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The Vaccinia Virus Complement Control Protein Modulates Adaptive Immune Responses during Infection[▽]

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Complement activation is an important component of the innate immune response against viral infection and also shapes adaptive immune responses. Despite compelling evidence that complement activation enhances T cell and antibody (Ab) responses during viral infection, it is unknown whether inhibition of complement by pathogens alters these responses. Vaccinia virus (VACV) modulates complement activation by encoding a complement regulatory protein called the vaccinia virus complement control protein (VCP). Although VCP has been described as a virulence factor, the mechanisms by which VCP enhances VACV pathogenesis have not been fully defined. Since complement is necessary for optimal adaptive immune responses to several viruses, we hypothesized that VCP contributes to pathogenesis by modulating anti-VACV T cell and Ab responses. In this study, we used an intradermal model of VACV infection to compare pathogenesis of wild-type virus (vv-VCPwt) and a virus lacking VCP (vv-VCPko). vv-VCPko formed smaller lesions in wild-type mice but not in complement-deficient mice. Attenuation of vv-VCPko correlated with increased accumulation of T cells at the site of infection, enhanced neutralizing antibody responses, and reduced viral titers. Importantly, depleting $CD8^+$ T cells together with $CD4^+$ T cells, which also eliminated T helper cell-dependent Ab responses, restored vv-VCPko to wild-type levels of virulence. These results suggest that VCP contributes to virulence by dampening both antibody and T cell responses. This work provides insight into how modulation of complement by poxviruses contributes to virulence and demonstrates that a pathogen-encoded complement regulatory protein can modulate adaptive immunity.

Complement is a critical component of the innate immune response and contributes to defenses against multiple viral pathogens, including poxviruses (5, 40). In response to the antiviral effects of complement, viruses have developed strategies to evade complement activation (4, 9, 27, 35). Vaccinia virus (VACV), the prototypic member of the poxvirus family, encodes a protein called the vaccinia virus complement control protein (VCP), which limits complement activation by inhibiting several early steps of the complement cascade (25, 26, 36). VCP limits complement activation by dissociating the C3 and C5 convertases, which are necessary to initiate and sustain activation of the cascade, and by acting as a cofactor for the serine protease factor I to promote degradation and inactivation of C3b and C4b (31, 36, 42, 45, 46, 48). *In vitro*, VCP has been shown to protect viral particles from complement-mediated neutralization (19, 20) and prevent complement-dependent lysis of infected cells (16).

Complement activation by the classical, lectin, and alternative pathways results in production of fragments with distinct antiviral properties (5, 58, 59). These include the proinflammatory peptides C3a and C5a, as well as C3b and C4b, which can mediate direct virus neutralization by opsonizing viral particles. Production of C3b also leads to downstream activation of C5 and formation of the membrane attack complex, which lyses infected cells and enveloped viral particles (5). Complement activation can also contribute to defense against viral infection by enhancing adaptive antibody (Ab) and T cell responses. Complement has been shown to enhance Ab responses to several viruses, including herpes simplex virus (HSV) (7, 14, 15, 55, 56), vesicular stomatitis virus (VSV) (44), and West Nile virus (WNV) (37, 38). Recently, it has become clear that complement is also important for eliciting optimal T cell responses to several viral pathogens (10, 24, 37, 50). During viral infection, complement-deficient mice exhibit reduced expansion and accumulation of $CD4^+$ and $CD8^+$ T cells following infection with influenza virus (24), WNV (37), or lymphocytic choriomengitis virus (LCMV) (50). Consistent with these findings, deficiency in the mammalian complement regulatory protein Daf-1 results in increased complement activation, which leads to enhanced $CD8^+$ T cell responses following infection with LCMV (10). Although the mechanisms by which complement enhances T cell-mediated immune responses have not been fully elucidated, it is clear that complement activation is important for generation of protective T cell-mediated immune responses.

Despite compelling evidence that complement activation enhances Ab and T cell responses (7, 10, 14, 15, 23, 24, 37, 44, 50, 55, 56), it is unknown whether inhibition of complement by pathogens can also limit adaptive immune responses. VCP has been characterized as a virulence factor (20), but the mechanisms by which it contributes to the pathogenesis of VACV *in*

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vivo have not been fully defined. VACV infection can be cleared by either Th-dependent Ab responses or $CD8^+$ T cell responses (1, 60). Since complement has been shown to enhance antiviral Ab and T cell responses, we hypothesized that VCP may contribute to pathogenesis by modulating anti-VACV Ab and T cell responses in a complement-dependent manner. In this study, we used an intradermal model of VACV infection to compare pathogenesis and immune responses generated by wild type virus (vv-VCPwt) and a virus specifically lacking VCP (vv-VCPko). Infection with vv-VCPko resulted in smaller lesions than vv-VCPwt and was associated with increased numbers of $CD8^+$ and $CD4^+$ T cells at the site of infection as well as enhanced helper T cell (Th)-dependent neutralizing Ab responses. However, in mice depleted of both $CD8^+$ and $CD4^+$ T cells, vv-VCPko was no longer attenuated, demonstrating that the ability of VCP to suppress adaptive immune responses contributes to its role in pathogenesis. Importantly, vv-VCPwt and vv-VCPko were equally pathogenic in $C3^{-/-}$ mice, indicating that VCP contributes to VACV pathogenesis in a complement-dependent manner.

MATERIALS AND METHODS

Mice. $C3^{-/-}$ mice on the C57BL/6 (B6) background were generously provided by J. D. Lambris (University of Pennsylvania) and were bred at the University of Pennsylvania. Age-matched wild-type C57BL/6 mice were used as controls (The Jackson Laboratory, Bar Harbor, ME). All experiments were performed with 6- to 9-week-old female mice, and all mice were maintained under specific-pathogen-free conditions. Experiments were conducted in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Viruses and infections. The construction of vv-VCPwt and vv-VCPko viruses has been described previously (16). These viruses are marker-free recombinants that were generated from the parental virus vSIGK-3, in which the VCP open reading frame was disrupted by insertion of a guanine phosphoribosyltransferase expression cassette (20, 25). All *in vivo* infections used virus that was purified twice through a 36% sucrose cushion (10 ml) at ~24,000 × *g* for 60 min. For ear pinna infections, 2×10^4 PFU of the indicated virus suspended in 10 μl of phosphate-buffered saline (PBS) were injected into the dorsal sides of the right and left ear pinnae by using a 29-gauge insulin syringe (Becton Dickinson). Lesion diameters were measured using digital calipers (Fisher Scientific). For intranasal (i.n.) challenge, mice were infected with 1×10^7 PFU of VACV strain WR suspended in 20 μl of PBS. Challenged mice were monitored daily for weight loss. Animals that lost 30% of their body weight were humanely sacrificed. In some studies, T cells were depleted by injecting mice intraperitoneally (i.p.) on day −2 and −1 before infection and on day 3 after infection with 0.2 mg/mouse anti-CD4 Ab (clone GK1.5; BioXcell) and/or 0.3 mg/mouse anti-CD8 Ab (clone 2.43) suspended in a total volume of 200 μl PBS. Undepleted control mice were injected with 200 μl PBS. The efficiency of T cell depletion was checked on day 8 postinfection in submaxillary lymph nodes. Lymph nodes were harvested, teased into a single-cell suspension, and passed through a 40-μm cell strainer. Cells were stained with anti-CD4 peridinin chlorophyll protein (clone RM4.5; BD PharMingen) and anti-CD8–fluorescein isothiocyanate (FITC) conjugate (clone 53-6.7; Biolegend) as described below and collected on a FACSCalibur apparatus. Treatment of mice resulted in depletion of ≥95% of $CD4^+$, $CD8^+$, or both subsets of T cells.

