Cross-competition of CD8+ T cells shapes the immunodominance hierarchy during boost vaccination

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CD8+ T cell responses directed against multiple pathogen-derived epitopes are characterized by defined immunodominance hierarchy patterns. A possible explanation for this phenomenon is that CD8+ T cells of different specificities compete for access to epitopes on antigen-presenting cells, and that the outcome of this so-called cross-competition reflects the number of induced T cells. In our study using a vaccinia virus infection model, we found that T cell cross-competition is highly relevant during boost vaccination, thereby shaping the immunodominance hierarchy in the recall. We demonstrate that competition was of no importance during priming and was unaffected by the applied route of immunization. It strongly depended on the timing of viral antigen expression in infected APCs, and it was characterized by poor proliferation of T cells recognizing epitopes derived from late viral proteins. To our knowledge, this is the first demonstration of the functional importance of T cell cross-competition during a viral infection. Our findings provide a basis for novel strategies for how boost vaccination to defined antigens can be selectively improved. They give important new insights into the design of more efficient poxviral vectors for immunotherapy.
Until now, most of the work on immunodominance has been done on viruses with relatively small genomes (10–20 kb). Large DNA viruses, like Herpes or Poxviruses (~200 kb), represent a great challenge for the analysis of immunodominance. However, they also offer the possibility to identify crucial mechanisms of immunodominance because the immune system exceedingly shapes the immune response by trimming it down to reactivity against a few epitopes. We and others recently identified HLA-A2– and H2-Kb/Db–restricted poxvirus determinants, which were derived from a large variety of proteins (10–15). Usage of these epitopes should allow us to identify and analyze the underlying mechanisms of immunodominance in poxviral T cell responses. We used different strains of vaccinia virus (VV; modified VV Ankara [MVA], chorioallantois VV Ankara [CVA], and Wyeth) for our infection experiments in C57BL/6 and HLA-A2 transgenic mice (HHD mice). In contrast to the replication-competent VV strains CVA and Wyeth, MVA is unable to replicate in mammalian cells. Therefore, the infection is abortive and is largely synchronized with the advantage to minimize an overlap of the phases of viral gene expression by cells that are infected at different time points during virus spreading. MVA is already used in clinical trials as a recombinant vector, and it is also considered as a next generation poxvirus vaccine. An interesting hallmark of poxviruses is the cascade-like course of antigen expression. There are three distinct phases of viral gene expression driven by distinct promoters with early, intermediate, and late activity. Previous work suggested that the immunogenicity of recombinant proteins produced by VV is influenced by the promoter driving the respective genes (16). Therefore, we presumed a specific impact of viral gene expression on the immunodominance hierarchy of early and late viral proteins.

Our experiments show that the induction and strength of primary CD8+ T cell responses against various VV-specific epitopes in a naive host is largely independent from simultaneous priming of T cells specific for other antigenic determinants delivered by the virus. However, during boost vaccinations, T cell cross-competition seems to be a major regulator of the expansion of virus-specific T cells. In particular, T cells recognizing determinants derived from late viral proteins had a clear disadvantage to proliferate during secondary responses.

However, the efficiency of boost vaccinations was strongly enhanced by using promoters that are active early during the viral life cycle. Our work has important implications for the use and the future design of viral vectors for immunotherapy.

