Difficulties and dangers of CEA-targeted immunotherapy against colorectal cancer

Bos, Rinke

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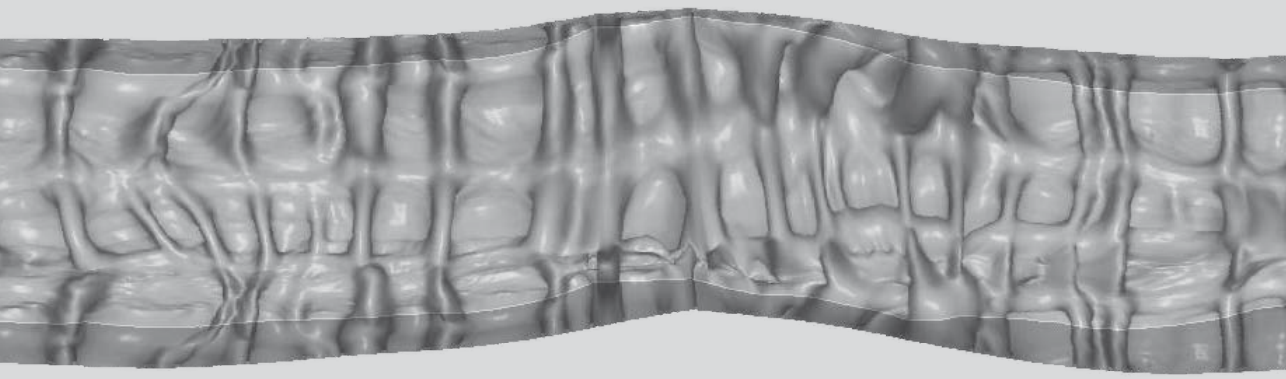

Different polarization of T-cell immunity by canarypox virus and DNA-based vaccines

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Abstract – Functional analysis of canarypox virus and DNA-based vaccines, both designed to elicit effective anti-tumor immunity against the same antigen (carcinoembryonic antigen; CEA) revealed two different effector mechanisms. DNA-CEA vaccination resulted in a CEA-specific Th1/CTL response and the efficacy of this vaccine against MC38-CEA relied on both CD4+ and CD8+ T-cell subsets. In contrast, ALVAC-CEA vaccinated mice feature CEA-specific CD4+ T cells and strong IgG responses. Here, tumor eradication depended on CD4+ cells, Fc receptor function and NK cells, suggesting that ADCC is the main effector mechanism induced by ALVAC-CEA. Notably, ALVAC-CEA vaccination induced high levels of both IFN-γ and IL-5 production, directed against the ALVAC vector. These responses exceeded the responses against CEA-specific CD4+ T-cell epitopes, showing that the response against the ALVAC vector dominated over the CEA-specific response. This dominating ALVAC specific response also negatively influenced the CEA-specific response induced by DNA-CEA vaccination, shown by co-injection of these two vaccines. This indicates that the character of the immune response against CEA is influenced by the dominant responses against ALVAC antigens. Interestingly, when OVA antigen was used as transgene-encoded antigen, ALVAC-OVA elicited stronger T-cell responses against the OVA CTL-epitope than DNA-OVA. Unexpectedly, also in this setting co-injection of ALVAC and DNA-based vaccines resulted in significantly lower CTL responses than those induced by ALVAC-OVA alone. These observations clearly demonstrate the importance of the choice of a vaccine that should not only be based on the type of antigen and the vector itself but should be chosen by studying the most suitable combination of these two components.

Introduction

Stimulation of immune responses against tumor-associated antigens can be achieved by the use of numerous vaccines, which can be divided into two groups. The first group consists of recombinant protein and peptide-based vaccines, which are comprised for 100% of the antigen of interest. The other group of vaccines are based on vectors that encode the target antigen. With the latter vaccines not only the transgene-encoded antigen of interest will be introduced but also the vector encoded antigens. When viral vectors or plasmids are used, immune responses might be induced against respectively viral antigens and plasmid backbone-derived epitopes [1]. In addition, vectors can influence the immune response via vector-associated components. For instance, CpG sequences present in plasmids or viral capsid proteins in viral vectors may manipulate the stimulation of immune responses. Also other components, like the A46R and A52R proteins of vaccinia virus can interfere with the immune system by suppressing host defence [2,3]. Vector associated components are particularly prominent for viral vectors and these might be dominating over the transgene-encoded antigen. This is most likely to occur when the antigen is a tumor-associated auto-antigen to which it might already be very difficult to induce effective immune responses.

We have shown previously that vaccination with ALVAC-CEA of C57BL/6 mice resulted in effective anti-tumor immunity against a CEA-positive tumor [4]. Protection against tumor outgrowth was only dependent on CD4+ cells and not CD8+ cells. This was somewhat unexpected regarding the fact that the tumor cells used (MC38) were MHC class I positive but MHC class II negative. In view of these intriguing data, we sought to understand the effector mechanisms induced by ALVAC-CEA vaccination. In addition, we compared tumor protection and T-cell responses after ALVAC-CEA vaccination with a DNA-based CEA vaccine in C57BL/6 mice.

