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Dissecting the Multifactorial Causes of Immunodominance in Class I–Restricted T Cell Responses to Viruses

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Summary

Following influenza virus infection, the numbers of mouse $T_{CD8}+$ cells responding to five different determinants vary more than 50-fold in primary responses but less so in secondary responses. Surprisingly, each determinant elicits a highly diverse and highly sensitive $T_{CD8}+$ response. Inefficient antigen processing by virus-infected cells accounts for the poor immunogenicity of just one of the subdominant determinants. Overexpressing class I–peptide complexes using vaccinia virus revealed that the poor immunogenicity of two subdominant determinants reflects limitations in T cell responses unrelated to TCR diversity or sensitivity. Despite greatly enhanced expression, the immunodominant determinant is actually less immunogenic when overexpressed by vaccinia virus. Immunodominance is also modulated by determinant-specific variations in the capacity of $T_{CD8}+$ to suppress responses to other determinants.

Introduction

Immunodominance is a central feature of $CD8^+$ T cell ($T_{CD8}+$) responses to pathogens (Zinkernagel and Doherty, 1979; Yewdell and Bennink, 1999). Few of the enormous number of peptides encoded by the pathogen elicit responses, and, even among the chosen few, responses are often skewed heavily to one or several peptides (termed immunodominant determinants [IDDs]) at the expense of the others (termed subdominant determinants [SDDs]) (Sercarz et al., 1993). A detailed accounting of immunodominance is essential to understanding cellular immunity, viral evasion mechanisms, and the rational design of vaccines.

It is not difficult to identify the factors that potentially contribute to immunodominance. These include the binding of peptides to class I molecules, the efficiency of antigen presentation, the presence of T cells capable of responding to a given complex, and immunodomination: the suppression of responses to SDDs by IDD-specific $T_{CD8}+$. The trick lies in weighing the relative contributions of each of these factors to immunodominance, as $T_{CD8}+$ responses ultimately reflect the mathematical product of the individual factors.

Studies of $T_{CD8}+$ have been greatly aided by the introduction of new methods that allow for the accurate quantitation of $T_{CD8}+$ responding to individual determinants (McMichael and O'Callaghan, 1998). Here, we use one of these methods, intracellular cytokine staining (ICS) (Jung et al., 1993), in conjunction with biochemical analysis and ER-targeted minigene-expressing recombinant vaccinia viruses (rVVs) to comprehensively examine the factors that account for the hierarchy of H-2 K^d-restricted $T_{CD8}+$ responses to influenza virus.

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Results

Establishing the Hierarchy: Quantitating Antiviral $T_{CD8}+$ Responses to Individual Determinants

Infection of BALB/c mice with PR8 influenza virus results in the priming of K^d-restricted memory $T_{CD8}+$ ($mT_{CD8}+$) to at least five determinants derived from two viral proteins (nucleoprotein [NP] and hemagglutinin [HA]) that can be detected by $T_{CD8}+$-mediated lysis following 7 day in vitro stimulation with synthetic peptides (Deng et al., 1997). To gain a more precise measure of the $T_{CD8}+$ response, we used ICS to quantitate the numbers of splenic $T_{CD8}+$ and peritoneal $T_{CD8}+$ that respond to each determinant at four intervals following infection, as determined using peptide-pulsed antigen-presenting cells (APCs).

Figures 1A and 1B demonstrate that responses of both peritoneal and splenic primary $T_{CD8}+$ ($pT_{CD8}+$) reach detectable levels between days 3 and 5 post infection, peaking on or about day 7 and declining by day 11. Confirming and extending our previous findings, $NP_{147-155}$-specific $T_{CD8}+$ were prevalent among both splenic and peritoneal $T_{CD8}+$. The next most prevalent $T_{CD8}+$ population was $HA_{518-526}$-specific $T_{CD8}+$, which was the only other population detected in the spleen in this experiment. Responses to NP_{39-47} were detected among peritoneal $T_{CD8}+$, while splenic and peritoneal responses to $NP_{218-226}$ and $HA_{462-470}$ were too close to background values to be reliable.