RESULTS

Priming of T cells is independent of T cell competition

To investigate the role of T cell cross-competition on shaping the T cell response to VV, we compared the immune responses against several replication-competent and -deficient strains, as well as recombinant MVA viruses. MVA constructs either expressed or lacked antigens bearing strongly immunogenic
epitopes compared with WT virus (MVA WT). We hypothesized that the quantity of induced T cells would be influenced by additional introduction or removal of immunodominant epitopes if T cell cross-competition was functionally important during priming. We compared the CD8+ T cell responses directed against a variety of virus-specific epitopes. Fig. 1 shows the relative numbers of T cells producing IFN-γ after in vitro restimulation with the respective peptides analyzed by intracellular cytokine staining of freshly isolated splenocytes. 8 d after infection of HLA-A2 transgenic (HHD) mice with MVA WT, T cells recognizing epitopes from late gene products (A6L6, H3L184, and I1L211) were dominating the response (Fig. 1 B). T cells recognizing epitopes derived from early proteins were subdominant (B22R79) or close to detection limit (<0.1% of CD8+; C7L24 and D12L25). Recombinant viruses additionally expressing human tyrosinase (MVA hTyr) or human Her-2/neu (MVA-Her-2/neu) induced a strong Tyr369+ or moderate Her-2/neu435+–specific response. However, the vector-specific response remained unchanged compared with MVA WT. To exclude a mouse strain–specific effect, we also analyzed C57BL/6 mice. In these mice, the cellular immune response against MVA WT is highly dominated by B8R20–specific T cells, which recognize a determinant derived from an early gene product, followed by A3L270– (late gene product), K3L49– (early gene product), and A8R189–specific (early gene product) T cells. Again, a recombinant virus expressing OVA (MVA P7.5 OVA) induced a strong additional OVA257–specific response in these mice without altering the frequencies of vector-specific T cells (Fig. 1 D). Tetramer staining showed similar results (unpublished data). We further constructed a mutant virus (MVA ΔB88R) with a deleted B8R gene (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070849/DC1) to investigate if the induction of B8R20–specific T cells possibly interfered with the primary induction of T cells specific for other VV epitopes. In vitro experiments confirmed similar growth kinetics for MVA ΔB88R and MVA WT (Fig. S2), excluding an attenuation of the deletion variant. MVA ΔB88R did not induce a B8R20–specific T cell response, and other vector-specific T cells did not compensate for that loss (Fig. 1 D). This was also seen at low doses (105 infectious units [IU]; unpublished data). From these experiments, we conclude that the primary induction of vector-specific T cells using MVA is not influenced by the priming of other vector-induced T cells. The time of expression (early vs. late) of antigens did not correlate with their position within the immunodominance hierarchy.

**Immunodominance hierarchy after secondary immunization correlates with viral gene expression**

Next, we wanted to analyze whether the timing of antigen expression (early vs. late viral genes) was relevant for the reactivation of memory T cells. When boosting HHD mice with MVA WT, memory T cells recognizing epitopes derived from late gene products such as H3L184 and I1L211 did not expand (Fig. 2 A). A6L6–specific T cells were not amplified compared with the primary response. Interestingly, early gene product B22R79–specific T cells expanded vigorously during the secondary immunization and became the dominant T cell population among the tested epitope specificities. This severe switch is surprising because it contradicts the prediction that T cells dominating the primary response should also dominate the secondary response if memory precursor frequencies were merely the critical factor for the immunodominance hierarchy of recall responses. In C57BL/6 mice, the primary response is dominated by T cells specific for B8R20, which is derived from an early gene product. If early antigen expression supports recall expansion, boosting of C57BL/6 mice with MVA WT should still support the recall expansion of B8R20–specific T cells. Indeed, boosting with MVA WT led to a strong expansion of B8R20–specific T cells (Fig. 2 B). From these experiments, we hypothesized (a) that T cells specific for early viral proteins might be able to suppress the expansion of other virus-specific T cells, and (b) that T cells specific for late viral proteins should have a disadvantage to proliferate and expand during recall responses.

**Figure 2.** Immunodominance hierarchy is changed after secondary immunization in HHD mice. (A) HHD or C57BL/6 mice were analyzed 8 d after prime (shaded bar) or boosted 35 d after prime and analyzed 6 d later (open bar). Mice were vaccinated i.p. with 108 IU. Only in HHD mice did B22R79–specific T cells increase in frequency; A6L6–, H3L184–, and I1L211–specific T cells do not proliferate during secondary immunizations. (B) In C57BL/6 mice, B8R20–specific T cells dominate the primary and secondary response. (C) Replication-competent virus CVA shows a shift similar to MVA in the immunodominance hierarchy. Results are representative of three independent experiments. n = 4.
Expansion of T cells specific for late gene products is broadly impaired

It has been shown that the route of vaccination can considerably change the outcome of immune responses toward vaccines and influences the immunodominance hierarchy (17). Therefore, we decided to include two clinically relevant application routes, intradermal and i.m. prime and boost immunizations, in our experiments. We found that irrespective of the administration route, the immunodominance hierarchy was conserved during priming, but again, T cells specific for late viral antigens had a disadvantage to expand during recall responses (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070849/DC1). Nevertheless, the T cell frequencies varied between different vaccination routes. The strongest responses could be measured in the spleen after i.p. priming, followed by i.m. and intradermal immunizations.

