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Modification of promoter spacer length in vaccinia virus as a strategy to control the antigen expression

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Vaccinia viruses (VACVs) with distinct early promoters have been developed to enhance antigen expression and improve antigen-specific CD8 T-cell responses. It has not been demonstrated how the length of the spacer between the coding region of the gene and its regulatory early promoter motif influences antigen expression, and whether the timing of gene expression can modify the antigen-specific CD4 T-cell response. We generated several recombinant VACVs based on the attenuated modified vaccinia Ankara (MVA) strain, which express GFP or the *Leishmania* LACK antigen under the control of an optimized promoter, using different spacer lengths. Longer spacer length increased GFP and LACK early expression, which correlated with an enhanced LACK-specific memory CD4 and CD8 T-cell response. These results show the importance of promoter spacer length for early antigen expression by VACV and provide alternative strategies for the design of poxvirus-based vaccines.

INTRODUCTION

Poxvirus-based vaccines are used to generate strong antigen-specific T-cell responses that control and clear pathogen infections (Ha *et al.*, 2008). Poxvirus vectors are thus being tested as candidate vaccines to express heterologous pathogen antigens, and are being further improved to potentiate antigen-specific immune responses (immunogenicity) (García-Arriaza & Esteban, 2014).

During a poxvirus infection, T cell immunogenicity correlates with antigen expression (Wyatt *et al.*, 2008). The quantity of antigen produced can affect the T-cell immunodominance hierarchy (Wilson & Hunter, 2008); that is, the distinct levels of immunogenicity of all antigens expressed by a virus (Yewdell & Bennink, 1999).

Recent studies of vaccinia virus (VACV) show that the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition by immune system cells (Moutaftsi *et al.*, 2009); 90 % of all antigens recognized by CD8 T-cells rank in the top 50 % in terms of mRNA temporal expression (Sette *et al.*, 2009). For this reason, efforts to develop new poxvirus vaccine candidates focus on using promoters to improve the timing of antigen expression and thus increase immune responses.

VACV gene expression is controlled by early, intermediate and late promoters (Yang *et al.*, 2011b, 2013). In mice, the timing of viral antigen expression correlates with better antigen-specific CD8 T-cell responses (Bronte *et al.*, 1997); early antigen-specific T-cells have a proliferative advantage over late antigen-specific T-cells (Kastenmuller *et al.*, 2007). Early VACV promoters such as pHyB (Baur *et al.*, 2010), psFJ1-10 (Isshiki *et al.*, 2014; Sato *et al.*, 2013) and PrMVA13.5-long (Wennier *et al.*, 2013), which bear different tandem early promoter elements, have been tested with different heterologous antigens; they were shown to be more effective compared to the widely used p7.5 and synthetic early late (pS) promoters (Chakrabarti *et al.*, 1997; Cochran *et al.*, 1985). A new LEO (late-early optimized) promoter, with an optimization of the early promoter motif, was also compared to pS and used to increase heterologous early antigen expression and antigen-specific CD8 T-cell responses (Di Pilato *et al.*, 2013). These promoters differ in their early motif sequence and in spacer lengths between the gene and the early promoter. It is nonetheless not known how the length of the spacer between the early promoter motif and the gene under promoter control affects the early antigen expression.

CD4 T-cells recognize VACV intermediate antigens better than early or late gene products (Yang *et al.*, 2011b). CD4 T-cells have a crucial role in mediating protection against a variety of pathogens such as human immunodeficiency virus (HIV), influenza, *Plasmodium falciparum* and *Leishmania* (Darrah *et al.*, 2007; Reece *et al.*, 2004; Román *et al.*, 2002; Younes *et al.*, 2003). In man and in mouse models of

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Leishmania major infection, multifunctional T-helper type 1 (Th1) CD4 T-cells correlate with vaccine-mediated protection (Darrah *et al.*, 2010; Macedo *et al.*, 2012). It is nevertheless unclear whether it is possible to potentiate antigen-specific CD4 T-cell responses by inducing heterologous early antigen expression during VACV infection. Modified vaccinia virus (MVA), a highly attenuated VACV, expressing LACK antigen (*Leishmania* homologue of receptors for activated C kinase), has been used as a safe, efficient vector able to induce a multifunctional, long-term, LACK-specific CD4 T-cell response (Sánchez-Sampedro *et al.*, 2012).

We generated several MVA vectors that express GFP or LACK antigens under the transcriptional control of LEO promoters with different spacer lengths. A LEO promoter with a spacer length of 160 nt increased GFP or LACK early expression compared to one with a spacer length of 38 nt. We show that enhancement of LACK early expression correlated with the length of the spacer, leading to increased LACK-specific CD4 and CD8 T-memory cell responses.

RESULTS

Promoter spacer length and GFP antigen expression

We constructed LEO promoters with different spacer lengths. The LEO promoter (termed here LEO38) has 29 nt of the late element, 15 nt of the early motif and a 38 nt multi-cloning site (MCS) spacer that represents the distance between the last nucleotide of the early core and the ATG of the gene under its control (Di Pilato *et al.*, 2013). We designed two new promoters, LEO99 and LEO160, with the same late-early LEO composition and two MCS spacers of 99 or 160 nt (Fig. 1a). The GFP ORF was cloned downstream of the LEO99 and LEO160 promoters into the VACV insertional pLZAW1 plasmid vector (Gómez *et al.*, 2007a, b) and introduced into the MVA thymidine kinase (*TK*) locus (Gómez *et al.*, 2007a, b). The MVA-LEO99-GFP and MVA-LEO160-GFP viruses were selected from MVA-WT by consecutive purification rounds (Gómez *et al.*, 2007b). Absence of MVA-WT contamination and correct GFP insertion in the *TK* locus were confirmed by PCR amplification (Fig. 1b).

