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VACCINES AND ANTIVIRAL AGENTS

Comparative Evaluation of the Vaccine Efficacies of Three Adenovirus-Based Vector Types in the Friend Retrovirus Infection Model

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ABSTRACT Adenovirus (AdV)-based vectors are popular experimental vaccine vectors, but despite their ability to induce strong immune responses, their application is impeded by widespread preexisting immunity against many AdV types that can impair or even abrogate the induction of transgene-specific immune responses. Therefore, the development of vectors based on AdV types with a low seroprevalence is important for effective AdV-based immunization in humans. We investigated the immunization efficacy of vectors based on AdV type 48 (Ad48) and Ad50 in the ovalbumin (ova) model as well as the Friend retrovirus (FV) model, which allows testing of the protective effect of vaccine-induced immunity. Using ova-encoding vectors, we found a significantly lower induction of ova-specific $CD8^+$ T cells and antibody responses by Ad48- and Ad50-based vectors than by Ad5-based vectors. Similarly, we found a reduced induction of FV-specific $CD8^+$ T cell responses in Ad48- and Ad50.Leader-Gag-immunized mice compared with that in Ad5-immunized mice; however, some of those mice were able to control the FV infection, and protection correlated with the level of neutralizing antibodies 10 days after FV challenge. Analyses of the AdV-specific antibodies and $CD8^+$ T cells induced by the individual AdV types revealed a high level of cross-reactivity, and the efficacy of Ad48-based immunization was impaired in Ad5-preimmune mice. Our results show that the immunity induced by Ad48- and Ad50-based vectors is reduced compared to that induced by Ad5 and is sufficient to control FV infection in only some of the immunized mice. A high level of cross-reactivity suggests that AdV preimmunity must be considered even when applying rare AdV-based vectors.

IMPORTANCE AdV-based vectors are important tools for the development of vaccines against a wide range of pathogens. While AdV vectors are generally considered safe and highly effective, their application can be severely impaired by preexisting immunity due to the widespread seroprevalence of some AdV types. The characterization of different AdV types with regard to immunogenicity and efficacy in challenge models is of great importance for the development of improved AdV-based vectors that allow for efficient immunization despite anti-AdV immunity. We show that the immunity induced by an Ad48-based vector is inferior to that induced by an Ad5-based vector but can still mediate the control of an FV infection in highly FV-susceptible mice. However, the efficacy of Ad48-based immunization was impaired in Ad5-preimmune mice. Importantly, we found cross-reactivity of both the humoral and cellular immune responses raised by the individual AdV types, suggesting that switching to a different AdV type may not be sufficient to circumvent preexisting anti-AdV immunity.

KEYWORDS $CD8^+$ T cells, adenovirus, immunization, preexisting immunity, retrovirus, vaccination, vector immunity, viral vector

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Adenovirus (AdV)-based vectors are popular tools in experimental vaccine development because of their immunogenicity, their ability to transduce a wide range of different dividing and nondividing cell types, the ease of their production, and a very good safety record in clinical vaccine trials to date. It had been cautioned from the beginning, however, that preexisting immunity against AdV would likely limit their use because of neutralization of the vector (1, 2), and disappointing results in clinical immunization studies have put AdV-based vectors under harsh scrutiny (3–5). Initially, AdV vectors were derived from human AdV type 5 (Ad5); unfortunately, the seroprevalence of this AdV type is particularly high. Therefore, the focus has shifted over the last decade to the use of different AdV types, such as human AdV types that are considered rare because of a lower overall seroprevalence (6–9), or simian AdV types, such as chimpanzee AdV (6, 10, 11).

Of the more than 70 known AdV types, only a small number have been tested in preclinical studies (6, 7, 9, 12–15), and apart from Ad5, only vectors based on human AdV types 4, 6, 26, and 35 and chimpanzee AdV types 3 and 63 have been advanced into clinical studies as vaccine candidates against Ebola virus (16, 17), *Plasmodium falciparum* (18, 19), *Mycobacterium tuberculosis* (20), influenza virus (21), hepatitis C virus (22), or human immunodeficiency virus (HIV) (23–26). For most of the rare AdV-based vectors, it has been shown that their immunogenicity is inferior to that of Ad5, resulting in reduced immune responses upon immunization with these vectors, although their efficacy in Ad5-preimmune mice is superior to the efficacy of an Ad5-based vaccine (7, 9, 12, 14, 15). Interestingly, this reduced immunogenicity has been shown to affect the induction of transgene-specific $CD8^+$ T cell responses (9, 10, 13, 14), but also, humoral immune responses have been shown to be reduced (7, 12, 15).

In the work presented here, we tested vectors based on rare AdV types in the Friend retrovirus (FV) infection model. FV is a murine retrovirus complex consisting of the two viruses Friend murine leukemia virus (F-MuLV) and spleen focus-forming virus (SFFV), infection with which leads to severe splenomegaly and erythroleukemia in susceptible mouse strains, while mice that are genetically resistant to FV-induced disease develop a chronic infection (27). We have worked with the FV model before and showed that it is very useful for the evaluation and improvement of AdV-based immunization strategies (28–34). While it was shown before that sterile protection from FV infection requires a complex immune response consisting of $CD8^+$ T cells, $CD4^+$ T cells, and neutralizing antibodies (35, 36), we demonstrated in an Ad5-based immunization that a high degree of protection can also be conferred either by the induction of $CD4^+$ T cells and humoral immune responses (28, 30, 33, 34) or by the sole induction of F-MuLV-specific $CD8^+$ T cells (31). Furthermore, we demonstrated that anti-AdV immune responses can influence the immunization outcome both in naive mice and in AdV-preimmune mice (32, 37).

We selected the two rare AdV types Ad48 of species D and Ad50 of species B and compared them with Ad5 (species C) for their potency as antiretrovirus vaccine vectors. The two AdV types Ad48 and Ad50 were selected for several reasons: they belong to AdV species different from Ad5 and they exhibit appreciable evolutionary distance with regard to both genome and hexon protein sequences (38, 39), indicating a potentially low cross-reactivity of Ad5-induced immune responses. Furthermore, they have been shown before to have a low seroprevalence (7), which should be a prerequisite for the selection of AdV types for the development of new AdV-based vectors. While Ad50 has been tested only for immunization of mice against the simian immunodeficiency virus (SIV) Gag protein (7) and was rather weakly immunogenic, Ad48 has been evaluated for its use for immunization against SIV (7), lymphocytic choriomeningitis virus (LCMV) (40), influenza virus (41), and *Trypanosoma cruzi* (42) and showed more promising results.

In the present study, we aimed to investigate in the FV infection model the protective effect of AdV-based immunization using Ad5 as well as the two rare AdV types Ad48 and Ad50 as vectors, testing them for their ability to induce transgene-specific $CD8^+$ and $CD4^+$ T cells as well as antibody responses and their efficacy in

conferring protection from FV challenge infection in the absence or presence of preexisting Ad5 immunity.

RESULTS

Evaluation of ova-specific CD8$^+$ T cell responses to AdV-based immunization. Vectors based on Ad48 and Ad50 have been shown to be less immunogenic than those based on Ad5 (7); as we have recognized that the F-MuLV Leader-Gag (GagL)-derived CD8$^+$ T cell epitope consisting of residues 85 to 93 of GagL ($GagL_{85–93}$) is rather weak and therefore subdominant in the presence of AdV-derived epitopes (31, 37), we first evaluated the Ad48- and Ad50-based vectors using chicken egg ovalbumin (ova) as the transgene. ova is a widely used and highly immunogenic model antigen, and immunization with an Ad5-based vector encoding ova (Ad5.ova) has been shown before to elicit potent CD8$^+$ T cell responses (32, 43).

CB6F1 mice were immunized twice in a 3-week interval with the Ad5-based vector Ad5.ova or the rare AdV-based vectors Ad48.ova and Ad50.ova, and at 2 weeks after each immunization, the CD8$^+$ T cell response to the peptide consisting of ova residues 257 to 264 ($ova_{257–264}$) was analyzed by major histocompatibility complex class I (MHC-I) dextramer staining and by intracellular cytokine staining. After the first immunization, significant amounts of $ova_{257–264}$-specific CD8$^+$ T cells were readily detectable in Ad5.ova-immunized mice, while $ova_{257–264}$-specific CD8$^+$ T cells were very low or undetectable after immunization with Ad48.ova or Ad50.ova (Fig. 1A). Similarly, *in vitro* restimulation with the $ova_{257–264}$ peptide resulted in a significant fraction of gamma interferon (IFN-γ)-producing CD8$^+$ T cells in Ad5.ova-immunized mice; interestingly, we also detected high levels of IFN-γ-producing CD8$^+$ T cells in some of the Ad48.ova-immunized mice, suggesting that the dextramer staining lacked some sensitivity, whereas no IFN-γ-producing CD8$^+$ T cells were detected in Ad50.ova-immunized mice (Fig. 1B). After the second immunization, the frequency of $ova_{257–264}$-specific CD8$^+$ T cells detected by dextramer staining was slightly increased in Ad5.ova-immunized mice compared with the levels after the first immunization, and $ova_{257–264}$-specific CD8$^+$ T cells were also detectable in all Ad48.ova- and most Ad50.ova-immunized mice. While the frequency was significantly higher in these mice after the boost immunization than in these mice after the prime immunization, it was still markedly lower than that in Ad5.ova-immunized mice (Fig. 1D); similar results were obtained when cells were restimulated with the $ova_{257–264}$ peptide and analyzed for IFN-γ expression (Fig. 1E).

