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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).

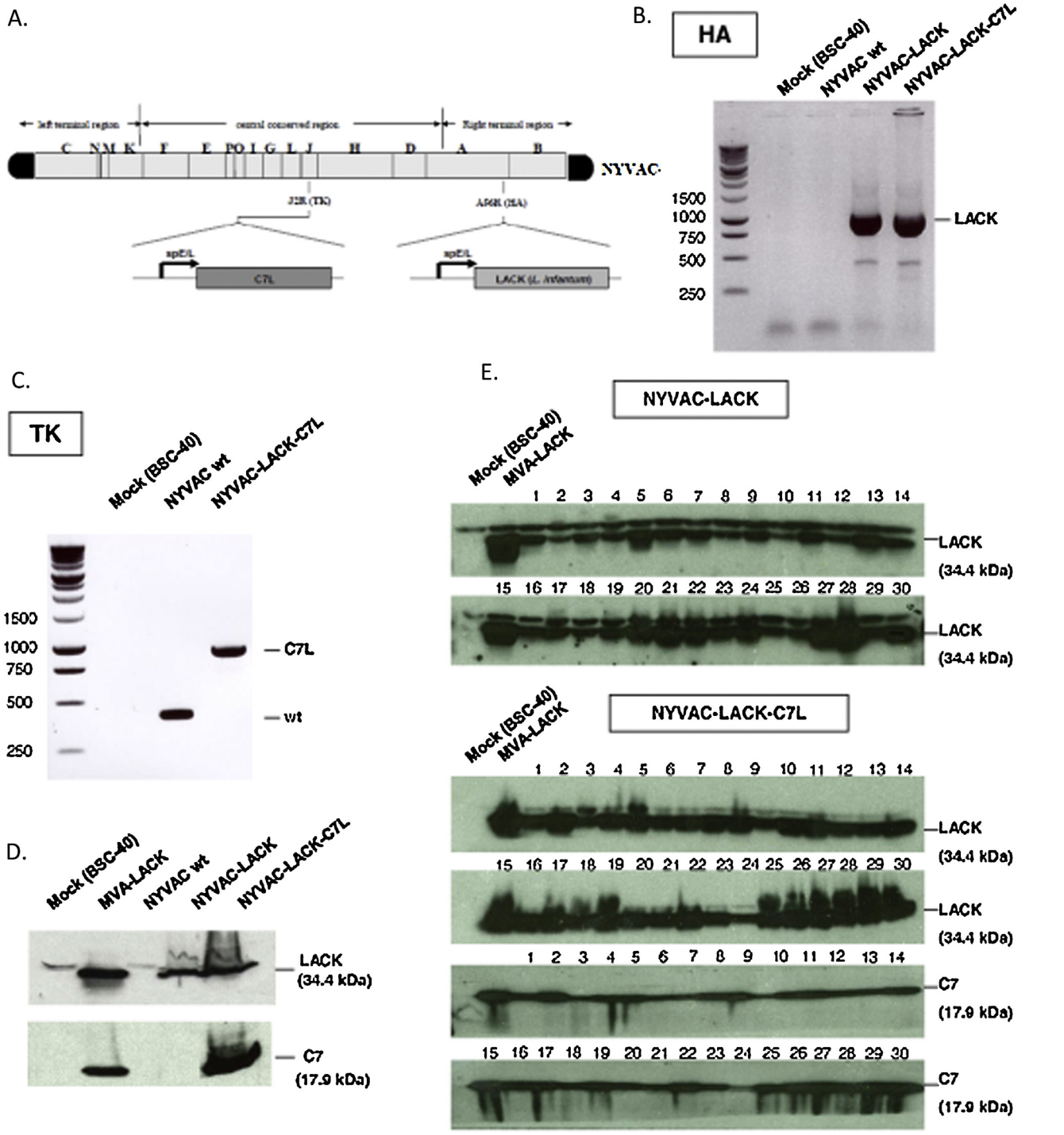

Fig. 1. Insertion, expression and stability of *LACK* and *C7L* by NYVAC vectors. (A) Organization of the *LACK* and *C7L* genes in the HA and TK loci of the NYVAC-LACK-C7L genome. (B) PCR analysis confirming full-length *LACK* insertion. In recombinant virus, a 1187-bp product is observed in HA insertion. (C) PCR analysis confirming full-length *C7L* insertion; in parental NYVAC, a 422-bp product was obtained, whereas in recombinant virus, a 951-bp product is observed in TK insertion. (D) Western blot analysis showing expression of heterologous proteins from infected BSC-40 cells. (E) Thirty individual plaques isolated from NYVAC-LACK- and NYVAC-LACK-C7L-infected cells at passage 13 were grown in BSC-40 cells. MVA-LACK-infected cells were used as control. Cells were infected with individual plaque isolates, lysed, proteins fractionated by 12% SDS-PAGE and analyzed by Western blot with a polyclonal anti-LACK or polyclonal anti-C7 antibody.

2.9. Statistical analyses

Statistical significance ($p < 0.05$ (*), $p < 0.005$ (**), or $p < 0.001$ (***)) of differences between viruses in growth curves in cultured cells and data from Luminex were determined by Student's *t*-test. Significance ($p < 0.05$ (*) or $p < 0.005$ (**)) of differences in lesion size between immunized mouse groups were determined by the Mann-Whitney test. For statistical analysis of ICS, we used an approach that corrects measurements for the response to medium alone (RPMI); the detailed procedure has been described (Nájera et al., 2010). All values used to analyze proportionate representation of responses are background-subtracted.

