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Prime Boost Vaccination Strategies: CD8 T Cell Numbers, Protection, and Th1 Bias

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Vaccination strategies involving priming with DNA and boosting with a poxvirus vector have emerged as a preferred combination for the induction of protective CD8 T cell immunity. Using IFN-γ ELISPOT and a series of DNA plasmid, peptide, and modified vaccinia Ankara (MVA) vaccine combinations, we demonstrate that the DNA/MVA combination was uniquely able to enhance IFN-γ secretion by Ag-specific CD8 T cells. However, CD8 T cell populations induced by DNA/MVA vaccination failed to show an enhanced capability to mediate protection in an IFN-γ-independent influenza challenge model. The DNA/MVA vaccine strategy was also not unique in its ability to induce high numbers of CD8 T cells, with optimal strategies simply requiring the use of vaccine modalities that individually induce high numbers of CD8 T cells. These experiments argue that rivals to DNA/poxvirus vaccination strategies for the induction of optimal protective CD8 T cell responses are likely to emerge. The Journal of Immunology, 2003, 170: 2599–2604.

Vaccine strategies that involve primary vaccinations with a DNA vaccine followed by boosting with a recombinant poxvirus vector encoding the same immunogen have emerged as favored approaches for generating protective CD8 T cell responses against a number of diseases including HIV (1–3), malaria (4, 5), and cancer (6). The immunological mechanisms underpinning the seemingly unparalleled ability of DNA/poxvirus vaccine combinations to induce enhanced levels of CD8 T cell-mediated protection have not been extensively explored. Several explanations have been proposed, and they include the induction by DNA/poxvirus combinations of immune responses with enhanced IFN-γ secretion phenotypes or Th1 bias (7). The ability to avoid induction of antivector responses by priming with a DNA vaccine has also been postulated to be important for an effective outcome from this vaccination strategy (8). Recently, evidence has also been presented suggesting that DNA/poxvirus vaccinations are able to induce high avidity CD8 T cell subsets (9).

To identify the factors underpinning the success of DNA/poxvirus vaccination strategies, we measured CD8 T cell numbers and CD8 T cell-dependent protection induced by a series of prime boost strategies using combinations of the following vaccine modalities: 1) a standard DNA plasmid vaccine (10), 2) a modified vaccinia Ankara (MVA)4 replication deficient poxvirus vector, and 3) synthetic peptide-based vaccines formulated with tetanus toxoid (TT) in the oil-in-water adjuvant, Montanide ISA 720 (M720) (11). The DNA and MVA vaccines both encode the murine polyepitope or polypeptide immunogen, which contains the following CD8 T cell epitopes: TYQRTRALV (TYQ) from the influenza nucleoprotein, RPQASGVYM (RPQ) from the lymphoblastoid choriomeningitis virus nucleoprotein, and SYIPSAEKI (SYI) from the Plasmodium berghei circumsporozoite protein (10). Epitope-specific CD8 T cell numbers were enumerated using IFN-γ ELISPOT technology, and the size of the IFN-γ spots produced by individual T cells was taken as a measure of the amount of IFN-γ secreted by each T cell (12). An influenza protection model was used to investigate the effect of different vaccination strategies on the capacity of a given number of vaccine-induced CD8 T cells to mediate protection. A crucial characteristic of this model is its relative insensitivity to IFN-γ (13, 14). Because elevated CD8 T cell-derived IFN-γ secretion has been shown to improve CD8 T cell-mediated protection in several systems (15, 16), this influenza challenge system was chosen to investigate whether DNA/MVA vaccination generates CD8 T cells with enhanced IFN-γ-independent protective capabilities. Several reports have illustrated that enhanced IFN-γ-independent protection can be mediated by CD8 T cells with high avidity/affinity (17–19).

