SHORT COMMUNICATION

Strikingly poor CD8+ T-cell immunogenicity of vaccinia virus strain MVA in BALB/c mice

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Vaccinia virus (VACV) strain MVA is a highly attenuated vector for vaccines that is being explored in clinical trials. We compared the CD8+ T-cell immunogenicity of MVA with that of a virulent laboratory strain of VACV (strain WR) in BALB/c mice by examining epitope-specific responses as well as estimating the total number of activated CD8+ T cells, irrespective of specificity. We found that MVA elicited total CD8+ T-cell responses that were reduced by at least 20-fold compared with strain WR in BALB/c mice. In C57Bl/6 mice, we also found a substantial difference in immunogenicity between these VACV strains, but it was more modest at around fivefold. Of note, the size of responses to the virulent WR virus was similar in both strains of mice suggesting that BALB/c mice can mount robust CD8+ T-cell responses to VACV. Although the data for total responses clearly showed that MVA overall is poorly immunogenic in BALB/c mice, we found one epitope for which strong responses were made irrespective of virus strain. Therefore, in the context of a vaccine, some recombinant epitopes may have similar immunogenicity when expressed from MVA and other strains of VACV, but we would expect these to be exceptions. These data show clearly the substantial difference in immunogenicity between MVA and virulent VACV strains and suggest that the impact of host genetics on responses to attenuated vaccine vectors like MVA requires more consideration.

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Since its use to eradicate smallpox, vaccinia virus (VACV) has attracted considerable interest as a recombinant vaccine vector, having a large capacity for foreign genes and inherent immunogenicity.1,2 As a result, vaccines based on highly attenuated strains of VACV, such as Modified Vaccinia Ankara (MVA), have entered clinical trials.3,4 Mouse models are invaluable for developing vaccines but are frequently limited to a single inbred strain of mice. Further, it is assumed that data from virulent VACV strains such as Western Reserve (WR) can be directly extrapolated to attenuated vectors, although virulence and antigen dose are linked to immunogenicity. In this context, the CD8+ T-cell (TCD8+) response to VACV has been most extensively investigated using C57Bl/6 mice and for VACV strain WR.5,6 Here we ask whether these data can be reasonably generalized to other strains of VACV, including MVA and to BALB/c mice, a commonly used inbred strain preferred for some infectious models.7,8

RESULTS AND DISCUSSION

First, we measured TCD8+ responses in BALB/c mice 7 days after immunization with VACV strains MVA, Copenhagen and WR using peptides published as H-2d-presented epitopes9,10 to restimulate splenocytes before intracellular staining for IFN-γ (IFN-γ ICS).11 This set of native VACV epitopes was used rather than a single native (or recombinant) epitope to obtain representative results and because previous studies have shown that changes in immunodominance can be misinterpreted as differences in immunogenicity.12 Immunization with WR resulted in larger TCD8+ responses than with Copenhagen and especially MVA for almost all peptides (Figure 1a). The only exception was E3140, which elicits a similar sized TCD8+ response for all strains of VACV. Comparisons between the virus strains were similar in mice > 30 days after immunization (not shown).

Lack of conservation of key epitopes across the VACV strains complicates the experiment above. In particular, the most immunogenic epitope in WR (F226(Y); SPYAAGYDL) exists as a variant (F226(G); SPGAAGYDL) in Copenhagen and MVA and in these viruses anti-F226 responses are far less prominent. A5275 and B249 are absent in MVA and C674 exist as two alternate variants (GFIRSLQTL in WR and SFIRSLQNI in Copenhagen and MVA). Both versions of this peptide were used in all IFN-γ ICS assays, but all the virus strains produced the same result and for simplicity we show data from homologous variants for each virus.

