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Vaccine-Induced Protection against Orthopoxvirus Infection Is Mediated through the Combined Functions of CD4 T Cell-Dependent Antibody and CD8 T Cell Responses

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ABSTRACT

Antibody production by B cells in the absence of CD4 T cell help has been shown to be necessary and sufficient for protection against secondary orthopoxvirus (OPV) infections. This conclusion is based on short-term depletion of leukocyte subsets in vaccinated animals, in addition to passive transfer of immune serum to naive hosts that are subsequently protected from lethal orthopoxvirus infection. Here, we show that CD4 T cell help is necessary for neutralizing antibody production and virus control during a secondary ectromelia virus (ECTV) infection. A crucial role for CD4 T cells was revealed when depletion of this subset was extended beyond the acute phase of infection. Sustained depletion of CD4 T cells over several weeks in vaccinated animals during a secondary infection resulted in gradual diminution of B cell responses, including neutralizing antibody, contemporaneous with a corresponding increase in the viral load. Long-term elimination of CD8 T cells alone delayed virus clearance, but prolonged depletion of both CD4 and CD8 T cells resulted in death associated with uncontrolled virus replication. In the absence of CD4 T cells, perforin- and granzyme A- and B-dependent effector functions of CD8 T cells became critical. Our data therefore show that both CD4 T cell help for antibody production and CD8 T cell effector function are critical for protection against secondary OPV infection. These results are consistent with the notion that the effectiveness of the smallpox vaccine is related to its capacity to induce both B and T cell memory.

IMPORTANCE

Smallpox eradication through vaccination is one of the most successful public health endeavors of modern medicine. The use of various orthopoxvirus (OPV) models to elucidate correlates of vaccine-induced protective immunity showed that antibody is critical for protection against secondary infection, whereas the role of T cells is unclear. Short-term leukocyte subset depletion in vaccinated animals or transfer of immune serum to naive, immunocompetent hosts indicates that antibody alone is necessary and sufficient for protection. We show here that long-term depletion of CD4 T cells over several weeks in vaccinated animals during secondary OPV challenge reveals an important role for CD4 T cell-dependent antibody responses in effective virus control. Prolonged elimination of CD8 T cells alone delayed virus clearance, but depletion of both T cell subsets resulted in death associated with uncontrolled virus replication. Thus, vaccinated individuals who subsequently acquire T cell deficiencies may not be protected against secondary OPV infection.

The vaccination campaign that culminated in eradication of smallpox is one of the most successful public health endeavors of modern medicine. The success of the smallpox vaccine is largely due to its being a live-virus vaccine that induces both cell-mediated and humoral immunity. Our understanding of immunity to smallpox in humans comes largely from prospective studies of the response to vaccinia virus (VACV) vaccination in humans (1–6) and from animal studies using closely related orthopoxviruses (OPV), such as VACV (7, 8), monkeypox virus (MPXV) (9–11), and ectromelia virus (ECTV) (12–15). ECTV is a natural mouse pathogen that causes mousepox, a disease very similar to smallpox, and undoubtedly one of the best small-animal models available for investigating immunity to and pathogenesis of OPV infections (12–14, 16, 17).

Virus control and recovery from primary OPV infections (17–19) or VACV vaccination (20–24) require both CD4 T cell-dependent antibody responses and effector T cell function. However, while antibody is also critical for protection against secondary OPV infection following vaccination, the role of T cells has been unclear. We and others have previously shown that control of OPV in vaccinated animals is dependent on neutralizing antibody, but not on CD4 or CD8 T cells (7, 25–27). Depletion of CD4, CD8, or both T cell subsets with monoclonal antibody (MAb) in vaccinated mice did not increase viral titers or reduce neutralizing antibody responses during a secondary challenge with ECTV (25, 27). Furthermore, neither the neutralizing antibody response nor virus control was shown to be affected by elimination of CD4 or CD8 T cells during secondary challenge in VACV-vaccinated macaques (26). In addition, passive transfer of immune serum to

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naive macaques was shown to protect against lethal MPXV infection (26). Finally, in experiments using replication-deficient VACV for vaccination of mice, antibody was found to be essential to protect against VACV-induced disease after secondary challenge, whereas CD4 or CD8 T cells were not required (7). Together, these studies suggested that antibody production by B cells alone is necessary and sufficient for protection against secondary OPV infections.

Generation of effective, high-affinity antibodies against most viral antigens is dependent on CD4 T cell help (28, 29). In the absence of CD4 T cell help, antibody of lower affinity is produced by extrafollicular antibody-secreting cells (ASC) without involving a germinal center (GC) response. T follicular helper (T_{FH}) cells, a specialized subset of CD4 T cells that provide help to cognate B cells, are necessary for GC formation and for GC B cells to proliferate and persist in GCs (30–34). GC B cells undergo somatic hypermutation, affinity maturation, and selection to produce high-affinity antibodies (30–33, 35). B cells that exit the GC become long-lived ASC or memory B cells. Long-lived ASC are terminally differentiated and continuously produce antibody without antigenic restimulation, whereas memory B cells differentiate into ASC on reencounter with antigen during a secondary infection (36). The proposition that CD4 T cells are not required for protection from secondary OPV challenge in vaccinated hosts suggests that persisting long-lived ASC, as well as any new extrafollicular ASC responses, are sufficient for protection.

The caveat for the mousepox (27), MPXV (26), and VACV (7) studies is that T cell subsets were depleted only during the acute phase (days 4 to 12) of a secondary virus challenge. It is possible, then, that continued MAb treatment, beyond 12 days, might reveal a role for one or both T cell subsets. In addition, in the MPXV study (26), macaques vaccinated 6 months earlier were treated with anti-CD4 MAb only once during secondary virus challenge, allowing the possibility that CD4 T cell numbers rebounded during the course of the experiment. It is likely that a threshold of neutralizing antibody produced by extrafollicular ASC during the acute phase of the secondary response is sufficient to restrict viral replication. However, whether this antibody alone is able to completely clear virus if CD4 T cell elimination is prolonged beyond the acute phase of virus challenge is not known. A related question is whether in the prolonged absence of CD4 T cells, CD8 T cells play any role in virus control, since no role for CD8 T cell function has been ascertained in an otherwise immunocompetent host. Finally, it is not clear whether the dose of virus encountered in a secondary challenge dictates the requirement for CD4 and/or CD8 T cells in virus control.