Virus growth curves. To assess the abilities of vv-VCPwt and vv-VCPko to replicate *in vitro*, we performed single-step and multistep growth curves. Confluent monolayers of BSC-1 cells were infected with 5 PFU/cell of the indicated virus diluted in minimal essential medium (MEM) containing 2.5% fetal bovine serum (FBS; medium referred to as 2.5% MEM) for single-step growth curves or with 0.05 PFU/cell for multistep growth curves. Two hours postinfection, the inoculum was replaced with fresh medium. At the indicated time points, cells were washed and harvested in 1 ml fresh 2.5% MEM. Cells were then freeze-thawed three times and sonicated to release virus, and titers were determined as described below.

Determination of viral titers in the ear. To determine viral titers, infected ears were removed after euthanizing mice, washed in 70% ethanol, allowed to dry, and then placed in 2.5% MEM. Ears were minced, freeze-thawed three times, and sonicated to release the virus. Serial 10-fold dilutions of supernatant from processed ears were performed in 2.5% MEM. Dilutions were plated on monolayers of BSC-1 cells and incubated at 37°C for 2 h. Medium was then removed, and the cells overlaid with 2.5% MEM–1% carboxymethylcellulose (CMC). The plates were incubated for 2 days at 37°C, and then plaques were visualized by crystal violet staining and counted.

Ab ELISAs and plaque reduction neutralization assay. B6 or $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko or uninfected naive mice were bled at various times after infection. For enzyme-linked immunosorbent assay (ELISA) experiments, 96-well Maxisorp Immuno plates (Nunc) were coated overnight at 4°C using whole-cell vaccinia virus-infected cell lysates in bicarbonate coating buffer as previously described (17, 57). After washes, plates were blocked in blocking buffer (PBS–1% BSA–0.05% Tween) for 1 h at room temperature (RT). Serial 2-fold dilutions of sera from B6 mice in blocking buffer were added in duplicate, and plates were incubated overnight at 4°C. Bound IgG or IgM was detected with either horseradish peroxidase-conjugated rabbit anti-mouse IgG H&L (Abcam) at 1:4,000 or a rat anti-IgM monoclonal antibody (BD Pharmingen) at 1:1,000. The plates with secondary antibodies were incubated for 1 h at 37°C, washed, and incubated with 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate for 20 min at RT, and absorbance was measured using an ELISA reader at 405 nm. For plaque reduction neutralization assays, heat-inactivated serum from individual mice in each group ($n = 4$) was diluted 1:100 and combined with ~100 PFU of the mature virus (MV) form of wild-type VACV. Virus and sera were incubated together for 2 h at 37°C and then added to confluent monolayers of BSC-1 cells in 12-well plates. Virus was incubated with cells at 37°C for 2 h and then overlaid with 2.5% MEM containing 1% CMC. Forty-eight hours later, plaques were visualized by staining with crystal violet and counted.

Cell isolation and flow cytometry. To isolate cells from the ears, amputated ears were washed in 70% ethanol and allowed to dry. Dorsal and ventral sheets were separated using forceps and incubated dermal side down on Dulbecco's modified Eagle's medium (DMEM) containing 0.5 mg/ml collagenase (Dispase; Roche) for 45 min at 37°C. Ears were then processed for 4 min in a Medimachine (BD Systems) using a 40-μm Medicon filter (BD Systems). Cells were recovered from the Medicon in complete tissue culture medium (CTCM; DMEM, 10% heat-inactivated FBS [HyClone], 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, and 1× antibiotic/antimycotic [Invitrogen]) by using a 10-ml sliptip syringe and passed through a 40-μm cell strainer. Cells were then pelleted, resuspended in 0.01 M Tris-HCl (pH 7.4) containing 0.84% (wt/vol) NH_4Cl to lyse red blood cells, and incubated for 5 min at room temperature. Cells were pelleted, resuspended in CTCM, and counted on a hemacytometer. Live cells were identified by trypan blue exclusion.

The frequencies of T cells in the ear were determined by flow cytometry. Cells were washed once with PBS and stained with Aqua Blue LIVE/DEAD fixable viability dye (Invitrogen) for 10 min on ice. Fc Block (BD Biosciences) was then added at 10 μg/ml and incubated for an additional 5 min on ice. For phenotypic analysis of T cells, cells were surface stained with the following Ab conjugates diluted in fluorescence-activated cell sorting (FACS) buffer (PBS, 1% FBS, 5 mM EDTA, 0.04% sodium azide) for 30 min on ice: anti-CD3 (with allophycocyanin [APC] or APC-Cy7; Biolegend), anti-CD4 (with phycoerythrin [PE]-Cy5.5; Caltag), anti-CD8 (Pacific Blue or efluor450; eBioscience), anti-CD45 (APC; Biolegend), anti-CD11b (PE-Cy5; Biolegend). Cells were then washed twice in FACS buffer and fixed with 1% paraformaldehyde. Fluorescence-minus-one (FMO) controls were also included and were used for gating. Antibody capture beads were used to prepare compensation tubes for each Ab. Samples were collected on an LSR II apparatus (BD Immunocytometry Systems) equipped with blue (488 nm), red (633 nm), violet (407 nm), and green (532 nm) lasers and analyzed using FlowJo software (TreeStar, Ashland, OR). T cells were identified by gating on single, live, $CD45^+$, $CD3^+$, $CD11b^-$ cells. CD4 and CD8 T cells were then identified as $CD4^+$ $CD8^-$ and $CD8^+$ $CD4^-$. The total numbers of $CD3^+$ $CD11b^-$, $CD4^+$, and $CD8^+$ cells were determined by calculating the frequencies of these cells in the total cell population and multiplying each frequency by the total number of cells isolated from infected ears.