Early GFP expression by MVA-LEO99-GFP and MVA-LEO160-GFP viruses was compared with that of the MVA-LEO38-GFP virus, which induces significantly more early GFP expression than MVA-pS-GFP (Di Pilato *et al.*, 2013). We infected human HeLa cells with the distinct MVA-GFP viruses at an m.o.i. of 10 p.f.u. $cell^{-1}$ and evaluated GFP expression in a time-course assay. In the first 6 h of infection, MVA-LEO160-GFP induced higher GFP levels compared to MVA-LEO99-GFP and MVA-LEO38-GFP virus, as determined by flow cytometry (Fig. 2a). To analyse early gene expression specifically, and to avoid contamination by late gene expression, the HeLa cells were treated for 6 h with cytosine arabinoside (AraC), an inhibitor of DNA replication. In these conditions of early phase MVA infection cycle arrest, MVA-LEO160-GFP induced more GFP expression than the other viruses (Fig. 2a). Median fluorescence intensity (MFI) analysis of HeLa GFP levels showed that in the first 6 h of infection, alone or with AraC, MVA-LEO160-GFP significantly increased HeLa GFP production compared to MVA-LEO99-GFP or MVA-LEO38-GFP (Fig. 2b). The difference in GFP expression between MVA-LEO160-GFP and MVA-LEO38-GFP was approximately twofold from 2 to 6 h post-infection (p.i.). During the same period, MVA-LEO99-GFP induced significantly more GFP expression than MVA-LEO38-GFP, indicating a correlation between spacer length and gene expression (Fig. 2b).

Results were similar for MVA-GFP viruses following infection of murine J774 macrophages and human THP-1 monocytes differentiated into macrophages (Fig. 2c, d). MVA-LEO160-GFP and MVA-LEO99-GFP significantly increased GFP levels compared to those induced by MVA-LEO38-GFP; the difference between MVA-LEO160-GFP and MVA-LEO99-GFP was statistically significant from 4 h p.i. (Fig. 2c, d).

To evaluate the contribution of early and late promoter motifs on GFP expression, we infected HeLa cells with the MVA viruses and analysed GFP levels at 8 h p.i., since the VACV replication cycle requires approximately 8 h (Fig. 2e). In this experiment, we included the pS promoter, which directs strong late gene expression (Baur *et al.*, 2010). MVA-LEO160-GFP and MVA-LEO99-GFP significantly increased GFP levels compared to those induced by MVA-LEO38-GFP (Fig. 2e). To determine whether the difference in GFP expression reflected distinct GFP mRNA levels induced by distinct viruses, we isolated total RNA from HeLa cells infected at an m.o.i. of 10 p.f.u. $cell^{-1}$ for 1 or 2 h. MVA-LEO160-GFP significantly increased *GFP* transcription compared with MVA-LEO38-GFP and MVA-LEO99-GFP; in turn, MVA-LEO99-GFP enhanced GFP mRNA levels compared with MVA-LEO38-GFP (Fig. 2f).

To determine whether infection with MVA-LEO160-GFP increased GFP expression *in vivo*, we infected BALB/c mice by intraperitoneal (i.p.) injection of 10^7 p.f.u. MVA-LEO38-GFP or MVA-LEO160-GFP. Since macrophages are one of the most susceptible cell types to MVA infection (Brandler *et al.*, 2010) and constitute approximately 30% of the total peritoneal cell yield (Ray & Dittel, 2010), only $GFP^+F4/80^{high}$ macrophages were analysed (Fig. 3a); both viruses showed similar infective capacity (>80%) (Fig. 3b). GFP^+ macrophages from MVA-LEO160-GFP-infected mice had significantly higher GFP levels than those from MVA-LEO38-GFP-infected mice at 4 h p.i. (Fig. 3c).

Promoter spacer length and LACK antigen expression

Considering the strength of the LEO160 promoter in inducing better early antigen expression among the promoters studied *in vitro*, we decided to compare its capacity to generate antigen-specific CD8 and CD4 T-cell responses with the LEO38 promoter. We cloned the *Leishmania* LACK ORF

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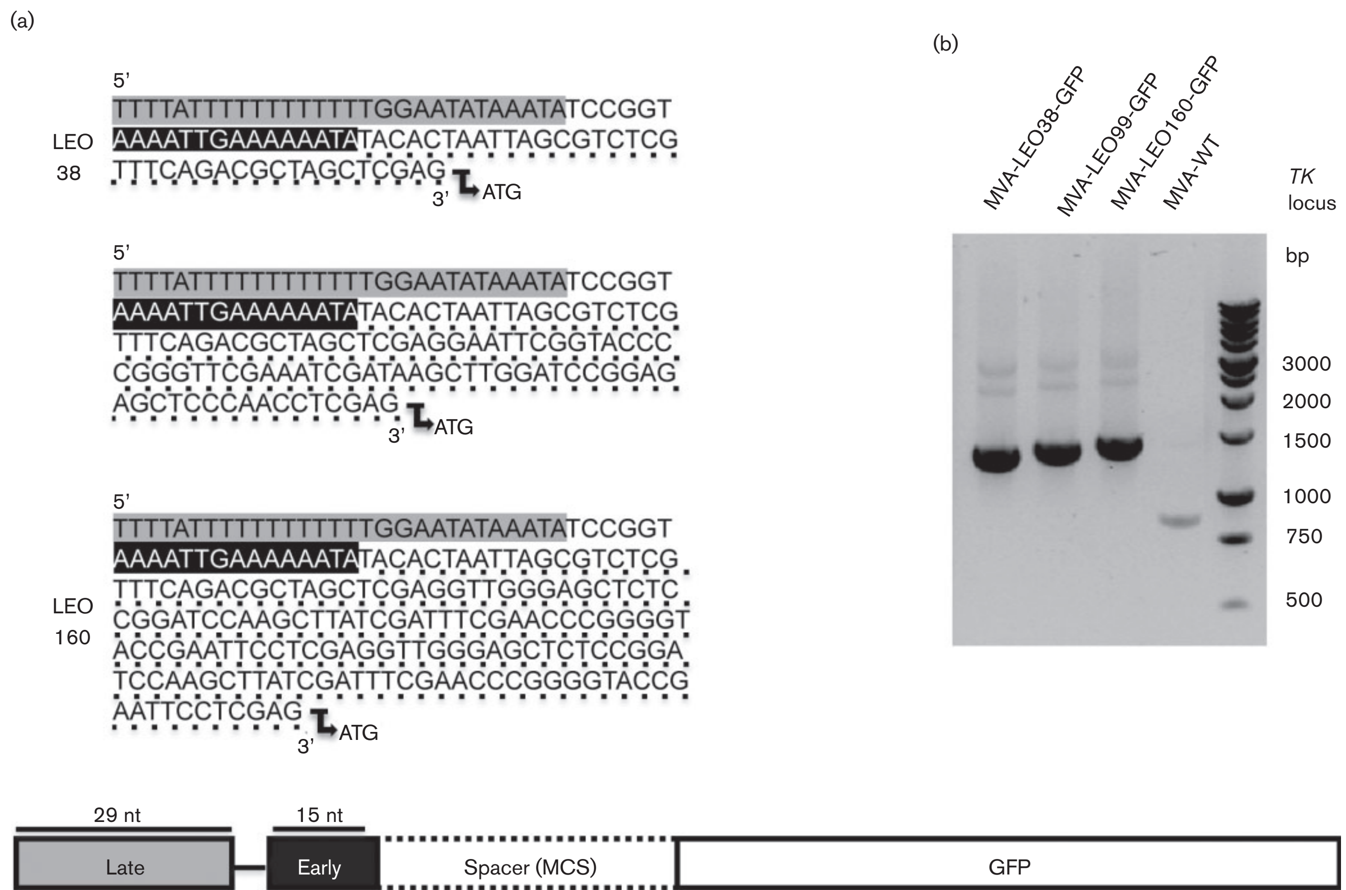