We show here that, although dispensable in the early stages of virus challenge, CD4 T cell help becomes necessary for sustained production of effective antiviral antibody response as the infection progresses. CD4 T cell help is necessary for expansion of GC B cells and virus-specific ASC with corresponding increases in virus-specific IgG and neutralizing antibody titers. In the absence of CD4 T cell help, antibody produced by extrafollicular ASC can contribute to the control of virus replication, but only in the presence of effector CD8 T cells. CD8 T cells play an important compensatory role in the control of virus in the absence of CD4 T cells, and vice versa. Importantly, prolonged depletion of both CD4 and CD8 T cell subsets results in 100% mortality due to uncontrolled virus replication.

MATERIALS AND METHODS

Viruses and cell lines. ECTV strain Moscow (ATCC VR 1374), referred to as ECTV-WT (wild type), and the thymidine kinase-deficient strain of ECTV-WT, designated ECTV-TK^{Δ} (37), were propagated as previously described (38). BS-C-1 cells (ATCC CCL26), used for virus stock preparation and viral plaque assays, were maintained in Eagle's minimum essential medium (Gibco) with 2 mM L-glutamine, antibiotics, and 10% fetal calf serum.

Mice. Female specific-pathogen-free C57BL/6J WT mice; gene knockout C57BL/6J mice deficient in perforin (Prf) (designated $Prf^{-/-}$) (39); and C57BL/6J mice deficient in Prf, granzyme A (GzmA), and GzmB (designated $Prf^{-/-}$ $GzmA^{-/-}$ $GzmB^{-/-}$) (27) were bred at the Australian National University Animal Breeding and Research Facility and used at 6 to 12 weeks of age. All animals were housed in individually ventilated cages, and experiments were performed in accordance with the recommendations of the Australian code of practice for the care and use of animals for scientific purposes and the Australian National Health and Medical Research Council Guidelines and Policies on Animal Ethics and approved by the Institutional Animal Ethics and Experimentation Committee.

Virus infection. For secondary-challenge experiments, mice were first immunized with 10^5 PFU of ECTV-TK^{Δ} given through the intraperitoneal (i.p.) route. Nine to 16 weeks later, the mice were anesthetized i.p. with tribromoethanol (200 to 240 mg/kg of body weight) and challenged with either 10^3 or 10^5 PFU of ECTV-WT in the flank of the left hind limb (hock). All animals were monitored daily for clinical signs of disease; weighed every 2 or 3 days; and, if they lost 25% of their original body weight, euthanized and recorded as dead the following day. The dose of ECTV-WT that results in a 50% lethal dose (LD_{50}) in naive C57BL/6 mice is 10^6 PFU (40), but doses of <1 LD_{50} (10^3 or 10^5 PFU) were used in this study to minimize lethality.

Leukocyte subset depletion and flow cytometry. Mice were primed and challenged as described above. Every alternate day starting from day −1 until either day 19 or day 34 postchallenge (p.c.), depending on the experiment, mice were injected i.p. with vehicle (phosphate-buffered saline [PBS]) or 0.5 to 1 mg MAb against CD4 T cells (clone GK1.5) and/or CD8 T cells (clone 2.43.1). Purified MAbs for *in vivo* depletion were purchased from BioXcell. The efficiency of cell subset depletion was assessed by flow cytometry and found to be routinely >98% for each population regardless of whether one or more antibodies were used for leukocyte depletion (data not shown). Flow cytometry was used to phenotype B cell subsets in the spleen. Splenocytes were first gated on live cells based on forward scatter and side scatter plots, and then, GC B cells were identified as B220-positive, Fas (CD95)-positive, and GL7-positive cells ($B220^+$ FAS^+ $GL7^+$) cells. Data were acquired on a BD LSR Fortessa flow cytometer with BD FACS Diva software and analyzed using FlowJo software version 9.5 (Tree Star, Inc.). All flow cytometry reagents were purchased from BD Biosciences and Biolegend.

Determination of viral load. Virus titers, expressed as $\log_{10}$ PFU per gram tissue, were determined on BS-C-1 monolayers using the conventional viral plaque assay, as described previously (13, 38). Low viral titers undetectable by viral plaque assay and the virus load in blood were measured by quantitative real-time PCR (qRT-PCR), as described previously (41). The viral genome copy number was correlated with the copy number of the late gene ECTV-Mos-156, which encodes the viral hemagglutinin (42). The limit of detection of viral genomes by qRT-PCR is 10 copies. One PFU of ECTV-WT is equivalent to 275 genome copies. Genome copy numbers above 10 are considered biologically significant, as the LD_{50} of ECTV-WT for the highly susceptible A/J mouse strain is 0.04 PFU, i.e., 11 genome copies. The LD_{50} of ECTV-WT for the resistant C57BL/6 mouse strain is 10^6 PFU (40).

PRNT. The plaque reduction neutralization test (PRNT), used to determine the virus-neutralizing activity of the antibody present in serum samples, was described previously (25, 27, 41). Sera were heat inactivated at 56°C for 30 min prior to use. The 50% plaque neutralization titer

