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Short Communication

New vaccinia virus promoter as a potential candidate for future vaccines

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Here we describe the design and strength of a new synthetic late-early optimized (LEO) vaccinia virus (VACV) promoter used as a transcriptional regulator of GFP expression during modified vaccinia Ankara infection. In contrast to the described synthetic VACV promoter (pS), LEO induced significantly higher levels of GFP expression *in vitro* within the first hour after infection, which correlated with an enhancement in the GFP-specific CD8 T-cell response detected *in vivo*, demonstrating its potential use in future vaccines.

Vaccines against intracellular human pathogens such as human immunodeficiency virus (HIV), hepatitis C virus, malaria and leishmania are designed to induce efficient antigen-specific T-cell immune responses that, in some cases, correlate with protection (Gómez *et al.*, 2012b; Good & Doolan, 2010; Sánchez-Sampedro *et al.*, 2012; Vijayan *et al.*, 2012; Yusim *et al.*, 2013). One of the most popular vectors selected to trigger such responses are the poxviruses, and, in particular, highly attenuated vaccinia virus (VACV) strains such as modified vaccinia Ankara (MVA), NYVAC and ALVAC have been used successfully against emergent infectious diseases and cancer in humans (Gómez *et al.*, 2012a).

It is known that the timing of expression of heterologous antigens in the VACV system affects the capacity to induce antigen-specific T-cell immune responses (Baur *et al.*, 2010) since the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition (Moutaftsi *et al.*, 2009). Considering that immunodominance is defined as the phenomenon whereby only a small fraction of all the possible epitopes from a particular pathogen elicits an immune response in an infected individual (Pasquetto *et al.*, 2005), it is possible to modulate such immunodominance hierarchy, changing the timing and the quantity of intracellular antigen production (Wilson & Hunter, 2008). In fact, it has been described that, in VACV, 90 % of the most recognized antigens by CD8 T-cells were ranked in the top 50 % in terms of mRNA expression (Sette *et al.*, 2009), and there is a correlation between viral gene expression and immunodominance hierarchy after a second immunization due to a mechanism of cross-competition between T-cells specific for early and late viral epitopes (Kastenmuller *et al.*, 2007).

Recently, after a deep analysis of the VACV transcriptome, two groups have defined two categories of early genes based on their temporal expression (Assarsson *et al.*, 2008; Yang *et al.*, 2010). Assarsson *et al.* (2008), using a genome tiling array approach, differentiated the immediate-early genes from the early genes, whereas Yang *et al.* (2010), using deep RNA sequence analysis, differentiated the E1.1 genes from E1.2 genes as subclusters of early genes and also defined a 15 nt consensus sequence (AAAANTGAAAA-NNNA) that corresponds to the core of early genes promoter. Both authors underline that there is a difference in the time of expression between the two early classes of genes, but Yang *et al.* (2010) avoided the terminology of immediate-early and early genes because both subclusters (E1.1 and E1.2) were transcribed in the presence of the protein synthesis inhibitor cycloheximide, while the transcription of early genes depends on the synthesis of one or a few immediate-early proteins (Honess & Roizman, 1975; Ross & Guarino, 1997; Salser *et al.*, 1970).

The core promoter of E1.1 genes more closely corresponds to the consensus sequence than those of E1.2 genes, suggesting that this difference could explain the readiness of E1.1 genes to be recognized by the transcription machinery before recognition of E1.2 genes (Yang *et al.*, 2010).

In an effort to select an optimized virus promoter for antigen expression, in this study we selected the first temporal early class of genes (immediate-early and E1.1) described by both groups (Table S1, available in JGV Online), comprising 45 genes in total, and performed a sequence alignment using a region of 200 nt upstream of the translation initiation site in order to define the

One supplementary table is available with the online version of this paper.

