Characterization of Antigen-Specific Immune Responses Induced by Canarypox Virus Vaccines


*J Immunol* 2007; 179:6115-6122; doi: 10.4049/jimmunol.179.9.6115

http://www.jimmunol.org/content/179/9/6115

---

**References**

This article cites 26 articles, 13 of which you can access for free at:

http://www.jimmunol.org/content/179/9/6115.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Characterization of Antigen-Specific Immune Responses Induced by Canarypox Virus Vaccines

Rinke Bos,* Suzanne van Duikeren,* Thorbold van Hall,* Marjolein M. Lauwen,* Mark Parrington,‡ Neil L. Berinstein,‡ Bryan McNeil,‡ Cornelis J. M. Melief,* J. Sjef Verbeek,‡ Sjoerd H. van der Burg,* and Rienk Offringa2*

Avipoxvirus-based vectors, such as recombinant canarypox virus ALVAC, are studied extensively as delivery vehicles for vaccines against cancer and infectious diseases. Effective use of such vaccines is expected to benefit from proper understanding of the interaction between these viral vectors and the host immune system. We performed preclinical vaccination experiments in a murine tumor model to analyze the immunogenic properties of an ALVAC-based vaccine against carcinoembryonic Ag (ALVAC-CEA), a tumor-associated autoantigen commonly overexpressed in colorectal cancers. The protective CEA-specific immunity induced by this vaccine consisted of CD4+ T cell responses with a mixed Th1/Th2 cytokine profile that were accompanied by potent humoral responses, but not by CEA-specific CD8+ CTL immunity. In contrast, protective immunity induced by a CEA-specific DNA vaccine (DNA-CEA) consisted of Th1 and CTL responses. Modification of the ALVAC-CEA vaccine through coinjection of DNA-CEA, admixture with CpG oligodeoxynucleotides, or supplementation with additional transgenes encoding a triad of costimulatory molecules (TRICOM) did not result in induction of CEA-specific CTL responses. Even though these results suggested that ALVAC does not elicit Ag-specific CTLs, immunization with ALVAC vaccines against other Ags efficiently induced CTL responses. Our data show that the capacity of ALVAC vaccines to elicit CTL immunity against transgene-encoded Ags critically depends on the presence of highly immunogenic CTL epitopes in these Ags. This consideration needs to be taken into account with respect to the design and evaluation of vaccination strategies that use ALVAC-based vaccine. The Journal of Immunology, 2007, 179: 6115–6122.

R
combinant avipoxviruses, including ALVAC (canarypox virus), are used as vehicles for the development of vaccination strategies against cancer, malaria, tuberculosis, and AIDS. Their inability to replicate in species other than birds provides an excellent safety barrier against vaccine-associated complications in humans. Despite this abortive infection, the Ags encoded by the foreign genes inserted into the ALVAC vector are expressed and become presented to the host immune system (1, 2). One of the potential applications of ALVAC concerns therapeutic vaccination against colorectal cancer through induction of immune responses against carcinoembryonic Ag (CEA), a tumor-associated autoantigen that is overexpressed by several cancer types of epithelial origin (3). The capacity of poxvirus-based vaccines to elicit CEA-specific antitumor immunity is studied extensively in both preclinical murine tumor models and cancer patients (4, 5). We have previously reported that ALVAC-CEA immunization of C57BL/6 mice induced protective immunity against an otherwise lethal dose of CEA-overexpressing tumor cells, MC38-CEA. Even though MC38-CEA tumor cells express MHC class I, but not MHC class II, protective immunity was found to depend on the CEA-specific CD4+ Th response. CD8+ CTL were found to be dispensable (6). The lack of involvement of CEA-specific CTL in tumor eradication prompted the question concerning which CD4+ T cell-dependent effector mechanisms would mediate tumor eradication in this setting and, furthermore, whether ALVAC-CEA immunization of C57BL/6 mice would be capable of inducing CEA-specific CTLs at all.

Our present study revealed that ALVAC-CEA immunization in this experimental setting elicited a CEA-specific CD4+ T cell response displaying a mixed Th1/Th2 profile that was accompanied by a potent CEA-specific humoral response, but not by any measurable CEA-specific CTL immunity. Accordingly, tumor clearance involved Ab-dependent effector mechanisms. Although the type of immunity elicited by ALVAC-CEA was protective in this mouse tumor model, it may not be optimal for antitumor activity in patients with colorectal cancer, in particular because one would expect anti-CEA Abs to be distracted from targeting the tumor due to the elevated levels of CEA in the circulation of these patients (3). We therefore investigated why ALVAC-CEA failed to induce a CEA-specific Th1/CTL response and tested whether modifications would empower this vaccine to elicit CEA-specific CTL immunity.