We next compared peak $pT_{CD8}+$ responses (day 7 following infection) with $mT_{CD8}+$ responses, using both ICS (panel C) and lytic activity to gauge responses (panel D). Peptide-specific $pT_{CD8}+$-mediated lysis is exclusively directed to $NP_{147-155}$, while ICS of the same population reveals responses to $NP_{147-155}$ as well as to SDDs $HA_{518-526}$ and NP_{39-47}. The percentage of responding $T_{CD8}+$ in this experiment is ~3-fold higher than that shown in panel A, which probably accounts for the detection of NP_{39-47}-specific splenic $pT_{CD8}+$.

$mT_{CD8}+$ specific for $NP_{147-155}$ and $HA_{518-526}$ were detected at 2- to 3-fold lower frequencies than $pT_{CD8}+$ (panel C). Surprisingly, the less vigorous primary responses to NP_{39-47} demonstrated a smaller decrease among $mT_{CD8}+$. Responses to $NP_{218-226}$ and $HA_{462-470}$ remained near the limits of detection by ICS. Following 7 day stimulation of $mT_{CD8}+$ with PR8 infected-P815 cells, however, lytic activity to all of the determinants could be detected (panel E). In additional experiments performed under similar conditions, ICS revealed $mT_{CD8}+$ specific for each of the determinants in day 7 in vitro cultures at frequencies consistent with the lytic activities (data not shown).