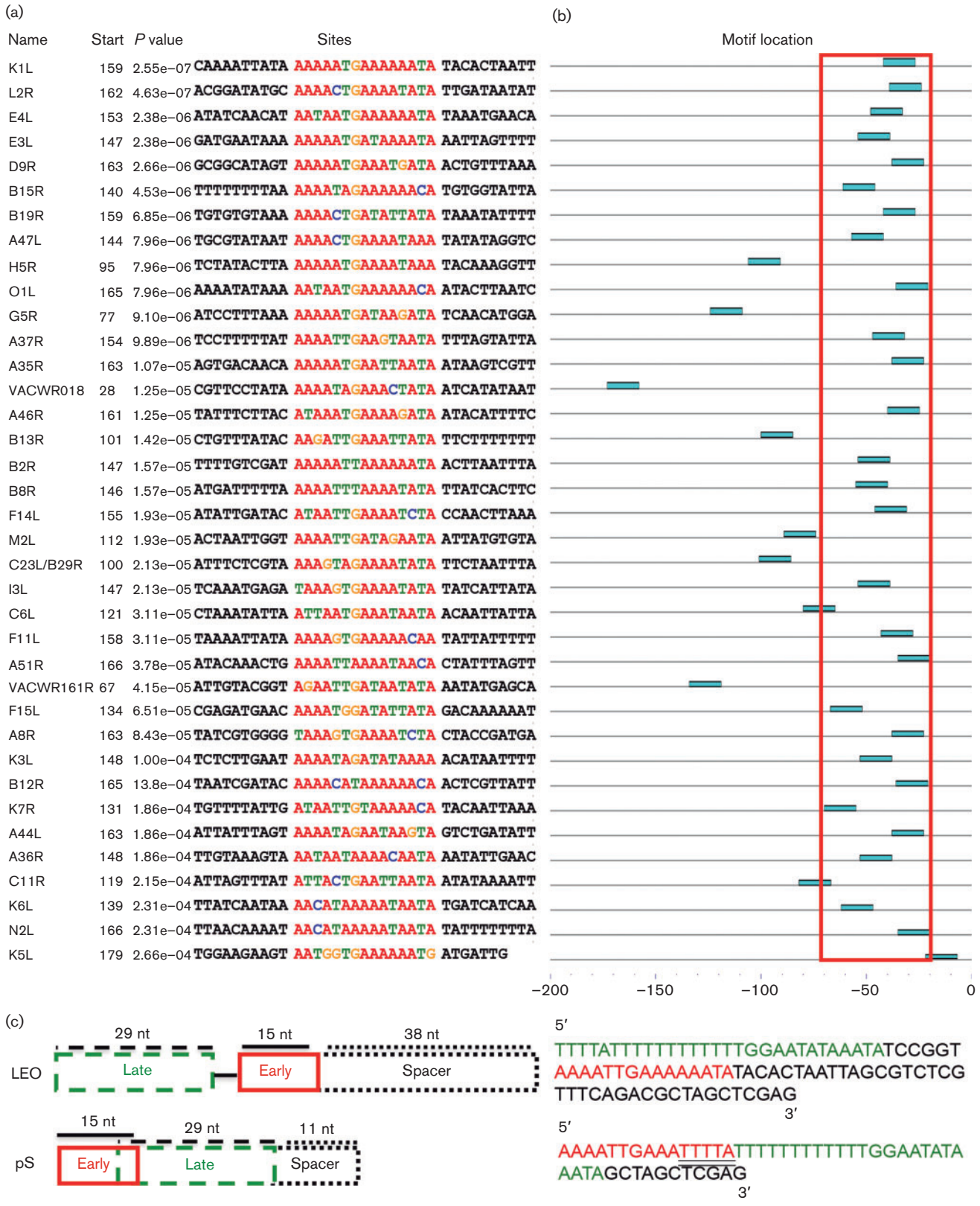

Fig. 1. Design of the synthetic LEO promoter. (a) Alignment of the core promoter sequence of 37 selected early genes using the MEME program. For the analysis, we used the 200 nt upstream of the start codons of the genes. The distribution of motif attributed was zero or unity for the sequence. The maximum width of the motif selected for the analysis was 20 nt. The *P* value

