JOURNAL OF VIROLOGY, July 2006, p. 6333–6338 Vol. 80, No. 13
0022-538X/06/$08.00+0 doi:10.1128/JVI.00115-06

Protective Immunity against Secondary Poxvirus Infection Is Dependent on Antibody but Not on CD4 or CD8 T-Cell Function

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Received 16 January 2006/Accepted 21 April 2006

Renewed interest in smallpox and the need for safer vaccines have highlighted our lack of understanding of the requirements for protective immunity. Since smallpox has been eradicated, surrogate animal models of closely related orthopoxviruses, such as ectromelia virus, have been used to establish critical roles for CD8 T cells in the control of primary infection. To study the requirements for protection against secondary infection, we have used a prime-challenge regime, in which avirulent ectromelia virus was used to prime mice that were then challenged with virulent ectromelia virus. In contrast to primary infection, T cells are not required for recovery from secondary infection, since gene knockout mice deficient in CD8 T-cell function and wild-type mice acutely depleted of CD4, CD8, or both subsets were fully protected. Protection correlated with effective virus control and generation of neutralizing antibody. Notably, primed mice that lacked B cells, major histocompatibility complex class II, or CD40 succumbed to secondary infection. Thus, antibody is essential, but CD4 or CD8 T cells are not required for recovery from secondary poxvirus infection.

There is an imperative need to understand protective immunity to smallpox in the face of the potential threat of a bioterrorist attack (12). Since variola virus (VARV), the causative agent of smallpox, has a restricted host range and is known to infect only humans, closely related orthopoxviruses, such as monkeypox virus and ectromelia virus (ECTV), have been used extensively in animal models to elucidate pathogenesis and immune response to infection. Currently, the best surrogate for VARV in a small animal model is ECTV, since it is infectious at very low doses, has a restricted host range, and causes severe disease with high mortality rates (8, 10). Further similarities between smallpox and mousepox, caused by ECTV, include virus replication and transmission, cytokine responses, and many aspects of pathology (8). Recent studies have also employed VARV and monkeypox virus infection in macaques (7, 14). However, these models utilize high doses of virus that are administered intravenously, and their usefulness is limited, since they only mimic postsecondary viremia and end stages of disease.

The cross-protection provided by vaccinia virus (VACV) to VARV infection has been exploited in the most successful pathogen eradication campaign to date (10). To circumvent the adverse side effects of current vaccines, it is important to elucidate the correlates of protective immunity in secondary poxvirus infection, which will assist with the design of safer, efficacious vaccines. Cross-reactive recall cytotoxic T-lymphocyte (CTL) and antibody responses are thought to eliminate virus in vaccinated individuals or those who have recovered from a primary infection. Studies with vaccinated individuals have shown that both T- and B-cell memory responses are long lived (1, 5, 11). However, it is still not clear which specific immune parameter(s) are essential for protection against smallpox (10). Indeed, recent findings from our laboratory (23) and others (7) have questioned the importance of CD8 T cells in recovery from secondary poxvirus infections, although they are known to be critical for recovery from primary infection (9, 16).

In this study we have used the mousepox model, a range of gene knockout (GKO) mice, in addition to acute T-cell subset depletion in wild-type (WT) mice, to evaluate the necessity of T- and B-cell function in the recovery from a secondary poxvirus infection. We show that CD8 T cells, or their effector functions, were not required and that recovery was associated with a neutralizing antibody response in mice lacking these functions. In contrast, mice that lacked B cells, major histocompatibility complex (MHC) class II, or CD40 succumbed to disease.

MATERIALS AND METHODS

Viruses. Plaque-purified ECTV Moscow (ATCC VR 1374), designated virulent ECTV, was propagated as previously described (4) and used to infect unprimed mice in a primary infection and challenge primed mice in a secondary infection. The thymidine kinase-deficient strain of ECTV (13), designated avirulent ECTV, was used to prime mice.