Next, we wanted to analyze whether the poor proliferation of T cells specific for late viral antigens during recall responses was caused by the lack of replication of MVA in vivo. Comparative analysis of the immune response 8 d after priming with MVA or the replication-competent parental strain CVA revealed a similar immunodominance hierarchy for both viruses (Fig. 2, A and C). Also, in CVA-immunized mice, A6L 6 was immunodominant, followed by H1L 211 - and H3L 184 -specific responses. Interestingly, we could detect a substantial response against B22R 79 8 d after immunization with CVA. 6 d after boosting, we again found suppression of T cell responses against late viral epitopes using this replication-competent virus (Fig. 2 C). Similar results were obtained in experiments using the replication-competent VV strain Wyeth (unpublished data). To exclude that the lack of recall proliferation was caused by a general malfunction of T cells specific for epitopes derived from late gene products, we boosted MVA-primed mice with the respective peptides. As seen in Fig. S4 (available at http://www.jem.org/cgi/content/full/jem.20070849/DC1), H3L 184 -specific T cells could be readily amplified in vivo when mice were revaccinated with H3L 184 peptide. Also, B22R 79 -specific T cells were expanded, when boosting with the respective peptide, yet to a lower extent. The frequency of C7L 74 -specific T cells did not exceed that of peptide-primed mice. In that respect, the expansion of MVA-primed T cells by single peptide revaccination resulted in a pattern that resembles the immunodominance hierarchy induced by MVA in the primary response (Fig. 1 B). This indicates that VV-specific T cells, which did not proliferate in recall responses to the virus, possibly lacked antigen-specific stimulation. Impairment of viral antigen processing or presentation might account for reduced or inhibited proliferation of T cells recognizing late gene product–derived epitopes during VV recall responses. To further analyze this issue, we decided to focus on the H3L late gene product.

Antigen presentation of late viral proteins is substantially delayed

As mentioned earlier, the viral life cycle of VV can be divided into three distinct phases: early, intermediate, and late viral gene expression. The interval between these phases is only about 1 h on the transcriptional level (18). To test if differences in viral transcription led to a relevant delay of the presentation of viral antigens, we established several T cell lines specific for VV early or late protein determinants. T cells recognizing early determinant B22R 79 were able to efficiently lyse infected target cells 3 h after infection (Fig. 3 A). In contrast, T cells with specificity for late viral proteins, such as H3L 184 -specific T cells were not able to recognize infected cells before 15 h after infection (Fig. 3 A). Specific 51 Cr release of MVA WT–infected (MOI 10) A375 target cells is shown (E/T ratio = 10:1). (A) H3L 184 -specific T cells do not substantially lyse infected target cells before 15 h after infection. (B) Peptide titration shows similar affinity of T cell lines, except for H3L 184 -specific T cells showing a higher affinity. (C) Infected LCL (MOI 10) was used for a kinetic analysis to stimulate IFN-γ production in several VV-specific T cell lines. MVA WT–infected cells already stimulate B22R 79 –specific (early gene) T cells 2 h after infection. H3L 184 -specific (late gene) T cells get stimulated 6 h after infection. A6L 6 - and H1L 211 -specific T cells (both late) or control cell line are not stimulated by MVA WT–infected cells. (D) LCL infected by recombinant virus MVA ΔH3L P7.5 H3L expressing the H3L gene under an early/late promoter rapidly induce IFN-γ production in H3L 184 -specific T cells. Data are representative of three independent experiments.
Timing of viral antigen expression regulates T cell expansion

