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NYVAC vector modified by *C7L* viral gene insertion improves T cell immune responses and effectiveness against leishmaniasis

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ABSTRACT

The NYVAC poxvirus vector is used as vaccine candidate for HIV and other diseases, although there is only limited experimental information on its immunogenicity and effectiveness for use against human pathogens. Here we defined the selective advantage of NYVAC vectors in a mouse model by comparing the immune responses and protection induced by vectors that express the LACK (*Leishmania*-activated C-kinase antigen), alone or with insertion of the viral host range gene *C7L* that allows the virus to replicate in human cells. Using DNA prime/virus boost protocols, we show that replication-competent NYVAC-LACK that expresses *C7L* (NYVAC-LACK-C7L) induced higher-magnitude polyfunctional $CD8^+$ and $CD4^+$ primary adaptive and effector memory T cell responses (IFNγ, TNFα, IL-2, CD107a) to LACK antigen than non-replicating NYVAC-LACK. Compared to NYVAC-LACK, the NYVAC-LACK-C7L-induced $CD8^+$ T cell population also showed higher proliferation when stimulated with LACK antigen. After a challenge by subcutaneous *Leishmania major* metacyclic promastigotes, NYVAC-LACK-C7L-vaccinated mouse groups showed greater protection than the NYVAC-LACK-vaccinated group. Our results indicate that the type and potency of immune responses induced by LACK-expressing NYVAC vectors is improved by insertion of the *C7L* gene, and that a replication-competent vector as a vaccine renders greater protection against a human pathogen than a non-replicating vector.

1. Introduction

Poxviruses are used extensively as vaccine vectors, due principally to the packing flexibility of their genome, which allows integration and expression of heterologous antigens, and to features such as low cost, stability, and ease of manufacture and administration. Despite these advantages, concerns regarding the safety of vaccinia virus (VACV) use during the smallpox immunization program (Lane et al., 1969; Redfield et al., 1987) emphasized the importance of developing highly attenuated strains as vaccine vectors for emerging infectious diseases and cancer (Gomez et al., 2011).

Two of the most extensively studied attenuated VACV strains are MVA (modified vaccinia virus Ankara) and NYVAC. NYVAC was derived from a plaque-cloned isolate of Copenhagen smallpox vaccine strain by selective deletion of 18 open reading frames involved in virulence, pathogenicity and host range regulation (Tartaglia et al., 1992). Interest in these vectors as vaccine candidates (Gomez et al., 2011) led us to develop NYVAC vectors that express a model antigen from a human pathogen, with distinct replication capacities in cultured cells. This included acquisition of a viral gene that confers a replication advantage on NYVAC in human cells. The model antigen, the *Leishmania* activated C-kinase (*LACK*) gene, is one of the most promising genes for leishmaniasis vaccination; it encodes a 36-kDa protein expressed in both parasite stages, is very immunogenic, is conserved in all *Leishmania* species (with 99% identity between *Leishmania major* and *Leishmania infantum* strains (Ahmed et al., 2004; Melby et al., 2001; Mougneau et al., 1995)), and shows protective results (Gonzalo et al., 2002, 2001; Tapia et al., 2003).

The *C7L* virus host range gene (Perkus et al., 1990) is responsible for biological differences between MVA and NYVAC attenuated strains (Najera et al., 2006). The C7 protein has anti-apoptotic functions (Najera et al., 2006); *C7L* and *K1L* (another host range gene) inhibit the antiviral effects of type I interferons (Meng et al., 2009), and the C7 N-terminal fragment is essential for C7 function (Terajima et al., 2013). Insertion of *C7L* into the genome of HIV antigen-expressing NYVAC increased immunogenicity against het-

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erologous antigens (Nájera et al., 2010). NYVAC-C7L safety studies in mice showed a restricted replication phenotype (Nájera et al., 2010); moreover, NYVAC vectors with inserted *C7L* and *K1L* have a good safety profile after intracranial inoculation of distinct NYVAC doses in newborn mice (Kibler et al., 2011).

We generated a NYVAC vector that expressed LACK antigen (NYVAC-LACK) and compared it with a NYVAC-LACK that also expressed *C7L* (NYVAC-LACK-C7L), which provides a replication advantage. In mice, we characterized T cell immunogenicity (primary adaptive, memory) and the protective effectiveness against *L. major* triggered by the heterologous DNA prime/NYVAC vector boost protocol, by comparing non-replicating NYVAC-LACK with replication-competent NYVAC-LACK-C7L.

2. Material and methods

2.1. Ethics statement

All mouse experiments were approved by the Ethics Committee for Animal Experimentation of the Centro Nacional de Biotecnología (CEEA CNB-CSIC; permit number 11037) in accordance with national and international guidelines for animal experimentation and with Royal Decree (RD 1201/2005).

2.2. Parasite strains and animals

For immunization studies, we used the WHOM/IR/-173 *L. major* strain, a kind gift from Dr Nicholas Glaichenhaus (CNSR, Val Bonne, France). Promastigotes were cultured at 27 °C in Schneider's Insect Medium supplemented with 20% fetal calf serum (FCS; both from Gibco BRL, UK) and antibiotics. Frozen stocks were thawed, cultured to the stationary phase, and used to infect BALB/c mice to maintain virulence. Aspirates from footpad lesions of infected mice were used to culture promastigotes for challenging vaccinated mice. Metacyclic promastigotes were purified as described (Sanchez-Sampedro et al., 2013). All mice were 6- to 8-week-old female BALB/c mice (Harlan), housed in pathogen-free conditions in the CNB-CSIC Animal Facility.