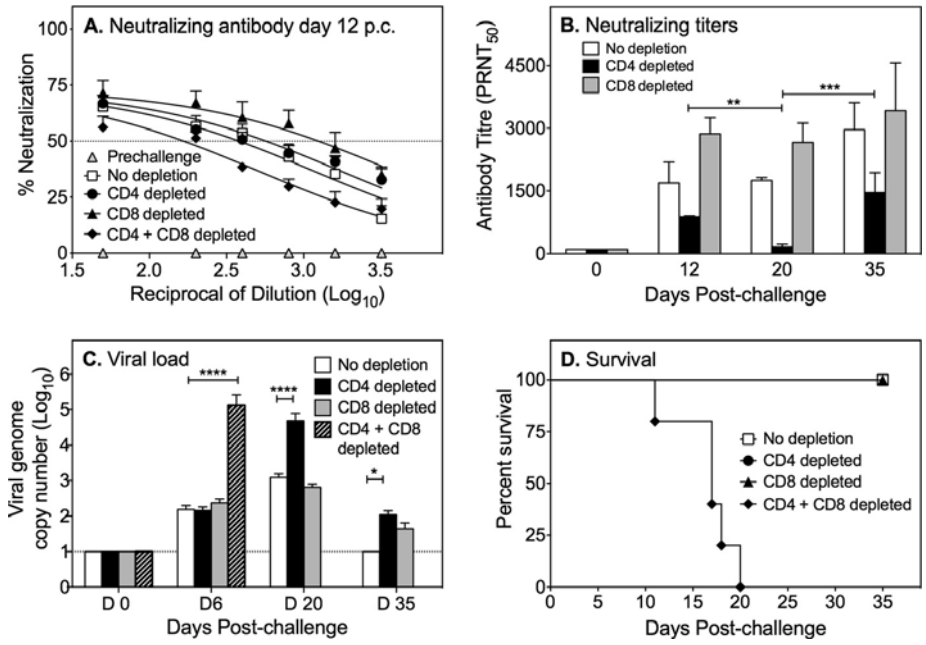

FIG 1 CD4 T cell-dependent neutralizing antibody titers inversely correlate with the viral load during secondary infection. (A) Virus-neutralizing activity in sera of mice 9 weeks after immunization with ECTV-TK$^{\Delta}$, 1 day before secondary challenge (Prechallenge), or 12 days after challenge with 10^3 PFU ECTV-WT (1,000-fold less than 1× LD_{50}) in groups depleted of CD4 T cells, CD8 T cells, or CD4 and CD8 T cells or with no depletion (No depletion). Mice were treated with anti-CD4, anti-CD8, or both MAbs every alternate day starting from day −1 until day 19 p.c. $P > 0.05$ for nondepleted compared with one or more T cell subset-depleted groups. Data shown are means and standard errors of the means (SEM). (B) $PRNT_{50}$s in sera of mice as in panel A, excluding those depleted of both T cell subsets, at 12, 20, and 35 days p.c. $P < 0.005$ for the nondepleted group compared with the CD4-depleted group (day 20); $P > 0.05$ for the nondepleted group compared with the T cell subset-depleted groups (day 35). **, $P < 0.01$, comparing titers in CD4-depleted groups at days 12 and 20 p.c.; ***, $P < 0.0005$, comparing titers in CD4-depleted groups at days 20 and 35 p.c. Data shown are means and SEM. (C) Virus genome copy numbers (means and SEM) in blood as measured by qRT-PCR. *, $P < 0.05$; ****, $P < 0.0001$. (D) Recovery of control mice (No depletion) and mice depleted of CD4, CD8, or both T cell subsets following challenge with 10^3 PFU virus. $P < 0.001$ between the CD4- and CD8-depleted group and all other groups. The results shown are from one of two independent experiments with 5 to 7 animals per group with similar outcomes.

($PRNT_{50}$) was taken as the reciprocal of the dilution of sera that caused a 50% reduction in the number of virus plaques compared with the number of plaques in the samples with sera from naive mice. The data were fitted with a nonlinear (four-parameter) function, and $PRNT_{50}$s were calculated by least-squares regression analysis.

ELISA and ELISPOT technique. ECTV-specific IgG and subtypes were quantified by enzyme-linked immunosorbent assay (ELISA), as described previously (25, 27, 41). The enzyme-linked immunospot assay (ELISPOT) for quantifying ECTV-specific ASC has been described in detail previously (41).

Statistical analysis. Statistical analyses of experimental data, as indicated, were performed using GraphPad Prism (GraphPad Software). A P value of <0.05 was taken to be significant. For the PRNT, data were fitted with a nonlinear (four-parameter) function, and $PRNT_{50}$ values were calculated by least-squares regression analysis. Individual interpolated $PRNT_{50}$ values were analyzed by 2-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) test for significance between groups. For the viral load, data were log transformed and analyzed by 2-way ANOVA, followed by Fisher's LSD test for significance between groups. Virus-specific IgG titers were analyzed by 2-way ANOVA, followed by Fisher's LSD test for significance between groups. The numbers of ASC in bone marrow and GC B cells in the spleen were analyzed by 1-way ANOVA, followed by Fisher's LSD test for significance between groups.

RESULTS

Neutralizing antibody responses rebound following cessation of anti-CD4 MAb treatment. To determine the effect of T cell subset depletion, up to and past the acute phase of the secondary virus challenge, we immunized mice with avirulent ECTV-TK$^{\Delta}$ (37). This immunization regime primes animals for robust and protective antiviral recall responses (25, 27, 43) comparable to those provided by VACV immunization (43). Nine to 16 weeks later, when memory was established, the mice were challenged with a sublethal dose of virulent ECTV-WT. One day prior to virus challenge, and every 2 days thereafter, the mice were treated with MAb to deplete CD4, CD8, or both T cell subsets. At 5 to 16 weeks postvaccination with ECTV-TK$^{\Delta}$, the neutralizing antibody titers are very low or below the level of detection. However, by 8 days p.c., high levels of neutralizing antibody are detectable (41).

At the time of secondary challenge (prechallenge), neutralizing antibody titers were not detected in vaccinated mice, but by day 12 p.c., they increased rapidly and substantially in all groups (Fig. 1A). At this time, the virus-neutralizing antibody titers were similar between control and CD4 T cell-depleted groups. However, continued depletion of CD4 T cells over 19 days resulted in a significant reduction in $PRNT_{50}$s, measured at day 20 p.c., compared to the control group (Fig. 1B), but the drop was nowhere near the neutralizing titers at the time of prechallenge, which were minimal (Fig. 1A). Cessation of anti-CD4 MAb treatment after day 19 p.c. resulted in a rebound of CD4 T cell numbers (not shown) and a concurrent increase in neutralizing antibody titers at day 35 p.c. (Fig. 1B). This drop in virus-neutralizing activity observed at day 20 p.c. and its subsequent increase at day 35 p.c. was inversely correlated with the viral load (Fig. 1C). That is, a decrease in neutralizing activity was correlated with an increase in the viral load, and vice versa (Fig. 1D). Thus, although the depletion