When we analyzed antibody levels, we found surprisingly low ova-specific antibody titers, with no detectable antibodies being found in any of the immunized mice after the first immunization (Fig. 1C) and only low levels of antibodies being found in half of the Ad5.ova- and Ad48.ova-immunized mice after the second immunization (Fig. 1F); no ova-binding antibodies were detected in Ad50.ova-immunized mice at any time point.

Induction of FV-specific CD8$^+$ T cell responses by different AdV vectors. The immunization experiments with ova-expressing Ad48 and Ad50 vectors confirmed a markedly reduced immunogenicity compared to that of Ad5. As we had seen an increase in the ova-specific CD8$^+$ T cell response after the boost immunization, we went ahead and constructed Ad48- and Ad50-based vectors encoding the F-MuLV Leader-Gag_{C1K} immunogen. The Leader-Gag_{C1K} immunogen is a modified protein where the amino acid flanking the $GagL_{85–93}$ epitope has been mutated to lysin to improve proteasomal degradation and thereby improve immunogenicity; contrary to the native Leader-Gag sequence, the modified Leader-Gag_{C1K} sequence allows for the induction of $GagL_{85–93}$-specific CD8$^+$ T cell responses from an AdV background (31).

To characterize the new Leader-Gag_{C1K}-encoding vectors, we transduced bone marrow-derived dendritic cells (DCs) *in vitro* and used them to stimulate transgenic $GagL_{85–93}$-specific CD8$^+$ T cells in a proliferation assay and for an analysis of the influence of transduction on the activation status of the DCs. As shown before (31), stimulation with Ad5.Leader-Gag_{C1K}-transduced DCs led to considerable proliferation of the CD8$^+$ T cells that was only slightly lower than that after stimulation with

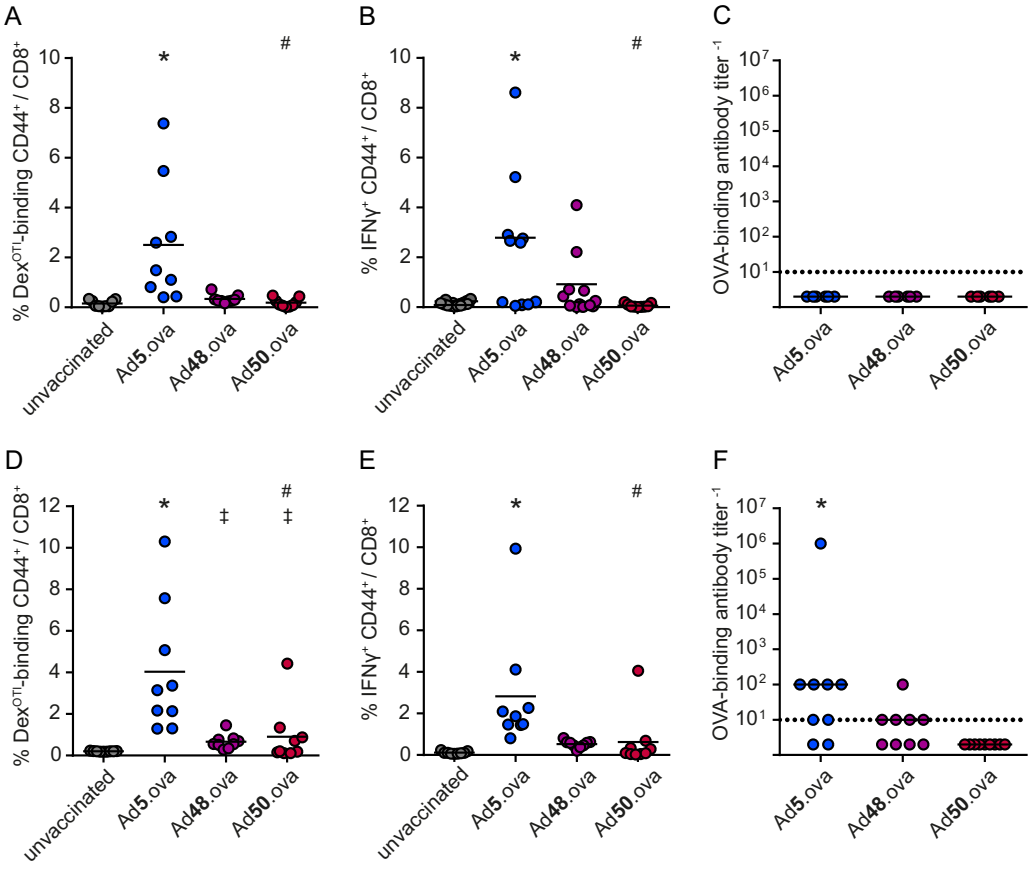

FIG 1 Induction of ova-specific CD8$^+$ T cell responses and binding antibodies after AdV-based immunization. CB6F1 mice were immunized twice by intramuscular injection of 10^9 vp of Ad5.ova, Ad48.ova, or Ad50.ova. Immunizations were conducted at a 3-week interval. (A, B, D, and E) CD8$^+$ T cell responses were analyzed by MHC-I dextramer (Dex) staining (A, D) and intracellular cytokine staining (B, E) 2 weeks after the first (A, B) and second (D, E) immunizations. (C, F) At the same time points, binding antibodies against ova were determined by ELISA. Data were acquired in three independent experiments with three mice per group per experiment. Each dot indicates an individual mouse. Solid lines indicate mean (A, B, D, E) or median (C, F) values. Dotted lines indicate the detection limit. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance. * and #, statistically significant differences ($P < 0.05$) from the results for the unvaccinated mice or the Ad5 reference group, respectively; ‡, statistically significant differences between the immune responses after the prime immunization and after the boost immunization.

peptide-loaded DCs (Fig. 2A and B). Proliferation of CD8$^+$ T cells was significantly lower when the cells were stimulated with Ad48.Leader-Gag$_{C1K}$- or Ad50.Leader-Gag$_{C1K}$-transduced DCs than when they were stimulated with Ad5.Leader-Gag$_{C1K}$, mirroring the *in vivo* results of AdV.ova immunization. When we analyzed the activation of DCs after AdV transduction using expression of CD86 and MHC-II as activation markers, we found that transduction with any of the three tested AdV types led to only a very low level of activation compared to that achieved with noninfected DCs, even though Ad50-transduced DCs showed significantly lower levels of expression of both CD86 and MHC-II than Ad5-transduced DCs (Fig. 2C and D).

To analyze the protective capacity of the different vector types, we immunized CB6F1 mice twice with vectors encoding Leader-Gag$_{C1K}$ (Ad5.Leader-Gag$_{C1K}$, Ad48.Leader-Gag$_{C1K}$, Ad50.Leader-Gag$_{C1K}$) and analyzed the GagL$_{85–93}$-specific CD8$^+$ T cell responses 2 weeks after the boost immunization by MHC-I tetramer staining (Fig. 3A). We saw, as before (31), that Ad5.Leader-Gag$_{C1K}$-immunized mice mounted a rather weak GagL$_{85–93}$-specific CD8$^+$ T cell response, which was detectable in only some of the immunized mice. Interestingly, the GagL$_{85–93}$-specific CD8$^+$ T cell response induced by Ad48.Leader-Gag$_{C1K}$ was comparable to that induced by Ad5.Leader-Gag$_{C1K}$; on the