Fig. 1. Schematic and insertion of LEO38, LEO99 and LEO160 promoters with the *GFP* gene into the MVA virus genome. (a) Sequence of LEO38, LEO99 and LEO160 promoters and schematic with the *GFP* gene. Late promoter element, grey box; early promoter motif, black box; spacer, white box with dashed line. (b) Promoter and *GFP* gene insertion into the MVA virus *TK* locus confirmed by PCR analysis. An 873 bp product was obtained in parental MVA, whereas in MVA-LEO38-GFP, MVA-LEO99-GFP and MVA-LEO160-GFP recombinant viruses, 1312, 1373 and 1434 bp products were observed, respectively.

downstream of LEO38 or LEO160 promoters (Fig. 4a). To demonstrate that differences in early antigen expression were spacer length-dependent and not spacer sequence-dependent, we modified the spacer MCS sequence and maintained the same nucleotide number in MVA-LEO160-LACK. The new VACV insertional pLZAW1 plasmid vectors were used to generate the MVA-LEO38-LACK and MVA-LEO160-LACK viruses, which were selected from MVA-WT by consecutive purification rounds (Gómez *et al.*, 2007b). Absence of MVA-WT contamination and correct LACK insertion in the *TK* locus were confirmed by PCR amplification (Fig. 4b).

The difference in LACK levels between these two viruses was tested by Western blot (Fig. 5a) in HeLa cells infected at an m.o.i. of 10 p.f.u. cell^{-1}. From 2 to 6 h p.i., alone or with AraC, MVA-LEO160-LACK significantly increased LACK expression (approximately twofold) compared with MVA-LEO38-LACK (Fig. 5b), which confirmed the results obtained for the GFP antigen. LACK levels were normalized to E3 (VACV constitutive early protein) levels to show that the difference in heterologous antigen expression was the result of distinct promoter strengths, and not of different virus infective capacities. We found no differences in E3 expression between the viruses (Fig. 5a). These *in vitro* and *in vivo* results indicate that promoter spacer length positively influences gene early expression under its control.

LACK antigen-specific memory T-cell responses

To determine whether increased LACK early expression enhances LACK-specific T-cell responses, we infected BALB/c mice i.p. with two doses of MVA-LEO38-LACK or MVA-LEO160-LACK virus, spaced by 1 week to avoid a high anti-VACV IgG antibody response (Hagensee *et al.*, 1995) that could impair the MVA infective capacities. This homologous immunization protocol, in which mice were virus-inoculated more than once, was used because it is reported that

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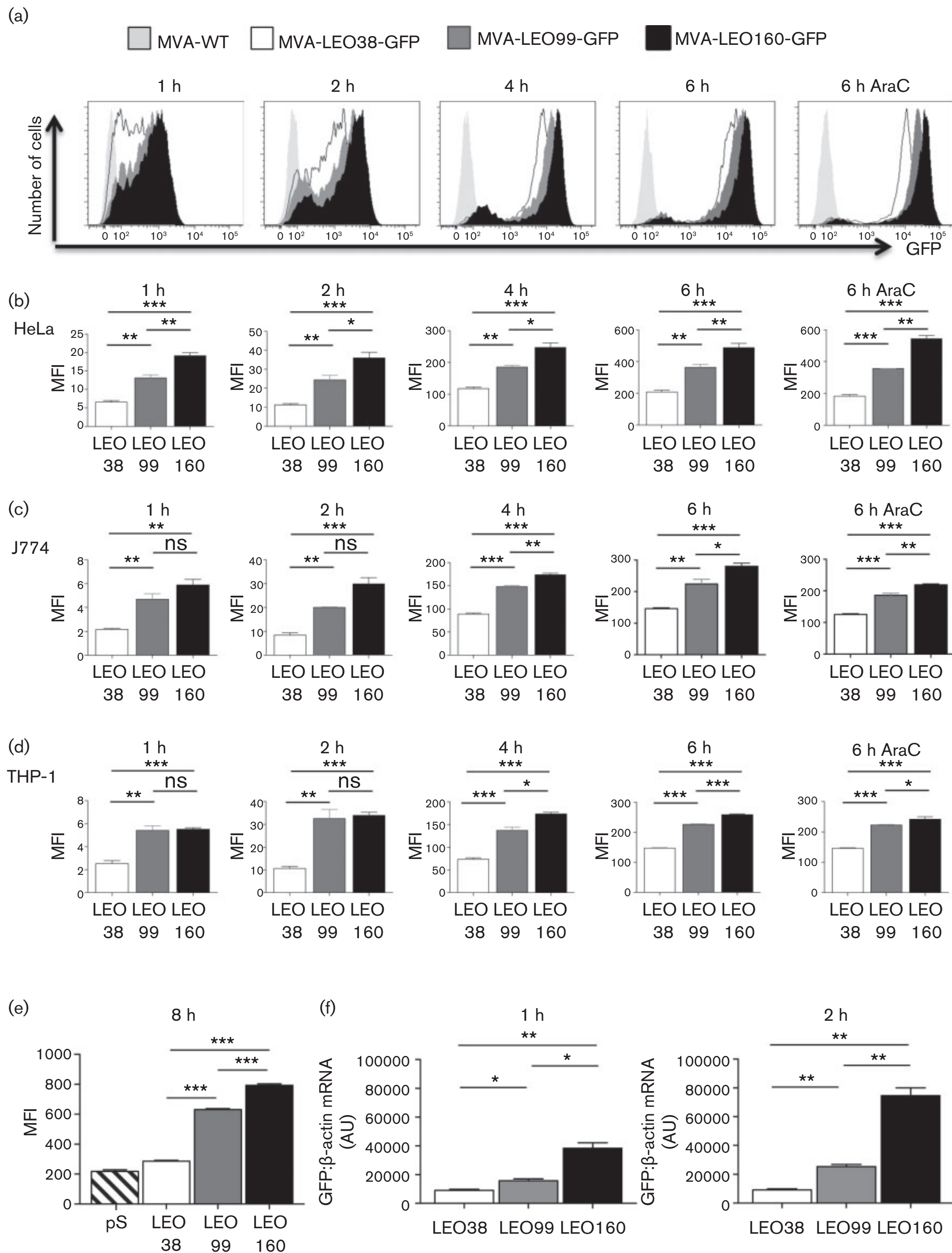