These data clearly illustrate, first, that lysis is a partial

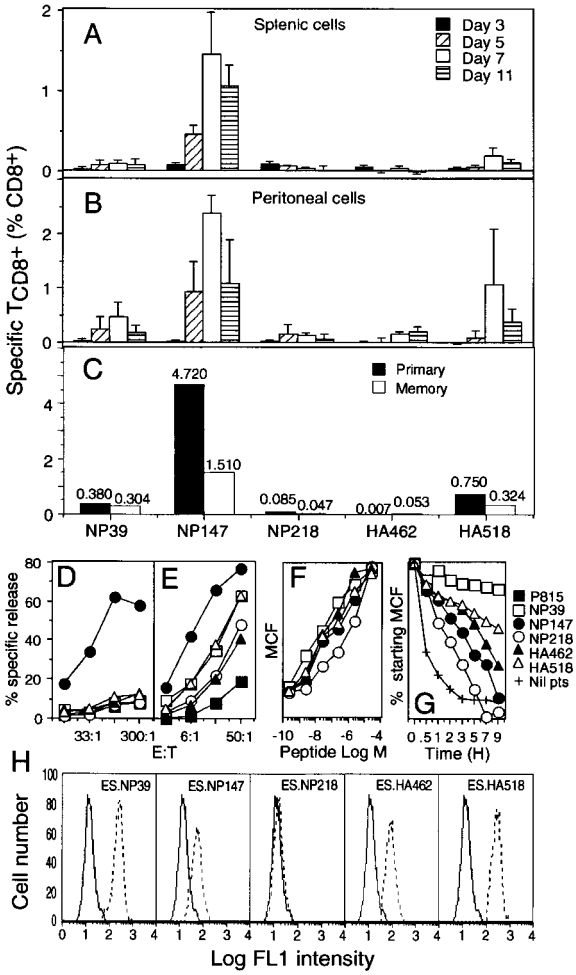

Figure 1. Hierarchy of K^d-Restricted Responses to PR8 Infection of BALB/c Mice

BALB/c mice were infected with PR8 by intraperitoneal injection, and 3, 5, 7, and 11 days later, splenic (A) and peritoneal (B) T_{CD8}+ from four individuals were analyzed by ICS after activation by peptide-sensitized APCs. Error bars correspond to the standard deviation of the values. Background values obtained with nonpeptide-stimulated cells were subtracted from each individual. In a separate experiment, splenic T_{CD8}+ obtained 7 days post infection were analyzed by ICS ([C], "primary") or tested directly ex vivo for lysis against peptide-sensitized ^{51}Cr-labeled cells at the indicated effector to target cell ratio (E:T) (D). Additionally, 21 days after priming, splenic T_{CD8}+ were either directly used for cytokine staining ([C], "memory") or stimulated by PR8-infected P815 cells for 7 days and tested for lysis against peptide-sensitized ^{51}Cr-labeled P815 cells (E). In (C), background values of .71% and .22% obtained by activating with nonpeptide-sensitized cells were subtracted, respectively, from values for pT_{CD8}+ and mT_{CD8}+ responses. In F, T2-K^d cells cultured at 26°C were incubated with peptides at the indicated concentrations for 1 hr, and K^d molecules remaining after incubation at 37°C for 2 hr determined by direct immunofluorescence and flow cytometry. Data are expressed as mean channel fluorescence (MCF). Similarly (G), T2-K^d cells incubated with a saturating concentration of peptide were washed and incubated at 37°C in the presence of BFA for the indicated time prior to direct staining. Data are expressed as the percentage of the initial MCF obtained with each peptide. Finally (H), T2-K^d cells were infected for 6 hr at 37°C with either a control rVV or rVVs coding various ER-targeted determinants. Cells were stained as above for K^d cell surface expression and analyzed by flow cytometry. Solid lines represent staining of cells infected with the control virus. Staining an mAb specific for an early VV encoded protein indicated that cells were infected to a similar extent by the VV vector (data not shown).

measure of pT_{CD8}+ responses and, second, that the numbers of T_{CD8}+ responding to individual determinants varies widely between determinants, establishing the immunodominance hierarchy in this system. Although the absolute numbers of responding T_{CD8}+ vary among individuals and experiments (within a 4-fold range over a large series of experiments), the ranking of determinants within the hierarchy was constant in a large number of animals examined in a number of different experiments. In what follows, we examine the contribution of the individual factors that contribute to immunodominance.

Peptide Affinity for K^d

We improved our previous examination of peptide binding to K^d (Deng et al., 1997) in two important ways. First, we analyzed the binding of Cys containing peptides ($NP_{218-226}$, NP_{39-47}) in the presence of a reducing agent, since sulfhydryl modification of the Cys residue on these peptides affects their binding to K^d (Chen et al., 1999). Second, we compared peptide off rates from cell surface K^d molecules, since this has been reported to correlate better with immunogenicity than the overall dissociation constant (van der Burg et al., 1996).

Peptide affinity and dissociation were measured using K^d-transfected T2 cells (a TAP-deficient human lymphoid-derived cell line) that were incubated overnight at 26°C to enhance expression of peptide-receptive cell surface molecules. Peptide affinity was estimated by adding decreasing amounts of peptides to cells and then shifting cells to 37°C for 2 hr to unfold K^d molecules lacking a peptide ligand. The expression of conformed cell surface K^d was determined by flow cytometry using a conformationally sensitive mAb conjugated to fluorescein. The concentration of peptide required for rescue of 50% of the K^d molecules provides an estimate of the dissociation constant (K_D) of the peptide–K^d interaction.

As shown in Figure 1F, all five peptides stabilized surface K^d molecules in a dose-dependent manner. Relative to $NP_{147-155}$, NP_{39-47}, $HA_{518-526}$, and $HA_{462-470}$ displayed higher affinity binding to K^d while $NP_{218-226}$ exhibited ~10-fold lower affinity (K_D estimates given in Table 1). These differences are reflected in the off rates, determined by pulsing cells with a saturating concentration of peptide, washing, and then incubating for increasing times at 37°C (Figure 1G).

