Optimal Long-Term Humoral Responses to Replication-Defective Herpes Simplex Virus Require CD21/CD35 Complement Receptor Expression on Stromal Cells

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Replication-defective herpes simplex virus (HSV) strains elicit durable immune responses and protect against virulent HSV challenge in mice, despite being unable to establish latent infection in neuronal cells. Mechanisms for generating long-lived immunity in the absence of viral persistence remain uncertain. In animals immunized with replication-defective HSV, durable serum immunoglobulin G (IgG) responses were elicited. Surprisingly, Western blot analyses revealed that the specificities of antiviral IgG changed over time, and antibody reactivity to some viral proteins was detected only very late. Thus, some of the durable IgG activity appeared to be contributed by either new or significantly enhanced antibody responses at late times. Following immunization, radiation bone marrow-chimeric mice lacking complement receptors CD21 and CD35 on stromal cells elicited only short-lived serum IgG and failed to mount recall responses to subsequent HSV exposure. Our results suggest that complement-mediated retention of viral antigens by stromal cells, such as follicular dendritic cells, is critical for optimal maintenance of antibody responses and B-cell memory following vaccination with replication-defective HSV.
replication-defective HSV induces durable immunity, we evaluated the antiviral IgG response in mice. We observed that the HSV protein specificities of the IgG response shifted over time, suggesting that the humoral response continued to evolve several months postinfection. To assess the role of antigen retention in this setting, we used a radiation-chimeric mouse model. In mice where CD21/CD35 was absent on stromal cells, antiviral serum IgG titers declined rapidly and recall responses to HSV were lost. We conclude that optimal maintenance of IgG responses following replication-defective HSV infection requires expression of CD21/CD35 on stromal cells.

MATERIALS AND METHODS

Cells and viruses. Vero cells (CCL-81; American Type Culture Collection) containing the HSV-2 U5, 5 and HSV-1 U29 genes (VS-29 cells) have been described previously (10). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 400 μg/ml G418, and 10% bovine calf serum (vol/vol) (DMEM-10). Cells were maintained in DMEM containing penicillin, streptomycin, l-glutamine, and 1% bovine calf serum (vol/vol) (DMEM-V) at 34°C until harvest.

Replication-defective HSV-2 strains d55, d29 (9, 12), and SBlacZ (10) have been described previously. The d55 mutant was generated from the HSV-2 186 WT strain by deletion of the UL5 gene. UL5 is an essential component of the viral helicase-primase complex and is required for viral DNA synthesis. The d29 mutant was generated in the 186 strain by deletion of the UL29 gene, which encodes the viral single-stranded DNA binding protein ICP8, which is also essential for viral DNA synthesis. The SBlacZ mutant was generated from the 186 strain by replacing the UL29 gene (ICP8 protein) with a fusion protein that contains the N-terminal 288 amino acids of ICP8 fused to the Escherichia coli β-galactosidase protein. All viruses were propagated on a complementing Vero-derived cell line, V5-29, which expresses the UL5 and UL29 gene products. Stocks of cell-free virus were prepared and their titers determined using V5-29 cells as described previously (19), and they were verified to be replication defective by titration on noncomplementing Vero cells. The attenuated, replication-competent HSV-2 186ΔKpn strain contains a deletion in the viral thymidine kinase (TK) gene (17) and was used in some experiments to compare the persistence levels of viral DNA following immunization. This virus was grown and its titer was determined using Vero cells.