Cell lines. MC57G (ATCC CRL-2295) cells, used for CTL assays, and BS-C-1 cells (ATCC CCL26), used for virus propagation and plaque assays, were maintained in Eagle's minimum essential medium (Gibco, Invitrogen Corp., Carlsbad, CA) with 2 mM L-glutamine, antibiotics, and 5% and 10% fetal calf serum, respectively.

Mice. Female specific-pathogen-free mice were bred at the Animal Services Division, John Curtin School of Medical Research, Canberra, Australia, and were used at 6 to 12 weeks of age. Animal experiments were performed according to approved institutional guidelines.

Mice deficient in CD8 T-cell effector functions used were B6.129S7-*Ifng*^{tm1Ts} (6), designated gamma interferon$^{-/-}$ (IFN-$\gamma^{-/-}$), C57BL/6-*Prf1*^{tm1Sdz}/J (15), designated Prf$^{-/-}$, B6.Cg-*Gzma*^{tm1Simn}*Gzmb*^{tm1Ley} (24), designated GzAB$^{-/-}$, B6.Cg-*Gzma*^{tm1Simn}*Gzmb*^{tm1Ley}*Prf1*^{tm1Sdz} (25), designated PrfGzAB$^{-/-}$, and B6.129P2-*B2 m*^{tm1Jae} (28), designated β2-m$^{-/-}$. Mice deficient in B cells, B6.129S2-*Igh-6*^{tm1Cgn} (18), designated μMT, and those deficient in MHC class II, B6.*H2-Aa*^{tm1Blt} (19), designated MHC II$^{-/-}$ and provided by H Bluethmann, were also used. Since all these GKO mice were on a C57BL/6 (B6) background or back-

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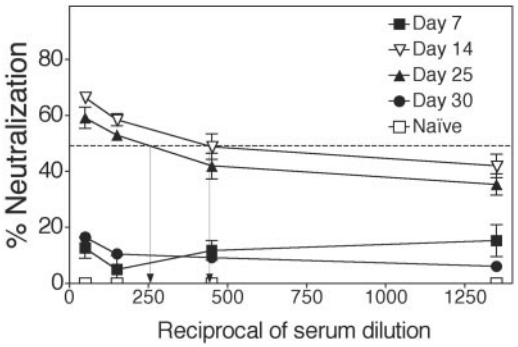

FIG. 1. Kinetics of the neutralizing-antibody response in B6 mice primed with avirulent ECTV. Groups of five female B6 mice were primed i.p. with avirulent ECTV. Serum samples were collected before priming (naive) and at the times indicated after priming. Virus-neutralizing activity was determined by the PRNT. Data shown are means ± standard errors of the means for five individual samples for each time point. There was no virus-neutralizing activity in sera of naive mice.

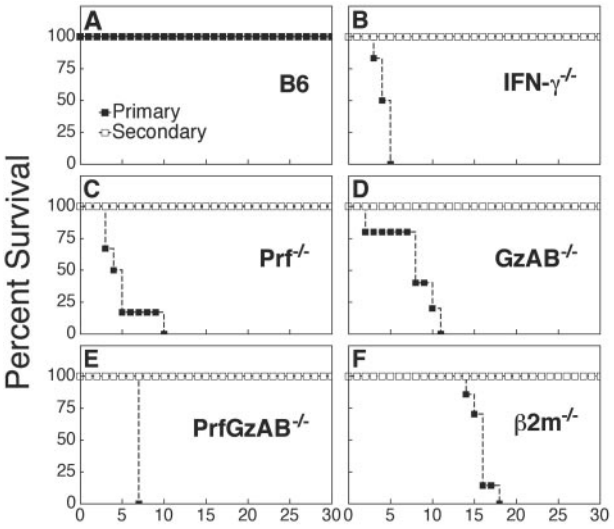

FIG. 2. Outcome of primary and secondary ECTV infection in mice lacking CD8 T cells or effector function. Groups of five female (A) WT, (B) IFN-$\gamma^{-/-}$, (C) $Prf^{-/-}$, (D) $GzAB^{-/-}$, (E) $PrfGzAB^{-/-}$, or (F) $\beta 2\ m^{-/-}$ mice were primed i.p. with avirulent ECTV and challenged s.c. with virulent ECTV (secondary, open squares). Unprimed mice from each strain were infected with virulent ECTV (primary, closed squares). Mice were observed for 30 days postchallenge (*x* axis). Data shown are representative of two independent experiments.