We found that the nature of the vector profoundly influences the type of immune response induced against the transgene-encoded antigen. Vaccinations with ALVAC-CEA resulted in a mixed Th1/Th2 response while DNA-CEA vaccinations induced Th1 and CTL responses. However, comparison of ALVAC versus DNA-based vaccination for another antigen (OVA) resulted in a completely different outcome, in that ALVAC-OVA induced a strong CTL response and neither of the vaccines induced specific CD4+ T-cell immunity. These results conclusively demonstrate that the outcome of a vaccination is not determined by the antigen or by the vector, but rather by the combination of these two components.

Materials and Methods

Mice. C57BL/6 Kh (B6, H-2^b) were bred in our own facilities (Leiden, The Netherlands). Fc receptor γ-chain knockout mice and C1q knockout mice were kindly provided by J.S. Verbeek (Leiden). The experiments were approved by the animal experimental commission (UDEC) of Leiden University.

Immunizations and tumor challenge experiments. Mice were vaccinated twice i.m. with a 2-week interval with 100 μg of plasmid pGT64 CEA B7-1 [5] or 3×10^7 pfu ALVAC-CEA (provided by Aventis Pasteur, Toronto, Canada) dissolved in 100 μl PBS. 4 days prior to each immunization, 80 μl 10 μM cardiotoxin 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 where 250.000 MC38-CEA tumor cells were injected s.c. in 200 μl PBS/0.5% bovine serum albumin. MC38-CEA cells (obtained from Dr. James Primus, Nashville, TN, USA) [6] were cultured as described previously [4]. CEA expression by MC38-CEA was regularly examined by cell surface staining with a CEA specific antibody (PARLAM4, Monosan, The Netherlands). Depletions of CD4+, CD8+, Gr-1+ or NK1.1+ cells were performed by i.p. injection of 25 μg GK1.5 antibody or 2.43 antibody, 200 μg RB6-8C5 antibody or 100 μg PK136 antibody respectively starting one week before the tumor challenge and was continued during the experiment.

Ova constructs. We prepared a gene construct encoding truncated OVA that is devoid of several signal sequences, but does contain K^b restricted CTL and I-A^b restricted T-helper epitopes. As a result of the removal of the signal sequences, the OVA antigen is cytoplasmic secreted. The segment encoding amino acids 242-378 was cloned by PCR.

In vitro analysis of T-cell responses. Splenocytes from immunized mice were tested for their responsiveness against CEA T-helper epitopes 1-5 [4], CTL epitope [7] and protein by flowcytometry. 2 Million splenocytes were incubated with 5 μg/ml antigen and brefeldin A (10μg/ml) for 12-16 hr. Fixation and staining procedures were done as described previously [8].

Antibody detection. CEA-specific antibodies were measured in sera from immunized mice two weeks after the last vaccination. Nunc Maxisorp immunoplates were coated with 2 μg/ml of CEA protein diluted in coating buffer and incubated o/n at 4°C. Non-specific binding sites were blocked with 100μl of PBS-0.05% Tween 20 (PBS-T)-1% BSA and incubated for 1 h at 37°C. After 4 washes with PBS-T, 100μl 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 above, 100μl of detection antibody 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 4 times, and 100μl of ABTS (25 mg 3-ethyl benzthiazoline-6-sulfonic acid dissolved in 10 ml citrate phosphate buffer pH 4.2) + H_2O_2 (3μl/10 ml) was added to each well. Absorbance values were read at 405 nm and the antibody titers were determined as the last serum dilution yielding absorbance values 5-fold higher than the pre-immune serum.

Cytokine secretion. Splenocytes from immunized mice were incubated with 5 mg/ml CEA T-helper epitopes, CTL epitope or inactivated ALVAC. Supernatant was analyzed 3 days later for Th1 and Th2 cytokines using a cytokine bead array (BD Pharmingen, Alphen aan den Rijn, The Netherlands).

Tetramer staining. Splenocytes from immunized mice were directly ex-vivo stained with anti-K^b-SIINFEKL for 30 min. at RT. After washing, cells were stained for CD8b positive cells and analyzed by FACS.

Results

ALVAC-CEA and DNA-CEA protect through different immune mechanisms

In our search for CEA-specific vaccines that could induce protective immunity against CEA-overexpressing tumors, we compared the anti-tumor efficacy of the canarypox virus-based vaccine ALVAC-CEA and the plasmid DNA-based vaccine DNA-CEA. As shown in Fig. 1, C57BL/6 mice immunized with two subsequent intramuscular (i.m.) doses of either ALVAC-CEA or DNA-CEA rejected an otherwise lethal dose of MC38-CEA tumor cells. Even though 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. The anti-tumor efficacy of ALVAC-CEA vaccination was abolished by in vivo depletion of the CD4+ T-cell subset, while depletion of the CD8+ T-cell subset did not diminish its protective effect (Fig. 1A).

