Immediate-Early Expression of a Recombinant Antigen by Modified Vaccinia Virus Ankara Breaks the Immunodominance of Strong Vector-Specific B8R Antigen in Acute and Memory CD8 T-Cell Responses[▽]

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Received 19 March 2010/Accepted 1 June 2010

Efficient T-cell responses against recombinant antigens expressed by vaccinia virus vectors require expression of these antigens in the early phase of the virus replication cycle. The kinetics of recombinant gene expression in poxviruses are largely determined by the promoter chosen. We used the highly attenuated modified vaccinia virus Ankara (MVA) to determine the role of promoters in the induction of CD8 T-cell responses. We constructed MVA recombinants expressing either enhanced green fluorescent protein (EGFP) or chicken ovalbumin (OVA), each under the control of a hybrid early-late promoter (pHyb) containing five copies of a strong early element or the well-known early-late p7.5 or pS promoter for comparison. In primary or cultured cells, EGFP expression under the control of pHyb was detected within 30 min, as an immediate-early protein, and remained higher over the first 6 h of infection than p7.5- or pS-driven EGFP expression. Repeated immunizations of mice with recombinant MVA expressing OVA under the control of the pHyb promoter led to superior acute and memory CD8 T-cell responses compared to those to p7.5- and pS-driven OVA. Moreover, OVA expressed under the control of pHyb replaced the MVA-derived B8R protein as the immunodominant CD8 T-cell antigen after three or more immunizations. This is the first demonstration of an immediate-early neoantigen expressed by a poxviral vector resulting in superior induction of neoantigen-specific CD8 T-cell responses.

The induction of potent T-cell responses in humans to control and eventually clear infections with human pathogens such as HIV, hepatitis B and C viruses, and papillomaviruses is considered important in the development of prophylactic and therapeutic vaccines against these pathogens (8, 25, 53). Furthermore, therapeutic vaccines against various cancers also require the induction of specific T cells (11, 19). Thus, the development of vaccines to induce efficient T-cell responses against a variety of target antigens remains an important goal. Live, replication-competent viral vectors are highly immunogenic but may pose safety problems, whereas inactivated vaccines have an excellent safety record but are relatively poor inducers of cellular immunity. Therefore, replication-restricted viral vectors are considered prime candidates for the induction of CD8 and CD4 T-cell responses because of their capacity to efficiently induce both humoral and cellular immune responses and their inherent safety even in immunocompromised individuals. The optimization of their CD8 T-cell-inducing capacity has been attempted by various approaches, including deletion of immunomodulatory genes and increasing the amount of neoantigen expression (15, 44, 54). In addition, numerous stud-

ies employing heterologous prime-boost regimens have been conducted, combining replication-competent as well as replication-deficient poxvirus vectors with DNA or nonpoxviral immunogens to enhance T-cell responses to the neoantigen (23, 43). Such heterologous prime-boost regimens continue to be favored as an immunization approach for induction of strong T-cell responses (26, 33). Here we describe an immunization approach based on modified expression kinetics of the neoantigen, using replication-restricted modified vaccinia virus Ankara (MVA) as the vaccine vector in a homologous prime-boost scheme.

MVA was generated by more than 570 passages on chicken embryo fibroblast (CEF) cells, starting with the parental chorioallantois vaccinia virus Ankara (CVA), a member of the genus *Orthopoxvirus* within the poxvirus family. MVA did not productively replicate in many mammalian cell lines, including a number of human cell lines, and had a highly attenuated phenotype *in vivo* (10, 18, 35). It was subsequently named MVA due to its novel properties, and it was shown that MVA could be administered safely to animals and humans (35, 45). MVA-BN was further developed from MVA of passage number 572 by limiting dilution and passaging and proved to be safe even in severely immunocompromised hosts (29, 46). Recent clinical studies using MVA-BN as a third-generation standalone smallpox vaccine confirmed its excellent safety profile in humans (50, 51) and underscored its potential as a safe vaccine and gene therapy vector.

Temporal regulation of neoantigen expression by replica-

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[▽] Published ahead of print on 10 June 2010.

tion-competent vaccinia virus vectors has been demonstrated to be crucial for the efficient induction of a neoantigen-specific T-cell response (6, 16). It was shown that not the promoter strength *per se* but early expression of the neoantigen in the viral replication cycle was necessary for the induction of an effective CD8 T-cell response (6). The early phase of the poxviral replication cycle comprises the first 2 h of infection, until the onset of viral DNA replication (37). In general, poxviral genes can be divided into four temporal classes of expression, termed early, intermediate, late, and constitutive (early-late). These classes are associated with the respective phases of the viral life cycle (7). By genome-wide transcription analysis of vaccinia virus (VACV), an additional temporal class of genes, expressed almost immediately after infection and named immediate-early genes, was recently defined (3). Expression of immediate-early genes starts between 0.5 and 1 h after infection, ahead of that of the early genes, and peaks 2 h after infection (3). No specific features in or around the predicted promoter sequences of immediate-early genes have been identified thus far as directing immediate-early expression.

