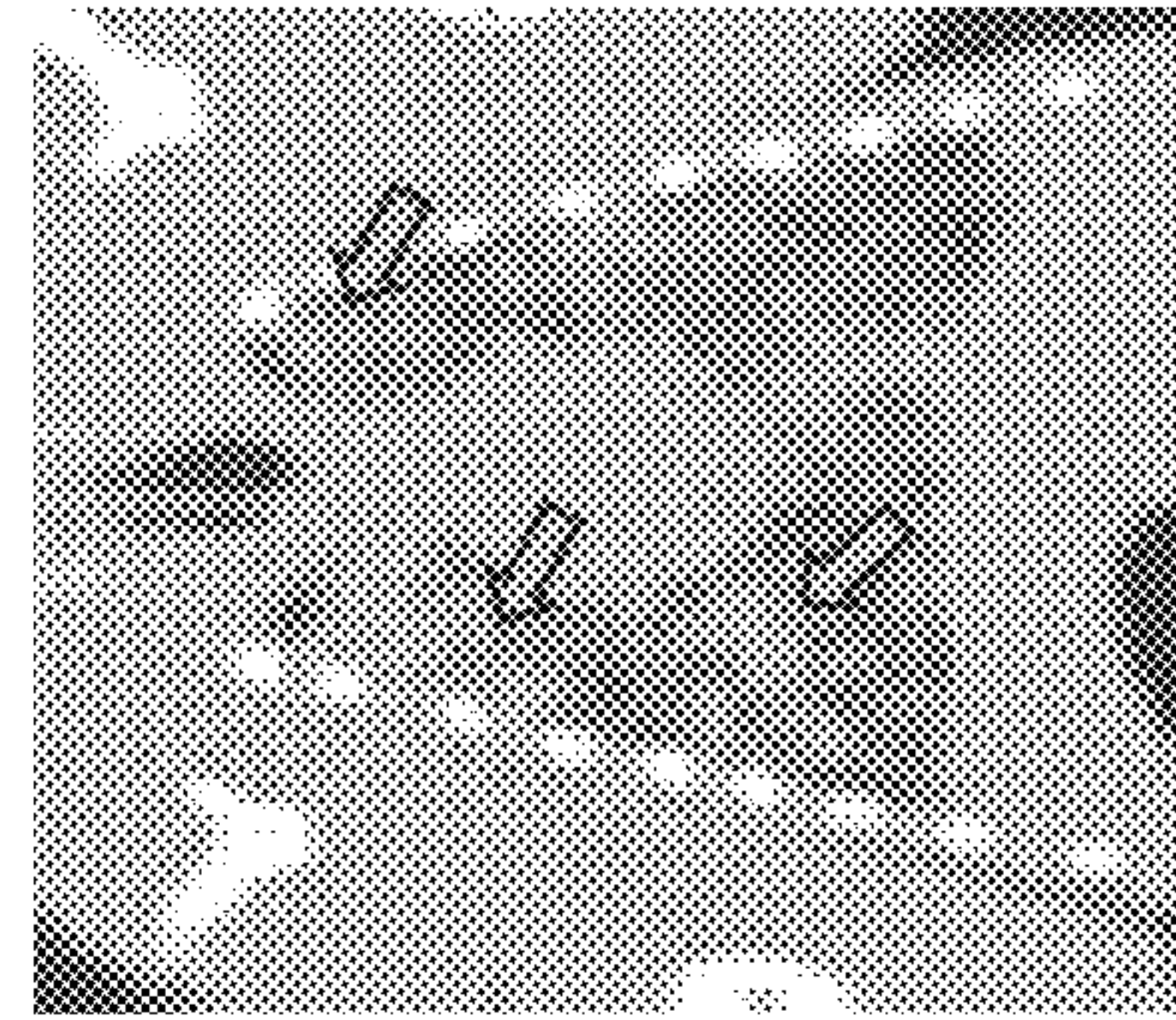
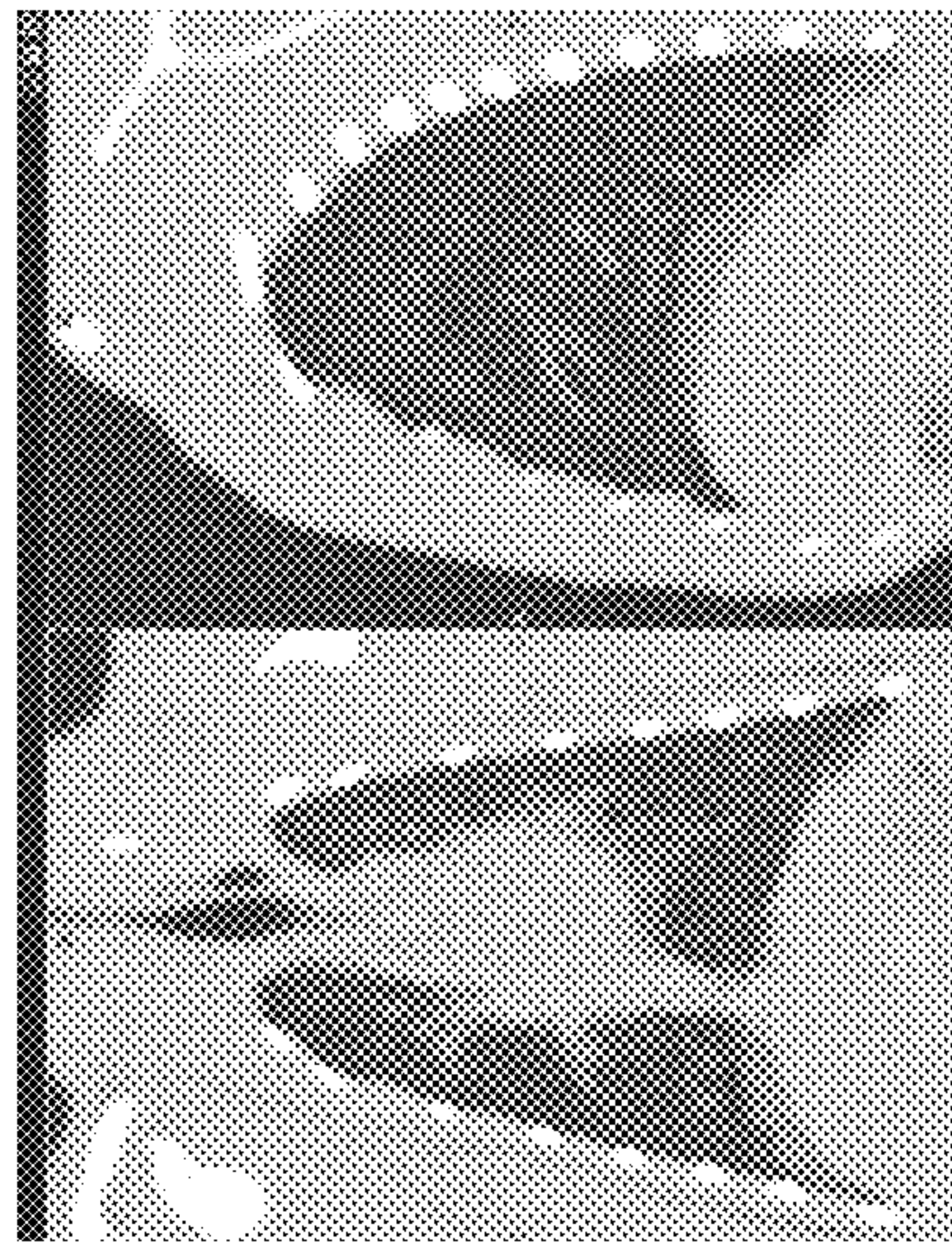
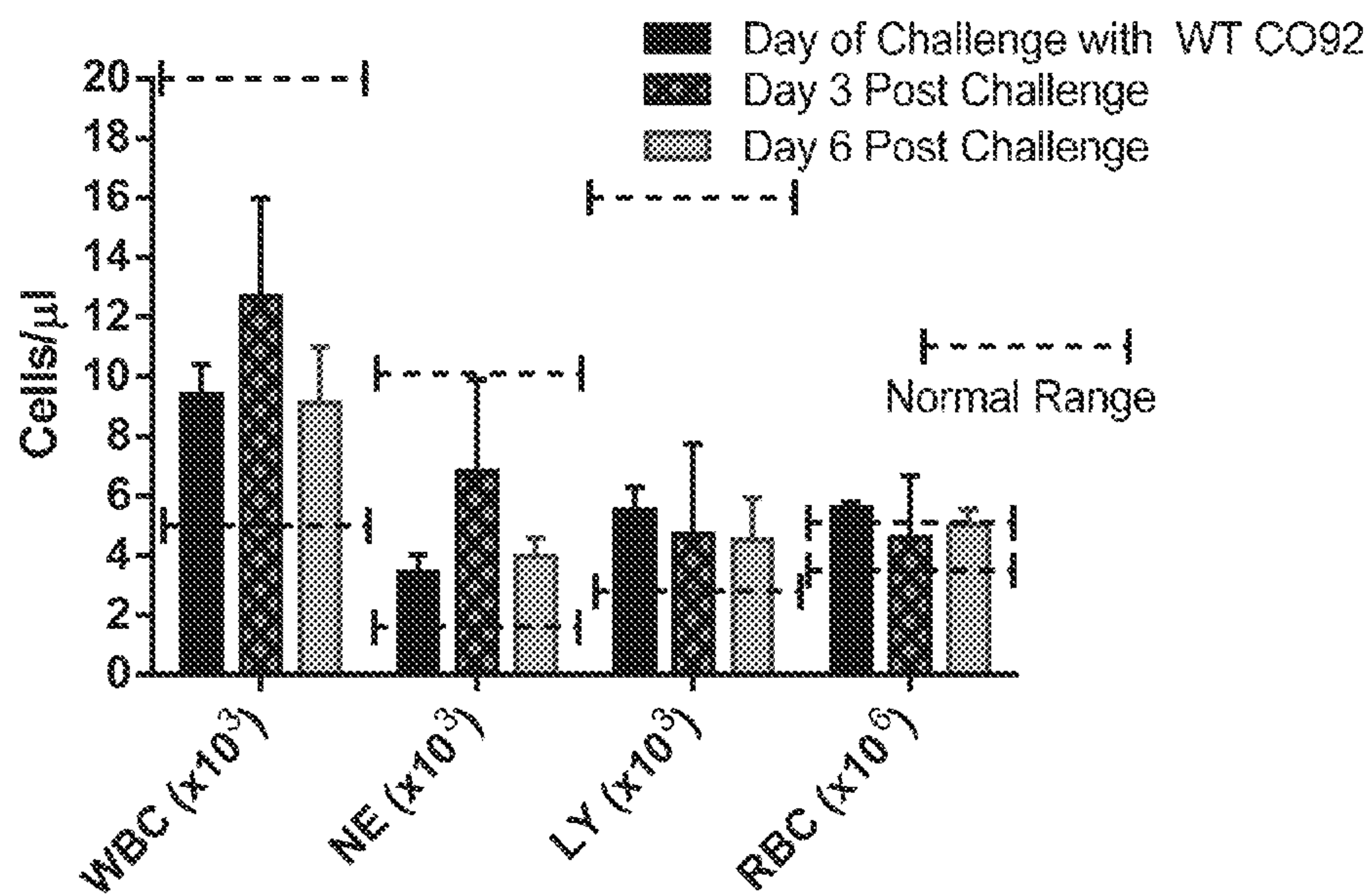