Analysis of anti-VACV T cell responses. Submaxillary draining lymph nodes (dLN) were isolated from mice infected with either vv-VCPko or vv-VCPwt and teased into single-cell suspensions. Cells were then resuspended in 0.01 M Tris-HCl (pH 7.4) containing 0.84% (wt/vol) NH_4Cl to lyse red blood cells, washed, and counted. To analyze VACV-specific gamma interferon (IFN-γ) responses, 1×10^6 cells were plated in 96-well plates and stimulated with the VACV immunodominant peptide $B8_{20-27}$ (TSYKFESV; Genscript) (51) for 6 h in the presence of Brefeldin A (BD GolgiPlug). Following stimulation, cells were washed once in FACS buffer and incubated with Fc Block. Cells were then surface stained with FITC-conjugated anti-CD8 Ab (BD Biosciences), fixed, permeabilized with CytoFix/CytoPerm (BD Biosciences), stained intracellularly

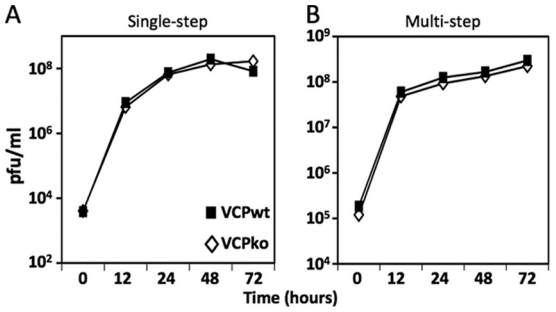

FIG. 1. Deleting VCP does not affect viral replication or spread *in vitro*. BSC-1 cells were infected with 5 PFU/cell (A) or 0.05 PFU/cell (B). The amount of virus produced was determined based on virus titers in infected cells at the indicated times after infection.

with Alexa-647-conjugated anti-IFN-γ Ab (BD Biosciences), and acquired using a FACSCalibur instrument (Becton Dickinson).

CD8 T cell responses were also analyzed by surface staining cells with $B8_{20–27}$ peptide-major histocompatibility complex (MHC) tetramer complexes (generously provided by E. John Wherry, Wistar Institute, Philadelphia, PA). Biotinylated MHC H-$2K^b$–peptide complexes were made as previously described (43) and were tetramerized using APC-conjugated streptavidin (Molecular Probes). Draining lymph node cells were isolated and stained directly *ex vivo*. Cells were incubated with Fc Block (BD Bioscience) and Aqua Blue viability dye (Invitrogen) as described above and surface stained with $B8_{20-27}$ tetramer, FITC-conjugated anti-CD62L, Pacific Blue-conjugated anti-CD8, Alexa-700-conjgated anti-CD3, PE–anti-CD44, and PE-Cy5.5–anti-CD4. Samples were collected on an LSRII instrument as described above.

Statistics. Statistical significance was determined using an unpaired Student's *t* test, and *P* values of less than 0.05 were considered significant.

RESULTS

Deletion of VCP does not affect viral replication *in vitro*. VCP has previously been shown to be dispensable for viral replication *in vitro* but to contribute to pathogenesis *in vivo* (20, 25). To confirm that construction of vv-VCPwt and vv-VCPko did not affect the replication capacities of these viruses, we performed single-step and multistep growth curves to assess the ability of these viruses to replicate and spread *in vitro*. vv-VCPwt and vv-VCPko replicated equally well in both of these assays (Fig. 1), demonstrating that deletion or rescue of VCP in our viruses did not affect *in vitro* viral replication

VCP contributes to pathogenesis. To assess whether VCP affects pathogenesis of VACV, we used an intradermal route of infection that closely resembles human VACV vaccination. Thus, although VACV is not a natural pathogen of mice, this route of infection and the dose of virus used mimics the use of this virus as a vaccine. B6 mice were infected intradermally (i.d.) in the ear pinna with 2×10^4 PFU of vv-VCPwt or vv-VCPko, and lesion sizes were measured over time. Lesion development occurred with similar kinetics in mice infected with either vv-VCPwt or vv-VCPko and peaked at day 10 postinfection. However, throughout the course of infection, mice infected with vv-VCPwt had significantly ($P < 0.01$) larger lesions than mice infected with vv-VCPko (e.g., 5.83 mm versus 3.66 mm on day 10) (Fig. 2A and C).

To determine if VCP's role in pathogenesis is dependent on complement, $C3^{-/-}$ mice were infected in the ear pinna with vv-VCPwt or vv-VCPko, and lesion sizes were monitored. In contrast to B6 mice, in which vv-VCPko formed smaller lesions

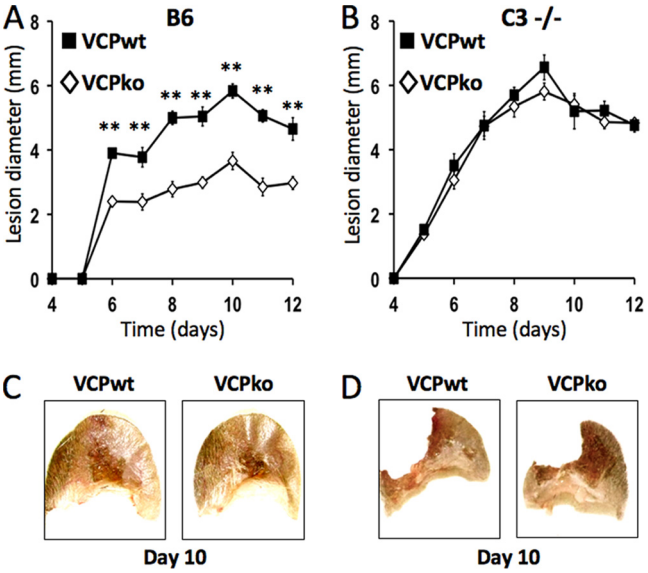

FIG. 2. VCPko is attenuated in wild-type but not $C3^{-/-}$ mice. (A and B) B6 ($n = 5$) (A) or $C3^{-/-}$ mice ($n = 4$ for vv-VCPwt and $n = 5$ for vv-VCPko) (B) were infected with 2×10^4 vv-VCPwt or vv-VCPko. Lesion sizes were measured over time. The differences in lesion sizes between vv-VCPwt and vv-VCPko were statistically significant on days 6 to 12 ($P < 0.01$). (C and D) Representative lesions from B6 (C) and $C3^{-/-}$ (D) mice are shown at day 10 after infection. **, $P < 0.01$ (paired Student's *t* test).

than vv-VCPwt, lesions formed by vv-VCPwt and vv-VCPko in $C3^{-/-}$ mice were similar at all time points. Since both viruses formed lesions of similar size in mice lacking a central component of the complement cascade, this indicates that VCP contributes to pathogenesis in a complement-dependent fashion (Fig. 2B and D). Although vv-VCPwt and vv-VCPko formed lesions of similar sizes in $C3^{-/-}$ mice, development of lesions in these mice was associated with dermal erosion beginning on day 8 postinfection (Fig. 2D), which suggests that complete complement deficiency results in some immunopathology after VACV infection.

Expression of VCP leads to increased viral titers. To begin to understand how VCP's ability to inhibit complement contributes to the difference in pathogenesis of vv-VCPwt and vv-VCPko, we examined viral titers in the ears after infection. Titers for ears from wild-type B6 mice infected with vv-VCPwt or vv-VCPko were determined on days 4, 6, 8, and 12 postinfection. There was no difference in viral titer at day 4 (Fig. 3) or day 6 (data not shown), suggesting that complement activation does not contribute to early control of viral replication in this model of poxvirus infection. However, at days 8 and 12, B6 mice infected with vv-VCPko had significantly lower titers of virus in the ear (Fig. 3A), indicating that these mice control viral replication faster than those infected with vv-VCPwt.