Fig. 2. *In vitro* GFP expression driven by LEO38, LEO99 and LEO160 promoters. (a) GFP expression in HeLa cells infected with MVA-WT, MVA-LEO38-GFP, MVA-LEO99-GFP or MVA-LEO160-GFP viruses (m.o.i. of 10 p.f.u. $cell^{-1}$) at 1, 2, 4 and 6 h p.i., and with AraC (40 μg ml^{-1}) at 6 h p.i. Graphs show fold increases in GFP MFI of MVA-GFP compared with MVA-WT viruses in HeLa (b), J774 (c) and THP-1 cells (d). (e) GFP expression in HeLa cells infected with MVA-WT, MVA-pS-GFP, MVA-LEO38-GFP, MVA-LEO99-GFP or MVA-LEO160-GFP viruses (m.o.i. of 5 p.f.u. $cell^{-1}$) at 8 h p.i. Graphs show fold increases in GFP MFI of MVA-GFP compared with MVA-WT viruses. Note *y*-axis scale difference for each condition. (f) GFP mRNA levels in HeLa cells infected with MVA-WT, MVA-LEO38-GFP, MVA-LEO99-GFP or MVA-LEO160-GFP

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viruses (m.o.i. of 10 p.f.u. cell^{-1}) at 1 or 2 h p.i. Bars show the ratio of GFP to β-actin mRNA levels. Arbitrary unit (AU) values show the mean±SEM of duplicates. One representative example is shown of two independent experiments assayed in triplicate. Values are shown as mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Ns=not statistically significant.

differential antigen presentation changes the T-cell immunodominance hierarchy between primary and secondary immunization (Crowe *et al.*, 2003) and that this hierarchy correlates with viral gene expression after secondary immunization (Kastenmuller *et al.*, 2007).

Since memory T-cell responses are essential for inducing long-term protection against pathogen infection (Darrah *et al.*, 2007; Reyes-Sandoval *et al.*, 2011; Schmidt *et al.*, 2008), at 66 days after the final virus dose, we measured the splenocyte T-cell memory profile by intracellular cytokine staining. Splenocytes from infected mice were stimulated with purified LACK protein to study LACK-specific CD4 T-cell responses, or with A20 cells previously nucleofected with the mammalian expression plasmid pcDNA-LACK to study LACK-specific CD8 T-cell responses. To determine the functional profile of T-cells, we measured levels of lysosomal-associated membrane protein-1 (LAMP-1/CD107a) as a surrogate marker for induction of killing, as well as IFNγ, TNFα and IL-2 levels. We defined the magnitude of the response as LACK-specific T-cells that expressed CD107a or the sum of the

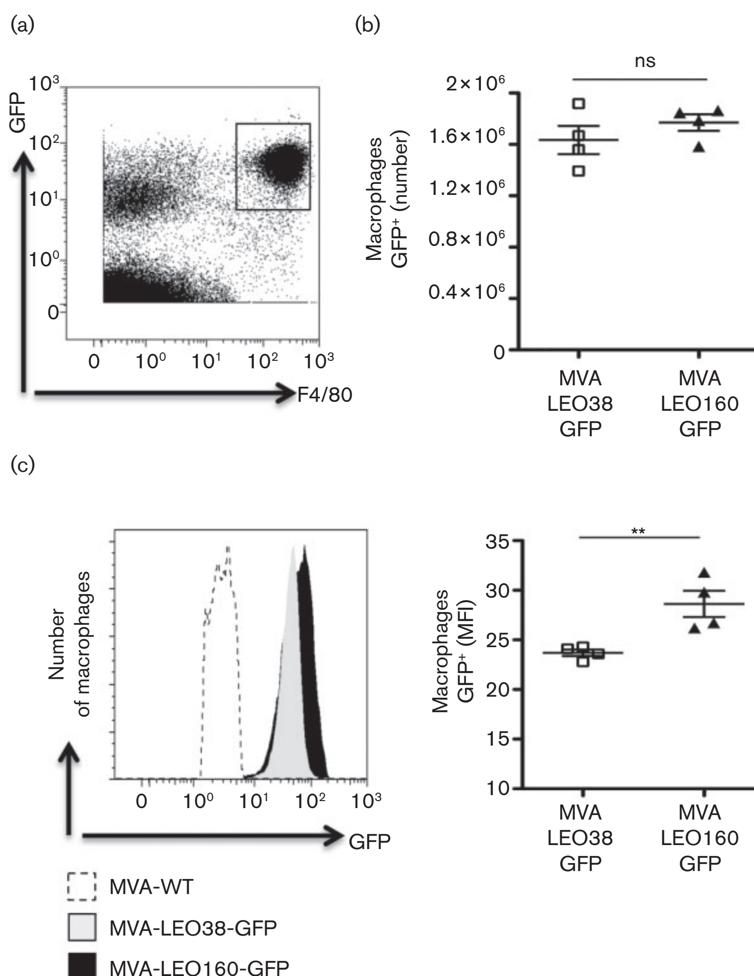

Fig. 3. *In vivo* macrophage GFP expression driven by LEO38 and LEO160 promoters. (a) FACS plot (F4/80 macrophage marker vs GFP) of peritoneal cells from MVA-LEO160-GFP-injected mice (10^7 p.f.u.). (b) Number of GFP$^+$ macrophages from the peritoneal cavity of MVA-LEO38-GFP- or MVA-LEO160-GFP-injected (10^7 p.f.u.) mice at 4 h p.i. (c) Expression (left panel) and MFI (right panel) of peritoneal GFP$^+$ macrophages at 4 h p.i. in MVA-LEO38-GFP- or MVA-LEO160-GFP-injected mice. Graph shows fold increases in these macrophages compared to MVA-WT-injected mice. Values are shown as mean±SEM; each point represents an individual mouse. Data are representative of two independent experiments. **$P<0.01$. Ns=not statistically significant.