Mice and bone marrow chimera construction. BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for the experiments shown in Fig. 2 and 3. C57BL/6 mice (CD45.1 allotype) served as WT controls and bone marrow donors for the generation of radiation chimeras (see Fig. 4). Mice deficient in complement receptors CD21 and CD35 (C2γ−/−), maintained on a C57BL/6 background (CD45.2 allotype), were used as controls and bone marrow donors for the generation of radiation chimeras. C57BL/6 mice, 2 to 3 months of age, were bred in-house at Harvard Medical School. Radiation chimeras were generated as described previously (38). Briefly, 6-week-old mice were lethally irradiated (2 × 650 rads from a cesium-137 source), anesthetized by isoflurane inhalation (Abbott Laboratories), infusèd with 1 × 106 WT bone marrow cells, and rested for 6 weeks prior to the experiments. Bone marrow chimeras were evaluated by measuring the percentage of CD45.1-positive peripheral blood mononuclear cells using flow cytometry. Successful chimeras were defined as animals with at least 95% donor bone marrow-derived peripheral blood mononuclear cells. All mice were housed at Harvard Medical School in a specific-pathogen-free facility. Study procedures complied with NIH guidelines and institutional reviews by the CBIR Institute and Harvard Medical School Animal Care and Use committees.

Viruses. Mice were inoculated by either the s.c. (s.c.), the intramuscular (i.m.), or the intraperitoneal (i.p.) route using a dose of 2 × 106 PFU of virus A volume prepared by dilution in sterile, endotoxin-free saline (Sigma). For initial experiments, groups of BALB/c mice were inoculated s.c. at week 0 either d55 or d29. At weeks 4 and 8, all mice were inoculated s.c. with SBlacZ. This immunization strategy reproducibly elicits protection against challenge infection and induces robust and durable IgG responses (7, 29).

For radiation chimeras experiments, C57BL/6 (WT), C2γ−/− mice reconstituted with WT bone marrow or with 5% C2γ−/− or 5% WT bone marrow, were inoculated with HSV-2 5B tk−/−, HSV-2 186ΔKpn, or HSV-2 5B tk−/−ΔlacZ at 1, 2, and 4 weeks. All mice were infected by the i.m. route using a dose of 2 × 106 PFU of SBlacZ at weeks 0, 4, and 8. Recall responses in these mice were measured following i.p. inoculation with SBlacZ at week 32 (6 months following the final immunization). The i.p. route was chosen to elicit maximal stimulation of memory B-cell responses.

Serum collection and ELISA. Blood was collected from each mouse prior to inoculation (week 0) and throughout the course of each experiment by tail vein puncture or retro-orbital bleeding. Sera were prepared using Microlainer brand serum separators (Becton Dickinson) and stored at −20°C prior to analysis. Enzyme-linked immunosorbent assays (ELISA) to determine antigen-specific IgG titers were completed as described previously (11). Briefly, 96-well Maxisorp microtiter plates (Nunc) were coated with HSV-2 (WT, C2γ−/−, or ΔlacZ) protein at 400 ng/volume containing 30 ng of DNA and 200 ng of Taqman PCR amplification and detection were performed on an ABI 7700 sequence detector (Applied Biosystems) using the HSV-2 TK gene-specific primers F145 (CTG TCC TTT TAT TGG CAT CGT CG) and R263 (GTC CAT CCG GTA GTC GC) and the fluorescence-labeled probe TK-pr (6-carboxyfluorescein-TTT GAA CTA AAC TCC CCC CAC CTC GC-6-carboxytetramethylrhodamine). Reactions were performed in a 50-μl volume containing 30 ng of DNA and Taqman universal PCR master mix (Applied Biosystems) with a final concentration of 250 nM of each primer and 200 nM of Taqman probe and amplified for 40 cycles. For the viral DNA standard, purified HSV-2 186 strain DNA was serially 10-fold diluted (from 1 × 108 to 1 copy) in the presence of 30 ng generic DNA derived from uninfected CV-1 cells. This standard curve indicated that the limit of detection for the assay was one copy.