FIG. 10A



**FIG. 11A** Immunized (Prime Boost)



**FIG. 11B** Control

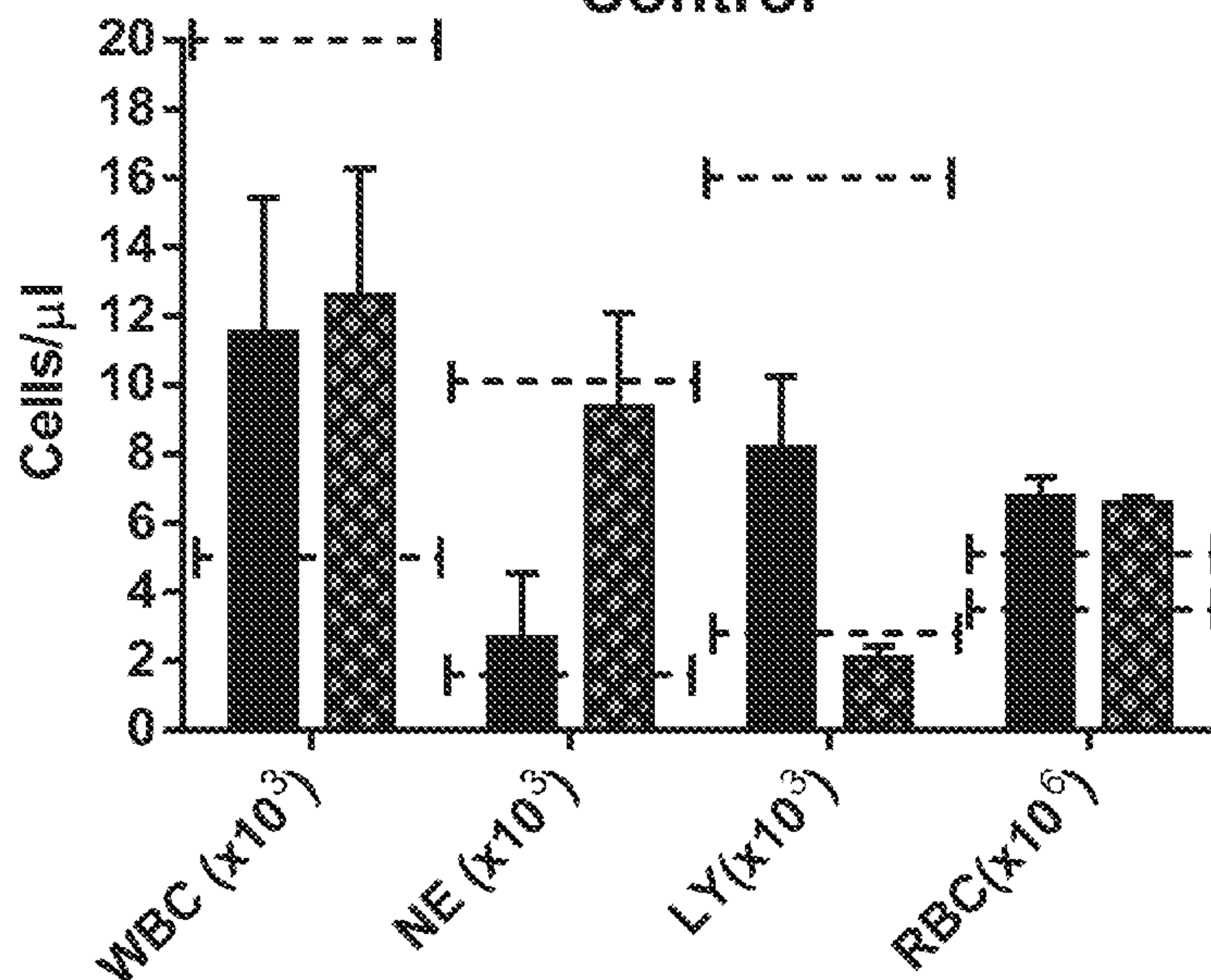




FIG. 12A

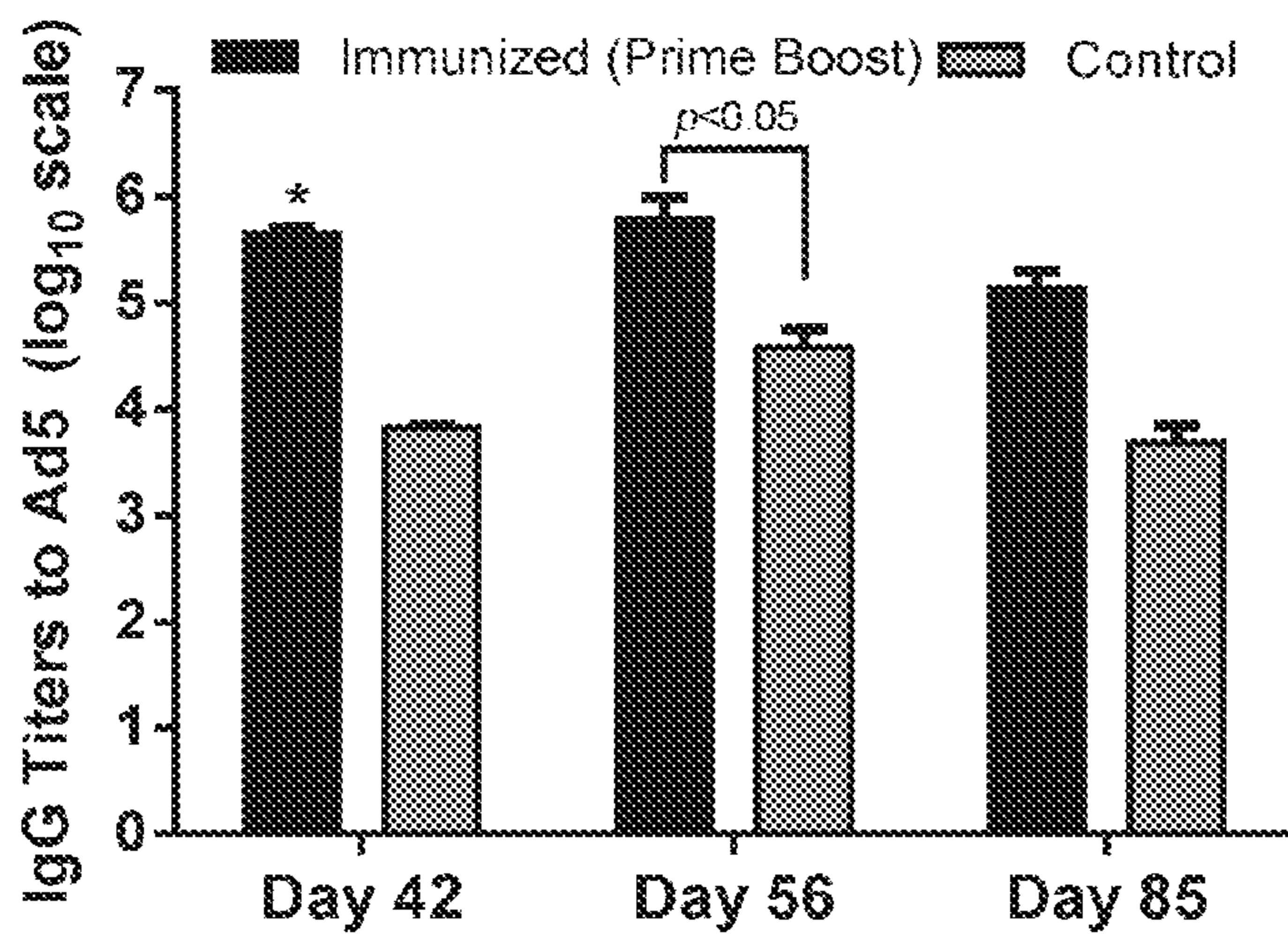


FIG. 12B

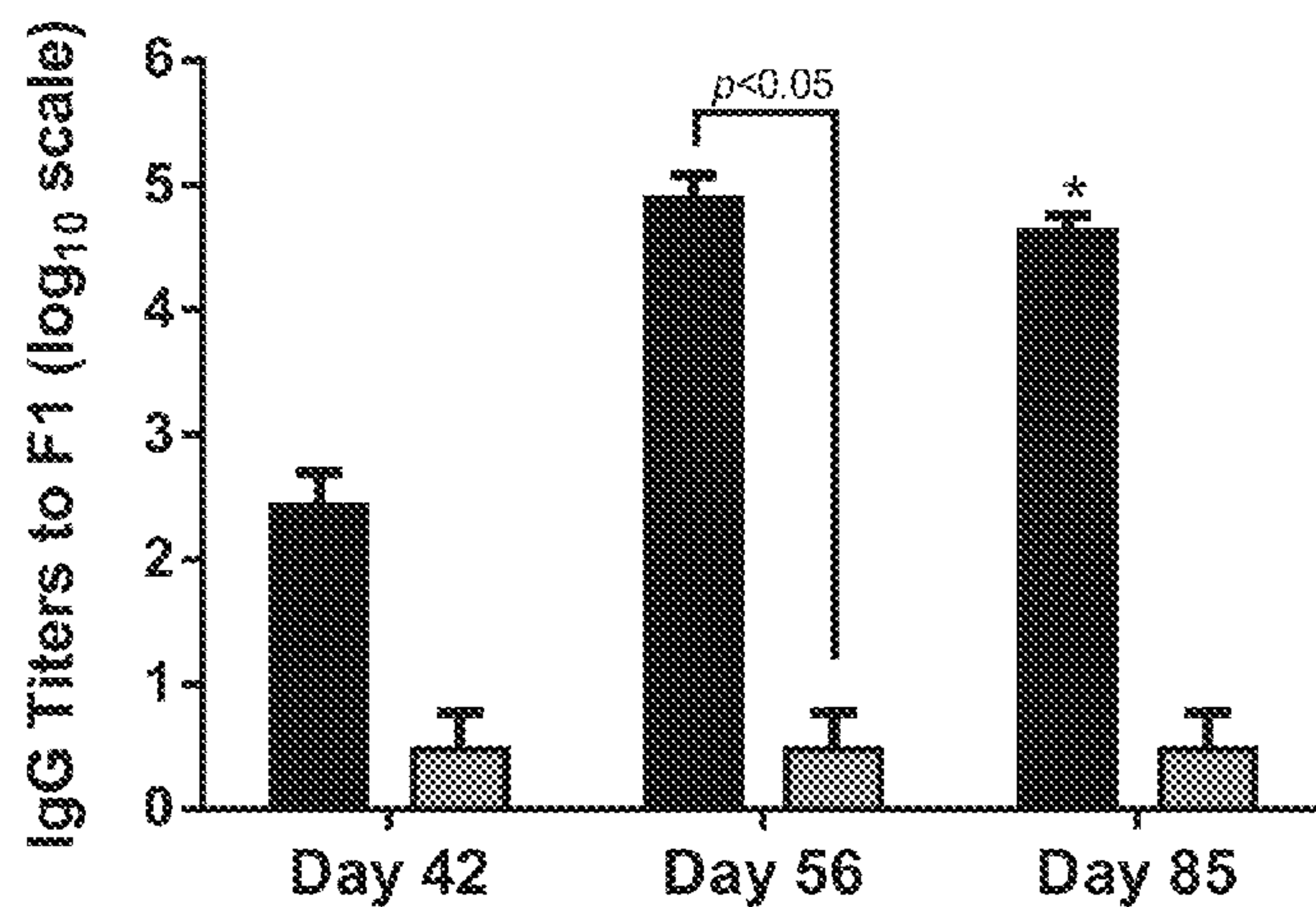


FIG. 12C

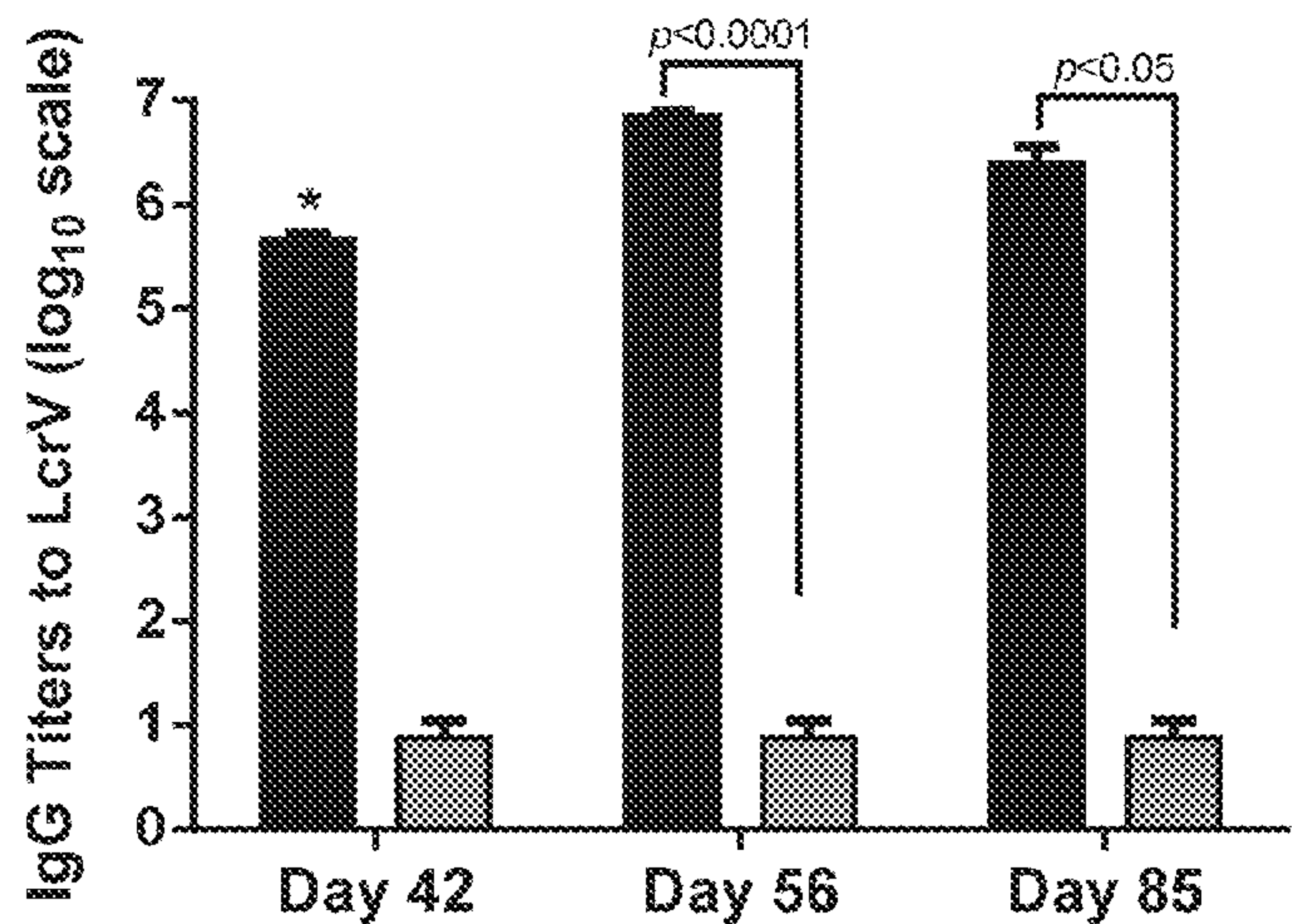




FIG. 12D

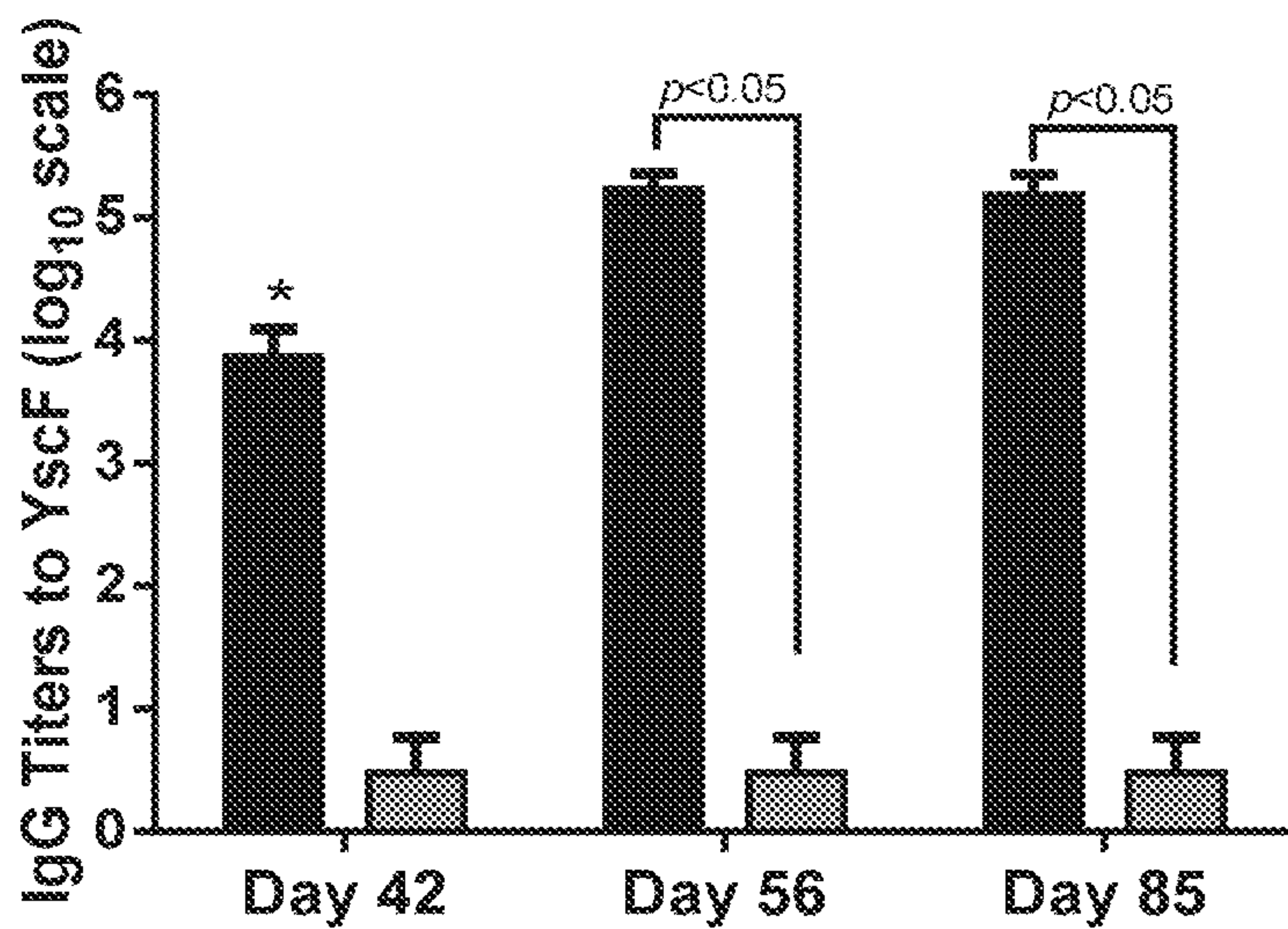


FIG. 12E

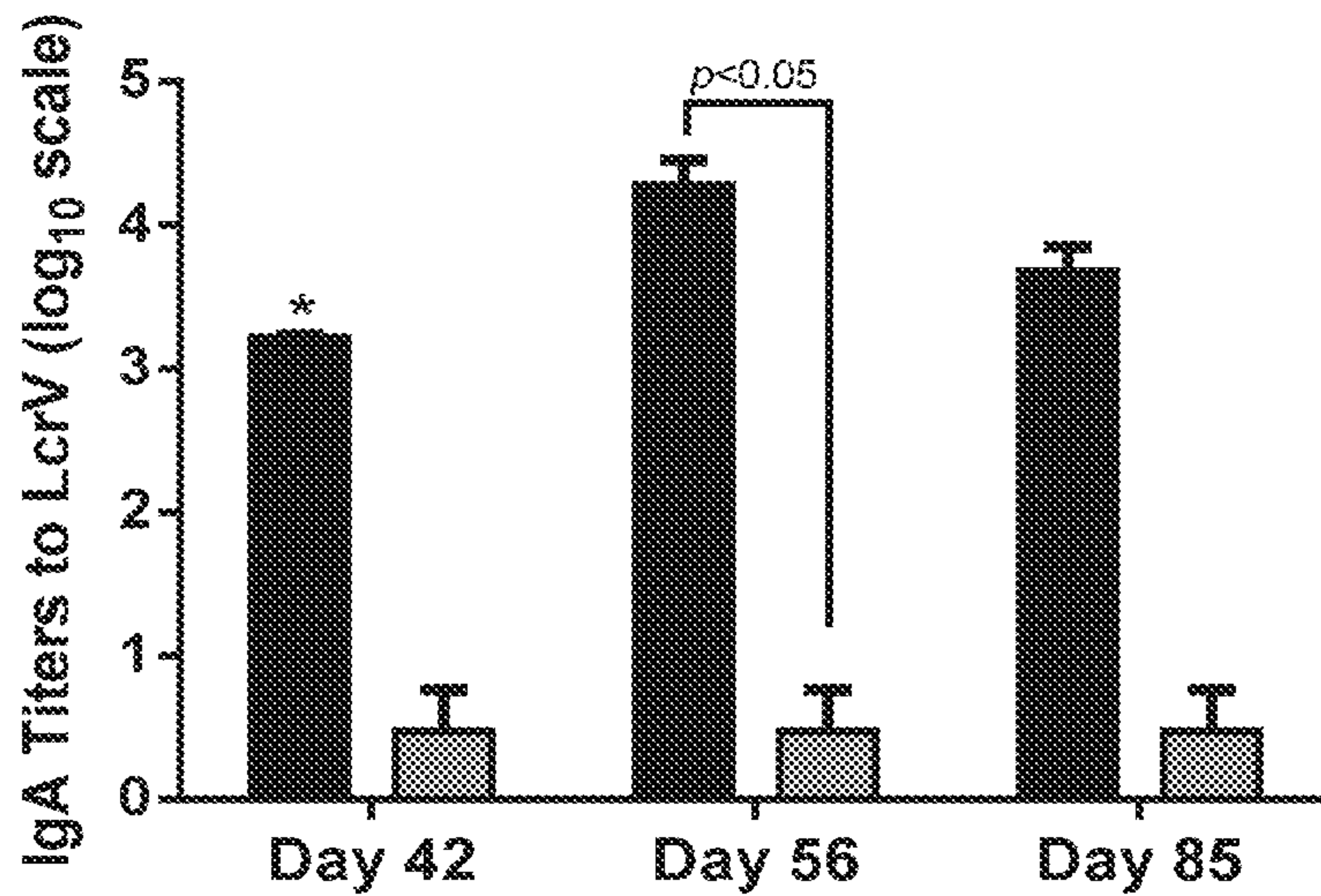


FIG. 13A

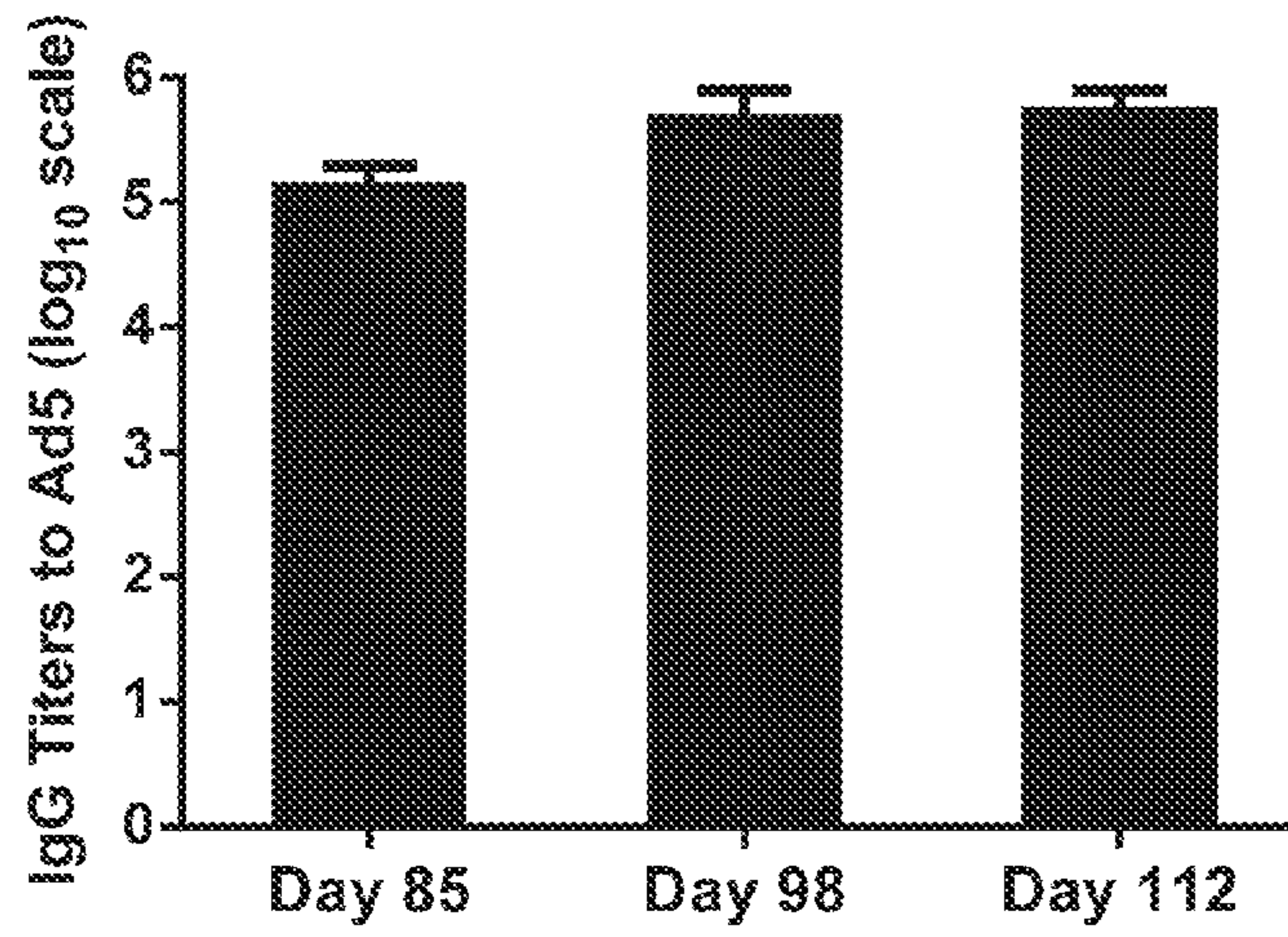


FIG. 13B

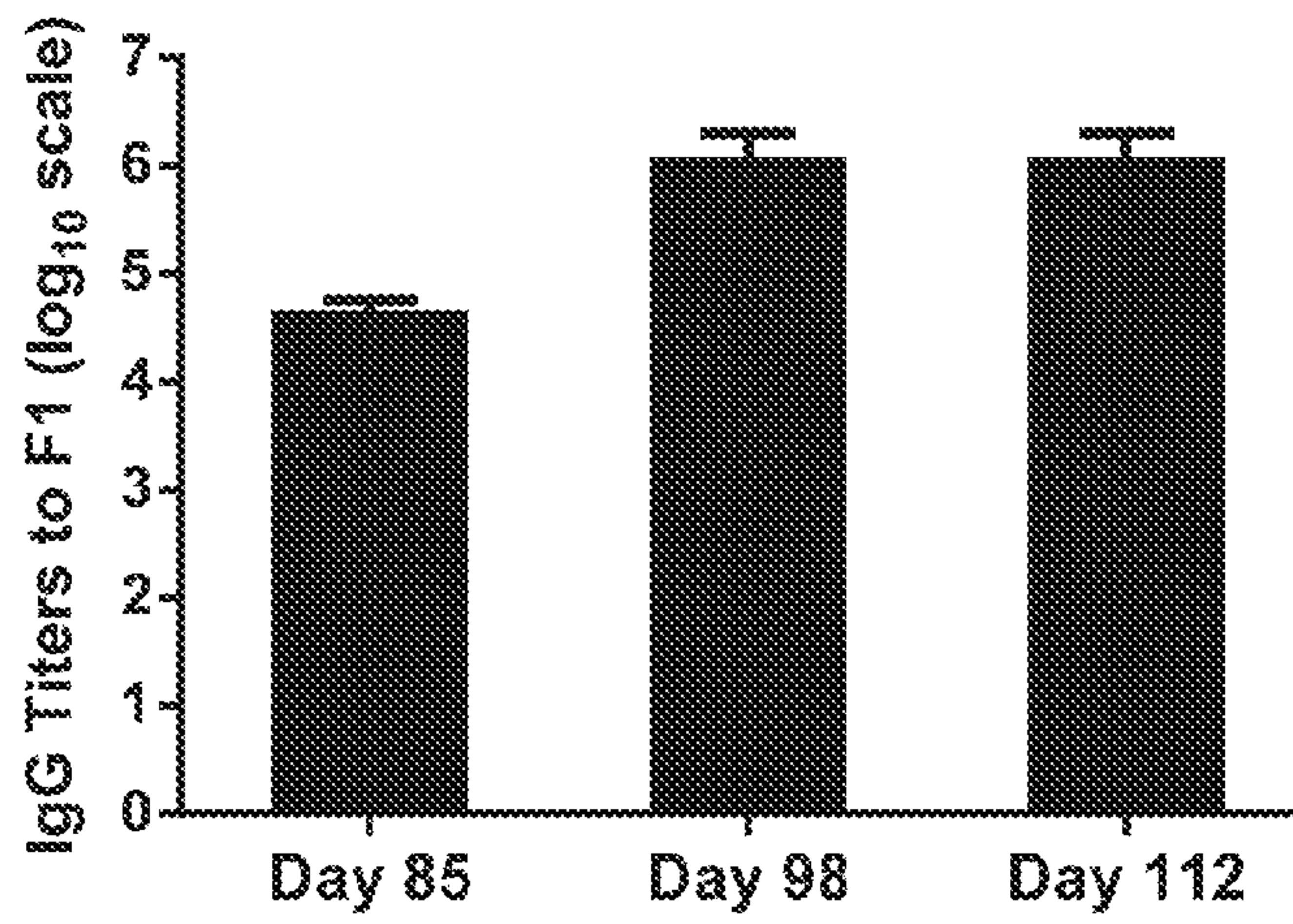


FIG. 13C

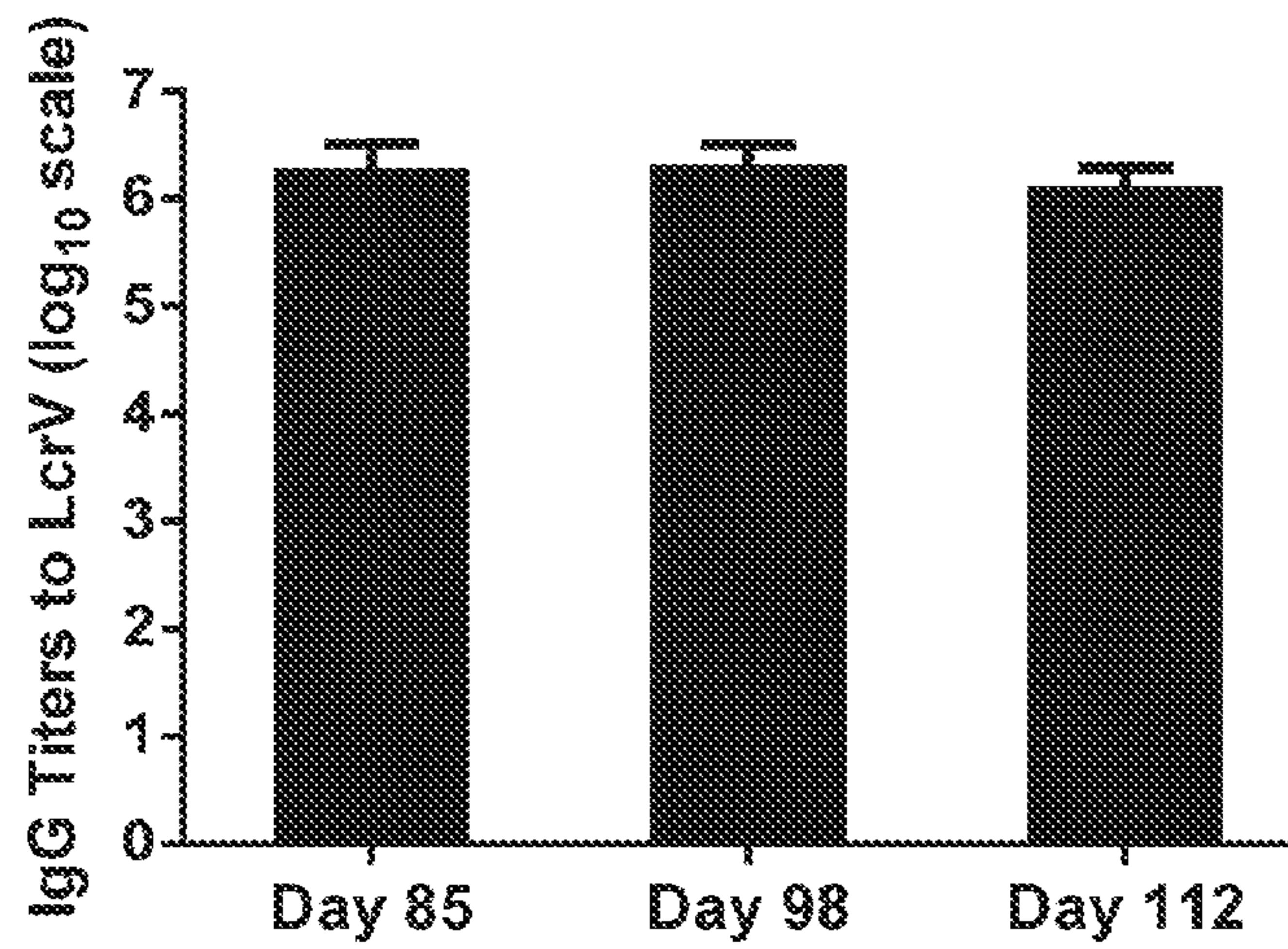


FIG. 13D

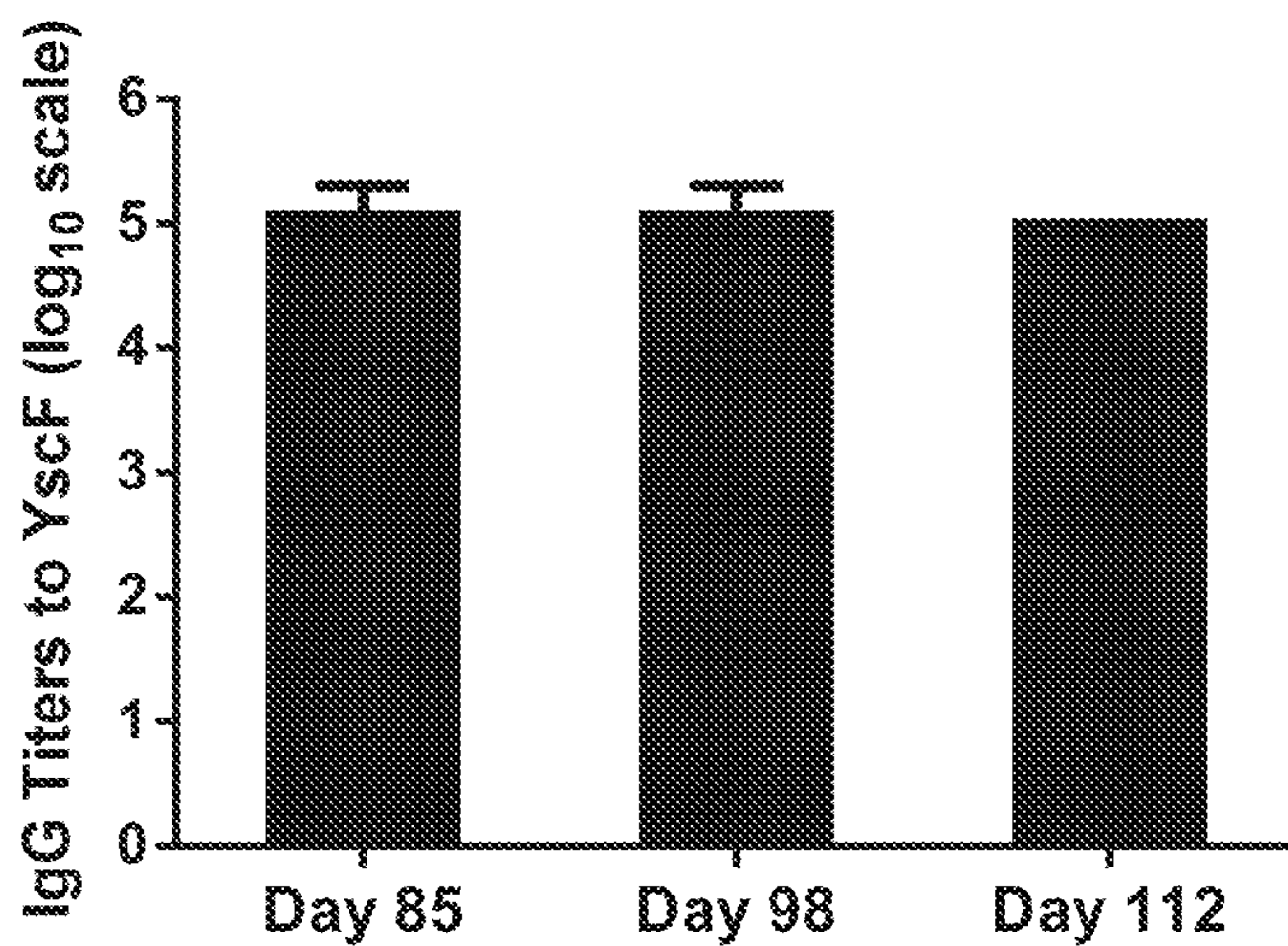


FIG. 13E

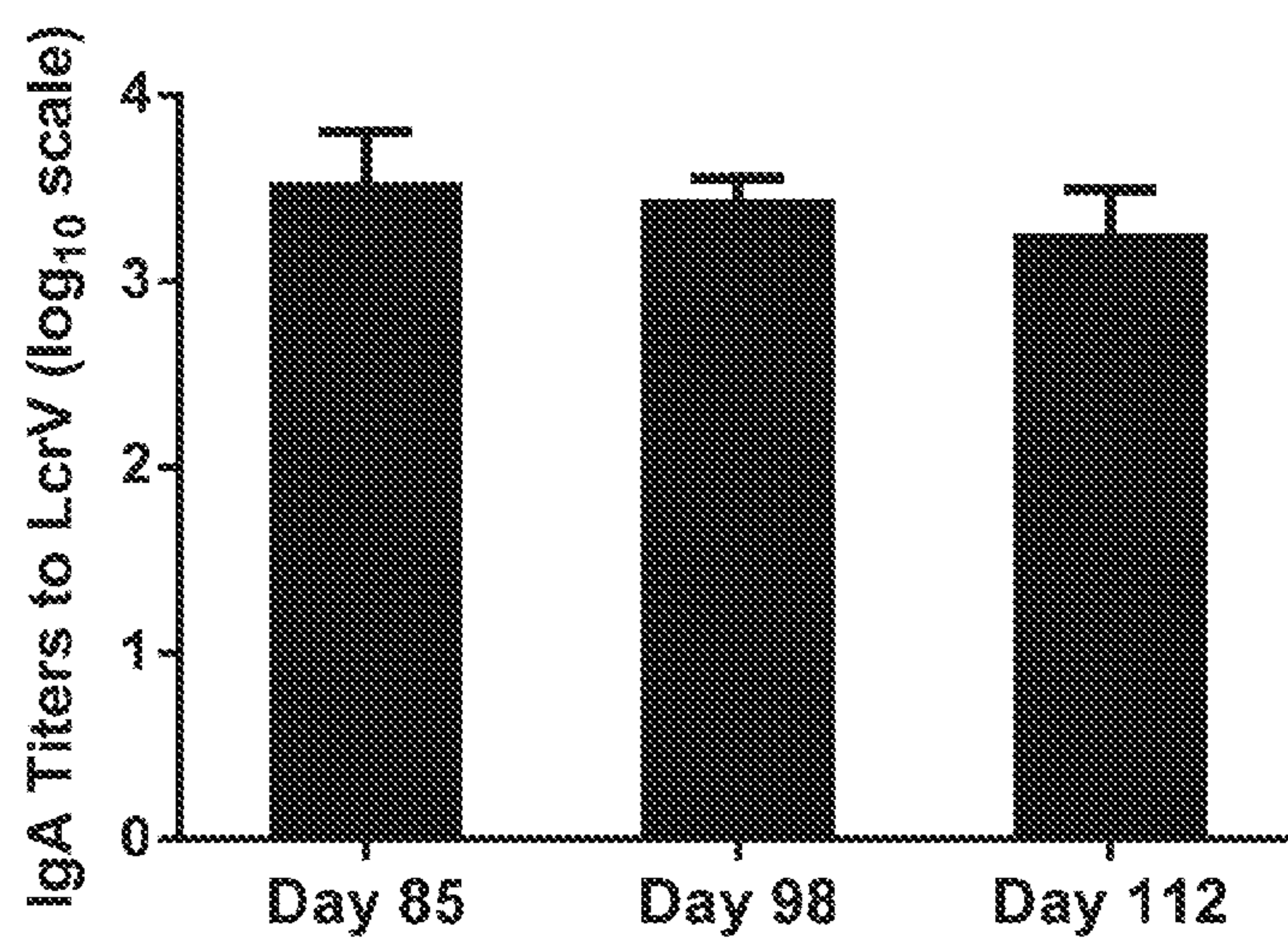
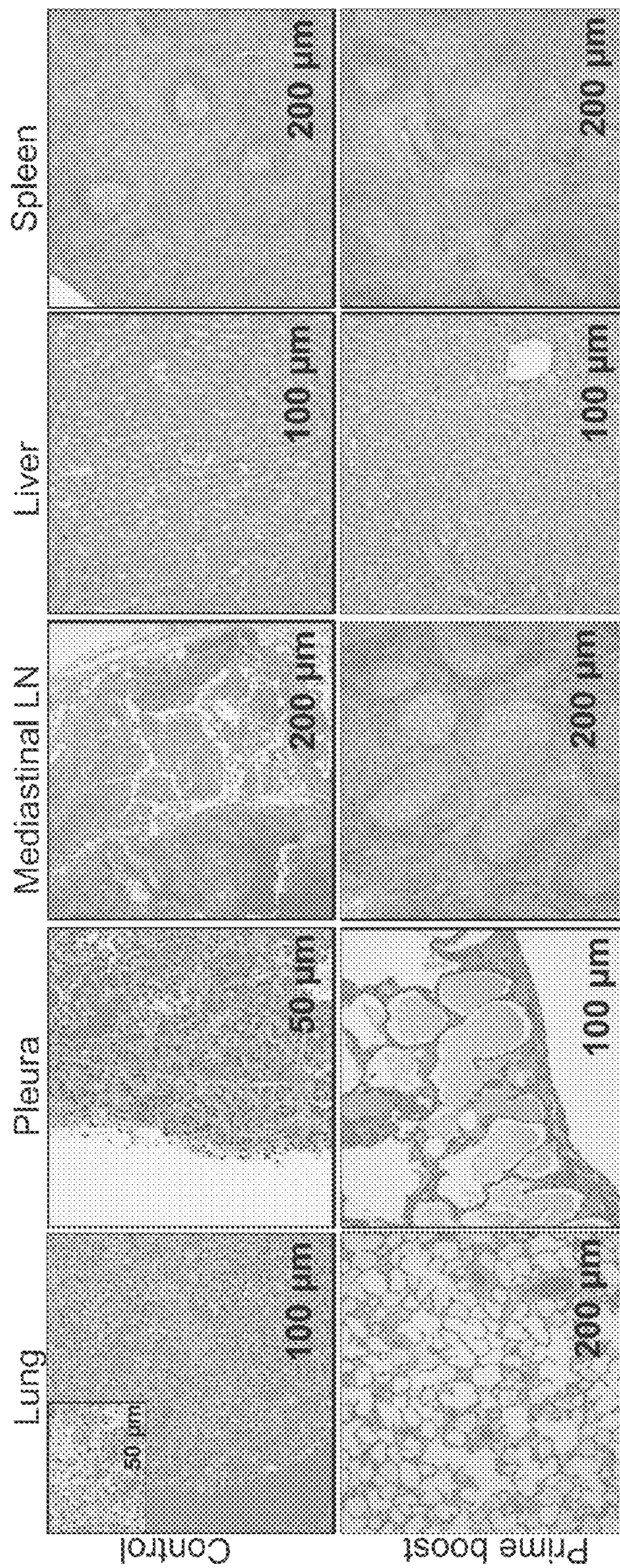




FIG. 14





**FIG. 15-01**

An example of a nucleotide sequence (SEQ ID NO:1) encoding the YscF protein domain SEQ ID NO:2:

```
ATGGCTAATTTCTCCGGGTCACAAAGGGCACATGACATTTGCCGATCTTGATGCCGTTGCCCAGA  
CTCTCAAGAAGCCTGCGGACGATGCCAACAAGGCAGTAAATGATTCATCGCAGCCCAGAAAGA  
CAAGCCTGACAATCCAGCACTCTTGGCCGACCTGCAACATAGTATCAACAAATGGTCTGTAATT  
TACAATATAAACTCTACCATTGTGCGGTCCATGAAAGATCTGATGCAGGGGATCCTGCAAAAAT  
TTCCC
```

An example of a YscF protein domain (SEQ ID NO:2):

```
MANFSGFTKGTDIADLDAVAQTLKKPADDANKAVNDSIAALKDKPDNPALLADLQHSINKWSVI  
YNINSTIVRSMKDLMQGILQKFP
```

An example of a nucleotide sequence (SEQ ID NO:3) encoding the mature F1 protein domain SEQ ID NO:4:

```
GCCGACCTTACAGCTAGTACCACTGCCACAGCAACGCTTGTAGAGCCTGCCCGAATCACCCCTGA  
CGTATAAGGAGGGGGCTCCAATCACATAATGGACAATGGAAACATCGATACCGAACTGCTGGT  
GGGGACCCTGACACTGGGTGGCTACAAGACCGGCACAACCTCCACATCCGTGAACTTCACCGAC  
GCCGCCGGCGATCCCATGTATCTCACATTCACTTCACAGGACGGCAACAATCATCAGTTCACCA  
CTAAGGTGATTGGCAAGGATTCAGAGACTTCGACATCTCTCCCAAGGTGAATGGCGAGAACCT  
CGTGGGGGACGACGTGGTACTGGCAACAGGTCCCAGGATTTCTTTGTCCGGTCCATTGGAAGC  
AAAGGGGGCAAGCTGGCAGCAGGAAAATACACCGACGCAGTTACAGTGACTGTGTCAAACCAG
```

An example of a mature F1 protein domain (SEQ ID NO:4):

```
ADLTASTTATATLVEPARITLTYKEGAPITIMDNGNIDTELLVGTLLTGGYKTGTTSTSVNFTD  
AAGDPMYLTFTSQDGNHQFTTKVIGKDSRFDI SPKVNGENLVGDDVVLATGSQDFVRSIGS  
KGGKLAAGKYTDAVTVTVSNQ
```

**FIG. 15-02**

An example of a nucleotide sequence (SEQ ID NO:5) encoding a LcrV protein domain SEQ ID NO:6:

```
ATGATCCGCGCCTACGAGCAAAATCCTCAGCACTTCATTGAAGACCTTGAGAAGGTGCGCGTGG
AGCAGCTCACAGGCCACGGTAGCAGTGTCTGGAGGAGCTTGTGCAGCTGGTGAAGGACAAGAA
TATCGATATTAGTATAAAAATACGATCCAAGGAAAGACTCTGAGGTGTTGCGGAACCGCGTTATT
ACCGACGATATTGAACTCCTGAAGAAAATCCTGGCCTATTTTTTGGCCAGAGGACGCTATCCTGA
AAGGGGGGCACTATGATAATCAGCTCCAAAATGGTATCAAACGGGTGAAAGAGTTCCTGGAGTC
TAGCCCAAATACTCAGTGGGAGCTGCGGGCCTTTATGGCTGTGATGCACCTTAGTCTGACAGCC
GATCGGATTGACGATGATATCCTTAAGGTGATCGTCGATAGCATGAACCATCATGGTGACGCAA
GAAGTAAACTGAGGGAGGAACTGGCCGAGCTGACTGCAGAGCTCAAAATCTATAGCGTCATACA
GGCCGAAATCAATAAGCACTTGAGCTCATCAGGCACCATTAACATCCACGACAAGTCCATTAAT
CTGATGGACAAAAATCTGTACGGATAACCGACGAGGAGATTTTCAAAGCGTCCGCCGAGTATA
AAATCCTCGAGAAAATGCCTCAGACAACTATACAGGTGGATGGTTCTGAAAAAAGATTGTTTC
TATAAAGGACTTCCTCGGGTCCGAGAACAAAAGGACCGGCGCACTGGGCAATCTCAAGAACTCA
TACAGTTATAATAAAGATAATAATGAGCTTTCCCATTTTGGCCACAACCTGCTCCGACAAAAGTA
GACCTCTGAACGACCTCGTGTCCCAAAGACAACACAGCTGAGTGATATAACCTCCAGGTTCAA
CTCAGCGATCGAGGCTTTGAACAGGTTTCATCCAGAAGTACGATTCAGTGATGCAGAGGCTGTTG
GATGATACTAGCGGTAAG
```

An example of a LcrV protein domain (SEQ ID NO:6):

```
MIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRKDSEVFANRVI
TDDIELLKKILAYFLPEDAILKGGHYDNQLQNGIKRVKEFLESSPNTQWELRAFMAVMHFSLTA
DRIDDDILKVIVDSMNHGHDARSKLREELAEELKIVSVIQAEINKHLSSSGTINIHDKSIN
LMDKNLYGYTDEEIFKASAEYKILEKMPQTTIQVDGSEKKIVSIKDFLGSNKRTGALGNLKN
YSYNKDNNELSHFATTCSDKSRPLNDLVSQKTTQLSDITSRFNSAIEALNRFIQKYDSVMQRL
DDTSGK
```



**FIG. 15-03**

An example of a nucleotide sequence (SEQ ID NO:7) encoding a fusion protein SEQ ID NO:8:

```
ATGGCTAATTTCTCCGGGTTACAAAAGGGCACTGACATTGCCGATCTTGATGCCGTTGCCCAGA
CTCTCAAGAAGCCTGCCGACGATGCCAACAAGGCAGTAAATGATTCCATCGCAGCCCTGAAAGA
CAAGCCTGACAATCCAGCACTCTTGGCCGACCTGCAACATAGTATCAACAAATGGTCTGTAATT
TACAATATAAACTCTACCATTGTGCGGTCCATGAAAGATCTGATGCAGGGGATCCTGCAAAAAT
TTCCCGCCGACCTTACAGCTAGTACCCTGCCACAGCAACGCTTGTAGAGCCTGCCCGAATCAC
CCTGACGTATAAGGAGGGGGCTCCAAFCACAATAAATGGACAATGGAAACAFCGATACCGAACFC
CTGGTGGGGACCCTGACACTGGGTGGCTACAAGACCCGGCACAACCTCCACATCCGTGAACTTCA
CCGACGCCGCCGGCGATCCCATGTATCTCACATTCACCTTCACAGGACGGCAACAATCATCAGTT
CACCCTAAGGTGATTGGCAAGGATTCCAGAGACTTCGACATCTCTCCCAAGGTGAATGGCGAG
AACCTCGTGGGGGACGACGTGGTACTGGCAACAGGTTCACAGGATTTCTTTGTCCGGTCCATTG
GAAGCAAAGGGGGCAAGCTGGCAGCAGGAAAATACACCGACGCAGTTACAGTACTGTGTCAA
CCAGATGATCCGCGCCTACGAGCAAAAATCCTCAGCACTTCATTGAAGACCTTGAGAAGGTGCGC
GTGGAGCAGCTCACAGGCCACGGTAGCAGTGTCTGGAGGAGCTTGTGCAGCTGGTGAAGGACA
AGAATATCGATATTAGTATAAAAATACGATCCAAGGAAAGACTCTGAGGTGTTCCGGAACCCGCT
TATTAACCGACGATAATTGAACTCCTGAAGAAAATCCTGGCCTATTTTTTTGCCAGAGGACGCTATC
CTGAAAGGGGGGCACTATGATAATCAGCTCCAAAATGGTATCAAACGGGTGAAAGAGTTCCTGG
AGTCTAGCCCAAATACTCAGTGGGAGCTGCGGGCCTTTATGGCTGTGATGCACCTTAGTCTGAC
AGCCGATCGGATTGACGATGATATCCTTAAGGTGATCGTCGATAGCATGAACCATCATGGTGAC
GCAAGAAGTAAACTGAGGGAGGAACFGGCCGAGCTGACTGCAGAGCTCAAAAATCTATAGCGTCA
TACAGGCCGAAATCAATAAGCACTTGAGCTCATCAGGCACCATTAACATCCACGACAAGTCCAT
TAATCTGATGGACAAAAATCTGTACGGATAACCGACGAGGAGATTTTCAAAGCGTCCGCCGAG
TATAAAAATCCTCGAGAAAATGCCTCAGACAACATAACAGGTGGATGGTTCTGAAAAAAGATTG
TTTTCTATAAAGGACTTCCTCGGGTCCGAGAACAAAAGGACCCGGCGCACTGGGCAATCTCAAGAA
CTCATACAGTTATAATAAAGATAATAATGAGCTTTCCCATTTTGGCACAACCTGCTCCGACAAA
AGTAGACCTCTGAACGACCTCGTGTCCAAAAGACAACACAGCTGAGTGATATAACCTCCAGGT
TCAACTCAGCGATCGAGGCTTTGAACAGGTTTCATCCAGAAGTACGATTCAGTGATGCAGAGGCT
GTTGGATGATACTAGCGGTAAG
```

An example of a fusion protein (SEQ ID NO:8):

```
MANFSGFTKGTDIADLDAVAQTLKKPADDANKAVNDSIAALKDKPDNPALLADLQHSINKWSVI
YNINSTIVRSMKDLMQGILQKFPADLTASTTATATLVEPARITLTYKEGAPITIMDNGNIDTEL
LVGTLTLGGYKTGTTSTSVNFTDAAGDPMYLTFTSQDGNHQTFTTKVIGKDSRDFDISPKVNGE
NLVGDDVVLATGSQDFVRSIGSKGGKLAAGKYTDVAVTVTSNQMIRAYEQNPQHFIEDLEKVR
VEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRKDSEVFANRVITDDIELLKKILAYFLPEDAI
LKGGHYDNQLQNGIKRVKEFLESSPNTQWELRAFMAVMHFSLTADRIDDDILKVIIVDSMNHHGD
ARSKLREELAELTAEIKIYSVIQAEINKHLSSSGTINIHDKSINLMDKNLYGYTDEEIFKASAE
YKILEKMPQTTIQVDGSEKKIVSIKDFLGSNKRTGALGNLKNSSYSYNKDNNELSHEFATTCSDK
SRPLNDLVSQKTTQLSDITSRFNSAIEALNRFIQKYDSVMQRLLDDTSGK
```

**FIG. 15-04**

An example of a nucleotide sequence (SEQ ID NO:9) encoding a fusion protein including linkers  
SEQ ID NO:10:

ATGGCTAATTTCTCCGGGTTACAAAGGGCACTGACATTGCCGATCTTGATGCCGTTGCCCAGA  
CTCTCAAGAAGCCTGCCGACGATGCCAACAAGGCAGTAAATGATTCCATCGCAGCCCTGAAAGA  
CAAGCCTGACAATCCAGCACTCTTGGCCGACCTGCAACATAGTATCAACAAATGGTCTGTAATT  
TACAATATAAACTCTACCATTGTGCGGTCCATGAAAGATCTGATGCAGGGGATCCTGCAAAAAT  
TTCCCGGGGGCGGGGTTCCGGGGGAGGCGGTAGTGGCGGCGGTGGATCAGCCGACCTTACAGC  
TAGTACCACCTGCCACAGCAACGCTTGTAGAGCCTGCCCGAATCACCCCTGACGTATAAGGAGGGG  
GCTCCAATCACAAATAATGGACAATGGAAACATCGATACCGAACTGCTGGTGGGGACCCCTGACAC  
TGGGTGGCTACAAGACCGGCACAACCTCCACATCCGTGAACCTCACCGACGCCGCCGGCGATCC  
CATGTATCTCACATTCACCTTACAGGACGGCAACAATCATCAGTTCACCACCTAAGGTGATTGGC  
AAGGATTCCAGAGACTTCGACATCTCTCCCAAGGTGAATGGCGAGAACCTCGTGGGGGACGACG  
TGGTACTGGCAACAGGTTCCCAGGATTTCTTTGTCCGGTCCATTGGAAGCAAAGGGGGCAAGCT  
GGCAGCAGGAAAATACACCGACGCAGTTACAGTGACTGTGTCAAACCAGGGAGGCGGTGGATCC  
GGAGGCGGAGGCTCAGGAGGCGGGGGGAGCATGATCCGCGCCTACGAGCAAAAATCCTCAGCACT  
TCATTGAAGACCTTGAGAAGGTGCCGCTGGAGCAGCTCACAGGCCACGGTAGCAGTGTCTCTGGA  
GGAGCTTGTGCAGCTGGTGAAGGACAAGAATATCGATATTAGTATAAAAATACGATCCAAGGAAA  
GACTCTGAGGTGTTCCGGAACCGCGTTATTACCGACGATATTGAACTCCTGAAGAAAATCCTGG  
CCTATTTTTTGGCAGAGGACGCTATCCTGAAAGGGGGGCACATGATAATCAGCTCCAAAATGG  
TATCAAACGGGTGAAAGAGTTCCTGGAGTCTAGCCCAAATACTCAGTGGGAGCTGCGGGCCTTT  
ATGGCTGTGATGCACITTAGTCTGACAGCCGATCGGATTGACGATGATATCCTTAAGGTGATCG  