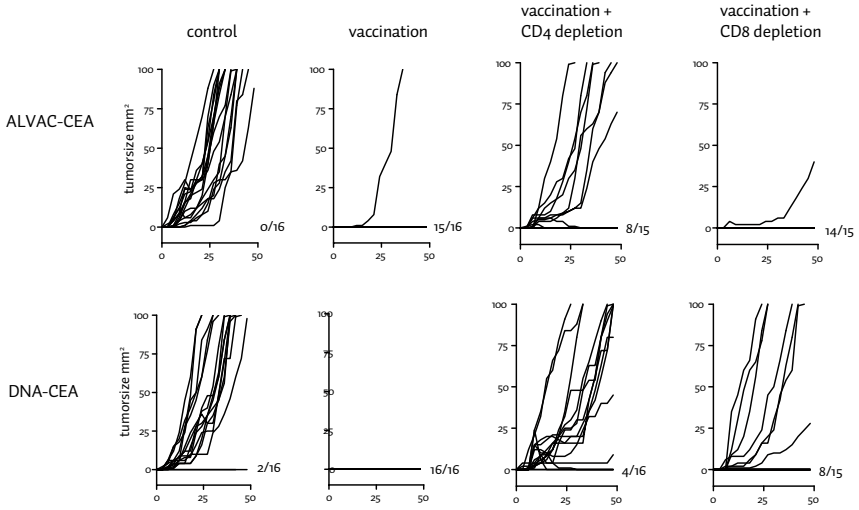

FIGURE 1. Involvement of CD4+ and CD8+ T-cell subsets in anti-tumor effect of ALVAC-CEA and DNA. C57BL/6 mice (15-16 per group) were vaccinated twice i.m. at a 2-week interval with 3×10^7 pfu of ALVAC-CEA (A) or with 100 μg of DNA-CEA (B). Two weeks after the last vaccination, mice were challenged s.c. with a dose of 2.5×10^5 MC38-CEA cells. Where indicated, mice were depleted for CD8+ T cells 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 one 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 anti-tumor efficacy of ALVAC-CEA vaccination ($P < 0.02$ log-rank test), whereas both CD4- and CD8-depletion significantly affected anti-tumor efficacy of DNA-CEA vaccination ($P < 0.003$ and 0.009, respectively).

These results are in accordance with our previous report [4], in which we showed this to be the case for mice that were vaccinated with ALVAC-CEA through the intravenous (i.v.) route. In contrast, the anti-tumor 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 anti-tumor effector mechanisms induced by ALVAC-CEA and DNA-CEA, we performed a detailed analysis of the CEA-specific T-cell responses elicited by these vaccines. The reactivity of splenocytes from vaccinated mice was assayed against CEA protein, against a pool of five synthetic 25-mer peptides that comprise previously defined I-A^b-restricted CD4+ Th epitopes [4], and against a synthetic 9-mer peptide that represents a previously defined H-$2D^b$-restricted CD8+ CTL epitope [7]. The CEA-specific responses by the CD4+ and CD8+ T-cell subsets were dissected 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 either the Th epitope pool or CEA protein, but this regime consistently failed to induce any CD8+ T-cell immunity against the CTL epitope (Fig. 2 A, B). Interestingly, CEA-specific responses induced by the DNA-CEA vaccine involved both CD4+ and CD8+ T cells (Fig. 2A, B).

The in vitro T–cell data show a clear correlation with the results of the tumor challenge experiments. ALVAC-CEA induces CEA-specific CD4+ T-cell immunity only and