The relevance of these findings to the association of naturally processed peptides with K^d molecules in the ER is fully supported by the next experiment. Recombinant vaccinia viruses expressing ER-targeted forms of the peptides (Deng et al., 1997) were tested for their abilities to enhance the expression of K^d molecules on surface of T2-K^d cells infected at 37°C (Figure 1H). The degree of rescue of K^d molecules matched the rank order of peptide affinity for K^d determined using synthetic peptides, with a single exception ($HA_{518-526}$ being slightly better than NP_{39-47}; this may be related to a decreased affinity of the mAb used for detection for complexes of K^d and NP_{39-47} with human β2m). These findings indicate that peptide association in the ER is similar to the binding of synthetic peptides to cell surface molecules and confirm that targeting peptides to the ER usually results in the generation of tens of thousands of class I–peptide complexes (Antón et al., 1997).

Table 1. Immunodominance by the Numbers

		A	B	C = (A×B)	D	E = (D/C)
Name	Sequence	K_A ($\times 10^7$ M^{-1})	K_L ($\times 10^{-12}$ M)	Threshold	Determinants Per Cell	Predicted Speed
NP_{39-47}	FYIQMCTEL	2.88	5.8	16.7	Low	Glacial
$NP_{147-155}$	TYQRTRALV	0.79	1.2	.94	808	860
$NP_{218-226}$	AYERMCNIL	0.22	5.8	1.27	~300	236
$HA_{462-470}$	LYEKVKSQL	1.68	3.6	6.05	4870	805
$HA_{518-526}$	IYSTVASSL	1.05	1.2	1.26	1616	1283

Column A shows the K_A derived from the peptide concentration calculated to rescue one-half of K^d molecules from the data in Figure 1F. Column B shows the peptide concentration required to achieve half maximal lysis of target cells in Figure 3. Column C is the product of columns A and B and provides a relative (but linear) comparison of the number of complexes per cell required for target cell lysis. Column D shows the number of peptides isolated per cell as estimated using the data in Figure 2. Column E results from dividing column D by column C and provides a prediction of the kinetics of sensitization for $T_{CD8}+$ lysis confirmed in Figure 2.

Antigen Processing

We next quantitated the numbers of peptide–K^d complexes produced by PR8-infected cells as previously described by Rammensee and colleagues (Falk et al., 1991) by HPLC purification of peptides present in acid extracts. With the exception of NP_{39-47}, we were able to detect each of the determinants in fractions that coeluted with the corresponding synthetic peptides (Figure 2). For $HA_{462-470}$ and $HA_{518-526}$, this provides the initial evidence that the naturally processed peptide is identical to the synthetic nonameric peptide that matches the K^d binding motif. We determined the efficiency of recovery of each of the peptides from cells (ranging from 10%–12%) by measuring the amount of peptide recovered in HPLC fractions from uninfected cells to which peptides were added during homogenization. Using this figure, along with the concentration of peptide calculated to be present in the fraction by comparison to a synthetic peptide standard curve, we could calculate the number of peptides recovered per cell (Table 1D).

There are a number of limitations to quantitating peptides by this method, including an inability to distinguish intracellular from cell surface complexes and a reliance on assumptions regarding peptide recovery efficiency and the identity of synthetic and naturally processed peptides. We therefore sought an independent means of testing the validity of the peptide quantitation. By multiplying the estimated peptide affinities for K^d by the peptide concentration required for target cell sensitization, it is possible to rank the relative number of complexes needed for $T_{CD8}+$ detection (Table 1, "threshold"). Dividing the number of determinants present in infected cells by this threshold value results in prediction of the velocity of presentation following infection. This prediction should be valid for different determinants presented from the same viral protein, and even between proteins, if the two proteins are expressed with similar kinetics.

The kinetics of complex formation were determined experimentally by adding BFA to cells at increasing intervals after PR8 infection to block export of nascent class I–peptide complexes from the ER (Antón et al., 1997). As seen in Figure 2, $HA_{462-470}$ reaches saturating concentrations first, followed by $NP_{147-155}$, $HA_{518-526}$, and $NP_{218-226}$. NP_{39-47} never reached saturating levels in this assay. With the minor exception of the slight tardiness of $HA_{518-526}$ (which is probably accounted for by a decrease in the sensitivity of the $HA_{518-526}$-specific $T_{CD8}+$ used in this assay), these findings agree with the predicted kinetics, thereby supporting the validity of our measurements of peptide affinity and complex formation. Importantly, they also demonstrate functionally that the production of NP_{39-47}, and to a lesser extent $NP_{218-226}$, are limiting relative to the other determinants in the context of the sensitivity of their cognate $T_{CD8}+$.