TCGATAGCATGAACCATCATGGTGACGCAAGAAGTAAACTGAGGGAGGAACTGGCCGAGCTGAC  
TGCAGAGCTCAAAAATCTATAGCGTCATACAGGCCGAAATCAATAAGCACTTGAGCTCATCAGGC  
ACCATTAACATCCACGACAAGTCCATTAATCTGATGGACAAAATCTGTACGGATATAACCGACG  
AGGAGATTTTCAAAGCGTCCGCCGAGTATAAAAATCCTCGAGAAAATGCCTCAGACAACATAACA  
GGTGGATGGTTCTGAAAAAAGATTGTTTTCTATAAAGGACTTCCTCGGGTCCGAGAACAAAAGG  
ACCGGCGCACTGGGCAATCTCAAGAACTCATACAGTTATAATAAAGATAATAATGAGCTTTCCC  
ATTTTGCCACAACCTGCTCCGACAAAAGTAGACCTCTGAACGACCTCGTGTCCCAAAGACAAC  
ACAGCTGAGTGATATAACCTCCAGGTTCAACTCAGCGATCGAGGCTTTGAACAGGTTTCATCCAG  
AAGTACGATTCAGTGATGCAGAGGCTGTTGGATGATACTAGCGGTAAG

An example of a fusion protein including linkers (SEQ ID NO:10):

MANFSGFTKGTDIADLDAVAQTLKKPADDANKAVNDSIAALKDKPDNPALLADLQHSINKWSVI  
YNINSTIVRSMKDLMQGILQKFPGGGGSSGGGSSGGGSSADLTASTTATATLVEPARITLTYKEG  
APITIMDNGNIDTELLVGTLLGGYKTGTTSTSVNFTDAAGDPMYLTFTSQDGNHQQFTTKVIG  
KDSRDFDISPKVNGENLVGDDVVLATGSQDFFVRSIGSKGGKLAAGKYTDAVTVTVSNQGGGGS  
GGGGSSGGGSMIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRK  
DSEVFANRVITDDIELLKKILAYFLPEDAILKGGHYDNQLQNGIKRVKEFLESSPNTQWELRAF  
MAVMHFSLTADRIDDDBILKVIIVDSMNHHGDARSKLREELAEELKIIYSVIQAEINKHLSSSG  
TINIHDKSINLMDKNLYGYTDEEIKFASAEYKILEKMPQTTIQVDGSEKKIVSIKDFLSENKR  
TGALGNLKNSSYNKDNNELSHFATTCSDKSRPLNDLVSQKTTQLSDITSRFNSAIEALNRFIQ  
KYDSVMQRLLDDTSGK



## METHODS FOR TREATING PLAGUE

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.**

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application *is a reissue of U.S. Pat. No. 10,076,562, which claims the benefit of U.S. Provisional Application Ser. No. 62/324,528, filed Apr. 19, 2016, which [is] are incorporated by reference herein.*

## GOVERNMENT FUNDING

This invention was made with government support under grant number AI071634, awarded by the NIH. The government has certain rights in the invention.

## SEQUENCE LISTING

This application contains a Sequence Listing electronically submitted via EFS-Web to the United States Patent and Trademark Office as an ASCII text file entitled "265-00920101-SequenceListing\_ST25.txt" having a size of 24 kilobytes and created on Jun. 22, 2017. The information contained in the Sequence Listing is incorporated by reference herein.

## SUMMARY OF THE APPLICATION

Provided herein are methods that include administering a first composition to a subject. The administration is to a mucosal surface, and in one embodiment the administration is by an intranasal route. The first composition includes a vector that has a polynucleotide encoding a fusion protein, where the fusion protein includes a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain. The method also includes administering a second composition to the subject by a different route, such as an intramuscular route. The second composition includes a fusion protein having the same three domains, and in one embodiment the fusion protein is the same one administered by an intranasal route. In one embodiment, the fusion protein is isolated. The second composition is administrated after the intranasal administration.

In one embodiment, the fusion protein includes at least one linker, where the linker is present between two of the domains. In one embodiment, the fusion protein includes a His-tag. In one embodiment, the vector is a replication defective adenovirus vector, such as a type-5 (Ad5). In one embodiment, the fusion protein includes the YscF protein, the mature F1 protein, and the LcrV protein. In one embodiment, the second administration is at least 7 days after the intranasal administration. In one embodiment, the subject is a human. In one embodiment, the administering confers immunity to plague, such as pneumonic plague, caused by *Yersinia pestis*.

As used herein, the term "protein" refers broadly to a polymer of two or more amino acids joined together by peptide bonds. The term "protein" also includes molecules which contain more than one protein joined by a disulfide

bond, or complexes of proteins that are joined together, covalently or noncovalently, as multimers (e.g., dimers, tetramers). Thus, the terms peptide, oligopeptide, and polypeptide are all included within the definition of protein and these terms are used interchangeably.

As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides, deoxynucleotides, peptide nucleic acids, or a combination thereof, and includes both single-stranded molecules and double-stranded duplexes. A polynucleotide can be obtained directly from a natural source, or can be prepared with the aid of recombinant, enzymatic, or chemical techniques. In one embodiment, a polynucleotide is isolated. A polynucleotide can be linear or circular in topology. A polynucleotide can be, for example, a portion of a vector, such as an expression or cloning vector, or a fragment.

As used herein, an "isolated" substance is one that has been removed from a cell and many of the proteins, nucleic acids, and other cellular material of its natural environment are no longer present. A substance may be purified, i.e., at least 60% free, at least 75% free, or at least 90% free from other components with which they are naturally associated. Proteins and polynucleotides that are produced by recombinant, enzymatic, or chemical techniques are considered to be isolated and purified by definition, since they were never present in a cell. For instance, a protein, a polynucleotide, or a viral particle can be isolated or purified.

As used herein, the terms "coding region," "coding sequence," and "open reading frame" are used interchangeably and refer to a nucleotide sequence that encodes a protein and, when placed under the control of appropriate regulatory sequences expresses the encoded protein. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end.

A "regulatory sequence" is a nucleotide sequence that regulates expression of a coding sequence to which it is operably linked. Nonlimiting examples of regulatory sequences include promoters, enhancers, transcription initiation sites, translation start sites, translation stop sites, transcription terminators, and poly(A) signals. The term "operably linked" refers to a juxtaposition of components such that they are in a relationship permitting them to function in their intended manner. A regulatory sequence is "operably linked" to a coding region when it is joined in such a way that expression of the coding region is achieved under conditions compatible with the regulatory sequence.

The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

It is understood that wherever embodiments are described herein with the language "include," "includes," or "including," and the like, otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.



Unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows immunoblot analysis of recombinant adenoviruses. Human lung epithelial cells A549 were infected with rAd5 constructs at 1000 v.p. per cell. Host cell lysates were harvested after 24 h p.i. An aliquot of the cell lysates was then resolved by SDS-PAGE and subjected to Western blot analysis by using mAb-LcrV antibody. Lane 1: Standard protein molecular weight markers in kilo-daltons (kDa). Lanes 2-4: A549 cells infected with rAd5-LcrV, rAd5-YFV and Ad5-empty, respectively. Lane 5: Purified rLcrV (50 ng). Lane 6: Purified rYFV (30 ng). The HRP-labeled anti-mouse secondary antibody and ECL Western blotting reagent kit (Millipore, Billerica, Mass.) was used for protein detection.