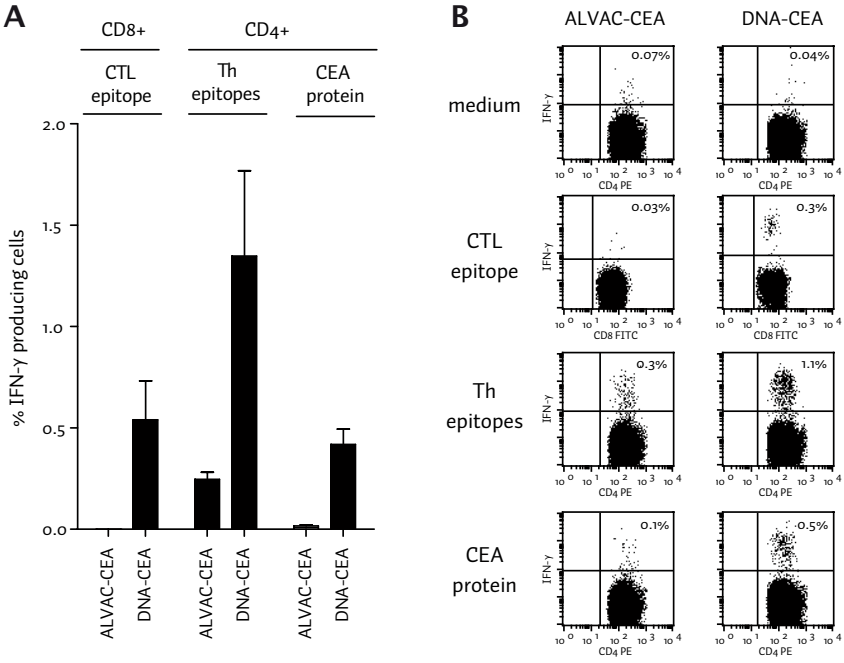

FIGURE 2. CEA-specific CD4+ and CD8+ T-cell responses induced by vaccination with ALVAC-CEA or DNA-CEA. A. C57BL/6 mice were immunized i.m. twice at a 2-wk interval with 3×10^7 pfu ALVAC-CEA or 100 μg DNA-CEA. Splenocytes were isolated 2 weeks after the last vaccination and assayed for IFN-γ production by intracellular cytokine staining in the presence of synthetic CEA peptide epitopes or CEA protein. Each bar depicts the mean percentage of IFN-γ producing CD4+/CD8+ T cells of a group of 5 mice. **B**. Dot plots of representative examples of each group of the experiment shown in A.

its protective anti-tumor effect depends on CD4+ T cells, not on CD8+ T cells. DNA-CEA induces CEA-specific CD4+ and CD8+ T cells, and the anti-tumor efficacy of this vaccine depends on both T-cell subsets.

Immune effector mechanisms mediating tumor 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. MC38-CEA, like most solid tumors, expresses MHC class I, but lacks MHC class II at its cell surface. Therefore, the CEA-specific CD4+ T-cell response induced by ALVAC-CEA cannot launch a direct attack against the tumor. 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 serum levels of anti-CEA IgG, which were primarily of the IgG2a subtype (Fig. 3).

To analyze whether the high levels of CEA-specific IgG induced by ALVAC-CEA contributed to the anti-tumor effector response, we performed tumor challenge experiments in Fc receptor γ-chain knockout mice. Due to the lack of the common γ-chain, these mice lack functional expression of the activating Fc-receptors FcγRI, FcγRIII, FcγRIV (and FcεRI) that are involved in, amongst others, antibody-dependent cellular cytotoxicity (ADCC) and Fc receptor-mediated release of inflammatory mediators [9]. Fig. 4 shows that the anti-tumor efficacy of ALVAC-CEA is greatly reduced in FcRγ-chain k.o. mice, while the protective effect of the DNA-CEA vaccine is not significantly affected by the lack of the FcRγ-chain. These results suggest that tumor eradication in ALVAC-CEA vaccinated mice is dependent on ADCC. We therefore examined the role in the anti-tumor response of NK cells and neutrophils, both of which express FcγRIII and can become activated upon binding of immune complexes comprising IgG1 and IgG2a [9]. Indeed, NK depletion of ALVAC-CEA vaccinated mice through treatment with an NK1.1-

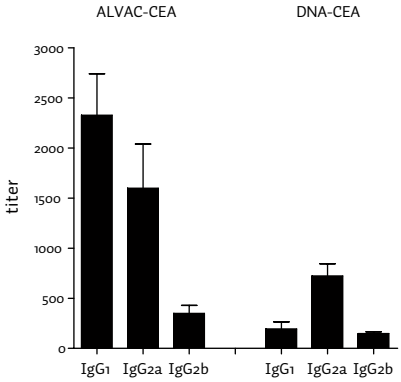

FIGURE 3. CEA-specific IgG responses induced by vaccination with ALVAC-CEA or DNA-CEA. C57BL/6 mice were immunized i.m. twice at a 2-week interval with respectively 3 x 10^7 pfu ALVAC-CEA or 100 μg DNA-CEA. Blood was drawn from the tail vein 2 weeks after the last vaccination and serum IgG titers were measured by ELISA. Each bar depicts the mean IgG titer of a group of 6 mice.