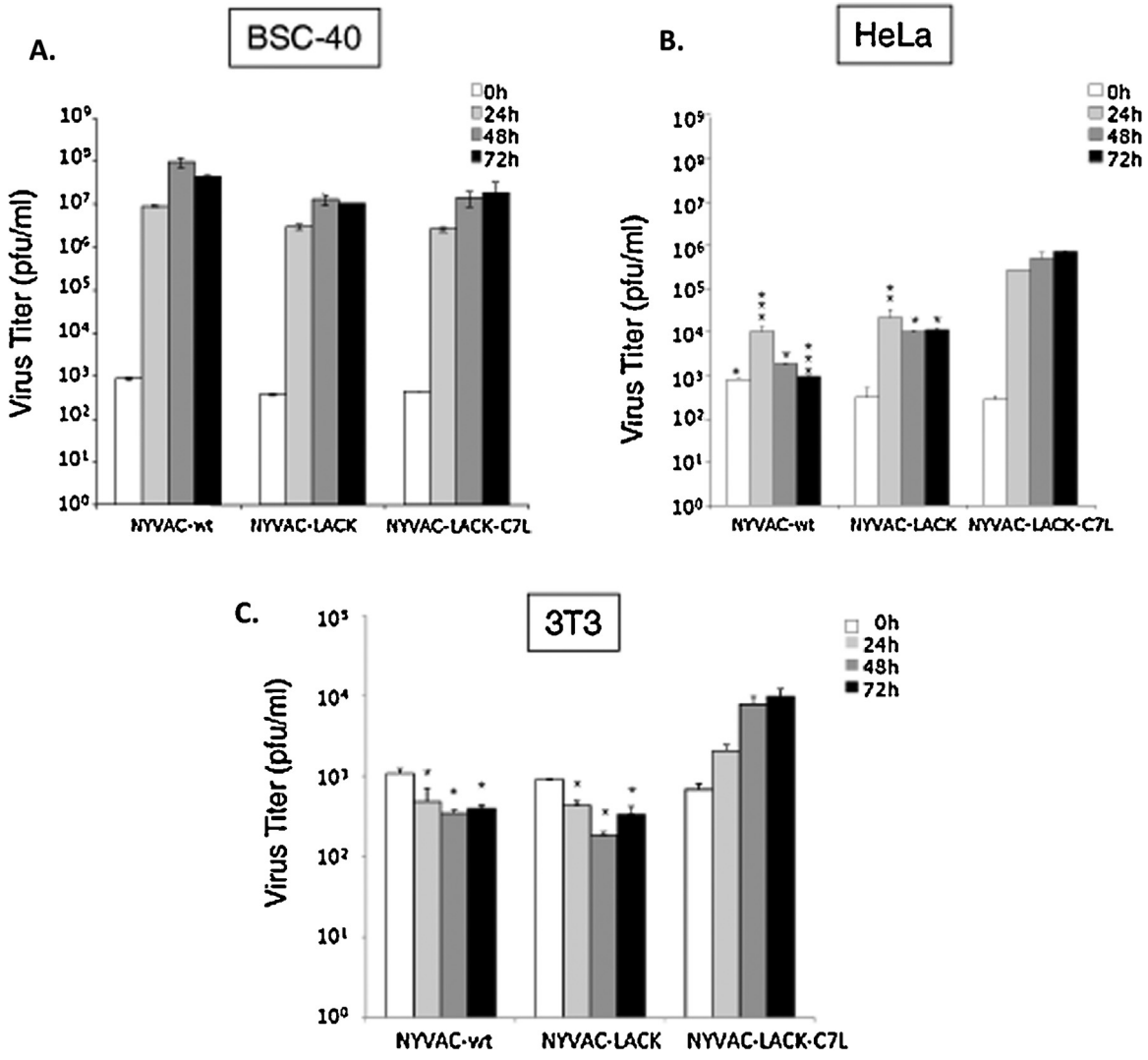

Fig. 2. *C7L* confers a growth advantage to NYVAC in human and murine cells. Comparative virus growth analysis in permissive and nonpermissive cell lines. (A) BSC-40 cell monolayers were infected (0.01 pfu/cell) with NYVAC-wt, NYVAC-LACK or NYVAC-LACK-C7L. Cells were collected at different times post-infection (0, 24, 48 and 72 h) and infective viruses were quantified by immunostaining assay of virus plaques. (B) HeLa cell monolayers were infected (0.01 pfu/cell) with NYVAC-wt, NYVAC-LACK or NYVAC-LACK-C7L; cells were collected and infective viruses quantified as in (A). (C) 3T3 cell monolayers were infected (0.01 pfu/cell) with NYVAC-wt, NYVAC-LACK or NYVAC-LACK-C7L; cells were collected and infective viruses quantified as in (A). Data from 2 different experiments. Error bars represent the SD in each graph. Statistical significance in comparison with NYVAC-LACK-C7L; Student's *t*-test; $p < 0.05$ (*), $p < 0.005$ (**) or $p < 0.001$ (***).

3. Results

3.1. *Generation of recombinant NYVAC expressing* L. infantum *LACK antigen and NYVAC-LACK expressing vaccinia virus C7*

To compare the immunogenic capacity and efficacy of NYVAC vectors, we developed two vectors that expressed the *Leishmania* LACK antigen, one bearing the *L. infantum LACK* gene inserted into the HA locus of the viral genome, and the other bearing in addition the vaccinia virus WR strain host range gene *C7L* in the TK locus (see Section 2 and Fig. 1A).

Correct insertion and purity of NYVAC-LACK and NYVAC-LACK-C7L viruses were confirmed by PCR and DNA sequence analysis. Viral DNA purified from BSC-40 cells infected with NYVAC-LACK or NYVAC-LACK-C7L was amplified with a set of primers that annealed to the HA and TK flanking sequences and with LACK internal primers (Supplementary Table S1 in the online version at DOI: 10.1016/j.virusres.2016.03.007). DNA extracted from cells infected with NYVAC-wt was used as control. The PCR product in NYVAC-LACK-infected cells amplified with a LACK forward primer and an HA reverse primer had a full-length size of 1187 bp (Fig. 1B), which indicates successful LACK insertion into the HA locus. PCR studies using HA forward and reverse primers showed no wild-type (wt) virus contamination in the preparation.

DNA extracted from NYVAC-LACK-C7L-infected BSC-40 cells amplified with TK forward and reverse primers produced a 422-bp PCR product (wt TK locus) and 951-bp (recombinant virus), which confirmed successful *C7L* insertion into the TK locus, with no wt virus contamination (Fig. 1C). Sequencing of the LACK insert in the NYVAC vectors confirmed that no mutations were introduced during vector generation. Heterologous expression of LACK and C7 proteins was confirmed in western blot with polyclonal rabbit anti-LACK and -C7 antibodies (Fig. 1D).

Genetic stability was defined after 13 passages in BSC-40 cells with 30 individual virus plaques picked and analyzed by western blot for LACK and C7 expression. All 30 plaques for both viruses (100%) expressed LACK and C7 proteins (in NYVAC-LACK-C7L) (Fig. 1E), indicating vector stability.