In contrast to B6 mice, viral titers in $C3^{-/-}$ mice were similar at all time points following infection with vv-VCPwt and vv-VCPko (Fig. 3B). While titers in B6 mice infected with vv-VCPwt decreased between day 8 and day 12, titers in $C3^{-/-}$ remained high (Fig. 3), suggesting that these mice have a reduced ability to control viral replication.

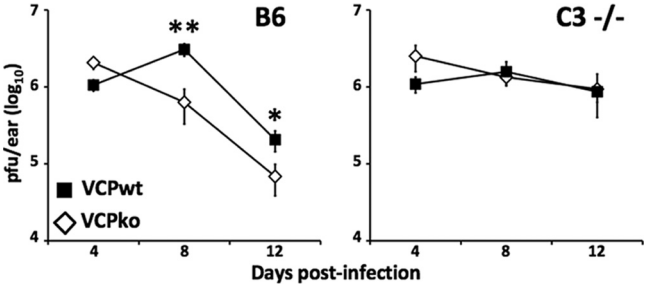

FIG. 3. Kinetics of viral titers in the ear. Ears from B6 or $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko were harvested at the indicated time points, and virus titers were determined ($n = 8$ ears/virus/time point for B6 mice; $n = 6$ for $C3^{-/-}$ mice on day 4; $n = 8$ for $C3^{-/-}$ mice on days 8 and 12). **, $P < 0.01$; *, $P < 0.05$. Data for B6 mice are representative of two independent experiments that included three to four mice per group. Data for $C3^{-/-}$ mice represent combined data from two experiments that included two to four mice per group.

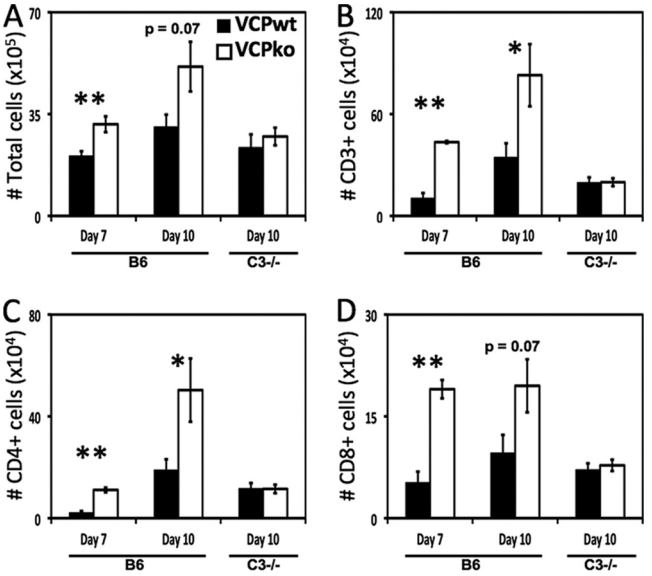

FIG. 4. Infection with vv-VCPko results in increased accumulation of T cells at the site of infection. Ears from B6 mice ($n = 4$ mice/group) or $C3^{-/-}$ mice ($n = 3$ mice/group for vv-VCPwt and 4 mice/group for vv-VCPko) infected with vv-VCPko or vv-VCPwt were harvested and cells isolated. Right and left ears from individual mice were pooled, but mice were analyzed separately. Viable cells were identified by trypan blue exclusion and counted on a hemacytometer (total cells, shown in panel A). Cells were stained for viability and T cell markers as described in Materials and Methods, and the frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ cells were determined using flow cytometry. Average numbers of total (A), $CD3^+$ (B), $CD4^+$ (C), and $CD8^+$ (D) cells are shown. Error bars represent standard errors of the means. **, $P < 0.01$; *, $P < 0.05$ (Student's t test). Results are representative of four independent experiments with B6 mice and three independent experiments with $C3^{-/-}$ mice.

VCP limits accumulation of T cells at the site of infection. Control of viral replication in mice infected with vv-VCPko coincides temporally with activation of adaptive immune responses. Although primary VACV infection can be cleared by antibody alone, $CD8^+$ T cells can mediate protection and are essential for controlling VACV infection in the absence of antibody (1, 60). Because complement has been shown to affect $CD4^+$ and $CD8^+$ T cell responses to several viruses (10, 23, 24, 37, 50), we compared numbers of T cells in the ears of mice infected with vv-VCPwt versus vv-VCPko (Fig. 4). Following intradermal infection with VACV, T cells are recruited to the site of infection by day 7 postinfection, and these responses peak at day 10 (22). To determine if VCP affected T cell responses at the site of infection, we used flow cytometry to examine accumulation of T cells in the ear on day 7 and day 10 after infection with vv-VCPko or vv-VCPwt. Infection with vv-VCPko was associated with increases in total numbers of cells (Fig. 4A) and in total numbers of $CD3^+$ cells (Fig. 4B) on both day 7 and day 10 after infection. On day 7 postinfection, vv-VCPko-infected mice had a 5-fold increase in $CD4^+$ T cells (Fig. 4C) and a 4-fold increase in $CD8^+$ T cells (Fig. 4D) in the ear. On day 10 after infection, mice infected with vv-VCPko had 2-fold increases in both $CD4^+$ and $CD8^+$ T cells (Fig. 4C and D). This finding demonstrates that in the absence of VCP, total numbers of $CD4^+$ and $CD8^+$ T cells are increased. Importantly, increased numbers of T cells at the site of vv-VCPko infection also coincided with decreases in viral titers in the ears of mice infected with this virus, suggesting that enhanced T cell accumulation may contribute to better control of viral replication. In contrast to B6 mice, $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko had similar numbers of $CD4^+$ and $CD8^+$ T cells in the ear at day 10 postinfection (Fig. 4). Taken together, these results show that VCP limits accumulation of $CD4^+$ and $CD8^+$ T cells at the site of infection in a complement-dependent manner.

Expression of VCP does not affect T cell priming or expansion. Complement is necessary for optimal T cell priming and expansion following infection with several viruses, including influenza virus (24), WNV (37), and LCMV (10). Thus, it is possible that the enhanced T cell response observed in the ear following infection with vv-VCPko is due to greater T cell expansion in the lymph node. To address this possibility, we used MHC class I tetramers specific for the VACV immunodominant peptide $B8_{20–27}$ (51) to monitor the antigen-specific T cell responses in the draining lymph nodes of mice infected with vv-VCPwt or vv-VCPko. We found no differences in the percentages or total numbers of B8-specific $CD8^+$ T cells at day 5 (Fig. 5A). On day 7, we observed only a small difference in frequency but not in total number of tetramer-positive (tet^+) $CD8^+$ T cells at day 7 (Fig. 5A and B). Since day 7 represents the peak of the anti-VACV T cell response in the draining LN, this result suggests that VCP has minimal effects on T cell priming and expansion. On day 10 postinfection, we found that the draining LN of mice infected with vv-VCPko had significantly lower percentages but only modestly reduced total numbers of B8-specific $CD8^+$ T cells than vv-VCPwt-infected mice (Fig. 5A and B). To confirm this result, we also restimulated T cells from infected mice with $B8_{20–27}$ and examined production of IFN-γ by $CD8^+$ T cells on day 10 after infection. We found that mice infected with vv-VCPko also had modestly reduced frequencies and total numbers of IFN-γ-positive $CD8^+$ T cells in the draining lymph nodes (Fig. 5C and D). Thus, mice infected with vv-VCPko have increased numbers of $CD8^+$ T cells at the site of infection and slightly reduced numbers of antigen-specific $CD8^+$ T cells in the draining LN. It is possible that this finding may reflect differences in