$pT_{CD8}+$ Response: Diversity

We examined the diversity of $pT_{CD8}+$ responding to each of the determinants using mAbs specific for the various Vβ gene products using micromagnetic beads coated with anti-fluorescein antibodies to bind $T_{CD8}+$ labeled with fluorescein-conjugated anti-Vβ mAbs. Positively and negatively selected $T_{CD8}+$ were characterized for Vβ expression by flow cytometry and for lytic activity against peptide-sensitized target cells.

Using $T_{CD8}+$ derived from PR8-infected mice and restimulated in vitro with PR8-infected cells, most lytic activity (~ 2/3) against peptide sensitized-cells is mediated by $V\beta8.1/2^+$ $T_{CD8}+$ (Figures 3A and 3B). The lytic activity of $V\beta8.1/2^+$ $T_{CD8}+$ is predominantly directed against $NP_{147-155}$, but this population also contains $T_{CD8}+$ specific for each of the four SDDs. $NP_{147-155}$-specific $T_{CD8}+$ still dominate the Vβ8.1/2 depleted cells (Figure 3B). This cannot be attributed to contamination by $V\beta8.1/2^+$ cells, which are reduced to 0.03% or less of the population as determined cytofluorographically (data not shown). $T_{CD8}+$ specific for three of the four SDDs (all but $HA_{462-470}$) are detected in this population at somewhat higher levels relative to $NP_{147-155}$-specific $T_{CD8}+$. Removal of $V\beta6^+$ and $V\beta8.3^+$ $T_{CD8}+$ from the $V\beta8.1/2^-$ cells (which account for 20% of the cells) selectively removed about half of the $NP_{218-226}$ activity and had little effect on other specificities (Figure 3C). Removal of $V\beta10^+$ cells from the $V\beta8.1/2^-$ $V\beta6^-$ population diminished $HA_{462-470}$ reactivity to undetectable levels, with little effect on the other populations (Figure 3D).

We confirmed and extended this analysis by Vβ staining of determinant-specific $T_{CD8}+$ identified by ICS following stimulation with PR8-infected P815 cells (Figure 3E). Most $NP_{147-155}$-specific $T_{CD8}+$ expressed Vβ8.1/2, with a substantial number expressing either Vβ6, Vβ7, or Vβ14, and some responding clones expressing Vβ2, Vβ4, Vβ8.3, Vβ9, Vβ10, Vβ11/12 (mAbs specific for each were mixed), or Vβ13. Remarkably, $T_{CD8}+$ responding to each of the SDDs were similarly diverse in their Vβ expression.

Based on this analysis, we conclude that the response to each of the five determinants is multiclonal. Through