The ability to induce CD8 T cells with an enhanced IFN-γ secretion phenotype was identified in these studies as the only advantage bestowed by the DNA/MVA vaccination strategy. The influenza model failed to show that DNA/MVA vaccination induced CD8 T cells with improved IFN-γ-independent protection, and other prime boost strategies were often able to generate higher numbers of CD8 T cells than DNA/MVA vaccination. These experiments illustrate that alternative prime boost strategies could rival DNA/poxvirus strategies for optimal induction of protective CD8 T cells.
Materials and Methods

MVA vaccines

The murine polytope (mpt) construct (20) was inserted into MVA-Bavarian Nordic (BN) (patent WO 0242480) under the control of a natural p7.5 promoter (MVAp7.5(mpt)) (21) or a strong synthetic promoter (sp) (MVAsp(mpt)) (22), which permits substantially enhanced Ag production (23). MVA expressing human tyrosinase (a melanoma Ag) via the p7.5 promoter was used as a control MVA (MVA control) (24). MVA-BN titers were determined using 50% tissue culture-infective dose (106 TCID50) as described previously (24). In our hands TCID50 and PFU values were broadly comparable for MVA-BN titers (data not shown). All MVA-BN vaccines were constructed and supplied by Bavarian Nordic (Martinsried, Germany).

Mice and vaccination

Female BALB/c (H-2d) mice (6–8 wk) were purchased from Animal Resources (Perth, Australia). Mice were immunized with the following: 1) recombinant MVA viruses at a dose of 106 TCID50 delivered by i.m. injection or intranasal (i.n.) delivery. For i.m. injections, mice were anesthetized with ketamine and xylazine (100 μl of ketamine 10 mg/ml: xylazine 2 mg/ml: water (1:1:1), per 10 g body weight), and 50 μl of MVA was injected into each quadriceps muscle. For i.n. vaccinations, mice were anesthetized with methoxyflurane (Medical Developments, Victoria, Australia), and 25 μl of MVA was introduced into each nostril. 2) Peptide vaccines were formulated with 10 μg of peptide (Mimotopes, Clayton, Australia) and 2.5 μg/ml tetanus toxoid (CSL, Parkville, Australia) emulsified in Montanide ISA 720 (M720) (SEPPIC, Paris, France) and were injected s.c. at the base of the tail as described (11). 3) A DNA vaccine encoding the murine polytope was administered twice (100 μg i.m. 50 μl into each quadriceps), 2 wk apart as described (20). Prime and boost immunizations were separated by a period of 3 wk (unless stated otherwise).

Ross River Virus T48 (RRV) (100% 50% cell culture-infective dose (CCID50)) was delivered in a volume of 200 μl to mice by i.p. injection (25).

IFN-γ ELISPOT

Three weeks after booster immunizations, mice were culled and splenocytes were analyzed directly ex vivo for epitope-specific CD8 T cell responses using an IFN-γ ELISPOT assay as described previously (26). The number and area of IFN-γ spots were measured using the KS ELISPOT reader (Zeiss, Halbermoos, Germany). In the analysis of spot areas, the Student residuals statistical technique was used to identify outliers, with each observation weighted by the inverse of the SE. A Bonferroni correction was applied to the p value to take account of the multiple tests (27).

Influenza challenge

Groups of mice were challenged by i.n. administration of influenza virus Mem71, which contains the hemagglutinin of A/Memphis/71/H3) and the neuraminidase of A/Bellamy/42 (N1), as described previously (10). Percent protection was calculated as the percentage reduction in lung influenza titers compared with control mice, which had been mock immunized with PBS. Using SAS for Windows (release 8.02), a Pearson’s correlation coefficient was calculated between mean values of the percentage protection and the log of the spot number, weighting each mean by the inverse of the variance of the measures.