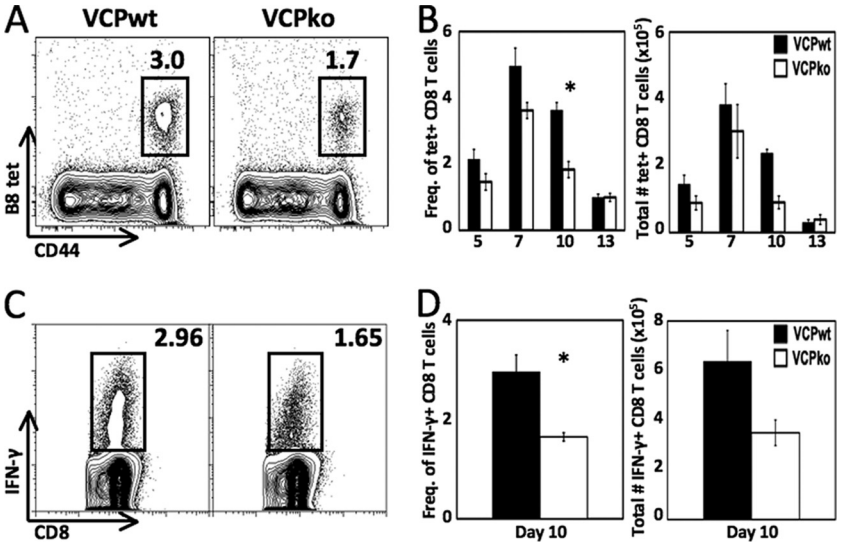

FIG. 5. VCP does not alter T cell priming in the draining lymph node. Cells were isolated from dLN of mice infected with either vv-VCPwt or vv-VCPko on the indicated days after infection and stained directly *ex vivo* with VACV tetramer specific for $B8_{20–27}$. (A) FACS plots gated on $CD8^+$ T cells display representative data from day 10 postinfection, showing differences in the frequencies of tet^+ CD8 T cells. Numbers are the percentages of tet^+, $CD44^+$ CD8 T cells and are representative of two experiments (three to four mice per group). (B) Plots gated on $CD8^+$ T cells and percentages representing the frequencies of IFN-γ^+ $CD8^+$ T cells. The graphs display the frequencies and total numbers of tet^+ $CD8^+$ T cells on the indicated days. (C) FACS plots gated on $CD8^+$ T cells, showing frequencies of IFN-γ^+ CD8 T cells in dLN on day 10 postinfection. (D) Graphs displaying the average frequencies and total numbers of IFN-γ^+ CD8 T cells in the dLN on day 10 postinfection. Data are representative of four experiments with three to five mice per group.

T cell trafficking or retention of T cells at the site of infection in mice infected with vv-VCPko. The modest differences observed in the $CD8^+$ T cell responses in the dLN suggest that VCP does not dramatically impact T cell priming. Defining the precise mechanism by which VCP limits T cell responses will require further investigation.

Infection with vv-VCPko induces better neutralizing Ab responses. Ab responses are important for clearance of primary VACV infection (1, 60). Further, complement activation is important for the development of optimal IgM and IgG antibody responses to several viruses (7, 38, 44). Therefore, we examined the effect of VCP expression on the development of anti-VACV IgM and IgG Ab responses by using a VACV-specific ELISA to measure levels of anti-VACV Abs following infection of B6 mice with vv-VCPwt and vv-VCPko. Infection with vv-VCPko resulted in significantly increased titers for both the IgM and IgG Ab responses on days 10 and 13 after infection compared to infection with vv-VCPwt (Fig. 6A and B).

To demonstrate the biological importance of this difference in IgM and IgG, we measured anti-VACV neutralizing Ab (nAb) responses by determining the ability of sera from infected mice to neutralize virus in an *in vitro* plaque reduction assay. Sera from B6 mice infected with vv-VCPko or vv-VCPwt had similar neutralization abilities at day 5 postinfection (Fig. 6C). However, on day 7 and day 10, sera from mice infected with vv-VCPko more effectively neutralized virus. By day 13, serum from mice infected with either vv-VCPwt or vv-VCPko was able to neutralize virus almost completely (Fig. 6C). The kinetics that we observed for nAb responses are consistent with a recent study examining early IgM and IgG responses in VACV-immunized mice (41). Although we did not observe differences in the levels of Ab by ELISA at times prior to day 10 (Fig. 6A and B), serum from mice infected with vv-VCPko was better able to neutralize virus on day 7 after infection (Fig. 6C). Thus, in addition to an early dampening of the magnitude of the anti-VACV Ab response, it is possible that VCP affects other aspects of the Ab response, such as Ab affinity. Importantly, the increase in nAb in sera from vv-VCPko-infected mice on day 7 coincided with the decrease in viral titer we saw in these mice (Fig. 3A), suggesting that enhanced nAb responses may contribute to control of viral replication. These differences in neutralization were not seen with samples taken from $C3^{-/-}$ mice. Sera from $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko had equivalent abilities to neutralize virus at all time points tested (Fig. 6C), demonstrating that the enhanced nAb response in B6 mice infected with vv-VCPko is dependent on complement.

$CD8^+$ T cells and $CD4^+$ T cells contribute to attenuation of vv-VCPko. The data presented so far suggest that expression of VCP by vv-VCPwt limits recruitment of T cells to the site of infection and dampens nAb responses. Since Th-dependent Ab responses and $CD8^+$ T cells can individually mediate protection against VACV (1, 60), the ability of VCP to modulate both of these responses may contribute to the attenuation of vv-VCPko. The Ab response to poxviruses is almost completely Th dependent (12, 47, 60). Therefore, depleting $CD4^+$ T cells also eliminates Ab responses (12, 47, 60). To address the contributions of enhanced nAb and $CD8^+$ T cell responses during infection with vv-VCPko, we depleted mice of $CD8^+$ and $CD4^+$ T cells individually or together and examined lesion sizes (Fig. 7A and B) and viral titers (Fig. 7C) following infection with vv-VCPwt or vv-VCPko. When mice were depleted of $CD4^+$ or $CD8^+$ T cells