FIG. 2A-2C shows protection conferred by immunization of mice with the purified recombinant proteins. Naïve mice (n=40) were immunized with either the mixture of three recombinant proteins (rYscF, rF1, and rLcrV, 25 µg/each) or 45 µg of the corresponding recombinant fusion protein (rYFV) via the i.m. route. The antigens were emulsified 1:1 in Alum adjuvant. One primary immunization and two identical boosters were given on days 0, 15 and 30. Naïve mice received the adjuvant only and served as a control. Mice were bled 14 days post last immunization and an ELISA was performed to examine IgG and its isotype antibody titers to the LcrV antigen (FIG. 2A). The P values were in comparison to the indicated groups and were based on Two-way ANOVA (IgG1 and IgG2a) with the Tukey's post hoc correction. The above immunized and control mice were then split into two sets and challenged on day 15 post immunization either subcutaneously (s.c.) with 8500 LD<sub>50</sub> (FIG. 2B) or intranasally (i.n.) with 800 LD<sub>50</sub> (FIG. 2C) of the WT CO92. The P values were in comparison to the control group and were based on Kaplan-Meier Curve Analysis.

FIG. 3A-3C shows immunization routes comparison in mice. Naïve mice (n=40) were either i.m. or i.n. immunized with one dose (8×10<sup>9</sup> v.p.) of rAd5-LcrV or rAd5-YFV vaccines. Animals received the same dose of Ad5-Empty which was split equally into i.m. injection and i.n. instillation, and served as a control. The above immunized and control mice were then divided into two sets and challenged on day 15 post immunization either subcutaneously (s.c.) with 60 LD<sub>50</sub> (FIG. 3A) or intranasally (i.n.) with 90 LD<sub>50</sub> (FIG. 3B) of the WT CO92. The P values were in comparison to the control group and were based on Kaplan-Meier Curve Analysis. Mice were also bled prior to the challenge to evaluate IgG antibody titers and that of its isotypes to LcrV by ELISA (FIG. 3C). The P values were in comparison to the indicated groups and were based on Two-way ANOVA (IgG1 and IgA) with the Tukey's post hoc correction.

FIG. 4A-4C shows protection conferred by immunization with the recombinant adenoviruses in mice that had pre-existing immunity to adenovirus. To establish pre-existing

immunity to adenovirus, naïve mice (n=40) received a single dose (8×10<sup>9</sup> v.p./100 µl) in both quadriceps (50 µl each) of the Ad5-Empty by i.m. injection 30 days prior to vaccination. Naïve mice receiving saline served as a control. Subsequently, mice were i.n. immunized with one dose (8×10<sup>9</sup> v.p.) of rAd5-LcrV or rAd5-YFV vaccines. Animals received the same dose of Ad5-Empty by i.n. instillation, and served as a negative control. The above mice were then divided into two sets and challenged on day 15 post immunization either subcutaneously (s.c.) with 24 LD<sub>50</sub> (FIG. 4A) or intranasally (i.n.) with 21 LD<sub>50</sub> (FIG. 4B) of the WT CO92. The P values were in comparison to the negative control group and were based on Kaplan-Meier Curve Analysis. Mice were also bled prior to the challenge to evaluate IgG antibody titers, titers to its isotypes, and IgA to LcrV by ELISA (FIG. 4C). The P values were in comparison to the indicated groups and were based on Two-way ANOVA with the Tukey's post hoc correction. The asterisks indicated statistical significance compared to the control (Ad5-Empty) mice for IgA levels by using multiple Student's t-test with the Holm-sidak post hoc test correction.

FIG. 5 shows prime-boost immunization provided better protection to mice against lethal WT CO92 aerosol challenge. PreAd-mice (groups of 20) were either i.n.-immunized with 8×10<sup>9</sup> v.p./40 µl of rAd5-YFV alone or in the combination with 10 µg of rYFV (emulsified 1:1 in Alum adjuvant) i.m. The immunization occurred two weeks apart. Naïve mice immunized with either 10 µg of rYFV (i.m.) or 8×10<sup>9</sup> v.p./40 µl (i.n.) of rAd5-YFV alone were used for comparison, and PreAd-mice without further immunizations served as a negative control. After 15 days post immunization, mice were challenged by the aerosol route with WT CO92 at a Dp of 6.34×10<sup>5</sup> CFU. The P values were in comparison to the negative control group and were based on Kaplan-Meier Curve Analysis.

FIG. 6A-6C. T cell mediated immune response in mice elicited by immunization with the rAd5-YFV vaccine alone or in combination with rYFV. PreAd-mice (n=10-25) were either i.n. immunized with 8×10<sup>9</sup> v.p./40 µl of rAd5-YFV alone or in the combination with 10 µg of rYFV (emulsified 1:1 in Alum adjuvant) i.m. The immunizations occurred two weeks apart. After 15 days post immunization, 20 mice from each immunized and 10 from control group were aerosol challenged with WT CO92 at a Dp of 4.62×10<sup>5</sup> CFU. The P values were in comparison to the negative control group or between groups (as indicated by the arrow) and were based on Kaplan-Meier Curve Analysis (FIG. 6A). On day 15 post last immunization, T cells were isolated separately from the spleens of remaining unchallenged 5 mice in each immunized group. The isolated T cells were co-cultured with γ-irradiated APCs pulsed or un-pulsed with F1-V fusion protein (100 µg/ml). The IFN-γ producing T cells were measured after 2 days of incubation with the APCs by using the enzyme-linked immunospot (Elispot) assay (FIG. 6B). T cell proliferation was assessed by measuring incorporation of [<sup>3</sup>H] thymidine on day 3 of co-culture with the APCs (FIG. 6C). The arithmetic means±standard deviations were plotted. Data were analyzed by using Two-way ANOVA with the Tukey's post hoc correction. The statistical significance was indicated by asterisks in comparison of the pulsed and un-pulsed T cells within each group or displayed by a horizontal line with the P value.

FIG. 7A-7C shows antibody responses in mice elicited by immunization with the rAd5-YFV vaccine alone or in combination with rYFV. Mice from different groups (FIG. 6A-6C) were also bled 15 days post immunization, and an ELISA was performed to examine IgG antibody titers, its



isotypes, and IgA to the F1 (FIG. 7A), LcrV (FIG. 7B) and YscF (FIG. 7C), respectively. The P values were in comparison to the indicated groups and based on Two-way ANOVA with the Tukey's post hoc correction. The asterisks indicated statistical significance compared to the control (Ad5-Empty) mice for IgA levels by using multiple Student's t-test with the Holm-sidak post hoc test correction.

FIG. 8A-8B-02 shows immunization of mice with the rAd5-YFV vaccine alone or in combination with rYFV provided protection against lethal primary aerosol and subsequent intranasal WT CO92 challenges. PreAd-mice (n=10) were either i.n.-immunized with  $8 \times 10^9$  v.p./40  $\mu$ l of rAd5-YFV alone or in the combination with 10  $\mu$ g of rYFV (emulsified 1:1 in Alum adjuvant) i.m. The immunizations occurred two weeks apart. PreAd-mice injected with Ad5-Empty served as a negative control. After 15 days post immunization, mice were first challenged with aerosolized WT CO92 at a Dp of  $4.62 \times 10^5$  CFU. After 32 days of the initial aerosol challenge, the survivals from the immunized groups along with five age-matched uninfected naïve mice were infected with 100 LD<sub>50</sub> of WT CO92 luc2 strain by the i.n. route. The deaths were recorded for the initial aerosol and then the subsequent intranasal challenge, and the percentages of survival were plotted (FIG. 8A). The P values were in comparison to the control group for each challenge and were based on Kaplan-Meier Curve Analysis. The animals were also imaged by IVIS for bioluminescence on day 3 after WT CO92 luc2 strain i.n. challenge (FIGS. 8B-01 and 8B-02). Panel B-I represented infected naïve mice as i.n. challenge control and the very right animal in this panel was uninfected image control. Panel B-II, animals immunized with the prime-boost strategy, and panel B-III, animals immunized with rAd5-YFV vaccine alone. The bioluminescence scale is within the figures and ranged from most intense (top of range) to least intense (bottom of range).

FIG. 9 shows the rAd5-YFV vaccine in combination with rYFV provided protection to NHPs with pre-existing adenovirus immunity against lethal aerosol challenge of WT CO92. To induce pre-existing adenovirus immunity, four NHPs were injected in the quadriceps muscle with  $5 \times 10^{10}$  v.p. of Ad5-Empty (day 0). On day 30, these NHPs were immunized by the intranasal route with  $1 \times 10^{11}$  v.p. of rAd5-YFV, followed by 50  $\mu$ g of rYFV boost (emulsified 1:1 in Alum adjuvant) via the i.m. route on day 42. Another four NHPs received saline only (without immunization) and served as a control. On day 85, the NHPs were challenged with WT CO92 by the aerosol route with a Dp ranging from  $1.32$  to  $8.08 \times 10^7$  CFU. The animals were euthanized when reached a clinical score  $\geq 8$  or at the termination of the experiment, and percentage of survival was plotted. The P values were in comparison to the NHP control group and are based on Kaplan-Meier Curve Analysis.

FIG. 10A-10C shows CT scans. NHPs were subjected to CT scan on day 42 (naïve and vaccinated) (FIG. 10A) and on day 88 (3 days post WT CO92 challenge) for the control NHPs (FIG. 10B) or day 167 (82 days post WT CO92 challenge) (FIG. 10C) for the immunized ones. The coronal and sagittal images of the lungs and their surrounding areas from representing NHPs were shown with the resolution of  $512 \times 512$  pixels. The image sharpness was optimized to soft tissue. The arrows indicated consolidation patches in the lungs of a representative infected control NHP.

FIG. 11A-11B shows hematologic analysis. Blood samples of immunized (FIG. 11A) and unimmunized control (FIG. 11B) NHPs were collected from the femoral veins and analyzed on the day of challenge with WT CO92 and on days 3 and 6 post challenge (days 88 and 91 post immuni-

zation and challenge) by using a Drew Scientific Hemavet 950 hematology system. WBC: white blood cells; NE: neutrophils; LY: lymphocytes. The arithmetic means  $\pm$  standard deviations of the cell counts/ $\mu$ l were plotted. The dotted lines indicated the physiological ranges for each of the corresponding parameters measured.

FIG. 12A-12E shows antibody responses in NHPs immunized with the rAd5-YFV vaccine in combination of rYFV. Four randomly selected NHPs were injected in the quadriceps muscle with  $5 \times 10^{10}$  v.p. of Ad5-Empty to induce pre-existing immunity (day 0). On day 30, these NHPs were immunized by the intranasal route with  $1 \times 10^{11}$  v.p. of rAd5-YFV, followed by 50  $\mu$ g of rYFV boost (emulsified 1:1 in Alum adjuvant) via the i.m. route on day 42. Another four NHPs received saline only (without immunization) and served as a control. On day 85, the NHPs were challenged with WT CO92 by the aerosol route. Blood samples were collected from the femoral veins of NHPs at various time points during the experiment. The total IgG titers to Ad5 (FIG. 12A), F1 (FIG. 12B), LcrV (FIG. 12C), and YscF (FIG. 12D) as well as IgA titers to LcrV (FIG. 12E) on days 42, 56, and 85 were evaluated by ELISA. The P values were in comparison to the indicated groups and were based on Two-way ANOVA with the Tukey's post hoc correction. The asterisks indicated statistical significance compared to the control (Ad5-Empty) mice by using multiple Student's t-test with the Holm-sidak post hoc test correction.

FIG. 13A-13E shows antibody responses of vaccinated NHPs after WT CO92 aerosol challenge. Four randomly selected NHPs were injected in the quadriceps muscle with  $5 \times 10^{10}$  v.p. of Ad5-Empty to induce pre-existing immunity (day 0). On day 30, these NHPs were immunized by the intranasal route with  $1 \times 10^{11}$  v.p. of rAd5-YFV, followed by 50  $\mu$ g of rYFV boost (emulsified 1:1 in Alum adjuvant) via the i.m. route on day 42. Another four NHPs received saline only (without immunization) and served as a control. On day 85, the NHPs were challenged with WT CO92 by the aerosol route. Blood samples were collected from the femoral veins of NHPs at various time points during the experiment from the immunized NHPs. The total IgG titers to Ad5 (FIG. 13A), F1 (FIG. 13B), LcrV (FIG. 13C), and YscF (FIG. 13D) as well as total IgA titers to LcrV (FIG. 13E) on days 85, 98 and 112 were evaluated by ELISA. Days 98 and 112 represented 14 and 28 days post WT CO92 challenge after immunization.

FIG. 14 shows histopathological analysis of tissues collected from NHP after WT CO92 aerosol challenge. Lungs, pleura, mediastinal lymph nodes, liver and the spleen tissues were collected from the control (3 or 4 day post WT CO92 challenge) and immunized NHPs (82 days post WT CO92 challenge) after euthanization and processed for histopathological analysis. The inset from lungs revealed the presence of coccobacilli, presumptively *Y. pestis*, by Gram staining. The magnification of each image is indicated.

FIG. 15-01-15-04 shows protein sequences and examples of nucleotide sequences encoding the proteins.

#### DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Provided herein are methods for using a fusion protein. The fusion protein includes at least three protein domains. The three domains are a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain. A fusion protein can be isolated, and optionally purified.



An example of a YscF protein domain is depicted at SEQ ID NO:2. Other examples of YscF protein domains include those having sequence similarity with the amino acid sequence of SEQ ID NO:2.

An example of a mature F1 protein domain is depicted at SEQ ID NO:4. Other examples of mature F1 protein domains include those having sequence similarity with the amino acid sequence of SEQ ID NO:4.

An example of a LcrV protein domain is depicted at SEQ ID NO:6. Other examples of LcrV protein domains include those having sequence similarity with the amino acid sequence of SEQ ID NO:6.

An example of a fusion protein is depicted at SEQ ID NO:8. The fusion protein depicted at SEQ ID NO:8 includes, from amino-terminal to carboxy-terminal end, a YscF domain, a mature F1 domain, followed by a LcrV domain; however, a fusion protein can include the three domains in any order. Thus, other fusion proteins have the domains in the order of, from amino-terminal to carboxy-terminal end, a LcrV domain, a YscF domain, followed by a mature F1 domain; a LcrV domain, a mature F1 domain, followed by a YscF domain; a YscF domain, a LcrV domain, followed by a mature F1 domain; a mature F1 domain, a YscF domain, followed by a LcrV domain; and a mature F1 domain, a LcrV domain, followed by a YscF domain. Other examples of a fusion protein include those having sequence similarity with the amino acid sequence of SEQ ID NO:8, and those having sequence similarity with any other fusion protein described herein.

A fusion protein described herein has immunological activity. "Immunological activity" refers to the ability of a protein to elicit an immunological response in a subject. An immunological response to a protein is the development in a subject of a cellular and/or antibody-mediated immune response to the protein. Usually, an immunological response includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells, directed to an epitope or epitopes of the protein. "Epitope" refers to the site on an antigen to which specific B cells and/or T cells respond so that antibody is produced. The immunological activity may be protective. "Protective immunological activity" refers to the ability of a protein to elicit an immunological response in a subject that prevents or inhibits infection by a *Yersinia* spp., such as *Yersinia pestis*. Whether a protein has protective immunological activity can be determined by methods known in the art such as, for example, the methods described in Example 1. For example, a protein described herein, or combination of proteins described herein, protects a subject against challenge with a *Yersinia pestis*.

Sequence similarity of two proteins can be determined by aligning the residues of the two proteins (for example, a candidate protein domain and a reference protein, e.g., a YscF protein domain such as SEQ ID NO:2, a mature F1 protein domain such as SEQ ID NO:4, a LcrV protein domain such as SEQ ID NO:6, or a fusion protein such as SEQ ID NO:8) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. A reference protein may be a protein described herein. A candidate protein is the protein being compared to the reference protein. A candidate protein may be isolated, for example, from a microbe such as a *Yersinia pestis*, or can be produced using recombinant techniques, or chemically or enzymatically synthesized.

When the candidate protein domain is present as part of a fusion protein, only those amino acids of the protein domain are compared with a reference protein. For instance, if the candidate protein is YscF and is part of a fusion protein, only those residues of the YscF domain of the fusion protein are aligned with a reference protein.

Unless modified as otherwise described herein, a pairwise comparison analysis of amino acid sequences can be carried out using the Blastp program of the BLAST 2 search algorithm, as described by Tatiana et al., (FEMS Microbiol Lett, 174, 247-250 (1999)), and available on the National Center for Biotechnology Information (NCBI) website. The default values for all BLAST 2 search parameters may be used, including matrix=BLOSUM62; open gap penalty=11, extension gap penalty=1, gap x\_dropoff=50, expect=10, wordsize=3, and filter on. Alternatively, proteins may be compared using the BESTFIT algorithm in the GCG package (version 10.2, Madison Wis).

In the comparison of two amino acid sequences, structural similarity may be referred to by percent "identity" or may be referred to by percent "similarity." "Identity" refers to the presence of identical amino acids. "Similarity" refers to the presence of not only identical amino acids but also the presence of conservative substitutions. A conservative substitution for an amino acid in a protein described herein may be selected from other members of the class to which the amino acid belongs. For example, it is known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can be substituted for another amino acid without altering the activity of a protein, particularly in regions of the protein that are not directly associated with biological activity. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Conservative substitutions include, for example, Lys for Arg and vice versa to maintain a positive charge; Glu for Asp and vice versa to maintain a negative charge; Ser for Thr so that a free —OH is maintained; and Gln for Asn to maintain a free —NH<sub>2</sub>.

Guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al. (1990, Science, 247:1306-1310), wherein the authors indicate proteins are surprisingly tolerant of amino acid substitutions. For example, Bowie et al. disclose that there are two main approaches for studying the tolerance of a protein sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selects or screens to identify sequences that maintain functionality. As stated by the authors, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require non-polar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie et al, and the references cited therein.

Guidance on how to modify the amino acid sequences of the protein domains disclosed herein can also be obtained by



producing a protein alignment of a reference protein (e.g., SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6) with other related polypeptides. For instance, the reference protein SEQ ID NO:2 can be aligned in a multiple protein alignment with other YscF proteins. Such an alignment shows the locations of residues that are identical between each of the proteins, the locations of residues that are conserved between each of the proteins, and the locations of residues that are not conserved between each of the proteins. By reference to such an alignment, the skilled person can predict which alterations to an amino acid sequence are likely to modify activity, as well as which alterations are unlikely to modify activity. Methods for producing multiple protein alignments are routine, and algorithms such as ClustalW (Larkin et al., 2007, ClustalW and ClustalX version 2, *Bioinformatics* 23(21): 2947-2948) and Clustl Omega (Sievers et al., 2011, *Molecular Systems Biology* 7: 539, doi:10.1038/msb.2011.75; Goujon et al., 2010, *Nucleic acids research* 38 (Suppl 2):W695-9, doi:10.1093/nar/gkq313).

Thus, as used herein, a candidate protein domain useful in the methods described herein includes those with at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% amino acid sequence similarity, or complete identity to a reference amino acid sequence.

Alternatively, as used herein, a candidate protein useful in the methods described herein includes those with at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% amino acid sequence similarity, or complete identity to the reference amino acid sequence.

In one embodiment, a fusion protein described herein includes a linker between one or more the protein domains. A linker is an amino acid sequence that joins protein domains in a fusion protein. A linker can be flexible or rigid, and in one embodiment is flexible. In one embodiment, a linker can be at least 3, at least 4, at least 5, or at least 6 amino acids in length. It is expected that there is no upper limit on the length of a linker used in a fusion protein described herein; however, in one embodiment, a linker is no greater than 10, no greater than 9, no greater than 8, or no greater than 7 amino acids in length. Many linkers are known to a skilled person (see Chen et al. 2013, *Adv. Drug Deliv. Rev.*, 65(10):1357-1369). Specific examples of linkers include GGGGS (SEQ ID NO:11). In one embodiment, a fusion protein can include more than one type of linker, e.g., one type of linker between a YscF protein domain and a mature F1 protein domain, and another type of linker between a mature F1 protein and a LcrV protein. In one embodiment, a fusion protein can include more than one linker between two protein domains, e.g., two GGGGS (SEQ ID NO:11) linkers or three GGGGS (SEQ ID NO:11) linkers between a YscF protein domain and a mature F1 protein domain. An example of a fusion protein having three GGGGS (SEQ ID NO:11) linkers between the domains is depicted at SEQ ID NO:10 (the amino acids corresponding to the linkers are underlined). This fusion protein includes, from amino-terminal to carboxy-terminal end, a YscF domain, a mature F1 domain, followed by a LcrV domain, with three GGGGS (SEQ ID NO:11) linkers between the

YscF domain and the mature F1 domain, and three GGGGS (SEQ ID NO:11) linkers between the mature F1 domain and the LcrV domain.

A fusion protein as described herein also can be designed to include one or more additional sequences such as, for example, the addition of C-terminal and/or N-terminal amino acids. In one embodiment, additional amino acids may facilitate purification by trapping on columns or use of antibodies. Such additional amino acids include, for example, histidine-rich tags that allow purification of proteins on nickel columns.

Also provided are polynucleotides encoding a fusion protein described herein that includes at least three protein domains. Given the amino acid sequence of a fusion protein described herein that includes at least three protein domains, a person of ordinary skill in the art can determine the full scope of polynucleotides that encode that amino acid sequence using conventional, routine methods. The class of nucleotide sequences encoding a selected protein sequence is large but finite, and the nucleotide sequence of each member of the class may be readily determined by one skilled in the art by reference to the standard genetic code, wherein different nucleotide triplets (codons) are known to encode the same amino acid. An example of a polynucleotide encoding a YscF protein domain is depicted at SEQ ID NO:1. An example of a polynucleotide encoding a mature F1 protein domain is depicted at SEQ ID NO:3. An example of a polynucleotide encoding a LcrV protein domain is depicted at SEQ ID NO:5. An example of a polynucleotide encoding a fusion protein is depicted at SEQ ID NO: 7.

A fusion protein described herein that includes at least three protein domains may include additional nucleotides flanking the coding region encoding the fusion protein. The boundaries of a coding region are generally determined by a translation start codon at its 5' end and a translation stop codon at its 3' end. In one embodiment, the additional nucleotides include vector nucleotides. In another embodiment, the additional nucleotides aid in expression of the fusion protein, such as expression for subsequent isolation and optional purification.

A polynucleotide that encodes a fusion protein described herein can be present in a vector. A vector is a replicating polynucleotide, such as a plasmid, phage, or cosmid, to which another polynucleotide may be attached so as to bring about the replication of the attached polynucleotide. Construction of vectors containing a polynucleotide described herein employs standard ligation techniques known in the art. See, e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989). A vector can provide for further cloning (amplification of the polynucleotide), e.g., a cloning vector, or for expression of the polynucleotide, e.g., an expression vector. The term vector includes, but is not limited to, plasmid vectors, viral vectors, cosmid vectors, and transposon vectors. A vector may be replication-proficient or replication-deficient. A vector may result in integration into a cell's genomic DNA.

Selection of a vector depends upon a variety of desired characteristics in the resulting construct, such as a selection marker, vector replication rate, and the like. Suitable host cells for cloning or expressing the vectors herein are prokaryotic or eukaryotic cells. Suitable eukaryotic cells include mammalian cells, such as yeast cells, murine cells, and human cells. Suitable prokaryotic cells include eubacteria, such as gram-negative organisms, for example, *E. coli*. Suitable eukaryotic cells include, but are not limited to, human embryonic kidney 293 (HEK293) cells.



An expression vector optionally includes regulatory sequences operably linked to a polynucleotide encoding the fusion protein. An example of a regulatory sequence is a promoter. A promoter may be functional in a host cell used, for instance, in the construction and/or characterization of a polynucleotide encoding a fusion protein described herein, and/or may be functional in the ultimate recipient of the vector. A promoter may be inducible, repressible, or constitutive, and examples of each type are known in the art. A polynucleotide encoding a protein described herein may also include a transcription terminator. Suitable transcription terminators are known in the art.

A vector introduced into a host cell optionally includes one or more marker sequences, which typically encode a molecule that inactivates or otherwise detects or is detected by a compound in the growth medium. Certain selectable markers may be used to confirm that the vector is present within the target cell. For example, the inclusion of a marker sequence may render the transformed cell resistant to an antibiotic, or it may confer compound-specific metabolism on the transformed cell. Examples of a marker sequence include, but are not limited to, sequences that confer resistance to kanamycin, ampicillin, chloramphenicol, tetracycline, streptomycin, neomycin, puromycin, hygromycin, DHFR, GPT, zeocin, histidinol, and others.

In one embodiment, the vector is an adenoviral vector. Adenoviruses are non-enveloped viruses 70-90 nm in diameter with an icosahedral capsid. Their genome is linear, double stranded DNA varying between 25-45 kilobases in size with inverted terminal repeats (ITRs) at both termini and a terminal protein attached to the 5' ends (Russell, 2000, *J Gen Virol.*, 90:1-20). Their genome also encompasses an encapsidation sequence (Psi), early genes, and late genes. The principal early genes are contained in the regions E1, E2, E3 and E4. Of these, the genes contained in the E1 region are required for viral propagation. The principal late genes are contained in the regions L1 to L5.