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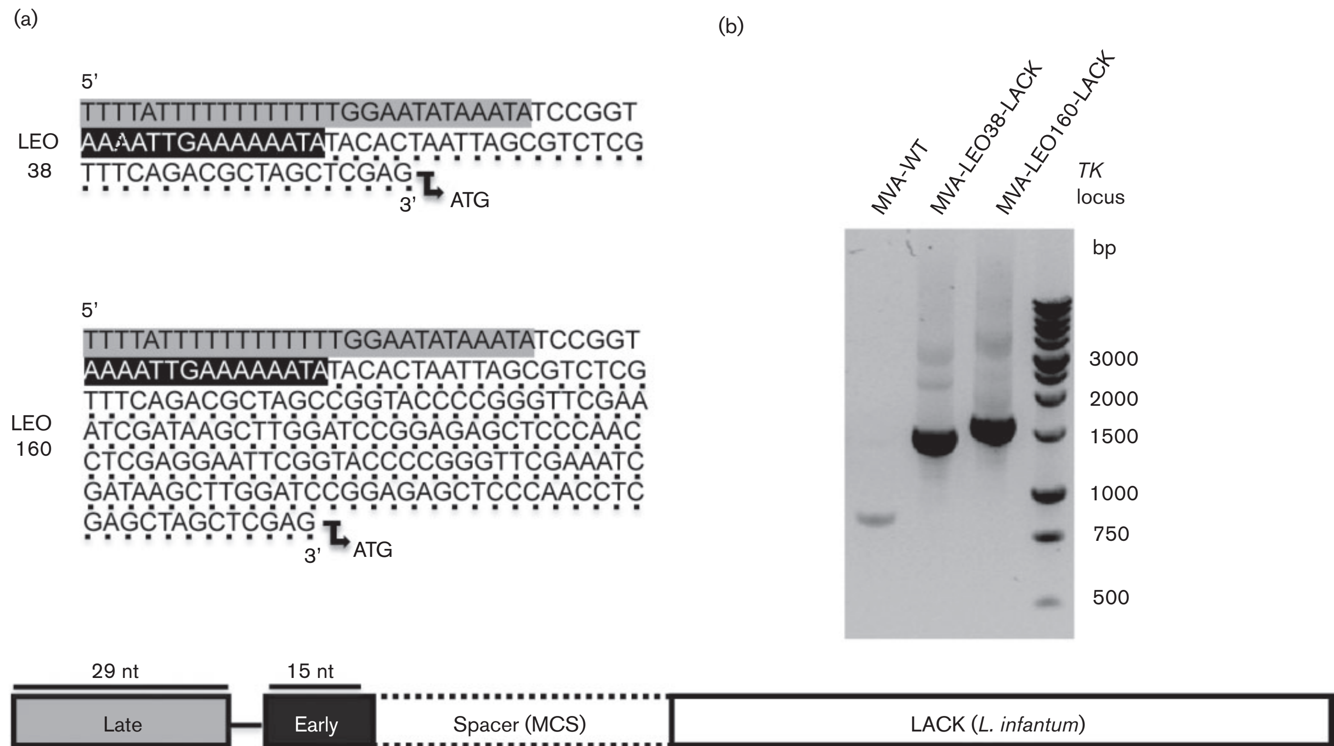

Fig. 4. Schematic and insertion of LEO38 and LEO160 promoters with the LACK gene into the MVA virus genome. (a) Sequences of LEO38 and LEO160 promoters and schematic with LACK gene. Late promoter element, grey box; early promoter motif, black box; spacer, white box with dashed line. (b) Promoter and LACK gene insertion into the MVA virus *TK* locus analysed by PCR. An 873 bp product was obtained in parental MVA, whereas in MVA-LEO38-LACK and MVA-LEO160-LACK recombinant viruses 1486 and 1608 bp products were observed, respectively.

frequencies of LACK-specific T-cells secreting IFNγ and/or TNFα and/or IL-2. Polyfunctionality, defined as T-cell capacity to express more than one of these four activation markers, determined the quality of the T-memory cell response.

MVA-LEO160-LACK induced significantly more LACK-specific IFNγ and/or TNFα and/or IL-2-secreting CD4 T-cells than MVA-LEO38-LACK, when splenocytes were stimulated with LACK protein (Fig. 6a). MVA-LEO160-LACK also generated significantly more LACK-specific CD107a-expressing CD4 T-cells than MVA-LEO38-LACK in virus-infected mice (Fig. 6b).

The quality of the LACK-specific CD4 T-cell response showed that compared with MVA-LEO38-LACK, MVA-LEO160-LACK induced a significant increase in quadruple activation marker-positive CD4 T-cells (Fig. 6c). The high polyfunctional profile generated was also represented by IFNγ, TNFα and IL-2 triple-positive CD4 T-cells (Fig. 6c); TNF-α single-positive CD4 T-cells were the third most representative group.

The LACK-specific CD8 T-cell profile showed that MVA-LEO160-LACK induced a significantly higher IFNγ and/or TNFα and/or IL-2$^+$ CD8 T-cell response to LACK antigen (Fig. 6d) compared with MVA-LEO38-LACK; this significant difference was also detected in LACK-specific CD107a$^+$ CD8 T-cells between the two groups (Fig. 6e). The polyfunctional CD8 T-cell profile showed a clear predominance of the IFNγ^+TNFα^+CD107a$^+$ subset, followed by the quadruple marker-positive population (Fig. 6f). All of these data indicate that increased LACK-specific CD4 and CD8 T-cell responses induced by MVA-LEO160-LACK correlate with the enhanced LACK antigen expression.

DISCUSSION

Two categories of VACV early genes have been defined based on their temporal expression (Assarsson *et al.*, 2008; Yang *et al.*, 2010); their promoter motif (consensus sequence) and their promoter spacers were also recently reported (Yang *et al.*, 2011a). To define the role of the promoter spacer in the optimization of early antigen expression, we designed two new promoters, LEO99 and LEO160, with distinct spacer lengths and compared them with the previously reported LEO (termed here LEO38) promoter (Di Pilato *et al.*, 2013).