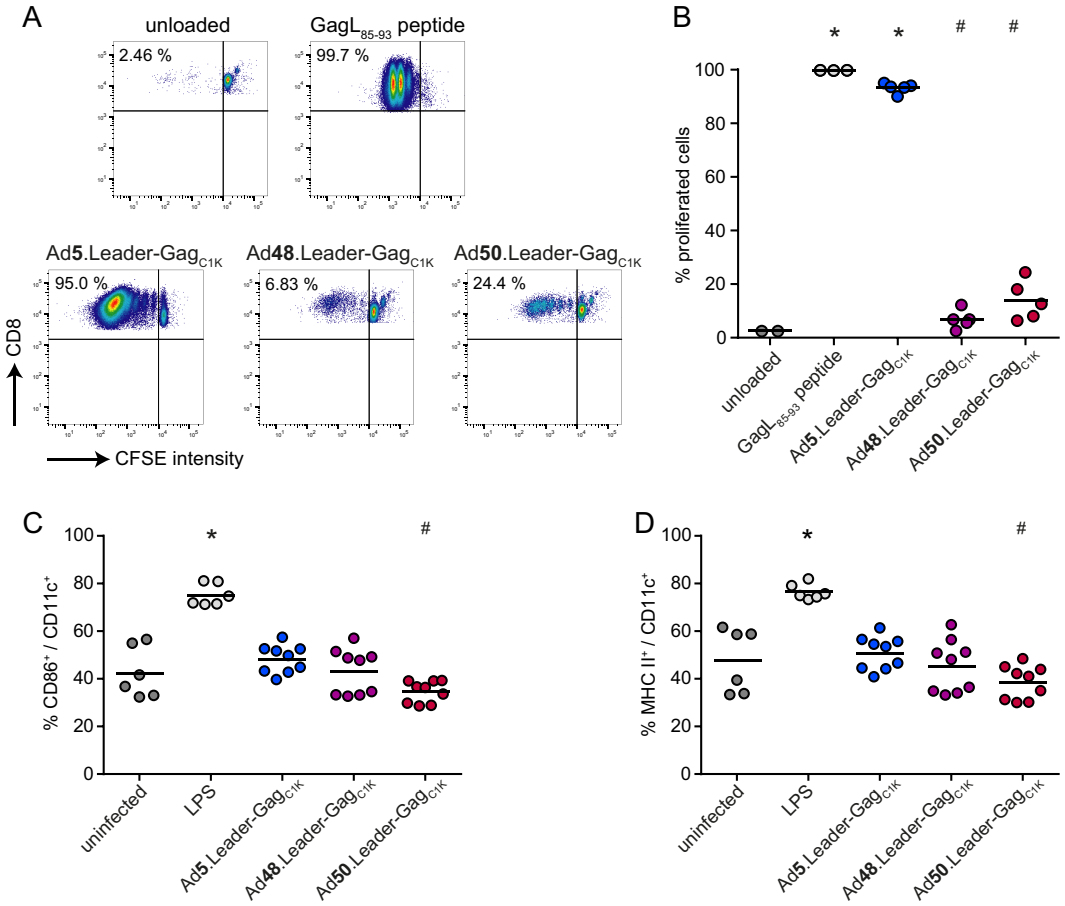

FIG 2 *In vitro* characterization of Ad5-, Ad48-, and Ad50-based vectors encoding Leader-Gag_{C1K}. Bone marrow-derived DCs were transduced with Ad5-, Ad48-, or Ad50-based vectors encoding Leader-Gag_{C1K} at an MOI of 10^3 and were coincubated with CFSE-labeled, naive $GagL_{85–93}$-specific $CD8^+$ T cells for 4 days. Unloaded DCs served as a negative control, and DCs loaded with the $GagL_{85–93}$ peptide served as a positive control. (A) Representative dot plots. (B) Quantitative results of the frequency of proliferated $CFSE^{low}$ $CD8^+$ T cells. (C and D) Transduced DCs were analyzed for the expression of the activation markers CD86 (C) and MHC-II (D) at 24 h after transduction by flow cytometry. The data shown in panels A and B were acquired in one experiment and are representative of those from three independent experiments. The data shown in panels C and D were acquired in two independent experiments. Lines indicate mean values. Data were analyzed by ordinary one-way analysis of variance and Tukey's multiple-comparison procedure for statistical significance. * and #, statistically significant differences ($P < 0.05$) from the results for unloaded DCs or Ad5-transduced DCs, respectively. LPS, lipopolysaccharide.

other hand, immunization with Ad50.Leader-Gag_{C1K} resulted in no detectable $GagL_{85–93}$-specific $CD8^+$ T cell responses.

Protection from FV infection after AdV-based immunization. To assess the protection induced by the AdV-based vaccines, mice were challenged with 500 spleen focus-forming units (SFFU) of FV 3 weeks after the second immunization. When the plasma viral load was analyzed 10 days after FV infection, unvaccinated mice had high viremia levels, while all mice immunized with Ad5.Leader-Gag_{C1K} had undetectable viral loads in plasma (Fig. 3B). Mice that were immunized with the Ad48- or Ad50-based vectors displayed a bimodal distribution of protected animals with no detectable virus in the plasma as well as mice with viral loads comparable to those in the unvaccinated control group.

At 3 weeks after FV challenge infection, mice were sacrificed and the spleen weights and spleen viral loads were determined. While all unvaccinated mice had severely enlarged spleens, the weights of the spleens of mice immunized with Ad5.Leader-Gag_{C1K} were clearly reduced, with most of the spleens showing no increase in weight at all (Fig. 3C). Four of 6 mice in the Ad48.Leader-Gag_{C1K} group were able to control the splenomegaly, whereas most Ad50-immunized mice had severely enlarged spleens. In

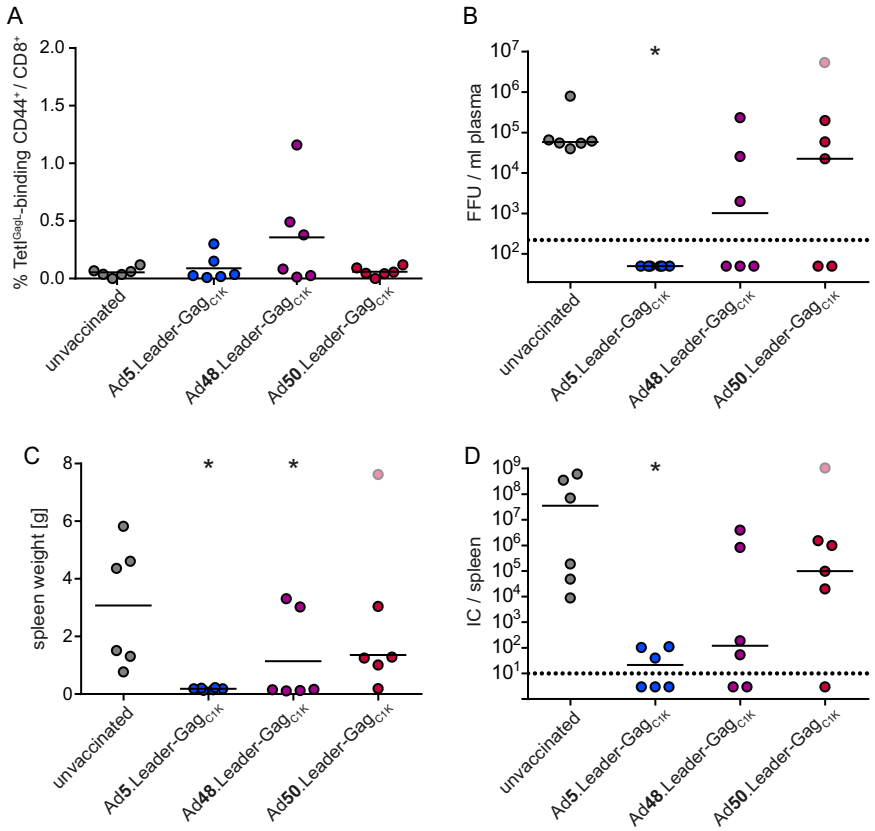

FIG 3 Induction of GagL$_{85–93}$-specific CD8$^+$ T cell responses after immunization with Ad5-, Ad48-, or Ad50-based vectors encoding Leader-Gag$_{C1K}$. CB6F1 mice were immunized twice in a 3-week interval by intramuscular injection of 10^9 vp of Ad5-, Ad48-, or Ad50-based vectors encoding F-MuLV Leader-Gag$_{C1K}$. (A) CD8$^+$ T cell responses were analyzed 2 weeks after the second immunization by MHC-I tetramer staining. At 3 weeks after the second immunization, mice were challenged with 500 SFFU of the FV complex. (B) Viral loads in plasma were analyzed 10 days after FV challenge. (C and D) At 21 days after challenge, spleen weights (C) and spleen viral loads (D) were analyzed. Data were acquired in two independent experiments with three mice per group per experiment. Each dot indicates an individual mouse. Lighter symbols indicate mice that were sacrificed according to predetermined termination criteria before the end of the experiment because of FV-induced disease; these symbols do not represent actual values. Solid lines indicate mean (A, C) or median (B, D) values. Dotted lines indicate the detection limit. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance. *, statistically significant differences ($P < 0.05$) from the results for the unvaccinated mice.

accordance with these results, the viral load in the spleens of all mice immunized with Ad5.Leader-Gag$_{C1K}$ and in the spleens of 4 of 6 mice immunized with Ad48.Leader-Gag$_{C1K}$ was lower than that in the spleens of the unvaccinated control mice and was undetectable in half of these mice, although the two splenomegalic mice from the Ad48.Leader-Gag$_{C1K}$ group also exhibited high viral loads in their spleens (Fig. 3D). Immunization with the Ad50-based vector resulted in protection in only one mouse that exhibited an undetectable spleen viral load, whereas the other mice had spleen viral loads that were comparable to those measured in the unvaccinated control group.

Induction of FV-specific antibody and CD4$^+$ T cell responses. It is interesting that mice immunized with Ad5.Leader-Gag$_{C1K}$ and Ad48.Leader-Gag$_{C1K}$, but not those immunized with Ad50.Leader-Gag$_{C1K}$, were rather well protected from FV challenge, even though the level of GagL$_{85–93}$-specific CD8$^+$ T cells was very low in all immunized mice. As FV-specific antibodies are also known to contribute to protection from FV challenge infection, we analyzed the antibody response after the second immunization with the AdV-based vaccines; however, the levels of FV-binding antibodies were fairly low in all mice (Fig. 4A).