of a site was computed from the match score of the site with the position-specific score matrix for the motif. Only sequences with $P<0.001$ are represented. (b) Alignment of all 37 sequences of 200 nt. The height of the motif 'block' is proportional to −log (*P* value) and the position in the panel corresponds to the motif location. The red box emphasizes the region between −70 and −20 nt upstream of the start codons of the genes. (c) Schematic representation and sequence of LEO and pS promoters. The double solid line represents the nucleotides shared by early and late fractions.

appropriate consensus sequence of the promoter of these genes and to determine the spacer between the core sequence and the start codon of the genes. Using the motif discovery program Multiple Em for Motif Elicitation (MEME), we observed that 82 % of the studied genes (37/45) contained the core of a promoter that closely corresponded to a newly defined consensus sequence, AAAANTGAAAAAATA (Fig. 1a), and in 73 % of them (27/37) the first nucleotide of the core was between positions −70 and −35 and the last nucleotide of the core was between positions −55 and −20 (Fig. 1a, b).

With all these data, we designed a new synthetic late-early optimized (LEO) VACV promoter that contained the same late part present in the widely used synthetic VACV pS promoter (Chakrabarti *et al.*, 1997) prior to the newly defined early consensus sequence, with a T in position 5, and followed by a spacer of 38 nt (Fig. 1c). The purpose of using this LEO promoter in the transcriptional control of a heterologous antigen is to increase the immediate expression of such an antigen during VACV infection. With this aim, we decided to compare the strength of the new LEO promoter with the viral synthetic early/late pS promoter (Chakrabarti *et al.*, 1997), since pS has been used successfully for the expression of high levels of heterologous antigens in vaccine candidates based on poxviruses (Gómez *et al.*, 2007a) and has been described previously as a better promoter than p7.5 and pC11R, and similar to pB8R, pA44L and pF11L in terms of gene expression levels during the first 8 h of infection (Orubu *et al.*, 2012). The pS promoter contains a different early motif that shares 5 nt with the following late part and has a spacer of 11 nt (Fig. 1c).

The new LEO promoter sequence was synthesized by GENEART GmbH (Regensburg) and inserted into the VACV insertional plasmid vector pLZAW1, which contains the right thymidine kinase (TK) flanking arm, a multicloning site, a T5NT sequence and the β-galactosidase reporter gene between two repetitions of the left TK flanking arm (Gómez *et al.*, 2007a, b). GFP expression was used as a readout in order to evaluate the promoter strength. For this purpose, the GFP gene was inserted into the pLZAW1 vector under the transcriptional control of the LEO (pLZAW1–LEO) or pS (pLZAW1–pS) promoter. These transfer vectors were used to generate the recombinant viruses MVA–LEO–GFP and MVA–PS–GFP following standard procedures described previously (Gómez *et al.*, 2007b). The purity of the recombinant viruses was assessed by PCR amplification of the insert in the TK locus. The viruses were grown in primary chicken embryo fibroblasts, purified through two 36 % (w/v) sucrose cushions and titrated by immunostaining assay as described previously (Ramírez *et al.*, 2000).

To define the temporal expression of GFP under the control of the two different promoters, non-permissive HeLa cells were infected at an m.o.i. of 5 with MVA–LEO–GFP or MVA–PS–GFP recombinant viruses. In the first 4 h of infection, cells infected with MVA–LEO–GFP expressed higher levels of GFP compared with cells infected with MVA–PS–GFP as determined by flow cytometry (Fig. 2a) and measured as mean fluorescence intensity (MFI) (Fig. 2b). At 8 h post-infection, no differences in the MFI between both viruses were observed, underlining that the strength of the late part of both promoters equals the fluorescence levels. These results were confirmed in a permissive chicken DF-1 cell line (data not shown).

