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Immunodominance of Poxviral-Specific CTL in a Human Trial of Recombinant-Modified Vaccinia Ankara

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Many recombinant poxviral vaccines are currently in clinical trials for cancer and infectious diseases. However, these agents have failed to generate T cell responses specific for recombinant gene products at levels comparable with T cell responses associated with natural viral infections. The recent identification of vaccinia-encoded CTL epitopes, including a new epitope described in this study, allows the simultaneous comparison of CTL responses specific for poxviral and recombinant epitopes. We performed detailed kinetic analyses of CTL responses in HLA-A*0201 patients receiving repeated injections of recombinant modified vaccinia Ankara encoding a string of melanoma tumor Ag epitopes. The vaccine-driven CTL hierarchy was dominated by modified vaccinia Ankara epitope-specific responses, even in patients who had not received previous smallpox vaccination. The only recombinant epitope that was able to impact on the CTL hierarchy was the melan-A26–35 analog epitope, whereas responses specific for the weaker affinity epitope NY-ESO-1157–165 failed to be expanded above the level detected in prevaccination samples. Our results demonstrate that immunodominant vaccinia-specific CTL responses limit the effectiveness of poxviruses in recombinant vaccination strategies and that more powerful priming strategies are required to overcome immunodominance of poxvirus-specific T cell responses. The Journal of Immunology, 2005, 175: 8431–8437.

Materials and Methods

Subjects and clinical trial protocol

The subjects were HLA-A*0201-positive patients with surgically treated melanoma who participated in a phase I clinical trial of recombinant plasmid DNA and MVA vaccines (3). DNA and MVA were engineered to express the following epitopes: A26–35 analog epitope (0.02–0.19% CD8+ cells), whereas no vaccine-driven responses specific for any of the other melanoma epitopes were observed (3). In this study, we have extended these results by carrying out simultaneous analyses of the magnitude and phenotype of MVA-specific cellular immune responses generated in these patients and CTL responses specific for the recombinant epitopes. We have demonstrated that MVA is capable of generating virus-specific cellular immune responses at levels similar to those generated by nonattenuated strains of vaccinia. We present detailed ex vivo kinetic and phenotypic analyses of vaccinia epitope-specific CTL responses, and report a direct comparison of epitope-specific CTL responses specific for recombinant gene products with responses specific for the viral vector. Demonstration that the vaccine-driven CTL hierarchy is dominated by poxviral-specific responses holds important implications for the future design of poxvirus-based recombinant vaccines.

Abbreviations used in this paper: MVA, modified vaccinia Ankara; ILD, ILDDNLKYV; KVD, KVDDTFYYV; CLT, CLTEYILWV.

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encode the same "Mel3" polyepitope string of seven melanoma tumor Ag MHC class I-restricted epitopes (13). Five of the epitopes were HLA-A*0201-restricted: tyrosinase 

\textsuperscript{9}9, melan-A\textsubscript{26–35} analog (substitution alanine to leucine at residue 27; Ref. 14), tyrosinase\textsubscript{163–172}, MAGE-3\textsubscript{271–279}, and NY-ESO-1\textsubscript{157–165}. All patients received four injections at two weekly intervals, and were randomized to either a heterologous "prime-boost" regimen of DNA and MVA, or a homologous MVA-only regimen (Fig. 1A). MVA.Mel3 was administered at a dose of \( 5 \times 10^6 \) PFU intradermally, and DNA.Mel3 at 1000 \( \mu \)g i.m. Two patients (09 and 11) were vaccinia-naive before the study (3). The trial was conducted according to the declaration of Helsinki, with approval from the relevant local ethics committees. All patients gave written informed consent.

**Immunoaassays**

Blood samples were collected at 16 time points (Fig. 1A). PBMC were separated, cryopreserved in 10% DMSO and 40% FCS in RPMI 1640 tissue culture medium, and stored in liquid nitrogen.

**Reagents**

Peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry in house, or purchased (Sigma-Genosys or Invitrogen Life Technologies). Tetrakers were synthesized as described previously (15). Anti-CD8a (PerCP) and anti-CD45R0 allophycocyanin were from BD Biosciences; anti-CD45RA (FITC), anti-CD27 (FITC), and anti-CD28 (allophycocyanin) were from BD Pharmingen.

