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Cellular and Humoral Immunity against Vaccinia Virus Infection of Mice

Rong Xu,* Aaron J. Johnson,* Denny Liggitt,† and Michael J. Bevan2*

Despite the widespread use of vaccinia virus (VV) as a vector for other Ags and as the smallpox vaccine, there is little information available about the protective components of the immune response following VV infection. In this study, protection against wild-type VV was evaluated in mice with respect to the relative contributions of CD8+ T cells vs that of CD4+ T cells and Ab. C57BL/6 mice primed with the Western Reserve strain of VV mount significant IgM and IgG Ab responses, specific cytotoxic T cell responses, IFN-γ responses in CD4+ and CD8+ T cells, and effectively clear the virus. This protection was abrogated by in vivo depletion of CD4+ T cells or B cells in IgH−− mice, but was not sensitive to CD8+ T cell depletion alone. However, a role for CD8+ T cells in primary protection was demonstrated in MHC class II−− mice, where depleting CD8+ T cells lead to increase severity of disease. Unlike control MHC class II−− mice, the group depleted of CD8+ T cells developed skin lesions on the tail and feet and had adrenal necrosis. Adoptive transfer experiments also show CD8+ T cells can mediate protective memory. These results collectively show that both CD4+ and CD8+ T cell-mediated immunity can contribute to protection against VV infection. However, CD4+ T cell-dependent anti-virus Ab production plays a more important role in clearing virus following acute infection, while in the absence of Ab, CD8+ T cells can contribute to protection against disease. The Journal of Immunology, 2004, 172: 6265–6271.

Vaccinia virus (VV)3 is a large DNA virus and is a member of the genus Orthopoxvirus of the family Poxviridae. Other orthopoxviruses include variola virus, the cause of smallpox, monkeypox virus, camelpox virus, ectromelia virus, and cowpox virus (1). These viruses are morphologically indistinguishable and antigenically related, such that previous infection with any one provides some protection against each member of the genus. This was the basis for cowpox (2) and later VV (3) being effective vaccines against smallpox. VV is the most studied of the poxvirus family and has played a central role in the eradication of smallpox. More recently VV also has been developed as an expression vector for foreign genes and as a live recombinant vaccine for infectious disease and cancer (4, 5). However, we have only limited knowledge about the immunologic correlates of its protective vaccination.

Both humoral and cellular immunity have been thought to play a role in protection against orthopoxviruses. This view is based on experience with individuals with either humoral or cellular immune defects who were unable to control VV infection (6). A number of studies indicate that Ab is essential to protect against orthopoxviruses. Vaccinia hyperimmune γ-globulin has efficacy in the prophylaxis of smallpox (7), and known envelope proteins from VV can elicit neutralizing Ab (8). More recently, results from mice immunized with modified vaccinia Ankara virus and Wyeth virus have shown the importance of Ab in protection against pathogenic VV infection and disease (9). Several studies have also analyzed the role of different cytokines during infection with VV: some cytokines increased virulence, as with IL-4, whereas others attenuated the infection, as with IFN-γ or IL-2 (10–12). Both NK cells and γδ T cells have been shown to play a role in innate resistance to VV infection (13, 14). The role of CD8+ T cell-mediated cellular immunity in protection in VV-immunized animals is still poorly characterized. Kinetic analysis of VV-specific T cell responses in BALB/c mice have shown that VV infection can induce a potent primary CD8+ T cell response as well as long-term memory (15). However, β2m−− mice almost completely lacking CD8+ T cells survived high-dose s.c. infection with similar lesion development as control mice (16). In addition, absence of either perforin- or Fas-dependent cytotoxicity did not affect clearance of a primary VV infection (17).

In the present study, using Ab-depleted and gene knockout mice we found that, following acute infection, the CD4+ T cell-dependent Ab response is more important for VV clearing. However, in a primary infection in the absence of CD4+ T cells and Ab, CD8+ T cells can play an important role in protecting mice from disease. In addition, both adoptive transfer of memory CD8+ T cells and depletion of memory CD8+ T cells from MHC class II−− mice show that CD8+ T cells can protect against secondary infection.