Next, we wanted to analyze whether the delayed antigen presentation of late viral proteins is related to the impaired proliferation of T cells specific for these proteins during boost vaccinations. We constructed a recombinant MVA in which the natural H3L gene has been knocked out (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070849/DC1) and reinserted into the viral genome under the control of a promoter (P7.5) with early/late activity (MVA ΔH3L P7.5 H3L). Northern blot analysis revealed that both viruses MVA WT and MVA ΔH3L P7.5 H3L express the H3L gene at comparable levels; but, in contrast to MVA WT, MVA ΔH3L P7.5 H3L already produces the H3 protein early during the viral life cycle (Fig. S6). In vitro, MVA ΔH3L P7.5 H3L-infected target cells induced IFN-γ in H3L184-specific T cells very early after infection (Fig. 3D). Importantly, H3L184-specific T cells were readily expanded in the secondary response when boosting with MVA ΔH3L P7.5 H3L (Fig. 4 A). Thereby, the absolute numbers of H3L184-specific T cells reached similar levels as B22R79-specific T cells (Fig. 4 B). Importantly, the responses against other epitopes (A6L6, I1L211, and B22R79) were comparable for both viruses (Fig. 4 and not depicted).
Cross-competition shapes immunodominance hierarchy

Thus far, we have shown that T cell cross-competition between T cells recognizing early or late viral products is functionally important for shaping the immunodominance hierarchy during recall responses. Next, we wanted to analyze whether T cells recognizing early epitopes also cross-compete with each other. In C57BL/6 mice, the B8R20-specific T cell response dominates both the primary and secondary response against VV. To analyze if B8R20-specific T cells were cross-competing with T cells of other specificities and impaired their expansion, we used MVA/H9004B8R for prime/boost immunizations (Fig. S1). As anticipated, despite the absence of B8R20-specific T cells, the expansion of A3L270- and C7L74-specific T cells was significantly amplified, as seen in secondary responses after 35 d. To confirm that T cells were able to cross-compete early after primary infection, we revaccinated MVA WT–primed mice with MVA WT or MVA ΔH3L P7.5 H3L. Indeed, similar to boosting at day 35 (Fig. 4), B22R79- and H3L184-specific T cells expanded when mice were boosted early (day 5) with MVA ΔH3L P7.5 H3L (Fig. 6 D and Fig. S7 D).

Cross-competition between T cells specific for early viral determinants

To analyze if B8R20-specific T cells were cross-competing with T cells of other specificities and impaired their expansion, we used MVA ΔB8R for prime/boost immunizations (Fig. S1). Indeed, similar to boosting at day 35 (Fig. 4), B22R79- and C7L74-specific T cells expanded. From day 4 or later, B22R79-specific T cells were significantly amplified, as seen in secondary responses after 35 d. To confirm that T cells were able to cross-compete early after primary infection, we revaccinated MVA WT–primed mice with MVA WT or MVA ΔH3L P7.5 H3L. Indeed, similar to boosting at day 35 (Fig. 4), B22R79- and H3L184-specific T cells expanded when mice were boosted early (day 5) with MVA ΔH3L P7.5 H3L (Fig. 6 D and Fig. S7 D).
viruses expressing the model antigen OVA driven by a promoter with exclusive early (MVA K1L OVA) or late (MVA P11 OVA) activity. As shown by Western blot analysis, infection with MVA K1L OVA led to a rapid production of OVA, even in the presence of Ara-C, which is an effective inhibitor of late viral gene expression. In contrast, the synthesis of OVA in MVA P11 OVA–infected cells is initiated later during viral infection and abrogated upon Ara-C treatment (Fig. 8A). After 24 h of infection, the overall amount of synthesized OVA is higher in MVA P11 OVA–infected cells (Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20070849/DC1). Interestingly, when measuring the processing and presentation of OVA by specific staining of OVA 257–loaded MHC-I complexes on RMA cells, we found a clear correlation with the time of expression, which depends on the promoter activity of the respective constructs (Fig. 8B).