**Ex vivo FACS staining and analysis**

Thawed PBMC were stained with PE-labeled tetramer and Abs as described previously (16). Small lymphocytes were gated according to forward/side scatter, and dead cells were excluded on the basis of atypical fluorescence. A detection threshold for a tetramer\textsuperscript{+} cell population was defined as 100 tetramer\textsuperscript{+} cells/10^6 CD8\textsuperscript{+} cells (0.01% CD8\textsuperscript{+}) (3).

**ELISPOT assay**

ELISPOT analysis of IFN-\( \gamma \) secretion (Mabtech) was performed as described previously (16). For analysis of peptide-specific responses, 5 \( \times 10^5 \) PBMC were incubated in duplicate wells overnight \( \pm 10 \mu M \) peptide. For analysis of cellular responses against whole MVA virus, 5 \( \times 10^7 \) PBMC were incubated in duplicate wells with 1 \( \times 10^5 \) autologous MVA-infected or uninfected prevaccination PBMC. Infection of cells was performed 18 h previously at a multiplicity of infection of 1:1, for 90 min, before washing, and overnight incubation. In some experiments, CD8\textsuperscript{+} cells were enriched using immunomagnetic separation (MACS; Miltenyi Biotec), and 1 \( \times 10^5 \) cells from enriched and depleted fractions were incubated in duplicate wells with 1 \( \times 10^5 \) infected PBMC. Infected prevaccination PBMC were also incubated alone, at 1 \( \times 10^5 \) cells/well, to determine background IFN-\( \gamma \) secretion by MVA-infected cells.

**Results**

**MVA efficiently generates antiviral cellular immune responses**

A schematic diagram of the study protocol is shown in Fig. 1A. Ex vivo ELISPOT analyses of immune responses in patients vaccinated with repeated injections of MVA.Mel3 demonstrated that all patients developed a vaccine-driven MVA-specific cellular immune response, above prevaccination levels, ranging from 85 to \( >1000 \) spots/10^6 PBMC (Fig. 1, B and C). The response was detectable ex vivo by day 14 after the first MVA.Mel3 injection (Fig. 1B). Subsequent injections of MVA.Mel3 resulted in continued boosting of the MVA-specific response, although responses tended to plateau after three injections (Fig. 1B). In general, the patients who received four injections of MVA.Mel3 developed a greater response than those who received two injections (Fig. 1C). Of note, one of the two vaccinia-naive patients (patient 09) developed one of the highest responses, with kinetics similar to the patients who had been previously vaccinated against smallpox (Fig. 1, B and C). Experiments in three patients (03, 10, 14), using CD8\textsuperscript{+}-enriched and CD8\textsuperscript{−}-depleted cellular fractions, demonstrated that the MVA-specific cellular immune response consisted of predominantly CD8\textsuperscript{T} T lymphocytes (Fig. 1D and data not shown).

**FIGURE 1.** MVA efficiently generates antiviral cellular immune responses. A, The clinical trial vaccination regimen. B, The kinetics of vaccine-driven cellular immune responses to whole MVA virus determined by ELISPOT are presented for patients who received four injections of MVA.Mel3. Arrows indicate MVA.Mel3 injections. C, The MVA-specific cellular immune responses, determined by ex vivo ELISPOT assay, are shown for prevaccination (■) and postvaccination samples for patients who received two (□), or four injections (●) of MVA.Mel3. D, For patient 14, the cellular response to whole MVA virus, determined by ex vivo ELISPOT, is shown for pre- and postvaccination time points. Whole PBMC were plated at 5 \( \times 10^5 \) cells/well, and CD8\textsuperscript{+} enriched and depleted fractions were plated at 1 \( \times 10^5 \) cells/well. Results do not include ELISPOT data from patient 5 and patient 8’s samples due to nonspecific IFN-\( \gamma \) secretion (patient 8) and shortage of frozen cells (patient 5).
Identification of a new vaccinia-encoded HLA-A*0201-restricted epitope