crossed to B6 at least 10 times, B6 WT mice were used as controls for these experiments. In addition, BALB/c.129P2-*Cd40*^{tm1Kik} (17) mice lacking CD40, designated $CD40^{-/-}$, and WT BALB/c mice were used.

Infection. For a primary infection, mice were infected subcutaneously (s.c.) in the left hind limb with 10^3 PFU of virulent ECTV. For a secondary infection, mice were primed with 10^5 PFU of avirulent ECTV given intraperitoneally (i.p.) and 35 days later challenged s.c. with 10^3 PFU of virulent ECTV. Mice were monitored for clinical signs of disease and survival.

CD4 and CD8 T-cell depletion. B6 mice were primed and challenged as above. On days −1, 1, 3, and 5 postchallenge (p.c.), mice were given phosphate-buffered saline (PBS) or 1 mg monoclonal antibody (MAb) against CD4 (clone GK1.5), CD8 (clone 2.43.1), or both. Efficiency of cell subset depletion was assessed by flow cytometry and found to be routinely >99%. On day 8 p.c., mice were sacrificed and organs and sera collected.

Cytotoxicity assays. The standard ^{51}Cr release assay (4) was used to determine ex vivo CTL activity, using ECTV-infected and uninfected MC57G target cells.

Anti-ECTV antibody determination. Serum samples were assayed by enzyme-linked immunosorbent assay (ELISA) for ECTV-specific immunoglobulin G (IgG) and IgG subtypes using purified ECTV as described previously (4, 23).

Plaque reduction neutralization test (PRNT). The PRNT, used to determine virus-neutralizing activity of the antibody present in serum samples, is described elsewhere (23). Briefly, serum samples were inactivated at 56°C for 30 min, and serial dilutions were made in PBS. Samples were incubated at 37°C for 1 h with 100 PFU of virulent ECTV before being adsorbed for 1 h on BS-C-1 cell monolayers. Overlay medium containing 1% methylcellulose and 2.5% fetal calf serum in Eagle's minimum essential medium was then carefully added. The plates were incubated at 34°C for 4 days, and resulting plaques were visualized by crystal violet staining. Heat-inactivated sera from uninfected mice were used as controls. The neutralization titer was taken as the reciprocal of the dilution of sera that caused a 50% reduction in the number of virus plaques over and above the number of plaques in the samples with sera from naive mice.

Statistical analysis. Data were analyzed using the nonparametric Mann-Whitney test and the statistical program GraphPad Prism (GraphPad Software, Inc.). A P value of <0.05 was taken to be significant.

RESULTS

GKO mice deficient in CD8 T cells or CD8 effector function are protected from virulent secondary ECTV infection. We have investigated the requirements for recovery from secondary poxvirus infection using a prime-challenge approach. To investigate the role of CD8 T-cell function, we employed B6 mice deficient in IFN-γ (IFN-$\gamma^{-/-}$), perforin ($Prf^{-/-}$), granzymes A and B ($GzAB^{-/-}$), perforin and granzymes A and B ($PrfGzAB^{-/-}$), or β2-microglobulin ($\beta 2\ m^{-/-}$) that lack CD8 T cells. Since these mutant animals are susceptible to wild-type ECTV (4, 16, 22), we used an avirulent, thymidine kinase-deficient, strain of ECTV (13) that replicates less efficiently than the wild-type virus but induces both cell-mediated and antibody responses, both of which were undetectable by 4 weeks postinfection (23). To ensure that mice were challenged at a time when there was no neutralizing antibody present, we first determined the kinetics of neutralizing activity elicited by the priming avirulent virus. The level and duration of neutralizing antibody responses are lower with the avirulent virus (Fig. 1) than with the wild-type virus (see Fig. 5A in the accompanying article [4a]). By day 30, the neutralizing antibody response is undetectable (Fig. 1). We therefore chose to challenge mice 35 days postpriming. Groups of primed and unprimed mice were challenged with virulent ECTV, resulting in virulent secondary and primary infections, respectively. The control WT B6 strain is genetically resistant to mousepox and effectively overcomes virulent primary and secondary infection (4) and (Fig. 2A). All GKO mice that had not been primed rapidly succumbed to virulent ECTV infection (Fig. 2B to F), consistent with results from previous studies (4, 16, 22). In contrast, all primed mice survived a secondary infection with no signs of morbidity (Fig. 2B to F), and this was clearly associated with their capacity to effectively control the viral load (Fig. 3A). Virus titers in organs of mice from a secondary infection were several orders of magnitude lower than those from a primary infection.