Results

CD8 T cell responses from individual vaccine modalities

The vaccine modalities used in this study were initially tested at different doses to determine the optimal doses and epitopes to be used in future experiments. As observed previously with recombinant murine polytope vaccines (i.e., DNA and poxvirus), IFN-γ ELISPOT analysis revealed a hierarchy of responses after vaccination that was not apparent after vaccination with peptide vaccines. Of the three epitopes, SYI emerged as the most immunodominant, followed by RQP, with TYQ being relatively subdominant (Ref. 26 and Fig. 1).

Murine vaccination experiments performed by others have used doses of recombinant MVA of as much as 106–2 × 108 PFU (24, 28). At a single dose of 5 × 108 TCID50, MVA-BN encoding the murine polytope under the synthetic promoter (MVAsp(mpt)) induced CD8 T cell responses of 250–300 IFN-γ spots per 106 splenocytes for the dominant epitopes (Fig. 1). These results are broadly comparable with responses reported by others (4). A single dose of 106 MVAsp(mpt) reliably induced TYQ responses of 10–15 IFN-γ spots per 106 splenocytes (Fig. 1). This relatively subdominant TYQ epitope and this relatively low dose of MVA were chosen for prime boost and protection studies so that enhancements in CD8 T cell activities might be readily observed.

The role of Th1 bias in prime boost immunization

DNA vaccines are reported to bias the immune response toward a Th1 phenotype, with enhanced T cell-derived IFN-γ production (29). To investigate whether DNA prime and MVA boost immunizations induce Ag-specific CD8 T cells with enhanced IFN-γ production, TYQ-specific CD8 T cells from the different prime boost vaccination regimes were analyzed by IFN-γ ELISPOT. To avoid any potential interference from anti-MVA Abs, MVA booster vaccinations were administered i.n. (30). The area of each ELISPOT spot was measured and was taken to represent a measure of the amount of IFN-γ secreted by each TYQ-specific CD8 T cell (12). Unlike conventional bulk IFN-γ assays, which measure secretion from a population of cells, this ELISPOT-based method allows IFN-γ secretion profiles to be measured for individual Ag-specific CD8 T cells.

Analysis of results from a series of prime boost combinations illustrated a trend where the mean spot area increased with the increasing number of Ag-specific CD8 T cells (Fig. 2). As the total number of CD8 T cells increases, the stochastic probability of having cells that secrete high amounts of IFN-γ might be expected to increase. Importantly, however, a clear exception to the trend was observed for a DNA prime followed by a MVAsp(mpt) boost immunization (Fig. 2, vaccination strategy 8), which showed a significantly higher mean spot area compared with the other vaccination strategies (p = 0.0055).

These results illustrate that a DNA prime and MVA boost immunization strategy, more than any other combination tested, increased the IFN-γ secretion of individual Ag-specific CD8 T cells.

Analysis of CD8 T cell numbers and protection

DNA prime and poxvirus boost immunizations have been postulated to elicit high avidity/affinity CD8 T cells with enhanced protective capabilities (9). To investigate whether DNA/MVA vaccination strategies enhance the protective capacity of individual CD8
T cells, a series of vaccination regimes were compared for their ability to induce CD8 T cells and to mediate protection against influenza. The MEM71 in vivo influenza challenge system was chosen because TYQ-specific CD8 T cell responses were previously shown to be protective (31). Furthermore, IFN-γ appears to have little impact on the level of infection in this model (13, 14), thus differences in IFN-γ secretion by Ag-specific CD8 T cells (see Fig. 2) are unlikely to complicate this analysis significantly.

Sixteen different vaccination regimes were used to immunize parallel groups of mice that were either: 1) challenged with MEM71 influenza or 2) used to enumerate vaccine-induced TYQ-specific CD8 T cell responses using IFN-γ ELISPOT. A significant correlation ($r = 0.86, p = 0.005$) emerged between the log of CD8 T cell numbers and vaccine-conferred protection against influenza challenge (Fig. 3). Importantly, DNA prime and MVA boost immunizations did not appear to provide better protection for the number of CD8 T cells than any other vaccination strategy (Fig. 3), combinations 7 and 8). Thus, these data do not support the notion that DNA prime and MVA boost immunizations induce CD8 T cells with superior IFN-γ-independent protective capabilities. One would expect vaccination strategies that produced CD8 T cells with superior protective capabilities to plot significantly above the correlation line, an outcome that might be expected if the immunization strategy induced CD8 T cells with high avidity/affinity.