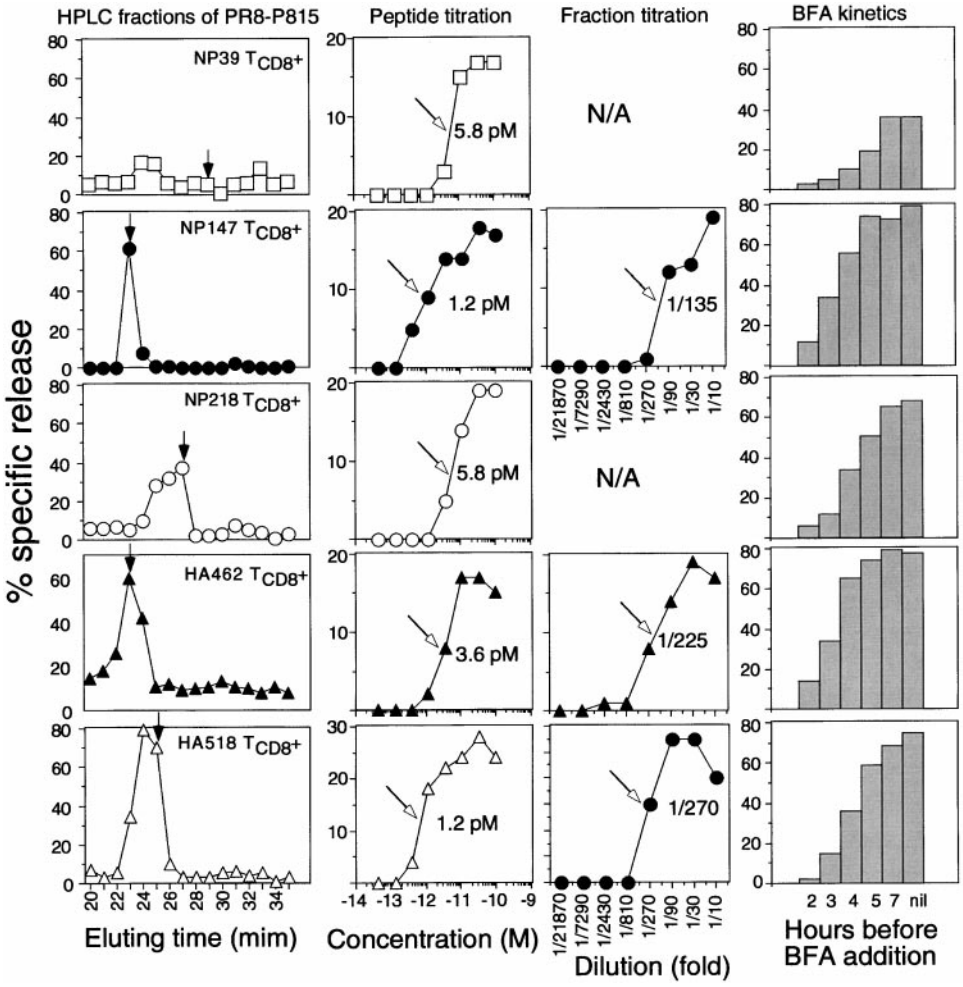

Figure 2. Quantitation of Peptide–K^d Complexes

Peptides were acid extracted from PR8-infected P815 cells and fractionated by HPLC, and the individual fractions were tested for their ability to sensitize cells for lysis by short-term $T_{CD8}+$ lines specific for the determinant indicated (left panel). Arrows point to the peak fractions in which corresponding synthetic peptides elute under identical conditions. $T_{CD8}+$ lines were also assayed for their dose response toward their cognate peptides (second panel from the left) and in the same assay against dilutions of fractions containing antigenic activity (third panel from the left; only the peak fraction is shown, but minor fractions were also titrated and used for the calculation in Table 1). Using data from the second and third panels in conjunction with the efficiencies of peptide recovery calculated by spiking peptides at 0.1 nM into uninfected cell lysates (ranging between 10%–12%), we calculated the number of peptides recovered per cell (Table 1). The first panel on the right shows the kinetics of presentation of the various determinants to short-term $T_{CD8}+$ lines that were serially diluted in the 96-well plates before the addition of ^{51}Cr-labeled P815 cells infected for 2 hr with PR8. BFA was added at a concentration of 10 μg/ml at this time ("2 h") and then for each of the next 5 hr. Supernatants were harvested 1 hr after the last BFA time point ("7 h"). On the assay day, $T_{CD8}+$ lines had similar dose response toward the respected peptide as shown in the middle panel (data not shown), with the exception of $T_{CD8}+$ specific for $HA_{518-526}$, which required ~3-fold more peptide to achieve similar levels of lysis as shown here. In other similar assays, $HA_{518-526}$ and $NP_{147-155}$ were presented with similarly rapid kinetics (data not shown).

repeated stimulation, it was possible to generate $NP_{147-155}$-specific $T_{CD8}+$ lines that express the following Vβ chains; 8.1/2, 8.3, 6, 10, 7, and 9 (data not shown). Importantly, the lines demonstrated nearly indistinguishable peptide dose responses (data not shown). This indicates that numerous widely different TCRs are capable of recognizing a single viral peptide with similarly high sensitivity.