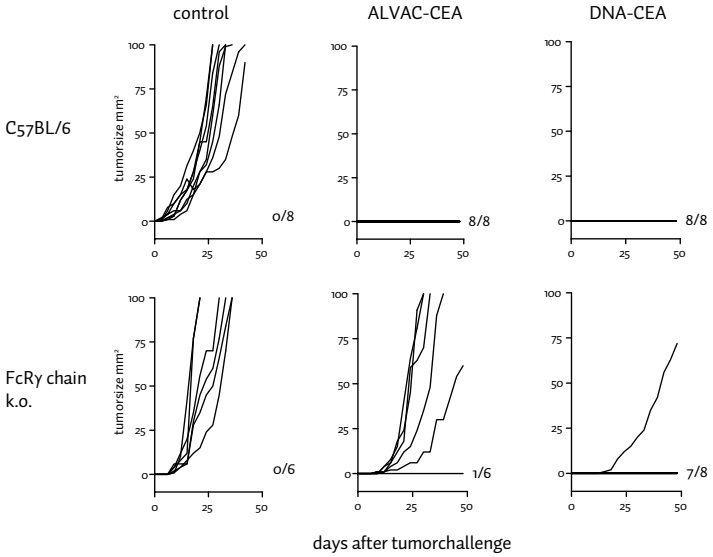

FIGURE 4. Anti-tumor efficacy of ALVAC-CEA vaccination is dependent on FcRγ-chains. C57BL/6 wild-type (**A**) and FcRγ-chain knockout (**B**) mice were immunized and challenged with MC38-CEA tumor cells as described in the legend to Fig. 1. Each line in the graph represents tumor growth over time in one 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. DNA-CEA vaccination resulted in significant protection of FcRγ-chain knock out mice from tumor growth as compared to ALVAC-CEA vaccinated and non-vaccinated groups ($P < 0.003$ and 0.0001, respectively). This experiment was performed twice with comparable outcome.

specific antibody abolished the protective anti-tumor effect of this vaccine (Fig. 5A). In contrast, depletion of neutrophils with a Gr-1-specific antibody did not significantly reduce anti-tumor efficacy of the ALVAC-CEA vaccine. Because antibodies also play a crucial role in activating the complement system, we assessed whether mice lacking an upstream component of the classical complement activation cascade would be able to induce an effective anti-tumor response upon vaccination with ALVAC-CEA. C1q knockout mice vaccinated with ALVAC-CEA and challenged with MC38-CEA showed significantly better survival then non-vaccinated C1q knockout mice (Fig. 5B), indicating that the complement cascade was not a crucial effector mechanism in tumor eradication.

Taken together, our data demonstrate that the anti-tumor 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, ALVAC-CEA vaccinated mice feature CEA-specific CD4+ T cells and strong IgG responses. Here, tumor eradication depends on CD4+ T cells, Fc-receptor function and NK cells, suggesting that ADCC is the main effector mechanism induced by ALVAC-CEA.

Impact of the ALVAC vector on CEA-specific immunity

Our data show that the vector used for immunization can have a profound impact on the character of the CEA-specific immune response. A prominent difference between the two vectors used is the fact that the ALVAC-CEA vaccine, in addition to transgene-

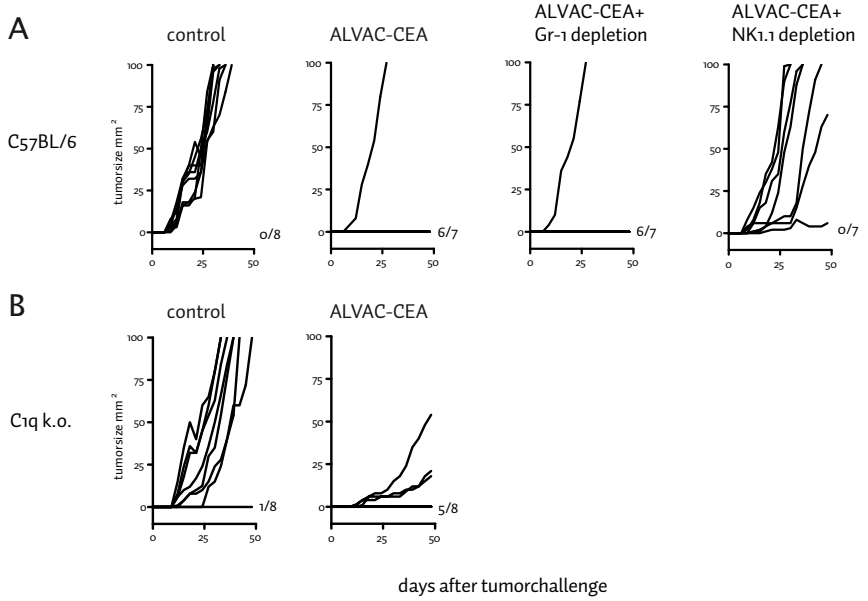

FIGURE 5. Important role for NK1.1+ cells, but not for neutrophils and complement, in anti-tumor efficacy of ALVAC-CEA vaccination. C57BL/6 wild-type (**A**) and C1q knockout (**B**) mice were immunized with ALVAC-CEA and challenged with MC38-CEA tumor cells as described in the legend to Fig. 1. Each line in the graph represents tumor growth over time in one 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. 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 in C57BL/6 wild-type mice that underwent Gr-1 depletion ($P < 0.003$).