Promoters which are active in both the early and late phases are usually employed to direct the expression of neoantigens in poxvirus vectors (47). A compact, synthetic promoter (pS) has been used widely to direct strong early as well as late gene expression (12, 24). The p7.5 promoter is another example of a classical early-late promoter (13) used for recombinant gene expression by VACV vectors. However, it has not been reported whether any of the widely used early-late promoters direct expression very early in infection which is comparable to that of the newly identified class of immediate-early genes and whether immediate-early gene expression might affect induction of CD8 T-cell responses. Furthermore, in most studies investigating the suitability of specific promoters for optimizing CD8 T-cell responses against the neoantigen, replication-competent vaccinia viruses were used. Since MVA is replication restricted, it is important to assess the properties of promoters with this vector. Here we describe the evaluation of a hybrid promoter (pHyb) containing pentameric early elements from the p7.5 promoter (13) and a late element from the A-type inclusion (ATI) promoter (21, 40). Such promoters, containing multiple early elements, have been reported to enhance early gene expression (20). We hypothesized that clustering of early elements might focus transcriptional complexes and favor immediate-early neoantigen expression. Indeed, pHyb directed immediate-early expression of a reporter gene encoding enhanced green fluorescent protein (EGFP). We found that control of ovalbumin (OVA) expression by pHyb significantly increased the OVA-specific CD8 T-cell response in mice after multiple immunizations compared to the response to pS-driven OVA expression and reversed the hierarchy of the initially immunodominant vector-derived B8R epitope and the OVA epitope in favor of the latter after three or more immunizations.

MATERIALS AND METHODS

Generation of MVA-BN recombinants. The pHyb promoter was assembled using a late element from the promoter directing the expression of the ATI protein in cowpox virus (21, 40). The five tandemly arranged early elements were derived from the p7.5 promoter and were modified at four nucleotide positions within the A-rich critical core region of 16 nucleotides, as described previously

(17). The natural p7.5 promoter used here consisted of a 104-bp DNA fragment containing the late and early promoter elements. The sequence of the strong synthetic early-late pS promoter comprised 40 nucleotides exactly matching the previously described sequence (12). Recombinant MVAs were generated using a cloned version of the genome of MVA-BN, a proprietary technology of Bavarian Nordic GmbH, in a bacterial artificial chromosome (BAC). Generation and mutagenesis of the MVA-BAC will be described in detail elsewhere. An OVA cDNA clone was kindly provided by Christian A. Mohr and U. Koszinowski, Munich, Germany. Briefly, the pHyb, p7.5, and pS promoter constructs were cloned upstream of the open reading frame for either OVA or EGFP. These expression cassettes were flanked with homology arms of approximately 45 nucleotides by PCR and were introduced into the intergenic region between the MVA136 and MVA137 genes by homologous recombination to obtain recombinant MVA-BACs. Infectious viruses were reconstituted from BACs by transfecting BAC DNA into BHK-21 cells and superinfecting them with Shope fibroma virus as a helper virus. After three passages on CEF cells, helper-free (confirmed by PCR) MVA-pHyb, MVA-pS, and MVA-p7.5 viruses expressing either EGFP or OVA under the control of the respective promoters were obtained.

Cell culture and cell cycle arrest by AraC. Primary CEF cells were prepared from 11-day-old embryos and cultured in VP-SFM (serum-free medium; Invitrogen, Karlsruhe, Germany). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (Invitrogen). Cells were infected with 10 50% tissue culture infective doses (TCID₅₀) per cell of the MVA recombinants expressing EGFP under the control of the indicated promoters. After the indicated times, infected cells were harvested by trypsinization and analyzed by flow cytometry for EGFP expression levels, using a BD LSR II flow cytometry analyzer (BD Biosciences, Heidelberg, Germany). Where indicated, cytosine arabinoside (AraC) was added to the medium throughout infection, at a final concentration of 40 µg/ml, to arrest MVA replication in the early phase.

Immunization of mice. Female C57BL/6 mice aged 6 to 8 weeks were purchased from Harlan Winkelmann, Germany. Mice were immunized via the intraperitoneal (i.p.) route with an inoculum of 200 µl containing 10⁸ TCID₅₀ of the respective MVA recombinants at weeks 0, 4, 8, and 22 for T-cell analysis and at weeks 0, 2, and 14 for analysis of anti-OVA and anti-MVA antibodies. Blood was taken via the tail vein at the indicated time points and processed as described below for analysis of CD8 T-cell responses. All animal experiments were approved by the government of Upper Bavaria (Regierung of Oberbayern) and were carried out in accordance with the guidelines for animal experiments of Bavarian Nordic GmbH.