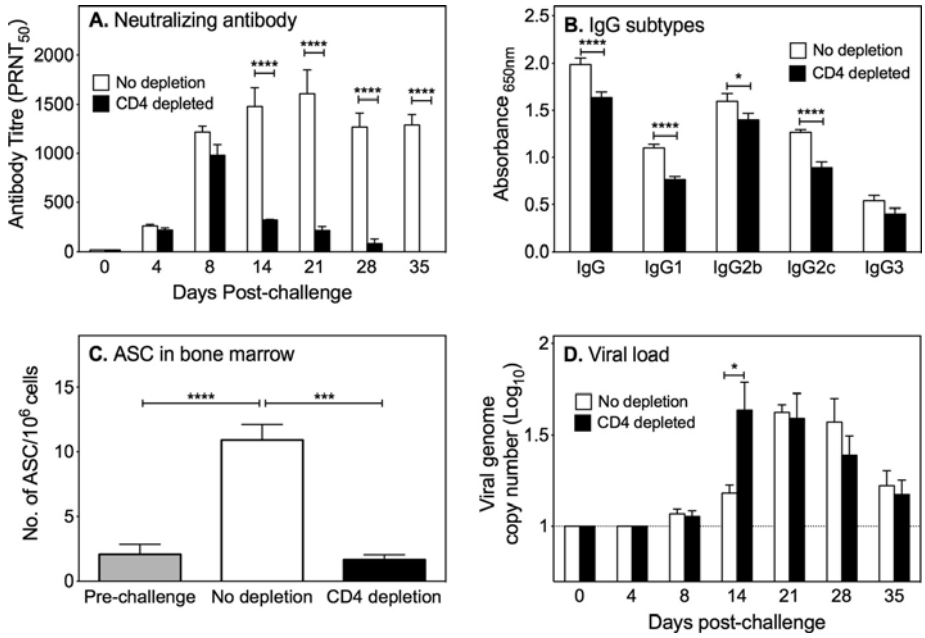

FIG 2 Effects of low-dose virus challenge on B cell responses and virus control in the continued absence of CD4 T cells. Mice were treated with anti-CD4 MAb every alternate day starting from day −1 until day 34 p.c. (A) $PRNT_{50}$s in sera of mice 16 weeks after immunization with ECTV-TK^{Δ} and challenge with 10^3 PFU ECTV-WT in control and CD4 T cell-depleted groups. ****, $P < 0.0001$ at days 14, 21, 28, and 35 p.c. in CD4-depleted compared to nondepleted groups. (B) ECTV-specific total IgG and IgG subclass titers measured by ELISA at day 35 p.c. *, $P < 0.05$; ****, $P < 0.0001$. (C) Numbers of ECTV-specific ASC in bone marrow at day 35 p.c. ***, $P < 0.0005$; ****, $P < 0.0001$. (D) Viral genome copy numbers (means and SEM) in blood as quantified by qRT-PCR. *, $P < 0.05$ for the nondepleted group compared with the CD4-depleted group at day 14 p.c. The results shown are from one of two independent experiments with 5 to 7 animals per group with similar outcomes. The error bars indicate SEM.

of CD4 T cells in the acute phase (up to day 12 p.c.) of secondary challenge did not affect the virus-neutralizing antibody, continued depletion up to day 19 p.c. did cause a drop in these levels. Furthermore, the cessation of CD4 T cell depletion resulted in a rebound of neutralizing antibody and reduction of the viral load (Fig. 1B and C).

Depletion of the CD8 T cell subset increased the neutralizing antibody response (Fig. 1A and B) and did not affect virus control (Fig. 1C). In contrast, when both CD4 and CD8 T cell subsets were depleted, neutralizing antibody titers were significantly reduced compared to the no-depletion control group, as well as groups depleted of only one subset (Fig. 1A). Furthermore, all animals in this double-depleted group succumbed to mousepox after 12 to 20 days (Fig. 1D). Death in these animals was a consequence of uncontrolled virus replication, evident as early as day 6 p.c. (Fig. 1C). Our data, therefore, suggest that at least one T cell subset is necessary for recovery of mice from a secondary challenge. CD8 T cells perform an important compensatory function to control virus in the absence of CD4 T cells. The reverse also holds; in the absence of CD8 T cell function, CD4 T cells and antibody are sufficient to control infection. It should be noted that there was no significant loss in protection when either CD4 or CD8 T cells were depleted.

Recall B cell responses diminish with prolonged CD4 T cell depletion. As indicated by the data in Fig. 1, continuous treatment with an anti-CD4 MAb over 3 weeks revealed a role for CD4 T cell help in the generation of protective recall antibody responses. Consistent with this, when CD4 T cells were continuously depleted for 34 days p.c., neutralizing antibody titers were reduced significantly beginning at day 14 and remained low at day 35, whereas titers in the control group increased over time (Fig. 2A). The drop in neutralizing antibody titers was associated with a reduction in virus-specific total IgG, as well as the IgG1, IgG2b, and IgG2c subclasses, in sera (Fig. 2B), contemporaneous with significantly reduced numbers of virus-specific ASC in the bone marrow (Fig. 2C). Despite the presence of virus-specific IgG at day 35 p.c. in the CD4 T cell-depleted group (Fig. 2B), the neutralizing activity was below the NT_{50} (Fig. 2A), suggesting a qualitative change in the antibody response. Although the decline in neutralizing antibody titers at day 14 correlated with an increase in the viral load, ECTV titers were otherwise comparable with those of the control group at all other time points, indicating that depletion of CD4 T cells alone did not compromise protection (Fig. 2D). These results, combined with those in Fig. 1, indicate that although dispensable in the early stages of virus challenge, CD4 T cell help becomes necessary for sustained production of effective antiviral antibody response as the infection progresses. They also indicate an important compensatory role for CD8 T cells in the control of virus, particularly in the absence of CD4 T cells.

CD8 T cells mediate protection in the absence of CD4 T cells, even with a higher-dose virus challenge. The preceding data established that CD8 T cells are able to compensate for the lack of CD4 T cells when the host is challenged with a low dose of virus (1,000-fold less than 1× LD_{50}). We questioned whether CD8 T cells might be sufficient to effectively control a higher dose of virus challenge in the absence of CD4 T cells. Challenge with a 100-fold-higher virus dose (10-fold less than 1× LD_{50}), along with continuous depletion of CD4 T cells over 34 days, significantly reduced the neutralizing antibody titers beginning at day 14 p.c. (Fig. 3A). As with the low-dose challenge (Fig. 1A and B), the neutralizing antibody response in the control group increased with time, but