RESULTS

Replication-defective HSV-2 recombinants elicit durable humoral responses. We have shown previously that replication-defective HSV-1 mutant viruses elicit durable immunity in mouse models (7, 31). Because of the potential utility of mutant HSV-2 strains as genital herpes vaccines (9, 12), we wanted to verify these observations using HSV-2 mutant strains. We examined the serum IgG response following immunization with replication-defective HSV-2 mutants derived from HSV-2 186 strain virus that are deleted for either the UL5 (a helicase-primase component protein gene) or the UL29 single-stranded DNA binding protein gene (Fig. 1). Both of these genes are essential for productive HSV infection (10). Mice were inoculated at week 0 by the s.c. route with a dose of 2 × 106 PFU of d55 or d29. At weeks 4 and 8, all mice were inoculated with a dose of 2 × 106 PFU of SBlacZ. We collected serum from each animal
every 2 weeks up to week 12 and measured antiviral IgG by ELISA (Fig. 2). Primary inoculation with either dl5 or dl29 virus generated a low-titer, anti-HSV IgG response by week 4. Secondary infection with 5BlacZ enhanced responses at weeks 6 and 8, and an additional boost with 5BlacZ further increased antiviral IgG titers by weeks 10 and 12. Unexpectedly, serum titers in the dl5 group were consistently lower than in the dl29-inoculated animals. This may indicate a difference in the ability of these viruses to produce viral antigen and induce primary IgG responses that were apparent only following a secondary exposure using 5BlacZ.

To assess the durability of these responses, we evaluated serum titers in these mice at weeks 16, 20, 30, 44, and 60. We observed that antiviral IgG titers peaked at relatively early times and then declined in all groups but stabilized by week 20 at approximately 30 to 50% of peak titers. Antibody titers were constant for at least 50 weeks postinfection, and these results paralleled our earlier findings of durable IgG antibody responses following inoculation of mice using replication-defective HSV-1 strains (7).

**Some IgG responses are enhanced at late times postinfection.** Productive HSV infection is characterized by the expression of at least 70 viral proteins. To assess the breadth of the IgG responses in these mice, we evaluated the HSV-2 antigen specificity using Western blot analysis. Pooled sera from the dl29-infected group of mice were used to probe membranes prepared using cell-free HSV-2 virion protein lysate (Fig. 3A). Despite the fact that ELISA titers remained relatively stable at late times, we observed that the antiviral IgG specificities were not static over this period. Three patterns of antibody responses, which we have labeled “continuous,” “declining,” and “late/enhanced,” were seen in these mice. Continuous IgG responses were usually generated immediately following infection and remained detectable for the duration of the experiment. This type of response could be seen for proteins with molecular masses of ~200 kDa and ~35 kDa. Declining responses were also induced early following infection but appeared to diminish over time (e.g., for proteins with molecular masses of ~180 kDa, ~120 kDa, ~110 kDa, and ~50 kDa). Late/enhanced IgG responses appeared to be significantly improved at later times in the absence of new infection (e.g., for proteins with molecular masses of ~70 kDa, ~60 kDa, and ~40 kDa).

A significant component of the host immune response to HSV is likely to be directed against nonstructural viral proteins that are highly expressed in infected cells and not incorporated efficiently into viral particles. Therefore, we repeated this Western blot analysis using membranes prepared with lysates from HSV-infected V5-29 cells (Fig. 3B). We observed similar patterns of responses regardless of the source of viral proteins. Continuous IgG responses were directed against proteins with molecular masses of ~200 kDa, ~110 kDa, ~80 kDa, ~65 kDa (Fig. 3B, broad band), ~60 kDa, and ~35 kDa. In addition, a late/enhanced IgG response against one protein with a molecular mass of ~55 kDa was observed. Next, we performed similar Western blot analyses using sera from the dl5 group of mice (Fig. 3C). In addition to several continuous responses in these animals (i.e., for proteins with molecular masses of ~110 kDa and 65 kDa), we observed late/enhanced responses to proteins with molecular masses of ~60 kDa and ~55 kDa. Taken together, these results indicated that the IgG responses evolved over an extended time following immunization with replication-defective HSV mutants. This might be due to maturation of IgG avidity, ongoing isotype class-switch recombination, or delayed expansion of low-magnitude cell populations. Regardless of the mechanism, this observation indicates that sufficient viral antigen may persist following inoculation to drive IgG evolution. We observed similar late/enhanced responses to a subset of viral proteins in both groups of mice, further implying that this result may be a general occurrence following infection with replication-defective HSV vectors.