Adenoviruses have been used as the basis for a variety of vectors which incorporate various coding regions. In each of these constructs, the adenovirus has been modified in such a way as to render it unable to replicate following gene transfer. Thus, available constructs are adenoviruses in which genes of the early region, adenoviral E1, E2A, E2B, E3, E4, or combinations thereof, are deleted and into the sites of which a DNA sequence encoding a desired protein can be inserted. One example of an adenoviral vector routinely used is adenovirus serotype 5 (Ad5). In the first Ad5 vectors, E1 and/or E3 regions were deleted enabling insertion of foreign DNA to the vectors (Danthinne and Imperiale, 2000, *Gene Ther.*, 7:1707-14; see also Rankii et al., U.S. Pat. No. 9,410,129, and Crouset et al., U.S. Pat. No. 6,261,807). Furthermore, deletions of other regions as well as further mutations have provided extra properties to viral vectors. An example of an adenovirus encoding a fusion protein described herein is disclosed in Clarke (US Patent Publication 2010/0209451). A viral vector, such as an adenoviral vector, can be present as a polynucleotide or as a polynucleotide inside a viral particle.

In one embodiment, a composition includes at least one fusion protein described herein. In one embodiment, a composition includes a vector encoding a fusion protein described herein. In one embodiment, the vector is an adenovirus vector, and the vector can be present in a viral particle. Unless a specific level of sequence similarity and/or identity is expressly indicated herein (e.g., at least 80% sequence similarity, at least 90% sequence identity, etc.), reference to the amino acid sequence of an identified SEQ

ID NO includes variants having the levels of sequence similarity and/or the levels of sequence identity described herein.

The compositions as described herein optionally further include a pharmaceutically acceptable carrier. "Pharmaceutically acceptable" refers to a diluent, carrier, excipient, salt, etc., that is compatible with the other ingredients of the composition, and not deleterious to the recipient thereof. Typically, the composition includes a pharmaceutically acceptable carrier when the composition is used as described herein. The compositions as described herein may be formulated in pharmaceutical preparations in a variety of forms adapted to the chosen route of administration, including routes suitable for stimulating an immune response to an antigen. Thus, a composition as described herein can be administered via known routes including, for example, orally, parenterally including intradermal, transcutaneous and subcutaneous, intramuscular, intravenous, intraperitoneal, etc., and topically, such as, intranasal, intrapulmonary, intradermal, transcutaneous and rectally, etc. It is foreseen that a composition can be administered to a mucosal surface, such as by administration to the nasal or respiratory mucosa (e.g., via a spray or aerosol), in order to stimulate mucosal immunity, such as production of secretory IgA antibodies, throughout the subject's body.

A composition described herein can be referred to as a vaccine. The term "vaccine" as used herein refers to a composition that, upon administration to a subject, will increase the likelihood the recipient is protected against a *Yersinia* spp., such as *Y. pestis*.

A composition as described herein may be administered in an amount sufficient to treat certain conditions as described herein. The amount of fusion protein or vector present in a composition as described herein can vary. In one embodiment, a dosage of viral particles containing a vector that encodes a fusion protein described herein can be at least  $1 \times 10^8$ , at least  $5 \times 10^8$ , at least  $1 \times 10^9$ , or at least  $5 \times 10^9$  viral particles, and no greater than  $1 \times 10^{10}$ , no greater than  $5 \times 10^{10}$ , no greater than  $1 \times 10^{11}$ , or no greater than  $5 \times 10^{11}$  viral particles. In one embodiment, a dosage of a fusion protein (e.g., intramuscular) described herein can be at least 0.01 micrograms (m), at least 0.1  $\mu$ g, at least 1  $\mu$ g, or at least 10  $\mu$ g, and no greater than 20  $\mu$ g, no greater than 50  $\mu$ g, or no greater than 100  $\mu$ g.

The formulations may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. Methods of preparing a composition with a pharmaceutically acceptable carrier include the step of bringing the active compound (e.g., a viral particle or fusion protein as described herein) into association with a carrier that constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

A composition can also include an adjuvant. An "adjuvant" refers to an agent that can act in a nonspecific manner to enhance an immune response to a particular antigen, thus potentially reducing the quantity of antigen necessary in any given immunizing composition, and/or the frequency of injection necessary in order to generate an adequate immune response to the antigen of interest. Adjuvants may include, for example, IL-1, IL-2, emulsifiers, muramyl dipeptides, dimethyl dioctadecyl ammonium bromide (DDA), avridine, aluminum hydroxide, magnesium hydroxide, oils, saponins, alpha-tocopherol, polysaccharides, emulsified paraffins,



ISA-70, RIBI, and other substances known in the art. It is expected that proteins as described herein will have immunoregulatory activity and that such proteins may be used as adjuvants that directly act as T cell and/or B cell activators or act on specific cell types that enhance the synthesis of various cytokines or activate intracellular signaling pathways. Such proteins are expected to augment the immune response to increase the protective index of the existing composition.

In another embodiment, a composition as described herein including a pharmaceutically acceptable carrier can include a biological response modifier, such as, for example, IL-2, IL-4 and/or IL-6, TNF, IFN- $\alpha$ , IFN- $\gamma$ , and other cytokines that effect immune cells. A composition can also include other components known in the art such as an antibiotic, a preservative, an anti-oxidant, or a chelating agent.

Also provided are methods of using the compositions described herein. The methods include administering to a subject an effective amount of a composition described herein. The subject can be, for instance, a human, a non-human primate (such as a cynomolgus macaque), a murine (such as a mouse or a rat), a guinea pig, or a rabbit.

In some aspects, the methods may further include additional administrations (e.g., one or more booster administrations) of the composition to the subject to enhance or stimulate a secondary immune response. A booster can be administered at a time after the first administration, for instance, one to eight weeks, such as two to four weeks, after the first administration of the composition. Subsequent boosters can be administered one, two, three, four, or more times annually. Without intending to be limited by theory, it is expected that in some aspects annual boosters will not be necessary, as a subject will be challenged in the field by exposure to microbes expressing proteins present in the compositions having epitopes that are identical to or structurally related to epitopes present on proteins of the composition administered to the subject.

In one embodiment, a method includes an administration of a vector that includes a coding region encoding a fusion protein described herein. The vector can be a viral vector, and the viral vector can be present in a viral particle. An example of a viral vector is an adenovirus. The administration of the vector can be topical, such as delivery to the nasal or respiratory mucosa. The administration of the vector can be followed by a booster administration of an isolated or purified fusion protein described herein. The booster can be parenteral, such as intramuscular, intradermal, or subcutaneous. Optionally, more than one administration of the vector can occur, and more than one administration of the fusion protein can occur.

In one aspect, the invention is directed to methods for producing an immune response in the recipient subject. An immune response can be humoral, cellular, or a combination thereof. Antibody produced includes antibody that specifically binds the fusion protein. A cellular immune response includes immune cells that are activated by the fusion protein. In this aspect, an "effective amount" is an amount effective to result in the production of an immune response in the subject. Methods for determining whether a subject has produced antibodies that specifically bind a fusion protein, and determining the presence of a cellular immune response, are routine and known in the art.

In one aspect the invention is also directed to conferring immunity to plague in a subject, including a human, caused by *Yersinia* spp., such as *Y. pestis*. The plague can be pneumonic, bubonic, or septicemic. Conferring immunity is

typically prophylactic—e.g., initiated before a subject is infected by a microbe causing plague, and is referred to herein as treatment of a subject that is "at risk" of infection. As used herein, the term "at risk" refers to a subject that may or may not actually possess the described risk. Thus, typically, a subject "at risk" of infection by a microbe causing plague is a subject present in an area where subjects have been identified as infected by the microbe and/or is likely to be exposed to the microbe even if the subject has not yet manifested any detectable indication of infection by the microbe and regardless of whether the subject may harbor a subclinical amount of the microbe. An example of a subject likely to be exposed to the microbe includes a subject in the armed forces deployed at a location where there is risk of exposure to *Y. pestis*, such as a weaponized *Y. pestis*. While the methods described herein are of use in prophylactic treatment, the methods can also be used to treat a subject after the subject is infected by the microbe. Accordingly, administration of a composition can be performed before, during, or after the subject has first contact with the microbe, and the subject can have or be at risk of having plague, such as pneumonic plague. Treatment initiated before the subject's first contact with the microbe can result in increased immunity to infection by the microbe.

In another aspect, the method is directed to treating one or more symptoms or clinical signs of certain conditions in a subject that can be caused by infection by a microbe causing plague including *Yersinia* spp., such as *Y. pestis*. As used herein, the term "symptom" refers to subjective evidence of a disease or condition experienced by the patient and caused by infection by a microbe. As used herein, the term "clinical sign" or, simply, "sign" refers to objective evidence of disease or condition caused by infection by a microbe. The method includes administering an effective amount of a composition described herein to a subject having a condition, or exhibiting symptoms and/or clinical signs of a condition, and determining whether at least one symptom and/or clinical sign of the condition is changed, preferably, reduced. Examples of symptoms and/or clinical signs caused by a microbe causing plague, such as *Y. pestis*, are known to the person skilled in the art. The successful treatment of infection by *Y. pestis* in a subject is disclosed in Example 1, which demonstrates the protection against plague disease caused by *Y. pestis* in a mouse model and cynomolgus macaques by administering a composition described herein. Mouse and cynomolgus macaques models are a commonly accepted model for the study of disease caused by *Y. pestis*.

Also provided herein is a kit for immunizing a subject to protect against plague. The kit includes a vector described herein, such as an adenoviral vector, which includes a coding region encoding a fusion protein described herein in a suitable packaging material in an amount sufficient for at least one administration. The kit also includes a fusion protein described herein, in a suitable packaging material in an amount sufficient for at least one administration. Optionally, other reagents such as buffers and solutions needed to administer the two compositions are also included. Instructions for use of the packaged materials are also typically included. As used herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods, generally to provide a sterile, contaminant-free environment. The packaging material may have a label which indicates that the materials can be used for conferring immunity to a subject. In addition, the packaging material contains instructions indicating how the materials within the kit are employed to immunize a subject



to protect against plague. As used herein, the term "package" refers to a container such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits the materials and other optional reagents. "Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

The present invention is illustrated by the following example. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

#### Example 1

Currently, no plague vaccine exists in the United States for human use. The capsular antigen (Caf1 or F1) and two type 3 secretion system (T3SS) components, the low calcium response V antigen (LcrV) and the needle protein YscF, represent protective antigens of *Yersinia pestis*. We used a replication-defective human type-5 adenovirus vector (Ad5) and constructed recombinant monovalent and trivalent vaccines (rAd5-LcrV and rAd5-YFV) that expressed either the codon-optimized lcrV or the fusion gene YFV (made up of ycsF, caf1 and lcrV). Immunization of mice with the trivalent rAd5-YFV vaccine by either the intramuscular (i.m.) or the intranasal (i.n.) route provided superior protection compared to the monovalent rAd5-LcrV vaccine against bubonic and pneumonic plague when animals were challenged with *Y. pestis* CO92. Pre-existing adenoviral immunity did not diminish the protective response, and the protection was always higher when mice were administered one i.n. dose of the trivalent vaccine (priming) followed by a single i.m. booster dose of the purified YFV antigen. Immunization of cynomolgus macaques with the trivalent rAd5-YFV vaccine by the prime-boost strategy provided 100% protection to animals that had pre-existing adenoviral immunity, against a stringent aerosol challenge dose of CO92. The vaccinated and challenged macaques had no signs of disease, and the invading pathogen rapidly cleared with no histopathological lesions. This is the first report showing the efficacy of an adenovirus-vectored trivalent vaccine against pneumonic plague in mouse and NHP models.

#### INTRODUCTION

*Yersinia pestis* is the causative agent of plague, and can be transmitted to humans via an infected flea bite or by direct inhalation of the aerosolized bacilli from an infected person or an animal (1, 2). Plague manifests itself in three major forms in humans, namely bubonic, septicemic, and pneumonic (2). Pneumonic plague is the most feared form due to its rapid onset and associated high mortality rate (1, 2). *Y. pestis* has been responsible for at least three pandemics in the past, which killed more than 200 million people (3). Current epidemiological records suggest 4,000 human plague cases annually worldwide (2). The emergence of multi-antibiotic resistant *Y. pestis* strains from plague patients, and the potential of malicious dissemination of recombinantly engineered bacteria as an airborne bio-weapon, necessitates the development of an effective pre-exposure and/or post-exposure prophylaxis treatment (1, 2).

Currently, no Food and Drug Administration (FDA)-licensed plague vaccine exists in the United States, and

recent efforts have focused on the development of recombinant subunit plague vaccines consisting of two well-characterized *Y. pestis* antigens, the F1 capsular antigen, and the type 3 secretion system (T3SS) component and effector LcrV (4-8). F1 encoded by the caf1 gene has a polymeric structure and confers bacterial resistance to phagocytosis (9). The F1-V-based vaccines are generally protective against pneumonic plague in rodents and non-human primates (NHPs), and are currently undergoing clinic trials (10-17). However, considering the natural existence of fully virulent F1 minus *Y. pestis* strains (18, 19) or those that have highly diverged LcrV variants (20, 21), such F1-V-based vaccines would most likely not provide optimal protection across all plague-causing *Y. pestis* strains in humans.

In an effort to search for new immunogenic antigens for the plague subunit vaccines, recent studies have shown that vaccination of mice with recombinant T3SS needle structure protein YscF (rYscF) provided protection to mice against subcutaneous injection of the fully virulent and encapsulated *Y. pestis* strain CO92, and against an intravenously injected pigmentation locus-negative *Y. pestis* KIM strain (22, 23).

In this study, we used a replication-defective human type-5 adenovirus vector (Ad5) to construct recombinant monovalent and trivalent (rAd5-LcrV and rAd5-YFV) vaccines that expressed either the lcrV or the fusion gene YFV (ycsF, caf1, and lcrV). We demonstrated the trivalent rAd5-YFV vaccine provided superior protection to immunized mice than the monovalent rAd5-LcrV vaccine against both bubonic and pneumonic plague, irrespective of whether or not the pre-existing adenoviral immunity was artificially developed in these animals. Most importantly, one dose of the trivalent rAd5-YFV vaccine by the intranasal (i.n.) route in conjunction with a single dose of the purified recombinant fusion protein rYFV by the intramuscular (i.m.) route in a prime-boost strategy, provided impressive (up to 100%) protection to both mice and cynomolgus macaques against high challenge doses of WT CO92 when given by the aerosol route. Vaccinated NHPs rapidly cleared the pathogen with no signs of disease and histopathological lesions in various organs.

#### Materials and Methods

**Bacterial strains and reagents.** *Y. pestis* CO92 strain (WT CO92) was isolated in 1992 from a fatal human pneumonic plague case and acquired through the BEI Resources, Manassas, Va. The bioluminescent WT *Y. pestis* CO92 luc2 strain (WT CO92 luc2), which contains the luciferase operon (luc or lux), allowing in vivo imaging of mice for bacterial dissemination in real time, was previously constructed in our laboratory (26, 27). *Y. pestis* strains were grown in heart infusion broth (HIB) medium (Difco, Voigt Global Distribution Inc., Lawrence, Kans.) at 26 to 28° C. with constant agitation (180 rpm) or on either 1.5% HIB agar or 5% sheep blood agar (SBA) plates (Teknova, Hollister, Calif.). For the aerosol challenge, WT CO92 was grown in HIB enriched with 0.2% xylose (DL-xylose; Sigma-Aldrich, St. Louis, Mo.) as we previously described (28). Luria-Bertani (LB) medium was used for growing *Escherichia coli* at 37° C. with agitation. Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, Wis.). Advantage cDNA PCR kits were purchased from Clontech (Palo Alto, Calif.). All digested plasmid DNA or DNA fragments from agarose gels were purified using QIAquick kits (Qiagen, Inc., Valencia, Calif.).

**Production and purification of recombinant proteins.** Genes encoding YscF, Caf1 (F1), and LcrV were amplified from the genome of WT CO92 by polymerase chain reaction (PCR) with the primer sets YscFHis\_F.cln (CA



CATATGAGTAACTTCTCTGGATTTACGAAAG, SEQ ID NO:12) and YscFHis\_R.cln (CA CTCGAGTGGGAACTTCTGTAGGATGCCTT, SEQ ID NO:13), Caf1His\_F.cln (CA CATATGAAAAAATCAGTTCCGTTATCG, SEQ ID NO:14) and Caf1His\_R.cln (CA CTCGAGTTGGTTAGATACGGTTACGGTTACAG, SEQ ID NO:15), LcrVHis\_F.cln (CA CATATGATTAGAGCCTACGAACAAAACCC, SEQ ID NO:16) and LcrVHis\_R.cln (CA GTCGACTTTACCAGACGTGTCATCTAGCAGAC, SEQ ID NO:17), respectively. The underlines denote the restriction enzyme sites in the primers. The amplified genes were individually cloned into the pET20b+ vector at the NdeI and XhoI restriction enzyme sites, which resulted in attaching a histidine (His)-Tag at the C-terminus of each of the gene products. In addition, the yscF, caf1, and lcrV fusion gene (YFV) was synthetically constructed by Epoch Biolabs, Inc. (Houston, Tex.) after codon optimization for *E. coli* by using Blue Heron Biotechnology (Bothell, Wash.) online service (<https://www.blueheronbio.com>). A flexible linker of 3× (GGGGS, SEQ ID NO:11) between YscF, Caf1 (F1), and LcrV domains was added to facilitate correct folding of the fusion protein. The fusion gene was similarly cloned into the pET20b+ vector with a His-Tag attached to the C-terminus of the YFV protein. Individual or the fusion genes were expressed from *E. coli* BL21(DE3) (New England BioLabs, Ipswich, Mass.) after induction with 0.5 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) for 4 h at 37° C. The recombinant proteins (rYscF, rF1, rLcrV, and rYFV) were then purified by using Ni<sup>2+</sup>-charged agarose (29). The recombinant F1 and LcrV fusion protein (rF1-V) was purchased from the BEI Resources, and used as a control for some of the experiments.

Construction of recombinant adenoviruses. The lcrV and the YFV fusion genes were codon optimized for expression in humans by using the Blue Heron Biotechnology online service, which also allowed us to optimize secondary structures of the corresponding RNAs and removal of unwanted sites for the restriction enzymes, except for those used for cloning purposes. The resulting constructs were designed to produce LcrV (37.2 kDa), as well as the YFV fusion protein consisting of YscF (9.5 kDa), mature form of F1 (15.6 kDa), and LcrV (37.2 kDa), interconnected via a flexible linker, as mentioned above. To improve expression of the corresponding genes, the Kozak consensus sequence was also placed upstream of the start codon. The constructs were then synthesized and verified via DNA sequence analysis by Epoch Biolabs, Inc. Each synthetic construct was cloned into pShuttleX vector (Clontech Laboratories, Inc., Mountain View, Calif.) under the control of a CMV promoter.

To generate recombinant adenoviruses, the above gene constructs with their CMV promoters were removed from the pShuttleX vector and cloned into the replication-defective human type-5 adenovirus plasmid vector Adeno-X (Clontech Laboratories, Inc.). The adenoviral constructs were created at the Baylor College of Medicine (BCM), Vector Development Laboratory, Houston, Tex. (available through the World Wide Web at the internet site maintained by the Vector Development Laboratory, for instance, [bcm.edu/research/advanced-technology-core-labs/lab-listing/vector-development/adenovirus-vectors](http://bcm.edu/research/advanced-technology-core-labs/lab-listing/vector-development/adenovirus-vectors)). The resulting recombinant plasmid vectors, Adeno-X/crV and Adeno-XYFV were transfected separately into human embryonic kidney 293 (HEK293) cells and the plaque formation was monitored. After small-scale expansion, eight plaques from each of the recombinant vector transfections were examined

for the production of target proteins by dot blot analysis of the infected whole cell lysates with a monoclonal antibody to LcrV (mAb-LcrV) (BEI Resources). The positive plaques were selected and designated as rAd5-LcrV and rAd5-YFV, respectively. The control adenovirus Ad5-CMV-Empty without recombinant gene insertion was purchased from the BCM Vector Development Laboratory, and designated as Ad5-empty.

The Ad5-empty, rAdS-LcrV, and the rAd5-YFV were then expanded on a large scale by using HEK293 cells in a chemically-defined, protein-free CD-293 medium (Thermo Fisher Scientific, Waltham, Mass.) and purified at the BCM Vector Development Laboratory under GLP (good laboratory practice) conditions, and used for the subsequent studies. To examine expression of the target protein-encoding genes in the stocked recombinant viruses, A549 human lung epithelial cells (American Type Culture Collection, Manassas, Va.) were infected with Ad5 constructs at 1000 viral particles (v.p.) per cell. The host cell lysates were harvested after 24 h post-infection (p.i.). An aliquot of the cell lysates was then resolved by SDS-PAGE and subjected to Western blot analysis with mAb-LcrV antibody. The purified rLcrV and rYFV antigens were used as controls. As shown in FIG. 1, the size of the major band detected in the A549 cell lysate infected with either the rAdS-LcrV (lane 2) or rAdS-YFV (lane 3) corresponded to the size of purified rLcrV (lane 5) or rYFV (lane 6). No band was detected in the A549 cell lysate infected with the Ad5-empty (lane 4). The multiple bands detected in lanes 2, 3, and 6 most likely represented degradation, or incomplete synthesis of the target proteins.

Animal studies. Six-to-eight-week old, female Swiss-Webster mice (17 to 20 g) were purchased from Taconic Laboratories (Germantown, N.Y.). All of the animal studies were performed in the Animal Biosafety Level (ABSL)-3 facility within the Galveston National Laboratory (GNL) under approved Institutional Animal Care and Use Committee (IACUC) protocols.

1) Induction of Pre-Existing Immunity to Adenovirus in Mice.

To establish pre-existing immunity to adenovirus, animals received a single dose of the Ad5-Empty by i.m. injection of  $8 \times 10^9$  v.p./100  $\mu$ l into both quadriceps (50  $\mu$ l each) 30 days prior to vaccination. Mice receiving saline (phosphate-buffered saline, PBS) served as a control. Blood was collected by the retro-orbital route before and 30 days after the Ad5-Empty injection, and microtiter plates pre-coated with 0.3  $\mu$ g/well of Ad5-empty were used to evaluate antibody titers to adenovirus. Animals with pre-existing adenovirus immunity were designated as PreAd-mice.

2) Immunization of Mice with the Recombinant Proteins or Recombinant Ad5 Constructs.

Naïve mice (40 per group) were immunized with either the mixture of three recombinant proteins (rYscF, rF1, and rLcrV, 25  $\mu$ g/each) or 45  $\mu$ g of the corresponding recombinant fusion protein (rYFV) via the i.m. route. The antigens were emulsified 1:1 in Imject Alum adjuvant (Pierce Companies, Dallas, Tex.). One primary immunization and two identical boosters were given on days 0, 15 and 30. Naïve mice receiving adjuvant alone served as a control. For the recombinant Ad5 constructs, naïve mice or preAd-mice (40 per group) were either i.m. or i.n. immunized with one dose ( $8 \times 10^9$  v.p.) of rAd5-LcrV monovalent or rAd5-YFV trivalent vaccine. Control animals (both naïve and preAd-mice) received the same dose of Ad5-empty via the same route as their corresponding immunized mice. In some cases, the dose of Ad5-Empty was split equally into i.m. injection and i.n. instillation for the control naïve mice. During i.m.



immunizations, the dose in a 100  $\mu$ l volume was equally split and injected into both quadriceps, while for the i.n. immunizations, the dose in 40  $\mu$ l was equally distributed into each of the nares of mice followed by 20  $\mu$ l of PBS wash.

3) Immunization of Mice with the Combination of rAd5-YFV and rYFV.

PreAd-mice (20 per group) were first i.n. immunized with  $8 \times 10^9$  v.p./40  $\mu$ l of rAd5-YFV trivalent vaccine and then followed (two weeks later) by i.m. immunization with 10  $\mu$ g rYFV (emulsified 1:1 in Alum adjuvant). PreAd-mice immunized with either 10  $\mu$ g of rYFV or  $8 \times 10^9$  v.p./40  $\mu$ l of rAd5-YFV alone were used for comparison, and PreAd-mice without further immunizations served as a negative control.

4) Evaluation of Antibody Titers in Mice.

Blood was collected by the retro-orbital route from all vaccinated and control mice at day 0 and after 12-15 days of last vaccination. Sera were separated and the antigen-specific antibodies were then evaluated. Briefly, ELISA plates were pre-coated with 200 ng/well of the recombinant proteins (e.g., rLcrV, rF1 or rYscF). Two-fold serially diluted sera was then added in the wells of the ELISA microtiter plates, followed by the addition of secondary horseradish peroxidase (HRP)-conjugated anti-mouse specific antibodies to IgG, its isotypes, and/or IgA. The ELISA was performed as we described previously (30).

5) T-Cell Responses.

T cells were isolated from splenocytes of PreAd-mice (n=5) immunized with either rAd5-YFV (i.n.,  $8 \times 10^9$  v.p) alone or in a prime-boost combination with rYFV (10 i.m.) on day 15 after the last immunization. The isolated T cells were co-cultured with  $\gamma$ -irradiated splenocytes from naïve mice (severed as antigen-presenting cells [APCs]) pulsed or un-pulsed with F1-V fusion protein, 100  $\mu$ g/ml. After 72 h of incubation, 1  $\mu$ Ci of [ $^3$ H] thymidine was added into each well, and the cells harvested 16 h later using a semi-automated sample harvester, FilterMate Harvester (PerkinElmer, Waltham, Mass.), followed by the measurement of radioactive counts (TopCount NXT, PerkinElmer) as we

challenge doses ranged from 24 to 8,500 LD<sub>50</sub> for the s.c. route and 21 to 800 LD<sub>50</sub> for the i.n. route. The presented dose (Dp) for the aerosol challenge was calculated to be in the range of 3.14 to  $6.34 \times 10^5$  colony forming units (CFU). The LD<sub>50</sub> of WT CO92 for Swiss-Webster mice is ~50 CFU for developing bubonic plague (s.c.), ~500 CFU for inducing pneumonic plague (i.n.), and ~Dp of 2100 CFU for the aerosol route (28, 32). For the re-challenge experiment(s), on day 32 after the initial WT CO92 aerosol challenge, the vaccinated mice that survived were infected i.n. with 100 LD<sub>50</sub> of the WT CO92 luc2 strain. The age matched naïve mice served as a control. The animals were imaged on day 3 p.i. with WT CO92 luc2 strain by using an in vivo imaging system (IVIS) 200 bioluminescent and fluorescence whole-body imaging workstation (Caliper Corp. Alameda, Calif.) in the ABSL-3 facility.