Compared with LEO38, the LEO99 and LEO160 promoters (with 99 and 160 nt spacers) increased early antigen

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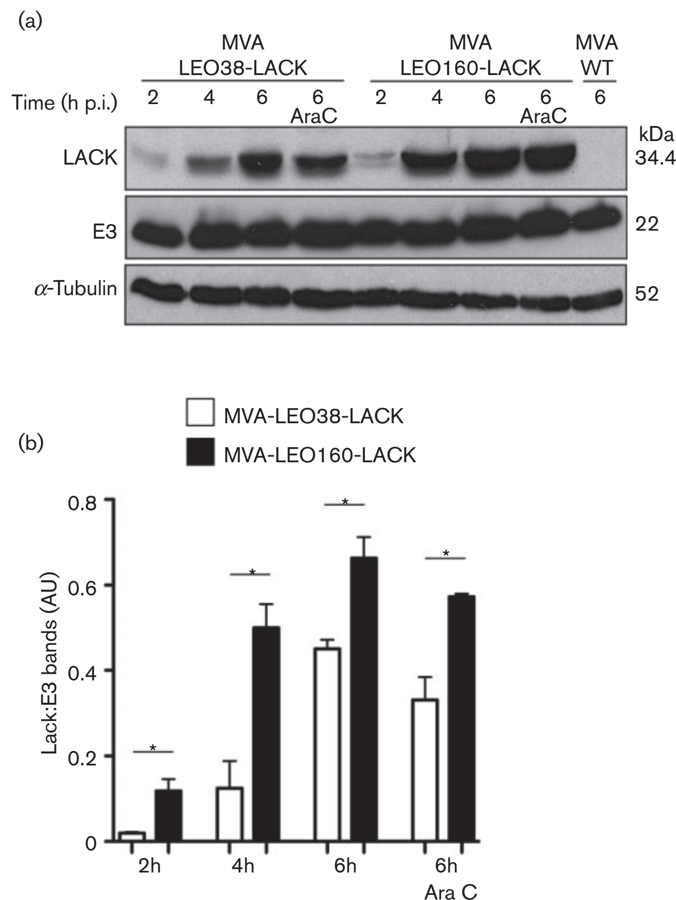

Fig. 5. *In vitro* LACK expression driven by LEO38 and LEO160 promoters. (a) LACK expression in HeLa cells infected with MVA-WT, MVA-LEO38-LACK or MVA-LEO160-LACK viruses (m.o.i. of 10 p.f.u. cell^{-1}) at 2, 4 and 6 h and with AraC at 6 h p.i. α-Tubulin was used as a protein loading control. (b) Bars show the ratio of LACK to E3 bands, quantified using ImageJ software. AU values show the mean ± SEM of two independent experiments. *$P<0.05$.

expression. The 50 nt window, from −70 nt to −20 nt before the ORF, is the most frequent site for the promoter motif of early genes and was used to generate the LEO promoter with a 38 nt spacer (Di Pilato *et al.*, 2013). This result suggests that VACV, the early genes of which rarely have spacers longer than 70 nt, does not alone induce the highest possible gene expression. The DNA footprint of VACV RNA polymerase ternary complexes is less than 50 nt (Hagler & Shuman, 1992). The VACV early transcription factor (Broyles *et al.*, 1988) and RNA polymerase-associated protein (RAP94) (Ahn *et al.*, 1994) mediate the promoter–RNA polymerase link. We hypothesized that spacers > 50 nt would offer greater space to the transcription machinery, possibly accelerating gene expression, and defined here that spacers with more than 99 nt offer advantages to early gene expression. These new long spacers are maintained in the virus during multiple passages in cells, indicating stability within the virus genome.

Attenuated VACV vectors (MVA, ALVAC and NYVAC poxvirus strains) are used as candidate vaccines for emerging infectious diseases and cancer (Gómez *et al.*, 2011). Several strategies have been developed to improve immunogenicity to heterologous antigens expressed by these vectors, including use of costimulatory molecules, administration of heterologous vectors, deletion of immunomodulatory viral genes and insertion of host range viral genes (García-Arriaza & Esteban, 2014; Gómez *et al.*, 2012, 2013). Early gene expression mediated by promoter optimization is another strategy to increase immunogenicity of the foreign antigen (Baur *et al.*, 2010). Although it is reported that VACV intermediate antigens are preferentially recognized by CD4 T-cells (Yang *et al.*, 2011b), we demonstrate that the early LACK expression driven by the early promoter of MVA-LEO160-LACK can positively influence LACK-specific CD4 T-cell responses. While antigen-specific T-cell responses can provide protective immunity against parasites (Belnoue *et al.*, 2004), in a *Leishmania* infection model, only CD4 T-cell immunogenicity correlated with this protection (Darrah *et al.*, 2007).

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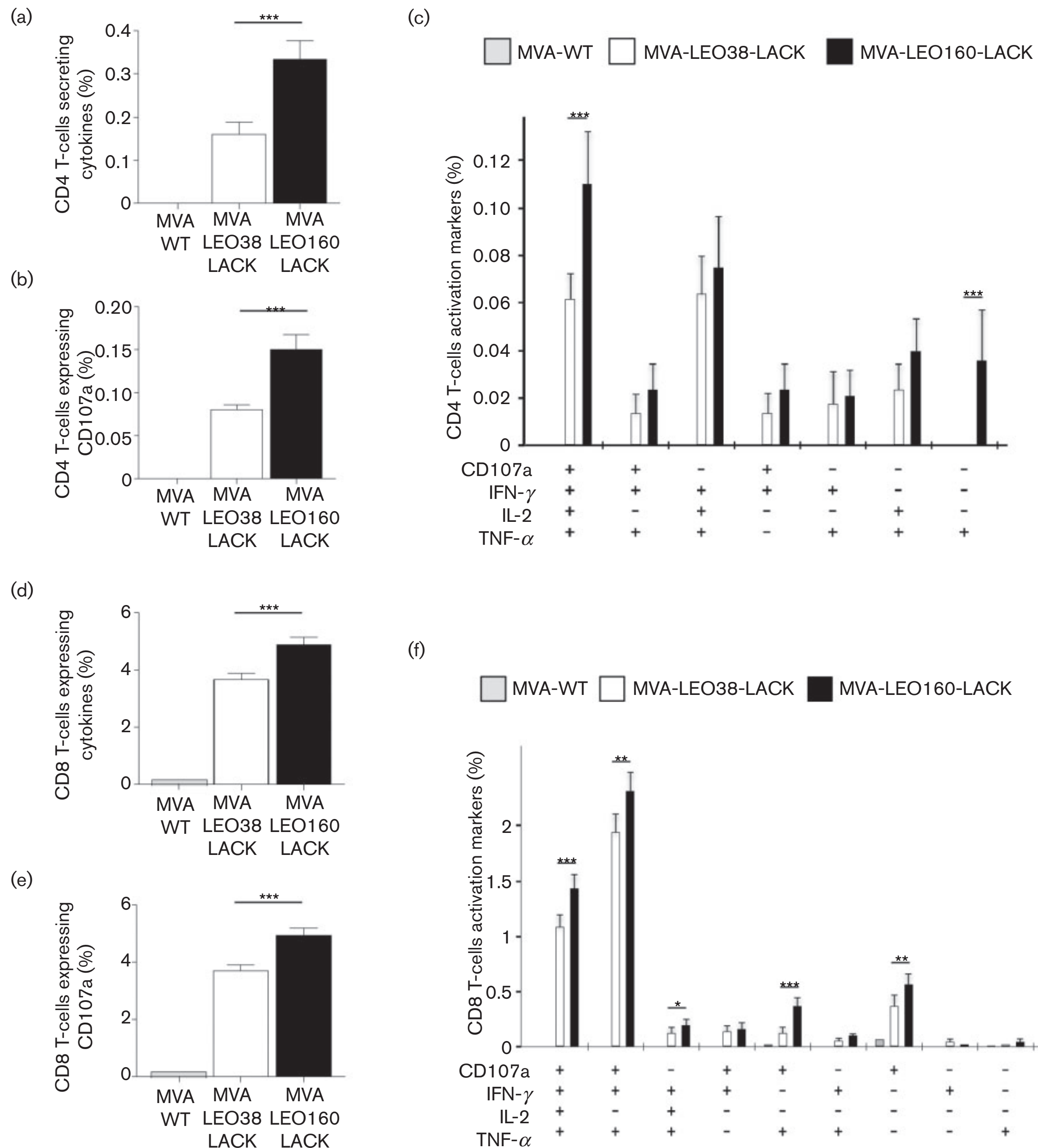