Timing of viral antigen expression is crucial for vaccination strategies

To investigate the influence of viral antigen expression on T cell cross-competition more closely, we constructed two viruses expressing the model antigen OVA driven by a promoter with exclusive early (MVA K1L OVA) or late (MVA P11 OVA) activity. As shown by Western blot analysis, infection with MVA K1L OVA led to a rapid production of OVA, even in the presence of Ara-C, which is an effective inhibitor of late viral gene expression. In contrast, the synthesis of OVA in MVA P11 OVA–infected cells is initiated later during viral infection and abrogated upon Ara-C treatment (Fig. 8A). After 24 h of infection, the overall amount of synthesized OVA is higher in MVA P11 OVA–infected cells (Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20070849/DC1). Interestingly, when measuring the processing and presentation of OVA by specific staining of OVA 257–loaded MHC-I complexes on RMA cells, we found a clear correlation with the time of expression, which depends on the promoter activity of the respective constructs (Fig. 8B).

2 h after infection, we could already detect a substantial A8R 189–specific T cell response induced by MVA ∆B8R, as the baseline and considered A3L 270–specific T cell response as an internal control. We then compared the K3L 6– and A8R 189–specific T cell response induced by recombinant viruses, which contain an additional moderate (MVA OVA ∆B8R containing OVA 257) or highly (MVA WT containing B8R 20) immunogenic epitope or both (MVA OVA containing OVA 257 and B8R 20). As shown in Fig. 7B, this stepwise additional induction of cross-competing T cells specific for early viral epitopes (OVA 257/B8R 20) leads to a gradually increased suppression of T cells specific for other early viral epitopes (K3L 6/A8R 189). Importantly, T cells specific for late viral epitopes (A3L 270) remain fully suppressed. Hence, we conclude that cross-competition is also functional among T cells specific for early viral proteins during recall responses.

Figure 6. Competition between T cells occurs early after priming. HHD mice were primed with MVA WT and boosted with the same virus (A and B) early at indicated days after priming, or at day 5 with MVA ∆H3L P7.5 H3L (C and D). (A) Schematic of prime/boost regimen. (B) Intracellular cytokine staining of splenocytes, comparing the VV-specific CD8+ T cell responses after priming (shaded bar) or 6 d after boosting (open bar). B22R 79–specific T cells are significantly increased when boosting 4 d after prime or later. (C) Schematic of prime/boost regimen. (D) H3L 184–specific T cell responses can be significantly amplified when using MVA ∆H3L P7.5 H3L (open bar) as compared with MVA WT (closed bar). Data are summary of three independent experiments. n = 6.

Figure 7. Early virus-specific T cells suppress the expansion of early and late virus-specific T cells. (A) Intracellular cytokine staining of splenocytes comparing MVA ∆B8R (open bar) with MVA WT (shaded bar) 6 d after homologous boost (day 35). Immunodominance hierarchy is changing in MVA ∆B8R–immunized mice favoring the expansion of K3L 6– and A8R 189– (both early genes) specific T cells over A3L 270– (late gene) specific T cells in the absence of B8R 20–specific T cells. (B) Intracellular cytokine staining of splenocytes comparing MVA ∆B8R with MVA OVA P7.5 ∆B8R, MVA WT, and MVA OVA P7.5 6 d after homologous boost (day 5). The expansion of K3L 6– and A8R 189–specific (both early genes) T cells is successively suppressed by gradual appearance of cross-competing B8R 20– and OVA 257–specific (both early genes) T cells, whereas A3L 270–specific (late gene) T cells remain fully suppressed. Data are representative of two independent experiments. n = 5.
Our study identified T cell cross-competition to be responsible for the dramatic switch in the epitope dominance patterns of VV-specific CD8\(^+\) T cells by comparing primary with secondary infections. Changes in immunodominance hierarchies of T cells have also been reported for other pathogens, such as influenza, herpes viruses, or LCMV (22–24). In the LCMV infection model, these changes have been attributed primarily to T cell exhaustion. In contrast, upon influenza virus infection, the changing epitope pattern has been attributed to differential antigen expression, reflecting the capacity of memory T cells to respond to non-dendritic cells (9).