Intracellular cytokine staining (ICCS). Immunized animals were bled from the tail vein, and 100 to 120 µl of blood per mouse was resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 4% FCS, 2 mM EDTA, and 2.5 U/ml heparin. Peripheral blood mononuclear cells (PBMCs) were prepared by lysing erythrocytes with red blood cell lysing buffer (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions. PBMCs were split into three aliquots and finally resuspended in RPMI-10% FCS containing 0.05 mM β-mercaptoethanol, 1 µl/ml GolgiPlug (BD Biosciences) to block secretion of cytokines via the exocytotic pathway, and 1 µg/ml of the OVA₂₅₇₋₂₆₈ SIINFEKL or B8R₂₀₋₂₇ TSYKFESV peptide or no peptide. Peptides were purchased from ProImmune (Oxford, United Kingdom). CD8 T-cell frequencies for the immunodominant H-2K^b-restricted TSYKFESV epitope, derived from amino acids 20 to 27 of the viral B8R early protein (49), were determined as a representative measure of vector-specific CD8 T-cell responses. PBMCs were incubated for 5 h at 37°C in 5% CO₂, harvested by centrifugation, resuspended in ice-cold PBS-10% FCS-2 mM EDTA, pH 7.4, and stored overnight at 4°C. The following day, PBMCs were stained with anti-CD8α-Pac-Blue, anti-CD4-PerCP-Cy5.5, and anti-CD62L-phycoerythrin (PE)-Cy7 antibodies and, in some experiments, with anti-CD127-allophycocyanin (APC) or anti-CD19-PerCP-Cy5.5 (all antibodies were from BD Biosciences). PBMCs were incubated with appropriate dilutions of the indicated antibodies for 30 min at 4°C in the dark. After being washed, cells were fixed and permeabilized by use of a Cytotfix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's instructions. After being washed, PBMCs were stained for intracellular gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), using a fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ antibody and a PE-conjugated anti-TNF-α antibody (BD Biosciences). The antibodies were diluted in Perm/Wash buffer (BD Biosciences), and the PBMCs were stained for 20 min at 4°C in the dark. After being washed, stained cells were analyzed by flow cytometry on a BD Biosciences LSR II system. Live PBMCs were identified by forward and side scatter characteristics. Approximately 20,000 PBMCs were acquired per sample, with about 50% of the cells from immunized mice being CD8⁺ CD19⁻.

MHC class I pentamer and dextramer staining. PBMCs were isolated as described above. Either the whole sample or an aliquot not used for ICCS was immediately subjected to staining of OVA- and B8R-specific CD8 T cells by anti-CD8 α -Pac-Blue and either major histocompatibility complex (MHC) class I pentamers (ProImmune) or MHC class I dextramers (Immudex, Copenhagen, Denmark) complexed with the respective H-2K^b binding peptides, i.e., SIINFEKL or TSYKFESV. OVA- and B8R-specific MHC class I pentamers were both labeled with APC, and the respective CD8 T-cell populations were stained in two separate reactions. MHC class I dextramers were labeled with either PE (SIINFEKL-dextramer) or APC (TSYKFESV-dextramer) and were combined for one staining reaction. After being washed, stained cells were analyzed by flow cytometry on a BD Biosciences BD LSR II system. Approximately 20,000 PBMCs were acquired per sample, with about 50% of the cells from immunized mice being CD8⁺ CD19⁻.

Enzyme-linked immunosorbent assay (ELISA) for detection of MVA-specific antibodies in mouse sera. Ninety-six-well plates were coated with crude extract of MVA-BN-infected CEF cells, essentially as described previously (50). Briefly, 2-fold serial dilutions of mouse sera were incubated for 1 h at room temperature (RT). If necessary, predilutions of mouse sera were prepared. For detection, the plates were incubated with a sheep anti-mouse IgG-horseradish peroxidase (HRP) detection antibody (Serotec) for 1 h at RT. 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich) was used as the substrate, and the reaction was stopped by adding 1 M H₂SO₄ (Merck). The optical density (OD) was measured at 450 nm with a Tecan F039300 Sunrise absorbance reader (Maennedorf, Switzerland).

ELISA for detection of OVA-specific antibodies in mouse sera. Ninety-six-well plates were coated with 0.25 μ g/well of chicken ovalbumin (Sigma-Aldrich). Twofold serial dilutions of mouse sera were incubated overnight at 4°C. If necessary, predilutions of mouse sera were prepared. Biotinylated donkey anti-mouse IgG(H+L) antibody (Dianova) was added to the plates for 2 h at RT. For the detection of biotinylated secondary antibody, streptavidin-HRP (Amersham Biosciences) was added to the plates and incubated for 2 h at RT. ABTS (Sigma-Aldrich)-0.03% H₂O₂-0.1 M citric acid was used to develop the assay. The OD was measured at 405 nm, with a reference wavelength of 492 nm, with a Tecan F039300 Sunrise absorbance reader.

Statistical analysis. Statistical analysis of data was done by using a two-way repeated-measures analysis of variance (ANOVA) if not indicated otherwise.

RESULTS

We constructed a hybrid early-late promoter, designated pHyb, containing a late element from the cowpox virus ATI promoter and five tandemly arranged early promoter elements (Fig. 1). The early promoter elements were based on the p7.5 promoter and were optimized further by use of published data (7, 12, 17). We compared pHyb with the widely used synthetic pS promoter, which directs high-level gene expression (12), and with the natural p7.5 promoter (13) (Fig. 1). These promoter constructs were cloned upstream of the open reading frames encoding either OVA or EGFP and introduced into the cloned genomes of MVA viruses by homologous recombination in *Escherichia coli*.