Non-human primate (NHPs) study. Cynomolgus macaques (2.5-3.5 kg, males) were purchased from Prelabs, Hines, Ill. The NHPs were sedated by the administration of ketamine i.m. during the procedures, and all of the studies were performed in the ABSL-3 facility under an approved IACUC protocol.

1) Induction of Pre-Existing Immunity to Adenovirus and Immunization.

To induce pre-existing immunity, four randomly selected NHPs were injected in the left quadriceps muscle with  $5 \times 10^{10}$  v.p./250  $\mu$ l of Ad5-Empty (day 0). After 30 days, these NHPs were i.n. immunized with  $1 \times 10^{11}$  v.p./500  $\mu$ l of rAd5-YFV, followed by 50  $\mu$ g/250  $\mu$ l of rYFV boost (emulsified 1:1 in Alum adjuvant) via the i.m. route on day 42. In the control group, four NHPs received 250-500  $\mu$ l of saline at days 0, 30 and 42 via the same routes as the immunized NHPs, and served as controls (Table 1). The nasal administration of rAd5-YFV was performed by using a Mucosal Atomization Device (MAD Nasal, Wolfe Tory Medical, Inc., Salt Lake City, Utah) that delivers intranasal medication in a fine mist, thus enhancing the absorption and improving bioavailability.

TABLE 1

NHP immunization and challenge timeline				
Group (size)	Induction of preexisting anti-adenovirus immunity (Day 0)	Prime vaccination (Day 30)	Boost with rYFV (Day 42)	Aerosol Challenge (Day 85)
Immunized (4)	$5 \times 10^{10}$ v.p./250 $\mu$ l Ad5-empty i.m. route	$1 \times 10^{11}$ v.p./500 $\mu$ l rAd5-YFV i.n. route (250 $\mu$ l per nostril)	50 $\mu$ g of the rYFV mixed with alhydrogel (250 $\mu$ l) given by the i.m. route	WT CO92 (Dp: 1.32 to $8.08 \times 10^7$ CFU)
Control (4)	Saline (250 $\mu$ l) i.m. route	Saline (500 $\mu$ l) i.n. route (250 $\mu$ l per nostril)	Saline (250 $\mu$ l) i.m. route	

previously described (31, 32). To measure interferon (IFN)- $\gamma$  producing T cells, the isolated T cells were incubated with the pulsed and un-pulsed APCs for 2 days and evaluated by the enzyme-linked immunospot (Elispot) assay (R&D Systems Inc., Minneapolis, Minn.).

6) Challenge and Re-Challenge.

Mice were challenged with WT CO92 on day 14-15 post last vaccination by either the subcutaneous (s.c.), i.n., or the aerosol route as we previously described (28, 33). Aerosolization was performed using a 6-jet Collison nebulizer attached to a whole-body mouse aerosol chamber. The

2) Aerosol Challenge.

The immunized and control NHPs were challenged with WT CO92 by the aerosol route on day 85 (Table 1). Briefly, WT CO92 was aerosolized with a 6-jet Collison nebulizer. The nebulizer was attached to a head-only NHP aerosol exposure box and real-time plethysmography was performed on each of the anesthetized NHP during aerosol challenge. The aerosol/plethysmography system was controlled by a Biaera AeroMP aerosol platform (Biaera Technologies, LLC Hagerstown, Md.) integrated with a respiratory inductive plethysmography (RIP) system (Data



Sciences International St. Paul, Minn.). Aerosol samples were collected during each animal exposure by using all glass BioSamplers to assure accurate aerosol delivery, and the corresponding Dps were calculated (28, 34).

The NHPs were monitored and evaluated closely for developing clinical signs of the disease. Clinical scores were provided after thorough examination of the animals by the veterinarian staff. The NHPs were euthanized when they were found with a clinical score of 8 and above. The parameters examined but not limited to included absence of grooming, decreased breathing, and non-responsive to human presence at cage side. All NHP exposures to aerosols of WT CO92 were performed in our ABSL-3 facility within the GNL in a specialized aerobiology suite equipped with a Class III biosafety glove cabinet.

### 3) Antibody Titers, Blood Cell Counts, and Bacterial Burden.

Blood samples were collected from the femoral veins of NHPs at various time points during the experiment. Antibody titers to Ad5, LcrV, F1, and YscF on days 0, 42, 56, 85, 98 and 112 were evaluated by ELISA as we described above. The last two time points (days 98 and 112) corresponded to days 14 and 28 after WT CO92 challenge. Blood cell counts were analyzed on the day of WT CO92 challenge (day 85) and on days 3 and 6 post challenge by using a Drew Scientific Hemavet 950 hematology system (Drew Scientific, Inc., Dallas, Tex.). The bacterial loads were evaluated by plating the blood samples which were drawn when control NHPs were euthanized (on day 3 or 4 post WT CO92 challenge) or at various time points (e.g., days 3, 6, 14, 28, 70, and 82) post WT CO92 challenge in the case of immunized NHPs.

### 4) Necropsy and Histopathological Analysis.

After euthanasia, necropsies were performed by the certified chief biocontainment veterinarian at UTMB. NHP organs, such as lungs, liver, spleen, and the lymph nodes (hilar, submandibular, and mediastinal) were removed and grossly examined. A portion of these organs was homogenized and plated for assessing bacterial load (35), while another portion was fixed in 10% neutral buffered formalin (33, 36) and tissues processed and sectioned at 5  $\mu$ m. The samples were mounted on slides and stained with hematoxylin and eosin (H&E). Sections from the lungs were also subjected to Gram stain to examine the presence of plague bacilli. Tissue lesions were scored on the basis of a severity scale, which correlated with estimates of lesion distribution and the extent of tissue involvement (minimal, 2 to 10%; mild, >10 to 20%; moderate, >20 to 50%; severe, >50%), as previously described (33, 36). The histopathological evaluation of the tissue sections was performed in a blinded fashion.

CT scans. CereTom NL 3000 (Neurologica, MA), which is an eight-slice tomography with high-contrast resolution of 0.6 mm (developed for human head imaging in ICU), was used. The image acquisition settings were: tube voltage, 100 kV; tube current, 5 mA; and axial mode with slice thickness of 1.25 mm. Image resolution was 512 $\times$ 512 pixels. The image sharpness was optimized to soft tissue.

Statistical analysis. Two-way analysis of variance (ANOVA) with the Tukey's post hoc test or the multiple Student's t-test with the Holm-sidak post hoc test correction was used for data analysis. We used Kaplan-Meier survival estimates for animal studies, and p values of  $\leq 0.05$  were considered significant for all of the statistical tests used.

### Results

Immunogenicity of rYFV fusion protein. Mice were i.m. immunized with either the mixture of recombinant proteins

(rYscF+rF1+rLcrV) or the fusion protein rYFV. Both recombinant proteins (rYFV or rYscF+rF1+rLcrV) conferred 100% protection to mice when challenged by either the s.c. route (8500 LD<sub>50</sub>, to induce bubonic plague) or the i.n. route (800 LD<sub>50</sub>, to induce pneumonic plague) with WT CO92, while developing significant antibody titers to LcrV (FIG. 2).

Protective immunity of the recombinant adenoviruses in both bubonic and pneumonic plague mouse models. Mice were immunized i.m. or i.n. with rAd5-LcrV monovalent or rAd5-YFV trivalent vaccines to evaluate their potential to protect animals from plague. Irrespective of the immunization route, mice that were administered rAd5-YFV trivalent vaccine displayed 100% protection when challenged with 60 LD<sub>50</sub> of WT CO92 in a bubonic plague model (FIG. 3A). However, only 50 to 55% of mice receiving the rAd5-LcrV monovalent vaccine were protected and all control mice died by day 11 p.i. (FIG. 3A). In a more stringent pneumonic plague model (90 LD<sub>50</sub> of WT CO92), animals vaccinated by the i.n. route with rAd5-YFV trivalent vaccine were 60% protected, while the survival rate declined to 10% when immunization occurred by the i.m. route (FIG. 3B). In comparison, either none or 20% of the animals immunized with the Ad5-LcrV monovalent vaccine survived when vaccination occurred by i.m. versus the i.n. route. Overall, these data indicated vaccines to be more effective when instilled by the i.n. route. The corresponding control mice (receiving Ad5-empty by the i.m. or the i.n. route) succumbed to infection by day 4 p.i. (FIG. 3B).

Higher antibody titers to LcrV were generally observed in mice that received the rAd5-YFV trivalent vaccine when compared to that of the rAd5-LcrV monovalent vaccine-immunized animals, reaching statistically significant levels for IgG1 in mice that were immunized by the i.n. route (FIG. 3C). In terms of immunization routes, i.n. vaccinated mice overall had superior antibody titers when compared to animals immunized by the i.m. route, reaching statistical significance for the production of IgG1 and IgA (FIG. 3C). Irrespective of the recombinant virus used and route of immunization, all of the vaccinated mice developed a more balanced Th1 and Th2 type antibody responses when compared to immunization of animals with the recombinant proteins (FIGS. 2A and 3C).

Pre-existing immunity to adenovirus in mice. The adenoviral antibody titers on day 30 after injection of Ad5-empty in mice ranged from 102,400 to 819,200. In a bubonic plague model, at a 24 LD<sub>50</sub> challenge dose, a similar level of protection (80 to 90%) was noted in mice immunized with rAd5-YFV trivalent vaccine, irrespective of whether or not pre-existing antibodies to adenovirus were developed (FIG. 4A). In contrast, the survival rate was 40% in mice without pre-existing Ad5 antibodies and only 10% in preAd-mice when immunization occurred with the rAd5-LcrV monovalent vaccine (FIG. 4A). In a pneumonic plague model (21 LD<sub>50</sub>), rAd5-YFV-immunized mice with or without pre-existing immunity to Ad5 exhibited a similar 55-60% survival rate which was much higher than in mice immunized with the rAd5-LcrV monovalent vaccine with or without pre-immunity to Ad5 (10-20% protection) (FIG. 4B). All of the control mice died on the indicated days in a bubonic or pneumonic plague model (FIGS. 4A and 4B).

Balanced Th1 and Th2 type antibody responses with robust titers to LcrV were observed across all immunized mice (FIG. 4C). However, two important observations were drawn from this study: 1) compared to rAd5-LcrV monovalent vaccine immunized mice, animals that were vaccinated with the rAd5-YFV trivalent vaccine generally developed



better antibody titers (both IgG and its isotypes as well as IgA) to LcrV, although some did not reach statistical significance (e.g., IgG1 and IgG2a in preAd-mice as well as IgA), and 2) mice without pre-existing adenoviral immunity developed slightly higher IgG and IgA antibody titers to LcrV compared to that of preAd-mice receiving the trivalent rAd5-YFV vaccination, although only total IgG and its isotopes reached statistical significance (FIG. 4C). As expected, none of the unimmunized control mice developed any detectable level of protective anti-LcrV antibodies, and, thus, succumbed to infection (FIGS. 4A and 4B). Importantly, in spite of slight lower antibody titers to LcrV in mice with pre-existing Ad5 antibodies, animals were similarly protected when the Ad5-YFV trivalent vaccine was administered by the i.n. route against challenge with WT CO92 in both bubonic and pneumonic plague models (FIGS. 4A and 4B).

Prime-boost and aerosol challenge. Our above data indicated that the trivalent rAd5-YFV vaccine was better than the monovalent rAd5-LcrV vaccine in providing protection to mice against *Y. pestis* infection. However, the overall protection rate did not reach 100% in the pneumonic plague model (FIGS. 3B and 4B). To enhance protection, a boost with rYFV (10  $\mu$ s) was administered to mice i.m. two weeks later following i.n. instillation of the rAd5-YFV trivalent vaccine. As shown in FIG. 5, mice immunized with only rAd5-YFV had a 70% survival rate after aerosol exposure of WT CO92, irrespective of whether or not pre-existing adenoviral immunity was developed. The preAd-mice vaccinated with the combination of rAd5-YFV and rYFV displayed a protection rate of 80% with an overall delayed death pattern after WT CO92 aerosol challenge at a Dp of  $6.34 \times 10^5$  CFU ( $\sim 302$  LD<sub>50</sub>). The rYFV-immunized mice alone (single dose, no boosts) had 5% survival, and all unimmunized preAd-mice died after aerosol exposure of the pathogen between days 3 to 5 p.i. (FIG. 5).

To further evaluate the potential of the prime-boost strategy, another set of immunized mice were exposed to a slightly lower WT CO92 aerosol challenge dose (Dp of  $4.62 \times 10^5$  CFU,  $\sim 220$  LD<sub>50</sub>). As shown in FIG. 6A, the preAd-mice first vaccinated with the rAd5-YFV trivalent vaccine and then boosted with rYFV, were 100% protected against developing pneumonic plague. On the other hand, preAd-mice that were vaccinated with only the rAd5-YFV trivalent vaccine showed 55% survival rate, with all the unimmunized preAd-mice succumbed to infection by day 3 post challenge.

In addition, 55-60% of T cells isolated from the prime-boost group of mice were IFN- $\gamma$  positive, while this number was only 30% for mice that were immunized with rAd5-YFV trivalent vaccine alone (FIG. 6B). However, there was no difference between the two immunized groups of mice (with or without the prime-boost) in terms of their T cell proliferative responses upon stimulation with the F1-V antigen (FIG. 6C).

In terms of antibody production, we noted that IgG, its isotypes, and IgA antibody titers to the three antigens (F1, LcrV, and YscF) were generally higher in the prime-boost group of mice over those animals that only received the rAd5-YFV trivalent vaccine. Further, a balanced Th1 and Th2-based antibody responses were observed (FIG. 7A-7C).

Continued protection of mice conferred by prime-boost vaccination strategy against the initial aerosol and then the subsequent intranasal WT CO92 challenge. In our subsequent experiment, preAd-mice were vaccinated with either the rAd5-YFV trivalent vaccine alone or with a rYFV boost. The preAd-mice receiving the Ad5 empty vector alone

served as a control. After the vaccination regimen, mice were subjected to WT CO92 aerosol challenge with still a relatively lower Dp ( $3.14 \times 10^5$  CFU,  $\sim 150$  LD<sub>50</sub>) as compared to the above two aerosol challenges (FIGS. 5 and 6A). As noted in FIG. 8A, 100% of the animals survived the initial challenge in all of the immunized groups, while 90% of the control mice died (FIG. 8A). After 32 days of the initial aerosol challenge, the survivals from the immunized groups were re-challenged with 100 LD<sub>50</sub> of WT CO92 luc2 strain by the i.n. route, and the age-matched uninfected naïve mice (n=5) served as a control. As shown in FIG. 8A, 80% of the mice were protected from developing plague in the rAd5-YFV-immunized group, while this protection was 100% when the prime-boost strategy was used. In contrast, all of the naïve re-challenge control mice succumbed to infection within day 4 p.i. The bioluminescent images showed that the plague bacilli disseminated from the initial infection site of lungs to the whole body in all 5 naïve control mice after day 3 p.i. (FIG. 8B-I). On the other hand, no animals were positive in the group that received vaccination by the prime-boost regimen (FIG. 8B-II). However, one mouse from the rAd5-YFV-immunized group was bioluminescent positive, with the organisms confined in the lungs (FIG. 8B-III). This bioluminescent-positive animal along with another one mouse in the same group, which did not show bioluminescence at the time of imaging (day 3 p.i.), eventually died, resulting in the overall survival rate of 80% in the rAd5-YFV immunized group of mice (FIG. 8A).

Evaluation of protection provided by the trivalent rAd5-YFV vaccine in cynomolgus macaques against aerosol challenge of WT CO92. Four NHPs were initially i.m. injected with Ad5-empty to generate pre-existing adenoviral immunity. This was followed by one dose of the rAd5-YFV by the i.n. instillation in the form of mist, and then one dose of the rYFV by the i.m. route. Four unimmunized NHPs served as a control (Table 1). These NHPs were then challenged with the aerosolized WT CO92 at Dp ranging from 1.32 to  $8.08 \times 10^7$  CFU ( $\sim 13,200$ - $80,800$  LD<sub>90</sub>, with 1 LD<sub>90</sub>=864 CFU (37)). No clinic signs were noted in the immunized group of NHPs, and the animals remained healthy and survived the WT CO92 challenge until euthanized at the end of the study (FIG. 9). The CT scans of immunized NHPs, that survived the WT CO92 challenge (FIG. 9) and euthanized on day 82 post challenge, did not display any abnormalities in the lungs and their surrounding areas when compared to the images of the animals before the WT CO92 challenge on day 85 (FIG. 10) (Table 1). In contrast, the control NHPs euthanized on day 3-to-4 post challenge, showed consolidation in both the right and the left lung, an indication of severe inflammation (FIG. 10).

Necropsy on immunized NHPs was performed 82 days after the WT CO92 challenge, and no gross abnormalities were observed, and the internal organs (lungs, liver, spleen and the lymph nodes) were all free of bacteria (Table 2). In contrast, all unimmunized control NHPs developed clinical signs of the disease as early as 36 h p.i. and reached a clinical score of 8 and higher on day 3-to-4 p.i. The control NHPs had cough, abnormal respiration, lethargy, and a hunched posture. Although we did not notice fever in these animals during the progression of the disease, it could be related to not continuously monitoring these NHPs by using telemetry. Necropsy of these animals revealed serous hemorrhagic fluid in the thorax with respiratory frothy serous discharge. Lungs were hyper-inflated with hemorrhagic frothy fluid, and the spleen, liver and the lymph nodes were enlarged. The highest bacterial loads ( $1.12$  to  $1.26 \times 10^9$  CFU/node) were noted in the hilar lymph nodes and lungs ( $2.22 \times 10^7$  to



1.06×10<sup>9</sup> CFU/g) followed by the liver (8.16×10<sup>6</sup> to 1.69×10<sup>7</sup> CFU/g), spleen (2.13 to 4.47×10<sup>6</sup> CFU/g) and submandibular lymph nodes (2.33×10<sup>5</sup> CFU/node). Only one animal showed bacteria in the blood with a count of 2500 CFU/ml, and no bacilli was detected in the other control NHPs (Table 2).

TABLE 2

NHP clinical score, bacterial loads and necropsy report				
NHP	Days Post Infection	Bacterial loads in blood/organs	Clinical Score	Necropsy Report
Control	3-4	Blood: 0-2500 CFU/ml Lung: 2.22 × 10 <sup>7</sup> -1.06 × 10 <sup>9</sup> CFU/g Liver: 8.16 × 10 <sup>6</sup> -1.69 × 10 <sup>7</sup> CFU/g Spleen: 2.13-4.47 × 10 <sup>6</sup> CFU/g Hilar lymph node: 1.12-1.26 × 10 <sup>9</sup> CFU/node Submandibular lymph node: 2.0-2.33 × 10 <sup>5</sup> CFU/node	≥8	External: Thin, pale, dehydrated and scruffy coat Respiratory: Frothy serous discharge; hyper-inflated with hemorrhagic frothy fluid (~50 mL) Lymphatic: Enlarged submandibular node Spleen: Firm and enlarged Liver: Firm, enlarged and rounded edges Locomotion: Lethargic Body Cavities: Serous hemorrhagic fluid in thorax (~50 mL)
Immunized	82	Negative for all the organs; blood samples were negative for bacteria as early as day 3 post infection	0	Normal

NHP blood cell counts and antibody titers. The changes in the blood cell counts in immunized NHPs versus the control after WT CO92 challenge are shown in FIG. 11. Only the lymphocyte (LY) counts in the control NHPs fell below the normal range by day 3 post WT CO92 challenge before they were euthanized. However, in the immunized NHPs, LY counts remained within the normal range on days 3 and 6 post WT CO92 challenge.

Both immunized and control NHPs showed some level of pre-existing Ad5 antibody titers (6,400-25,600) on day 0 as a consequence of naturally acquired infection with adenoviruses. The anti-Ad5 titer was increased to 409,600 on day 30 in immunized NHPs after receiving the rAd5-Empty injection, and continued to climb up slightly on days 42 and 56 as a result of immunization with rAd5. The anti-Ad5 antibody titer was maintained at a similar level to that observed on day 0 in the control NHPs (FIG. 12A). No pre-existing anti-LcrV, anti-F1, and anti-YscF antibodies were detected in both the groups of NHPs before immunization (data not shown). However, high antibody titers to three *Y. pestis*-specific antigens (e.g., F1, LcrV, and YscF) were noticed in all of the immunized NHPs (FIG. 12B-12E). Compared to the antibody titers on day 42, the antigen specific IgG antibodies increased ~10 fold for LcrV and YscF, but nearly 1000 fold for F1 on day 56 (FIG. 12B-12D). Thus, boost on day 30 with rYFV (Table 1) led to increase in antibody titers. These antigen-specific antibody titers slightly decreased on day 85 (the day of challenge). A similar trend was observed for the anti-LcrV IgA antibody titers, which were increased ~10 fold on day 56 after the rYFV boost (FIG. 12E). Compared to all three antigen-specific IgG antibody titers, the anti-LcrV titers were the highest followed by anti-YscF and anti-F1 across the course of immunization, and the difference could reach up to 1000 fold (anti-LcrV vs anti-F1 on day 42) (FIGS. 12B and 12C). After WT CO92 aerosol challenge, anti-F1 IgG titers were further boosted, while sustaining IgG titers for LcrV and YscF, and IgA LcrV titers up to 28 days post WT CO92 challenge (overall day 112 after initiation of vaccination) (FIG. 13A-13E).

NHP histopathological analysis. As shown in FIG. 14, the unimmunized control NHPs showed marked acute inflammatory reactions in the lungs, pleura, and the mediastinal lymph nodes. Specifically, multifocal hemorrhage and diffused supportive inflammation were observed in the lungs with no alveolar spaces. Similar changes were also observed

in pleura and mediastinal lymph nodes of these unimmunized NHPs. Furthermore, tissue sections from the lungs with Gram staining revealed the presence of bacteria, presumably *Y. pestis* (FIG. 14, inset). Interestingly, the liver and the spleen tissues of unimmunized NHPs showed normal morphological characteristics in spite of higher bacterial loads (Table 2), indicating that pneumonic changes are the primary cause of death in control groups. In the immunized NHP group, the lungs, pleura, mediastinal lymph nodes, and the liver were normal, and the lungs had alveolar spaces. The only notable and expected changes observed in the prime-boost group was the hyperplasia of lymphoid follicles in mediastinal lymph nodes and the spleen. These changes can mainly be attributed to reaction of vaccination.

## DISCUSSION

Historically, vaccination has not only been one of the most significant advances in healthcare, but also a cost-effective means of public health intervention. The high mortality rate associated with pneumonic plague, the potential use of *Y. pestis* as a biological weapon, and the current lack of a FDA approved plague vaccine highlight the importance of our studies.

Previously, the plague vaccine licensed in the U.S. (sold under the name of USP) was a formaldehyde-killed preparation of the highly virulent 195/P strain of *Y. pestis*; however, the production of this vaccine was discontinued in 1999. The vaccination regimen included a course of injections over a period of 6 months, and then the annual boosters (38, 39). The vaccine was effective against bubonic plague, but protection against pneumonic plague was uncertain. The incidence of side effects, such as malaise, headaches, elevated body temperature, and lymphadenopathy was high; and the vaccine was expensive (40). A live-attenuated vaccine based on *Y. pestis* pigmentation locus negative EV76 strains is also available in some parts of the world where plague is endemic (1). These types of vaccines have existed since the first half of the 20<sup>th</sup> century and have proven effective against both subcutaneous and inhalation challenges with *Y. pestis*. However, the EV76-based vaccines are



not genetically uniform and are also highly reactogenic (41), and, hence, would not meet the standards for FDA approval.

The major problems encountered in developing live-attenuated vaccines are inadequate attenuation, particularly in immunocompromised individuals, and the potential to revert back to the virulent phenotype. Efforts have been made to generate well-characterized and rationally-designed attenuated plague vaccines. For example, mutations that effectively attenuate *Salmonella* such as *aroA*, *phoP*, *htrA* and *lpp* genes, were introduced in *Y. pestis*, but these mutations had only a limited effect on *Y. pestis* virulence (33, 42-44). Similarly, a deletion of the *Y. pestis* global regulator gene *rovA*, significantly attenuated the bacterium during subcutaneous infection, but this mutant was only slightly attenuated when given via an intranasal or the intraperitoneal route (45). Recently, a highly attenuated  $\Delta lpp$   $\Delta msbB$   $\Delta ail$  triple mutant, which was deleted for genes encoding Braun lipoprotein (Lpp), an acetyltransferase (MsbB), and the Attachment Invasion Locus (Ail), was constructed (27). Mice immunized with this triple mutant via either the intranasal, subcutaneous, or the intramuscular route, were protected from lethal WT CO92 challenge, and thus could be an excellent vaccine candidate (27, 35). This triple mutant was subsequently excluded from the CDC select agent list in May 2016. However, further evaluation of the efficacy of this triple mutant in higher animal models is warranted.