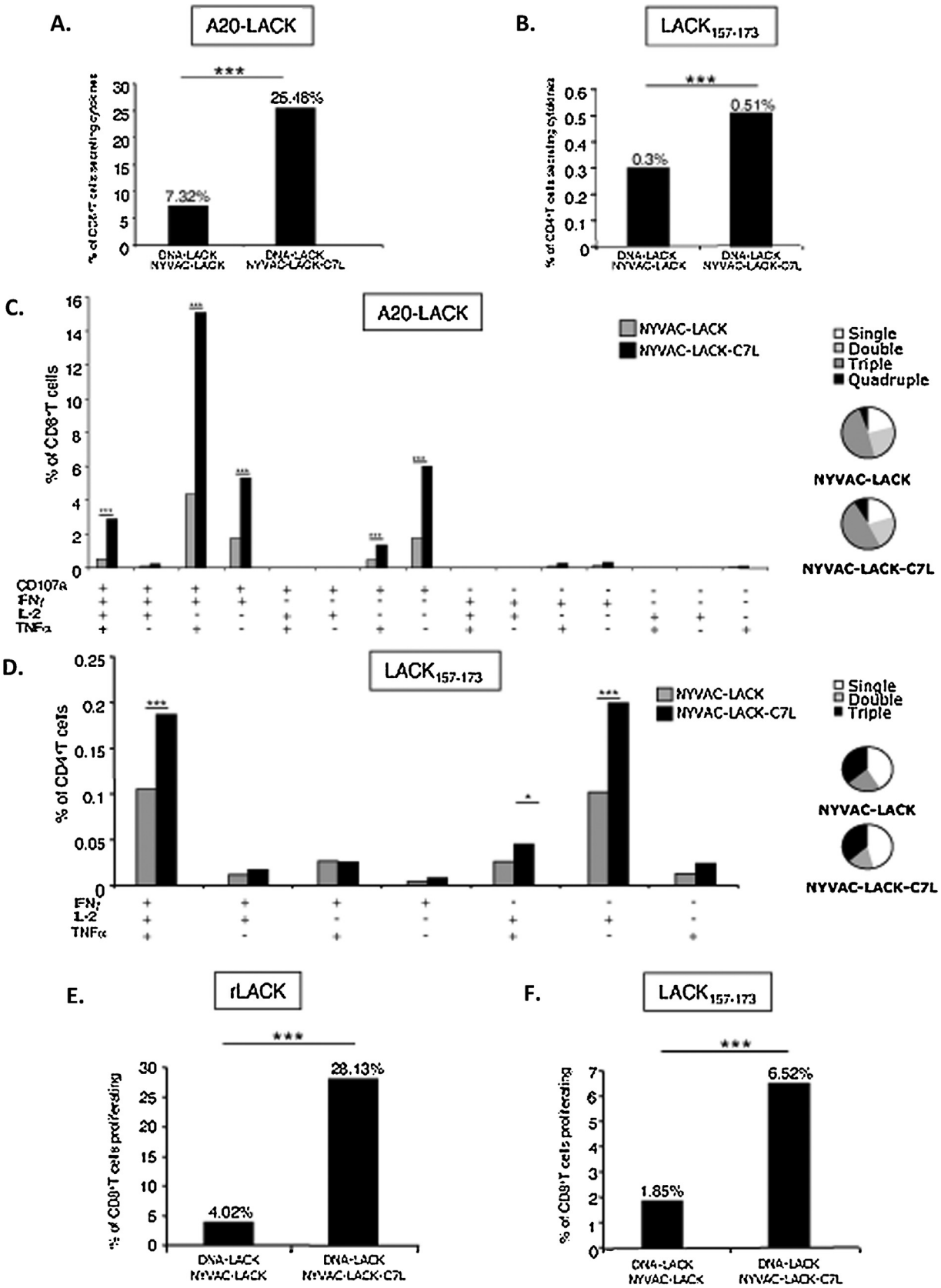

Fig. 3. Primary adaptive immune response and proliferation of T cells induced in mice immunized with NYVAC-LACK vectors. BALB/c mice (4 per group), 6–8 weeks of age, were primed intradermally (i.d.) in the abdomen with 100 μg DNA-LACK or DNA-Φ in 100 μl per mouse. At day 14, mice were boosted intraperitoneally (i.p) with 2×10^7 pfu/mouse NYVAC-LACK, NYVAC-LACK-C7L, or NYVAC-wt virus. Mice were sacrificed 11 days after boost. Total magnitude is shown of $CD8^+$ T cell responses in splenocytes restimulated with A20-LACK cells (A) or $CD4^+$ T cells restimulated with $LACK_{157\text{-}173}$ peptide (B). T cells were gated and analyzed for IFNγ, TNFα and/or IL-2 production and for CD107a. NYVAC-wt background was subtracted before representation (NYVAC control values are <0.05% of cytokine-secreting T cells). Cytokine production by A20-LACK-specific $CD8^+$ T cells (C) or by $LACK_{157\text{-}173}$ peptide-specific $CD4^+$ T cells (D). Cytokine combinations are indicated on the *x*-axis, and percentages of cytokine-producing T cells on the *y*-axis. Pie diagrams show the quality of the response measured as the relative quantity of single-, double-, triple- or quadruple-cytokine-producing cells. Splenocytes were CFSE-stained, antigen-stimulated for 6 days and stained with antibodies to cell surface antigens. CFSE-negative, proliferating $CD8^+$ T cells are shown after stimulation with rLACK protein (E) or $LACK_{157\text{-}173}$ peptide (F). Data representative of three independent experiments; ICS Statistics; $p < 0.05$ (*), $p < 0.001$ (***).

3.2. Growth advantage of NYVAC-LACK-C7L over NYVAC-LACK in human and murine cell lines

To confirm that LACK expression did not affect virus growth in vitro but that *C7L* was essential, we prepared growth curves for cell lines in which NYVAC is replicative (BSC-40) or non-replicative (HeLa and 3T3). We infected BSC-40, HeLa and 3T3 cell monolayers with NYVAC-wt, NYVAC-LACK or NYVAC-LACK-C7L. Cells were collected at various times post-infection (0, 24, 48 and 72 h) and infective viruses were titrated and quantified by immunostaining assay of virus plaques. Viral growth was similar between parental and recombinant NYVAC viruses in BSC-40 cells (Fig. 2A). In contrast, there were differences in virus growth kinetics in HeLa and 3T3 cells when the *C7L* gene was inserted into the NYVAC genome (Fig. 2B and C), with more than one-log higher virus titers than for NYVAC-wt or NYVAC-LACK infections.

3.3. NYVAC-LACK-C7L induces higher magnitude primary adaptive T cell immune responses than NYVAC-LACK

We compared the immunogenic characteristics of the NYVAC vectors in a mouse model, using an immunization protocol based on DNA and VACV vectors (heterologous prime/boost protocol) that induces higher T cell activation and protection against leishmaniasis than homologous vector immunization (Gonzalo et al., 2001; Pérez-Jiménez et al., 2006; Sanchez-Sampedro et al., 2013). To characterize the NYVAC vector-triggered immune responses, we inoculated mice as described (see Section 2). Eleven days post-boost, we used ICS to measure the primary adaptive response in splenocytes stimulated in vitro with A20-LACK cells or with $LACK_{157-173}$, a peptide essential for Th2 response development and linked to susceptibility (Launois et al., 2007).

To study the contribution of $CD8^+$ and $CD4^+$ T cells to the overall LACK-specific immune responses, we summed the frequency of A20-LACK-specific $CD8^+$ T cells that produced the degranulation marker CD107a and/or secreted TNFα, IL-2 and/or IFNγ (Fig. 3A), or of $LACK_{157-173}$ peptide-specific $CD4^+$ T cells that secreted TNFα, IL-2 and/or IFNγ (Fig. 3B). Responses were negligible in mice immunized with empty vector (DNA-Φ) and NYVAC-wt, whereas LACK-specific $CD8^+$ and $CD4^+$ T cells were induced in all groups that received DNA and LACK-expressing recombinant viral vectors. The DNA-LACK/NYVAC-LACK-C7L protocol induced a higher magnitude $CD8^+$ response (25.48%) than DNA-LACK/NYVAC-LACK (7.32%). Results were similar for $CD4^+$ responses, for which DNA-LACK/NYVAC-LACK-C7L induced a higher magnitude response (0.51%) than DNA-LACK/NYVAC-LACK (0.3%).

To assess the quality of the T cell populations triggered, we measured TNFα, IL-2, IFNγ and CD107a simultaneously. Exploration of all possible cytokine/CD107a combinations yielded 15 LACK-specific $CD8^+$ and seven $CD4^+$ T cell populations. The distinct NYVAC recombinant viruses induced the same $CD8^+$ T cell populations, with a high polyfunctional profile represented mainly by triple-positive cells that produced CD107a, IFNγ and TNFα (Fig. 3C). Other populations triggered by the heterologous protocol were single-positive cells that produced CD107a alone, double-positive cells with IFNγ or TNFα, or quadruple-positive cells that produced CD107a, IL-2, IFNγ and TNFα. Some differences were nonetheless observed in the magnitude of the response; NYVAC-LACK-C7L induced the highest magnitude LACK-specific $CD8^+$ T cell response in all populations compared with those induced by NYVAC-LACK.

To analyze the phenotype of the primary adaptive response to $LACK_{157-173}$, we evaluated the frequency of TNFα-, IL-2- and/or IFNγ-secreting cells after in vitro stimulation with $LACK_{157-173}$ peptide. No specific $CD8^+$ T cell responses were detected. The $CD4^+$ T cell response to $LACK_{157-173}$ peptide showed a highly polyfunctional profile, represented mainly by TNFα-, IL-2- and IFNγ-secreting cells (Fig. 3D). NYVAC-LACK-C7L-induced $CD4^+$ T cell responses were higher in magnitude than those induced by NYVAC-LACK. NYVAC-LACK-C7L vaccination led to increased frequency of IL-2 single-producer T cells. Splenocytes from distinct A20-LACK- or $LACK_{157-173}$-stimulated groups showed similar polyfunctional profiles, measured as relative quantity of single, double, triple or quadruple cytokine-producing cells (Fig. 3C, D).

We assessed the proliferative capacity of these $CD8^+$ and $CD4^+$ T cells after 6 days stimulation. rLACK protein induced more $CD8^+$ T cell proliferation in DNA-LACK/NYVAC-LACK-C7L (28.13%) than in DNA-LACK/NYVAC-LACK splenocytes (4.02%) (Fig. 3E), as was also the case for $LACK_{157-173}$ peptide (DNA-LACK/NYVAC-LACK-C7L, 6.52%; DNA-LACK/NYVAC-LACK, 1.85%) (Fig. 3F). Proliferating $CD4^+$ T cells were negligible with rLACK or with $LACK_{157-173}$ peptide.

3.4. NYVAC-LACK-C7L elicits polyfunctional effector memory $CD4^+$ and $CD8^+$ T cells with higher magnitude responses compared with NYVAC-LACK

As memory T cell responses are crucial for long-term protection against parasitic infection, we studied vaccine-elicited memory responses at 53 days post-boost in splenocytes from immunized mice stimulated with A20-LACK cells or $LACK_{157-173}$. The DNA-LACK/NYVAC-LACK-C7L protocol induced a higher overall $CD8^+$ T cell response (15.3%) than DNA-LACK/NYVAC-LACK (4.6%) (Fig. 4A); the total $CD4^+$ T cell response was also slightly higher in DNA-LACK/NYVAC-LACK-C7L (0.19%) than in those that received DNA-LACK/NYVAC-LACK-vaccinated mice (0.13%) (Fig. 4B).