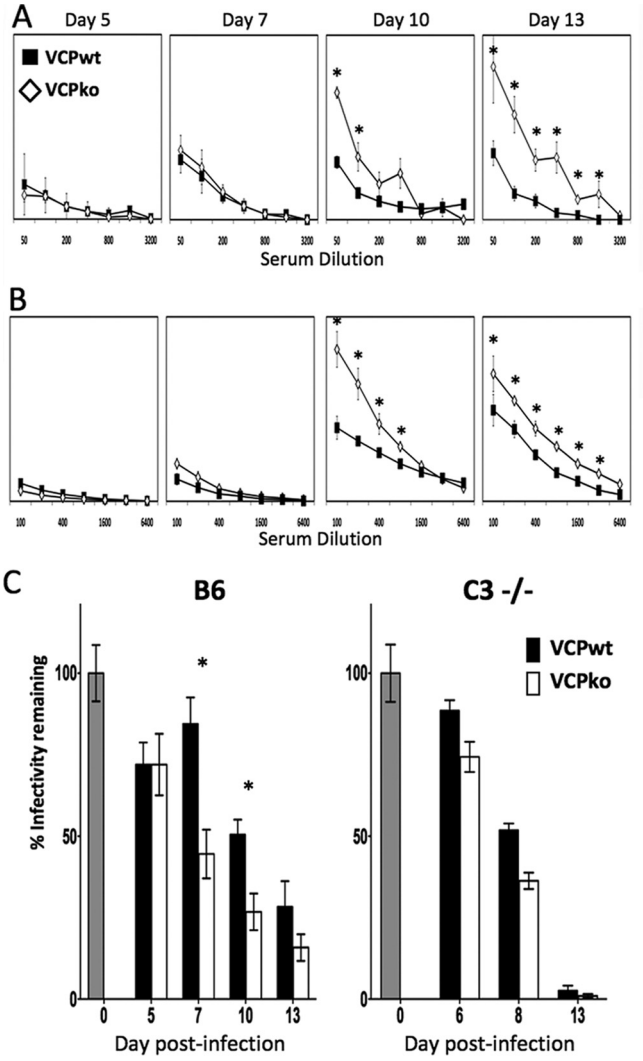

FIG. 6. Infection with vv-VCPko results in increased neutralizing Ab responses. The amount of VACV-specific IgM (A) or IgG (B) in sera from wild-type B6 mice infected with vv-VCPwt or vv-VCPko was determined by ELISA with VACV-infected cell lysate. Graphs represent average values ± standard errors of the means for heat-inactivated sera from individual mice ($n = 4$ mice/virus/time point) at the indicated dilutions. (C) The ability of sera to neutralize MV was tested by using a standard plaque reduction neutralization assay. Wild-type B6 or $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko were bled on the indicated days. Heat-inactivated serum from individual mice was incubated with MV for 2 h, and virus titers were determined on BSC-1 cells. Graphs represent percentages of infectivity remaining after incubation with serum, ± standard errors of the means. Values for infected mice are normalized to heat-inactivated serum pooled from eight naïve mice and were analyzed in triplicate. Statistical significance was determined using a paired Student's *t* test. *, $P < 0.05$. Results are representative of two similar experiments.

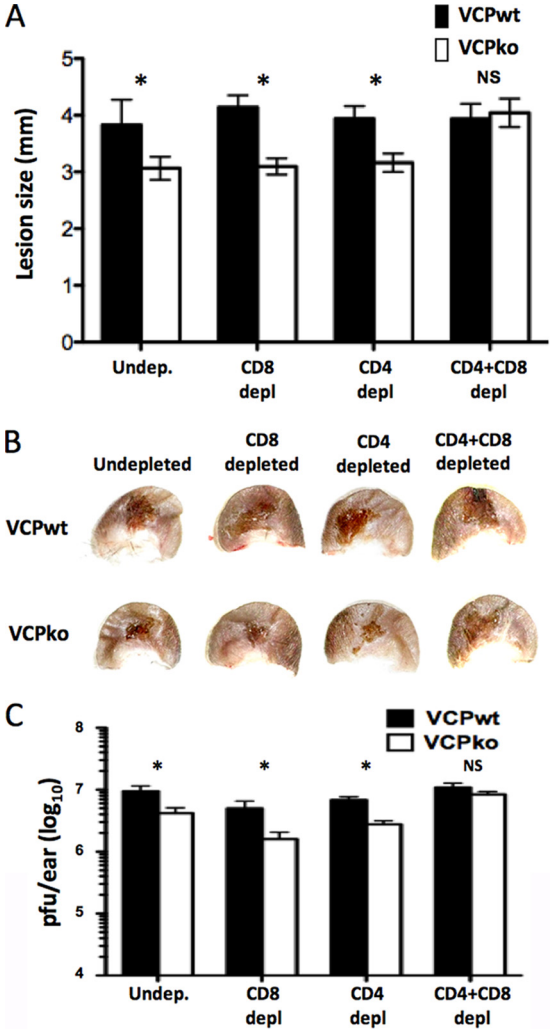

FIG. 7. Depletion of $CD4^+$ and $CD8^+$ T cells together, but not individually, restores virulence of vv-VCPko. Mice were depleted of $CD4^+$ and or $CD8^+$ T cells prior to infection with vv-VCPwt or vv-VCPko, and lesion sizes were monitored. (A) Average lesion sizes on day 8 postinfection ± standard errors of the means. (B) Photomicrographs showing lesions that represent the median lesion sizes of each group. (C) Average viral titers in the ear on day 8 postinfection ± standard errors of the means ($n = 4$ mice/group). *, $P < 0.05$. Results are representative of two independent experiments each testing 4 mice/group.

individually, infection with vv-VCPko was still associated with formation of smaller lesions. However, when $CD4^+$ and $CD8^+$ T cells were depleted together, vv-VCPko-infected mice developed lesions that were the same size as in mice infected with vv-VCPwt (Fig. 7A and B) and had similar viral titers in the ear on day 8 postinfection (Fig. 7C). As has been demonstrated in several previous studies (12, 47, 60), depletion of $CD4^+$ T cells was associated with an absence of VACV-specific Ab responses (data not shown).

Taken together, these data show that depletion of both $CD4^+$ and $CD8^+$ T cells restores the virulence of vv-VCPko, and they strongly suggest that VCP contributes to pathogenesis by limiting both Ab and T cell responses.

Immunization with vv-VCPko provides enhanced protection against challenge. Vaccination with VACV provides protection against subsequent infection with antigenically related poxviruses, including variola virus (VARV), monkeypox virus (MPXV), and ectromelia virus (ECTV) (13). Protection against secondary poxvirus infection is primarily mediated by Abs, but $CD8^+$ T cell responses also contribute to control of viral replication and dissemination (1, 8, 11, 12, 33, 34, 60, 61).