GKO mice deficient in CD8 T cells or CD8 effector function generate neutralizing antibody during secondary ECTV infection. Since mice controlled a secondary infection even in the absence of CD8 T-cell function, we investigated whether antibody contributed to recovery. As with the B6 mice (Fig. 1), there was no detectable anti-ECTV neutralizing antibody in prechallenge sera of all primed mice (data not shown). Upon

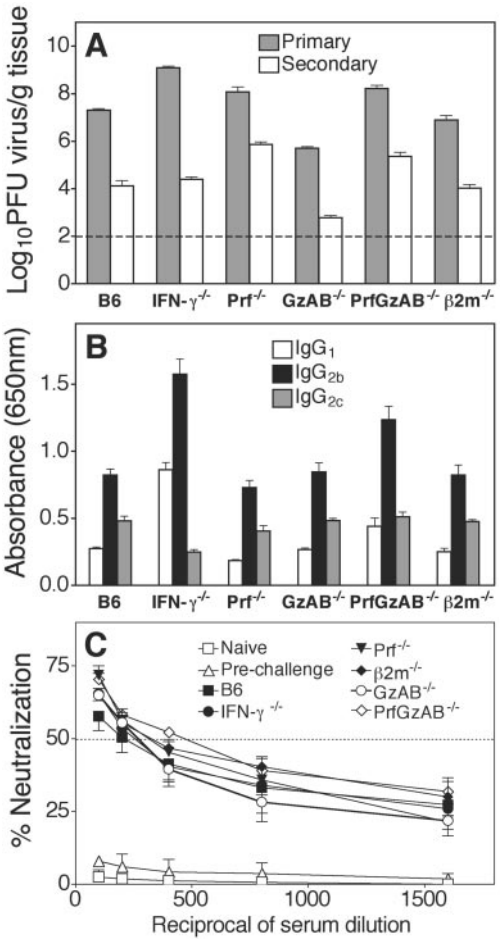

FIG. 3. ECTV titers in spleen and serum antibody profiles following primary and secondary infection. (A) Groups of five female primed (secondary) and unprimed (primary) WT, IFN-$\gamma^{-/-}$, $Prf^{-/-}$, $GzAB^{-/-}$, $PrfGzAB^{-/-}$, and $\beta 2\ m^{-/-}$ mice were challenged with virulent ECTV. On day 5 p.c., mice were sacrificed and organs collected for determination of viral titers. The broken line indicates the limit of detection of the assay, which was 100 PFU. Data shown are means ± standard errors of the means of viral titers from one of two independent experiments. Sera were collected from a separate group of primed mice at 2 weeks p.c. and used to determine (B) ECTV-specific IgG subtypes by ELISA at a serum dilution of 1:200 and (C) virus-neutralizing activity by PRNT. Data shown are means ± standard errors of the means for five individual samples per group. There was no virus-neutralizing activity in naive or prechallenge sera of primed mice in any strain. For clarity, only titers of naive and prechallenge B6 sera are depicted here. Data shown in each panel are from one of two separate animal experiments.

secondary ECTV challenge, all strains of animals responded rapidly with the generation of IgG of various subtypes (Fig. 3B) that had significant virus-neutralizing activity, equivalent to that of B6 control mice (Fig. 3C). These data are consistent with a role for antibody in virus control during secondary infection, even in the absence of CD8 T-cell function.