Materials and Methods

Mice
C57BL/6 Kh (B6, H-2b) were bred in our Leiden University Medical Center facilities (Leiden, The Netherlands). Fc receptor γ-chain knockout mice and C1q knockout mice were provided by J. S. Verbeek (Leiden University Medical Center, Leiden, The Netherlands). Fc receptor γ-chain knockout mice and C1q knockout mice were provided by J. S. Verbeek (Leiden University Medical Center, Leiden, The Netherlands).

1 Abbreviations used in this paper: CEA, carcinoembryonic Ag; HPV, human papillomavirus; ADCC, Ab-dependent cellular cytotoxicity; ODN, oligodeoxynucleotide.

Received for publication July 19, 2007. Accepted for publication August 27, 2007.

* Address correspondence and reprint requests to Dr. Rienk Offringa, Department of Immunohematology and Blood Transfusion, Tumor Immunology Group, E3-Q, Leiden University Medical Center, Albinusdreef 2, 2333 RA Leiden, The Netherlands. E-mail address: R.Offringa@LUMC.nl

1 This work was supported by Sanofi Pasteur Toronto and by Grant UL 2000-2035 from the Dutch Cancer Society.

2 Address correspondence and reprint requests to Dr. Rienk Offringa, Department of Immunohematology and Blood Transfusion, Tumor Immunology Group, E3-Q, Leiden University Medical Center, Albinusdreef 2, 2333 RA Leiden, The Netherlands. E-mail address: R.Offringa@LUMC.nl

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

www.jimmunol.org
Medical Center, Leiden, The Netherlands). The experiments were approved by the animal experimental committee of Leiden University Medical Center.

Vaccine components

Immunizations were performed with plasmid pGT64 CEA, which encodes both CEA and murine CD80 (7) or ALVAC-CEA-CD80, or ALVAC-CEA-TRICOM. The latter two ALVAC vaccines were engineered to express either murine CD80 (B7-1) or a triad of murine costimulatory molecules: CD80, ICAM-1, and CD48, the murine high-affinity ligand of CD2. Before our comparative vaccination studies, we ensured that the ALVAC batches used did not differ essentially in quality and, in particular, that the ratio between the number of ALVAC-DNA genomes (genome equivalents) and viral particles was comparable. Furthermore, by infection of HeLa cells with titrated amounts of different ALVAC preparations and measuring CEA expression levels, we have shown that the batches of ALVAC-CEA, ALVAC-CEA-CD80, and ALVAC-CEA-TRICOM used in our experiments induced CEA expression on infected cells with comparable efficiency (data not shown). In some experiments ALVAC was coinjected with CpG oligodeoxynucleotides (ODN) 1826, which is a 20-mer containing two CpG motifs (TTCATGACGTCTCTGA CGTT). The latter was provided by Coley Pharmaceutical and used at 50 μg/injection.

For the construction of ALVAC-OVA and DNA-OVA vaccines, a gene construct encoding OVA aa 242–358 was cloned by PCR. This sequence is devoid of the various signal sequences present in full-length OVA, but does encompasses the H-2Kb-restricted CTL epitope (OVA257–264) (8). As a result of the removal of the signal sequences, the OVA Ag is expressed as a cytoplasmic protein that is not secreted (9).

ALVAC-SB contains a gene construct that encodes four defined CTL epitopes and an Ab epitope that are arranged in a string-of-beads fashion and are spaced by triple alanine sequences. We previously described this gene construct in the context of a recombinant adenovirus construct named rAd1 (10). The sequence of the gene product is the following: MNAAA-SGPSNTPPEI-AAA-RAHYNIVTF-AAA-AIYKKSQHM-AAA-VNIRNC CYI-AAA-SEQKLISEDELNN. The underlined sequences correspond to the following epitopes, respectively: an H-2Dd-restricted epitope from the adenovirus type 5 (Ad5) E1A protein; an H-2Db-restricted epitope from the human papillomavirus (HPV) type 16 E7 protein; an H-2Kb-restricted epitope from the murine wild-type p53 protein; and an H-2Db-restricted epitope from the Ad5 E1B protein; and a human c-Myc-derived sequence recognized by mAb 9E10 (10).

Immunizations and tumor challenge experiments

Mice were vaccinated twice i.m. within a 2-wk interval with 100 μg of plasmid pGT64 CEA B7-1 (7) or with 3 × 10^6 PFU ALVAC-CEA (providing by S. Pastoret) dissolved in 100 μl of PBS. At 4 days before each immunization, 80 μl of 100 μM cardotoxin was injected i.m. Two weeks after the last vaccination, spleens were isolated for in vitro tests or mice were used for tumor challenge experiments in which 250,000 MC38-CEA tumor cells were s.c. injected in 200 μl of PBS/5% BSA. MC38-CEA (11) were cultured as previously described (6). CEA expression by MC38-CEA was regularly examined by cell surface staining with a CEA-specific Ab (PARLAM4, Monosan). In vivo depletion of CD4^+ and CD8^+ T cells. DNA-CEA induces CEA-expression on infected cells with comparable efficiency (data not shown). In some experiments ALVAC was coinjected with CpG oligodeoxynucleotides (ODN) 1826, which is a 20-mer containing two CpG motifs (TTCATGACGTCTCTGA CGTT). The latter was provided by Coley Pharmaceutical and used at 50 μg/injection.