It is also apparent from this analysis that DNA/MVA combinations do not have a monopoly on induction of high numbers of CD8 T cells as assessed by IFN-γ ELISPOT. In fact, priming with peptide followed by MVA produced substantially higher numbers of TYQ-specific CD8 T cells (Fig. 3, combinations 10 and 11), perhaps largely because of the more efficient induction of TYQ-specific CD8 T cells during priming (see Fig. 5).

The role of vector responses in prime and boost immunization

The ability of DNA prime and MVA boost and peptide prime and MVA boost to raise large numbers of CD8 T cells may be because of the near exclusive induction during priming of responses specific for the immunogen rather than induction of antivector responses (32). The subsequent MVA boost might then preferentially expand the pre-existing immunogen-specific CD8 T cells rather than expanding vector responses. To test whether avoidance of vector responses is important for induction of large numbers of CD8 T cells, a control MVA or an irrelevant virus, RRV, was codeivered with a primary immunization of the peptide vaccine. Neither codeivered MVA nor the contemporaneous virus infection significantly affected the efficiency of TYQ-specific CD8 T cell induction by the peptide vaccine (Fig. 4, Prime). These findings argue that induction of anti-MVA vector responses do not significantly compete with induction of immunogen-specific CD8 T cell responses during priming. Importantly, the addition of MVA or RRV to the primary vaccination also did not significantly affect the outcome after peptide prime and MVA boost (Fig. 4, Prime boost). Thus, avoiding immune competition between vector and immunogen during priming, by using modalities that induce few antivector responses, does not appear to be a crucial component of effective prime boost vaccination strategies. This experiment does not address the separate and well established problem of antivector Ab responses inhibiting subsequent immunizations with the same vector. This issue was avoided in these experiments by i.n. delivery of MVA booster immunizations (30).
one of the following: 1) a s.c. injection with 10 μg of TYQ/M720/T and an i.p. injection of 10^5 CCID_{50} of the alphavirus, RRV. TYQ-specific CD8 T cell numbers were determined by ELISPOT 3 wk later. Prime Boost, a parallel group of mice were immunized as described under Prime and were given a single i.n. injection of 10^6 CCID_{50} of MVAsp(mpt) as a booster immunization after 8 wk. TYQ-specific CD8 T cells were enumerated as above.

Optimal induction of CD8 T cell numbers can be achieved using different prime boost strategies

To determine whether the optimal prime boost strategy for induction of high numbers of CD8 T cells remains constant, irrespective of the epitope being tested, mice were primed with the following: 1) a peptide vaccine containing a mixture of three peptides (TYQ, SYI, and RPQ); or 2) DNA(mpt), which encodes these three epitopes; or 3) MVAsp(mpt), which similarly encodes these epitopes. All of the mice were then given a booster immunization of MVAsp(mpt) delivered i.m. Surprisingly, the following occurred: 1) the TYQ-peptide prime and MVAsp(mpt) boost strategy induced more TYQ-specific CD8 T cells than the other two prime boost combinations (Fig. 5A, TYQ), 2) the 2× DNA(mpt)/MVAsp(mpt) induced more RPQ-specific CD8 T cells than the other combinations (Fig. 5A, RPQ), and 3) MVAsp(mpt)/MVAsp(mpt) induced the highest number of SYI-specific responses (Fig. 5A, SYI). Thus, the optimal prime boost strategy for induction of high numbers of CD8 T cells appeared to depend on the epitope being tested.

Can responses raised by single vaccine modalities predict the outcome following prime boost?