Yewdell’s group, which works extensively on immunodominance in the influenza model, recently challenged this interpretation (8), and came to the conclusion that cross-presentation could also account for changes in dominance patterns. Furthermore, they demonstrated that immunodominance hierarchies were independent of perforin or Fas-mediated lysis in the secondary response, and are therefore not connected to APC killing or CD8\(^+\)/H11001 T cell cross-competition. La Gruta et al. elegantly shifted epitopes within the same viral infection context to study immunodominance (25). They found that the epitope hierarchies were a result of antigen dose and the size of the preexisting T cell pool. Competitive interactions, as demonstrated in our study, seemed to have only little impact in their model. Removal or addition of immunogenic epitopes in influenza virus had no effect during priming, but led to

**DISCUSSION**

In our study, we identified T cell cross-competition to be responsible for the dramatic switch in the epitope dominance patterns of VV-specific CD8\(^+\) T cells by comparing primary with secondary infections. Changes in immunodominance hierarchies of T cells have also been reported for other pathogens, such as influenza, herpes viruses, or LCMV (22–24). In the LCMV infection model, these changes have been attributed primarily to T cell exhaustion. In contrast, upon influenza virus infection, the changing epitope pattern has been attributed to differential antigen expression, reflecting the capacity of memory T cells to respond to non-dendritic cells (9).

**Figure 8.** Timing of viral antigen expression is essential during secondary responses. (A) Western blot analysis of Hela cells infected with MVA K1L OVA or MVA P11 OVA in the presence (+) or absence (-) of Ara-C, which is a specific inhibitor of late viral gene expression. Cell lysates were harvested at 0, 5, or 12 h after infection. (B) For relative quantification of SIINFEKL-H2-K\(^b\) complexes on infected cells, 25-D1.16 antibody was used. Mean fluorescence intensity of positive cells is shown. (C) Intracellular cytokine staining of splenocytes comparing MVA K1L OVA with MVA P11 OVA 8 d after prime or 6 d after boost, which was performed 35 d after priming with MVA P7.5 OVA, to allow for comparable memory T cell frequencies at the time of the second immunization. Mice were primed and boosted i.p. 5 d later with different MVA constructs. (D) 6 d after homologous boost, mice were challenged with different doses of L. monocytogenes-OVA (2 \times 10^5 = shaded bar; 5 \times 10^5 = open bar) i.v. and numbers of viable L. monocytogenes in spleen were determined 2 d later. nd = not detectable.
compensation or suppression of other T cells during boosting, respectively (26, 27). The authors attributed their findings to effects of antibodies or limiting amounts of antigen. In our study, however, we could link the immunodominance pattern arising during secondary vaccinations to the presence of primed T cells. A reason for potential differences between VV and other infectious agents could be the high-level gene expression promoted by VV, leading to high antigen amounts. Hence, competition mediated by limited antigenic resources seems to be unlikely. Consistent with that view, a mathematical model of T cell competition recently predicted that a large set of different epitopes, such as that found for VV infection, should decrease T cell competition (28). However, under these conditions, high affinity of T cells and high expression levels of single epitopes would increase the chances for T cell competition. Hence, when immunizing with VV, the APC itself or resources of the APC other than the peptide–MHC complexes may become the limiting factor for competing T cells. Besides costimulatory or adhesion molecules, this could involve cytokines or access to APC. Another possibility is that memory T cells could be able to silence APCs after a certain number of interactions. Further work will be necessary to elucidate the cellular and molecular basis of T cell cross-competition.