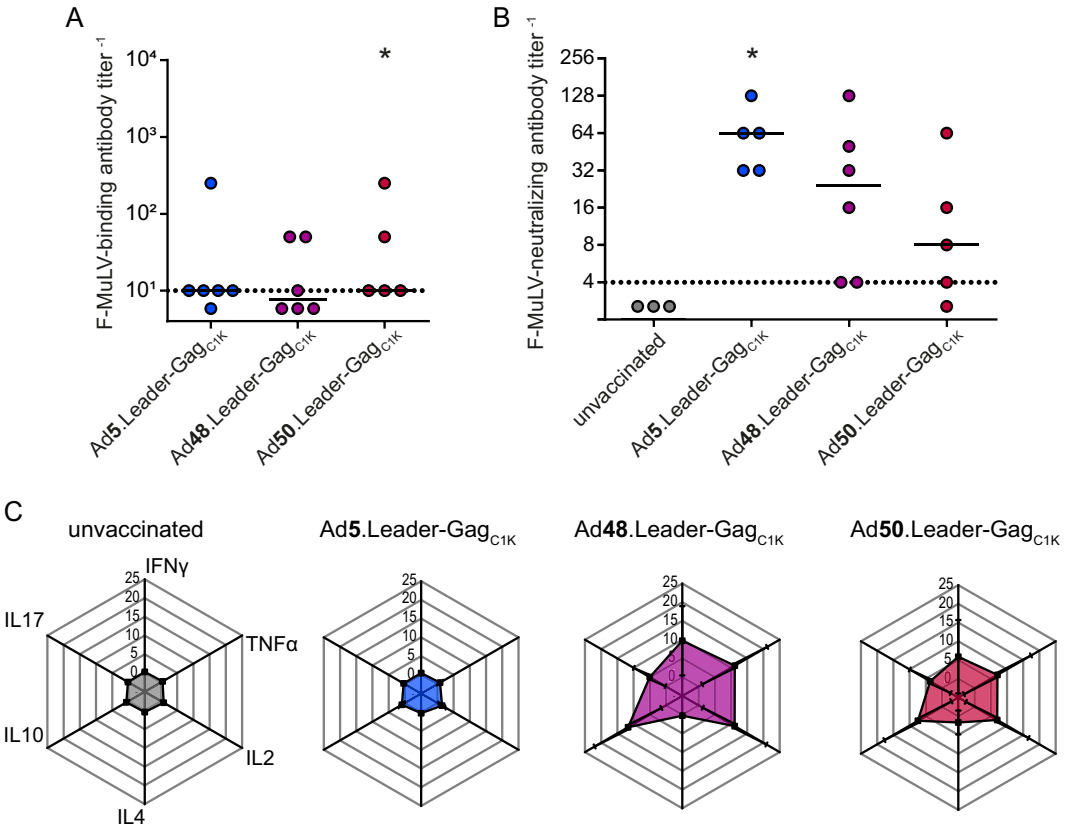

FIG 4 Induction of F-MuLV-specific antibodies and CD4$^+$ T cells by immunization with Ad5-, Ad48-, or Ad50-based vectors. CB6F1 mice were immunized twice in a 3-week interval by intramuscular injection of 10^9 vp of Ad5-, Ad48-, or Ad50-based vectors encoding F-MuLV Leader-Gag$_{C1K}$. (A) At 2 weeks after the second immunization, F-MuLV-binding antibody levels were determined by ELISA. At 3 weeks after the second immunization, the mice were challenged with 500 SFFU of the FV complex. (B) F-MuLV-neutralizing antibodies were analyzed in plasma samples collected 10 days after FV challenge. At 3 weeks after FV infection, spleen cells were stained for the production of the indicated cytokines after *in vitro* restimulation with Gag-derived peptides. Graphs represent the frequency of cytokine-positive CD44$^+$ CD4$^+$ T cells. (A, B) Data were acquired in two independent experiments. Each dot indicates an individual mouse. Solid lines indicate median values. Dotted lines indicate the detection limit. (C) Data were acquired in one experiment with five mice per group. The graphs show mean values and standard deviations. All axis scales are as indicated for the IFN-γ axis, and the axis labels of all radar charts are as indicated for the first radar chart. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance. *, statistically significant differences ($P < 0.05$) from the results for unvaccinated mice.

We had observed good protection before for Ad5.Leader-Gag$_{C1K}$ (31) and attributed this strong protection, in spite of low CD8$^+$ T cell responses, to the induction of Leader-Gag-specific CD4$^+$ T cells, which may contribute help to the vaccine-induced as well as intrinsic immune response upon FV challenge infection. To analyze if the immunization would lead to improved responses upon FV challenge, we analyzed the F-MuLV-neutralizing antibody titers 10 days after FV infection. While unvaccinated mice had not mounted a neutralizing antibody response at this time point, mice immunized with Ad5.Leader-Gag$_{C1K}$ had mounted significantly higher neutralizing antibody responses, whereas mice immunized with Ad48.Leader-Gag$_{C1K}$ showed a slightly lower neutralizing antibody response (Fig. 4B). Most mice that were immunized with Ad50.Leader-Gag$_{C1K}$ had detectable neutralizing antibodies, but their levels tended to be lower than those in the other AdV-immunized mice. These findings correlate well with the strong protection that we observed in Ad5.Leader-Gag$_{C1K}$- and Ad48.Leader-Gag$_{C1K}$-immunized mice. In fact, when we performed a Spearman ranked correlation analysis, we found a good correlation of neutralizing antibody responses on day 10 postchallenge (p.c.) and spleen virus loads on day 21 p.c. ($r_s = -0.70$; data not shown).

To evaluate the contribution of Gag-specific CD4$^+$ T cells to the control of FV infection, we analyzed the cytokine production of CD4$^+$ T cells isolated from spleens

21 days after FV challenge infection after *in vitro* restimulation with Gag-derived peptides (Fig. 4C). Interestingly, we found no apparent difference in the cytokine production of $CD4^+$ T cells derived from unvaccinated or Ad5.Leader-Gag_{C1K}-immunized mice, but there was a strong tendency toward increased frequencies of IFN-γ-, tumor necrosis factor alpha (TNF-α)-, interleukin-2 (IL-2)-, IL-10-, and IL-17-producing $CD4^+$ T cells in mice immunized with the Ad48-based vector, and the tendency toward increased frequencies of these cytokines was a little less in mice immunized with the Ad50-based vector. These results suggest that a stronger Gag-specific $CD4^+$ T cell response induced by Ad48 and Ad50 may contribute to the protection from FV challenge infection and compensate to some extent for the inferior potency with regard to $CD8^+$ T cell priming.

Taken together, these results show that in spite of their lower immunogenicity, the Ad48- and Ad50-based vectors can confer some protection from FV infection to highly susceptible mice, with Ad48 proving more effective than Ad50.

Induction of AdV-specific immune responses by AdV-based immunization. Since vectors based on rare adenoviruses are investigated as potential substitutes for Ad5-based vectors due to the high prevalence of Ad5-specific antibodies in the human population, we were interested in the extent to which 2-fold immunization with vectors based on Ad5, Ad48, or Ad50 would induce type-specific and cross-reactive antibodies. Therefore, we performed an enzyme-linked immunosorbent assay (ELISA) and *in vitro* neutralization assays with plasma samples collected 2 weeks after the second immunization to assess the levels of antibodies induced by each vector type.

Ad5-binding antibodies were mostly induced after immunization with the Ad5-based vectors, with no apparent differences between the vectors encoding different transgenes being seen, but Ad5-binding antibodies were also detectable at lower levels in mice immunized with Ad48- or Ad50-based vectors (Fig. 5A). In contrast to this, neutralizing antibodies against Ad5 were exclusively found in mice immunized with Ad5-based vectors (Fig. 5B). Ad48-binding antibodies were induced at very low levels in most Ad48-immunized mice, but half of the mice immunized with Ad5- or Ad50-based vectors also had Ad48-binding antibody levels at the detection limit (Fig. 5C). In contrast to the low binding antibody levels, Ad48-immunized mice exhibited surprisingly high Ad48-neutralizing antibody titers, but none of the mice immunized with the Ad5- or Ad50-based vectors exhibited an Ad48-neutralizing antibody response (Fig. 5D). Interestingly, Ad50-binding antibodies were detected in plasma samples from almost all immunized mice: while the Ad50-immunized mice tended to have the highest Ad50-binding antibody titers, most Ad5- or Ad48-immunized mice had readily detectable Ad50-binding antibody titers (Fig. 5E). The Ad50-neutralizing capacity of sera from most mice was rather low; of the Ad50-immunized mice, only two Ad50.ova-immunized mice had detectable Ad50-neutralizing antibody titers, but intriguingly, sera from most Ad48-immunized mice also showed Ad50-neutralizing activity, whereas Ad5-immunized mice had no Ad50 cross-neutralizing antibodies (Fig. 5F).