To check if higher levels of antigen expression at early times post-infection correlated with an enhancement of the antigen-specific T-cell immune responses, we performed an immunization protocol using MVA–LEO–GFP and MVA–PS–GFP. BALB/c mice ($n=8$ per group) were injected by the intraperitoneal route with two doses of 1×10^7 p.f.u. of each virus with an interval of 1 week between the two inoculations. As a negative control, we used the parental virus MVA–WT. At 11 and 53 days after the last immunization, mice were sacrificed and spleens were processed for intracellular cytokine staining assay to study CD8 T-cell primary and memory immune responses, respectively. The splenocytes were stimulated for 6 h with 10 μg ml^{-1} of the GFP peptide HYLSTQSAL ($GFP_{200–208}$) (Centro Nacional de Biotecnología Proteomics Facility, Spain), which is able to induce a specific CD8 T-cell response, as reported previously (Gambotto *et al.*, 2000). The functional profile of the vaccine-induced T-cell response was determined by measuring the intracellular expression of the cytokines IFN-γ, IL-2 and TNF-α, as well as the exposure of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) on the surface of activated antigen-specific CD8 T-cells as a surrogate marker for induction of killing.

The magnitude of the CD8 T-cell response was defined as the number of CD8 T-cells expressing IL-2 and/or IFN-γ and/or CD107a and/or TNF-α, and the polyfunctionality as the capacity of CD8 T-cells to express more than one of these four activation markers. In primary and memory immune responses, the magnitude and polyfunctionality of the vaccine-induced GFP-specific CD8 T-cell immune response were significantly higher in mice infected with MVA–LEO–GFP than in those infected with MVA–PS–GFP (Fig. 3). In primary immune responses, the absolute frequencies of quadruple, triple, double and single GFP-specific CD8 T-cell populations were significantly higher in mice infected with MVA–LEO–GFP compared with MVA–PS–GFP-infected mice (Fig. 3c), whereas in memory immune responses only