Surprisingly, T cell cross-competition has been rarely documented so far. Marrack’s group demonstrated T cell cross-competition by using peptide-pulsed DC and transferred TCR transgenic T cells (29). They found that the degree of competition depended on the affinity of responding T cells, but was much less efficient than competition among T cells with the same epitope specificity (30). In the LCMV model, T cell cross-competition is believed to be functionally unimportant (5). However, in this particular study, cross-competition was only analyzed during the priming phase. Our work demonstrates that cross-competition toward VV is active during the secondary response and depends on immediate T cell effector function (presence of primed T cells). This notion is supported by Kedl et al., who succeeded in demonstrating T cell cross-competition by using primed T cells (29). Additionally, we found that T cells were able to crosscompete 3 d after priming. This correlates with the development of cytotoxicity of naive T cells after exposure to APCs, which was detectable after 48 h and was fully present after 72 h (20). In a bacterial model, Wong and Pamer showed that CTL activity developed within 72 h, and that these T cells probably eliminated APCs, thereby regulating antigen presentation and, consequently, T cell priming and expansion (31). Interestingly, Belz et al. recently showed that during secondary influenza infections, T cells terminate the antigen presentation in a perforin-dependent manner (32). However, in the influenza model, perforin, granzyme B, or FAS–FASL interaction did not seem to influence the immunodominance hierarchy (7, 8). Other models showed that early onset of IFN-γ production by CD8+ T cells correlated with the immunodominant responses (33). Further work will be necessary to define which features of effector T cells are crucial to execute cross-competition.

The primary response to the replication-competent VV strain CVA was similar compared with MVA, apart from a substantial response against B22R79. Interestingly, we could also induce and detect an increased B22R79-specific response by performing short-interval prime/boost experiments with MVA. This, in a way, imitates replication caused by repeated infections during the priming phase. In that respect, the primary response to a replication-competent VV could be interpreted as a dynamic process of priming and boosting of T cells. This is in contrast to persistent chronic viral infections, where T cell exhaustion is a factor for changing immunodominance hierarchies. As recently shown, using LCMV as a model for a chronic viral infection, CD8+ T cells undergo extensive peptide-dependent division independent of IL-7 or -15. This suggests that these CD8+ T cells go through a fundamentally different pattern of differentiation compared with memory CD8+ T cells that develop after an acute infection (34). In addition to A*0201 transgenic mice, the suppression of secondary T cell responses against late viral epitopes by T cells recognizing early viral epitopes could also be confirmed in C57BL/6 mice, and seems to be a characteristic feature of VV infection. A similar connection between immunodominance and the kinetics of viral protein expression has been observed using LCMV (24, 35). Indeed, in an elegant model using hemisplenectomized mice and analysis of TCR–β motifs, Bousso et al. found that the timing of recruitment of individual T cell clones contributes more to the immune responses than their precursor frequency (36).

In contrast to T cells specific for H3L184 or I1L211, which do not proliferate during secondary responses, the expansion of T cells specific for A6L6 is not fully suppressed when the boost is performed late in the memory phase (day 35). This might be explained by comparatively higher T cell numbers against A6L6, which cannot be controlled as easily by competing T cells recognizing early epitopes. After short-interval prime/boost experiments (day 5), the A6L6-specific T cell response is fully suppressed, possibly because during priming, the T cell size is increased compared with the memory phase (day 35), and therefore competition is enhanced. Nevertheless, we assume that the amount of B22R79-specific T cells induced after priming is probably too low to completely outcompete A6L6, H3L184, and I1L211-specific T cells. Additionally, ~2% B22R79-specific T cells (Fig. 2) cannot fully account for the total VV-specific response after secondary immunization, which is ~30–40% of all CD8+ T cells (11). Therefore, it is intriguing to speculate that one or more yet unidentified HLA-A*0201-restricted epitopes might exist.

It can be expected that T cells recognizing epitopes derived from early viral proteins confer better protection against infection. Because VV replication is completed at ~8 h after infection, T cells specific for late viral proteins are likely to be activated too late to confer any protection because of delayed antigen presentation in infected cells. In this context, T cell cross-competition, and the subsequent preferential expansion of T cells specific for early proteins, reflects the host’s ambition to rapidly clear viral infections. We speculate that this
finding might also apply to other large DNA viruses, such as herpes viruses. Interestingly, a recent study analyzing T cells in CMV-infected humans described that the number of immediate early protein 1–specific (IE-1), and not pp65–specific (a late viral protein), T cells correlated with protection from disease (37).