Genetically susceptible mice are protected from virulent secondary mousepox. A serious complication of VACV vaccination in patients with defective cell-mediated immunity has been progressive vaccinia (3), and therefore, a major effort is being made towards the design of safer vaccines. Our results

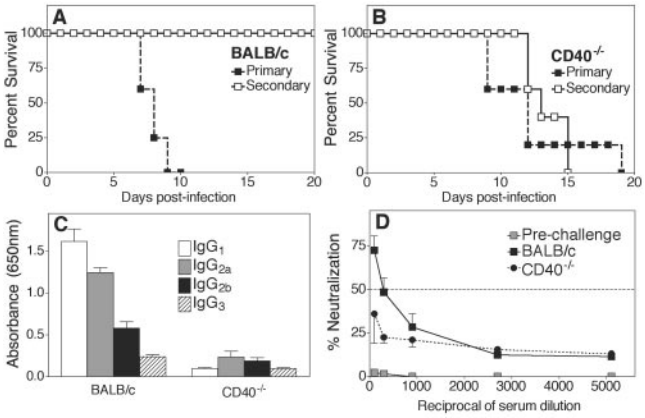

FIG. 4. Response to primary and secondary ECTV infection in BALB/c WT and $CD40^{-/-}$ mice. Groups of five female primed (secondary, unfilled squares) or unprimed (primary, filled squares) (A) BALB/c or (B) $CD40^{-/-}$ mice were challenged as in Fig. 2. Data shown are representative of two independent experiments. Sera collected from separate groups of primed mice at 2 weeks p.c. were used to determine (C) ECTV-specific IgG subtypes by ELISA and (D) virus-neutralizing activity by PRNT as described in the legend to Fig. 3. Data shown are means ± standard errors of the means for five individual samples per group and are from one of two separate animal experiments.

suggest that it is possible to vaccinate individuals with defective CD8 T-cell responses to induce protective immunity against virulent poxvirus infections using highly avirulent virus. Indeed, BALB/c mice that are genetically susceptible to mousepox, due to defects in IFN-γ and antiviral CTL responses (4), were protected against mousepox if they were first primed, whereas unprimed littermates succumbed (Fig. 4A).

Mice lacking CD40, MHC class II, or B cells succumb to virulent secondary infection. Although CD8 T-cell function was not critical in a secondary infection, CD4 T-cell help for B-cell function mediated through CD40 was important for an effective antiviral antibody response, since priming did not protect $CD40^{-/-}$ mice (on a BALB/c background) against virulent secondary challenge (Fig. 4B). Unlike the WT BALB/c animals (Fig. 4A), $CD40^{-/-}$ mice died regardless of whether it was a primary or secondary infection (Fig. 4B). WT BALB/c mice responded to secondary ECTV challenge by generating antibody of various IgG subtypes with neutralizing activity, whereas $CD40^{-/-}$ mice did not (Fig. 4C and D). Engagement of CD40 on B cells by CD40L on CD4 T cells is crucial for T-cell–B cell interaction, antibody isotype switching to typical T-dependent antigens, and B-cell memory (17, 21). $CD40^{-/-}$ mice do not synthesize isotype-switched antibody to typical T-dependent antigens and lack germinal centers and B-cell memory (17). Our data indicate that CD4 T-cell help for antibody production is an important component of the protective response. Further support for this comes from the finding that MHC $II^{-/-}$ mice that lack CD4 T cells were unable to control either a primary or secondary ECTV infection (Fig. 5A).