encoded antigen CEA, comprises and encodes multiple canarypox virus proteins. Because viral proteins are expected to be highly immunogenic, we also examined the vaccination-induced responses 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-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, compare left and right hand panels). The cytokine profiles of the responses elicited by DNA-CEA and ALVAC-CEA correspond very well with the immune effector responses that mediate the anti-tumor

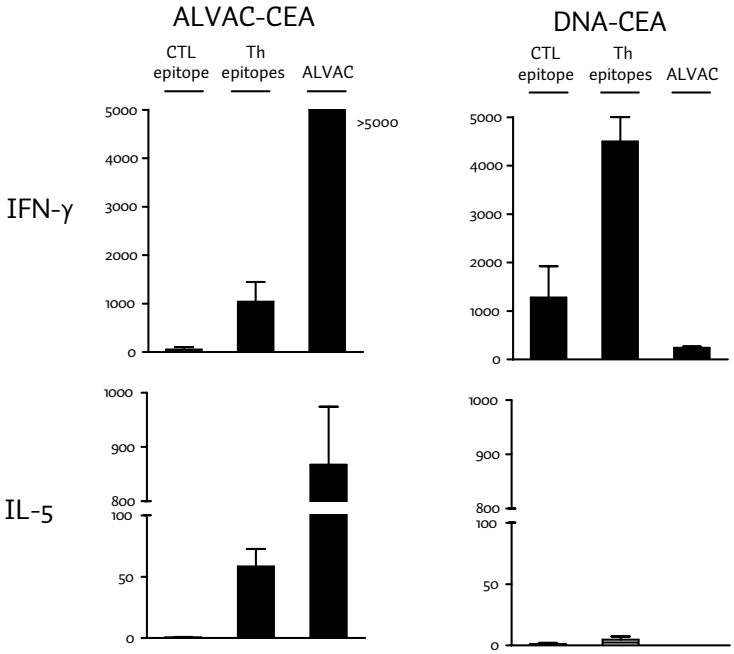

FIGURE 6. Specificity and cytokine profile of T-cell response induced by ALVAC-CEA or DNA-CEA. C57BL/6 mice were immunized with ALVAC-CEA or DNA-CEA as described in the legend to Fig. 1. Splenocytes were isolated two weeks after the last vaccination and incubated for 3 days with synthetic CEA peptide epitopes or inactivated ALVAC. Culture supernatants were tested for the presence of cytokines by cytokine bead array analysis. Each bar depicts the mean results of a group of 3 mice. Data are shown for IFN-γ and IL-5.

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 CD4+ T cells and IgG antibodies (Fig. 3, 4 and 5) and accordingly this immune response features a mixed Th1/Th2 profile that is supportive of humoral responses.

The virus-vector 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 antigens. To investigate this possibility, mice were co-immunized with DNA-CEA and ALVAC control vector, and the cytokine profiles of the CEA and ALVAC-specific responses were analyzed. Co-injection of ALVAC largely precluded the induction of CEA-specific CTL responses by DNA-CEA, and also resulted in lower CEA-specific Th1 responses (Fig. 7). Similar results were obtained when co-immunization experiments were performed with ALVAC-CEA instead of the ALVAC empty vector (data not shown). Notably, this effect of ALVAC co-injection was not only observed when ALVAC and DNA-CEA were administered as a mixture in the same site (Fig. 7;

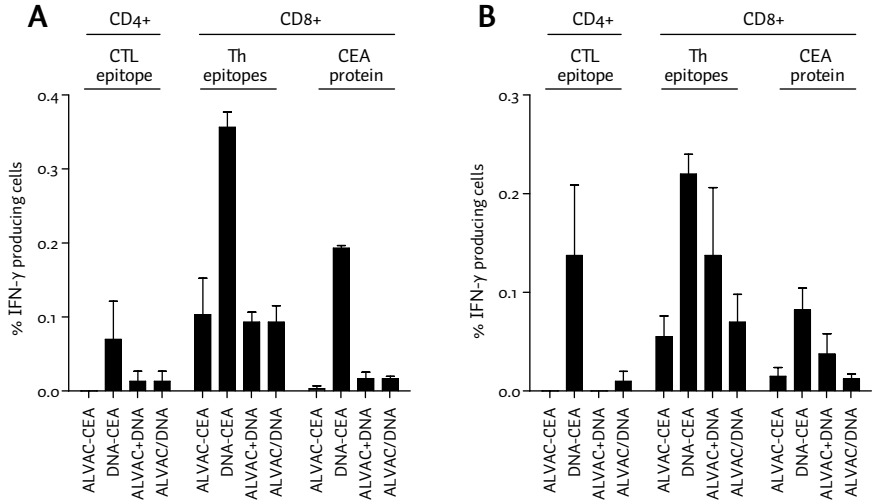

FIGURE 7. ALVAC-induced T-cell responses dominate in ALVAC/DNA co-immunization. C57BL/6 mice were immunized with ALVAC-CEA, DNA-CEA or a combination of DNA-CEA and the ALVAC control vectors as described in the legend to Fig. 1. Mice immunized with both vaccines received the combination in both limbs (DNA+ALVAC) or separate injections of ALVAC and DNA-CEA in the left and right limb respectively (ALVAC/DNA). Analysis of antigen-specific T-cell reactivity was performed as described for Fig. 2A. Each bar depicts the mean results of a group of 3 mice. **A** and **B** depict 2 separate experiments.