$pT_{CD8}+$ and $mT_{CD8}+$ Responses: Effects of Increasing Complex Numbers on APCs

The cytofluorographic detection K^d molecules following infection of T2-K^d cells with rVVs expressing ER-targeted peptides corresponds to tens of thousands of cell surface complexes. Using this panel of rVVs expressing ER-targeted peptides, we could compare immunogenicity under conditions in which class I peptide complexes far exceed levels of naturally presented peptides (Figure 4A). Each rVV elicited similarly vigorous VV-specific response 7 days following immunization with 11%–13% of $T_{CD8}+$ activated by VV-infected cells as determined by ICS (ES-$NP_{147-155}$ elicited a lower response in the experiment shown, but this was not consistently observed between experiments). Despite the overexpression of the influenza peptides, the response to even the IDD, $NP_{147-155}$, which constituted 1.6% of $T_{CD8}+$ cells, constituted only 12% of the VV-specific response (Figure 4A).

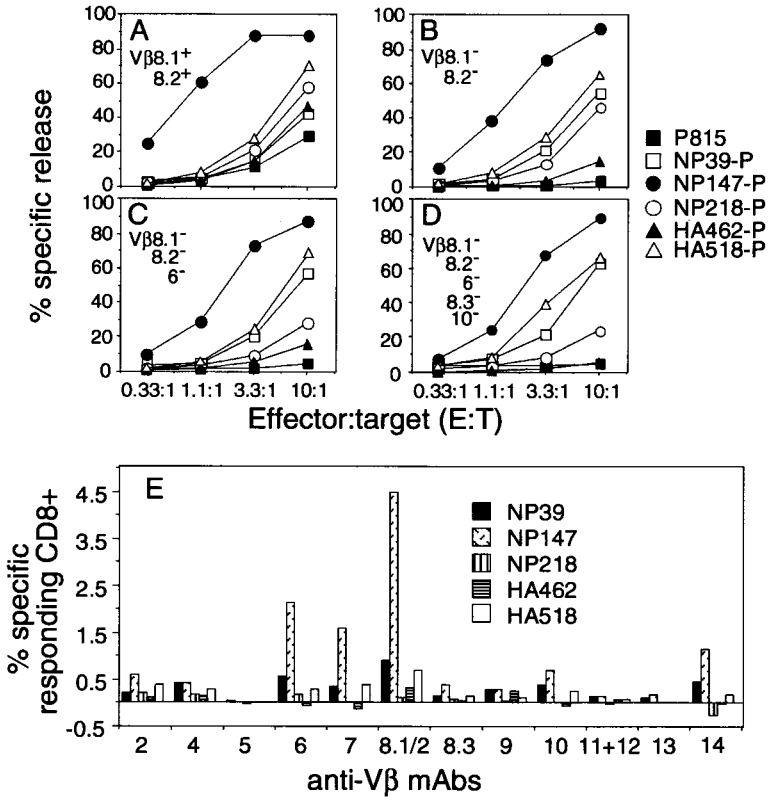

Figure 3. Diversity of $T_{CD8}+$ Responses

Splenocytes from PR8 primed animals were restimulated in vitro for 7 days with PR8-infected P815 cells and IL-2, and live cells recovered from Ficoll-Hypaque (>95% $CD8^+$ as determined by flow cytometry) were positively selected for expression of Vβ8.1/8.2 (A) or depleted sequentially of cells expressing Vβ8.1/8.2 (B) Vβ6 (C) and Vβ8.3/Vβ10 (D). Selected populations were tested for their abilities to lyse peptide-sensitized ^{51}Cr-labeled P815 cells at the indicated E:T ratio. In (E), similarly in vitro–stimulated splenocytes were activated by specific peptide-pulsed P815, and $CD8^+$ IFNγ-expressing cells identified by ICS were further stained with the indicated Vβ-specific mAb.