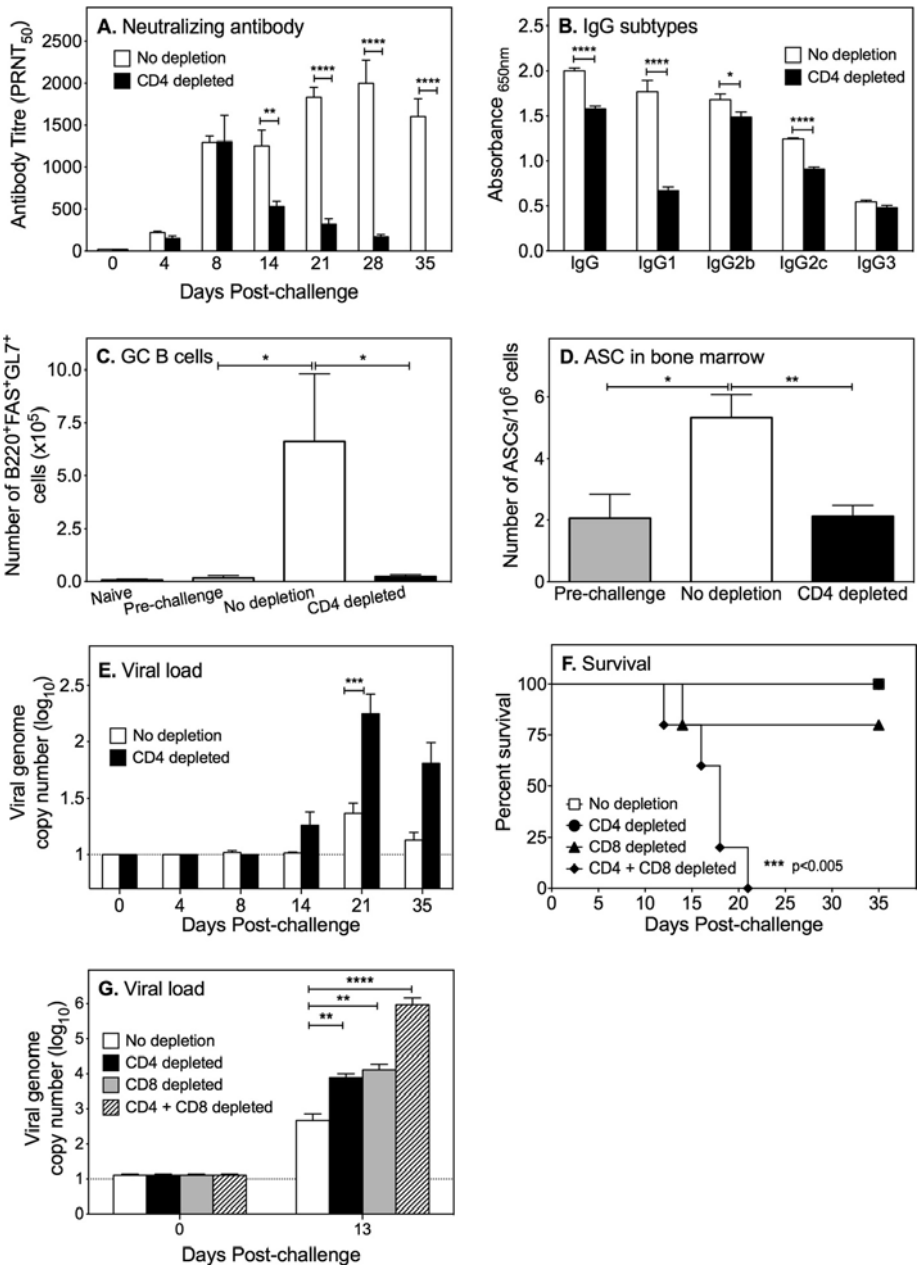

FIG 3 Effects of high-dose virus challenge on B cell responses and virus control in the continued absence of CD4 T cells. Mice were treated with anti-CD4 MAb every alternate day starting from day −1 until day 34 p.c. (A) $PRNT_{50}$ in sera of mice 16 weeks after immunization with ECTV-TK^{Δ} and challenge with 10^5 PFU ECTV-WT (10-fold less than $1\times LD_{50}$) in control and CD4 T cell-depleted groups. **, $P < 0.005$; ****, $P < 0.0001$. (B) ECTV-specific total IgG and IgG subclass titers measured by ELISA at day 35 p.c. *, $P < 0.05$; ****, $P < 0.0001$. (C) Numbers of GC B cells in the spleen at day 35 p.c. *, $P < 0.05$ for the prechallenge or CD4-depleted group compared with the no-depletion group. (D) Numbers of ECTV-specific ASC in bone marrow at day 35 p.c. *, $P < 0.05$; ** $P < 0.005$. (E) Viral genome copy numbers (means and SEM) in blood as quantified by qRT-PCR. ***, $P < 0.0005$. (F) Recovery of control mice (No depletion) and mice depleted of CD4, CD8, or both T cell subsets following challenge with 10^5 PFU virus. $P < 0.0005$ for the no-depletion or CD4 T cell-depleted group compared with the CD4- and CD8-depleted group; $P > 0.05$ for the no-depletion group compared with the CD4 or CD8 T cell-depleted group. (G) Viral genome copy numbers (means and SEM) in blood at day 13 p.c. **, $P < 0.005$; ****, $P < 0.0001$. The results shown are from one of two independent experiments with 5 to 7 animals per group with similar outcomes. The error bars indicate SEM.

levels in CD4 T cell-depleted mice decreased to below the $PRNT_{50}$ at day 35 p.c. Absence of CD4 T cells also resulted in reduced ECTV-specific total IgG, IgG1, IgG2b, and IgG2c (Fig. 3B) and numbers of GC B cells in the spleen (Fig. 3C) and virus-specific ASC in the bone marrow (Fig. 3D). As was the case with low-dose virus challenge (Fig. 2), in the CD4 T cell-depleted group, virus-neutralizing activity was below the NT_{50}s (Fig. 2A) despite the presence of virus-specific IgG at day 35 p.c. (Fig. 2B).

Diminution of B cell responses in CD4 T cell-depleted mice directly correlated with increased viremia, which persisted at day 35 p.c. (Fig. 3E). Virus genomes were also detectable in the blood of the nondepleted group, although the copy numbers were at