ALVAC + DNA), but also when these vaccines were injected separately in opposite limbs (Fig. 7; 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 takes place at the systemic level. Furthermore, this effect does not require linkage of the CEA and ALVAC antigens within a single vaccine formulation or vector.

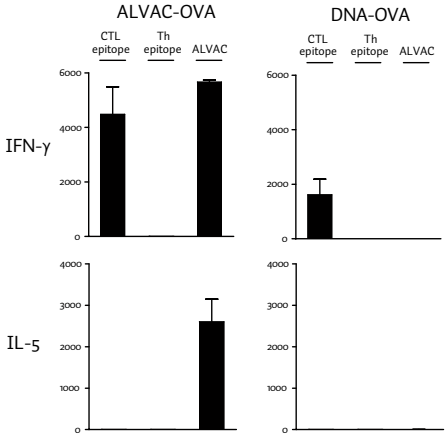

FIGURE 8. Specificity and cytokine profile of T-cell response induced by ALVAC-OVA or DNA-OVA. C57BL/6 mice were immunized with ALVAC-OVA or DNA-OVA as described in the legend to Fig. 1. Cytokine profile of OVA and ALVAC-specific T-cell immunity was assayed as described in the legend to Fig. 6. Each bar depicts the mean results of a group of 3 mice.

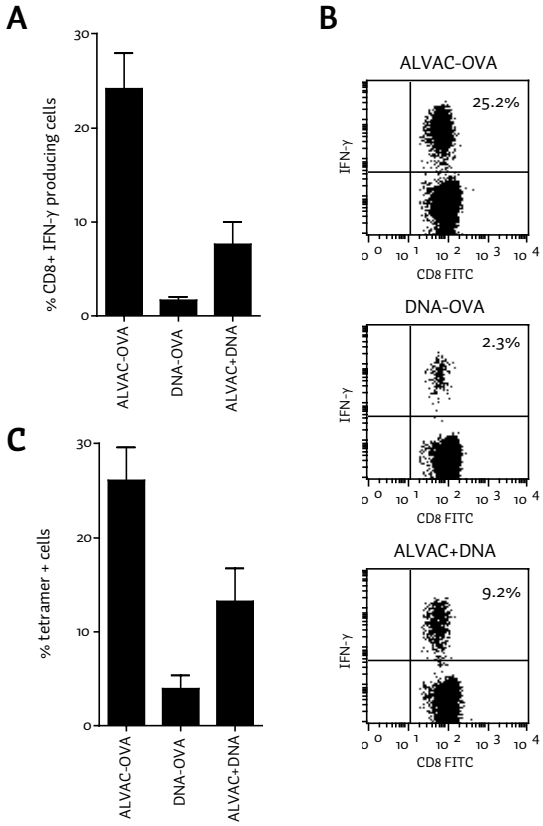

FIGURE 9. Induction of potent OVA-specific CTL immunity by ALVAC-OVA. C57BL/6 mice were immunized with ALVAC-OVA, DNA-OVA or a combination of these vaccines as described in the legend to Fig. 1. Splenocytes were isolated two weeks after the last vaccination, after which the cells were directly assayed for IFN-γ production by o/n intracellular cytokine staining in the presence of synthetic OVA CD4+ or CD8+ peptide epitopes (**A**, **B**), or by staining with H-2K^b/OVA tetramers (**C**). Each bar in panels A and C depicts the mean results of a group of 4 mice. Panel B shows a representative example of the data that make up panel A.

Impact of the ALVAC vector on ovalbumin-specific immunity

The profound differences between the immune responses induced by ALVAC-CEA and DNA-CEA prompted us to study the impact of the vaccine vector on the immune response against another, commonly used model antigen: ovalbumin (OVA). The T-cell response against OVA in C57BL/6 mice is directed against two well-defined epitopes: the H-2K^b-restricted CTL epitope (OVA$_{257-264}$) and the I-A^b-restricted Th epitope (OVA$_{323-339}$). On the basis of our results with CEA-specific vaccines, we had expected DNA-OVA to be superior in the induction of OVA-specific Th1/CTL immunity as compared to ALVAC-OVA. Surprisingly, we found that ALVAC-OVA elicited stronger T-cell responses against the OVA CTL epitope than DNA-OVA (Fig. 8). The magnitude of the OVA-specific CTL response induced by ALVAC-OVA, and the extent in which it differs from the DNA-OVA induced response, becomes even more apparent upon analysis of freshly isolated splenocytes with either intracellular IFN-γ staining or MHC-tetramer staining

(Fig. 9). After two subsequent immunizations with ALVAC-OVA, approximately 25% of the CD8+ T cells in the secondary lymphoid organs are specific for the CTL epitope $OVA_{257-264}$. The CTL numbers induced by two vaccinations with DNA-OVA are approximately 10-fold lower.