As noted above, the F226(G) variant of the immunodominant F226(Y) epitope in WR elicits relatively weak TCD8+ responses from Copenhagen and even more so from MVA, but it is not clear to what extent this is due to the epitope variant or the virus strain background. To distinguish between these possibilities and to allow matching of a known immunodominant epitope between WR and MVA, we made recombinants of both, engineered such that they expressed the alternate variant of the F226 epitope. WR F2G had the usual dominant WR F226(Y) epitope replaced with F226(G), and MVA F2Y had the reverse replacement. After immunizing mice with these...
viruses, we found that replacement of the F226(Y) epitope of WR with F226(G) in WR F2G, substantially reduced F226-specific responses, but other specificities were not altered (Figure 1b). Conversely, the introduction of F226(Y) to MVA, while improving F226-specific TCD8+ responses compared with MVA, produced a response that was less than a quarter of that elicited by WR. Indeed WR F2G induced a significantly higher F226-specific response than MVA F2Y, although it expressed subdominant and immunodominant variants, respectively ($P = 0.0256$). Summing up the responses to the conserved epitopes tested in this experiment (including only the appropriate variant of F226) showed that MVA F2Y was much less immunogenic than WR, although it shared the immunodominant F226(Y) epitope (Figure 1c). Similarly, WR F2G remained substantially more immunogenic than MVA, despite both viruses containing the subdominant F226(G) epitope. These data suggest that MVA is inherently poorly immunogenic in BALB/c mice compared with WR.

To ensure that the results above were not unwittingly biased by the set of epitopes that we had available, we used an epitope-independent method of measuring TCD8+ responses. Upon activation of TCD8+ cells, IFN-γ expression by these viruses was compromised in their ability to respond to VACV in general. To examine this, we used a surrogate measure of the total acute anti-VACV response in BALB/c mice and impact of sequence variation in the immunodominant F226 peptide. Groups of BALB/c mice were immunized with $1 \times 10^6$ PFU of VACV i.p. and 7 days later peptide-specific TCD8+ cell responses were measured using IFN-γ ICS. The net percentages (and s.e.m.) of TCD8+ cells from BALB/c mice infected with VACV WR, Copenhagen (Cop) or MVA that produce IFN-γ in ex vivo stimulations with the indicated peptides, respectively ($P = 0.01$, Mann–Whitney test). The difference between WR and MVA was even more striking at 20-fold. Less virulent strains of VACV have been found previously to induce lower responses in C57Bl/6 mice, but MVA was not tested and the differences were not as large as we show here in BALB/c mice. To address this, we used MVA F2Y that had poor immunogenicity like MVA but allowed the use of F226(Y)-loaded DimerX (similar to peptide-MHC-tetramer) reagents to identify a population of virus-specific TCD8+ that could be compared with similar cells from WR-infected mice (Figure 2c). After MVA F2Y and WR immunization, 61.4 and 93.5% of F226(Y)-specific TCD8+ responses were able to be identified as activated according to their CD62L and GzmB profile, respectively (Figure 2c). This suggests our method underestimates numbers of TCD8+ primed by both viruses in BALB/c mice, but more so for MVA than WR. Taking these different levels of underestimation into account, the difference in total response elicited by these viruses was ~20-fold.

Figure 1 Peptide-specific TCD8+ responses to different strains of VACV in BALB/c mice and impact of sequence variation in the immunodominant F226 peptide. Groups of BALB/c mice were immunized with $1 \times 10^6$ PFU of VACV i.p. and 7 days later peptide-specific TCD8+ cell responses were measured using IFN-γ ICS. (a) Average percentages (and s.e.m.) of TCD8+ cells from BALB/c mice infected with VACV WR, Copenhagen (Cop) or MVA that produce IFN-γ in ex vivo stimulations with the indicated peptides. (b) Average percentages (and s.e.m.) of TCD8+ cells from BALB/c mice infected with VACV WR, WR F2G, MVA or MVA F2Y that produce IFN-γ in ex vivo stimulations with the indicated peptides. (c) Sum of TCD8+, cell responses to the seven epitopes conserved across all strains shown in B (including only the appropriate F226 variant). Data are means and s.e.m. from two independent experiments, each with $n = 5–10$ (*$P<0.01$, Mann–Whitney test).
However, we did note that BALB/c mice made poorer responses to strain Copenhagen than did C57Bl/6 mice (around 2-fold less). It is possible that BALB/c mice need relatively higher levels of infection to generate the same $T_{CD8^+}$ response compared with C57Bl/6 mice and the high virulence of WR masks this defect. Understanding differences in immune responses to virulent viruses across mouse strains is complicated because it is not possible to dissect the roles of virus burden, antigen loads and inflammation, all of which will vary with virus replication and spread. However, for a non-replicating virus such as MVA, which causes no pathology and has limited spread, these complications are not a problem. Therefore, the simplest explanation of these data is that BALB/c mice have a simple defect in priming $T_{CD8^+}$ responses to MVA compared with C57Bl/6 mice.