The heterologous vaccination protocol using DNA-LACK and the two NYVAC recombinant viruses induced similar $CD8^+$ T cell populations, represented mainly by triple-positive cells that produced CD107a and secreted TNFα and IFNγ, as well as quadruple-positive cells (Fig. 4C). NYVAC-LACK-C7L induced a higher magnitude of triple- and quadruple-positive $CD8^+$ T cells than NYVAC-LACK. The differences in vector ability to trigger $CD8^+$ T cell responses were clearly shown by IFNγ and TNFα flow cytometry profiles after splenocyte stimulation with A20-LACK cells or $LACK_{157-173}$ compared with A20 cells alone or RPMI alone as controls (Supplementary Fig. S1 in the online version at DOI: 10.1016/j.virusres.2016.03.007).

The vaccine-induced $CD4^+$ T cell responses to $LACK_{157-173}$ peptide were represented mainly by triple-positive cells secreting TNFα, IL-2 and IFNγ and double-positive cells secreting TNFα and IL-2 (Fig. 4D). $LACK_{157-173}$-stimulated splenocytes from NYVAC-LACK- and NYVAC-LACK-C7L-vaccinated groups showed similar response magnitude and high polyfunctional profiles (Fig. 4D, pie diagram).

We analyzed the proliferative capacity of these memory $CD8^+$ and $CD4^+$ T cells after 6 days stimulation. rLACK protein induced stronger $CD8^+$ T cell proliferation in the DNA-LACK/NYVAC-LACK-C7L- (17.65%) than in the DNA-LACK/NYVAC-LACK-immunized group (2.54%) (Fig. 4E), also the case for $LACK_{157-173}$ peptide (DNA-LACK/NYVAC-LACK-C7L, 5.04%; DNA-LACK/NYVAC-LACK, 1.75%) (Fig. 4F). In the case of primary adaptive immunity analysis, we did not detect any proliferating $CD4^+$ T cells.

Supernatants of proliferating T cells were analyzed by Luminex for Th1 (IFNγ, TNFα, IL-2) and Th2 (IL-4, IL-10, IL13) cytokines. We observed a strong immune conversion towards the Th2 $LACK_{157-173}$ peptide that induces reduced levels of IL10 in comparison with IFNγ. This is more patent when stimulating with rLACK protein, where we observed lower Th2 than Th1 cytokine levels (Supplementary Table S2 in the online version at DOI: 10.1016/j.virusres.2016.03.007); the insertion of C7L also slightly reduced IL-10 levels ($p < 0.05$ (*)).

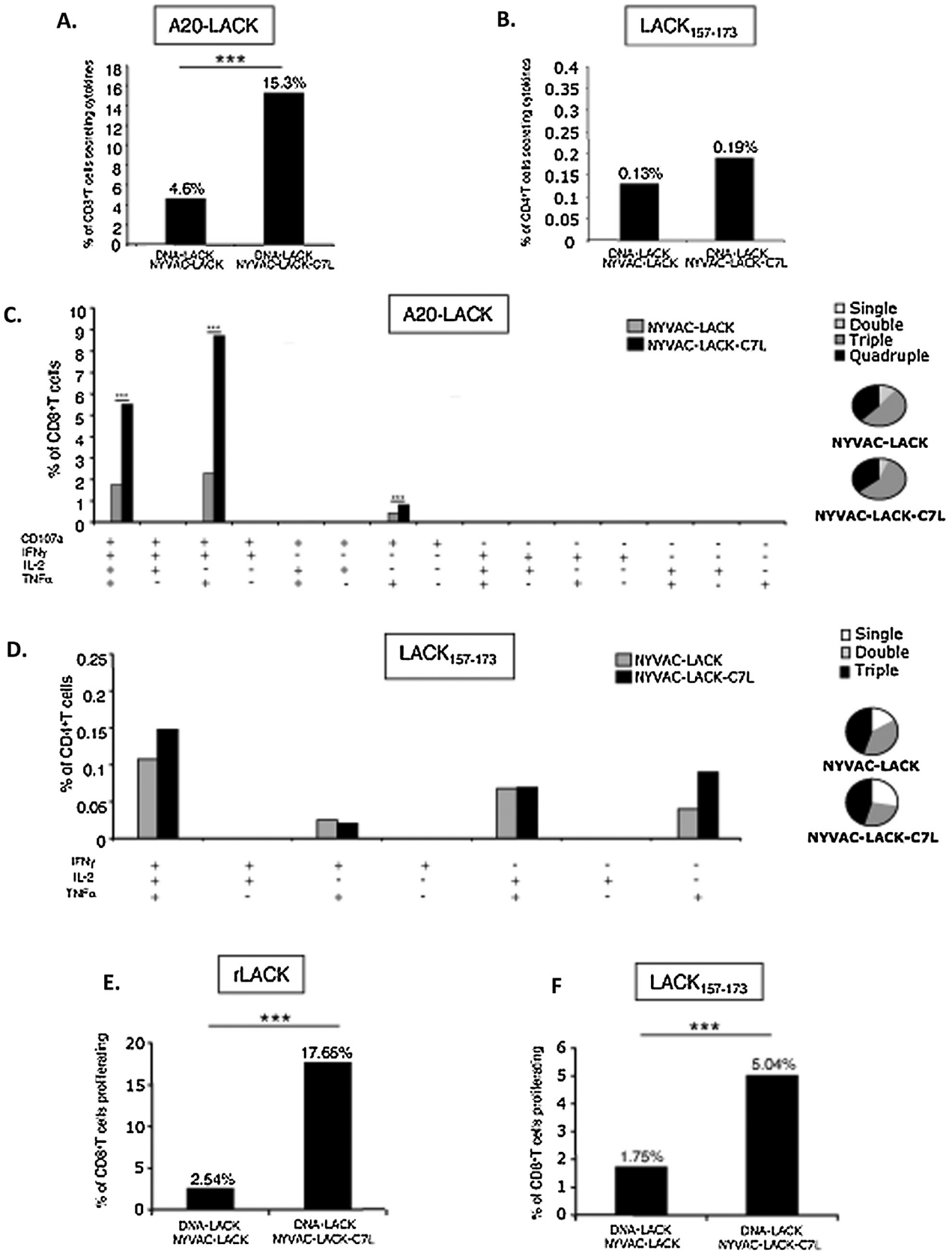

Fig. 4. Memory immune responses and proliferation of T cells induced in mice immunized with NYVAC-LACK vectors. BALB/c mice (4 per group) were immunized as in Fig. 3 and sacrificed 53 days after boost. Analysis of the magnitude of antigen-specific memory $CD4^+$ and $CD8^+$ T cell responses in splenocytes restimulated with A20-LACK cells (A) or $LACK_{157-173}$ peptide (B). T cells were gated and analyzed for IFNγ, TNFα and/or IL-2 production and for CD107a. NYVAC-wt background was subtracted before representation. Cytokine production by A20-LACK-specific $CD8^+$ T cells (C) or $LACK_{157-173}$ peptide-specific $CD4^+$ T cells (D). Cytokine combinations are indicated on the *x*-axis, and percentages of cytokine-producing T cells on the *y*-axis. Pie diagrams show the quality of the response measured as the relative quantity of single-, double-, triple- or quadruple-cytokine-producing cells. (E, F) Splenocytes were CFSE-stained, antigen-stimulated for 6 days and stained with antibodies to cell surface antigens. CFSE-negative, proliferating $CD8^+$ T cells after stimulation with rLACK protein (E) or $LACK_{157-173}$ peptide (F). Data representative of three independent experiments; ICS Statistics; $p < 0.001$ (***).