Expression of EGFP in untreated HeLa cells, which are nonpermissive for MVA-BN (46), was similar with all three promoters 16 h after infection (Fig. 2A). In untreated CEF cells, which are permissive for MVA, the pS and p7.5 promoters directed a higher level of total EGFP expression than the pHyb promoter (Fig. 2B). To analyze early gene expression specifically, the MVA infection cycle was arrested in its early phase by treatment with AraC for 16 h. Under these conditions, the pHyb promoter directed much higher EGFP expression than the pS and p7.5 promoters (Fig. 2A and B). In fact, the levels of EGFP in MVA-pHyb-eGFP-infected cells were not influenced by AraC. This suggested that the tandem arrangement of the five early elements in pHyb (Fig. 1) was responsible for the observed increase in early neoantigen expression.

FIG. 1. Sequence and schematic representation of the arrangement of early and late promoter elements in p7.5, pS, and pHyb. (A) Schematic representation of p7.5, pS, and pHyb promoters. Early and late promoter elements are not drawn to scale. (B) Nucleotide sequences of the p7.5, pS, and pHyb promoters. The region where the p7.5 early promoter element has been optimized is boxed in the p7.5 sequence, and the optimized sequence is shown below. Single solid line, late promoter element; double line, early promoter element.

A kinetic analysis of EGFP expression in HeLa cells showed that the pHyb promoter directed protein expression within the first 30 min of infection, whereas significant EGFP expression from the p7.5 and pS promoters was not detectable before 60 min (Fig. 2C). Moreover, the pHyb promoter directed higher levels of EGFP expression throughout the first 6 h of infection (Fig. 2C). The expression levels from the p7.5 and pS promoters reached those induced by the pHyb promoter only late in infection, after more than 6 h. Late in infection, pS-driven EGFP expression was the highest of the three promoters tested (Fig. 2C). Hence, at all time points between 0.5 and 6 h after infection, expression of EGFP from the pHyb promoter was significantly higher than that achieved with the p7.5 and pS promoters, and detectable EGFP expression started earlier.

Analysis of CD8 T-cell responses. CD8 T-cell responses against recombinant OVA expressed under the control of the p7.5, pS, and pHyb promoters were determined in C57BL/6 mice after one, two, three, and four immunizations with 10⁸ TCID₅₀ of recombinant MVA per mouse. The OVA-specific CD8 T-cell response was determined by ICCS for IFN- γ after stimulation with the K^b-restricted, OVA-derived peptide SIINFEKL. To monitor the CD8 T-cell response to the MVA vector, CD8 T cells recognizing the immunodominant CD8 T-cell epitope TSYKFESV, encoded by the poxviral early gene B8R (49), were quantified. Figure 3A to C show the gating strategy and representative ICCS and MHC class I dextramer

FIG. 2. Expression of EGFP directed by recombinant MVAs. Recombinant MVAs containing the EGFP open reading frame under the control of the indicated promoters were used to infect HeLa (A and C) and CEF (B) cells at a multiplicity of infection of 5. (A and B) Cells were either treated with cytosine arabinoside (+AraC) or left untreated (–AraC) during infection. Cells were harvested at 16 h p.i. and analyzed by flow cytometry for EGFP expression. (C) HeLa cells were infected with MVA-p7.5-eGFP (p7.5), MVA-pS-eGFP (pS), or MVA-pHyb-eGFP (pHyb) or incubated with medium (mock). At the indicated times after infection, cells were harvested and analyzed by flow cytometry for EGFP expression. The experiments were repeated independently at least two times. MFI, mean fluorescence intensity.

staining of B8R- and OVA-specific CD8 T cells. One week after the first immunization, we observed similar proportions of OVA-specific CD8 T cells, independent of the promoter used (Fig. 3D). The slightly larger numbers of OVA-specific CD8 T cells observed after the second immunization with MVA-pHyb-OVA than those seen with MVA-pS-OVA and MVA p7.5-OVA (Fig. 3E) were not statistically significant ($P = 0.27$ and 0.62 , respectively). B8R-specific CD8 T-cell responses did not differ significantly between recombinant MVAs after the first and second immunizations ($P > 0.5$) (Fig. 3D and E).

After the third immunization with MVA-pHyb-OVA, we observed significantly stronger OVA-specific CD8 T-cell responses than those after triple immunization with MVA-pS-OVA ($P < 0.01$) or MVA-p7.5-OVA ($P < 0.001$) (Fig. 3F). In contrast, there were no significant differences in the proportion of B8R-specific CD8 T cells for mice immunized with MVA-pHyb-OVA compared to mice treated with the two other MVA constructs ($P > 0.19$).