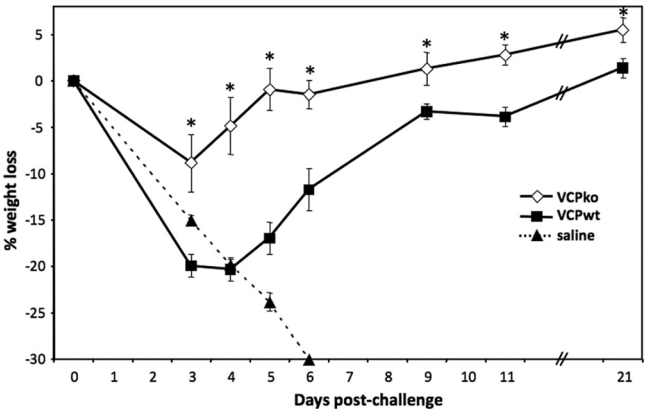

FIG. 8. Increased protection from lethal challenge in mice vaccinated with vv-VCPko. Mice were immunized in the ear pinna with 2×10^4 PFU of vv-VCPwt, vv-VCPko, or saline ($n = 5$ mice/group). Thirty days after immunization mice were challenged i.n. with 1×10^7 PFU of strain WR. Results show average weight loss following challenge ± the standard errors of the means and are representative of two experiments. *, $P < 0.05$.

Since primary infection with vv-VCPko induced stronger Ab and T cell responses, we hypothesized that it may also provide better protection against lethal challenge when used to vaccinate mice. To test this, we immunized mice by the intradermal route by injecting a total of 4×10^4 PFU of vv-VCPwt or vv-VCPko into the ear pinnae. Immunized mice were challenged 30 days later, and weight loss was monitored. Mice that had been immunized with vv-VCPko lost less weight and recovered more rapidly than mice immunized with vv-VCPwt (Fig. 8). This suggests that immunization with vv-VCPko increases protective immunity. Since vv-VCPko is also attenuated during primary infection, viruses that do not express VCP may serve as safer, more effective vaccine vectors.

DISCUSSION

VCP has previously been shown to increase VACV virulence in intradermal models of VACV infection in rabbits and guinea pigs (20). Our data recapitulate this finding but also extend our understanding of VCP's role in pathogenesis. We show here that VCP limits protective adaptive immune responses to VACV by dampening nAb responses and limiting accumulation of $CD4^+$ and $CD8^+$ T cells at the site of infection, resulting in increased viral replication. Importantly, the use of $C3^{-/-}$ mice showed that these effects of VCP are dependent on an intact complement system, and thus they demonstrate for the first time that pathogen-encoded complement regulatory proteins can modulate adaptive immunity in a complement-dependent manner.

Complement activation can contribute to direct and indirect control of viral replication by opsonizing and/or lysing viral particles or infected cells and by enhancing adaptive immune responses. To begin to understand how VCP contributes to virulence *in vivo*, we first examined the kinetics of viral replication. We observed that mice infected with vv-VCPko controlled viral replication better than mice infected with vv-VCPwt, but only at the later time points examined (days 8 and 12); viral titers were similar at day 4 (Fig. 2) and day 6 (data not shown). The observation that vv-VCPko-infected mice exhibited better viral control only late in infection suggests that VCP contributes to pathogenesis by limiting adaptive immune responses to VACV. Indeed, we found that vv-VCPko-infected mice had increased numbers of $CD4^+$ and $CD8^+$ T cells at the site of infection (Fig. 3) and enhanced nAb responses (Fig. 4) by day 7 postinfection. These results are consistent with several viral infection models that have demonstrated that complement contributes to control of viral infection by inducing adaptive immune responses (10, 23, 24, 37, 38, 44, 50, 55, 56).

A recent study using ECTV to examine the role of complement in poxvirus infection showed that infection of $C3^{-/-}$ mice with ECTV resulted in elevated viral titers as early as day 2 in the spleen and day 4 in the liver and the blood (40). In contrast to that study, we found that at such early time points there was no difference in viral titers in B6 or $C3^{-/-}$ mice infected with vv-VCPwt or vv-VCPko. However, one key difference in the ECTV model is that this mouse-specific virus replicates extensively in the mouse and spreads to the draining lymph nodes and then through the blood to seed the liver and the spleen. In contrast to ECTV, after intradermal inoculation, VACV spreads relatively poorly in mice and remains localized to the dermis (52). The blood contains very high levels of complement, leading to rapid opsonization of pathogens and enhancement of complement receptor-mediated recognition and phagocytosis (18). Thus, while complement limits early viral dissemination via the blood in a systemic model of poxvirus infection, our results indicate that it may not contribute to initial control of localized VACV replication in the intradermal model.

Although we show that VCP limits accumulation of T cells at the site of infection in a complement-dependent manner, our studies do not define the mechanism by which VCP affects T cell responses. However, our findings are consistent with several models of viral infection that have demonstrated that $C3^{-/-}$ mice have decreased numbers of T cells at sites of viral infection, including the lungs of mice infected with influenza virus (24) and the brains of mice infected with WNV (37). In both the influenza virus and WNV models, defects in T cell accumulation at sites of infection correlate with reduced T cell priming and/or proliferation (24, 37). However, when we examined VACV-specific T cell responses in the dLN of infected mice, we found only very slight differences in the CD8 T cell responses to vv-VCPko and vv-VCPwt at day 5 and day 7. This result suggests that complement may not contribute to T cell priming and expansion in our infection model. We did observe a difference in CD8 T cell responses on day 10 postinfection; at this time point, mice infected with vv-VCPko had fewer tet^+ and IFN-γ^+ CD8 T cells in the dLN. It is possible that VCP alters T cell trafficking to or retention at the site of infection. Although complement has been shown to affect T cell priming in numerous models, it can also influence T cell responses in other ways. For instance, C3 deficiency has been shown to alter lymphocyte survival (49). Furthermore, inhibiting the C5a receptor can limit recruitment of T cells to the lungs of mice infected with influenza virus without affecting T cell priming (23). Thus, there are multiple alternative mechanisms by which VCP may limit CD8 T cell responses during VACV infection. One possibility is that VCP suppresses local complement activation, and by doing so dampens inflammatory responses at the

site of infection, leading to decreased infiltration of leukocytes. This possibility is consistent with a previous study showing that the cowpox ortholog of VCP, inflammation modulatory protein (IMP), reduces recruitment of inflammatory cells to the site of infection (39). Furthermore, mammalian complement regulatory proteins have also been shown to limit accumulation of leukocytes at sites of inflammation. For instance, administering soluble complement receptor 1 (sCR1) to mice has been shown to limit T cell-mediated delayed-type hypersensitivity responses in skin by limiting the influx of T cells to the site of antigen challenge (53, 54).

Recent efforts to better define the antiviral functions of Abs during poxvirus infection have revealed an important role for complement in Ab-mediated protection (2, 3). Benhnia et al. showed that monoclonal Abs (MAbs) specific for the EV protein B5 that are able to initiate complement-mediated lysis of infected cells and complement-dependent neutralization of EV particles *in vitro* provide better protection against VACV challenge following passive immunization (2, 3). Furthermore, the enhanced protection provided by these MAbs *in vivo* was reduced when mice that had been passively immunized were depleted of complement prior to challenge, demonstrating that enhanced protection depended on the ability of MAbs to activate complement (3). Our data demonstrate that VCP limits the early nAb antibody response, but it is also likely that VCP reduces complement-mediated neutralization by nAbs that are produced. The ability of VCP to dampen the magnitude of the nAb response and limit the effector functions of Ab that are produced may both contribute to VCP's role in pathogenesis. These findings may explain why viral titers in $C3^{-/-}$ mice remain high on day 12. T cell accumulation in $C3^{-/-}$ mice is reduced but is not absent (Fig. 3). $C3^{-/-}$ mice also develop nAb responses, although with somewhat delayed kinetics (Fig. 4). Thus, it is possible that the combination of decreased T cell accumulation, reduced nAb production, and the inability of these Abs to mediate complement-dependent neutralization and lysis due to the absence of C3 may contribute to an inability of these mice to control viral replication.