The crucial contribution of antibody in recovery from a secondary poxvirus infection is further underscored by the failure of μMT mice, deficient in B cells, to recover from an ECTV challenge despite priming (Fig. 5B). μMT mice completely recovered from the avirulent virus used for priming,

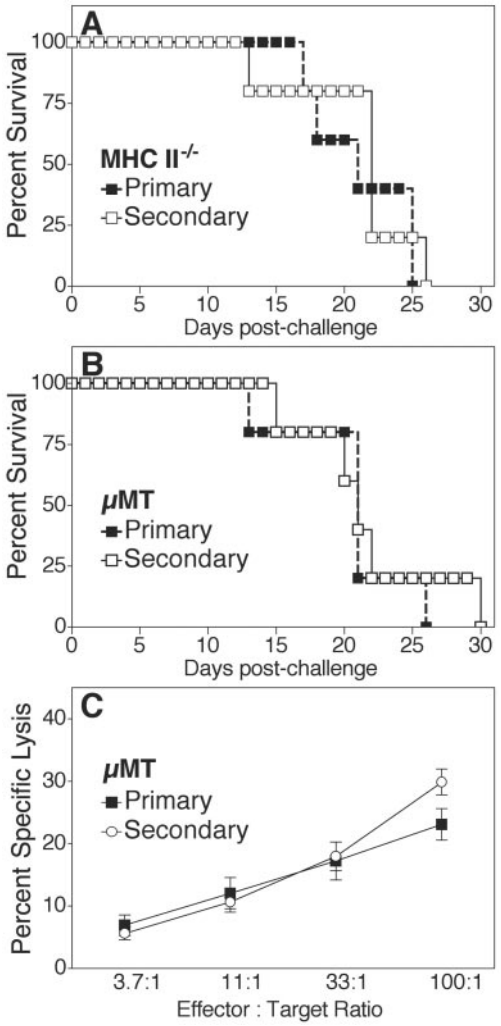

FIG. 5. Responses to primary and secondary ECTV infection in mice lacking B cells or CD4 T cells. Groups of five female primed (secondary) or unprimed (primary) (A) MHC $II^{-/-}$ mice or (B) μMT mice were challenged as in Fig. 2. (C) Splenic CTL activity was measured in separate groups of primed and unprimed μMT mice at 8 days p.c.

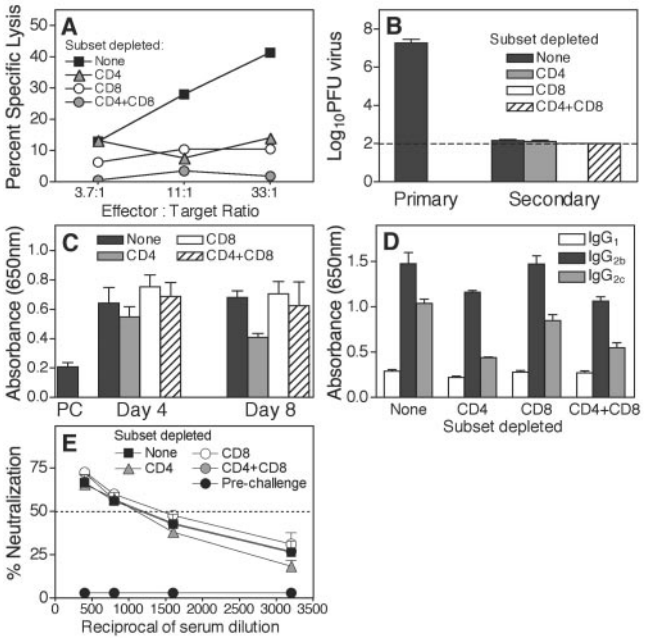

FIG. 6. Effect of CD4 and/or CD8 depletion on CTL activity, viral load, and antibody profile in primed B6 WT mice. Groups of five female B6 mice were primed and challenged as described for Fig. 2. At days −1, 1, 3, and 5 p.c., mice were treated with PBS or MAb to CD4, CD8, or both subsets. Mice were bled prechallenge (designated PC), at day 4 p.c. and then sacrificed on day 8 p.c., and spleens and sera were collected. (A) CTL activity was measured in pooled splenocytes from five mice in each group. (B) Viral titers in the spleens were determined. Titers in spleens of untreated mice at 8 days after primary infection are included for comparison and are significantly higher than those in a secondary challenge. (C) ECTV-specific IgG and (D) IgG subtypes levels were measured by ELISA as described for Fig. 3B. (E) The PRNT was used to measure virus-neutralizing activity of the sera collected. Data shown are means ± standard errors of the means from five samples per group. There was no virus-neutralizing activity in prechallenge sera of primed mice from any treatment group.