Materials and Methods

Virus

The VV Western Reserve strain was purchased from the American Type Culture Collection (Manassas, VA), grown in HeLa cells, and titered in BS-C-40 cells (18).

Mice and infection

C57BL/6, C57BL/6-Igh−−/− mice (also called IgH−−−), CD8−−−, and Rag1−−− mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II−− mice on C57BL/6 background were purchased from Taconic Farms (Germantown, NY). All mice were females and used at 9–12 wk of age. Depletion of CD4+ or CD8+ cells was done by i.p. injection with 200 μg of mAb on days 3 and...
Depletion was verified by flow cytometric analysis of peripheral blood cells to be >98%. In some experiments, continuous depletion was done by i.p. injection with 200 μg of specific Ab once per week after the first two injections. NK1.1+ cells were depleted by i.p. treatment with 500 μg of cloning PK135 on day 2 before infection (clone GK1.5 for CD4 and clone 2.43 for CD8). Depletion was monitored by i.p. injection with VV at a multiplicity of infection of 10⁶ PFU of VV. Effector responses were analyzed in the spleens of infected mice on day 7 postinfection (PI), while memory responses were analyzed at least 60 days after infection. For protection assays, mice were vaccinated once (i.p. with VV 10⁶ PFU) and monitored for 2 wk with measurement of individual body weights every 2 days and scoring signs of illness. Clinically impaired mice with severe systemic infection and having lost >25% of body weight were euthanized. Tissues were collected into 10% neutral-buffered Formalin and H&E-stained sections prepared using standard procedures. The mean change in body weight was calculated as percentage of the mean weight for each group on the day of challenge.

**Cell surface staining and flow cytometry**

Single-cell suspensions of splenocytes from mice were stained in PBS containing 0.5% BSA and 0.2% sodium azide (FACS buffer) with mAbs specific for CD8 (clone 53-6.7), CD4 (clone RM4-4). Cells were washed with FACS buffer and fixed in PBS containing 2% parafomaldehyde. Samples were acquired on a BD Biosciences FACSCalibur flow cytometer (San Jose, CA.), and analysis was performed using CellQuest software (BD Biosciences). All Abs were purchased from BD PharMingen (San Diego, CA.).

**Cell line and in vitro infections**

LB27.4 cells are B cell hybridoma cells that express both MHC class I and II proteins of the H-2<sup>b</sup> haplotype. These cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS, 2 mM l-glutamine, and antibiotics. Cells were infected with VV at a multiplicity of infection of ~5 and harvested between 8 and 12 h after infection. To stimulate for intracellular cytokine staining, cells were then resuspended in complete RPMI 1640 and used to stimulate mouse splenocytes. For CTL assays, these cells were labeled with ⁵¹Cr for 1 h, washed three times, and used as target cells in a 5-h ⁵¹Cr release assay, as previously described (19). The percent specific lysis is calculated as the percent lysis of VV-infected targets minus uninfected targets.

**Intracellular cytokine stimulation and staining**

Single-cell suspensions of splenocytes were prepared from immunized Ly5.2<sup>+</sup> mice and used as effector cells. In a 96-well flat-bottom plate, either 1 × 10⁵ Ly5.2<sup>+</sup> splenocytes were incubated with irradiated 3 × 10⁵ uninfected SV40 or VV-infected splenocytes from Ly5.1<sup>+</sup> mice. All stimulations were performed overnight at 37°C, then with 5 h of further culture in the presence of brefeldin A (Golgi Plug; BD PharMingen) and intracellular cytokine staining was performed as previously described (19). First, the cell surface was stained with anti-CD8 (clone 53-6.7) and anti-CD4 (clone RM4-4) mAbs. The cells were then fixed and permeabilized (Cytofix/ Cytoperm kit; BD PharMingen) and subsequently stained intracellularly with anti-IFN-γ. Samples were fixed in PBS containing 2% parafomaldehyde and analyzed as described above. All Abs were purchased from BD PharMingen.