Despite the strong OVA-specific CTL response, we could not detect immunity against the OVA Th epitope in either ALVAC-OVA or DNA-OVA immunized mice (Fig. 8). 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 (Fig. 8). In case of the OVA antigen, 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 DNA-OVA (Fig. 8 and 9) or other OVA-specific vaccine formulations like peptide-based vaccines that we have used so far (data not shown). In view of our finding that co-injection of ALVAC inhibited the induction of CEA-specific Th1/CTL immunity by DNA-CEA (Fig. 7), we examined the effect of DNA-OVA co-injection on induction of OVA-specific CTL by ALVAC-OVA. Unexpectedly, also in this setting, co-administration of ALVAC and DNA-based vaccines resulted in CTL levels that were significantly lower than those induced by the best of the two vaccine formulations, which in this case is ALVAC-OVA (Fig. 9).

In conclusion, we have shown that the nature of the vaccine vector can profoundly influence the character of the immune response induced against the transgene-encoded antigen of interest. The performance of a given vaccine is not determined by either the vector or transgene-encoded antigen alone, but is clearly dictated by the vector-antigen combination. Furthermore, our data argue against co-administration of two different vector-based vaccines as a way to improve the performance of sub-optimal vaccines.

Discussion

This study provides a comprehensive comparison of two vaccines targeting a tumor-specific antigen, CEA, which is considered for targeting of human cancers. Our data indicate that the nature of the vector strongly affects the character of the immune response induced against the transgene-encoded antigen. ALVAC-CEA vaccination resulted in a vigorous anti-ALVAC Th1/Th2 response and a lower CEA-specific CD4+, but not CD8+ T-cell response, which also displayed a mixed Th1/Th2 profile. In addition, immunization with ALVAC-CEA elicited strong CEA-specific IgG responses and tumor eradication depended on Fc-receptor function and NK cells, suggesting that ADCC is the main effector mechanism induced by ALVAC-CEA.

Notably, the anti-ALVAC Th1/Th2 response did not only have impact on the endogenous CEA-specific response, but also even overruled the CEA-specific Th1/CTL response induced by a DNA-CEA vaccine. It is therefore likely that the character of the CEA-specific immune response is dictated by that of the dominating anti-ALVAC response. In fact, it is not very surprising, but rather physiological that the ALVAC-based vaccine

triggers mixed Th1/Th2 immunity, because both cellular and humoral immunity are important for combating viral infections. Importantly, the ALVAC-induced Th1/Th2 profile is not prohibitive for the induction of CTL immunity, because ALVAC-based vaccines do induce CTL responses against the viral proteins (data not shown). This is not the case for CEA, because this is an antigen containing strong CD4+ T-cell epitopes but probably a low affinity CD8+ T-cell epitope (data not shown). However, immunizations with ALVAC encoding OVA, which has a CTL epitope ($OVA_{257-264}$) with a very high affinity for MHC class I [10], resulted in a Th1/Th2 anti-ALVAC response, but also in a vigorous OVA specific IFN-γ producing CD8+ T-cell response. Unfortunately, such strong CTL epitopes are not always available for immunotherapy of cancer. It must be noted that in our studies only non-self antigens were tested, because both CEA and OVA-specific vaccinations were tested in C57BL/6 mice. Notably, the anti-ALVAC response might even be more dominating when self-antigens are targeted.

We have shown that co-injection of ALVAC-CEA and DNA-CEA has a negative effect on the CEA-specific immune response induced by DNA-CEA vaccination and we demonstrated that this was due to the strong Th1/Th2 anti-ALVAC response compared to the lower CEA-specific response. Unexpectedly, when ALVAC-OVA was combined with DNA-OVA, the combination of these two vaccines also resulted in a reduction of the T-cell response. To delineate this mechanism further investigation by additional co-immunization experiments is required.

Together, these data suggest that a mixture of two vaccines always leads to antigenic competition and/or additional mechanisms that negatively influence the immune response and plead in favour for heterologous prime-boost vaccinations instead of vaccine combinations. It is evident from our studies that both the vector and the antigen of interest can influence the character of the immune response. Most importantly, the combination of the vector and the antigen will crucially determine the nature of the immune response. This must be taken into account in the design of new vaccines for immunotherapy.

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