**Maintenance of serum IgG requires CD21/CD35 on stromal cells.** The apparent ongoing evolution of the humoral immune response indicated that antigen persistence might play an important role in the generation of durable immunity following infection by replication-defective HSV. To address this directly, we used a radiation bone marrow chimera model in which Cr2+/myeloid cells were transferred into Cr2−/−mice. We generated chimeric mice by reconstituting irradiated Cr2−/−mice with wild-type bone marrow (WTBM→Cr2−/−).
The percentages of chimerism were examined by flow cytometry to distinguish donor cells (CD45.1 positive) from recipient cells (CD45.2 positive) and were greater than 98% in all mice (data not shown). Mice were inoculated at weeks 0, 4, and 8 with a dose of $2 \times 10^6$ PFU of 5B\textit{lacZ} virus by the i.m. route. Sera were collected regularly, up to week 32, and antiviral IgG titers were determined using ELISA (Fig. 4A). Following infection, WT mice elicited IgG responses at weeks 3 through 10 that were similar in kinetics and magnitude to those in previous experiments (Fig. 2). Cr2\textsuperscript{−/−} mice displayed impaired humoral responses to HSV, as observed previously (11). This result was consistent with the loss of essential costimulatory signals provided by CD21/CD35 on B cells (1, 13). WTBM\textsuperscript{−}Cr2\textsuperscript{−/−} chimeras induced robust IgG responses that were comparable to those in WT controls through at least 10 weeks. Between weeks 10 and 13, we observed a decline in serum IgG titers from peak values in all groups. The rates of IgG loss, however, were more rapid in the WTBM\textsuperscript{−}Cr2\textsuperscript{−/−} chimeric animals. By week 22, only WT mice retained significant antiviral serum IgG (approximately 35% of peak titers). These results indicated that CD21/CD35 on radiation-resistant cells, likely stromal cells, played an important role in the maintenance of IgG responses at later times following replication-defective HSV infection.

Stromal cell CD21/CD35 is critical for IgG recall responses. To assess memory B-cell activity at late times postinfection, we boosted the mice by immunization with 5B\textit{lacZ} virus and

![FIG. 3. Specificities of serum IgG responses change at late times following inoculation. Sera were collected as indicated in Fig. 2, and equivalent volumes from each mouse were pooled for use in Western blot assays to determine the breadth of IgG reactivities to HSV-2 proteins. Samples from the \textit{dl29} group (A and B) or the \textit{dl5} group (C) were used to probe nitrocellulose membranes prepared using cell-free HSV-2 virion protein lysate (A) or HSV-2 infected V5-29 cell lysate (B and C) as described in Materials and Methods. The times of serum collection (in weeks) are indicated at the top. Molecular masses (in kDa) of marker proteins for each blot are shown on the left. Continuous (C), declining (D), and late/enhanced (E) responses, as described in Results, are indicated to the right of each blot.](image)

![FIG. 4. Serum IgG responses to replication-defective HSV infection require stromal cell CD21/CD35. (A) WT mice (n = 7) (black circles), WTBM\textsuperscript{−}Cr2\textsuperscript{−/−} chimeras (n = 5) (white squares and dashed line), and Cr2\textsuperscript{−/−} mice (n = 5) (white triangles) were inoculated with $2 \times 10^6$ PFU of 5B\textit{lacZ} by the i.m. route at weeks 0, 4, and 8 (black triangles). Sera were collected at the indicated times, and anti-HSV-2 IgG titers were determined by ELISA. Mean reciprocal IgG titers $\pm$ SEMs are shown. (B) Recall responses in these mice were evaluated by i.p. inoculation with $2 \times 10^6$ PFU of 5B\textit{lacZ} at week 32 (white triangle). Serum IgG titers were determined by ELISA, and results are shown as means $\pm$ SEMs.](image)
measured their ability to elicit recall responses (Fig. 4B). To ensure a maximal recall response in these animals, we inoculated mice by the i.p. route at week 32 and collected sera at weeks 33 and 34 for ELISA analysis. WT mice primed 6 months earlier by the i.m. route responded to the i.p. boost with robust antiviral IgG titers that were greater than previous peak activity titers. Cr2<sup>−/−</sup> mice failed to elicit significant antiviral IgG following this additional exposure. Notably, the WTBM—Cr2<sup>−/−</sup> chimeric mice also failed to mount a significant recall response to this late HSV boost. These results suggested that the population of reactive memory B cells was significantly diminished in the absence of CD21/CD35 expression on stromal cells.