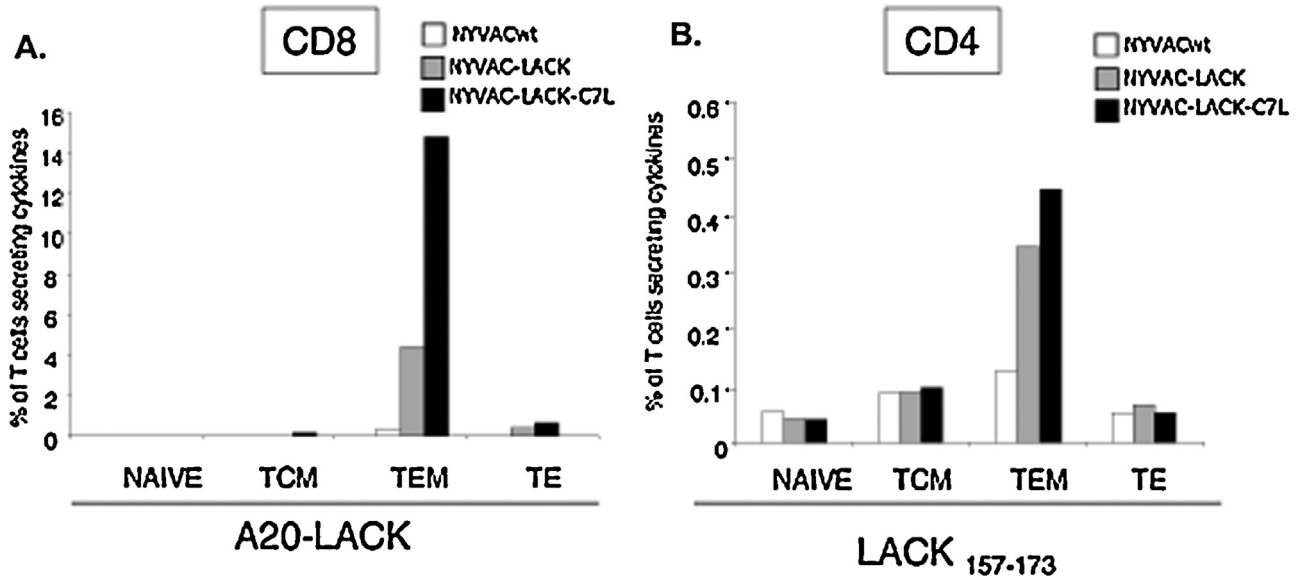

Fig. 5. Phenotype of memory immune response populations induced in mice immunized with NYVAC-LACK vectors. BALB/c mice (4 per group) were immunized as in Fig. 3 and sacrificed 53 days after boost. Phenotype of antigen-specific memory $CD8^+$ T cells in splenocytes restimulated with A20-LACK cells (A) or specific $CD4^+$ T cells in splenocytes restimulated with $LACK_{157-173}$ peptide. Memory T cells were classified as central memory ($CD62L^+CD127^+$; TCM), effector memory ($CD62L^-CD127^+$; TEM), effector ($CD62L^-CD127^-$; TE) or naïve ($CD62L^+CD127^-$). Data were analyzed by first gating cytokine expression in $CD4^+$ or $CD8^+$ populations and including all cytokine-producing cells in "CD4-producing cytokines" or "CD8-producing cytokines" Boolean gates. $CD4^+$ or $CD8^+$ populations were separated based on CD62L and CD127 staining. Percentages indicate the frequencies of T cells secreting IFNγ and/or TNFα and/or IL-2 and/or CD107a. Data representative of three independent experiments.

To differentiate between the LACK-specific memory populations elicited by heterologous immunization with the distinct recombinant viruses, we stained splenocytes for CD4, CD8, CD127 and CD62L surface markers. We used ICS to evaluate TNFα, IL-2 and IFNγ secretion and CD107a production after *in vitro* stimulation with A20-LACK (Fig. 5A) or $LACK_{157-173}$ (Fig. 5B). Both $CD8^+$ (A20-LACK) and $CD4^+$ ($LACK_{157-173}$) antigen-specific T cell responses showed mainly an effector memory phenotype, with highest $CD8^+$ T cell activation in A20-LACK cell-stimulated NYVAC-LACK-C7L splenocytes.

3.5. *NYVAC-LACK-C7L triggers greater protection against* Leishmania *infection than NYVAC-LACK*

To determine whether the differences in primary adaptive and memory immune responses between NYVAC-LACK and NYVAC-LACK-C7L affected protection against leishmaniasis, we tested vaccine effectiveness in the highly susceptible BALB/c mice, which develop cutaneous lesions after s.c. inoculation of parasites (Von Stebut et al., 2003). Groups of 10 mice vaccinated following the DNA prime/NYVAC boost protocol were challenged in the footpad 53 days after boost with 5×10^4 metacyclic (PNA-purified) *L. major* promastigotes. Vaccinated mouse groups showed distinct degrees of reduction in lesion size (Fig. 6A, left). Statistical differences between immunization groups were analyzed by the Mann-Whitney test (Fig. 6A, right). At endpoint, the reduction in the DNA-LACK/NYVAC-LACK-vaccinated group (29%) versus the DNA-φ/NYVAC-wt virus group did not present a statistically significant difference ($p = 0.07$); the difference versus the PBS group presented a statistically significant difference (32.79%; $p = 0.0156$). The largest reductions in lesion size were observed in the DNA-LACK/NYVAC-LACK-C7L group compared with DNA-φ/NYVAC-wt (56.9%; $p = 0.0019$) and PBS/PBS groups (59.12%; $p = 0.0048$). The protection triggered by NYVAC-LACK-C7L in comparison with NYVAC-LACK showed a statistically significant difference (39.17%; $p = 0.043$) (Fig. 6A). Overall, the mouse vaccination studies showed that recombinant NYVAC-LACK-C7L was superior to NYVAC-LACK for protection against leishmaniasis.

4. Discussion

Vaccine-bearing VACV vectors are widely used as candidates against a broad spectrum of diseases (Jacobs et al., 2009). MVA and NYVAC are two of the most advanced poxvirus vaccine vectors (Gomez et al., 2011). Although a replication-competent NYVAC is being considered for HIV vaccine, information is limited on the protective capacity of these vectors for human diseases (Kibler et al., 2011). In this study, we used a leishmaniasis disease model to compare the immunogenicity and protective capacity of NYVAC vectors with different replication capacities in human cells. We generated two NYVAC recombinant vectors, one expressing *L. infantum* LACK antigen (NYVAC-LACK) and one expressing LACK and the host range gene *C7L* (NYVAC-LACK-C7L). We compared vector capacity to trigger $CD8^+$ and $CD4^+$ T cell immune responses, as well as effectiveness in the highly susceptible mouse BALB/c leishmaniasis model (Garcia-Arriaza et al., 2014; Gomez et al., 2007, 2013).

NYVAC-LACK is non-replicative, whereas NYVAC-LACK-C7L is replicative in murine 3T3 and human HeLa cells. NYVAC-C7L has a restricted replication phenotype (Nájera et al., 2010) and NYVAC recombinants with inserted host range genes *C7L* and *K1L* have a good safety profile after intracranial inoculation of newborn mice with NYVAC (Kibler et al., 2011). The introduction of *C7L* into the NYVAC genome affects replication capacity but does not increase virulence, and the vector safety profile is maintained (Kibler et al., 2011).