In vitro analysis of T cell responses

Splenocytes from immunized mice were tested for their responsiveness against CEA Th epitopes I–5 (6), the CTL epitope with peptide sequence CGIQNSVSA (12) and protein by flow cytometry. A total of 2 × 10^6 splenocytes were incubated with 5 μg/ml Ag and brefeldin A (10 μg/ml) for 12–16 h. Fixation and staining procedures were done as previously described (13).

Ab detection

CEA-specific Abs were measured in sera from immunized mice 2 wks after the last vaccination. Nunc Maxisorp immunoplates were coated with 2 μg/ml CEA protein diluted in coating buffer and incubated overnight at room temperature. Nonspecific binding sites were blocked with 50 μl of PBS-0.05% Tween 20 (PBS-T)/1% BSA and incubated for 1 h at 37°C. After four washes with PBS-T, 50 μl of serial dilutions of mouse serum in PBS-T/1% BSA was added and the plates were incubated for 1 h at 37°C. After washing as described, 50 μl of detection Ab in diluted 1/500 in PBS-T/1% BSA was added to the appropriate wells and incubated for 1 h at 37°C. Plates were washed four times, and 50 μl of ABTS (25 mg of 3-ethyl benzthiazoline 6-sulfonic acid) dissolved in 10 ml of citrate phosphate buffer (pH 4.2) plus H2O2 (5 μl/5 ml) was added to each well. Absorbance values were read at 415 nm and the Ab titers were determined as the last serum dilution yielding absorbance values 5-fold higher than the preimmune serum.

Cytokine secretion

Splenocytes from immunized mice were incubated with 5 μg/ml CEA Th epitopes, CTL epitope, or inactivated ALVAC. Supernatants were analyzed 3 days later for Th1 and Th2 cytokines using a cytokine bead array (BD Pharmingen).

Results

ALVAC-CEA and DNA-CEA protect against distinct immune mechanisms

We previously observed that protective immunity against the CEA-overexpressing tumor MC38-CEA that is induced in C57BL/6 mice by immunization with ALVAC-CEA critically depends on CD4^+ T cells, but does not require CD8^+ T cells (6). This observation was somewhat unexpected because MC38-CEA is a nonlymphoid tumor cell line expressing MHC class I, but lacking MHC class II. To investigate whether the nature of this anti-tumor immune response would be dictated by characteristics of the tumor cell MC38, the tumor Ag CEA, or the mode of vaccination, we compared the effect of ALVAC-CEA with that of a plasmid DNA-based vaccine encoding the same Ag (DNA-CEA). As shown in Fig. 1, B6 mice immunized with two subsequent i.m. doses of either ALVAC-CEA or DNA-CEA rejected an otherwise lethal dose of MC38-CEA tumor cells. Although we found these two vaccines to protect against tumor outgrowth with similar efficiency, T cell depletion studies revealed that the underlying immune effector mechanisms differed considerably. In accordance with our previous report, in which we administered ALVAC-CEA through the i.v. route (6), the antitumor efficacy of i.m. ALVAC-CEA vaccination was abolished by in vivo depletion of the CD4^+ T cell subset, whereas depletion of the CD8^+ T cell subset did not diminish its protective effect (Fig. 1A). In contrast, the antitumor efficacy of DNA-CEA vaccination was severely compromised by depletion of either the CD4^+ or the CD8^+ T cell subset (Fig. 1B).

In view of the differences in antitumor effector mechanisms induced by ALVAC-CEA and DNA-CEA, we dissected the CEA-specific T cell responses elicited by these vaccines. The reactivity of splenocytes from vaccinated mice was assayed against a pool of five synthetic 25-mer peptides that comprise previously defined I-A^b-restricted CD4^+ Th epitopes (6), and against a synthetic 9-mer peptide that represents a previously defined H-2Dd-restricted CD8^+ CTL epitope (12) (data not shown). The CEA-specific responses by the CD4^+ and CD8^+ T cell subsets were discerned by combining intracellular staining for IFN-γ with surface staining for CD4 and CD8. ALVAC-CEA vaccination induced CD4^+ T cell responses measurable in the presence of the Th epitope pool, but this regimen consistently failed to induce any CD8^+ T cell immunity against the CTL epitope (Fig. 2). In contrast, CEA-specific responses induced by the DNA-CEA vaccine involved both CD4^+ and CD8^+ T cells (Fig. 2). The in vitro T cell data closely match those of the tumor challenge experiments. ALVAC-CEA induces CEA-specific CD4^+ T cell immunity only and its protective antitumor effect depends on CD4^+ T cells, not on CD8^+ T cells. DNA-CEA induces CEA-specific CD4^+ and CD8^+ T cells, and the antitumor efficacy of this vaccine depends on both T cell subsets.
Immune effector mechanisms mediating antitumor protection by ALVAC-CEA