Fig. 6. Vaccine-induced LACK-specific CD4 T- and CD8 T-memory cell responses in MVA-WT-, MVA-LEO38-LACK- or MVA-LEO160-LACK-infected mice. The response was measured 66 days after the last immunization in mice ($n=4$ in each group). (a) Percentage of LACK-specific CD4 T-cells secreting cytokines after two virus doses (10^7 p.f.u. mouse^{-1}) spaced by 1 week. Values are the sum of the percentages of IL-2- and/or IFNγ- and/or TNFα-secreting CD4 T-cells. (b) Percentage of LACK-specific CD4 T-cells expressing CD107a. (c) Functional profile of LACK-specific CD4 T-memory cells. Combinations are shown of the responses (*x*-axis) and percentages of functionally distinct populations (*y*-axis). (d) Percentage of LACK-specific CD8 T-cells secreting cytokines. Values are the sum of the percentages of IL-2- and/or IFNγ- and/or TNF-α-secreting CD8 T-cells. (e) Percentage of LACK-specific CD8 T-cells expressing CD107a. (f) Functional profile of LACK-specific CD8 T-memory cells. Combinations are shown of the responses (*x*-axis) and percentages of functionally distinct populations (*y*-axis). Values are shown as mean ± CI. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Following *Leishmania major* infection, BALB/c mice show principally a Th2 response, due to the Th2 immunogenicity of the LACK antigen (Mougneau *et al.*, 1995). As it is associated with greater susceptibility to *Leishmania* infection in BALB/c mice (Launois *et al.*, 2007), a Th2 response must be avoided when using a LACK vaccine strategy in this strain.

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Conversely, the Th1 response protects against leishmaniasis (Darrah *et al.*, 2007, 2010). Th1 immune responses were triggered by MVA-LEO38-LACK and MVA-LEO160-LACK, with clear differences in magnitude and polyfunctionality. We found that MVA-LEO160-LACK increased the number of IL-2^{+}IFNγ^{+}TNFα^{+} CD4 Th1 cells. Compared with MVA-LEO38-LACK, MVA-LEO160-LACK induced more antigen-specific CD4 T-effector memory cells, which are necessary for protection against *Leishmania* reinfection (Peters *et al.*, 2014). MVA-LEO160-LACK also enhanced CD107a^{+} memory CD4 T-cells to LACK. CD107a^{+} CD4 T-cells are resistant to HIV-1 infection, which implies that these cells could control the infection (Terahara *et al.*, 2014). Compared with MVA-LEO38-LACK, we also demonstrated that MVA-LEO160-LACK increased the magnitude of polyfunctional CD8 T-cells that express IL-2 and IFNγ; these cell subsets have a role in maintaining Th1 polarization of CD4 T-cells (Gurunathan *et al.*, 2000), limiting the natural Th2 response (Uzonna *et al.*, 2004).

Although the role of memory CD8 T-cells against *Leishmania* remains to be defined, CD8 T-memory cells have a clear protective function against parasites (Reyes-Sandoval *et al.*, 2011; Rigato *et al.*, 2011). The capacity of the MVA-LEO160-LACK viral vector to increase CD8 and CD4 T-memory cell responses against a heterologous antigen could be useful in other infection models where a robust presence of both T-cell subtypes has been associated with protection and control of disease (Hansen *et al.*, 2009, 2011).

In summary, we have identified how VACV promoters with different spacer lengths can be used to enhance the levels of foreign antigens in infected cells. We defined *in vitro* and *in vivo* the strength of MVA-LEO160, as a valid strategy to induce early heterologous antigen expression. In mouse studies, the magnitude and the quality of the CD4 and CD8 T-memory responses defined the improved immune properties of this vector. Based on its capacity to increase early antigen expression and antigen-specific CD4 and CD8 T-cell responses, MVA-LEO160 is a prototype for the design of new poxvirus-based vaccine vectors.

METHODS

Mice and injections. BALB/c mice (6–8 weeks old) were purchased from Harlan (Interfauna Ibérica). For the homologous immunization protocol to assay T-cell immunogenicity, mice received two intraperitoneal (i.p.) injections (one every 7 days) of 10^7 p.f.u. of MVA-WT, MVA-LEO38-LACK or MVA-LEO160-LACK. Animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of the Centro Nacional de Biotecnologia (CNB-CSIC, Madrid, Spain) in accordance with national and international guidelines and Royal Decree (RD 1201/2005) (permit number 11037, 13012).