Our study of the type of immune response triggered after mouse immunization with the two NYVAC vectors focused mainly on $CD8^+$ and $CD4^+$ T cell populations following heterologous vaccination protocols with DNA-LACK priming and boost with NYVAC-LACK or NYVAC-LACK-C7L virus. The primary adaptive response elicited was characterized by a high frequency of polyfunctional LACK-specific $CD8^+$ T cell responses, with a majority of $IFN\gamma^+TNF\alpha^+CD107a^+$ cells. These activated T cells produce IFNγ and TNFα, crucial cytokines that synergize (Cote-Sierra et al., 2002; Liew et al., 1991) to combat *Leishmania* infection (Kima and Soong, 2013; Murray et al., 2000; Wilhelm et al., 2001), and express CD107a, a marker of cytotoxic degranulating cells (Novais

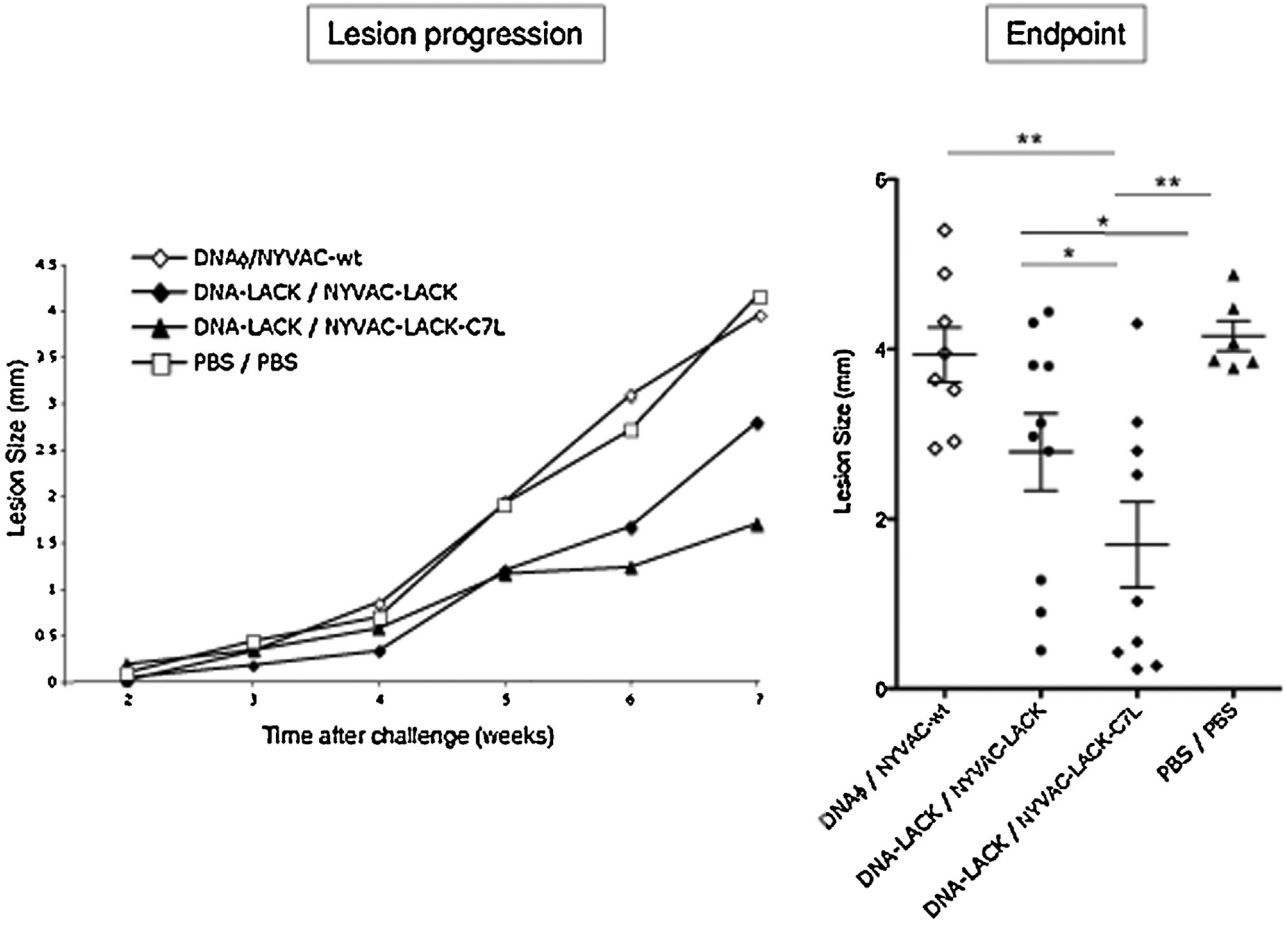

Fig. 6. Reduced lesion size in mice vaccinated against leishmaniasis with NYVAC-LACK vectors. BALB/c mice (10 per group) were immunized as in Fig. 3 and challenged as described (see Section 2). Left, lesion development after challenge with 5×10^4 *L. major* metacyclic promastigotes in the right footpad 53 days after boost. Right, individual lesion size at endpoint. Horizontal bars indicate mean values. Data representative of three independent experiments; Mann-Whitney test; $p < 0.05$ (*), $p < 0.005$ (**).

et al., 2013). Re-stimulated T cells also showed high proliferation capacity, thereby demonstrating that the vaccine triggered large numbers of high quality, cytotoxic, LACK-specific $CD8^+$ T cells.

We detected polyfunctional $LACK_{157-173}$-specific $CD4^+$ T cells, as half of the population coexpressed IL-2, IFNγ and TNFα. These cells are of interest, given the immune conversion from a Th2 response (Launois et al., 1997) towards a high quality Th1 response to the epitope, and because polyfunctional $CD4^+$ antigen-specific T cells are defined as a correlate of protection (Darrah et al., 2007; Glennie et al., 2015; Macedo et al., 2012; Matos et al., 2013; Peters et al., 2014). Although previous studies showed a Th2 response after immunizing with recombinant vaccinia viruses encoding LACK antigen (Stober et al., 2005), we observed a clear Th1 immune conversion in supernatants of proliferating T cells analyzed for Th1 and Th2 cytokines. The peptide induced lower IL-10 levels of in comparison with IFNγ· We also noticed that the insertion of C7L slightly reduces IL-10 cytokine levels and increases IL-2 and TNFα in comparison with parental NYVAC-LACK virus. When stimulating with rLACK protein, we observed even lower levels of Th2 compared with Th1 cytokines.

The activated T cell populations showed strong antigen-specific memory responses. These cells were of the effector memory phenotype, since the LACK-specific $CD8^+$ and $CD4^+$ T cell populations were mainly $CD62L^-CD127^+$. The effector memory populations were divided into low frequency, high quality $CD4^+$ T cells and high frequency, LACK-specific $CD8^+$ T cells that maintained polyfunctionality, cytotoxic profile, and proliferative capacity; the latter are relevant for long-term protective immunity against parasites (Rigato et al., 2011; Schmidt et al., 2008).

We found differences between NYVAC vectors in the magnitude of the overall response; NYVAC-LACK-C7L elicited statistically significant stronger LACK-specific $CD8^+$ and $CD4^+$ cellular immune responses than the replication-restricted NYVAC-LACK, as reported for HIV antigens (Nájera et al., 2010). There were notable differences between LACK-specific $CD8^+$ and $CD4^+$ T cells, with an approximately 2-log higher frequency of $CD8^+$ T cells.

Recent research on *Leishmania braziliensis* shows that, due to their cytolytic activity, $CD8^+$ T cells drive development of metastatic lesions in cutaneous leishmaniasis (Novais et al., 2013; Novais and Scott, 2015). $CD8^+$ T cells are nonetheless crucial for long-term protective immunity to parasites (Kaushal et al., 2014; Reyes-Sandoval et al., 2011; Rigato et al., 2011). These cells also promote development of $CD4^+$ Th1 cells rather than the typical, disease-promoting Th2 response in natural infections (Uzonna et al., 2004). For some *leishmania* species such as *L. major*, $CD8^+$ T cells contribute to resistance against primary and secondary infections (Novais and Scott, 2015). The immunoregulatory role of $CD8^+$ T cells after DNA-LACK vaccination and the maintenance of $CD4^+$ T cell responses are also described (Gurunathan et al., 2000). These data support a possible in vivo $CD8^+$ T cell requirement for long-term protection of LACK-vaccinated mice.

Our data also suggest that differences in $CD8^+$ and $CD4^+$ T cell population frequencies can lead to vaccine success or failure, as indicated by immunization with different VACV strains such as MVA, M65 and M101 (Sanchez-Sampedro et al., 2013), and

reinforced in the present study using NYVAC-LACK and NYVAC-LACK-C7L viruses.