The protective effect of ALVAC-CEA vaccination does not rely on MHC class I-restricted CD8\(^+\) T cell immunity, arguing that other CEA-specific effector mechanisms must play a crucial role in controlling tumor growth. Because CEA is expressed at the tumor cell surface, we tested the involvement of the CEA-specific humoral response. We found ALVAC-CEA immunization to induce high levels of anti-CEA serum IgG, primarily of the IgG1 and IgG2a subtypes (Fig. 3). Vaccination with DNA-CEA induced much lower titers of anti-CEA IgG, which were primarily of the IgG2a subtype (Fig. 3). To analyze whether the

**FIGURE 1.** Involvement of CD4\(^+\) and CD8\(^+\) T cell subsets in antitumor effect of ALVAC-CEA and DNA-CEA. C57BL/6 mice (15–16 per group) were i.m. vaccinated twice at a 2-wk interval with 3 \(\times\) 10\(^7\) PFU of ALVAC-CEA (A) or with 100 \(\mu\)g of DNA-CEA (B). Two weeks after the last vaccination, mice were s.c. challenged with a dose of 2.5 \(\times\) 10\(^7\) MC38-CEA cells. Where indicated, mice were depleted for CD8\(^+\) or CD4\(^+\) T cells, depletion starting 5 days before tumor challenge. Tumor size was measured every 3 days and mice were sacrificed when tumor size exceeded 100 mm\(^2\). Each line in the graph represents tumor growth over time in a single mouse. The horizontal lines represent one or more mice in which no tumor growth was detected. The numbers at the right of these lines indicate the fraction of mice in each group that were tumor-free at the end of the experiment. CD4 depletion significantly reduced antitumor efficacy of ALVAC-CEA vaccination (\(p < 0.02\) log-rank test), whereas both CD4 and CD8 depletion significantly affected antitumor efficacy of DNA-CEA vaccination (\(p < 0.003\) and \(p < 0.009\), respectively).

**FIGURE 2.** CEA-specific CD4\(^+\) and CD8\(^+\) T cell responses induced by vaccination with ALVAC-CEA or DNA-CEA. A, C57BL/6 mice were i.m. immunized twice at a 2-wk interval with 3 \(\times\) 10\(^7\) PFU ALVAC-CEA or 100 \(\mu\)g of DNA-CEA. Splenocytes were isolated 2 wk after the last vaccination and assayed for IFN-\(\gamma\) production by intracellular cytokine staining in the presence of synthetic CEA peptide epitopes. Horizontal bar depicts the mean percentage of IFN-\(\gamma\)-producing CD4\(^+\)/CD8\(^+\) T cells of a group of five mice. B, Dot plots of representative examples of each group of the experiment shown in A.
high levels of CEA-specific IgG induced by ALVAC-CEA contributed to the antitumor effector response, we performed tumor challenge experiments in Fc receptor γ-chain knockout mice. Due to the absence of the common γ-chain, these mice lack functional expression of the activating Fc receptors FcγRI, FcγRIII, FcγRIV (and FcγRI) that are involved in, among other activities, Ab-dependent cellular cytotoxicity (ADCC) and Fc receptor-mediated release of inflammatory mediators (14). Fig. 4 shows that the antitumor efficacy of ALVAC-CEA vaccination is greatly reduced in Fc receptor γ-chain knockout mice, whereas the protective effect of the DNA-CEA vaccine is not significantly affected. These results suggest that tumor eradication in ALVAC-CEA vaccinated mice is dependent on ADCC. We therefore examined the role in the antitumor response of NK cells and neutrophils, both of which express FcγRIII and can become activated upon binding of immune complexes comprising IgG1 and IgG2a (14). Indeed, NK depletion of ALVAC-CEA-vaccinated mice through treatment with an NK1.1-specific Ab abolished the protective antitumor effect of this vaccine (Fig. 5A). In contrast, depletion of Gr-1+ leukocytes including neutrophils with a Gr-1-specific Ab did not significantly reduce antitumor efficacy of the ALVAC-CEA vaccine. Because Abs also play a crucial role in activating the complement system, we assessed whether mice lacking C1q, an upstream component of the classical complement activation cascade, would be able to induce an effective antitumor response upon vaccination with ALVAC-CEA. C1q knockout mice vaccinated with ALVAC-CEA and challenged with MC38-CEA showed significantly better survival than nonvaccinated C1q knockout mice (Fig. 5B), indicating that the complement cascade played no more than a minor role in tumor eradication.