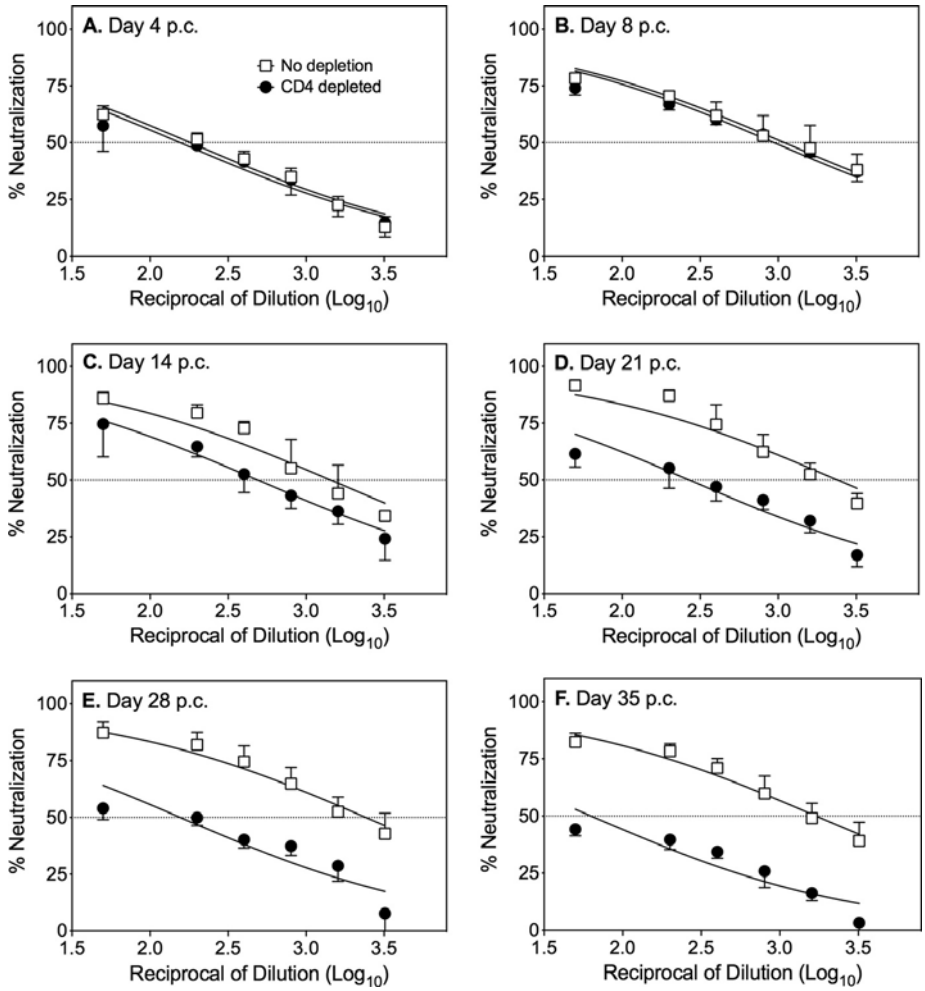

FIG 4 Kinetics of virus-neutralizing antibody response generation in the presence or absence of CD4 T cells during secondary challenge with high-dose ECTV-WT. These are the same data shown in Fig. 3A, but the dose-response neutralizing curves for individual days for each animal group are shown to demonstrate that neutralizing activity is detectable in sera at day 35 p.c. Shown is virus-neutralizing activity in sera of mice 16 weeks after immunization with ECTV-TK$^{\Delta}$ and challenge with 10^5 PFU ECTV-WT (10-fold less than 1× LD_{50}) in control and CD4 T cell-depleted groups at days 0, 8, 14, 21, 28, and 35 p.c. (n = 5 mice per group). The neutralizing antibody response decays with time in the absence of CD4 T cell help. The data were fitted with a nonlinear (four-parameter) function, and $PRNT_{50}$s were calculated by least-squares regression analysis. Individual interpolated $PRNT_{50}$ values were analyzed by 2-way ANOVA followed by Fisher's LSD test for significance between nondepleted and CD4-depleted groups. $P < 0.0001$ for days 14, 21, 28, and 35 p.c. between nondepleted and CD4-depleted groups. The error bars indicate SEM.

least 10-fold lower (Fig. 3E). Animals depleted of only CD4 T cells did not succumb to mousepox, presumably due to the presence of CD8 T cells, which kept viral loads in check in this group (Fig. 3F). Likewise, there was no significant loss of protection when only CD8 T cells were depleted. Indeed, depletion of both subsets resulted in 100% mortality (Fig. 3F), consistent with overwhelming viral loads (Fig. 3G). Despite the significant drop in $PRNT_{50}$s at day 35 p.c. in CD4 T cell-depleted mice (Fig. 3A), low levels of virus-neutralizing antibody activity, likely produced by extrafollicular ASC and long-lived ASC, were still detectable in sera, as shown in Fig. 4. These are the same data as in Fig. 3A, but the dose-response neutralizing curves for individual days p.c. for all groups are shown.

The results indicate that CD4 T cell help is necessary for expansion of GC B cells, with corresponding increases in IgG and neutralizing antibody titers. Without CD4 T cell help, ASC numbers in the bone marrow did not increase above the numbers of long-lived ASC that are generated by the primary response following immunization (Fig. 3D, prechallenge group). Thus, antibody produced by extrafollicular ASC and long-lived ASC, generated through priming, are not sufficient to control virus in the absence of both T cell subsets. The data also show that CD8 T cells can compensate for the lack of CD4 T cells when the host is challenged with a higher dose of virus.

Virus control by CD8 T cells, in the absence of CD4 T cells, during secondary infection requires perforin and granzymes. The granule exocytosis pathway of CD8 T cells, consisting of Prf and Gzms, is important for control of primary ECTV infection (27, 40, 44–46). Mice deficient in Prf or GzmA and GzmB are highly susceptible to primary ECTV infection (27, 40, 44) and succumb to a virus dose of 10^3 PFU. However, if first immunized with ECTV-TK$^{\Delta}$, as in this study (Fig. 5A), they overcome a secondary challenge with virulent virus (27). We investigated whether these effector molecules are responsible for CD8 T cell function that controls the virus load during secondary challenge in the absence of CD4 T cells. Mice deficient in Prf (Prf$^{-/-}$) alone