and no virus was detectable at the time of challenge (data not shown). The inability of these animals to control virulent ECTV infection was not due to defects in the memory CD8 CTL response, since μMT mice generated strong and comparable CTL activity, relative to that of control mice, in both primary and secondary infections (Fig. 5C). Indeed, there is an absolute requirement for antibody production in recovery from primary mousepox (4a, 9) (Fig. 5B).

WT mice do not require CD4 and CD8 T cells for recovery from a virulent secondary infection. In order to distinguish the consequences of T-cell deficiency for priming from its effect during a secondary infection and to account for potential compensatory mechanisms in GKO strains that are protected in a secondary infection, we acutely depleted CD4, CD8, or both T-cell subsets with MAb in primed B6 mice so that the absence of these subsets was confined only to the secondary response.

Elimination of CD4, CD8, or both subsets in primed mice abrogated the MHC class I-restricted CTL response (Fig. 6A). Notably, the absence of CD4 and CD8 T cells did not affect the ability of B6 mice to effectively control secondary infection (Fig. 6B). Further, neither subset was required for the rapid production of total virus-specific IgG (Fig. 6C) or the profile of IgG subtypes produced (Fig. 6D). Significantly, antibody generated in the absence of CD4 T-cell help still had potent virus-neutralizing activity (Fig. 6E), comparable to titers for control mice.

DISCUSSION

Current vaccines used to immunize against smallpox have unacceptable complication rates, particularly in immunocompromised individuals. Identifying the essential correlates of protective immunity in secondary poxvirus infection will help with the design of safer, efficacious vaccines against smallpox. Recall CTL and antibody responses are long lived and therefore are thought to play an important role in vaccinated individuals or those who have recovered from primary smallpox. However, recent findings from our laboratory (23) and others

(7) have questioned the importance of CD8 T cells in recovery from secondary poxvirus infections.

We have shown here that protection during a secondary poxvirus infection does not require CD8 T cells or their effector function but correlated strongly with generation of neutralizing antibody. GKO mice deficient in CD8 T-cell function and WT mice depleted of CD4, CD8, or both subsets were fully protected against virulent ECTV challenge. Importantly, primed mice that lacked B cells, MHC class II, or CD40 succumbed to secondary infection.

In contrast to our results with the ECTV model and to findings with the monkeypox model (7), recovery from a secondary VACV infection does not require B-cell function (26, 27). Mice deficient in B cells, CD4 cells, or MHC class II molecules were fully protected against lethal VACV challenge if they were vaccinated with the highly attenuated modified vaccinia Ankara strain (26). Only mice that lacked both MHC class II and class I molecules, and therefore were deficient in CD4 and CD8 T cells, were not protected. A study by Belyakov and colleagues has shown that in the absence of B cells, vaccinated mice challenged with virulent VACV became sick and then recovered, alluding to a possible role for antibody (2). However, the usefulness of VACV infection in mice as a model for smallpox, in comparison to that of ECTV, is unsatisfactory, since the pathogenesis, disease progression, and outcome of infection are unlike that of VARV.

Many early clinical studies on smallpox suggest a protective role for antibody in vaccinated individuals and patients that had recovered from an infection (10). More recent studies with smallpox vaccinees indicate that serum antibody responses remained stable between 1 and 75 years after infection, whereas antiviral T-cell responses declined slowly, with a half-life of 8 to 15 years (5, 11). Nevertheless, it should be noted that CD4 T-cell memory is maintained for much longer (1), and this may be important for maintenance of long-term B-cell memory and protective recall responses. Our studies using CD40$^{-/-}$ mice (Fig. 4B) and MHC II$^{-/-}$ mice (Fig. 5A) that lack CD4 T cells highlight the importance of CD4 T-cell help for generation of antibody in recovery from secondary poxvirus infection.