Taken together, our data demonstrate that the antitumor effects of ALVAC-CEA and DNA-CEA vaccination rely on clearly distinct immune mechanisms. CD8+ T cells are crucial for tumor eradication in DNA-CEA vaccinated mice. In contrast, the antitumor efficacy of ALVAC-CEA vaccination depends on CD4+ T cells, Fc receptor function and NK cells, suggesting that ADCC is the main effector mechanism involved.

Impact of the ALVAC vector on CEA-specific immunity

The distinct antitumor effector mechanisms induced by ALVAC-CEA and DNA-CEA vaccination prompted us to examine the cytokine profile of the CEA-specific T cell responses. Because ALVAC-CEA comprises multiple canarypox virus proteins, we also analyzed the vaccine-induced response against these vector components. Splenocytes from mice immunized with either ALVAC-CEA or DNA-CEA were cultured in the presence of CEA peptide epitopes or inactivated ALVAC, after which the culture supernatants were analyzed for the presence of secreted cytokines. Cytokine bead array analysis of Th1/Th2 cytokines showed that in particular the Th1 cytokine IFN-γ and the Th2 cytokine IL-5 were secreted. In accordance with the data in Fig. 2, DNA-CEA vaccination induced CEA-specific T cell activity against both the CD4+ Th epitopes and the CD8+ CTL epitope, whereas ALVAC-CEA vaccination induced immunity against the CD4+ Th epitopes only. Furthermore, as anticipated, ALVAC-CEA induced very strong T

**FIGURE 3.** CEA-specific IgG responses induced by vaccination with ALVAC-CEA or DNA-CEA. C57BL/6 mice were immunized i.m. twice at a 2-wk interval with \(3 \times 10^7\) PFU ALVAC-CEA or 100 μg of DNA-CEA, respectively. Blood was drawn from the tail vein 2 wk after the last vaccination, and serum IgG titers were measured by ELISA. Data depict the mean IgG titer of each group of six mice.

**FIGURE 4.** Antitumor efficacy of ALVAC-CEA vaccination is dependent on Fc receptor γ-chains. C57BL/6 wild-type (A) and Fc receptor γ-chain knockout (B) mice were immunized and challenged with MC38-CEA tumor cells as described in Fig. 1. Lines represent tumor growth over time in one mouse. Each horizontal line represents one or more mice in which no tumor growth was detected. The number at the right of this line indicates the fraction of mice in each group that were tumor-free at the end of the experiment. DNA-CEA vaccination resulted in significant protection of Fc receptor γ-chain knockout mice from tumor growth as compared with ALVAC-CEA-vaccinated and nonvaccinated groups (\(p < 0.003\) and 0.0001, respectively). This experiment was performed twice with comparable outcomes.
cell reactivity against ALVAC vector components (Fig. 6). Interestingly, the responses against CEA Th epitopes induced by DNA-CEA were associated with IFN-γ secretion only, whereas the responses against CEA Th epitopes induced by ALVAC-CEA involved secretion of both IFN-γ and IL-5. This mixed Th1/Th2 cytokine profile was also found for the ALVAC-specific responses induced by ALVAC-CEA (Fig. 6). The cytokine profiles of the responses elicited by DNA-CEA and ALVAC-CEA correspond very well with the immune effector responses that mediate the antitumor effect of these vaccines. The efficacy of DNA-CEA vaccination involves the induction and action of CEA-specific CD8\(^+\) T cells (Fig. 1), and accordingly this immune response is associated with a Th1-type cytokine profile that optimally supports the induction and maintenance of CTL immunity. In contrast, the efficacy of ALVAC-CEA vaccination involves the induction and action of CEA-specific CD8\(^+\) T cells and IgG Abs (Figs. 3–5), and accordingly this immune response features a mixed Th1/Th2 profile that is more supportive of humoral responses.

The virus-specific responses induced by ALVAC-CEA are markedly stronger than the CEA-specific responses (Fig. 6). It is therefore conceivable that the character and polarization of the immune response against CEA is influenced by the dominant responses against ALVAC Ags. We initially considered the possibility that immunization with lower amounts of ALVAC might result in a lower viral load and, thereby, might favor the attention of the immune system toward the transgene-encoded Ag CEA. However, immunizations with lower ALVAC-CEA titers did not result in the enhancement of CEA-specific CD4\(^+\) and/or CD8\(^+\) T

**FIGURE 5.** Important role for NK1.1\(^+\) cells, but not for neutrophils and complement, in antitumor efficacy of ALVAC-CEA vaccination. A, C57BL/6 wild-type (A) and C1q knockout (B) mice were immunized with ALVAC-CEA and challenged with MC38-CEA tumor cells as described in Fig. 1. Each line in the graph represents tumor growth over time in one mouse. The horizontal line represents one or more mice in which no tumor growth was detected. The number at the right of this line indicates the fraction of mice in each group that were tumor-free at the end of the experiment. Where indicated, mice were depleted for Gr-1\(^+\) cells or NK1.1\(^+\) cells, depletion starting 5 days before tumor challenge. ALVAC-CEA vaccination resulted in significant reduction of tumor development in C1q knockout mice (p < 0.003), C57BL/6 wild-type mice (p < 0.003), and C57BL/6 wild-type mice that underwent Gr-1 depletion (p < 0.003).