**Evaluation of viral persistence in inoculated mice.** The previous results argued that retention of viral antigen on stromal cells, such as FDC, was necessary for long-term antibody responses. Another possible explanation for this observation was persistent viral infection in these animals. Therefore, we examined the levels of HSV DNA present in muscle tissue from two WT mice 5 months following infection with 5BlacZ using a sensitive real-time PCR assay. In duplicate assays, only very low levels of HSV DNA (two to six molecules per sample) were observed in muscle at the site of inoculation with 5BlacZ (Table 1). HSV-2 DNA was not detected reproducibly in distal tissues from these mice (i.e., spleen and liver). Similar results were obtained following inoculation with the attenuated, replication-competent 186ΔKpn strain, which contains a deletion of the viral thymidine kinase gene. All tissues from a mock-treated animal were negative for HSV-2 DNA in these experiments. These data indicated that neither replicating nor nonreplicating strains of HSV-2 established ongoing infection efficiently. Instead, the limited amounts of viral DNA detected are more consistent with a model in which long-term antibody responses are maintained by mechanisms other than viral persistence, such as antigen retention on FDC.

**DISCUSSION**

We have shown here that immunization with replication-defective HSV-2 strains can elicit durable serum IgG responses and that antigen retention by CD21/CD35 appears to be very important for maintenance of long-term humoral immunity. We observed that the IgG response to replication-defective HSV-2 in WT mice was not static and that qualitative changes in antibody reactivity to viral proteins occurred at late times postinfection. In particular, many viral-antigen-specific IgG responses were generated at early times and were maintained over the course of 1 year postinfection. Other responses were generated early but appeared to diminish over this time. Finally, a subset of responses was enhanced at late times. This indicated that a portion of the durable antibody response to replication-defective HSV infection was due to antibody activity that was either new or significantly enhanced late after infection. Inoculation of WTBM—Cr2<sup>−/−</sup> chimeric mice indicated that complement receptors CD21 and/or CD35 expressed on stromal cells play an important role in maintaining serum IgG titers and memory B-cell responses following immunization. Together, these results indicate that antigen retention by CD21/CD35 is a necessary component of the durable antibody response elicited by replication-defective HSV mutants.

Durable serum antibody responses to viral infection can be explained by antigen-independent mechanisms mediated by the generation of long-lived, antibody-producing PCs (34, 35) or by antigen-dependent mechanisms that require continued stimulation of memory B cells by immune complexes trapped on FDC (2, 3) that are present in the spleen or lymph node (14, 18, 22). The continuous responses that we observed might arise by either mechanism. Similarly, the declining responses might be merely a less stable activity resulting from either turnover of short-lived PCs (34) or a loss of memory B cells due to the decay of trapped antigen or diminished B-cell stimulation (8, 15). Therefore, the differences we detected between continuous and declining responses may indicate variation in B-cell or PC generation quantity or quality at early times following infection. The late/enhanced responses that we observed following replication-defective HSV-2 infection, however, allow us to speculate on the importance of antigen retention in this setting, because generation of new B-cell responses, expansion of existing low-magnitude populations, or ongoing IgG avidity maturation would not be expected to occur in the absence of antigen. Detection of these responses at late times provides compelling evidence that viral antigen remains accessible for an extended time following infection.