We screened 26 vaccinia-derived peptides, which we have identified as candidate HLA-A*0201-restricted epitopes, by screening large numbers of vaccinia-encoded peptides, predicted by computer algorithms to bind to HLA-A*0201 molecules (17). Ex vivo ELISPOT assays and tetramer staining demonstrated responses specific for the peptide ILDDNLYKV (ILD) (Fig. 2, A and B). Consistent with these results, we have recently demonstrated that T cell response specific for the ILD peptide can be detected in HLA-A2 transgenic mice (17). The ILD epitope is encoded within a 434-aa protein of unknown function, which has a high degree of sequence homology between different Orthopoxviruses (Fig. 2C). ILD is the fourth vaccinia-encoded CTL epitope to be identified, for which responses have been demonstrated in ex vivo analyses of human PBMC. Two epitopes (KVDDTFYYV (KVD) and CLTEYILWV (CLT)) are also HLA-A*0201-restricted and were identified in subjects recently vaccinated with a licensed smallpox vaccine (12). A fourth HLA-A*0201-restricted candidate epitope SLSAYIIRV (SLS) has been identified in HLA-A2 transgenic mice (17).

FIGURE 2. Identification of a new vaccinia-encoded epitope. A, Ex vivo ELISPOT analysis of postvaccination PBMC from patients (04 and 07) demonstrated peptide-specific IFN-γ secretion for ILD. The percentages of IFN-γ-secreting cells as proportion of total CD8+ cells (determined by flow cytometry) are shown. B, Ex vivo ILD tetramer staining of PBMC from postvaccination time points for patients 04 and 07, and an HLA-A*0201-negative donor are shown. The percentage of tetramer+ cells in the CD8+ population is shown. C, The ILD epitope is conserved between different Orthopoxviruses. The protein containing the ILD epitope is highly conserved, exhibiting a high degree of amino acid sequence homology between Vaccinia Copenhagen GSR (AAA48060.1), MVA 074R (AAB96493.1), monkeypox GSR (NP_536501.1), and variola HSR (NP_042111.1). The ILD epitope is conserved in all proteins. The ILD epitope is highlighted.

FIGURE 3. Vaccinia epitope-specific CTL responses out-compete the expansion of CTL specific for melan-A26-35. Time courses of vaccinia, melan-A26-35, and NY-ESO-1157-165-specific T cell responses are shown. Epitope-specific responses determined by ex vivo tetramer analysis are presented as tetramer+ cells/10^6 CD8+ cells. Arrows indicate the MVA.Mel3 injections. A, Arm A: patients who received the DNA/MVA prime-boost protocol. B, Arm B: patients who received four injections of MVA.Mel3. Patients’ samples without an ex vivo detectable melan-A26-35 and/or NY-ESO-1157-165 response were not included in the analysis. Results from patient 14 were included, because patient 14’s vaccinia-specific responses were further characterized in Fig. 1D.
POXVIRAL-SPECIFIC CTL RESPONSES IN A HUMAN CLINICAL TRIAL

mice, for which epitope-specific CTL have been generated by in vitro priming of PBMC obtained from a donor vaccinated against smallpox as a child (18).

**MVA epitope-specific CTL responses**

This clinical trial provided the opportunity to analyze the kinetics of MVA-specific CTL responses during acute infection and to follow these responses with time. We performed ex vivo tetramer analyses of CTL responses specific for the ILD, KVD, and CLT epitopes at multiple time points from the clinical trial patients (Fig. 3). Vaccine-driven CTL responses specific for at least one of these epitopes were demonstrated in all of the patients analyzed. Although vaccinia-specific CTL were not detectable in any prevaccination samples, responses were detectable as early as 14 days after the first MVA.Mel3 injection. The range of magnitudes of peak responses as a percentage of total CD8+ cells were as follows: KVD, 0.02–0.79%; CLT, 0.02–0.2%; and ILD, 0.02–0.6% (Fig. 3). In some patients, the combined frequencies of CTL specific for all three epitopes reached 1% of total CD8+ cells (patients 09 and 10).

For each patient, the CTL responses for each of the epitopes followed the same kinetic pattern, albeit at different magnitudes (Fig. 3). The epitope hierarchy varied between patients, but in general the KVD and ILD epitopes were dominant over the CLT epitope. This finding is consistent with human studies of CMV, EBV, and influenza, where immunodominance hierarchies of viral-specific CTL restricted by a given MHC class I allele vary between individuals (19–21). In each patient, once the epitope hierarchy was established, it was maintained throughout the study period. Although two of the patients (10 and 14) demonstrated continued boosting of vaccinia epitope-specific CTL responses with each of four MVA.Mel3 injections, the remaining patients developed a plateau or decline after the third or fourth doses.