**FIGURE 6.** Specificity and cytokine profile of T cell response induced by ALVAC-CEA or DNA-CEA. C57BL/6 mice were immunized with ALVAC-CEA or DNA-CEA as described in Fig. 1. Splenocytes were isolated 2 wks after the last vaccination and incubated for 3 days with synthetic CEA peptide epitopes or inactivated ALVAC. Culture supernatants were tested for the presence of cytokines by cytokine bead array analysis. Data are the mean cytokine secretion of each group of three mice, shown for IFN-γ and IL-5.
FIGURE 7. The cytokine profile induced by ALVAC-CEA cannot be changed by several modifications of the vaccine. C57BL/6 mice were immunized with ALVAC-CEA, DNA-CEA, or a combination of ALVAC empty vector and DNA-CEA (A), as described in Fig. 1. Mice immunized with both vaccines received the combination in both limbs (DNA+ALVAC) or separate injections of ALVAC and DNA-CEA in the left and right limb, respectively (ALVAC/DNA). Similar immunizations were also performed with ALVAC-CEA-CD80 and ALVAC-CEA-TRICOM (B). Analysis of Ag-specific T cell reactivity was assayed for IFN-γ production by intracellular cytokine staining in the presence of synthetic CEA peptide epitopes as described for Fig. 2A. Data shown are the mean percentage of each group of three mice. Results are from two separate experiments with similar outcome.

cell responses (data not shown). To further investigate the impact of ALVAC-CEA on polarization of T cell immunity, mice were coimmunized with DNA-CEA and ALVAC control vector, and the cytokine profiles of the CEA and ALVAC-specific responses were analyzed. Coinjection of ALVAC largely precluded the induction of CEA-specific CTL responses by DNA-CEA, and also resulted in lower CEA-specific Th1 responses (Fig. 7A). Similar results were obtained when coimmunization experiments were performed with ALVAC-CEA instead of the ALVAC empty vector (data not shown). Notably, this effect of ALVAC coinjection was not only observed when ALVAC and DNA-CEA were administered as a mixture in the same site (Fig. 7A, ALVAC+DNA), but also when these vaccines were injected separately in opposite limbs (Fig. 7A, ALVAC/DNA). Thus, the impact of ALVAC on the character and polarization of the CEA-specific response is not confined to the local draining lymph nodes, but also acts at the systemic level. Furthermore, this effect does not require linkage of the CEA and ALVAC Ags within a single vaccine formulation or vector.

One of the reasons why DNA-CEA vaccine is more efficient in inducing Th1/CTL type immunity than ALVAC-CEA may be that naked plasmid DNA is rich in CpG sequences that can trigger innate immunity through TLR9. Especially in C57BL/6 mice, TLR9 ligands, such as synthetic oligomers comprising CpG oligodeoxynucleotides sequences, were shown to constitute excellent vaccine adjuvants for the induction of Th1/CTL immunity (see references in Ref. 15). However, our finding that a mixture of ALVAC-CEA with DNA-CEA vaccine did not result in enhancement of the CEA-specific Th1/CTL response (Fig. 7A), suggested that in vivo stimulation of TLR9 at the vaccination site could not overrule the dominant features of ALVAC-induced immunity. Accordingly, we found that a mixture of ALVAC-CEA with CpG oligodeoxynucleotides similarly did not result in increased CEA-specific Th1/CTL immunity (data not shown). Reports by others have suggested that incorporation into the vector of genes that encode costimulatory molecules, such as CD80 (B7-1), ICAM-1, and CD2 ligand (human LFA-3 or murine CD48), can improve the antitumor efficacy of ALVAC and other poxvirus-based CEA vaccines (see references in Ref. 16). Furthermore, the CEA-encoding plasmid DNA used in our experiments (DNA-CEA) also encompasses the gene-encoding murine CD80 (see Materials and Methods). We therefore examined the characteristics of CEA-specific T cell responses induced by ALVAC-CEA vaccines that incorporated either the gene for murine CD80 (ALVAC-CEA-CD80) or the genes for all three aforementioned murine costimulatory proteins (ALVAC-CEA-TRICOM) (see Materials and Methods). As shown in Fig. 7B, we found the CEA-specific T cell responses induced by vectors encoding either CD80 or TRICOM to be indistinguishable from those elicited by the parental ALVAC-CEA vaccine.