While the above conventional vaccine strategies have focused on live-attenuated or killed bacterial approaches, a new method in the development of vaccines uses platform technologies to overcome some of the challenges in vaccine design. The adenoviral vector system has been successfully used as a vaccine platform for a number of pathogens, including *Y. pestis* (46, 47), with several advantages: 1) the adenoviral genome is well characterized with the capability of integrating  $\geq 6$ -kb of the potential insert size for delivering multiple antigens; 2) the replication-defective Ad5 vector has been developed for gene therapeutic applications at a wide range of doses, with minimal side effects; and 3) adenoviruses have a broad tropism infecting a variety of dividing and non-dividing cells. Studies have shown that adenoviruses transfer genes effectively to APCs in vivo to promote rapid and robust humoral and cellular immune responses to the transgene products (48-55). In addition, adenoviruses can be grown to high titers in tissue culture cells and can be applied systemically as well as through mucosal surfaces, and are relative thermostable to facilitate their clinical use.

Our rAd5-YFV trivalent vaccine had an average yield of  $1 \times 10^{16}$  v.p. per batch in a cell suspension culture in CD 293 Medium. The vaccine was free of proteins, serum, and animal-derived components, thus making it suitable for a broad range of prophylactic and therapeutic use. Compared to a favored Th2 response in mice immunized with rYFV or a mixture of rYscF, rLcrV, and rF1 (given with alum which skews the immune response to Th2) (FIG. 2A), a more balanced Th1- and Th2-based antibody response was observed in mice immunized with the rAd5 vaccines (FIGS. 3C, 4C, and 7A-7C). Indeed, Ad5 has been shown to promote Th1 response (47). As expected, intranasal administration of rAd5-LcrV monovalent and rAd5-YFV trivalent vaccines elicited IgA production in immunized animals (both mice and NHPs), and most importantly, mice immunized with rAd5-YFV alone or in a prime-boost vaccination strategy, exhibited a robust T cell proliferative responses (FIG. 6C). These features suggest superiority of Ad5-based vaccines over the rF1-V-based subunit vaccines, as the

protection of the latter vaccines is largely dependent on systemic antibody responses without mucosal and cellular immune components. Interestingly, although generally a higher IgG antibody titer was observed across all mice immunized intranasally when compared to animals immunized intramuscularly with the recombinant adenoviruses, the protection rate was indistinguishable during the development of bubonic plague. However, subtle differences in protection were noted depending upon of the route of immunization of mice in a pneumonic plague model (FIGS. 3A and 3B), which further highlighted the importance of mucosal immunity during the development of pneumonic plague.

Pneumonic plague begins with an anti-inflammatory state (i.e., first 24 to 36 h after infection), which is characterized by a delay in the inflammatory cell recruitment to the lungs and production of pro-inflammatory cytokines and chemokines (56). Therefore, a plague vaccine should be able to stimulate a strong mucosal immunity to overcome this initial immune suppression in the host (57). In our future studies, we plan to discern the role of mucosal immune response (e.g., IgA) that is triggered by the rAd5-YFV vaccine in protection.

Compared to the monovalent rAd5-LcrV vaccine, the trivalent rAd5-YFV vaccine not only mounted higher anti-LcrV antibody titers (both IgG and IgA) (FIGS. 3C and 4C) but also generated immune responses to the F1 and YscF (FIG. 7), which correlated with better protection of animals against both bubonic and pneumonic plague (FIGS. 3A and 3B, 4A and 4B, and 5). In addition, LcrV was more immunogenic than F1 and YscF in both mice and NHPs that were immunized with the trivalent rAd5-YFV vaccine (FIGS. 7 and 12). In contrast, the antibody titers to F1 were the lowest among the three examined antigens in the rAd5-YFV-immunized NHPs (FIG. 12). The difference in immunogenicity may be attributed to the nature of each of the antigens; however, conformation of the antigens in the fusion protein may also play a role, especially as higher anti-LcrV antibody titers were observed in the rAd5-YFV-immunized mice than in rAd5-LcrV vaccinated animals. Alternatively, the presence of other two antigens could augment antibody production to LcrV.

Previously, a rAd5 (designated as rAdsecV) expressing a human Igk secretion (sec) signal fused to 1crV was reported (46). The rAdsecV produced a secreted form of LcrV and elicited specific T cell responses as well as high IgG titers in sera, which protected mice from a lethal intranasal challenge of *Y. pestis* CO92 in a single intramuscular immunization (46). Although there is no direct comparison, the AdsecV provided better protection (80-100%) in mice than our monovalent rAd5-LcrV vaccine (~20%) (FIGS. 3B and 4B), indicating that the secreted form of LcrV might be more immunogenic in mice. However, different species of mice (Swiss-Webster versus BALB/c) and challenge doses were used in these studies (46). In our initial study, a rAd5 expressing the Igk secretion signal fused to YFV was successfully created; however, we found that the secreted YFV (sYFV) was toxic to HEK 293 cells, which prevented large-scale expansion of this construct (data not shown).

There are several established plague models using NHPs, such as the langur monkey (58), African green vervets (59, 60), baboons (61, 62), and rhesus macaque (63, 64). However, the current recommendations from FDA and the National Institute of Allergy and Infectious Disease to support plague therapeutic and vaccine studies is a cynomolgus macaque (*Macaca fascicularis*) (CM) pneumonic plague model (65). In addition, the lethal dose of *Y. pestis*



has been established for aerosol challenge of CMs with the standard CO92 strain, and this model was utilized in protection studies including F1-V-based subunit vaccines for the past several years as well as in most recent studies (65-72). Importantly, CMs exhibit a clinical course of the disease similar to that described in humans (73).

Indeed, we observed the unimmunized NHPs after WT CO92 aerosol challenge had cough, respiratory changes, lethargy, and hunched posture, as well as typical pneumonic lesions in the lungs (FIG. 14). However, no fever was observed during the course of infection. This is in contrast to the most recent report that the onset of fever was predominant across all CMs infected with *Y. pestis* (72). This highlights the importance of using telemetry to observe physiological parameters in a real-time manner. Our study did not employ telemetry, while the other report measured body temperature in real time and the temperature of 1.5° C. above the baseline was considered fever (72). One notable finding of our study was that a significant increase in the antibody titer was noted in immunized NHPs, especially to F1, after rYFV boost as well as after WT CO92 challenge (FIG. 12 and FIG. 13). These data indicated memory B cell evoked recall responses. Similarly, a predominant hyperplasia of lymphoid follicles was observed in the immunized NHPs in mediastinal lymph nodes and spleen for as long as 82 days after the WT CO92 challenge (FIG. 14), suggesting a sustained immune response was developed in these NHPs, which could be pivotal in long-term protection of animals against plague. Our studies also indicated that by using the prime-boost strategy in CMs, higher antibody responses were generated compared to animals that were immunized with only rAd5-YFV (FIG. 12). An average antibody titers of  $\sim 1.7 \times 10^6$  for LcrV,  $\sim 4.3 \times 10^4$  for F1 and  $\sim 1.2 \times 10^5$  for YscF, were mounted when animals were immunized following the prime-boost strategy. These antibody titers were sufficient for providing complete protection to CMs against high aerosol challenge doses of *Y. pestis* CO92, although the role of cell-mediated immunity in protection should also be considered.

One of the major concerns of adenoviral vectors for vaccine development is the pre-existing immunity to Ad5 (in  $\sim 95\%$  of the human population) that could lessen the efficacy of the vaccine. Currently, most of the efforts to overcome the concerns regarding neutralizing antibodies have been focused on identifying alternative serotypes of adenovirus (74, 75). While some groups have reported favorable results with this approach, it offers only a short-term solution, as new adenoviral vector adaptation will result in the generation of neutralizing antibodies through widespread use. On the other hand, a number of studies indicated that administration of Ad5-vectored vaccines via the i.n. route might overcome pre-existing immunity against the Ad5 vector (76-79). We did observe slightly lower *Y. pestis* antigen-specific antibody titers in mice with the pre-existing adenoviral immunity than those animals without the pre-existing adenoviral immunity when mice were i.n.-immunized with either the rAd5-LcrV or the rAd5-YFV vaccine (FIG. 4C). However, the protection conferred in mice against *Y. pestis* challenge was similar in both groups of mice irrespective of the pre-existing adenoviral immunity (FIGS. 4A and 4B). Most importantly, NHPs with pre-existing adenoviral immunity and immunized with the rAd5-YFV vaccine, plus a boost of rYFV, were fully protected from a high aerosol challenge dose of WT CO92 (FIG. 9).

In addition to YscF, other *Y. pestis* antigens such as the T3SS components YpkA, YopH, YopE, YopK, YopN, as well as a subunit of pH 6 antigen and purified LPS were studied

for their immunogenic efficacies against plague infection, but did not generate promising results (80). The only protection was observed in mice vaccinated with YopD, a protein involved in the delivery of T3 SS effectors into the host cell (81). However, YopD-vaccination provided protection only against the non-encapsulated bacilli but not against the encapsulated *Y. pestis* CO92 strain.

As the most promising plague subunit vaccines currently under development are primarily dependent on only two antigens F1 and LcrV, the incorporation of a new antigen YscF may help in formulating a better vaccine against all human plague causing-strains as we showed using the bacteriophage T4-based platform (82). Furthermore, the adenoviral vector has been demonstrated to have adjuvant activities as well as the ability to promote cellular immunity (51, 83, 84). In this regard, our trivalent rAd5-YFV vaccine has unique advantages as a plague vaccine. Our further studies will include in depth characterization of cell-mediated immune responses in vaccinated CMs.

#### CITATIONS

- Smiley S T. 2008. Current challenges in the development of vaccines for pneumonic plague. *Expert Rev Vaccines* 7:209-221.
- Sun W, Roland K L, Curtiss R, 3rd. 2011. Developing live vaccines against plague. *J Infect Dev Ctries* 5:614-627.
- Perry R D, Fetherston J D. 1997. *Yersinia pestis*—etiologic agent of plague. *Clin Microbiol Rev* 10:35-66.
- Cornelis G R. 2002. *Yersinia* type III secretion: send in the effectors. *J Cell Biol* 158:401-408.
- Powell B S, Andrews G P, Enama J T, Jendrek S, Bolt C, Worsham P, Pullen J K, Ribot W, Hines H, Smith L, Heath D G, Adamovicz J J. 2005. Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. *Biotechnol Prog* 21:1490-1510.
- Alvarez M L, Pinyerd H L, Crisantes J D, Rigano M M, Pinkhasov J, Walmsley A M, Mason H S, Cardineau G A. 2006. Plant-made subunit vaccine against pneumonic and bubonic plague is orally immunogenic in mice. *Vaccine* 24:2477-2490.
- Williamson E D, Flick-Smith H C, Waters E, Miller J, Hodgson I, Le Butt C S, Hill J. 2007. Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates. *Microb Pathog* 42:11-21.
- Cornelius C A, Quenee L E, Overheim K A, Koster F, Brasel T L, Elli D, Ciletti N A, Schneewind O. 2008. Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague. *Infect Immun* 76:5588-5597.
- Baker E E, Somer H, Foster L W, Meyer E, Meyer K F. 1952. Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. *J Immunol* 68:131-145.
- Rosenzweig J A, Jejelowo O, Sha J, Erova T E, Brackman S M, Kirtley M L, van Lier C J, Chopra A K. 2011. Progress on plague vaccine development. *Appl Microbiol Biotechnol* 91:265-286.
- Quenee L E, Ciletti N, Berube B, Krausz T, Elli D, Hermanas T, Schneewind O. 2011. Plague in Guinea pigs and its prevention by subunit vaccines. *Am J Pathol* 178:1689-1700.
- Quenee L E, Ciletti N A, Elli D, Hermanas T M, Schneewind O. 2011. Prevention of pneumonic plague in



- mice, rats, guinea pigs and non-human primates with clinical grade rV10, rV10-2 or F1-V vaccines. *Vaccine* 29:6572-6583.
13. Lin J S, Kummer L W, Szaba F M, Smiley S T. 2011. IL-17 contributes to cell-mediated defense against pulmonary *Yersinia pestis* infection. *J Immunol* 186:1675-1684.
  14. Smiley S T. 2008. Immune defense against pneumonic plague. *Immunol Rev* 225:256-271.
  15. Agar S L, Sha J, Foltz S M, Erova T E, Walberg K G, Baze W B, Suarez G, Peterson J W, Chopra A K. 2009. Characterization of the rat pneumonic plague model: infection kinetics following aerosolization of *Yersinia pestis* CO92. *Microbes Infect* 11:205-214.
  16. Williamson E D, Packer P J, Waters E L, Simpson A J, Dyer D, Hartings J, Twenhafel N, Pitt M L. 2011. Recombinant (F1+V) vaccine protects cynomolgus macaques against pneumonic plague. *Vaccine* 29:4771-4777.
  17. FDA. 2012. African Green monkey (*Chlorocebus aethiops*) animal model development to evaluate treatment of pneumonic plague.
  18. Sha J, Endsley J J, Kirtley M L, Foltz S M, Huante M B, Erova T E, Kozlova E V, Popov V L, Yeager L A, Zudina I V, Motin V L, Peterson J W, DeBord K L, Chopra A K. 2011. Characterization of an F1 deletion mutant of *Yersinia pestis* CO92, pathogenic role of F1 antigen in bubonic and pneumonic plague, and evaluation of sensitivity and specificity of F1 antigen capture-based dipsticks. *J Clin Microbiol* 49:1708-1715.
  19. Quenee L E, Cornelius C A, Ciletti N A, Elli D, Schneewind O. 2008. *Yersinia pestis* *cafI* variants and the limits of plague vaccine protection. *Infect Immun* 76:2025-2036.
  20. Anisimov A P, Dentovskaya S V, Panfertsev E A, Svetoch T E, Kopylov P, Segelke B W, Zemla A, Telepnev M V, Motin V L. 2010. Amino acid and structural variability of *Yersinia pestis* LcrV protein. *Infect Genet Evol* 10:137-145.
  21. Motin V L, Pokrovskaya M S, Telepnev M V, Kutyrev V V, Vidyaeva N A, Filippov A A, Smirnov G B. 1992. The difference in the lcrV sequences between *Y. pestis* and *Y. pseudotuberculosis* and its application for characterization of *Y. pseudotuberculosis* strains. *Microb Pathog* 12:165-175.
  22. Matson J S, Durick K A, Bradley D S, Nilles M L. 2005. Immunization of mice with YscF provides protection from *Yersinia pestis* infections. *BMC Microbiol* 5:38.
  23. Swietnicki W, Powell B S, Goodin J. 2005. *Yersinia pestis* Yop secretion protein F: purification, characterization, and protective efficacy against bubonic plague. *Protein Expr Purif* 42:166-172.
  24. Lathem W W, Price P A, Miller V L, Goldman W E. 2007. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science* 315:509-513.
  25. Doll J M, Zeitz P S, Ettestad P, Bucholtz A L, Davis T, Gage K. 1994. Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. *Am J Trop Med Hyg* 51:109-114.
  26. Sha J, Rosenzweig J A, Kirtley M L, van Lier C J, Fitts E C, Kozlova E V, Erova T E, Tiner B L, Chopra A K. 2013. A non-invasive in vivo imaging system to study dissemination of bioluminescent *Yersinia pestis* CO92 in a mouse model of pneumonic plague. *Microb Pathog* 55:39-50.
  27. Tiner B L, Sha J, Kirtley M L, Erova T E, Popov V L, Baze W B, van Lier C J, Ponnusamy D, Andersson J A,

- Motin V L, Chauhan S, Chopra A K. 2015. Combinational deletion of three membrane protein-encoding genes highly attenuates *Yersinia pestis* while retaining immunogenicity in a mouse model of pneumonic plague. *Infect Immun* 83:1318-1338.
28. Agar S L, Sha J, Foltz S M, Erova T E, Walberg K G, Parham T E, Baze W B, Suarez G, Peterson J W, Chopra A K. 2008. Characterization of a mouse model of plague after aerosolization of *Yersinia pestis* CO92. *Microbiology* 154:1939-1948.
  29. Suarez G, Sierra J C, Kirtley M L, Chopra A K. 2010. Role of Hcp, a type 6 secretion system effector, of *Aeromonas hydrophila* in modulating activation of host immune cells. *Microbiology* 156:3678-3688.
  30. van Lier C J, Tiner B L, Chauhan S, Motin V L, Fitts E C, Huante M B, Endsley J J, Ponnusamy D, Sha J, Chopra A K. 2015. Further characterization of a highly attenuated *Yersinia pestis* CO92 mutant deleted for the genes encoding Braun lipoprotein and plasminogen activator protease in murine alveolar and primary human macrophages. *Microb Pathog* 80:27-38.
  31. Sha J, Kirtley M L, van Lier C J, Wang S, Erova T E, Kozlova E V, Cao A, Cong Y, Fitts E C, Rosenzweig J A, Chopra A K. 2013. Deletion of the Braun lipoprotein-encoding gene and altering the function of lipopolysaccharide attenuate the plague bacterium. *Infect Immun* 81:815-828.
  32. van Lier C J, Sha J, Kirtley M L, Cao A, Tiner B L, Erova T E, Cong Y, Kozlova E V, Popov V L, Baze W B, Chopra A K. 2014. Deletion of Braun lipoprotein and plasminogen-activating protease-encoding genes attenuates *Yersinia pestis* in mouse models of bubonic and pneumonic plague. *Infect Immun* 82:2485-2503.
  33. Sha J, Agar S L, Baze W B, Olano J P, Fadl A A, Erova T E, Wang S, Foltz S M, Suarez G, Motin V L, Chauhan S, Klimpel G R, Peterson J W, Chopra A K. 2008. Braun lipoprotein (Lpp) contributes to virulence of yersiniae: potential role of Lpp in inducing bubonic and pneumonic plague. *Infect Immun* 76:1390-1409.
  34. Guyton A C. 1947. Measurement of the respiratory volumes of laboratory animals. *Am J Physiol* 150:70-77.
  35. Tiner B L, Sha J, Ponnusamy D, Baze W B, Fitts E C, Popov V L, van Lier C J, Erova T E, Chopra A K. 2015. Intramuscular immunization of mice with a live-attenuated triple mutant of *Yersinia pestis* CO92 induces robust humoral and cell-mediated immunity to completely protect animals against pneumonic plague. *Clin Vaccine Immunol* doi:10.1128/CVI.00499-15.
  36. Agar S L, Sha J, Baze W B, Erova T E, Foltz S M, Suarez G, Wang S, Chopra A K. 2009. Deletion of Braun lipoprotein gene (*lpp*) and curing of plasmid pPCP1 dramatically alter the virulence of *Yersinia pestis* CO92 in a mouse model of pneumonic plague. *Microbiology* 155:3247-3259.
  37. Warren R, Lockman H, Barnewall R, Krile R, Blanco O B, Vasconcelos D, Price J, House R V, Bolanowski M A, Fellows P. 2011. *Cynomolgus* macaque model for pneumonic plague. *Microb Pathog* 50:12-22.
  38. Russell P, Eley S M, Hibbs S E, Manchee R J, Stagg A J, Titball R W. 1995. A comparison of Plague vaccine, USP and EV76 vaccine induced protection against *Yersinia pestis* in a murine model. *Vaccine* 13:1551-1556.
  39. Titball R W, Williamson E D. 2001. Vaccination against bubonic and pneumonic plague. *Vaccine* 19:4175-4184.
  40. Titball R W, Williamson E D. 2004. *Yersinia pestis* (plague) vaccines. *Expert Opin Biol Ther* 4:965-973.



41. Cui Y, Yang X, Xiao X, Anisimov A P, Li D, Yan Y, Zhou D, Rajerison M, Carniel E, Achtman M, Yang R, Song Y. 2014. Genetic variations of live attenuated plague vaccine strains (*Yersinia pestis* EV76 lineage) during laboratory passages in different countries. *Infect Genet Evol* 26:172-179.
42. Oyston P C, Dorrell N, Williams K, Li S R, Green M, Titball R W, Wren B W. 2000. The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*. *Infect Immun* 68:3419-3425.
43. Oyston P C, Russell P, Williamson E D, Titball R W. 1996. An *aroA* mutant of *Yersinia pestis* is attenuated in guinea-pigs, but virulent in mice. *Microbiology* 142 (Pt 7):1847-1853.
44. Williams K, Oyston P C, Dorrell N, Li S, Titball R W, Wren B W. 2000. Investigation into the role of the serine protease HtrA in *Yersinia pestis* pathogenesis. *FEMS Microbiol Lett* 186:281-286.
45. Cathelyn J S, Crosby S D, Latham W W, Goldman W E, Miller V L. 2006. *RovA*, a global regulator of *Yersinia pestis*, specifically required for bubonic plague. *Proc Natl Acad Sci USA* 103:13514-13519.
46. Chiuchiolo M J, Boyer J L, Krause A, Senina S, Hackett N R, Crystal R G. 2006. Protective immunity against respiratory tract challenge with *Yersinia pestis* in mice immunized with an adenovirus-based vaccine vector expressing V antigen. *J Infect Dis* 194:1249-1257.
47. Tatsis N, Ertl H C. 2004. Adenoviruses as vaccine vectors. *Mol Ther* 10:616-629.
48. Boyer J L, Kobinger G, Wilson J M, Crystal R G. 2005. Adenovirus-based genetic vaccines for biodefense. *Hum Gene Ther* 16:157-168.
49. Barouch D H, Nabel G J. 2005. Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* 16:149-156.
50. Bessis N, GarciaCozar F J, Boissier M C. 2004. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther* 11 Suppl 1:S10-17.
51. Molinier-Frenkel V, Lengagne R, Gaden F, Hong S S, Choppin J, Gahery-Segard H, Boulanger P, Guillet J G. 2002. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. *J Virol* 76:127-135.
52. Hackett N R, Kaminsky S M, Sondhi D, Crystal R G. 2000. Antivector and antitransgene host responses in gene therapy. *Curr Opin Mol Ther* 2:376-382.
53. Song W, Kong H L, Traktman P, Crystal R G. 1997. Cytotoxic T lymphocyte responses to proteins encoded by heterologous transgenes transferred in vivo by adenoviral vectors. *Hum Gene Ther* 8:1207-1217.
54. Wilson J M. 1996. Adenoviruses as gene-delivery vehicles. *N Engl J Med* 334:1185-1187.
55. Tripathy S K, Black H B, Goldwasser E, Leiden J M. 1996. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat Med* 2:545-550.
56. Latham W W, Crosby S D, Miller V L, Goldman W E. 2005. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc Natl Acad Sci USA* 102:17786-17791.
57. Do Y, Didierlaurent A M, Ryu S, Koh H, Park C G, Park S, Perlin D S, Powell B S, Steinman R M. 2012. Induction

- of pulmonary mucosal immune responses with a protein vaccine targeted to the DEC-205/CD205 receptor. *Vaccine* 30:6359-6367.
58. Chen T H, Meyer K F. 1965. Susceptibility of the langur monkey (*Semnopithecus entellus*) to experimental plague: pathology and immunity. *J Infect Dis* 115:456-464.
59. Hallett A F, Isaacson M, Meyer K F. 1973. Pathogenicity and immunogenic efficacy of a live attenuated plague vaccine in vervet monkeys. *Infect Immun* 8:876-881.
60. Chen T H, Elbert S S, Eisler D M. 1976. Immunity in plague: protection induced in *Cercopithecus aethiops* by oral administration of live, attenuated *Yersinia pestis*. *J Infect Dis* 133:302-309.
61. Stacy S, Pasquali A, Sexton V L, Cantwell A M, Kraig E, Dube P H. 2008. An age-old paradigm challenged: old baboons generate vigorous humoral immune responses to LcrV, a plague antigen. *J Immunol* 181:109-115.
62. Byvalov A A, Pautov V N, Chicherin Iu V, Lebedinskii V A, Evtigneev V I. 1984. Effectiveness of revaccinating hamadryas baboons with NISS live dried plague vaccine and fraction I of the plague microbe. *Zh Mikrobiol Epidemiol Immunobiol* 4:74-76.
63. Ransom J P, Krueger A P. 1954. Chronic pneumonic plague in *Macaca mulatta*. *Am J Trop Med Hyg* 3:1040-1054.
64. Finegold M J, Petery R F, Berendt R F, Adams H R. 1968. Studies on the pathogenesis of plague: blood coagulation and tissue responses of *Macaca mulatta* following exposure to aerosols of *Pasteurella pestis*. *Am J Pathol* 53:99-114.
65. Van Andel R, Sherwood R, Gennings C, Lyons C R, Hutt J, Gigliotti A, Barr E. 2008. Clinical and pathologic features of cynomolgus macaques (*Macaca fascicularis*) infected with aerosolized *Yersinia pestis*. *Comp Med* 58:68-75.
66. Williamson E D, Flick-Smith H C, Waters E, Miller J, Hodgson I, Le Butt C S, Hill J. 2007. Immunogenicity of the rF1+rV vaccine for plague with identification of potential immune correlates. *Microb Pathog* 42:11-21.
67. Mett V, Lyons J, Musyichuk K, Chichester J A, Brasil T, Couch R, Sherwood R, Palmer G A, Streatfield S J, Yusibov V. 2007. A plant-produced plague vaccine candidate confers protection to monkeys. *Vaccine* 25:3014-3017.
68. Cornelius C A, Quenee L E, Overheim K A, Koster F, Brasel T L, Elli D, Ciletti N A, Schneewind O. 2008. Immunization with recombinant V10 protects cynomolgus macaques from lethal pneumonic plague. *Infect Immun* 76:5588-5597.
69. Welkos S, Norris S, Adamovicz J. 2008. Modified caspase-3 assay indicates correlation of caspase-3 activity with immunity of nonhuman primates to *Yersinia pestis* infection. *Clin Vaccine Immunol* 15:1134-1137.
70. Mizel S B, Graff A H, Sriranganathan N, Ervin S, Lees C J, Lively M O, Hantgan R R, Thomas M J, Wood J, Bell B. 2009. Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates. *Clin Vaccine Immunol* 16:21-28.
71. Koster F, Perlin D S, Park S, Brasel T, Gigliotti A, Barr E, Myers L, Layton R C, Sherwood R, Lyons C R. 2010. Milestones in progression of primary pneumonic plague in cynomolgus macaques. *Infect Immun* 78:2946-2955.
72. Fellows P, Price J, Martin S, Metcalfe K, Krile R, Barnewall R, Hart M K, Lockman H. 2015. Character-



- ization of a Cynomolgus Macaque Model of Pneumonic Plague for Evaluation of Vaccine Efficacy. *Clin Vaccine Immunol* 22:1070-1078.
73. Pitt M L. Non-human primates as a model for pneumonic plague. 2004. In: *Animal Models and Correlates of Protection for Plague Vaccines Workshop*.
74. Barouch D H, Pau M G, Custers J H, Koudstaal W, Kostense S, Havenga M J, Truitt D M, Sumida S M, Kishko M G, Arthur J C, Koriath-Schmitz B, Newberg M H, Gorgone D A, Lifton M A, Panicali D L, Nabel G J, Letvin N L, Goudsmit J. 2004. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* 172:6290-6297.
75. Nanda A, Lynch D M, Goudsmit J, Lemckert A A, Ewald B A, Sumida S M, Truitt D M, Abbink P, Kishko M G, Gorgone D A, Lifton M A, Shen L, Carville A, Mansfield K G, Havenga M J, Barouch D H. 2005. Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys. *J Virol* 79:14161-14168.
76. Zhang J, Jex E, Feng T, Sivko G S, Baillie L W, Goldman S, Van Kampen K R, Tang D C. 2013. An adenovirus-vectored nasal vaccine confers rapid and sustained protection against anthrax in a single-dose regimen. *Clin Vaccine Immunol* 20:1-8.
77. Croyle M A, Patel A, Tran K N, Gray M, Zhang Y, Strong J E, Feldmann H, Kobinger G P. 2008. Nasal delivery of an adenovirus-based vaccine bypasses pre-existing immunity to the vaccine carrier and improves the immune response in mice. *PLoS One* 3:e3548.
78. Xu Q, Pichichero M E, Simpson L L, Elias M, Smith L A, Zeng M. 2009. An adenoviral vector-based mucosal vaccine is effective in protection against botulism. *Gene Ther* 16:367-375.
79. Yu J R, Kim S, Lee J B, Chang J. 2008. Single intranasal immunization with recombinant adenovirus-based vaccine induces protective immunity against respiratory syncytial virus infection. *J Virol* 82:2350-2357.
80. Benner G E, Andrews G P, Byrne W R, Strachan S D, Sample A K, Heath D G, Friedlander A M. 1999. Immune response to *Yersinia* outer proteins and other *Yersinia pestis* antigens after experimental plague infection in mice. *Infect Immun* 67:1922-1928.
81. Andrews G P, Strachan S T, Benner G E, Sample A K, Anderson G W, Jr., Adamovicz J J, Welkos S L, Pullen J K, Friedlander A M. 1999. Protective efficacy of recombinant *Yersinia* outer proteins against bubonic plague caused by encapsulated and nonencapsulated *Yersinia pestis*. *Infect Immun* 67:1533-1537.
82. Tao P, Mahalingam M, Kirtley M L, van Lier C J, Sha J, Yeager L A, Chopra A K, Rao V B. 2013. Mutated and bacteriophage T4 nanoparticle arrayed F1-V immunogens from *Yersinia pestis* as next generation plague vaccines. *PLoS Pathog* 9:e1003495.