When we measured the footpad lesion size as an indicator of vaccine effectiveness, NYVAC-LACK-C7L-vaccinated mice showed higher lesion reduction compared with NYVAC-LACK-vaccinated mice. Our pre-challenge findings showed that those vectors that induced the highest responses in terms of polyfunctional $CD8^+/CD4^+$ T cells and T effector memory phenotype correlated with more successful control of the infection.

Our studies confirm an enhanced immunogenicity by insertion of the *C7L* gene into the NYVAC vector within a *L. major* model, as previously reported in the case of HIV antigens (Nájera et al., 2010). Moreover, here we showed in a challenge model that this augmented immunogenicity correlated with higher degree of protection against a pathogen.

Further improvements in NYVAC-LACK-C7L might include the selective removal of immune evasion genes, as described for HIV antigen-expressing MVA and NYVAC vectors (Esteban, 2009; Gomez et al., 2011).

5. Conclusion

Our findings provide evidence that NYVAC-LACK and NYVAC-LACK-C7L vectors preferentially trigger LACK-specific $CD8^+$ T cell responses, with a reduced $CD4^+$ T cell response. Both vectors induced primary adaptive and effector memory responses that were polyfunctional. The difference between the vectors lies mainly in the magnitude of the responses they induce, which was greater in the case of NYVAC-LACK-C7L than NYVAC-LACK. The type and potency of immune responses induced by LACK-expressing NYVAC vectors are thus improved by *C7L* insertion, as this replication-competent vector rendered greater protection against the pathogen than the non-replicating vector.

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References

Ahmed, S.B., Bahloul, C., Robbana, C., Askri, S., Dellagi, K., 2004. A comparative evaluation of different DNA vaccine candidates against experimental murine leishmaniasis due to *L. major*. Vaccine 22 (13-14), 1631–1639.

Cote-Sierra, J., Bredan, A., Toldos, C.M., Stijlemans, B., Brys, L., Cornelis, P., Segovia, M., de Baetselier, P., Revets, H., 2002. Bacterial lipoprotein-based vaccines induce tumor necrosis factor-dependent type 1 protective immunity against *Leishmania major*. Infect. Immun. 70 (1), 240–248.

Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W.B., Davey, D.F., Flynn, B.J., Hoff, S.R.T., Andersen, P., Reed, S.G., Morris, S.L., Roederer, M., Seder, R.A., 2007. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. Nat. Med. 13 (7), 843.

Didierlaurent, A., Ramirez, J.C., Gherardi, M., Zimmerli, S.C., Graf, M., Orbea, H.A., Pantaleo, G., Wagner, R., Esteban, M., Kraehenbuhl, J.P., Sirard, J.C., 2004. Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-a2-restricted cytotoxic T-cell responses. Vaccine 22 (25-26), 3395–3403.

Esteban, M., 2009. Attenuated poxvirus vectors MVA and NYVAC as promising vaccine candidates against HIV/AIDS. Hum. Vaccin. 5 (12), 867–871.

Garcia-Arriaza, J., Cepeda, V., Hallengard, D., Sorzano, C.O., Kummerer, B.M., Liljestrom, P., Esteban, M., 2014. A novel poxvirus-based vaccine, MVA-cHIKV, is highly immunogenic and protects mice against chikungunya infection. J. Virol. 88 (6), 3527–3547.

Glennie, N.D., Yeramilli, V.A., Beiting, D.P., Volk, S.W., Weaver, C.T., Scott, P., 2015. Skin-resident memory CD4+ T cells enhance protection against *Leishmania major* infection. Journal Exp. Med. 212 (9), 1405–1414.

Gomez, C.E., Najera, J.L., Jimenez, E.P., Jimenez, V., Wagner, R., Graf, M., Frachette, M.J., Liljestrom, P., Pantaleo, G., Esteban, M., 2007. Head-to-head comparison on the immunogenicity of two HIV/AIDS vaccine candidates based on the attenuated poxvirus strains MVA and NYVAC co-expressing in a single locus the HIV-1BX08 gp120 and HIV-1(IIIB) Gag-Pol-Nef proteins of clade B. Vaccine 25 (15), 2863–2885.

Gomez, C.E., Najera, J.L., Krupa, M., Perdiguero, B., Esteban, M., 2011. MVA and NYVAC as vaccines against emergent infectious diseases and cancer. Curr. Gene Ther. 11 (3), 189–217.

Gomez, C.E., Perdiguero, B., Cepeda, M.V., Mingorance, L., Garcia-Arriaza, J., Vandermeeren, A., Sorzano, C.O., Esteban, M., 2013. High, broad, polyfunctional, and durable T cell immune responses induced in mice by a novel hepatitis C virus (HCV) vaccine candidate (MVA-HCV) based on modified vaccinia virus Ankara expressing the nearly full-length HCV genome. J. Virol. 87 (13), 7282–7300.

Gonzalo, R.M., Rodriguez, J.R., Rodriguez, D., Gonzalez-Aseguinolaza, G., Larraga, V., Esteban, M., 2001. Protective immune response against cutaneous leishmaniasis by prime/booster immunization regimens with vaccinia virus recombinants expressing *Leishmania infantum* p36/LACK and IL-12 in combination with purified p36. Microbes Infect. 3 (9), 701–711.

Gonzalo, R.M., del Real, G., Rodriguez, J.R., Rodriguez, D., Heljasvaara, R., Lucas, P., Larraga, V., Esteban, M., 2002. A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the *Leishmania infantum* P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. Vaccine 20 (7-8), 1226–1231.

Gurunathan, S., Stobie, L., Prussin, C., Sacks, D.L., Glaichenhaus, N., Iwasaki, A., Fowell, D.J., Locksley, R.M., Chang, J.T., Wu, C.Y., Seder, R.A., 2000. Requirements for the maintenance of Th1 immunity in vivo following DNA vaccination: a potential immunoregulatory role for CD8+ T cells. J. Immunol. 165 (2), 915–924.

Jacobs, B.L., Langland, J.O., Kibler, K.V., Denzler, K.L., White, S.D., Holechek, S.A., Wong, S., Huynh, T., Baskin, C.R., 2009. Vaccinia virus vaccines: past, present and future. Antiviral Res. 84 (1), 1–13.

Kaushal, H., Bras-Goncalves, R., Negi, N.S., Lemesre, J.L., Papierok, G., Salotra, P., 2014. Role of CD8(+) T cells in protection against *Leishmania donovani* infection in healed Visceral Leishmaniasis individuals. BMC Infect. Dis. 14, 653.

Kibler, K.V., Gomez, C.E., Perdiguero, B., Wong, S., Huynh, T., Holechek, S., Arndt, W., Jimenez, V., Gonzalez-Sanz, R., Denzler, K., Haddad, E.K., Wagner, R., Sekaly, R.P., Tartaglia, J., Pantaleo, G., Jacobs, B.L., Esteban, M., 2011. Improved NYVAC-based vaccine vectors. PLoS One 6 (11), e25674.

Kima, P.E., Soong, L., 2013. Interferon gamma in leishmaniasis. Front. Immunol. 4, 156.

Lane, J.M., Ruben, F.L., Neff, J.M., Millar, J.D., 1969. Complications of smallpox vaccination, 1968. N. Engl. J. Med. 281 (22), 1201–1208.

Launois, P., Maillard, I., Pingel, S., Swihart, K.G., Xénarios, I., Acha-Orbea, H., Diggelmann, H., Locksley, R.M., MacDonald, H.R., Louis, J.A., 1997. IL-4 rapidly produced by V beta 4 V alpha 8CD4+ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. Immunity 6 (5), 541.

Launois, P., Pingel, S., Himmelrich, H., Locksley, R., Louis, J., 2007. Different epitopes of the LACK protein are recognized by V beta 4 V alpha 8CD4+ T cells in H-2b and H-2d mice susceptible to *Leishmania major*. Microbes Infect. 9 (11), 1260.