We also analyzed the $CD8^+$ T cell response against the AdV types after immunization; here, we used spleen cells obtained from mice immunized with the ova-encoding vectors and performed an enzyme-linked immunosorbent spot (ELISpot) assay using either the $ova_{257-264}$ peptide or AdV-derived peptides consisting of DNA-binding protein (DBP) residues 418 to 426 ($DBP_{418-426}$) and hexon protein residues 486 to 494 ($Hexon_{486-494}$) of the respective AdV types. These $CD8^+$ T cell epitopes show a moderate degree of conservation, with one or three amino acids differing in $DBP_{418-426}$ (Ad5, FALSNAEDL; Ad48, FGLSNAEDL; Ad50, FGMANAEDL) and four amino acids differing in $Hexon_{486-494}$ (Ad5, KYSPSNVKI; Ad48 and Ad50, KYTPANVTL). As seen before in the dextramer and intracellular cytokine stainings, the ELISpot assay showed a strong $CD8^+$ T cell response to the $ova_{257-264}$ peptide in Ad5.ova-immunized mice; this response was slightly lower in Ad48-immunized mice and significantly lower in Ad50-immunized mice (Fig. 6). When cells were stimulated with the Ad5-derived $DBP_{418-426}$ peptide, most Ad5-immunized mice showed a specific response, and also, 4 of 6 Ad48-immunized mice had a low but specific response, in contrast to the findings for

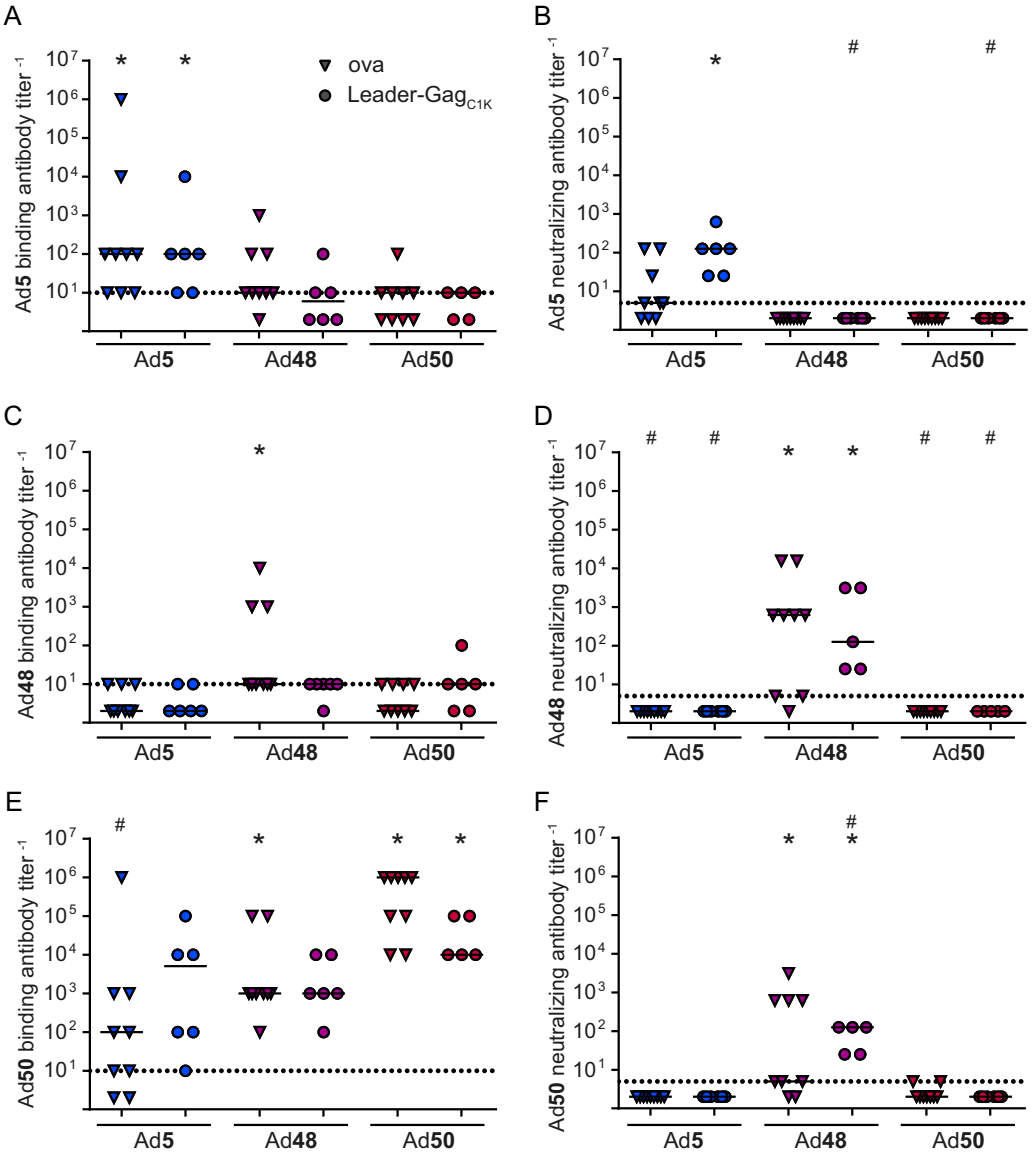

FIG 5 Cross-reactivity of AdV-specific binding and neutralizing antibodies induced by AdV-based immunization. Plasma from mice immunized with Ad5-, Ad48-, or Ad50-based vectors encoding ova or Leader-Gag$_{C1K}$ were collected 2 weeks after the boost immunization. Binding antibodies against all three vectors were determined by ELISA (A, C, E), and neutralizing antibodies were analyzed by *in vitro* neutralization assay (B, D, F). Data were acquired in two independent experiments. Each dot indicates an individual mouse. Solid lines indicate median values. Dotted lines indicate the detection limit. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance. * and #, statistically significant differences ($P < 0.05$) from the results for unvaccinated mice or the respective AdV reference group, respectively.

the Ad50-immunized mice, none of which had a response that was above the detection cutoff. The CD8$^+$ T cell responses to the Ad48-derived DBP$_{418-426}$ peptide were very low or undetectable in most mice of all groups; the CD8$^+$ T cell responses to the Ad50-derived DBP$_{418-426}$ peptide, on the other hand, were comparable to the responses to the Ad5-derived DBP$_{418-426}$ peptide, with a remarkably high response by cells from Ad5-immunized mice being seen, but we also observed the reactivity of cells from Ad48-immunized mice, whereas the responses for most samples from Ad50-immunized mice again remained below the cutoff. The CD8$^+$ T cell response to the Hexon$_{486-494}$ peptides was rather low overall, with only a few mice recognizing the Ad5- or the Ad48/Ad50-derived peptide sequence.

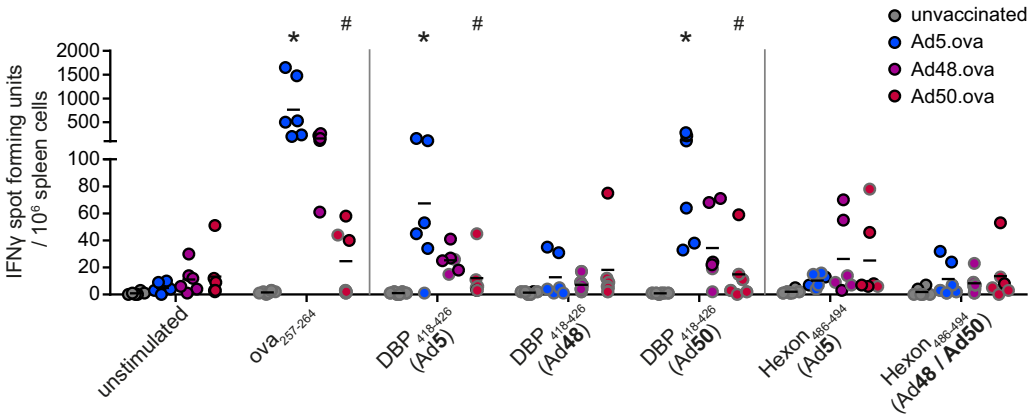

FIG 6 Induction of AdV-specific CD8$^+$ T cell responses by AdV-based immunization. Spleen cells were collected 3 weeks after the boost immunization from mice that were immunized twice with 10^9 vp Ad5.ova, Ad48.ova, or Ad50.ova. Spleen cells were subjected to an ELISpot assay and stimulated with the $ova_{257-264}$ peptide or with the $DBP_{418-426}$ or $Hexon_{486-494}$ peptide derived from the respective AdV types, as indicated. IFN-γ spot-forming units were analyzed after 42 h of stimulation. Data were acquired in two independent experiments. Each dot indicates an individual mouse, and solid lines indicate mean values. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance. * and #, statistically significant differences ($P < 0.05$) from the results for unvaccinated mice or for Ad5.ova-immunized mice, respectively.

Taken together, these data demonstrate that a high level of cross-reactivity against Ad50-derived sequences is observed both for antibody and for CD8$^+$ T cell responses induced by the other AdV types; interestingly, Ad48-induced antibodies showed strong neutralizing activity against Ad50, whereas considerable CD8$^+$ T cell cross-reactivity was observed for Ad5-induced CD8$^+$ T cells.