**ELISA**

At the indicated time points, VV-specific serum Ab titers were determined by a solid-phase ELISA as described previously (20). VV-infected HeLa lysate diluted in PBS was used as Ag to coat a 96-well flat-bottom Titer-plates (Flow Laboratories, McLean, VA), 100 μl/well containing 5 × 10⁶ PFU of VV overnight at 4°C. HRP-conjugated goat Abs against mouse IgM and IgG were purchased from Caltag Laboratories (Burlingame, CA) and ABTS (Sigma-Aldrich, St. Louis, MO) was used as substrate. The VV-specific Ab titer was determined by subtracting the absorbance number binding to HeLa lysate-coated plates from that binding to VV-HeLa lysate-coated plates. Relative ELISA units were expressed as the reciprocal of the highest dilution showing an OD reading >2 SDs from the OD reading of serum samples from naive mice.

**Ovary VV titer assay**

Mice were sacrificed on certain days after infection. Ovaries were removed, washed, and stored in 1 ml of DMEM (with 2.5% FCS) at −70°C. Pairs of ovaries from individual mice were homogenized, freeze-thawed three times, and sonicated for 2 min with a pause every half minute using an ultrasonic cleaner 1210 Branson (Danbury, CT). Serial dilutions were made and the virus titers were then determined by plaque assay on confluent SV40 cells.

**Results**

**Essential role of CD4<sup>+</sup> T cells and B cells in VV clearance**

To assess immune control of VV infection, a number of approaches were taken to deplete mice of subsets of lymphocytes and a variety of assays were used to assess VV pathology. C57BL/6 mice were depleted of CD4<sup>+</sup> or CD8<sup>-</sup> T cells by repeated injection of opsonizing mAb and injected with 2 × 10⁸ PFU of VV. In this experiment, none of the mice lost >10% of starting body weight (data not shown). Viral load in the ovaries was determined on days 7, 14, and 20 PI (Fig. 1A). Control B6 mice, as well as CD4<sup>-</sup> or CD8-depleted mice, showed high virus titers at day 7. By day 14 it was apparent that CD4-depleted mice were compromised in their ability to clear virus; they harbored 100-fold more PFU than control or CD8-depleted mice, and retained high levels at day...
20 PI. The CD8-depleted mice showed a 2-fold higher viral titer in ovaries than controls at day 20, but this was not statistically significant.

In addition to the group of mAb-depleted mice, IgH−/− mice were included in this experiment (Fig. 1A). Similar to CD4-depleted mice, these mice retained high virus titers at day 14 PI. This suggests that the deficiency in the CD4-depleted mice may be explained by their inability to mount an effective humoral response.

Other groups of gene knockout mice were also tested for their ability to control primary VV infection (Fig. 1B). CD8−/− mice that lack CD8 lineage cells, MHC class II−/− mice that lack CD4 lineage cells, and Rag.1−/− mice that cannot make T cells or B cells were tested. Rag.1−/− mice were unable to control infection as they rapidly lost body weight after day 6 and they were euthanized at day 10 PI. In contrast, the MHC class II−/− mice lost up to 10% body weight very early PI, but recovered the weight by day 8 PI. VV titers in ovaries were determined on day 14 PI, revealing that the MHC class II−/− mice (similar to the anti-CD4 mAb-treated mice) retained high levels of virus. Mice lacking CD8 T cells showed 1 log higher VV titers than wild-type mice in this experiment. However, in a second experiment there was no difference in recovered PFU between control and CD8−/− mice, and overall the difference was not statistically significant.

Cell-mediated and humoral immunity to VV

Following infection of control C57BL/6 mice, CD4 and CD8 T cells are able to mount significant VV-specific responses as measured by intracellular staining for IFN-γ (Fig. 2). At day 7 PI, 4% of CD4s and 20% of CD8s respond specifically to VV-infected cells.

The T cell response to VV was also measurable in 51Cr release assays using splenocytes as effectors at day 7 PI (Fig. 3). Cytotoxicity toward VV-infected target cells was not diminished in anti-CD4 mAb-depleted mice. Thus, the primary CD8+ cytotoxic T cell response to this virus is not dependent on CD4+ T cells. Splenocytes from mice that had been depleted of CD8+ cells had no demonstrable cytotoxicity toward VV-infected target cells which express both MHC class I and class II, suggesting that the bulk of activity was mediated by CD8+ T cells. IgH−/− mice that lack B cells showed a higher level of specific cytolyis than wild-type mice, a result that is possibly explained by the smaller spleen size and higher fraction of T cells among splenocytes in these mice.