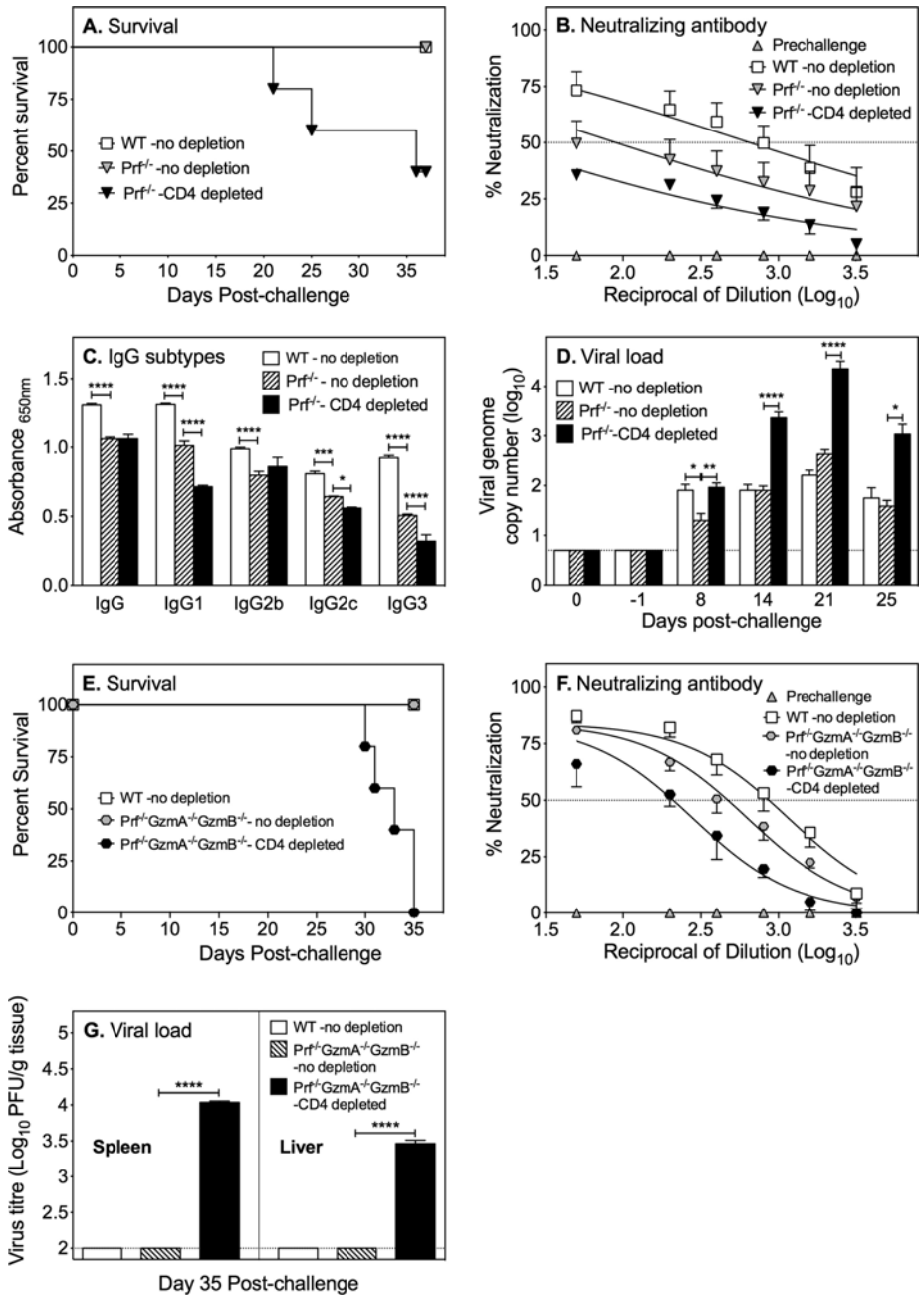

FIG 5 ECTV-immune Prf$^{-/-}$ and Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice succumb to mousepox in the absence of CD4 T cells. Mice were immunized with ECTV-TK$^{\Delta}$ and 16 weeks later challenged with 10^3 PFU ECTV-WT. One group in each experiment was treated with anti-CD4 MAb every alternate day starting from day −1 until day 34 p.c. (A) Vaccinated Prf$^{-/-}$ mice succumb to ECTV-WT secondary challenge in the absence of CD4 T cells. $P < 0.05$ for the nondepleted groups compared with the CD4-depleted group; $P < 0.05$ for the Prf$^{-/-}$ group compared to the WT. (B) Virus-neutralizing activity in sera of WT and Prf$^{-/-}$ mice. $P < 0.05$ for the nondepleted Prf$^{-/-}$ group compared with the CD4-depleted Prf$^{-/-}$ group; $P < 0.05$ for the WT compared with the Prf$^{-/-}$ group. (C) ECTV-specific total IgG and IgG subclass titers measured by ELISA at day 35 p.c. *, $P < 0.05$; ***, $P < 0.0005$; ****, $P < 0.0001$. (D) Viral genome copy numbers (means and SEM) in blood as quantified by qRT-PCR. *, $P < 0.05$; **, $P < 0.005$; ****, $P < 0.0001$. (E) Vaccinated Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice succumb to secondary challenge with ECTV-WT in the absence of CD4 T cells. $P < 0.0005$ for nondepleted groups compared with the CD4-depleted group. (F) Virus-neutralizing activity in sera of WT and Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice. $P < 0.05$ for the nondepleted Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ group compared with the CD4-depleted group. (G) Virus recovered from the spleens and livers of Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice depleted of CD4 T cells that died on day 35 p.c. ****, $P < 0.0001$. The results shown are from one of two independent experiments with 5 to 7 animals per group with similar outcomes. The error bars indicate SEM.

or in Prf and GzmA and -B (Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$) were used.

ECTV-TK$^{\Delta}$-primed Prf$^{-/-}$ mice fully recovered from a secondary virus challenge (Fig. 4A), as we have shown previously (27). In contrast, Prf$^{-/-}$ mice depleted of CD4 T cells succumbed to mousepox beginning at day 21, with 60% mortality by day 35. Virus-neutralizing antibody titers were significantly lower in Prf$^{-/-}$ mice than in WT animals at day 21 p.c., and CD4 T cell

depletion further reduced titers (Fig. 5B). Prf$^{-/-}$ mice produced significantly reduced levels of total IgG and all IgG subclasses, and CD4 T cell depletion further decreased IgG, IgG1, IgG2c, and IgG3 levels (Fig. 5C), with corresponding increases in the viral load (Fig. 5D). The 2 surviving mice that were euthanized at day 35 had greater than 10^4 viral genome copies in the blood, whereas virus was below the limit of detection in the control groups.

As Prf is only one of many effector molecules that CD8 T cells utilize to eliminate virus-infected cells, we used Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice. ECTV-TK$^{\Delta}$-primed Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice fully recovered from a secondary virus challenge, as shown previously (27), but elimination of CD4 T cells resulted in 100% mortality (Fig. 5E). Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ mice also had lower neutralizing antibody levels than WT mice, and titers dropped further in the absence of CD4 T cell help (Fig. 5F). The death of Prf$^{-/-}$ GzmA$^{-/-}$ GzmB$^{-/-}$ animals depleted of CD4 T cells was likely a consequence of a high viral load, as virus was recovered from organs of animals that were found dead on day 35 (Fig. 5G). In contrast, virus was below the limit of detection in all other groups sacrificed on day 35 (Fig. 5G). Taken together, the data established that the granule exocytosis pathway of CD8 T cells is necessary for control of secondary ECTV infection and becomes critical and evident in the absence of CD4 T cells.

DISCUSSION

T cell responses are critical for recovery of a host from primary OPV infections. It has long been recognized that individuals with T cell deficiencies given the smallpox vaccine can develop adverse reactions, including progressive vaccinia, which can be lethal without any intervention (reference 20; reviewed in references 21 and 24). Animal models of OPV infections have also been used to demonstrate essential roles for T cells in recovery from primary infection (17, 19, 22, 47–49).