Note that our results demonstrate that it was possible to

significantly increase the number of OVA-specific CD8 T cells after two previous immunizations with the homologous MVA construct. At this time point, the vector-specific CD8 T-cell response against the immunodominant B8R antigen had already reached a very high level, which increased only moderately after the third immunization (Fig. 3D to F). The ability of the MVA vector to boost neoantigen-specific CD8 T-cell responses in the presence of strong antivector immunity was independent of the promoter ($P < 0.001$ for OVA-specific CD8 T cells after three versus two homologous immunizations with each of the three recombinant MVA constructs). The proportion of OVA-specific CD8 T cells was exceptionally high after three immunizations with MVA-pHyb-OVA. Up to 20% of all CD8 T cells were OVA specific 6 days after the third immunization (Fig. 3F). Equal proportions of OVA-specific CD8 T cells and B8R-specific CD8 T cells were detected by ICCS on day 6 after the third immunization with MVA-pHyb-OVA (Fig. 3F). This was due partly to the decrease in relative numbers of B8R-specific CD8 T cells, suggesting that expansion of primed OVA-specific CD8 T cells was stimulated with a higher efficiency (Fig. 3F).

The ratio of OVA- to B8R-specific CD8 T cells was significantly different after 3 immunizations with MVA-pHyb-OVA from those after immunization with the other two constructs (Fig. 3G) ($P = 0.014$ versus pS and $P = 0.002$ versus p7.5; Student's t test). The enhancing effect of pHyb was apparent after 2 immunizations (Fig. 3G) but was not statistically significant at this time point. Taken together, the $CD8_{OVA}/CD8_{B8R}$ ratios indicate that the pHyb promoter exerts its advantage in booster immunizations, particularly after the second booster. The $CD8_{OVA}/CD8_{B8R}$ ratios also suggest that the pS promoter is indeed less efficient than the pHyb promoter but has an advantage over the p7.5 promoter (Fig. 3G).

When OVA-specific CD8 T-cell responses were analyzed at different time points after the third immunization, we found that OVA-specific CD8 T cells were immunodominant at specific time points after the boost, as determined by ICCS (Fig. 4, top panels). Reversal of immunodominance was generally observed 4 to 6 days after the third immunization (Fig. 4 and data not shown). In contrast, even after four immunizations with MVA-pS-OVA, B8R-specific CD8 T cells remained immunodominant (Fig. 5C and data not shown). These results were confirmed by employing the MHC class I dextramer staining technique. Using this approach, we observed higher levels of OVA- than B8R-specific CD8 T cells at all time points after the third immunization with MVA-pHyb-OVA, but never with the p7.5 or pS promoter (Fig. 4, bottom panels). Thus, only the pHyb promoter was able to reverse CD8 T-cell immunodominance in favor of the pHyb-driven neoantigen.

CD8 T-cell memory. In the early memory phase, 28 days after the third immunization with MVA-pHyb, OVA-specific CD8 T cells still outnumbered B8R-specific CD8 T cells and reached significantly higher numbers than those in mice immunized three times with MVA-pS-OVA (Fig. 5A) ($P = 0.036$ using Student's t test). Analysis of long-term CD8 T-cell memory in the blood of mice 13 weeks after the third immunization demonstrated that OVA-specific CD8 T-cell numbers were still significantly higher with MVA-pHyb-OVA than with MVA-pS-OVA (Fig. 5B) ($P = 0.019$) and MVA-p7.5-OVA ($P = 0.002$). This result was confirmed by staining with MHC

FIG. 3. Analysis of OVA- and MVA-specific CD8 T-cell responses induced by recombinant MVAs. (A) Gating strategy for analysis of OVA- and B8R-specific CD8 T cells. Representative examples of gated CD8⁺ CD19⁻ cells analyzed by MHC class I dextramer staining (B) and ICCS (C) are shown for B8R- and OVA-specific CD8 T cells. Leukocytes from animals immunized with MVA (empty vector) and leukocytes incubated without peptide (no pept.) served as controls. Numbers in parentheses indicate the frequencies of dextramer⁺ or IFN-γ⁺ cells among CD8⁺ CD19⁻ cells. (D) MVA-p7.5-OVA (p7.5), MVA-pS-OVA (pS), and MVA-pHyb-OVA (pHyb) were used to immunize C57BL/6 mice i.p., using a dose of 10⁸ TCID₅₀ per mouse. Mice were subjected to a second (E) and a third (F) i.p. injection 4 and 8 weeks after the first immunization, respectively. Leukocytes from blood were analyzed 6 to 8 days after the 1st immunization and 6 days after the 2nd and 3rd immunizations for induction of OVA-specific and vector-specific (B8R) CD8 T-cell responses. Quantitation of antigen-specific CD8 T cells was done by ICCS for IFN-γ after 5 h of restimulation and gating on CD4⁻ CD8⁺ or CD19⁻ CD8⁺ lymphocytes. The data shown in panels D to F are the arithmetic means (plus SEM) of the percentages of OVA- and B8R-specific cells among total CD8 T cells. (G) The percentages of OVA (CD8_{OVA})- and B8R (CD8_{B8R})-specific CD8 T cells were used to calculate the mean ratio from the individual ratios of CD8_{OVA} to CD8_{B8R} T cells for each mouse. The log₁₀ of the ratios was used to calculate the standard error (SEM). The data points in panels D to G show combined results of two independent experiments and are representative of 10 or 11 mice per group.