Excluding CFA and coimmunization experiments, a total of 17 prime boost combinations have been tested (Figs. 2 and 5), and the outcomes after immunization with each individual vaccine modality have also been measured (Fig. 1). A linear regression analysis was undertaken to determine whether the outcome from prime boost (P/B) immunizations might be predicted from knowledge of the immune responses generated when the prime (P) and boost (B) immunization modalities are given alone. Using the weighted least squares method, the best equation to predict the outcome (P/B) based on P and B emerged to be P/B = 1.34 + 1.48P + 0.99B, with a resulting correlation coefficient between the observed and predicted outcome of r = 0.87 (p < 0.0001) (Fig. 5B). The 1.48 (p = 0.006) and 0.99 (p = 0.012) coefficient values were not significantly different from 1, with the 95% confidence limits being 0.51–2.46 and 0.26–1.72, respectively. To test for a synergistic effect between P and B, the linear regression was repeated with a P × B term. The coefficient for the P × B term was −0.00105, and the associated p value was 0.8351. The data thus provided no evidence for the existence of a synergistic effect between P and B and argue that CD8 T cell responses following prime boost may be simply additive.

Discussion

This study indicates that the main advantage of the DNA prime MVA boost vaccination strategy is the induction of CD8 T cells with enhanced IFN-γ secretion phenotypes. The DNA/MVA strategy did not have the monopoly on induction of high numbers of
CD8 T cells, with other vaccine strategies outperforming this combination (Figs. 2 and 3). An analysis of 16 different vaccine combinations also failed to provide any evidence for the induction by DNA/MVA of CD8 T cells with improved IFN-γ-independent protective capacities (Fig. 3), as might be expected if high avidity T cells were induced by this strategy. Surprising was the observation that the best prediction for the number of CD8 T cells induced by prime and boost immunizations was a simple sum of the responses induced when the prime was used alone plus the responses induced when the boosting modality was used alone.

The ELISPOT assay was used to show that the IFN-γ production per CD8 T cell was on average significantly higher after DNA/MVA immunization than after other vaccination strategies. The ability of DNA vaccines to induce CD8 T cells that produce more IFN-γ per cell can likely be attributed to the presence of cytidine-phosphate-guanosine (CpG) motifs in the DNA plasmids (29). The cytokine secretion phenotype of CD8 T cells may be determined during priming, and importantly, the phenotype appears to be largely retained upon expansion of the memory CD8 T cell pool (33). Thus, DNA priming may establish a memory pool with enhanced Th1 bias, which is then effectively expanded by the poxvirus boost. Current evidence would indicate that use of immunostimulatory CpG oligonucleotides as adjuvants in primary immunizations should impart the same Th1 enhancement as priming with DNA (34). However, it remains to be established whether the Th1 bias imposed on CD8 T cells by DNA/MVA vaccination or by CpG adjuvants is influenced by the following: 1) the immunodominance of the CD8 T cell epitope and/or 2) the nature of the CD4 T cell responses induced during vaccination. Although enhanced IFN-γ secretion appears to be of little protective value against influenza (Fig. 2; confirming previous reports, Refs. 12 and 13), there is little doubt that for other diseases including cancer, HIV, and the malaria liver stage, enhanced CD8 T cell IFN-γ secretion is likely to mediate substantial improvements in protection (35–37).