To test this hypothesis directly, we used a bone marrow chimera model. The fact that maintenance of serum IgG titers and B-cell recall activity required CD21/CD35 expression on stromal cells provides strong support for a model in which the durable immunity generated by replication-defective HSV in mice is largely antigen dependent. Association of viral proteins with FDC would allow naïve B cells to encounter antigen and/or memory B cells generated in low abundance during initial infection to reengage antigen, thereby enhancing immunity without requiring ongoing infection. In the absence of efficient FDC-mediated antigen trapping in WTBM—Cr2<sup>−/−</sup> chimeric mice, early serum antibody titers were comparable to

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<tr>
<th>Virus</th>
<th>Tissue</th>
<th>vDNA (copies/sample)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
<td>Liver</td>
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<td>Muscle</td>
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<tr>
<td>5BlacZ (no. 2)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0, 1</td>
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<td>Liver</td>
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<td></td>
<td>Muscle</td>
<td>2, 6</td>
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<tr>
<td>186ΔKpn&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Mock</td>
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<sup>a</sup> Replicate samples from independent assays.

<sup>b</sup> Replication-defective strain.

<sup>c</sup> Replication-competent strain.

**TABLE 1.** Real-time PCR quantification of HSV DNA at 5 months postinfection.

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those in WT mice, indicating that induction of humoral responses was not altered significantly. Serum IgG titers, however, declined rapidly, and recall responses were lost in these chimeric animals, indicating specific defects in antibody and memory B-cell maintenance.

Additional experiments will be required to identify the viral proteins recognized by the late/enhanced IgG responses. The predominance of these responses in purified virion lysates, however, indicates that they may be components of the virus particle. Potential candidates include the structural proteins VP19c (53 kDa) and VP22a (40 kDa) or the tegument proteins VP16 (55 kDa) and VP22 (38 kDa). It is unclear why an immune response to these proteins might differ from those of other viral antigens, though it may indicate that virus particles are preferentially captured and/or stabilized on stromal cells following acute infection. Ongoing viral infection might also provide a continued source of antigen over this extended period. We observed very low levels of viral DNA in muscle 5 months after inoculation with replication-defective HSV-2, but we did not detect viral RNA transcripts for either the ICP4 or the ICP27 immediate early proteins (data not shown) and therefore do not believe that persistent viral infection can explain the durable immunity seen in these studies. Regardless of the source of the viral proteins, our results demonstrate that complement-mediated retention of antigen by receptors CD21 and CD35 on stromal cells plays a significant role in IgG durability.

Although it could be argued that the amount of viral protein generated during a single-round infection by a replication-defective virus is significantly reduced and that humoral responses in this context may be more dependent on complement activity, we have observed a similar dependence on complement for induction of IgG responses following WT HSV infection (11, 37, 38). Those studies suggested that the amount of viral antigen generated during natural HSV infection is limiting or inefficiently presented to B cells without the assistance of these innate factors. Our current results are consistent with a model in which opsonization by complement protein C3 and subsequent trapping of immune complexes by CD21/CD35 on FDC are a necessary step in the maintenance of IgG responses to replication-defective HSV, indicating that optimal durable immunity is dependent on in vivo antigen retention.

In conclusion, we have shown that the serum IgG antibody response following infection with replication-defective HSV-2 is durable but not static over time. Analyses of WTBM→Cr2−/− chimeric mice indicated that CD21/CD35 expression on radiation-resistant stromal cells was necessary to maintain serum IgG titers. Furthermore, these chimeric mice failed to mount a recall response to subsequent HSV-2 infection, indicating a severe defect in B-cell memory. Our data support a model in which antigen retention on FDC is necessary to maintain optimal serum IgG and memory B-cell responses following replication-defective HSV infection. This may allow ongoing stimulation of naïve and memory B cells and replenishment of a short-lived, antibody-producing plasma cell population. Additional experiments will be required to distinguish these possibilities. We observed only low levels of HSV-specific antibodies in Cr2−/− mice, which were generated presumably in the absence of significant antigen trapping by stromal cells and receptor costimulation provided by Cr2 on B cells. It is apparent, however, that optimal long-term IgG responses in this experimental situation require Cr2 expression on stromal cells, which likely functions to allow viral antigen retention by FDC. A more detailed understanding of this CD21/CD35-mediated mechanism may assist in the design and development of vaccines that induce durable and protective immune responses.

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