The serum Ab response against VV was also monitored in mAb-depleted and gene knockout mice. Control and CD8-depleted mice had high levels of specific IgM and IgG responses measured at days 7 and 14 PI, respectively (Fig. 4A). In contrast, CD4-depleted mice showed a weaker IgM response and no specific IgG response could be detected. In a separate experiment, MHC class II−/− mice showed the same deficiency in the humoral response as CD4-depleted mice (Fig. 4B). Thus, MHC class II-restricted activity of

FIGURE 2. IFNγ production by VV-specific CD4 and CD8 T cells following primary infection. C57BL/6 mice (Ly5.2+) were injected i.p. with 2 × 106 PFU of VV. Splenocytes collected at day 7 PI were stimulated with uninfected or VV-infected Ly5.1+ splenocytes for 12 h in vitro. Intracellular IFN-γ staining was performed to determine the frequencies of virus-specific CD4 and CD8 T cells. The percentages of Ly5.2+ CD4 or CD8 T cells that were IFN-γ+ are indicated in the upper right corners.

FIGURE 3. Direct ex vivo cytotoxic activity from VV-immunized mice. Splenocytes were isolated from C57BL/6 control, CD4, or CD8 mAb-depleted and IgH−/− mice 7 days after VV injection (2 × 106 PFU i.p.). Cytotoxicity was assayed on 51Cr-labeled uninfected or VV-infected LB27.4 target cells. Data show the average of three mice. The percent specific lysis is calculated as the percent lysis of VV-infected targets minus that of uninfected targets which was 3% or less at the highest 200:1 E:T ratio.

FIGURE 4. VV-specific IgM and IgG Ab responses following primary immunization. Mice were immunized on day 0 with 2 × 106 PFU of VV delivered i.p. A. Control C57BL6 and mice depleted of CD4 or CD8 T cells with mAb were bled for IgM titers on day 7 and for IgG titers on day 14. B. Control and MHC class II−/− mice were bled on days 7 and 30 for IgM and IgG assays. Data show the average of four mice per group. Naive represents serum from unimmunized C57BL/6 mice.
CD4 T cells is required to mount a robust antivaccinia humoral response.

**CD8 T cells can mediate protection**

Our data so far have shown that, in the face of a strong Ab response requiring B cells and CD4 T cells, there is at best only a slight contribution of CD8 cells to primary protection against VV infection (Fig. 1). This is despite the fact that CD8 T cells in a normal mouse mount a response measured by cytotoxicity and IFN-γ secretion (Fig. 2). To study the potential efficacy of this CD8 response in primary protection, we used MHC class II−/− mice that are defective in the humoral response. The mice were either left untreated or depleted of CD8+ T cells by injection of mAb. As shown previously in Fig. 1, MHC class II−/− mice showed only a transient loss of body weight following infection. This contrasted with the progressive loss of weight in CD8-depleted mice (Fig. 5A). These mice showed ruffled fur, lethargy, and other signs of pathology and were euthanized at day 16 PI. The depleted mice showed numerous pocks and lesions on the tail and feet that became detectable by day 10 and peaked around day 12. Fig. 4B illustrates the presence of pocks on the tails of mice from untreated, infected MHC class II−/− mice and CD8-depleted mice at