In addition to a requirement for T cell responses in recovery of the host during primary infection, antibody plays a key role in complete virus clearance (18, 19, 46). Furthermore, a number of studies have used animal models of OPV infection to demonstrate that passive transfer of vaccinia immune globulin (VIG) is protective in immunocompetent hosts, i.e., as long as T cells are also present (22, 49). In humans, passive transfer of VIG to contacts of smallpox patients was shown to result in a reduction in smallpox incidence by nearly 70% (50, 51). As most of these individuals had been either previously vaccinated or revaccinated at the time of VIG treatment, it is not clear whether VIG transfer alone was sufficient to provide complete protection. While there are no data available on the immune status of the remaining 30% of treated individuals who succumbed to smallpox, at least 4 were newborns without a fully developed immune system (50, 51). In a more recent study by Edghill-Smith et al., transfer of VIG to naive, immunocompetent macaques was shown to provide complete protection from a lethal primary MPXV infection (26). While these studies provide evidence that passive transfer of VIG protects against OPV infections, there is as yet no evidence to suggest that endogenous T or B cell responses were not involved in conferring protection. It can be envisaged that passive transfer of VIG to immunocompetent hosts significantly reduces the viral load, allowing time for endogenous cell-mediated immunity and antibody responses to be generated and to contribute to virus clearance.

While antibody is also critical for protection against secondary OPV infection following vaccination, the roles of CD4 and CD8 T cells have been unclear. Previous studies in which CD4 T cells and/or CD8 T cells were depleted during the acute phase of a secondary OPV challenge in vaccinated mice or macaques suggested that antibody and B cells alone are sufficient for protection (7, 26, 27). Recent evidence from our laboratory indicates that innate immunity also plays a key role during the first 8 days p.c. NK cells, granulocytes, and plasmacytoid dendritic cells are important for virus control, and their essential roles become apparent in the absence of CD4 and CD8 T cell subsets (41). In the current study, through prolonged CD4 T cell depletion, we have established that CD4 T cell help is essential for a GC response in order to sustain production of high titers of neutralizing antibody as the infection progresses. CD4 T cell help is necessary for expansion of GC B cells in the spleen and ASC numbers in the bone marrow, with corresponding increases in IgG and neutralizing antibody titers. It is unclear, however, whether the reduced GC B cell and ASC numbers in the absence of CD4 help might be due to a lack of proliferation or altered survival.

In the absence of CD4 T cells, antibody produced by extrafollicular ASC can control virus replication as long as effector CD8 T cells are also present. Nonetheless, at a 100-fold-higher challenge dose, these effector mechanisms were not very effective in virus clearance. It is conceivable that if CD4 T cell depletion was extended beyond 34 days p.c. and/or the challenge dose increased even further (e.g., $>1\times$ LD$_{50}$), antibody produced by extrafollicular ASC and CD8 T cells might not be sufficient to control the virus. Furthermore, the fact that animals succumbed to disease after 10 days p.c. when both CD4 and CD8 T cells were eliminated suggests that innate immunity is not effective in controlling virus beyond the acute stage of a secondary infection (41).

Elimination of CD4 T cells resulted in a substantial drop in neutralizing antibody titers, associated with significant reductions in IgG1, IgG2b, and IgG2c levels. IgG subclasses in mice vary in their capacities to mediate effector functions due to differential affinities of individual subclasses for specific activating and inhibitory IgG Fc receptors (FcR) (52). While there are significant differences between the mouse and human IgG subclasses and FcR (52), this finding may also apply to human IgG subclasses and FcR. A preponderance of IgG2a (IgG2c in C57BL/6 mice) produced against a number of viral infections in mice is suggestive of an important role for this subclass of antibody (53). It is not known whether a reduction in neutralizing antibody titers is due to a drop in IgG1, IgG2b, IgG2c, or all 3 subclasses in our study. However, each of the subclasses can contribute to antiviral mechanisms, and induction of IgG2a and IgG1 is a far better correlate for vaccine efficacy and protection (54). In addition to providing help to cognate B cells for antibody production, CD4 T cells can also contribute both directly and indirectly to virus control through other mechanisms (17). Cytotoxic CD4 T cells can mediate direct antiviral activity through Prf-mediated cytolysis, although they are not as efficient as CD8 T cells (55). As CD4 T cells are required for generation of optimal cytotoxic CD8 T cell responses to ECTV (17), they can indirectly influence virus control.

CD8 T cells can regulate high-affinity antibody and autoantibody production through interaction with Qa-1^{+} T$_{FH}$ cells involving, in part, Prf-dependent mechanisms (56). Elimination of CD8 T cells, including those that regulate B cell responses, may therefore be expected to augment the antibody response (Fig. 1A to C). However, our data also indicate that deficiency in Prf results

in nonoptimal antiviral antibody response generation (Fig. 5B and C). The granule exocytosis pathway is utilized mainly by CD8 T cells and natural killer cells and to a lesser extent by other lymphocyte subsets, including CD4 T cells. Therefore, a deficiency in the Prf pathway is likely to influence the functions of all Prf-expressing cells and, as a consequence, the immune response that is generated. A lack of cytolytic activity by these effector cells resulting in a reduced amount of viral antigen can also potentially modulate the antibody response. Since NK cells play an important role in optimizing the anti-ECTV antibody and B cell responses (41), it is possible that Prf contributes to this process.

The success and efficacy of the vaccine that led to eradication of smallpox may be attributed to the fact that it is a live-virus vaccine, which induces long-lived cell-mediated and humoral immunity (57, 58). Nonetheless, there are significant safety concerns with the previous and currently licensed live-virus smallpox vaccines (21, 59, 60). As a result, safer vaccination strategies are being investigated. One approach has been to use specific outer membrane proteins of extracellular and/or intracellular virions (61, 62), DNA encoding those membrane proteins (63, 64), or secreted immune response modifier proteins (65) for immunization. Such an approach has been shown to induce strong antibody responses and to provide either partial or complete protection against lethal virus challenge. Immunization with OPV DNA and/or protein subunit vaccines (61–65) will likely involve major histocompatibility complex (MHC) class II-restricted CD4 T cell responses to provide cognate (66, 67) or noncognate (68) help to B cells for induction of GC responses, high-affinity antibody production, and B cell memory (31–33, 35). While it is not yet known whether OPV subunit vaccines can induce long-lived antibody responses like the live-virus vaccine, our data with whole-virus vaccination indicate that CD4 T cell help will be required for generation of effective recall antibody responses. Furthermore, in the event that antibody titers drop below the threshold neutralizing levels in vaccinated individuals who acquire deficiencies in the CD4 T cell compartment, such as those with HIV/AIDS, virus-specific effector CD8 T cells will be required to control virus. Under such circumstances, subunit vaccines that incorporate both B and CD8 T cell determinants will be more effective in conferring protection.

In summary, we have established that CD4 T cell-dependent antibody responses and effector CD8 T cells are essential for recovery from secondary OPV infection in vaccinated animals. Our findings may provide an explanation as to why the live-VACV vaccine, which induces both cell-mediated and humoral immunity, was very effective in providing protection against smallpox. It is conceivable that the requirement for CD4 T cell help for protective-antibody generation during a secondary infection also applies to viruses other than OPV.

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