Immunization in preexisting Ad5 immunity. Vectors based on rare AdV types also hold the promise to be effective in the presence of preexisting Ad5 immunity. Therefore, we compared the potency of our new Ad48- and Ad50-based vectors with that of an Ad5 vector in mice that were preimmune against Ad5.

To establish Ad5 immunity, CB6F1 mice were immunized twice with 10^9 viral particles (vp) of Ad5.empty, which does not encode any transgene (32). Following this preimmunization, mice were immunized twice with the Ad5-, Ad48-, and Ad50-based vectors encoding Leader-Gag_{C1K}. At 2 weeks after the second Leader-Gag_{C1K} immunization, we analyzed the $GagL_{85-93}$-specific CD8$^+$ T cell responses (Fig. 7A). As expected, the $GagL_{85-93}$-specific CD8$^+$ T cell response that we observed after immunization of prenaive mice with Ad5.Leader-Gag_{C1K} was abrogated in Ad5-preimmune mice; interestingly, 2 out of 5 Ad5-preimmune mice that were immunized with Ad48.Leader-Gag_{C1K} were able to mount an appreciable $GagL_{85-93}$-specific CD8$^+$ T cell response. On the other hand, we did not observe any $GagL_{85-93}$-specific CD8$^+$ T cell response in any Ad50.Leader-Gag_{C1K}-immunized mice, irrespective of the Ad5 preimmunization. To evaluate the protection from FV challenge infection, mice were infected with 500 SFFU of FV 3 weeks after the second Leader-Gag_{C1K} immunization, and we analyzed the viral loads in plasma 10 days after FV infection (Fig. 7B) and the spleen weights (Fig. 7C) and spleen viral loads (Fig. 7D) on day 21 after FV infection. In spite of the positive findings regarding the induction of CD8$^+$ T cells by immunization with Ad48.Leader-Gag_{C1K} in Ad5-preimmune mice, we found that neither Ad5- nor Ad48-immunized, Ad5-preimmune mice were able to control the FV infection to the same extent as immunized, Ad5-prenaive mice. We observed a slight trend toward improved control in Ad50-immunized, Ad5-preimmune mice compared to that in Ad5-prenaive mice; however, these mice also did not exert any strong control over FV infection.

Taken together, our results put the usefulness of Ad48- or Ad50-based vectors in preexisting Ad5 immunity into question. A full evaluation will require further experimentation, possibly also with different immunogens.

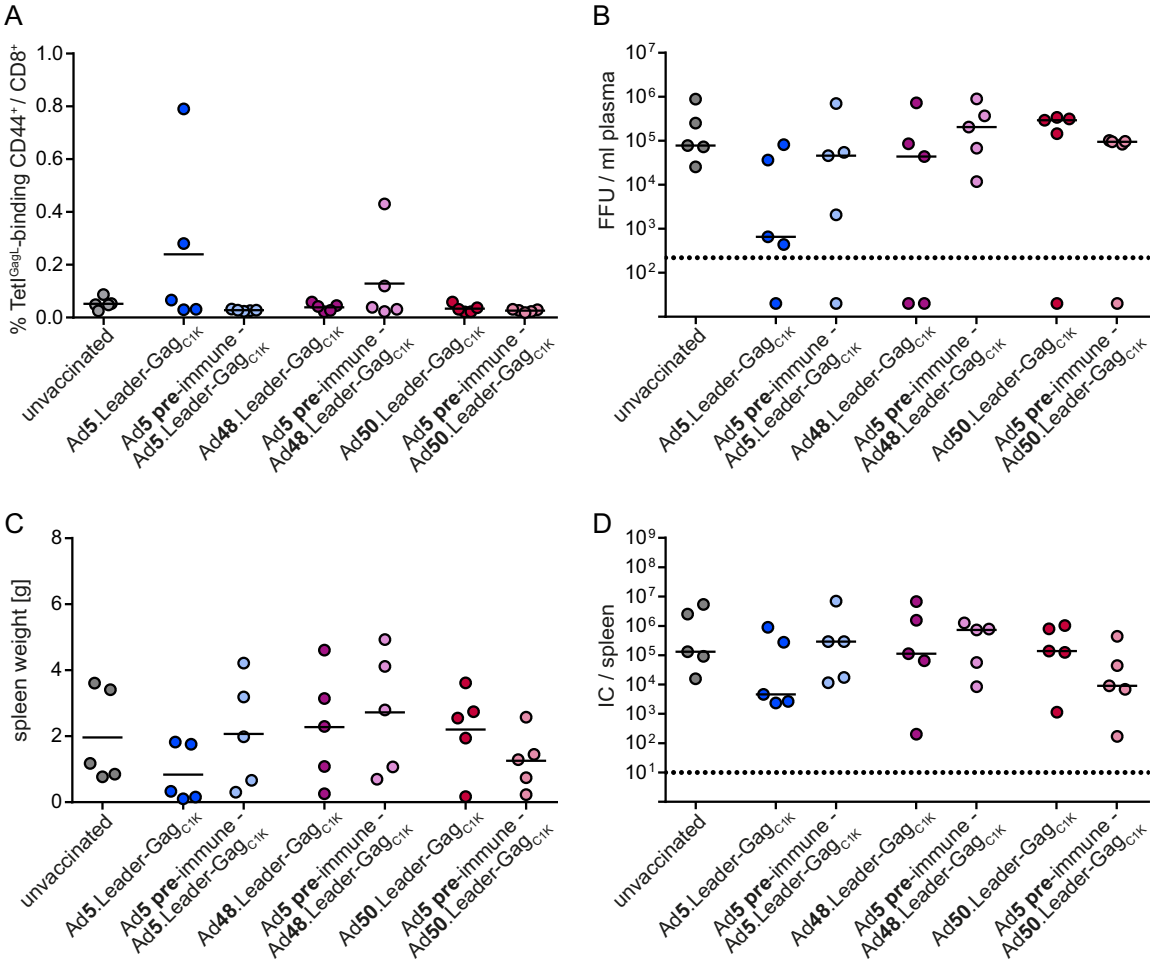

FIG 7 Immunization in the presence of Ad5 immunity. CB6F1 mice were immunized twice in a 3-week interval by intramuscular injection of 10^9 vp of Ad5-, Ad48-, or Ad50-based vectors encoding F-MuLV Leader-Gag$_{C1K}$, with or without two prior immunizations with 10^9 vp of the transgene-free Ad5 vector Ad5.empty. (A) CD8$^+$ T cell responses were analyzed 2 weeks after the second Leader-Gag$_{C1K}$ immunization by MHC-I tetramer staining. At 3 weeks after the second immunization, mice were challenged with 500 SFFU of the FV complex. (B) Viral loads in plasma were analyzed 10 days after FV challenge. (C and D) At 21 days after challenge, spleen weights (C) and spleen viral loads (D) were analyzed. Data were acquired in one experiment with five mice per group. Each dot indicates an individual mouse. Solid lines indicate mean (A, C) or median (B, D) values. Dotted lines indicate the detection limit. Data were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for statistical significance.

DISCUSSION

AdV-based vectors are very potent tools in experimental immunization; however, preexisting immunity can severely impair their efficacy, and therefore, the development of vectors based on alternative AdV types with a low seroprevalence is considered an important approach to effective AdV-based vaccines. However, AdV types other than Ad5 have mostly been described to exhibit reduced immunogenicity, and therefore, not all AdV types are able to induce potent transgene-specific immune responses (7).

In agreement with previous findings, we observed in both the ova and the FV models that Ad48- and Ad50-based vectors induced lower CD8$^+$ T cell responses than the Ad5-based vector. This is well in line with previous reports, where it was similarly found that Ad48 and, even more so, Ad50 were less immunogenic than Ad5 (7). Interestingly, it was described that Ad48 induced only very low transgene-specific antibody titers (7); while we observed overall rather low levels of binding antibodies after immunization against ova or F-MuLV, the levels of anamnestic F-MuLV-neutralizing antibodies after FV challenge infection were comparable in the mice immunized with the Ad5- and Ad48-based vectors. The ability of mice to mount a neutralizing antibody response correlated with their ability to control FV loads, even

though the CD8$^+$ T cell responses induced by the Leader-Gag$_{C1K}$-encoding AdV vectors were very low. This observation is well in line with our previous observation that incomplete immune responses can also confer a high degree of protection from FV challenge (28, 30–34). While it has been recognized that a complex immune response comprising antibodies, CD4$^+$ T cells, and CD8$^+$ T cells is required for complete protection in immunization experiments with attenuated F-MuLV (35, 36), we showed before that the exclusive induction of CD8$^+$ T cells (31) or the induction of CD4$^+$ T cells and antibodies without the induction of CD8$^+$ T cells can confer a very high level of protection (30, 34).

It is interesting to note that immunization with the Ad48-based vector conferred better protection than immunization with the Ad50-based vector against FV infection, even though Ad50-based vectors resulted in the better induction of ova-specific CD8$^+$ T cells as well as the more potent stimulation of CD8$^+$ T cells *in vitro*. Our analysis of the CD4$^+$ T cell response suggests that Ad48 may be more effective than Ad50 at inducing transgene-specific CD4$^+$ T cell responses which contribute to protection by providing help to the anamnestic antibody and CD8$^+$ T cell responses upon challenge infection.