2.3. Cells, plasmids and viruses

Primary chicken embryo fibroblasts (CEF), African green monkey kidney cells (BSC40), human HeLa cells, mouse embryonic fibroblast cells (3T3), A20 cells (H-2d haplotype) and splenocytes were cultured as described (Sanchez-Sampedro et al., 2013).

The DNA-LACK plasmid is the previously characterized mammalian expression plasmid pCIneo-LACK (Perez-Jimenez et al., 2006). The empty plasmid pCIneo (Promega) was used as control (DNA-Φ). Both plasmids were purified using the EndoFree Plasmid Purification kit (Qiagen).

For LACK and C7L recombinant viruses, vaccinia insertion plasmids PCAR2-LACK and PCyA20-C7L were generated and purified with a plasmid purification kit (Quiagen). Viruses used included NYVAC-wt (kindly provided by Sanofi-Pasteur) and the recombinant NYVAC and NYVAC-C7L viruses expressing *L. infantum* LACK antigen in the viral hemagglutinin (HA) locus. Viruses were grown in CEF cells; purification and titration were described (Didierlaurent et al., 2004; Ramsay et al., 1997).

2.4. Construction of vaccinia virus recombinants expressing L. infantum *LACK antigen (NYVAC-LACK and NYVAC-LACK-C7L)*

The gene that encodes *L. infantum* LACK protein and the vaccinia virus *C7L* gene were inserted following an infection/transfection protocol (Sanchez-Sampedro et al., 2013), using vaccinia plasmids pCAR2-LACK and pCyA20-C7L to allow insertion into the HA and TK (thymidine kinase) loci, respectively.

2.5. Reagents

L. infantum rLACK protein was expressed and purified by affinity chromatography as described (Sanchez-Sampedro et al., 2012). The $LACK_{157\text{-}173}$ peptide (FSPSLEHPIVVSGSWDN) was chemically synthesized by the CNB Proteomics Service.

2.6. Immunization and parasite challenge

Eighteen 6- to 8-week-old female BALB/c mice/group were primed intradermally (i.d.) in the abdomen with 100 μg DNA-LACK or empty DNA-Φ in 100 μl PBS/mouse. On day 14, mice were boosted intraperitoneally (i.p.) with 2×10^7 plaque-forming units (pfu)/mouse of NYVAC-LACK, NYVAC-LACK-C7L, non-recombinant NYVAC-wt virus or PBS. We used 8 mice from each group to analyze primary adaptive and memory responses. At 8 weeks post-boost, the remaining 10 mice/group received a subcutaneous (s.c.) challenge in the right hind footpad using a Micro-Fine 0.5 ml 30 G needle (BD Biosciences). Parasite dose was 5×10^4 metacyclic peanut agglutinin (PNA)-purified *L. major* promastigotes in 10 μl PBS.

2.7. Intracellular cytokine staining assay (ICS)

Spleens from vaccinated mice were harvested and 4×10^6 splenocytes (erythrocyte-depleted by addition of 0.1 M NH_4Cl, 5 min on ice) were stimulated with distinct antigens and protocols, including rLACK antigen (25 μg/ml final concentration), $LACK_{157\text{-}173}$ peptide (4 μg/ml), A20 cells nucleofected with pCIneo-LACK plasmid (A20-LACK) or empty pCIneo plasmid at a 1:10 A20:splenocyte ratio, or RPMI 1640 medium alone. Cells were nucleofected with 6 μg plasmid 24 h before stimulation using a 4D nucleofector (Lonza). Splenocytes were then incubated 6 h in RPMI with 10% FCS, in the presence of GolgiPlug and monensin (BD Biosciences). After stimulation, cells were stained for surface and intracellular markers as described (Sanchez-Sampedro et al., 2013). The following antibodies were used for primary adaptive and memory studies: CD3-PE-CFS94 (145-2C11), CD4-APC-Cy7 (GK1.5), CD8-V500 (53-6.7), CD107a-AlexaFluor488 (eBio1D4B), CD62L-AlexaFluor700 (MEL-14), CD127-PerCP-Cy5.5 (A7R34), IFNγ-PECy-7 (XMG1.2), TNFα-PE (MP6-XT22) and IL-2-APC (JES6-5H4). Dead cells were excluded using the violet LIVE/DEAD stain kit (Invitrogen).

Cells were acquired using Gallios flow cytometer (Beckman Coulter) and data analyses were performed with FlowJo software version 8.5.3 (Tree Star). The number of events recorded ranged from 5×10^5 to 10^6.

2.8. Proliferation assay

Erythrocyte-depleted splenocytes (10^6) from vaccinated mice were harvested, washed with phosphate-buffered saline (PBS), resuspended to 10^6 cells/ml final concentration in PBS and stained with CellTrace CFSE (Life Technologies, Invitrogen). Cells were washed and resuspended for stimulation in RPMI with 10% FCS containing 0.5 μg/ml anti-CD28 antibody and 0.03 U/ml interleukin-2 (IL-2; BD Biosciences)) with 25 μg/ml rLACK or 4 μg/ml $LACK_{157\text{-}173}$ peptide. Cells were stimulated for 6 days before surface staining, which was performed as for the ICS assay using the following antibodies: CD3-APC (145-2C11), CD4-AlexaFluor700 (RM4-5), CD8-PECy7 (53-6.7) (Perez-Jimenez et al., 2006).