FIG. 4. Kinetics of OVA- and MVA-specific CD8 T-cell responses after three immunizations with recombinant MVAs. MVA-p7.5-OVA (p7.5), MVA-pS-OVA (pS), and MVA-pHyb-OVA (pHyb) were used to immunize groups of 5 or 6 C57BL/6 mice i.p. at a dose of 10^8 TCID₅₀ per mouse three times at 4-week intervals. Leukocytes from blood were analyzed 4, 6, and 8 days after the third immunization for induction of OVA-specific and vector-specific (B8R) CD8 T-cell responses. Antigen-specific CD8 T cells were quantified by ICCS for IFN- γ after 5 h of restimulation (upper panels) and by MHC class I dextramer staining (lower panels). Cells were gated for CD19⁻ CD8⁺ lymphocytes. Leukocytes from animals immunized with MVA (empty vector) and leukocytes incubated without peptide (no pept.) served as controls. The data shown are the percentages of OVA- and B8R-specific cells among total CD8 T cells. Arithmetic means (plus SEM) for 5 or 6 mice per group are shown.

class I pentamers detecting B8R- and OVA-specific CD8 T cells (data not shown). Irrespective of the type of promoter, approximately 70 to 80% of all OVA-specific CD8 T cells detected by MHC class I pentamer staining were of the effector memory phenotype (CD62L⁻ CD127⁺) 12 weeks after the third immunization (data not shown). In conclusion, pHyb was able to induce a strong and long-lasting CD8 T-cell response against the neoantigen, and the proportions of OVA- and B8R-specific cells found in the early phase after the third immunization with the three MVA constructs were essentially preserved in the memory phase.

Fourteen weeks after the third immunization, mice were boosted one more time with the respective MVA constructs. Of all constructs, MVA-pHyb-OVA again induced the largest

number of OVA-specific CD8 T cells compared to pS ($P = 0.013$) and p7.5 ($P = 0.004$) (Fig. 5C). Notably, immunization with recombinant MVA-OVA in the memory phase was able to preferably boost responses to OVA with all types of promoters used, whereas B8R-specific responses appeared to remain static or even slightly decreased (Fig. 5C). This result was observed consistently, but the underlying mechanism remains unclear at present. Quantitative and temporal competition is likely to play a major role in this phenomenon, since B8R-specific responses against the parental nonrecombinant MVA vector increased after the fourth immunization, from 25.7% to 33.2% of all CD8 T cells (data not shown). This result appeared to be specific for CD8 T cells in blood, since the proportion of B8R-specific CD8 T cells in the spleen after the

FIG. 5. OVA- and MVA-specific memory CD8 T cells. Leukocytes from C57BL/6 mice immunized with MVA-p7.5-OVA, MVA-pS-OVA, and MVA-pHyb-OVA were prepared and analyzed 28 days (A) and 13 weeks (B) after the third immunization by ICCS for IFN- γ to quantify OVA- and B8R-specific CD8 T cells. (C) Mice immunized three times were vaccinated a fourth time with the respective OVA-expressing MVA recombinants 14 weeks after the third immunization. Mice were analyzed by IFN- γ ICCS for OVA- and B8R-specific CD8 T cells in blood 6 days after the fourth immunization. Leukocytes from the immunized animals were incubated without peptide to serve as controls (no pept.). Arithmetic means (plus SEM) for combined numbers from two independent experiments are shown, with 8 to 11 mice per group (in total).

FIG. 6. Humoral immune responses against OVA and MVA in immunized mice. Groups of five mice were immunized via the i.p. route with 10^8 TCID₅₀ of MVA-pHyb-OVA or MVA-pS-OVA at weeks 0, 2, and 14. Blood was collected 2 weeks after each immunization for analysis of OVA- and MVA-specific antibodies in serum by ELISA. The data shown are mean group titers $\pm$ SEM for OVA-specific (A) and MVA-specific (B) total antibody.

fourth immunization increased with all MVA constructs (data not shown). Similar to the case for OVA-specific CD8 T cells in blood, the percentage of OVA-specific CD8 T cells in the spleen after the fourth immunization was clearly highest for the pHyb promoter construct compared to the other promoter constructs (data not shown).