Several reports have shown that the avidity/affinity of CD8 T cell populations can be manipulated in vitro (38, 17–19). By restimulating in vitro with low levels of peptide epitope, high avidity/affinity CD8 T cell populations can be expanded that show enhanced IFN-γ-independent protective capacities after adoptive transfer into mice (17–19). Although we did not measure T cell avidity/affinity directly, we showed that different vaccination strategies did not readily or significantly influence the IFN-γ-independent protective capacities of CD8 T cell populations in vivo. Epitope levels did not appear to influence the outcome, with CD8 T cell responses induced by DNA and MVAp7.5 vaccines (delivering low levels of Ag) or peptide and MVAsp vaccines (delivering relatively higher levels of Ag) failing to show significant differences in their protective capabilities. Assuming that high avidity/affinity CD8 T cells should show enhanced protection in the influenza challenge system, these experiments might argue that different vaccine strategies do not readily influence the functional avidity/affinity of CD8 T cell populations in vivo. Although this view is not supported by conclusions from some studies (9, 39, 40), these latter investigations did not control for differences in T cell numbers and/or IFN-γ secretion and/or used readouts involving in vitro restimulation. Conceivably, the vaccine modalities used in the current study already induce CD8 T cells with maximal functional avidity/affinity. Thus, in the absence of any low avidity/affinity CD8 T cell induction, we may have been unable to detect differences in IFN-γ-independent protection. In either event, our data does not support the view that DNA prime and MVA boost is uniquely capable of inducing CD8 T cells with significantly improved IFN-γ-independent protective activities.

The coinduction of antivector responses during priming did not appear to significantly influence the generation of epitope-specific CD8 T cell responses following priming or after prime boost immunizations (Fig. 5). These experiments do not support the notion that for prime boost to be effective, the priming vaccine modality must avoid induction of antivector responses. Recent use of recombinant adenovirus as an effective priming modality supports this conclusion (41). The results shown in Fig. 5 do not address the separate issue of whether antivector neutralizing Ab responses generated by priming immunizations are able to inhibit effective boosting by the same vector. This issue has been addressed by others (42), and any such potential influences were avoided in the current study by using i.n. MVA booster immunizations (30). In addition, recent studies have shown that the 10⁶ CCID₅₀ MVA dose used here fails to induce sufficient anti-MVA Ab responses to interfere with subsequent MVA booster immunizations (P. Chaplin, unpublished observations).

The current study has demonstrated that there are other prime boost strategies besides DNA/MVA that can induce high levels of protective CD8 T cells. However, perhaps the most surprising outcome is the observation that the number of CD8 T cells induced following prime boost immunizations approximates to the simple addition of the responses seen when the prime and boost vaccination modalities were administered individually. This 1 + 1≈2 concept contrasts with Ab responses following prime boost immunizations where substantial synergistic affects are obtained (7). The 1 + 1≈2 notion is based on IFN-γ ELISPOT data and might be compromised because this assay most likely enumerates effector/effector memory CD8 T cells rather than the central memory CD8 T cells, which are the cells believed to expand upon re-exposure to Ag (43). However, the size of the effector/effector memory CD8 T cell expansion often appears to be related to the number of central memory CD8 T cells generated (44). Either way, the 1 + 1≈2 concept might best be viewed as a broad approximation illustrating the following: 1) prime boost strategies including DNA/MVA do not produce exceptional CD8 T cell expansions, and 2) the best method for induction of high numbers of CD8 T cells is to combine vaccine modalities that individually induce high levels of CD8 T cells.

In the current study the priming and boosting vaccine modalities did not share Ag-specific CD4 T cell epitopes. In contrast, prime boost strategies using vaccines encoding whole Ags would also amplify Ag-specific CD4 T cell responses, and such responses are known to influence maintenance and recall of CD8 T cell responses and contribute to protection (45, 46). How Ag-specific (and vaccine vector-specific) CD4 T cell responses influence the outcome of different prime boost vaccination strategies remains to be explored. In addition, recent simian-human immunodeficiency virus studies have reported that protection after MVA only was similar to protection seen after DNA/MVA vaccination, suggesting that strategies that optimize postchallenge immune-cell trafficking may be as valuable as those that induce high numbers of CD8 T cells (47).

The increased regulatory and manufacturing complexities associated with the licensing and registering of a vaccine product comprising two different vaccination modalities may hinder the development of heterologous prime boost vaccine products. The current study may facilitate the design of improved prime boost vaccine strategies and aid in the development of effective vaccine products that use only a single vaccination modality.

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