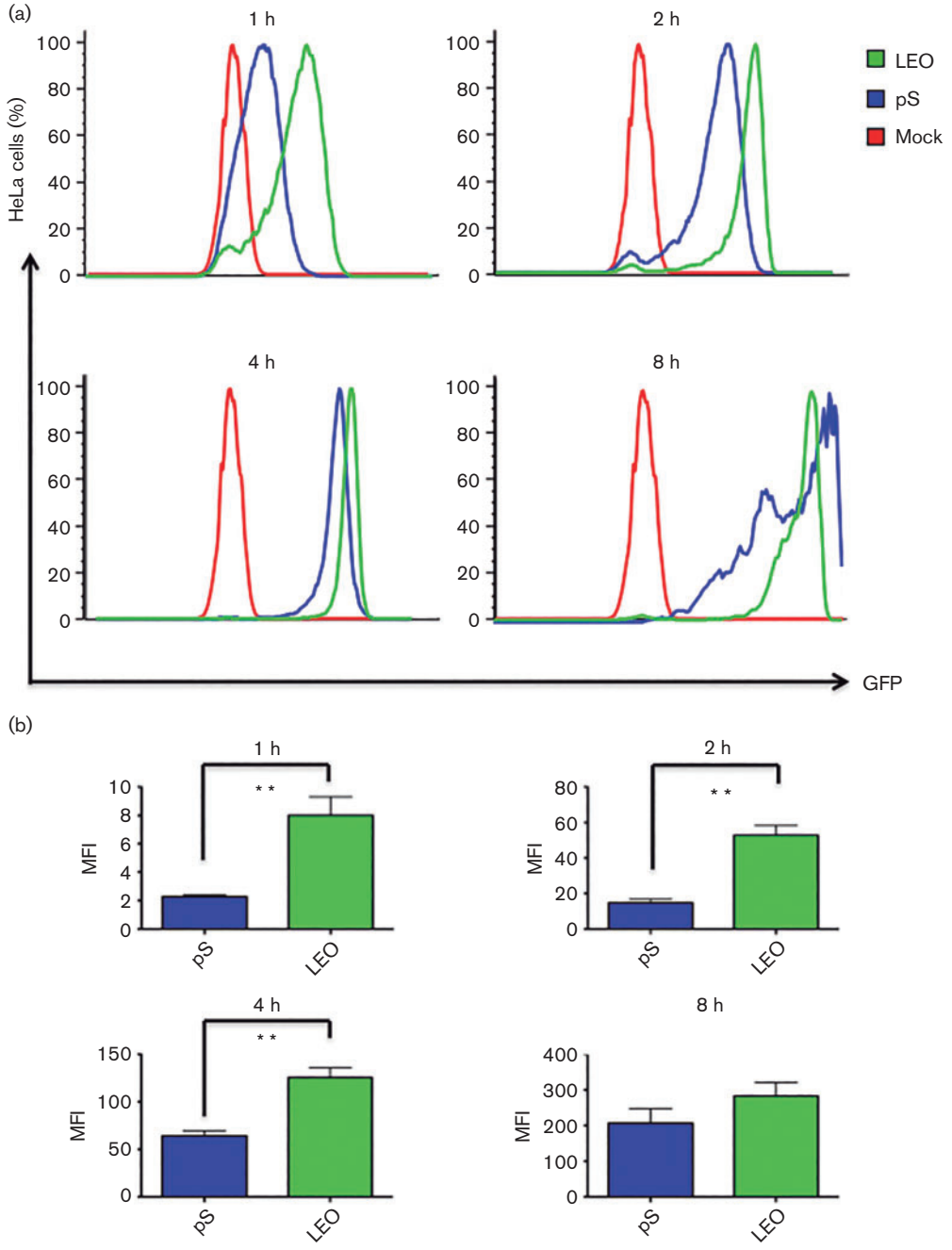

Fig. 2. GFP expression levels driven by LEO or pS promoters. (a) Expression of GFP in HeLa cells infected with MVA–LEO–GFP (LEO) or MVA–PS–GFP (pS) viruses at an m.o.i. of 5 at 1, 2, 4 and 8 h post-inoculation. (b) Mean fluorescence intensity (MFI) value of GFP expression compared with mock cells. The values represent the mean of three different experiments. $^{**}P<0.01$.

the quadruple CD8 T-cell subset was increased significantly (Fig. 3d). These results demonstrated that the enhanced immediate expression of the heterologous GFP antigen increased the quality of the antigen-specific T-cell response.

The LEO promoter represents the first example of a VACV synthetic promoter designed after bioinformatics analysis that shows an enhanced antigen expression within the first hour after infection and, importantly, is able to generate a significant increase in the antigen-specific CD8 T-cell immune response compared with that of the commonly used pS. This novel promoter represents an excellent prototype for future use to potentiate the expression of