Humoral immune response. To determine whether pHyb affects the induction of antibodies against OVA, sera of mice were analyzed for OVA-specific antibody titers after four immunizations. MVA-pS-OVA- and MVA-pHyb-OVA-immunized mice had very similar OVA-specific antibody titers at this time point (5.21 ± 0.06 [mean $\pm$ standard error of the mean {SEM}] versus 5.10 ± 0.12), when OVA-specific CD8 T-cell numbers were clearly higher in the MVA-pHyb-OVA-immunized mice (Fig. 5C). To evaluate whether differences might occur after fewer immunizations, we determined OVA-specific antibody titers in sera obtained from C57BL/6 mice every 2 weeks after immunizations at weeks 0, 2, and 14 with MVA-pS-OVA and MVA-pHyb-OVA. Both constructs induced high OVA-specific antibody responses after the second and third immunizations. OVA-specific antibody titers of MVA-pS-OVA-immunized mice were approximately 5- to 10-fold higher than those of mice immunized with MVA expressing OVA under the control of the pHyb promoter after two and three immunizations ($P = 0.044$) (Fig. 6A). In contrast, MVA-specific serum antibody titers of mice immunized with the two constructs were largely similar after the second and third immunizations (Fig. 6B), indicating that humoral immunity to the vector backbone was induced similarly by both MVA-pHyb-OVA and MVA-pS-OVA. Although OVA-specific antibody titers appeared higher than those against MVA antigen (Fig. 6), the two ELISA systems are not comparable due to the nature of the poxviral particles and the purified protein (OVA) antigen used as coating agents in our solid-phase assays. This is expected to lead to different coating characteristics and available antigenic epitopes. In conclusion, the pHyb promoter favored the CD8 T-cell response to the neoantigen, but this

promoter was not superior to the pS promoter for induction of neoantigen-specific antibodies.

DISCUSSION

The pHyb-driven EGFP product, detected as early as 30 min postinfection (p.i.), is the first demonstration of a recombinant immediate-early protein in pox virology. Three immunizations with MVA-pHyb-OVA resulted in an increased neoantigen-specific CD8 T-cell response compared to those with conventional poxviral promoters. Furthermore, three or more homologous immunizations with MVA-pHyb-OVA reversed the CD8 T-cell immunodominance hierarchy in favor of OVA.

The category of immediate-early genes was recently proposed to be added to the existing temporal classes of early, intermediate, late, and early-late transcribed poxviral genes (3). Immediate-early gene transcriptional activity was first detected between 0.5 and 1 h p.i., with a peak at 2 h p.i. (3). However, no defined nucleotide sequence motifs have been identified that distinguish these promoters from the well-known early promoters, and the presence of a temporal class of immediate-early proteins translated from these immediate-early transcripts has not been proven (3, 17). Although we have not determined whether mRNA expression kinetics of pHyb-driven EGFP or OVA would match the reported definition of an immediate-early gene (3), we considered pHyb an immediate-early promoter because it was possible to detect protein expression within 30 min after infection.

The p7.5 promoter was derived from the C23L/B29R gene of VACV (1, 13). Interestingly, the early onset of expression of the C23L/B29R gene resembled that of immediate-early gene transcripts, although C23L/B29R was not assigned to the immediate-early class of genes (3). Because p7.5 was unable to drive immediate-early translation but induced slightly earlier EGFP expression than that induced by pS (Fig. 2), we reasoned that pentamers of an optimized p7.5 early promoter element might focus transcriptional complexes and favor immediate-early neoantigen expression (Fig. 1). This was based on the fact that a single optimized early p7.5 element was not sufficient to direct immediate-early expression (data not shown). We refrained from extending the chosen repetitive nucleotide sequences further, as poxviral genomes are prone to mutations and genomic alterations due to the recombinogenic potential of the virus (4, 22). We did not observe any changes in the promoter sequences of pHyb analyzed after 20 passages in CEF cells (data not shown). Hence, the pHyb promoter with five direct repeats is stable. This confirms earlier reports describing similar tandem array promoters (20, 27) and the stability of MVA vector constructs containing multiple copies of a short version of the ATI promoter (48). Therefore, we defined the first immediate-early promoter for neoantigen expression by poxviral vectors that stably directed protein expression within 30 min of infection.

It was recently demonstrated that cross-competition occurs not only between CD8 T cells specific for early versus late expressed antigens but also between CD8 T cells specific for different early viral antigens (28). We have classified the pHyb promoter as an immediate-early promoter, whereas the B8R promoter directing the expression of the immunodominant VACV CD8 T-cell antigen has been classified as an early

promoter (3). It is therefore tempting to speculate that a shift in antigen expression kinetics from early to very early times in the viral infection cycle might provide an advantage for the respective CD8 T-cell specificity in recall responses. Although this temporal difference is relatively small, it might become relevant under conditions of a substantial antipoxvirus CD8 T-cell immunity. This might explain the finding that the major advantage of pHyb is discernible after 3 or 4 immunizations but is scarcely discernible after secondary immunization and not at all discernible after primary immunization. A vaccine-specific immune response represents an estimated 30 to 40% of all CD8 T cells after primary infection in mice and humans (36, 38). Even if it is considered that a vaccine-specific recall response might double the number of reactive CD8 T cells, the stunning 20% of pHyb-induced OVA-specific IFN- γ -secreting CD8 T cells would represent more than 25% of the total vaccine response. After three immunizations with MVA-pHyb-OVA, we found a surprisingly high proportion of CD8 T cells recognizing only two CD8 T-cell epitopes, derived from B8R and OVA, adding up to ~60% of the cells in the case of the MVA-pHyb-OVA construct and exceeding 50% with the two other MVA-OVA recombinants (Fig. 4). For primary infections of C57BL/6 mice with lymphocytic choriomeningitis virus (LCMV), a similarly large number (60%) of CD8 T cells recognizing two viral epitopes was reported (39). Clearly, vaccinia virus expresses a much larger number of antigenic determinants than LCMV and induces a more diverse CD8 T-cell response, with ~50 epitopes making up 95% of the total primary response in C57BL/6 mice (38). The extraordinarily large numbers of OVA- and B8R-specific CD8 T cells that we observed occurred after three or more homologous immunizations with high doses of MVA. Presumably, competition between different CD8 T-cell specificities becomes more selective upon repeated immunization, favoring dominant antigens as opposed to subdominant determinants. Thus, the MVA-OVA-specific CD8 T-cell response appeared to become highly focused toward fewer epitopes after repeated immunizations. A similar focusing of the CD8 T-cell response was demonstrated previously for secondary responses against dominant epitopes of LCMV, *Listeria monocytogenes*, and cellular antigens (5, 9, 42).