Our data demonstrate that the ALVAC vector has a profound impact on the character of the immune response against the transgene-encoded CEA, which cannot be overruled by either coinjection of DNA vaccine or CpG oligodeoxynucleotides, nor by complementing ALVAC vaccines with genes encoding powerful costimulatory molecules.

ALVAC immunization elicits CTL immunity against highly immunogenic epitopes

Although none of the ALVAC-CEA immunization protocols tested elicited CEA-specific CD8+ T cell immunity, the ALVAC-induced Th1/Th2 profile does not appear to be prohibitive for CTL activation, in that the T cell responses against the ALVAC vector component, although dominated by CD4+ T cell immunity, comprised an, albeit modest, CD8+ component (Fig. 8A). This finding suggested that the capacity of ALVAC vaccines to induce CTL immunity may be limited to highly immunogenic CTL epitopes, such as those comprised by the viral vector. We therefore studied the impact of the ALVAC context on the immune response against the commonly used model Ag OVA, which contains a potent H-2Kb-restricted CTL epitope (OVA257–264) (8). Interestingly, we found ALVAC-OVA to elicit a very strong T cell response against the OVA CTL epitope (Fig. 8B). The ALVAC-OVA-immunized mice, like their ALVAC-CEA-immunized counterparts, did show a strong T cell response against ALVAC that was associated with the secretion of both IFN-γ and IL-5 (data not shown). In case of the OVA Ag, however, the presence of this ALVAC-specific Th1/Th2 response did not preclude the induction of a very strong CTL response. On the contrary, the immune context provided by ALVAC vaccination appears to be optimal for induction of OVA-specific CTL immunity, in that these responses exceed those induced by an OVA-encoding DNA vaccine or synthetic peptide-based formulations that we tested in parallel (data not shown).

Further evidence of the capacity of ALVAC vaccines to elicit Ag-specific CTL immunity was obtained through immunizations with a construct comprising a string-of-beads arrangement of four well-defined CTL epitopes (ALVAC-SB), derived from the human

Downloaded from http://www.jimmunol.org/ by guest on June 9, 2017
FIGURE 8. Induction of CTL immunity by ALVAC-based vaccines. C57BL/6 mice were immunized with ALVAC-CEA (A), ALVAC-OVA (B), or ALVAC-SB (C) as described in the Fig. 1. Splenocytes were isolated 2 wks after the last vaccination, after which the cells were directly assayed for IFN-γ production by overnight intracellular cytokine staining in the presence of CEA CD4+ and CD8+ peptide epitopes, inactivated ALVAC (A) or CD8+ peptide epitopes derived from OVA, Ad5 E1A, HPV16 E7, wild-type murine p53, or Ad5 E1B (B and C). The latter four epitopes are encoded by ALVAC-SB, as described in Materials and Methods. Data are the mean results of each group of 3 (A) and 5 (B and C) mice, respectively.

Ad5 E1A and E1B, from the HPV16 E7 Ag, and from wild-type murine p53 (3) as described in Materials and Methods. As shown in Fig. 8C, ALVAC-SB elicits CTL responses against the Ad5 E1A and E1B epitopes, but not against the HPV16 E7 and p53 epitopes. This result is in concordance with our previous findings that the E1A and E1B epitopes were highly immunogenic, whereas the E7 epitope was much less immunogenic, and a proper repertoire for the p53 epitope is lacking in normal mice as a result of self-tolerance (17–19).

In conclusion, the capacity of ALVAC-based vaccines to elicit CD8+ T cell immunity against the transgene-encoded Ag of interest strongly depends on the nature of this Ag, in particular on the presence of highly immunogenic CTL epitopes in the Ag.

Discussion

ALVAC and DNA-based vaccines encoding the tumor Ag CEA elicit Ag-specific immune responses with distinct characters. Whereas DNA-CEA induces Th1/CTL-type immunity, ALVAC-CEA induces Th1/Th2-responses that are associated with strong humoral immunity. The propensity of ALVAC-CEA to elicit Th1/Th2-type immunity, instead of a Th1/CTL response, could not be changed by modifying the vaccine through coinjection of DNA-CEA, admixture with CpG oligodeoxynucleotides, or supplementation with additional transgenes encoding costimulatory molecules. Even though our experiments with ALVAC-CEA suggested that ALVAC-based vaccines may not be suitable for inducing CTL immunity against transgene-encoded Ags, immunization with ALVAC-OVA resulted in impressive OVA-specific CTL responses. Furthermore, immunization with ALVAC-SB, comprising a minigene, which encodes a string-of-beads arrangement of CTL epitopes, resulted in induction of CTL immunity against the two most immunogenic peptides within this multi-epitope construct. Therefore, the Th1/Th2 cytokine profile induced by ALVAC immunization is not prohibitive for induction of CTL immunity, provided the CTL epitopes concerned are highly immunogenic.