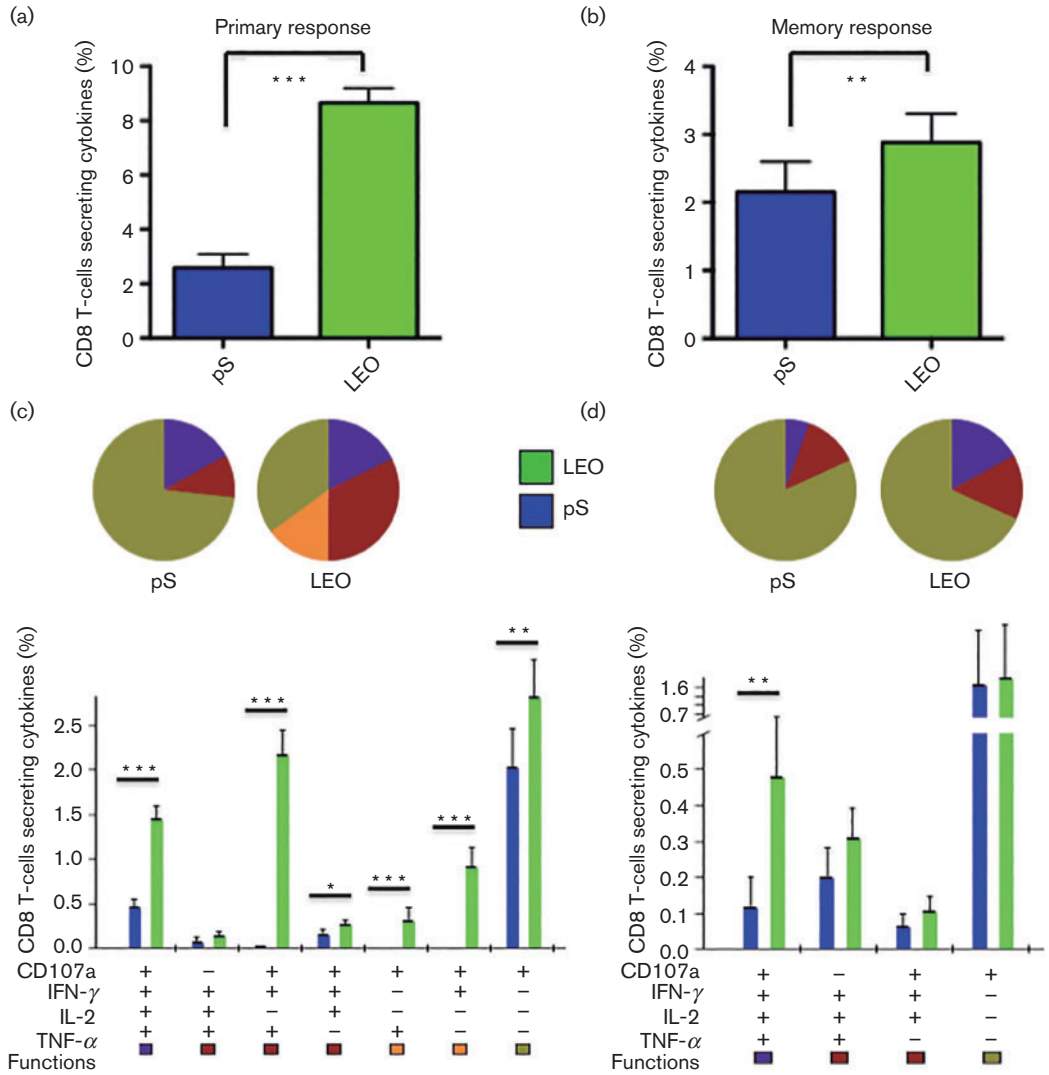

Fig. 3. Vaccine-induced GFP-specific CD8 T-cell immune responses in mice infected with MVA–PS–GFP (pS) or MVA–LEO–GFP (LEO) viruses. (a, b) Magnitude of the CD8 positive T-cell primary (a) and memory (b) immune responses of pooled splenocytes. The total value represents the sum of the percentages of CD8-positive T-cells secreting IL-2 and/or IFN-γ and/or CD107a and/or TNF-α. (c, d) Functional profile of primary (c) and memory (d) GFP-specific CD8 T-cells. The combinations of the responses are shown on the *x*-axis and the percentages of the functionally distinct populations are shown on the *y*-axis. The combinations with percentages <0.1 are not shown. Responses are grouped and colour coded on the basis of the number of functions. The non-specific responses obtained in mice infected with the control MVA–WT were subtracted in the total magnitude and in all combinations of CD8 response. The bars of the columns represent the confidence intervals of one experiment repeated twice. The statistical analysis used has been reported previously (García-Arriaza *et al.*, 2010). ***$P<0.001$, **$P<0.01$, *$P<0.05$.

antigens from different pathogens and to generate safe VACV recombinant-based vaccines able to induce potent immune responses that prevent development of the disease.

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