VCP primarily prevents complement-mediated neutralization of Ab-sensitized virions *in vitro* (19, 21). The observation that mice infected with vv-VCPko have lower viral titers only at later times during infection may also indicate that VCP limits VACV neutralization after induction of virus-specific IgM and IgG. It was recently shown that natural IgM Abs can bind to poxviruses and initiate the classical pathway in the absence of specific IgM and IgG (40). However, since VCP is not present until infected cells begin secreting proteins, it is possible that early in infection there is not enough VCP to prevent complement-enhanced neutralization by natural IgM, rendering vv-VCPwt and vv-VCPko equally susceptible to complement-mediated neutralization early in infection.

Protection against primary VACV infection is dependent on Ab responses but can also be mediated by $CD8^+$ T cells if humoral responses are absent (1, 60). To determine whether enhanced $CD8^+$ T cell responses or Ab responses contributed to the attenuation of vv-VCPko, we depleted $CD4^+$ and $CD8^+$ T cells (Fig. 5). $CD4^+$ T cell help is critical for induction of VACV-specific Abs, and depleting these cells abrogates IgM and IgG Ab responses to the virus (1, 47, 60). When either $CD4^+$ or $CD8^+$ T cells were depleted individually, vv-VCPko still formed smaller lesions and had reduced viral titers. However, when both of these subsets were depleted together, vv-VCPko formed lesions that were the same size as those formed by vv-VCPwt and replicated to a similar level (Fig. 5). Based on our findings, we propose that when $CD8^+$ T cells are depleted, enhanced nAb responses induced by infection with vv-VCPko are sufficient to reduce viral titers. Similarly, when only $CD4^+$ T cells are depleted, which also abrogates virus-specific Ab responses, increased accumulation of $CD8^+$ T cells at the site of vv-VCPko infection reduces viral titers. This model is consistent with several studies that have shown that protection against VACV is primarily Ab mediated but can be mediated by $CD8^+$ T cells if Ab is absent (1, 60). This model also implies that the ability of VCP to modulate $CD8^+$ T cell responses without also dampening Ab responses would not increase pathogenesis, since loss of either Ab or $CD8^+$ T cells alone did not restore virulence of vv-VCPko (Fig. 5). An important caveat of our study is that we have not directly addressed the role of Ab; in our experiments loss of Ab is secondary to the absence of $CD4^+$ T cells. Providing help to B cells is the primary function attributed to $CD4^+$ T cells during VACV infection, since mice lacking $CD4^+$ T cells have the same phenotype as B cell-deficient mice (60). However, $CD4^+$ T cells have additional functions beyond providing B cell help, including production of proinflammatory cytokines. It is presently unclear whether $CD4^+$ T cells have direct antiviral activity during VACV infection, although it is possible that they represent an important source of proinflammatory cytokines during intradermal infection. Thus, it may be possible that depletion of both $CD4^+$ and $CD8^+$ T cells eliminates two major sources of inflammatory cytokines, leading to increased viral replication.

Following i.p. or i.n. infection, VACV can be cleared by either Th-dependent Ab responses or $CD8^+$ T cells independently (1, 60). Our data suggest that during an intradermal infection, the decrease in viral titers in vv-VCPko-infected mice at day 8 is due to enhanced nAb and/or $CD8^+$ T cells. Furthermore, these responses can control viral replication independently, since elimination of either one alone is insufficient to restore virulence of vv-VCPko (Fig. 5). This suggests that i.d. VACV infection is controlled by similar mechanisms as are i.p. and i.n. infections.

Interestingly, although depletion of $CD4^+$ and $CD8^+$ T cells restored virulence of vv-VCPko, depletion of both of these subsets did not further increase the virulence of vv-VCPko and vv-VCPwt at day 8 postinfection; lesions sizes and viral titers in mice depleted of $CD4^+$ and $CD8^+$ T cells were similar to those in intact mice infected with vv-VCPwt (Fig. 5). Several studies have shown that the amount of VACV replication that can occur in skin is limited. This has been consistently reported for i.d. infection in the ear pinna (52) and in flank skin (28). Thus, the level of viral replication observed for vv-VCPwt in undepleted mice may represent the maximum level of viral replication that can occur in skin. This concept is also consistent with the observation that viral replication is sustained in $C3^{-/-}$ mice as late as day 12 after infection but is not associated with an increase in viral titer beyond levels observed at the peak of viral replication (Fig. 2).

In addition to its effects on the adaptive immune response to primary VACV infection, we have also shown that vaccination

with vv-VCPko provides better protection against lethal VACV challenge than vaccination with vv-VCPwt. While it may seem counterintuitive that infection with vv-VCPko, which results in lower viral replication, would provide better vaccine-induced immunity, our data suggest that increased complement activation due to the absence of VCP enhances Ab and T cell responses, resulting in better protection. Defining the precise mechanisms by which vaccination with vv-VCPko enhances protective immunity will be an interesting area of future investigation.

The ability of VCP to limit adaptive immune responses to VACV may have implications for infection with other orthopoxviruses that cause human disease. Both VARV and MPXV express orthologs of VCP that have been shown to regulate complement activation *in vitro* (30–32, 46, 48). The MPXV homolog of VCP (known as MoPICE) is thought to contribute to virulence because it is expressed by the more virulent Central African strain of this virus but is not found in the less pathogenic West African strain (6, 29). Additionally, complement-deficient mice succumb to infection with ECTV (40), demonstrating the importance of complement in protection against pathogenic poxvirus infection. In light of our results, it would be interesting to determine whether the MPXV and ECTV VCP orthologs (MoPICE and EMICE, respectively) also affect adaptive immune responses to these viruses.

VACV serves as a useful model for assessing the function of poxvirus immunomodulatory proteins *in vivo*, and the intradermal model of infection mimics human intradermal vaccination. However, it should be noted that VACV is not a natural mouse pathogen and causes milder disease in mice than ECTV, which causes lethal smallpox-like disease in mice. Therefore, it will be particularly interesting to examine the effects of deletion of the ECTV complement regulatory protein on pathogenesis in the mousepox infection model.

Our findings are consistent with several studies that have demonstrated that complement activation contributes to control of viral infection by modulating T cell and Ab responses. However, none of these studies addressed the ability of pathogen-encoded complement regulatory proteins to modulate adaptive immune responses during infection. We have shown that VCP contributes to virulence in a complement-dependent manner and that expression of this protein by VACV decreases T cell accumulation at the site of infection and dampens neutralizing Ab responses.

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