In preliminary analyses of four patients, we did not detect vaccine-driven SLS-specific CTL (data not shown), and this epitope was not examined further. Recently, vaccine-driven SLS-specific CTL have been detected ex vivo in melanoma patients receiving recombinant MVA-transduced dendritic cells (22), possibly reflecting the influence of Ag-processing pathways on immunodominance hierarchies.

Our preclinical studies confirmed that each of the Mel3 recombinant epitopes is properly processed and presented to epitope-specific CTL clones (13). In the clinical trial, CTL specific for the melan-A26–35 analog were generated in ~50% of the patients (3). However, no vaccine-driven CTL responses specific for any of the other Mel3-encoded epitopes were demonstrated, even after in vitro restimulation (3). Using ex vivo tetramer analyses, we then performed a direct comparison of the CTL responses specific for the vaccinia-encoded and recombinant melan-A26–35 epitopes (Fig. 3). Significantly, with the exception of three patients (02, 04, and 08), CTL responses specific for the vaccinia-encoded epitopes were dominant over those for melan-A26–35. Generally, the melan-A26–35-specific responses followed the same kinetics as vaccinia-specific responses. However, in patient 10 who demonstrated continued boosting of vaccinia epitope-specific responses with each of the four MVA.Mel3 injections, the melan-A26–35-specific response was not boosted by repeated MVA.Mel3 injections.

**NY-ESO-1157–165-specific responses failed to be boosted by MVA.Mel3**

In one patient (patient 12), NY-ESO-1157–165-specific CTL were detectable ex vivo in prevaccination samples (0.15% CD8+ cells) (Fig. 4A). The pre-existing NY-ESO-1157–165-specific CTL were phenotypically Ag experienced and functionally active, as defined by proliferation in the absence of professional APCs (Fig. 4B) and ex vivo peptide-specific IFN-γ secretion (Fig. 4C). However, surprisingly, no vaccine-driven expansion of these cells was demonstrated over the course of the trial (3), whereas two injections of MVA were sufficient to expand the ILD-specific response, which outnumbered the NY-ESO-1157–165-specific response (Figs. 3 and 4A).

**Phenotypic analysis of MVA epitope-specific CTL**

We performed detailed phenotypic analyses of KVD and CLT tetramer+ CTL at multiple study time points for patients 09 (vaccinia-naive) and 14 (vaccinated against smallpox). We examined the expression of isoforms of the CD45R molecule, a critical component of the Ag receptor signal transduction machinery in lymphocytes, as well as the costimulatory molecule receptors, CD27 and CD28. The phenotypic profiles were the same in both patients for both epitopes. The profiles of KVD-specific CTL from multiple time points for patient 09 and patient 14 are shown in Fig. 5. Early postvaccination epitope-specific CTL were CD45RA+/CD45R0−.
with increasing proportions of CD45RA+/H11001+/CD45R0- cells with subsequent injections, consistent with our previous phenotypic analyses of melan-A_{26...35}-specific responses (3). Analysis of expression of CD27 and CD28 demonstrated early dual positivity with increasing proportions of CD27+CD28- and subsequently dual negative CD27+CD28- populations. This result is consistent with expression patterns described in CMV infection (23), and supports a putative model of T cell differentiation, beginning with CD27+CD28+ precursor or early differentiated cells and progressing through to CD27+CD28- differentiated T cells (23), and with previous findings that CD45RA+/CD27-CD28- cells are Ag-driven effector cells resulting from multiple rounds of cell division (23).

Discussion
The level of protective immunity against smallpox provided by MVA, and many of the other attenuated strains of vaccinia, is unknown because they entered clinical use toward the end of the smallpox eradication program and were not used in endemic areas. In animal challenge studies, MVA has been shown to produce similar levels of protective immunity as nonattenuated strains of vaccinia (24). However, the development of candidate smallpox vaccines relies on measures of immunogenicity. We have previously demonstrated that, despite its degree of attenuation, MVA is capable of generating strong anti-viral Ab responses (3). In this study, we have extended these results by demonstrating that repeated doses of 5 \times 10^7 PFU MVA efficiently generate antiviral (predominantly CD8+)-cellular immune responses, at levels similar to those described in humans (10–12).