**FIGURE 5.** CD8+ T cell-mediated protection in VV-infected MHC class II−/− mice. MHC class II−/− mice (four mice per group) were either left as control or depleted CD8+ T cells with anti-CD8 Ab. Mice were immunized with VV (2 × 106 PFU i.p.) on day 0. A, Changes in body weight are expressed as relative to the weight observed at day 0. Values are the means for each group. B, Pocks (denoted by arrows) on tails of day 13-immunized mice. Right panel, The average numbers of tail pocks in each group. C, VV-specific IgM Ab response. Serum samples were collected on day 10 PI. D, Adrenal necrosis in VV-infected, CD8-depleted MHC class II−/− mice. Representative H&E-stained sections from control MHC class II−/− and CD8-depleted MHC class II−/− mice at day 16 PI. Upper panels, Magnification, ×10; lower panels, enlargement of indicated areas.
day 13 PI. Some of the controls had a few small pocks on their tails, but did not develop lesions. The low level of IgM Ab produced by MHC class II−/− mice at day 10 PI was not affected by the depletion of CD8 cells (Fig. 4C). No detectable IgG response was observed in either group of mice. All mice were examined at day 16 PI for VV titers in ovaries and for other signs of infection. Control and CD8-depleted MHC class II−/− mice showed similar viral titers in ovaries at roughly 10^7 PFU/ovary. We also compared histopathologic changes in tissues from the two groups. In addition to the external appearance and numbers of pocks and lesions, a clear difference was found in the adrenals (Fig. 5D). Acute, focal extensive, and multifocal parenchymal cell degeneration and necrosis was observed in the zona fasciculata of the adrenal cortex, only in the CD8-depleted MHC class II−/− mice. This necrotic change lacked any inflammatory cell component and was not accompanied by vascular lesions. Unequivocal inclusion bodies were not recognized in affected adrenal glands. The necrosis occurred despite the fact that similar, low viral titers were observed in adrenals of both groups. Thus, the mechanism of injury to the adrenals and the way in which CD8 T cells protect against this pathology are unclear at present.

**Memory CD8+ T cells protect against secondary challenge**

We asked whether previously primed MHC class II−/− mice had protective immunity against a second challenge with VV and the nature of this protection. At 60 days PI, MHC class II−/− mice had no virus detectable in the ovary. Groups of primed MHC class II−/− mice were left as control or were depleted of CD8+ T cells by repeated mAb injection. These mice, plus naive MHC class II−/− mice as well as primed mice that received injections of a NK cell-depleting mAb, were challenged with a high dose of VV and viral titers in ovary were determined 3 days later (Fig. 6). At this early time point, naive MHC class II−/− mice contained high virus titers in the ovaries, while previously primed “memory” mice had <100 PFU/ovary. At least part of this protection is mediated by CD8+ T cells, since their depletion just before challenge resulted in much higher viral titers in the ovaries. These CD8-depleted memory mice, however, did control virus better than naive mice, suggesting that other forms of memory protection, such as IgM Ab, may contribute to immunity in these animals. Primed MHC class II−/− mice that were depleted of NK cells just before challenge showed no decrease in protection measured as PFU in the ovary.

We further addressed the ability of memory CD8 T cells to mediate protective immunity by adoptive transfer. C57BL/6 mice were immunized with 2 × 10^6 PFU of VV i.p. and sacrificed after 3 mo. At this time, 4% of spleen CD8 T cells were VV specific as determined by in vitro stimulation and IFN-γ staining. Serum collected from the same group of mice contained 10^3 relative ELISA units of VV-specific IgG (data not shown). Splenic CD8 T cells were purified by no-touch magnetic bead separation, with purity ranging from 80 to 90% in different experiments. The contaminating cells were not CD4 T cells or B cells and included up to one-third γδ T cells which have not been shown to mediate protective memory against VV. Either 2 × 10^7 purified CD8 T cells or 300 μl of serum from these memory mice were then adoptively transferred to naive C57BL/6 recipients. These mice along with one group of control mice were then challenged with 2 × 10^6 PFU of VV i.p., and viral control was examined in the ovary on day 6. As shown in Fig. 7, mice that received memory CD8 T cells had a 100-fold lower virus titer than that of control mice. The transfer of 2 × 10^7 immune CD8 T cells mediated considerably more control of viral infection than did 300 μl of transferred serum, as these recipients contained higher levels of virus in the ovary.

**Discussion**

Immunization with VV results in long-lasting protection against smallpox and was the approach used to eliminate natural smallpox infections worldwide. This success was achieved without a detailed understanding of immune responses to poxviruses. In this article, we have studied T cell protection against VV in mice after acute primary infection and in previously infected mice rechallenged with VV. Our results suggest that CD4+ T cells and MHC class II-mediated immunity are essential for clearing VV during acute infection. B cell-deficient mice show similar failure to clear VV as CD4-depleted mice. In line with others, we attribute the inability of CD4+ T cell-depleted, MHC class II−/− and IgH−/− mice to clear a primary VV infection to their inability to make any switched IgG response and a weaker (or absent) IgM response.