The reason underlying the distinct immunogenicity of different AdV types is not yet well understood. A positive role of stimulatory motifs for Toll-like receptor 9 in the AdV genomic DNA has been suggested (44), but also, empty AdV virions stimulate innate immune responses, indicating the presence of other factors (45). Both the fiber protein (46) and the hypervariable regions in the hexon protein (47) have been implicated in the induction of strong proinflammatory responses. Surprisingly, the stronger proinflammatory responses were associated with unfavorable effects on transgene-specific immunity (47), whereas it had been assumed in the past that the stronger immunogenicity of the vector itself would have an adjuvant effect on the immunization outcome (48, 49). When we analyzed the activation of DCs by the different AdV types, we could not see striking differences; curiously, the activation of DCs, as estimated by the expression of CD86 and MHC-II, was the lowest after Ad50 transduction, whereas CD8$^+$ T cell proliferation was the lowest after stimulation with Ad48-transduced DCs. Overall, our results confirm that complex mechanisms underlie the potency of different AdV types for the induction of CD8$^+$ T cell responses and their efficacy in conferring protection. For a clear definition of correlates of immunogenicity, a more large-scale approach that includes a multitude of distinct AdV types will be necessary and may provide important insights in the future.

Our results show that the application of rare AdV types as vaccine vectors can also be severely impaired by preexisting immunity against other AdV types, which can be explained by the fact that we found a considerable degree of cross-reactivity of antibodies and CD8$^+$ T cell responses (summarized in Fig. 8). The usefulness of any rare AdV type-based vectors therefore has to be scrutinized with respect to the robustness in the presence of any preexisting AdV immunity.

Other reports on rare AdV-based vectors showed only neutralizing AdV-specific antibodies, whereas we also analyzed binding antibodies and found surprisingly high levels of cross-specific antibody binding. We have demonstrated before that we already observed detrimental effects of preexisting Ad5 immunity even when neutralizing antibody levels were very low (32), suggesting that high levels of binding, nonneutralizing antibodies may also interfere with the induction of transgene-specific CD8$^+$ T cell responses, implying that high levels of cross-specific binding antibodies may similarly be problematic.

The level of cross-neutralization was much lower than the level of cross-binding antibodies, and we mostly observed moderate cross-neutralization of Ad48 toward Ad50. Low levels of cross-neutralization of Ad50 have also been reported before for Ad35-immunized mice (7), and similarly, cross-neutralization of Ad11- and Ad35-induced neutralizing antibodies has been shown (14), clearly demonstrating that the cross-reactivity of antibodies induced by specific AdV types can occur and has to be considered in the selection of AdV types for use as immunization vectors. Importantly,

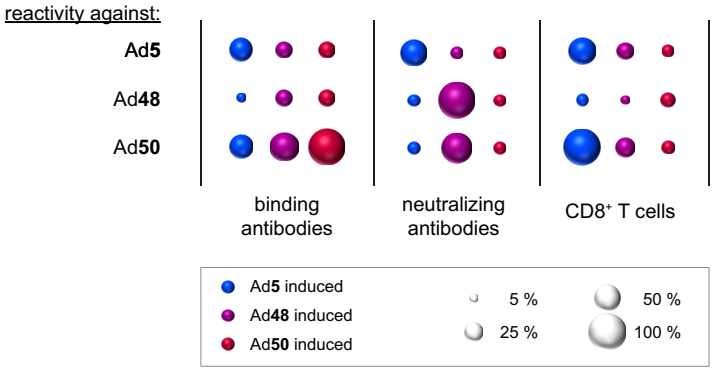

FIG 8 Summary of cross-reactivity. The reactivity of binding antibodies, neutralizing antibodies, and $CD8^+$ T cells, as shown in Fig. 5 and 6, relative to the highest reactivity for the respective parameter is shown. Log-transformed median values were used for calculation of relative antibody reactivity; mean values of IFN-γ spot-forming units after DBP-derived peptide stimulation were used for calculation of relative $CD8^+$ T cell reactivity. The bubble color indicates the AdV type used for immunization; the bubble size indicates the degree of relative reactivity.

not only humoral immune responses but also cellular immune responses can affect vaccination efficacy. It has been demonstrated before that cellular immune responses exhibit a high degree of cross-reactivity against not only other human AdV types but also nonhuman AdV types (50). We demonstrated here that Ad5-induced $CD8^+$ T cells reacted equally well to an Ad5-derived epitope sequence and a rather divergent, Ad50-derived epitope sequence, implying that rare AdV-based vectors may harbor epitopes that would lead to the elimination of transduced cells in preexisting Ad5 immunity by Ad5-induced $CD8^+$ T cells, thereby abrogating vaccine efficacy.

Our results demonstrate that different AdV types exhibit distinct properties and potentials as vaccine vectors, and the choice of vaccine vector should be guided by both the desired type of immune response and the degree of cross-reactivity exhibited by immune cells and antibodies induced by prevalent AdV types. In spite of the low efficacy with regard to the induction of $CD8^+$ T cells, Ad48 conferred protection from FV challenge infection in 4 of 6 mice and showed the least potential of being recognized by cross-reactive immune responses induced by the other types, suggesting that AdV types that are not obviously strongly immunogenic might be considered potential vaccine vectors. However, the impaired efficacy in preexisting Ad5 immunity puts the usefulness of the vectors tested here into question.

MATERIALS AND METHODS

Cells and cell culture. The human embryonic kidney cell line 293A (Microbix Biosystems, Toronto, ON, Canada) and the human lung carcinoma cell line A549 (ATCC CCL-185; LGC Standards) were propagated in Dulbecco's modified Eagle medium (DMEM) with high glucose. A murine fibroblast cell line from *Mus dunni* mice (51) was maintained in RPMI medium (Invitrogen/Gibco, Karlsruhe, Germany). The cell culture media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen/Gibco), 50 μg/ml gentamicin (Invitrogen/Gibco), and 20 μg/ml ciprofloxacin (Fresenius Kabi, Austria). The cell lines were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Adenoviral vectors. The following adenoviral vectors have been described before. Ad5.ova (43) encodes chicken egg ovalbumin. Ad5.Leader-Gag_{C1K} (31) encodes a modified full-length version of the F-MuLV clone FB29-derived Leader-Gag protein with an amino acid exchange at position 94 (Y94K). Ad5.GFP (52) encodes enhanced green fluorescent protein (GFP) from *Aequorea victoria* jellyfish. Ad5.empty does not encode any transgene sequences (32).

Ad48- and Ad50-based vectors were created using the AdApt system (kindly provided by Dan Barouch, Harvard Medical School, Boston, MA [7]). Briefly, the transgenes of interest, ova and Leader-Gag_{C1K}, were cloned into the pAdApt48 and pAdApt50 plasmids, and the resulting transgene-containing plasmids were cotransfected into 293A cells with pWe.Ad48-pIX-rIT or with pBR.Ad50.pIX-Nhe and pBR.Ad50.Sfi-rIT, respectively, for reconstitution of the vectors. Non-transgene-containing pAdApt48 and pAdApt50 plasmids were used for reconstitution of the non-transgene-encoding vectors Ad48.empty and Ad50.empty.

Adenoviral vectors were purified with a Vivapure AdenoPACK 100 kit or a Vivapure AdenoPACK 20 kit (Vivascience, Hannover, Germany). The adenovirus particle concentrations were determined by

spectrophotometry as described previously (53) and expressed as the number viral particles (vp) per milliliter; infectivity was verified in a 50% tissue culture infective dose assay.

The identity of the expression cassettes of the various adenoviral vectors was verified by sequencing, and transgene expression was confirmed by Western blot analysis.

Mice. Female CB6F1 hybrid mice (BALB/c × C57BL/6 F1; H-2$^{b/d}$ Fv1$^{b/b}$ Fv2$^{r/s}$ Rfv3$^{r/s}$) were purchased from Charles River Laboratories (Sulzfeld, Germany). T cell receptor-transgenic C57BL/6 mice expressing a T cell receptor specific for the F-MuLV Gag leader-derived epitope GagL$_{85–93}$ have been described before (54) and were bred in the animal facility of the Institute for Virology at the University Hospital Essen.

The mice were used when they were between 8 and 9 weeks of age. The mice were treated in accordance with national law and the institutional guidelines of the University Hospital Essen, Essen, Germany. The study was approved by the Northrhine-Westphalia State Office for Nature, Environment and Consumer Protection (LANUV NRW).

***In vitro* proliferation assay.** Bone marrow-derived dendritic cells (DCs) were prepared as described before (55) and transduced with Ad5-, Ad48-, or Ad50-based vectors encoding Leader-Gag$_{C1K}$ at a multiplicity of infection (MOI) of 10^3. CD8$^+$ T cells from T cell receptor-transgenic mice were isolated from spleen cells by magnetic cell sorting using CD8 microbeads (Miltenyi, Bergisch-Gladbach, Germany) and labeled with 1.5 μM carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher Scientific, Schwerte, Germany). Transduced DCs and CFSE-labeled CD8$^+$ T cells were coincubated for 4 days at an initial ratio of 1:2.5. CFSE intensity was analyzed by flow cytometry after staining of the cells with BrillianViolet421 (BV421)-anti-CD8 (BioLegend, Fell, Germany).