The finding that ALVAC-based vaccine triggers mixed Th1/Th2 immunity, as opposed to responses with a strong Th1 bias, can most likely be attributed to the fact that both cellular and humoral immunity are important for combating viral infections. Therefore, the predisposition of ALVAC-based vaccines to elicit a mixed Th1/Th2 profile should not be interpreted as an immune escape mechanism, but rather as an intrinsic feature of the natural immune response against this virus. Importantly, these Th1/Th2 responses are largely directed against ALVAC vector Ags (Fig. 6). It is conceivable that the character of the CEA-specific immune response is dictated by that of the dominating response against the vector Ags, particularly when the transgene-encoded Ag does not comprise highly immunogenic CTL epitopes. The CEA CTL epitope CGIQNSVSA (12), although containing the anchor residues compatible with H-2Db binding, displays only intermediate MHC class I binding (data not shown). Furthermore, CEA is a glycoprotein, and this could further limit efficient presentation of its CTL epitope. For another glycosylated Ag, MUC-1, the efficiency of processing into MHC class I and the resulting strength of CTL immunity were shown to be inversely correlated with the degree of glycosylation (20). Notably, the availability of highly immunogenic T cell epitopes within a given Ag depends on the repertoire of MHC molecules expressed by the immunized subject, implying that vaccination with ALVAC-CEA may elicit CEA-specific CTL responses in mice strains expressing a different set of MHC class I molecules.

Our present studies were performed in mice for which CEA is a foreign Ag, suggesting that induction of CEA-specific T cell immunity by the vaccines studied would be more difficult in a host that expresses CEA as an autoantigen. Indeed, we found that ALVAC-CEA vaccination of CEA transgenic mice only elicited weak CD4+ T cell immunity against subdominant CEA CD4+ T cell epitopes distinct from those detected in nontransgenic mice (6). This difference in CEA-specific T cell responses is largely due to clonal deletion of CEA-specific T cells in the thymus of CEA transgenic mice (6). The magnitude and breadth of these T cell responses in CEA transgenic mice was not significantly increased by applying various homologous or heterologous prime-boost protocols that involved CEA-specific vaccines based on protein, DNA, ALVAC, or vaccinia virus. Accordingly, these vaccination regimens were ineffective in causing rejection of MC38-CEA tumors in CEA transgenic mice (our unpublished data). Paradoxically, work by other researchers showed that optimized immune intervention protocols involving vaccination with CEA-encoding poxviruses did protect these CEA transgenic mice against outgrowth of CEA-positive tumors (16, 21, 22). However, these studies did not involve detailed analysis of the CEA-specific T cell responses. It is therefore unclear to which extent CEA-specific immunity contributed to the antitumor effects observed. In fact, recent papers indicated that T cell responses against a foreign, retroviral epitope contributed significantly to this antitumor immune response (21–23). In contrast, work by yet another laboratory demonstrated that CEA-specific vaccination of CEA transgenic mice, using DNA and recombinant adenovirus-based vaccines, did elicit clear-cut CEA-specific CD8+ T cell immunity that was instrumental in preventing outgrowth of CEA overexpressing tumors (12, 24, 25). Notably, the CEA transgenic mice used by Primus and colleagues (11) express lower levels of CEA than mice used by our group (26), suggesting that the CEA-specific T cell repertoire in the former mice was less affected by self-tolerance.

In conclusion, our data demonstrate that the capacity of ALVAC vaccines to elicit CTL immunity against transgene-encoded Ags depends on the presence of immunogenic CTL epitopes in such Ags. In the case of Ags with strong CD8+ T cell epitopes, ALVAC can induce high levels of CTLs, but in the case of Ags with weaker
epitopes, the Th1/Th2 bias of ALVAC may limit the CTL response. The latter limitations cannot be overcome by supplementation of ALVAC with genes encoding costimulatory molecules, or admixture with strong Th1-polarizing adjuvants such as CpG ODN. This information is highly relevant for the various ongoing vaccine development programs against cancer and infectious diseases that make use of ALVAC or closely related viral vectors as delivery tools (1, 2, 4).

Acknowledgments
We thank Kees Franken for providing recombinant CEA and Drs. Peter Kuppen, Ton Schumacher, Pamela Dunn, Thorsten Vogel, and Gavin Zealy for scientific discussions.

Disclosures
Neil Berinstein, Mark Parrington & Bryan McNeil: employees of Sanofi-Pasteur Ltd.; Rienk Offringa & Cornelis J.M. Melief: recipients of research support under collaborative agreement with Sanofi Pasteur Ltd.

References