83. Jones F R, Gabitzsch E S, Xu Y, Balint J P, Borisevich V, Smith J, Peng B H, Walker A, Salazar M, Paessler S. 2011. Prevention of influenza virus shedding and protection from lethal H1N1 challenge using a consensus 2009 H1N1 H A and N A adenovirus vector vaccine. *Vaccine* 29:7020-7026.
84. Patel A, Zhang Y, Croyle M, Tran K, Gray M, Strong J, Feldmann H, Wilson J M, Kobinger G P. 2007. Mucosal delivery of adenovirus-based vaccine protects against Ebola virus infection in mice. *J Infect Dis* 196 Suppl 2:S413-420.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. Supplementary materials referenced in publications (such as supplementary tables, supplementary figures, supplementary materials and methods, and/or supplementary experimental data) are likewise incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

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          260          265          270
Cys Ser Asp Lys Ser Arg Pro Leu Asn Asp Leu Val Ser Gln Lys Thr
          275          280          285
Thr Gln Leu Ser Asp Ile Thr Ser Arg Phe Asn Ser Ala Ile Glu Ala
          290          295          300
Leu Asn Arg Phe Ile Gln Lys Tyr Asp Ser Val Met Gln Arg Leu Leu
          305          310          315          320
Asp Asp Thr Ser Gly Lys
          325

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<210> SEQ ID NO 7
<211> LENGTH: 1686
<212> TYPE: DNA
<213> ORGANISM: artificial

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&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: A nucleotide sequence encoding a fusion protein domain SEQ ID NO:8

&lt;400&gt; SEQUENCE: 7

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atggctaatt tctccgggtt cacaaagggc actgacattg cccgatcttga tgccggtgcc      60
cagactctca agaagcctgc ggacgatgcc aacaaggcag taaatgattc catcgcagcc      120
ctgaaagaca agcctgacaa tccagcactc ttggccgacc tgcaacatag tatcaacaaa      180
tggtctgtaa tttacaatat aaactctacc attgtgcggt ccatgaaaga tctgatgcag      240
gggatcctgc aaaaatttcc cgccgacctt acagctagta cactgccac agcaacgctt      300
gtagagcctg cccgaatcac cctgacgtat aaggaggggg ctccaatcac aataatggac      360
aatggaaaca tcgataccga actgctggtg gggaccctga cactgggtgg ctacaagacc      420
ggcacaacct ccacatccgt gaacttcacc gacgccgccc gcgatcccat gtatctcaca      480
ttcacttcac aggacggcaa caatcatcag ttcaccacta aggtgattgg caaggattcc      540
agagacttcg acatctctcc caaggtgaat ggcgagaacc tcgtggggga cgacgtggta      600
ctggcaacag gttcccagga tttctttgtc cggtcattg gaagcaaagg gggcaagctg      660
gcagcaggaa aatacaccga cgcagttaca gtgactgtgt caaaccagat gatccgcgcc      720
tacgagcaaa atcctcagca cttcattgaa gaccttgaga aggtgcgctg ggagcagctc      780
acaggccacg gtagcagtgt cctggaggag cttgtgcagc tggggaagga caagaatac      840
gatattagta taaaatacga tccaaggaaa gactctgagg tgttcgcgaa ccgcggttatt      900
accgacgata ttgaactcct gaagaaaatc ctggcctatt ttttgccaga ggacgctatc      960
ctgaaagggg ggcactatga taatcagctc caaaatggta tcaaacgggt gaaagagttc     1020
ctggagtcta gcccaaatac tcagtgggag ctgcgggcct ttatggctgt gatgcacttt     1080
agtctgacag cccgatcggat tgacgatgat atccttaagg tgatcgtcga tagcatgaac     1140
catcatggtg acgcaagaag taaactgagg gaggaactgg cccgagctgac tgcagagctc     1200
aaaatctata gcgtcataca ggccgaaatc aataagcact tgagctcatc aggcaccatt     1260
aacatccacg acaagtccat taatctgatg gacaaaaatc tgtacggata taccgacgag     1320
gagatthtca aagcgtccgc cgagtataaa atcctcgaga aaatgcctca gacaactata     1380
caggtggatg gttctgaaaa aaagattggt tctataaagg acttctcgg gtccgagaac     1440
aaaaggaccg gcgcactggg caatctcaag aactcataca gttataataa agataataat     1500
gagctttccc attttgccac aacctgctcc gacaaaagta gacctctgaa cgacctcgtg     1560
tcccaaaaga caacacagct gagtgatata acctccaggt tcaactcagc gatcgaggct     1620
ttgaacaggt tcatccagaa gtacgattca gtgatgcaga ggctggttga tgatactagc     1680
ggtaag                                             1686

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&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 562

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: An example of a fusion protein

&lt;400&gt; SEQUENCE: 8

```

Met Ala Asn Phe Ser Gly Phe Thr Lys Gly Thr Asp Ile Ala Asp Leu
1           5           10           15

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Asp Ala Val Ala Gln Thr Leu Lys Lys Pro Ala Asp Asp Ala Asn Lys
20           25           30

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Ala	Val	Asn	Asp	Ser	Ile	Ala	Ala	Leu	Lys	Asp	Lys	Pro	Asp	Asn	Pro
		35					40					45			
Ala	Leu	Leu	Ala	Asp	Leu	Gln	His	Ser	Ile	Asn	Lys	Trp	Ser	Val	Ile
	50					55					60				
Tyr	Asn	Ile	Asn	Ser	Thr	Ile	Val	Arg	Ser	Met	Lys	Asp	Leu	Met	Gln
65					70					75					80
Gly	Ile	Leu	Gln	Lys	Phe	Pro	Ala	Asp	Leu	Thr	Ala	Ser	Thr	Thr	Ala
				85					90					95	
Thr	Ala	Thr	Leu	Val	Glu	Pro	Ala	Arg	Ile	Thr	Leu	Thr	Tyr	Lys	Glu
			100					105					110		
Gly	Ala	Pro	Ile	Thr	Ile	Met	Asp	Asn	Gly	Asn	Ile	Asp	Thr	Glu	Leu
		115					120					125			
Leu	Val	Gly	Thr	Leu	Thr	Leu	Gly	Gly	Tyr	Lys	Thr	Gly	Thr	Thr	Ser
	130					135					140				
Thr	Ser	Val	Asn	Phe	Thr	Asp	Ala	Ala	Gly	Asp	Pro	Met	Tyr	Leu	Thr
145					150					155					160
Phe	Thr	Ser	Gln	Asp	Gly	Asn	Asn	His	Gln	Phe	Thr	Thr	Lys	Val	Ile
				165					170					175	
Gly	Lys	Asp	Ser	Arg	Asp	Phe	Asp	Ile	Ser	Pro	Lys	Val	Asn	Gly	Glu
			180					185					190		
Asn	Leu	Val	Gly	Asp	Asp	Val	Val	Leu	Ala	Thr	Gly	Ser	Gln	Asp	Phe
		195					200					205			
Phe	Val	Arg	Ser	Ile	Gly	Ser	Lys	Gly	Gly	Lys	Leu	Ala	Ala	Gly	Lys
	210					215					220				
Tyr	Thr	Asp	Ala	Val	Thr	Val	Thr	Val	Ser	Asn	Gln	Met	Ile	Arg	Ala
225					230					235					240
Tyr	Glu	Gln	Asn	Pro	Gln	His	Phe	Ile	Glu	Asp	Leu	Glu	Lys	Val	Arg
				245					250					255	
Val	Glu	Gln	Leu	Thr	Gly	His	Gly	Ser	Ser	Val	Leu	Glu	Glu	Leu	Val
			260					265					270		
Gln	Leu	Val	Lys	Asp	Lys	Asn	Ile	Asp	Ile	Ser	Ile	Lys	Tyr	Asp	Pro
		275					280					285			
Arg	Lys	Asp	Ser	Glu	Val	Phe	Ala	Asn	Arg	Val	Ile	Thr	Asp	Asp	Ile
	290					295					300				
Glu	Leu	Leu	Lys	Lys	Ile	Leu	Ala	Tyr	Phe	Leu	Pro	Glu	Asp	Ala	Ile
305					310					315					320
Leu	Lys	Gly	Gly	His	Tyr	Asp	Asn	Gln	Leu	Gln	Asn	Gly	Ile	Lys	Arg
				325					330					335	
Val	Lys	Glu	Phe	Leu	Glu	Ser	Ser	Pro	Asn	Thr	Gln	Trp	Glu	Leu	Arg
			340					345					350		
Ala	Phe	Met	Ala	Val	Met	His	Phe	Ser	Leu	Thr	Ala	Asp	Arg	Ile	Asp
		355					360					365			
Asp	Asp	Ile	Leu	Lys	Val	Ile	Val	Asp	Ser	Met	Asn	His	His	Gly	Asp
	370					375					380				
Ala	Arg	Ser	Lys	Leu	Arg	Glu	Glu	Leu	Ala	Glu	Leu	Thr	Ala	Glu	Leu
385					390					395					400
Lys	Ile	Tyr	Ser	Val	Ile	Gln	Ala	Glu	Ile	Asn	Lys	His	Leu	Ser	Ser
				405					410					415	
Ser	Gly	Thr	Ile	Asn	Ile	His	Asp	Lys	Ser	Ile	Asn	Leu	Met	Asp	Lys
			420					425					430		
Asn	Leu	Tyr	Gly	Tyr	Thr	Asp	Glu	Glu	Ile	Phe	Lys	Ala	Ser	Ala	Glu
		435					440					445			



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Tyr Lys Ile Leu Glu Lys Met Pro Gln Thr Thr Ile Gln Val Asp Gly  
 450 455 460  
 Ser Glu Lys Lys Ile Val Ser Ile Lys Asp Phe Leu Gly Ser Glu Asn  
 465 470 475 480  
 Lys Arg Thr Gly Ala Leu Gly Asn Leu Lys Asn Ser Tyr Ser Tyr Asn  
 485 490 495  
 Lys Asp Asn Asn Glu Leu Ser His Phe Ala Thr Thr Cys Ser Asp Lys  
 500 505 510  
 Ser Arg Pro Leu Asn Asp Leu Val Ser Gln Lys Thr Thr Gln Leu Ser  
 515 520 525  
 Asp Ile Thr Ser Arg Phe Asn Ser Ala Ile Glu Ala Leu Asn Arg Phe  
 530 535 540  
 Ile Gln Lys Tyr Asp Ser Val Met Gln Arg Leu Leu Asp Asp Thr Ser  
 545 550 555 560  
 Gly Lys

<210> SEQ ID NO 9  
 <211> LENGTH: 1776  
 <212> TYPE: DNA  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: A nucleotide sequence encoding a fusion protein  
 including linkers SEQ ID NO:10

<400> SEQUENCE: 9

atggctaatt tctccgggtt cacaaagggc actgacattg cccgatcttga tgccggtgcc 60  
 cagactctca agaagcctgc ggacgatgcc aacaaggcag taaatgattc catcgcagcc 120  
 ctgaaagaca agcctgacaa tccagcactc ttggccgacc tgcaacatag tatcaacaaa 180  
 tggctctgtaa tttacaatat aaactctacc attgtgcggt ccatgaaaga tctgatgcag 240  
 gggatcctgc aaaaatttcc cgggggcccggg ggttcccggg gaggcggtag tggcggcggg 300  
 ggatcagccg accttacagc tagtaccact gccacagcaa cgcttgtaga gcctgcccga 360  
 atcacccctga cgtataagga gggggctcca atcacaataa tggacaatgg aaacatcgat 420  
 accgaactgc tgggtggggac cctgacactg ggtggctaca agaccggcac aacctccaca 480  
 tccgtgaact tcaccgacgc cgccggcgat cccatgtatc tcacattcac ttcacaggac 540  
 ggcaacaatc atcagttcac cactaagggtg attggcaagg attccagaga cttcgacatc 600  
 tctcccaagg tgaatggcga gaacctcgtg ggggacgacg tggactggc aacagggttc 660  
 caggatttct ttgtccggtc cattggaagc aaagggggca agctggcagc aggaaaatac 720  
 accgacgcag ttacagtac tgtgtcaaac cagggagcgc gtggatccgg aggcggaggc 780  
 tcaggagcgc gggggagcat gatccgcgcc tacgagcaaa atcctcagca cttcattgaa 840  
 gaccttgaga aggtgcccgt ggagcagctc acaggccacg gtagcagtgt cctggaggag 900  
 cttgtgcagc tggatgaagga caagaatata gatattagta taaaatacga tccaaggaaa 960  
 gactctgagg tgttcgcaaa ccgcgttatt accgacgata ttgaactcct gaagaaaatc 1020  
 ctggcctatt ttttgccaga ggacgctatc ctgaaagggg ggcactatga taatcagctc 1080  
 caaaatggta tcaaacgggt gaaagagttc ctggagtcta gcccataac tcagtgggag 1140  
 ctgcgggcct ttatggctgt gatgcacttt agtctgacag cccgatccgat tgacgatgat 1200  
 atccttaagg tgategtcga tagcatgaac catcatggty acgcaagaag taaactgagg 1260  
 gaggaactgg ccgagctgac tgcagagctc aaaatctata gcgtcataca ggccgaaatc 1320  
 aataagcact tgagctcatc aggcaccatt aacatccacg acaagtccat taatctgatg 1380



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gacaaaaatc tgtacggata taccgacgag gagattttca aagcgtccgc cgagtataaa 1440
atcctcgaga aaatgcctca gacaactata caggtggatg gttctgaaaa aaagattggt 1500
tctataaagg acttctcgg gtccgagaac aaaaggaccg gcgcaactggg caatctcaag 1560
aactcataca gttataataa agataataat gagctttccc attttgccac aacctgctcc 1620
gacaaaagta gacctctgaa cgacctcgtg tcccaaaaga caacacagct gagtgatata 1680
acctccaggt tcaactcagc gatcgaggct ttgaacaggt tcatccagaa gtacgattca 1740
gtgatgcaga ggctggttga tgatactagc ggtaag 1776

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&lt;210&gt; SEQ ID NO 10

&lt;211&gt; LENGTH: 592

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: artificial

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: An example of a fusion protein including linkers

&lt;400&gt; SEQUENCE: 10

```

Met Ala Asn Phe Ser Gly Phe Thr Lys Gly Thr Asp Ile Ala Asp Leu
1           5           10           15
Asp Ala Val Ala Gln Thr Leu Lys Lys Pro Ala Asp Asp Ala Asn Lys
20           25           30
Ala Val Asn Asp Ser Ile Ala Ala Leu Lys Asp Lys Pro Asp Asn Pro
35           40           45
Ala Leu Leu Ala Asp Leu Gln His Ser Ile Asn Lys Trp Ser Val Ile
50           55           60
Tyr Asn Ile Asn Ser Thr Ile Val Arg Ser Met Lys Asp Leu Met Gln
65           70           75           80
Gly Ile Leu Gln Lys Phe Pro Gly Gly Gly Gly Ser Gly Gly Gly Gly
85           90           95
Ser Gly Gly Gly Gly Ser Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr
100          105          110
Ala Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly
115          120          125
Ala Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu
130          135          140
Val Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr
145          150          155          160
Ser Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe
165          170          175
Thr Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly
180          185          190
Lys Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn
195          200          205
Leu Val Gly Asp Asp Val Val Leu Ala Thr Gly Ser Gln Asp Phe Phe
210          215          220
Val Arg Ser Ile Gly Ser Lys Gly Gly Lys Leu Ala Ala Gly Lys Tyr
225          230          235          240
Thr Asp Ala Val Thr Val Thr Val Ser Asn Gln Gly Gly Gly Gly Ser
245          250          255
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Met Ile Arg Ala Tyr Glu
260          265          270
Gln Asn Pro Gln His Phe Ile Glu Asp Leu Glu Lys Val Arg Val Glu
275          280          285

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Gln Leu Thr Gly His Gly Ser Ser Val Leu Glu Glu Leu Val Gln Leu  
 290 295 300  
 Val Lys Asp Lys Asn Ile Asp Ile Ser Ile Lys Tyr Asp Pro Arg Lys  
 305 310 315 320  
 Asp Ser Glu Val Phe Ala Asn Arg Val Ile Thr Asp Asp Ile Glu Leu  
 325 330 335  
 Leu Lys Lys Ile Leu Ala Tyr Phe Leu Pro Glu Asp Ala Ile Leu Lys  
 340 345 350  
 Gly Gly His Tyr Asp Asn Gln Leu Gln Asn Gly Ile Lys Arg Val Lys  
 355 360 365  
 Glu Phe Leu Glu Ser Ser Pro Asn Thr Gln Trp Glu Leu Arg Ala Phe  
 370 375 380  
 Met Ala Val Met His Phe Ser Leu Thr Ala Asp Arg Ile Asp Asp Asp  
 385 390 395 400  
 Ile Leu Lys Val Ile Val Asp Ser Met Asn His His Gly Asp Ala Arg  
 405 410 415  
 Ser Lys Leu Arg Glu Glu Leu Ala Glu Leu Thr Ala Glu Leu Lys Ile  
 420 425 430  
 Tyr Ser Val Ile Gln Ala Glu Ile Asn Lys His Leu Ser Ser Ser Gly  
 435 440 445  
 Thr Ile Asn Ile His Asp Lys Ser Ile Asn Leu Met Asp Lys Asn Leu  
 450 455 460  
 Tyr Gly Tyr Thr Asp Glu Glu Ile Phe Lys Ala Ser Ala Glu Tyr Lys  
 465 470 475 480  
 Ile Leu Glu Lys Met Pro Gln Thr Thr Ile Gln Val Asp Gly Ser Glu  
 485 490 495  
 Lys Lys Ile Val Ser Ile Lys Asp Phe Leu Gly Ser Glu Asn Lys Arg  
 500 505 510  
 Thr Gly Ala Leu Gly Asn Leu Lys Asn Ser Tyr Ser Tyr Asn Lys Asp  
 515 520 525  
 Asn Asn Glu Leu Ser His Phe Ala Thr Thr Cys Ser Asp Lys Ser Arg  
 530 535 540  
 Pro Leu Asn Asp Leu Val Ser Gln Lys Thr Thr Gln Leu Ser Asp Ile  
 545 550 555 560  
 Thr Ser Arg Phe Asn Ser Ala Ile Glu Ala Leu Asn Arg Phe Ile Gln  
 565 570 575  
 Lys Tyr Asp Ser Val Met Gln Arg Leu Leu Asp Asp Thr Ser Gly Lys  
 580 585 590

<210> SEQ ID NO 11  
 <211> LENGTH: 5  
 <212> TYPE: PRT  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Linker

<400> SEQUENCE: 11

Gly Gly Gly Gly Ser  
1 5

<210> SEQ ID NO 12  
 <211> LENGTH: 33  
 <212> TYPE: DNA  
 <213> ORGANISM: artificial  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Primer



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<400> SEQUENCE: 12  
cacatatgag taacttctct ggatttacga aag 33

<210> SEQ ID NO 13  
<211> LENGTH: 31  
<212> TYPE: DNA  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 13  
cactcgagtg ggaacttctg taggatgcct t 31

<210> SEQ ID NO 14  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 14  
cacatatgaa aaaaatcagt tccgttatcg 30

<210> SEQ ID NO 15  
<211> LENGTH: 34  
<212> TYPE: DNA  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 15  
cactcgagtt ggtagatagc ggtagcggtt acag 34

<210> SEQ ID NO 16  
<211> LENGTH: 31  
<212> TYPE: DNA  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16  
cacatatgat tagagcctac gaacaaaacc c 31

<210> SEQ ID NO 17  
<211> LENGTH: 34  
<212> TYPE: DNA  
<213> ORGANISM: artificial  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 17  
cagtcgactt taccagacgt gtcacatagc agac 34

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What is claimed is:

1. A method comprising:

administering a first composition to a subject by an intranasal route,

wherein the first composition comprises a vector comprising a polynucleotide encoding a fusion protein, wherein the fusion protein comprises a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain; and

administering a second composition to the subject by an intramuscular route,

55

wherein the second composition comprises the fusion protein, wherein the fusion protein is isolated, and wherein the intramuscular administration is after the intranasal administration.

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2. The method of claim 1 wherein the fusion protein comprises at least one linker, wherein the linker is present between two of the domains.

65

3. The method of claim 1 wherein the fusion protein comprises a His-tag.

4. The method of claim 1 wherein the vector is a replication defective adenovirus vector.

55

5. The method of claim 4 wherein the defective adenovirus vector is type-5 (Ad5).

6. The method of claim 1 wherein the fusion protein comprises the YscF protein, the mature F1 protein, and the LcrV protein.

7. The method of claim 1 wherein the intramuscular administration is at least 7 days after the intranasal administration.

8. The method of claim 1 wherein the subject is a human.

9. The method of claim 1 wherein the administering confers immunity to plague caused by *Yersinia pestis*.

10. The method of claim 9 wherein the plague is pneumonic plague or bubonic plague.

11. A method comprising:

administering a composition to a subject by an intranasal route,

wherein the composition comprises a vector comprising a polynucleotide encoding a fusion protein,

wherein the fusion protein comprises a YscF protein domain, a mature F1 protein domain, and a LcrV protein domain; and

56

administering a second administration of the composition to the subject by an intranasal route.

12. The method of claim 11 wherein the fusion protein comprises at least one linker, wherein the linker is present  
5 between two of the domains.

13. The method of claim 11 wherein the fusion protein comprises a His-tag.

14. The method of claim 11 wherein the vector is a replication defective adenovirus vector.

10 15. The method of claim 14 wherein the defective adenovirus vector is type-5 (Ad5).

16. The method of claim 11 wherein the fusion protein comprises the YscF protein, the mature F1 protein, and the LcrV protein.

15 17. The method of claim 11 wherein the subject is a human.

18. The method of claim 11 wherein the administering confers immunity to plague caused by *Yersinia pestis*.

20 19. The method of claim 18 wherein the plague is pneumonic plague or bubonic plague.

\* \* \* \* \*