Following acute VV infection, virus-specific Ab appears to be the most important effector for clearing virus or preventing virus replication. Depletion of CD8 T cells by mAb or gene knockout has little effect on the course of primary infection. There are numerous mechanisms utilized by specific Abs to destroy poxviruses: First, Ab can directly bind to the virus, causing aggregation and obstructing viral absorption and internalization into the cell. Furthermore, Abs can bind to the virus, allowing for complement-mediated lysis or opsonization, resulting in phagocytosis. Abs can
also bind the infected host cell, leading to Ab-dependent cell-mediated cytotoxicity. In addition to their role in B cell activation and switching, activated CD4 T cells also produce several cytokines, such as IFN-γ, TNF-α, and IL-2, and provide costimulatory molecules, like CD40 ligand, for activation of APCs. With regard to this, we saw no effect of CD4 depletion on the CD8+ cytotoxic response at day 7 PI.

The antiviral function of CD8 T cells in natural resistance against exposure of VV is still unclear. Spriggs et al. (16) showed that β2m–/– mice, which lack CD8 T cells, survived infection with high virus doses similar to those of control mice. In this report, the course of infection appeared indistinguishable from controls except that less virus-specific IgG Abs were produced. We have observed that in C57BL/6 mice both CD4 and CD8 T cells are highly activated at day 7 PI and that CD8 T cells show specific CTL function ex vivo. Despite the massive CD8 response, we have found that both CD8-depleted C57BL/6 mice and CD8–/– mice cleared virus at similar rates as control mice. This suggests that activated CD8 T cells are not essential for virus clearance following acute infection. However, in the absence of humoral or CD4+ T cell-mediated immunity, a protective function of CD8+ T cells against VV can be demonstrated. We report that MHC class II–/– mice infected with a sublethal dose of VV can survive and finally clear virus, but only through the action of CD8+ T cells. This indicates that CD8 T cells play a role in preventing weight loss following VV infection and in reducing mortality. The mechanism of this protection by CD8s, whether it is via cytotoxicity or cytokine based, is unclear and needs to be studied further.

MHC class II–/– mice that have been depleted of CD8 T cells develop several lesions during primary infection. They have numerous pocks on the feet and tail, and the adrenal cortex showed extensive necrosis. This adrenal necrosis was also observed in VV-infected Rag1–/– mice (data not shown). This may be the first report of adrenal damage following VV infection. It has been reported that adenoviruses have particular tropism for the parenchymal cells in the zona fasciculata (21). The adrenal gland is also frequently affected in the absence of humoral or CD4-dependent Ab responses at day 7 PI. Moreover, we report that depletion of CD8 T cells in immunized BALB/c mice, roughly 5% of CD8 T cells were virus specific and stably maintained for >300 days. More recently, two CD8 T cell epitopes restricted by human MHC class I were identified (29). It was found that the frequency of VV-specific CD8 T cells was detectable for many years following vaccination immunization. The protective function of these memory CD8 T cells has not been studied yet. Recently, Belyakov et al. (9) reported that depletion of CD8 T cells in immunized BALB/c mice was not sufficient to render these mice susceptible to rechallenge with VV (9). In the absence of antibody, we have found that memory CD8 T cells are able to provide protection from VV rechallenge in MHC class II–/– mice. Furthermore, we report that depletion of CD8 T cells from memory MHC class II–/– mice significantly reduces resistance to VV. We further confirmed that memory CD8 T cells have the ability to protect naive mice by adoptive transfer to naive recipients. Several factors may account for the differences in interpretations in the potential role of CD8 immunity: First, Belakov et al. (9) transferred fewer CD8 T cells which were purified by a positive sort, while we used CD8 T cells purified with a negative sort. Second, different routes of immunization were used and, third, we determined the protective function by in vivo virus load, whereas they monitored weight loss. Although it seems likely that in healthy animals, CD4-dependent Ab responses are most important in controlling initial infection and in providing long-term protective immunity, our results suggest that in cases of deficiencies in either B cells or CD4 T cells, the CD8 response could be vitally important in controlling VV infection.

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