Activation of DCs was analyzed by flow cytometry at 24 h after transduction with AdV vectors after staining with phycoerythrin (PE)/Dazzle594-anti-CD11c (BioLegend), BV605-anti-CD86 (BioLegend), and PE-anti-MHC-II (BD Pharmingen, Heidelberg, Germany).

Immunization. CB6F1 mice were immunized with 1×10^9 vp of the respective adenoviral vectors diluted in 30 μl of phosphate-buffered saline (PBS) by intramuscular injection into the Musculus gastrocnemius. Prime-boost immunizations were performed in a 3-week interval.

FV and challenge infection. An uncloned, lactate dehydrogenase-elevating virus (LDV)-free FV stock was obtained from BALB/c mouse spleen cell homogenate (10%, wt/vol) 14 days after infection with a B-tropic, polycythemia-inducing FV complex (56). CB6F1 mice were infected with 500 spleen focus-forming units (SFFU) diluted in 100 μl PBS by intravenous injection into the tail vein.

Viremia assay. At 10 days postchallenge (p.c.), plasma samples from CB6F1 mice were obtained, and viremia was determined in a focal infectivity assay (57). Serial dilutions of plasma were incubated with *M. dunni* cells for 3 days under standard tissue culture conditions. When the cells reached ~100% confluence, they were fixed with ethanol and labeled first with F-MuLV Env-specific monoclonal antibody 720 (58) and then with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig antibody (Dako, Hamburg, Germany), followed by aminoethylcarbazole (Sigma-Aldrich, Deisenhofen, Germany) as the substrate to detect foci. The foci were counted, and the number of focus-forming units (FFU) per milliliter of plasma was calculated.

IC assay. At 21 days after challenge, FV-infected animals were sacrificed by cervical dislocation, the spleens were removed and weighed, and single-cell suspensions were prepared. Serial dilutions of isolated spleen cells were seeded onto *M. dunni* cells and incubated under standard tissue culture conditions for 3 days. When the cells reached 100% confluence, they were fixed with ethanol and stained as described above for the viremia assay. The resulting foci were counted, and the numbers of infectious centers (IC) per spleen were calculated.

Binding antibody ELISA. For the analysis of ova- or F-MuLV-binding antibodies, MaxiSorp ELISA 96-well plates (Nunc, Roskilde, Denmark) were coated with ova protein (5 μg/ml; Sigma-Aldrich, Munich, Germany) or whole F-MuLV antigen (5 μg/ml), respectively. For the analysis of adenovirus-binding antibodies, the plates were coated with 3.9×10^8 viral particles per well of Ad5.empty, Ad48.empty, or Ad50.empty. After coating, the plates were blocked with 10% fetal calf serum in PBS and incubated with plasma dilutions. Binding antibodies were detected using a polyclonal rabbit anti-mouse HRP-coupled anti-mouse immunoglobulin antibody and tetramethylbenzidine (TMB+) as the substrate (both were from Dako GmbH, Germany). Plasma dilutions were considered positive if the absorption was 2-fold higher than that obtained with plasma from naive mice.

Neutralizing antibody assay. To detect F-MuLV-neutralizing antibodies, serial dilutions of heat-inactivated (30 min, 56°C) plasma in PBS were mixed with purified F-MuLV and guinea pig complement (Sigma-Aldrich, Munich, Germany), incubated at 37°C for 60 min, and then added to *M. dunni* cells that had been plated at a density of 7.5×10^3 cells per well in 24-well plates the day before. Seventy-two hours later, cells were stained as described above for the viremia assay. Dilutions that resulted in a reduction of foci by 75% or more were considered neutralizing.

For the analysis of adenovirus-neutralizing antibodies, dilutions of Ad5.GFP, Ad48.GFP, or Ad50.GFP were mixed with serial plasma dilutions and incubated at 37°C for 1 h and then added to A549 cells that had been plated in flat-bottom 96-well plates at a density of 1×10^4 cells/well the day before. Five days later, the plates were analyzed for GFP expression by fluorescence microscopy. Plasma dilutions were considered neutralizing when the number of GFP-expressing cells was reduced by 75% or more.

MHC-I tetramer and dextramer staining of transgene-specific CD8$^+$ T cells. ova$_{257–264}$-specific CD8$^+$ T cells or GagL$_{85–93}$-specific CD8$^+$ T cells were analyzed in peripheral blood 2 weeks after immunization. After lysis of erythrocytes, blood cells were stained with an allophycocyanin (APC)-coupled MHC-I dextramer containing the H-2K^b-restricted ova epitope SIINFEKL (Immudex, Copenhagen, Denmark) or with a PE-coupled MHC-I tetramer containing the H-2D^b-restricted F-MuLV Gag-Leader epitope

AbuAbuLAbuLTVFL (in which the cysteine residues of the original amino acid sequence of $GagL_{85–93}$ [CCLCLTVFL] were replaced by aminobutyric acid [Abu] to prevent disulfide bonding [59]; MBL, Woburn, MA), respectively, and with BV421-anti-CD8 (BioLegend) and BV510-anti-CD44 antibodies (Becton, Dickinson) and fixable viability dye eFluor 780 (FVD-eF780; eBioscience, Frankfurt, Germany). Data were acquired on an LSR II flow cytometer (Becton, Dickinson, Mountain View, CA) and analyzed using FlowJo software (TreeStar, Ashton, OR).

Intracellular cytokine staining. For the analysis of effector molecules of $ova_{257–264}$-specific $CD8^+$ T cells or F-MuLV $GagL_{85–93}$-specific $CD8^+$ T cells, blood samples were collected 2 weeks after immunization and subjected to erythrocyte lysis. Cells were stimulated for 6 h *in vitro* with 1 μg/ml of the $ova_{257–264}$ peptide (SIINFEKL) or 1 μg/ml of the $GagL_{85–93}$ peptide (AbuAbuLAbuLTVFL; modified from the original sequence, CCLCLTVFL, with Abu) in the presence of 2 μg/ml brefeldin A. Cells were stained with BV421-anti-CD8, BV510-anti-CD44, and fluorescein isothiocyanate (FITC)-anti-gamma interferon (IFN-γ) (eBioscience).

For the analysis of cytokine production by F-MuLV Gag-specific $CD4^+$ T cells, spleen cells were stimulated *in vitro* for 42 h in the presence of 10 μg/ml Gag-derived peptides (Gag from residues 225 to 239, QSSLYPALTSPLNTK; Gag from residues 260 to 274, PPPYRDPGPPSPDGN; Gag from residues 492 to 506, AMSFIWQSAPDIGRK [60]) in the presence of 10 units/ml of IL-2, followed by an additional 6 h of incubation after addition of 2 μg/ml of brefeldin A. Cells were stained with BV605-anti-CD4 (BioLegend), BV510-anti-CD44, FVD-eF780, FITC-anti-IL-10 (Invitrogen), PE-anti-TNF-α (BioLegend), PE/Dazzle594-anti-IFN-γ (BioLegend), PE-Cy7-anti-IL-17 (Invitrogen), APC-anti-IL-4 (eBioscience), and eFluor450-anti-IL-2 (eBioscience).

Data were acquired on an LSR II flow cytometer (Becton, Dickinson, Mountain View, CA) and analyzed using FlowJo software (TreeStar, Ashton, OR).

ELISpot assay analysis of $CD8^+$ T cell responses. $CD8^+$ T cell responses to the ova-derived peptide $ova_{257–264}$ or the AdV-derived peptides $DBP_{418–426}$ (for Ad5, FALSNAEDL; for Ad48, FGLSNAEDL; for Ad50, FGMANAEDL) and $Hexon_{486–494}$ (for Ad5, KYSPSNVKI; for Ad48 and Ad50, KYTPANVTL) were analyzed by ELISpot assay. Spleen cells were obtained from immunized mice, and single-cell suspensions were prepared. Spleen cells (10^6) were cultivated in the presence of 1 μg/ml of the $ova_{257–264}$ peptide or 10 μg/ml of the AdV-derived peptides for 42 h on ELISpot assay plates that had been precoated with an anti-IFN-γ capture antibody (mouse IFN-γ ELISpot^{plus} kit; Mabtech, Nacka Strand, Sweden). The plates were stained according to the manufacturer's instructions, and the number of spots was determined using an AID ELISpot assay reader (Autoimmun Diagnostika, Straßberg, Germany).

Statistical analyses. Statistical analyses were performed using the software GraphPad Prism (version 6; GraphPad Software, La Jolla, CA), testing with the ordinary one-way analysis of variance and Tukey's multiple-comparison procedure or the Kruskal-Wallis one-way analysis of variance on ranks and Dunn's multiple-comparison procedure for the comparison of data from more than two groups and the Wilcoxon matched-pairs signed-rank test to analyze the increase in $CD8^+$ T cell responses from the prime to the boost immunization.

ACKNOWLEDGMENTS

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