However, it is of interest that total responses to MVA, as measured by ELISPOT assays using MVA-infected cells (Fig. 1, B and C) were significantly lower than MVA responses to single MVA epitopes, as measured by tetramer staining (Fig. 3). This observation is of importance because it suggests that ELISPOT assays may underestimate the degree to which the antivaccinia response dominates in humans and underscores the need for the identification of additional poxvirus epitopes and/or better methods for measuring total responses to poxviruses.
We have conducted detailed ex vivo kinetic and phenotypic analyses of vaccinia epitope-specific CTL responses and have demonstrated that the vaccine-driven CTL hierarchy is dominated by poxviral-specific responses. In a proportion of vaccinated patients, MVA-specific responses reached a plateau after repeated MVA injections. This observation supports the notion that vaccination strategies involving sequential injections with the same viral vector are limited by the development of neutralizing Abs or cellular responses that abrogate vaccine “take.” An additional possibility is that the peak of vaccinia responses observed in some patients (i.e., patients 7 and 9) may be due to competition between MVA-specific T cells specific for different MVA epitopes. We and others (13, 25) have demonstrated that CTL competition for Ag recognition on the surface of APCs may lead to the immunodominance of higher-frequency CTL populations. It is therefore possible that repeated injections of MVA could lead to the expansion of immunodominant MVA-specific T cell response(s), out-competing T cell responses specific for the ILD, KVD, and CLT MVA epitopes. Differences in the patients’ HLA haplotype may explain why this effect is more pronounced in some patients observed rather than in all patients.

The mechanisms controlling immunodominance still remain unclear. It is known that some of the variables that influence immunodominance include the following: 1) peptide MHC class I binding affinity; 2) efficacy of TCR triggering by the MHC class I/peptide complex; 3) efficiency of Ag processing and presentation; 4) T cell repertoire; and 5) competition between T cells at the surface of APCs (26). There are several features of the melan-A26-35 epitope that are likely to have contributed to its dominance over the other Mel3 recombinant epitopes, and to the fact that it was the only recombinant epitope that was able to impact on the vaccinia epitope-dominated hierarchy. First, unlike the other Mel3-encoded epitopes, the melan-A26-35 analog has a high peptide-binding affinity to HLA-A*0201 molecules (14). Second, healthy individuals as well as melanoma patients have high frequencies of melan-A26-35-specific CTL (16). The three patients in whom the vaccinia-specific CTL responses were not dominant over the melan-A26-35-specific response were the two melan-A26-35 responders in the prime-boost group (patients 02 and 08) and the patient with the highest prevaccination melan-A26-35-specific CTL frequency (patient 04). The presence of a prevaccination-primed CTL response specific for the low-affinity NY-ESO-1157–165 epitope in patient 12 (3) was not sufficient to overcome the immunodominance of poxviral-specific responses. These results are consistent with the low-binding affinity of the NY-ESO-1157–165 epitope (27), and with the poor immunogenicity of this epitope in A2-transgenic mice (compared with a superagonist agonist with a substitution of cysteine to valine at position 9 of the peptide) (27).

The insights gained from the results presented in this study have important implications for the future design of recombinant viral vaccines. Our results demonstrate that vaccinia-specific CTL responses dominate the outcome of recombinant poxviral vaccine-driven CTL responses, and outnumber melan-A26-35-specific CTL even in vaccinia-naive patients. These results strongly suggest that recombinant poxviruses should only be used in combination with strategies that successfully prime strong Ag-specific T cell responses. Further identification of poxviral CD8+ and CD4+ epitopes, including those restricted by other HLA alleles and encoded by Avipox viruses, will assist future vaccine design and monitoring. Ultimately, the efficiency with which vaccinia and other large viruses (such as adenoviruses) generate CTL responses may limit their success as backbone delivery vectors in recombinant vaccine strategies.

Advances in molecular technology have permitted the design of synthetic protein vaccines, providing immunotherapy with a degree of specificity that has not been possible using traditional vaccines based on live attenuated pathogens or whole inactivated organisms. Such specificity is providing a platform for the design of T cell therapy for complex disease processed such as autoimmune and cancer (28). The combined use of subunit vaccines with compounds that activate innate responses such as TLR ligands and α-galactosylceramide, which initiates NKT cells (29, 30), should be exploited in future vaccine strategies, in an effort to overcome the limitation of immunodominant viral-specific CTL responses.

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Disclosures

The authors have no financial conflict of interest.

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