Liew, F.Y., Li, Y., Yang, D.M., Severn, A., Cox, F.E., 1991. TNF-α reverses the disease-exacerbating effect of subcutaneous immunization against murine cutaneous leishmaniasis. Immunology 39, 304.

Macedo, A.B., Sanchez-Arcila, J.C., Schubach, A.O., Mendonca, S.C., Marins-Dos-Santos, A., de Fatima Madeira, M., Gagini, T., Pimentel, M.I., De Luca, P.M., 2012. Multifunctional CD4 T cells in patients with American cutaneous leishmaniasis. Clin. Exp. Immunol. 167 (3), 505–513.

Matos, I., Mizenina, O., Lubkin, A., Steinman, R.M., Idoyaga, J., 2013. Targeting antigens to dendritic cells in vivo induces protective immunity. PLoS One 8 (6), e67453.

Melby, P.C., Yang, J., Zhao, W., Perez, L.E., Cheng, J., 2001. *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect. Immun. 69 (8), 4719–4725.

Meng, X., Jiang, C., Arsenio, J., Dick, K., Cao, J., Xiang, Y., 2009. Vaccinia virus K1L and C7L inhibit antiviral activities induced by type I interferons. J. Virol. 83 (20), 10627–10636.

Mougneau, E., Altare, F., Wakil, A.E., Zheng, S., Coppola, T., Wang, Z.E., Waldmann, R., Locksley, R.M., Glaichenhaus, N., 1995. Expression cloning of a protective *Leishmania* antigen. Science 268 (5210), 563–566.

Murray, H.W., Jungbluth, A., Ritter, E., Montelibano, C., Marino, M.W., 2000. Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment. Infect. Immun. 68 (11), 6289–6293.

Nájera, J.L., Gómez, C.E., García-Arriaza, J., Sorzano, C.O., Esteban, M., 2010. Insertion of vaccinia virus C7L host range gene into NYVAC-B genome potentiates immune responses against HIV-1 antigens. PLoS One 5 (6), e11406-e11406.

Najera, J.L., Gomez, C.E., Domingo-Gil, E., Gherardi, M.M., Esteban, M., 2006. Cellular and biochemical differences between two attenuated poxvirus vaccine candidates (MVA and NYVAC) and role of the C7L gene. J. Virol. 80 (12), 6033–6047.

Novais, F.O., Scott, P., 2015. CD8+ T cells in cutaneous leishmaniasis: the good, the bad, and the ugly. Semin. Immunopathol. 37 (3), 251–259.

Novais, F.O., Carvalho, L.P., Graff, J.W., Beiting, D.P., Ruthel, G., Roos, D.S., Betts, M.R., Goldschmidt, M.H., Wilson, M.E., de Oliveira, C.I., Scott, P., 2013. Cytotoxic T cells mediate pathology and metastasis in cutaneous leishmaniasis. PLoS Pathog. 9 (7), e1003504.

Pérez-Jiménez, E., Kochan, G., Gherardi, M.M., Esteban, M., 2006. MVA-LACK as a safe and efficient vector for vaccination against leishmaniasis. Microbes Infect. 8 (3), 810.
Perez-Jimenez, E., Kochan, G., Gherardi, M.M., Esteban, M., 2006. MVA-LACK as a safe and efficient vector for vaccination against leishmaniasis. Microbes Infect.
Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., Paoletti, E., 1990. Vaccinia virus host range genes. Virology 179 (1), 276–286.
Peters, N.C., Pagan, A.J., Lawyer, P.G., Hand, T.W., Henrique Roma, E., Stamper, L.W., Romano, A., Sacks, D.L., 2014. Chronic parasitic infection maintains high frequencies of short-lived Ly6C + CD4+ effector T cells that are required for protection against re-infection. PLoS Pathog. 10 (12), e1004538.
Ramsay, A.J., Leong, K.H., Ramshaw, I.A., 1997. DNA vaccination against virus infection and enhancement of antiviral immunity following consecutive immunization with DNA and viral vectors. Immunol. Cell Biol. 75 (4), 382.
Redfield, R.R., Wright, D.C., James, W.D., Jones, T.S., Brown, C., Burke, D.S., 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. N. Engl. J. Med. 316 (11), 673–676.
Reyes-Sandoval, A., Wyllie, D.H., Bauza, K., Milicic, A., Forbes, E.K., Rollier, C.S., Hill, A.V., 2011. CD8+ T effector memory cells protect against liver-stage malaria. J. Immunol. 187 (3), 1347–1357.
Rigato, P.O., de Alencar, B.C., de Vasconcelos, J.R., Dominguez, M.R., Araujo, A.F., Machado, A.V., Gazzinelli, R.T., Bruna-Romero, O., Rodrigues, M.M., 2011. Heterologous plasmid DNA prime-recombinant human adenovirus 5 boost vaccination generates a stable pool of protective long-lived CD8(+) T effector memory cells specific for a human parasite, *Trypanosoma cruzi*. Infect. Immun. 79 (5), 2120–2130.
Sanchez-Sampedro, L., Gomez, C.E., Mejias-Perez, E., Sorzano, C.O., Esteban, M., 2012. High quality long-term CD4+ and CD8+ effector memory populations stimulated by DNA-LACK/MVA-LACK regimen in *Leishmania major* BALB/c model of infection. PLoS One 7 (6), e38859.
Sanchez-Sampedro, L., Gomez, C.E., Mejias-Perez, E., Perez-Jimenez, E., Oliveros, J.C., Esteban, M., 2013. Attenuated and replication-competent vaccinia virus strains M65 and M101 with distinct biology and immunogenicity as potential vaccine candidates against pathogens. J. Virol. 87 (12), 6955–6974.
Schmidt, N.W., Podyminogin, R.L., Butler, N.S., Badovinac, V.P., Tucker, B.J., Bahjat, K.S., Lauer, P., Reyes-Sandoval, A., Hutchings, C.L., Moore, A.C., Gilbert, S.C., Hill, A.V., Bartholomay, L.C., Harty, J.T., 2008. Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. Proc. Natl. Acad. Sci. U. S. A. 105 (37), 14017.
Stober, C.B., Lange, U.G., Roberts, M.T., Alcami, A., Blackwell, J.M., 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine *Leishmania major* infection. J. Immunol. 175 (4), 2517–2524.
Tapia, E., Pérez-Jiménez, E., López-Fuertes, L., Gonzalo, R., Gherardi, M.M., Esteban, M., 2003. The combination of DNA vectors expressing IL-12 + IL-18 elicits high protective immune response against cutaneous leishmaniasis after priming with DNA-p36/LACK and the cytokines, followed by a booster with a vaccinia virus recombinant expressing p36/LACK. Microbes Infect. 5 (2), 73.
Tartaglia, J., Perkus, M.E., Taylor, J., Norton, E.K., Audonnet, J.C., Cox, W.I., Davis, S.W., van der Hoeven, J., Meignier, B., Riviere, M., et al., 1992. NYVAC: a highly attenuated strain of vaccinia virus. Virology 188 (1), 217–232.
Terajima, M., Urban, S.L., Leporati, A.M., 2013. The N-terminus of vaccinia virus host range protein C7L is essential for function. Virus Genes 46 (1), 20–27.
Uzonna, J.E., Joyce, K.L., Scott, P., 2004. Low dose *Leishmania major* promotes a transient T helper cell type 2 response that is down-regulated by interferon gamma-producing CD8+ T cells. Journal Exp. Med. 199 (11), 1559–1566.
Von Stebut, E., Ehrchen, J.M., Belkaid, Y., Kostka, S.L., Molle, K., Knop, J., Sunderkotter, C., Udey, M.C., 2003. Interleukin 1alpha promotes Th1 differentiation and inhibits disease progression in *Leishmania major*-susceptible BALB/c mice. J. Exp. Med. 198 (2), 191–199.
Wilhelm, P., Ritter, U., Labbow, S., Donhauser, N., Rollinghoff, M., Bogdan, C., Korner, H., 2001. Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. J. Immunol. 166 (6), 4012–4019.