The pHyb-driven OVA-derived SIINFELK peptide replaced the vector-derived B8R peptide epitope TSYKFESV as the immunodominant determinant of MVA-pHyb-OVA. In some experiments, four immunizations with MVA-pHyb-OVA were required to achieve a ratio of OVA- to B8R-specific CD8 T cells of >1 (data not shown). The observed reversal of immunodominance cannot be explained by different affinities of the two peptides for the MHC molecule. The OVA peptide has a comparable or even somewhat lower affinity for H-2K^b than the B8R peptide TSYKFESV (34, 49). Reversal of immunodominance was not observed with any of the other promoters used. Thus, the very early kinetics of pHyb-driven OVA expression provides the most likely explanation for enhanced OVA-specific CD8 T-cell responses and the reversal of immunodominance. We observed a tendency toward lower proportions of B8R-specific CD8 T cells in MVA-pHyb-OVA- versus MVA-pS-OVA- and MVA-p7.5-OVA-immunized mice. In fact, proportions of B8R-specific CD8 T cells appeared to be correlated inversely with the proportions of OVA-specific CD8

T cells, suggesting that these specificities compete in a way that is strongly influenced by the type of promoter used for OVA expression. Since the strong OVA-specific CD8 T-cell response observed with MVA-pHyb-OVA was only partly at the cost of the B8R-specific response, the neoantigen most likely also competed successfully with CD8 T-cell specificities directed against other antigenic epitopes encoded by MVA.

Homologous prime/boost immunizations using a single poxviral vector have frequently been disfavored due to reports describing inefficient cytotoxic T-lymphocyte (CTL) induction under conditions of preexisting poxvirus-specific immunity (14, 41) or inferiority of homologous versus heterologous prime/boost regimens (2, 43, 52). Many of these studies have either employed replicating vaccinia virus (14, 30, 41) or used relatively low doses of MVA. Note that reported data indicate that preexisting immunity affects the humoral neoantigen-specific immune response to a higher degree than the CD8 T-cell response (31). Our study demonstrates the remarkable efficiency of an MVA-based homologous prime-boost regimen in expanding neoantigen-specific CD8 T cells, even after three or four homologous boosts. This result is in agreement with our studies demonstrating larger responses against CTL epitopes expressed by recombinant MVA in a two-dose homologous immunization scheme than those induced by DNA prime and MVA boost immunization (unpublished data). An important prerequisite to achieve this high efficiency with MVA is most likely the use of a high dose of this vector. The feasibility of inoculating individuals with high doses without safety problems and without serious adverse reactions is one of the major advantages of MVA (51). Therefore, our study emphasizes the utility of MVA as a vaccine vector in homologous prime-boost regimens. Neoantigen-specific CTL expansion was particularly effective when the neoantigen was under the control of a strong immediate-early promoter. Previous studies comparing different promoters for expression of neoantigens have been conducted mainly with replication-competent vaccinia virus strains. Overall expression levels of EGFP in CEF cells, which are permissive for MVA, were highest for the pS promoter among all promoters we tested, including p7.5, pIL (32), and pATI, whereas they were comparable in nonpermissive HeLa cells (Fig. 2 and data not shown). This suggests that promoters used to induce CD8 T cells against neoantigens might yield different results in replication-competent and replication-restricted poxvirus vectors.

In conclusion, we showed that a replication-restricted poxviral vector with a promoter directing very early expression of the neoantigen induces superior neoantigen-specific CD8 T-cell responses. Thus, our results have implications particularly for the design of replication-restricted poxvirus-based vaccine vectors to be used in prime-boost vaccination approaches for inducing CD8 T-cell immunity against chronic viral infections, such as infections with HIV, human papillomavirus, and hepatitis C virus, or against cancers.

ACKNOWLEDGMENTS

Bavarian Nordic GmbH is the funder of this study.

We thank Susanne Lukassen and Claudia Mnich for excellent technical assistance in generating the recombinant MVA viruses and Jutta Kramer and the whole vaccine generation department for producing highly pure viral stocks. We thank Martin Schletz and Heike Ryl for

help with the ELISAs and Niels Wulff for help with the statistical analysis of the data.

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