To determine whether the difference in anti-MVA $T_{CD8^+}$ numbers in the two mouse strains could be resolved at a higher dose, we compared responses in mice immunized with the standard $1 \times 10^6$ PFU with a 10-fold higher dose (Figure 2e). The number, but not percent of $T_{CD8^+}$, was significantly increased in BALB/c mice given $1 \times 10^7$ PFU, but the response remained around a third of that elicited by the standard dose in C57Bl/6 mice. In C57Bl/6 mice, no significant increase in response was seen for the higher dose, supporting the notion that $T_{CD8^+}$ responses in BALB/c mice might be more dependent on high doses of VACV antigen. They also underscore the defect of BALB/c mice in making $T_{CD8^+}$ responses to MVA.

When examining individual epitopes, the poor immunogenicity we describe here was not apparent for one epitope. Responses to E3140 were comparable in all the strains of VACV tested, and this suggests that, for at least one epitope, MVA can prime good $T_{CD8^+}$ responses. This most likely explains the apparent superiority of a recombinant MVA over a thymidine kinase-negative WR in priming $T_{CD8^+}$ to an encoded β-galactosidase epitope when the opposite result was found for VACV-specific responses. The interpretation in that case was that the induction of modest antivector and good antirecombinant antigen responses is a desirable general characteristic of recombinant MVA vaccines. However, in the light of the data here, perhaps the previous result reflects a fortuitous choice of foreign antigen rather than a general principle. Looking across the native epitopes we tested (for example, Figure 1b), it is clear that E3140 is an exception rather than the rule, and there is no reason to expect that recombinant antigens in a vaccine would follow a different pattern.

In summary, MVA elicits many fewer $T_{CD8^+}$ than WR in BALB/c and C57Bl/6 mice, but this was especially notable in BALB/c mice. The contrast between these mouse strains highlights the importance...
of host genetic background in responses to attenuated vaccine vectors such as MVA.

METHODS

Viruses
The unmodified VACV used were low passage stocks grown in BHK-21. WR refers to WR (NIH TC-adapted), ATCC VR-1354, sequence accession AV243312.1. MVA refers to ‘clone 1’ characterized extensively in Wyatt et al. that was derived at NIH from an original passage collected after 572 passages in primary chick cells in 1974. Both were provided by Bernard Moss (NIH).

Mice and infections
Female C57Bl/6 and BALB/c mice greater than 8 weeks of age were housed, and experiments were done according to ethical requirements and under an approval from the Australian National University Animal Ethics and Experimentation Committee. Mice were injected via the intraperitoneal (i.p.) route with 1 x 10^6 PFU of virus in 200 μl PBS except in Figure 2e where some mice received 1 x 10^5 PFU.

T<sub>CD8</sub> assays
Mice were euthanized 7 days after infection and single-cell suspensions of spleens used in the following assays. (A) Splenocytes were cultured with synthetic peptides at 0.1 μM in the presence of brefeldin A for 4 hours before surface staining for CD8 (clone 53-6.7) and with F2 26(Y)-loaded H-2Ld:Ig fusion protein (DimerX; BD Biosciences, San Jose, CA, USA) pre-bound with synthetic peptides at 0.1 μM. Intermediates were enriched and identified through the use of a fusion protein peptide were engineered using a transient dominant method in which unstable intermediates were enriched and identified through the use of a fusion protein between enhanced green fluorescent protein (GFP) and Bsd<sup>+</sup> (confers resistance to blasticidin). The sequences used to encode the F2<sub>26</sub> epitope were altered to include identifying restriction enzyme sites and some additional changes to allow each recombinant to be distinguished from its parent by PCR, but the only amino-acid changes were the desired glycine and tyrosine substitutions.

Generation of VACVs with the alternate F<sub>226</sub> peptide
Recombinant VACV WR expressing the alternate F<sub>226</sub> peptide SPGAGYDL and recombinant VACV MVA expressing the alternate F<sub>226</sub> peptide were engineered using a transient dominant method in which unstable intermediates were enriched and identified through the use of a fusion protein between enhanced green fluorescent protein (GFP) and Bsd<sup>+</sup> (confers resistance to blasticidin). The sequences used to encode the F<sub>226</sub> epitope were altered to include identifying restriction enzyme sites and some additional changes to allow each recombinant to be distinguished from its parent by PCR, but the only amino-acid changes were the desired glycine and tyrosine substitutions.

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