In summary, we have demonstrated that the expansion of virus-specific CD8+ T cells was regulated by T cell cross-competition favoring T cells that are able to rapidly detect infected cells. Therefore, the outcome of this competition was heavily influenced by the timing of antigen expression, but independent of the route of vaccination or the ability of a virus to replicate. In immunotherapy using recombinant viral vectors, a successful expansion of the desired T cell response can be strongly impinged by cross-competing, vector-specific T cells. We show that this impairment can be compensated by expressing target antigens early during the viral life cycle, allowing antigen-specific T cells to successfully cross-compete. Thereby, we are able to improve the T cell responses against target antigens and simultaneously decrease the vector-specific response. The identification of other factors determining the outcome of T cell cross-competition and, ultimately, the elucidation of the molecular and cellular mechanism behind T cell cross-competition will allow for designing improved vaccines.

MATERIALS AND METHODS

Mice and vaccination. HLA-A*0201-transgenic, H2Db−/−, β2m−/−, HD (38) or C57BL/6 mice were derived from in-house breeding under specific pathogen-free conditions following institutional guidelines. Only female mice between 8 and 12 wk of age were used. Mice were vaccinated with 103 or 104 IU MVA. For peptide vaccination, mice were immunized s.c. with 0.1 mg peptide and 10 ng of synthetic CpG1668. Mice were killed on the indicated days after vaccination, and spleens were harvested to be analyzed by ICS.

Cell lines. RMA cells were provided by F.A. Lemonnier (Institute Pasteur, Paris, France). Lymphoblastoid B cell line RL-LCL (HLA-A*0201 positive) was established in our laboratory, A375 human melanoma cells (CRL-1619) was established in our laboratory, A375 human melanoma cells (CRL-1619) were purchased from American Type Culture Collection. Cell lines were cultured with RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Viruses. The VV strains New York City Board of Health (Wyeth) and Health, Bethesda, MD). CVA at second passage on chicken embryo fibroblasts (CEF) or MVA (clone isolated F6) at 582nd passage on CEF were used for this study. Replication-competent VV and MVA were routinely cultured with RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Quantification of antigen-specific CD8+ T cell responses. Splenocytes from vaccinated HHD mice were stimulated with either the HLA-A*0201 restricted human tyrosinase peptide Tyr393-Her-2/neu(393), the vaccinia-specific peptides derived from H3L446, A6L4, H1L16, D12K110, C7L7, B22R9, (13), or a control peptide (Flu M1) for 5 h. C57BL/6 mice were stimulated with either H-2Kb− or H-2Db− restricted VV-specific peptides derived from A3L290, B8K300, BR9, C3L45, OVA1, or a control peptide (β-Gal45) for 5 h. 1 mg/ml Brefeldin A (Sigma-Aldrich) was present throughout stimulation, RMA cells were infected for 30 min at MOI 20 on ice and then shifted to 37°C. At indicated time points, cells were harvested and stained with anti-SIINFEKL-Kb antibody (25-D1.16).

Statistical analysis. All statistical analysis was performed using Excel Software. Results are expressed as the mean ± the SD. Differences between groups were analyzed for statistical significance using two-tailed student t test.

Online supplemental material. Fig. S1 shows the construction of BR-deficient MVA. Fig. S2 shows the multistep viral growth curve on CEF cells. Fig. S3 shows that the immunodominance hierarchy is changed after secondary immunization in HHD mice. Fig. S4 shows IFN-γ production of VV-specific T cells from freshly isolated splenocytes of MVA WT (104 IU)-primed virus-infected anemia (37).
and peptide-revaccinated HHD mice. Fig. S5 shows construction of H3L-deficient MVA revertant for the H3L gene. Fig. S6 shows that MVA ΔH3L (PT5) H3L-expressing vaccine H3L gene early during the viral life cycle. Fig. S7 shows that competition between T cells occurs early after priming. Fig. S8 shows the total amount of OVA-expressed and vector-specific responses induced by different MVA constructs. Additional information is provided in a Supplemental materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070489/DC1.

We would like to thank A. Dick and S. Ecke for their outstanding performance during their bachelor thesis. We also thank K.M. Huster for critical reading of the manuscript and helpful discussion.

This work was supported by the Deutschen Forschungsgemeinschaft SFB 456 (B7). The authors have no conflicting financial interests.

Submitted: 9 March 2007
Accepted: 19 July 2007

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