The use of GKO mice has at least two potential shortcomings when investigating recall responses. First, it is not possible to distinguish between the consequences of T-cell deficiency on priming from its effect during a secondary infection. This is particularly relevant to strains of mice that were susceptible to both primary and secondary infection. These strains, namely, MHC II$^{-/-}$, CD40$^{-/-}$, and μMT, all have defects in antibody production. Second, compensatory mechanisms may contribute to recovery in those GKO strains that are protected in a secondary infection, such as mice deficient in CD8 T cells or effector functions. This was our rationale for inclusion of T-cell elimination studies in which we depleted CD4, CD8, or both T-cell subsets with MAb in primed B6 mice, so that the absence of these subsets was confined only to the secondary phase. These experiments definitively established that the absence of both CD4 and CD8 T cells did not affect the ability of B6 mice to effectively control secondary infection or the rapid production of virus-specific IgG. Importantly, antibody generated in the absence of CD4 T-cell help still had potent virus-neutralizing activity.

Thus, unlike the case with CD40$^{-/-}$ mice (Fig. 4B), MHC II$^{-/-}$ (Fig. 5A) and μMT (Fig. 5B) mice, in which antibody production was defective both in the priming and challenge phases, results from experiments in which depletion of CD4 T cells in WT mice was confined only to the challenge phase indicated that CD4 help was not required for antibody production during a secondary response. The mechanism by which neutralizing antibody is generated in the absence of CD4 help is not clear, and its elucidation merits further study.

We have shown here that CD4 and CD8 T cells do not contribute to virus control (Fig. 6B) and that B-cell function, through the production of neutralizing antibody, is essential for recovery from secondary ECTV infection (Fig. 5 and 6E). A recent study on monkeypox demonstrated that depletion of CD4 or CD8 T cells did not affect vaccine-induced protection from lethal monkeypox; however, depletion of B cells rendered animals susceptible to monkeypox, further underscoring the importance of antibodies in mediating recovery. However, in addition to concerns with the monkeypox model requiring high doses of virus given intravenously and mimicking only postsecondary viremia and end-stage disease, a major pitfall of the study is that at the time of challenge, animals had high antibody levels with significant neutralization activity (7). The ECTV model used in this study has the advantage that it more closely resembles VARV infection in humans, and the mode of priming we have employed stimulates innate CTL and antibody responses. Furthermore, unlike the virulent wild-type virus, the avirulent ECTV used in this study is cleared rapidly from primed mice, which no longer have demonstrable neutralizing antibody at the time of challenge (23) (Fig. 1 and 3C). Thus, in contrast to the above study (7), our data are not confounded by the possibility that secondary virus infection is controlled by preexisting neutralizing antibody.

Studies on VACV have identified that antibodies are directed against both the extracellular enveloped virion and the intracellular mature virion. Lustig and colleagues recently demonstrated that antibodies to either intracellular mature virion or extracellular enveloped virion proteins protect against lethal VACV infection, but combinations provided better protection (20). Although we have not measured antibody responses to the two forms of ECTV specifically, this merits further investigation.

This study has delineated some important requirements for recovery from secondary poxvirus infection. Data obtained from primed GKO mice established that CD8 T cells or CD8 effector functions were not necessary for recovery, but antibody was essential. Absence of CD4 T cells during a secondary response abolished the antiviral CTL response, indicating that it is strictly helper T cell dependent. However, neither CD4 nor CD8 T-cell subsets were required for production of virus-neutralizing antibody. Our data suggest that it would be possible to design highly attenuated vaccines that provide effective protection against virulent poxvirus infection even in immunodeficient individuals.

ACKNOWLEDGMENTS

This work was supported by grants from the Howard Hughes Medical Institute and National Health and Medical Research Council of Australia.

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