Cells. Human epithelial cervix adenocarcinoma cells (HeLa), immortalized chicken embryo fibroblast cells (DF-1; both from the American Type Culture Collection) and primary chicken embryo fibroblasts (CEF; Intervet) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS. Human monocytic THP-1 cells, murine macrophage J774.G8 cells and B lymphocytes derived from a reticulum cell sarcoma (A20; all from ATCC) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 50 µM 2-mercaptoethanol and 10% (v/v) FCS. THP-1 cells were differentiated into macrophages by treatment with 5 ng phorbol 12-myristate 13-acetate ml^{-1} (Sigma-Aldrich) for 24 h before use. Cells were cultured in humidified air, 5% (v/v) CO_2 at 37 °C.

Viruses. The recombinant MVA-GFP and MVA-LACK viruses were constructed using GFP as a fluorescent marker and β-galactosidase as a reporter gene, respectively. DF-1 cells were infected with 0.01 p.f.u. per cell MVA-WT (1 h) and transfected with VACV insertional plasmid vectors pLZAW1-LEO (Di Pilato *et al.*, 2013), pLZAW1-LEO99 or pLZAW1-LEO160 using Lipofectamine (Invitrogen). Recombinant viruses were selected from progeny virus by consecutive rounds of plaque purification, as reported (Gómez *et al.*, 2007a, b). *In vitro* virus infections were performed in medium with 2% (v/v) FCS. All viruses were grown in primary CEF cells, purified through two 36% (w/v) sucrose cushions and titres were determined by immunostaining plaque assay in DF-1 cells, as described (Ramírez *et al.*, 2000).

Plasmids. The plasmid transfer vectors pLZAW1-LEO99 and pLZAW1-LEO160 were obtained by inserting part of the pGEM-7Zf(-) (Promega) multi-cloning site (MCS) into the pLZAW1-LEO plasmid. The plasmid transfer vectors pLZAW1-LEO-GFP, pLZAW1-LEO99-GFP, pLZAW1-LEO160-GFP, pLZAW1-LEO-LACK and pLZAW1-LEO160-LACK were obtained by cloning the GFP or *Leishmania infantum* LACK sequences into the pLZAW1-LEO, pLZAW1-LEO99 or pLZAW1-LEO160 plasmids.

Protein. *L. infantum* LACK protein was expressed as a fusion protein with an N-terminal histidine tag using the *Escherichia coli* strain BL21 pLysS transformed with the pRSET-B-LACK plasmid (Gonzalez-Aseguinolaza *et al.*, 1999), which allows affinity chromatography purification on a Ni^{2+} column. LACK expression was induced by addition of isopropyl thio-β-D-galactoside (37 °C).

PCR. Viral DNA was extracted by the SDS-proteinase K method (Perdiguero *et al.*, 2013). Primers TK-L and TK-R were used for PCR analysis of the *TK* locus, as described (Gómez *et al.*, 2007a), to confirm GFP and LACK gene insertion. The primers GFP-Fwd CCGCTCGAGATGGCTAGCAAAGGAGAAGAAC and GFP-Rev AACTGCAG TTCTCAAGCTATGCATCCAAC, the primers LACK-Fwd CTAGCTAGCTCGAGAT GAACTACGAGGGTCACC and LACK-Rev AACTGCAGTTACTCGGCGTCGGAG ATGG, and the primers MCS-Fwd CCGCTCGAGGAATTCGGTACCCC and MCS-Rev CCGCTCGAGGTTGGGAGCTCTCC were used for GFP, LACK and MCS amplification, respectively.

Real-time PCR. Total RNA was isolated using an RNeasy kit (Qiagen). After DNase I (Roche) digestion, 1 µg of RNA was reverse transcribed using SuperScript III RT (Invitrogen). Quantitative PCR was performed on a 7500 real-time PCR system using the Power SYBR green PCR master mix (both from Applied Biosystems). The primers GFP-Fwd CCTGAAGTTCATCTGCACTA and GFP-Rev TGTCTTGTAGTTGCCGTCA, β-actin-Fwd CCCAGCACAATGAAG-ATCAA and β-actin-Rev CGATCCACACGGA GTACTTG were used for real-time PCR.

Western blot. HeLa cells were lysed in Laemmli buffer, extracts were fractionated by 8% (v/v) SDS-PAGE and then analysed by Western blot using rabbit polyclonal anti-LACK, anti-E3 (both from the Centro Nacional de Biotecnología) or anti-tubulin antibodies (Cell Signalling).

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Nucleofection assay. A20 cells were nucleofected using 4D-Nucleofector (Lonza) and an Amaxa SF Cell Line kit (Lonza). Cells were nucleofected with 6 μg of pCIneo-LACK plasmid; pCIneo-ϕ was used as a negative control. At 24 h post-nucleofection, A20 cells were used to restimulate splenocytes from infected mice (1 : 10 A20 : splenocyte ratio).

Flow cytometry. For intracellular cytokine staining, erythrocyte-depleted splenocytes were rested overnight, resuspended in RPMI 1640 with 10% (v/v) FCS and 1 μg Golgiplug ml^{-1} (Becton Dickinson), monensin (eBioscience) and anti-CD107a (1D4B; BD). After restimulation with 25 μg ml^{-1} LACK protein or A20 cells (6 h, 37 °C), splenocytes were stained for surface markers using anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD62L (MEL-14) (all from BD) and anti-CD127 (A7R34) (eBioscience). Cells were then fixed, permeabilized (Cytofix/Cytoperm kit; BD) and stained intracellularly with anti-IL-2 (JES6-5H4), anti-IFNγ (XMG 1.2) and anti-TNFα (MP6-X722) (all from BD). Peritoneal exudate cells, obtained after injection of cold PBS into previously infected mice, were counted and stained with anti-F4/80 (BM8, eBioscience). Dead cells were stained using a violet LIVE/DEAD stain kit (Invitrogen) in all flow cytometry analyses. Cells were acquired using a GALLIOS (Beckman Coulter) or LSRII (BD) flow cytometer; data analyses were performed with FlowJo software version 8.5.3 (Tree Star). Boolean combinations of single functional gates were created with FlowJo to determine the frequency of each response based on all possible combinations of cytokines or of differentiation marker expression.

Statistical analysis. For statistical analysis of the T-cell response to LACK antigen, we used an approach that corrects measurements for medium response and allows calculation of confidence intervals (CI) and *P* values of hypothesis tests (Nájera *et al.*, 2010). Only antigen response values significantly larger than the corresponding RPMI condition are shown. Background values were subtracted from all values used to allow analysis of proportional representation of responses. Distribution was analysed and presented using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov. For statistical analysis of antigen expression, Student's *t*-test was applied to compare the viruses used.

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