Since VV infection results in a ~2-fold increase in the number of $T_{CD8}+$, as compared with a ~1.3-fold increase following PR8 infection, to compare absolute numbers of responding cells, the percent of responding cells following VV infection must be multiplied by 1.5. Thus, in absolute numbers, the response to $NP_{147-155}$ induced by ES-$NP_{147-155}$ was ~2-fold less than that induced by PR8.

Using the response to $NP_{147-155}$ as a means of normalizing PR8 and VV-ES-X-induced responses, responses to ES-NP_{39-47} and $NP_{218-226}$ demonstrate, respectively, 10- and 3-fold enhancements, while the response to $HA_{518-526}$ is unchanged (Figure 4A). Most impressively, the response to $HA_{462-470}$ remains undetectable. When the same $T_{CD8}+$ were tested for lysis of peptide-sensitized target cells, there was a close correlation between lytic activity and the number of cytokine-secreting cells (Figure 4B).

The abilities of rVVs encoding ER-targeted peptides to prime for $mT_{CD8}+$ responses 6 weeks following immunization were similarly examined (Figure 5A). The number of $NP_{147-155}$-specific $mT_{CD8}+$ was ~50% of the value following PR8 infection. As with the $pT_{CD8}+$, NP_{39-47}-specific $mT_{CD8}+$ demonstrated the greatest increase relative to the PR8-induced response (6-fold), even surpassing the number of $NP_{147-155}$ $mT_{CD8}+$. In the same terms, the relative proportion of $HA_{462-470}$ $mT_{CD8}+$ increased 3-fold, while the $HA_{518-526}$ $mT_{CD8}+$ response is unaffected and $NP_{218-226}$ may even decrease, as it is now below the limit of detectability.

These findings indicate that overexpression of subdominant determinants can increase their immunogenicity but that the effect varies considerably with the determinant and between $pT_{CD8}+$ versus $mT_{CD8}+$.

Effect of Preexisting $mT_{CD8}+$ on the Immunodominance Hierarchy

The response of $mT_{CD8}+$ in vivo was examined by immunizing mice with VV-ES-X, challenging 40 days later with PR8, and measuring $T_{CD8}+$ activation ex vivo 7 days later using peptide-sensitized APCs (Figure 5B). This resulted in the expansion of each of the primed $mT_{CD8}+$ populations specific for the corresponding determinant. Priming with ES-$NP_{147-155}$ had only a marginal enhancing effect on the numbers of $NP_{147-155}$-responding $T_{CD8}+$ relative to unprimed mice. These cells were clearly different functionally from $pT_{CD8}+$, however, since they blocked the PR8-induced expansion of primary $T_{CD8}+$ to each of the other determinants. Such $mT_{CD8}+$-mediated suppression is a well-known feature of immunodomination (Bennink and Doherty, 1981; Jamieson and Ahmed, 1989). $mT_{CD8}+$ specific for the other determinants demonstrated an intermediate phenotype and their capacity to block responses to unprimed determinants varied considerably and in an interesting manner.

$mT_{CD8}+$ specific for $HA_{518-526}$, $HA_{462-470}$, or $NP_{218-226}$ blocked responses to unprimed SDDs and partially inhibited responses to $NP_{147-155}$. The degree of inhibition of $NP_{147-155}$ $T_{CD8}+$ was proportional to the number of $mT_{CD8}+$ responding to the priming determinant. Despite their abundance, NP_{39-47}-specific $mT_{CD8}+$ only blocked $NP_{147-155}$-specific response to a similar extent as $HA_{518-526}$-specific $T_{CD8}+$. Moreover, they did not block the primary response to $HA_{518-526}$, while conversely $HA_{518-526}$ $mT_{CD8}+$ blocked the response to NP_{39-47}. Indeed, $pT_{CD8}+$ responding to any of the determinants was able to profoundly block NP_{39-47}-specific $T_{CD8}+$. The lytic activity of $mT_{CD8}+$ on target cells sensitized with the different

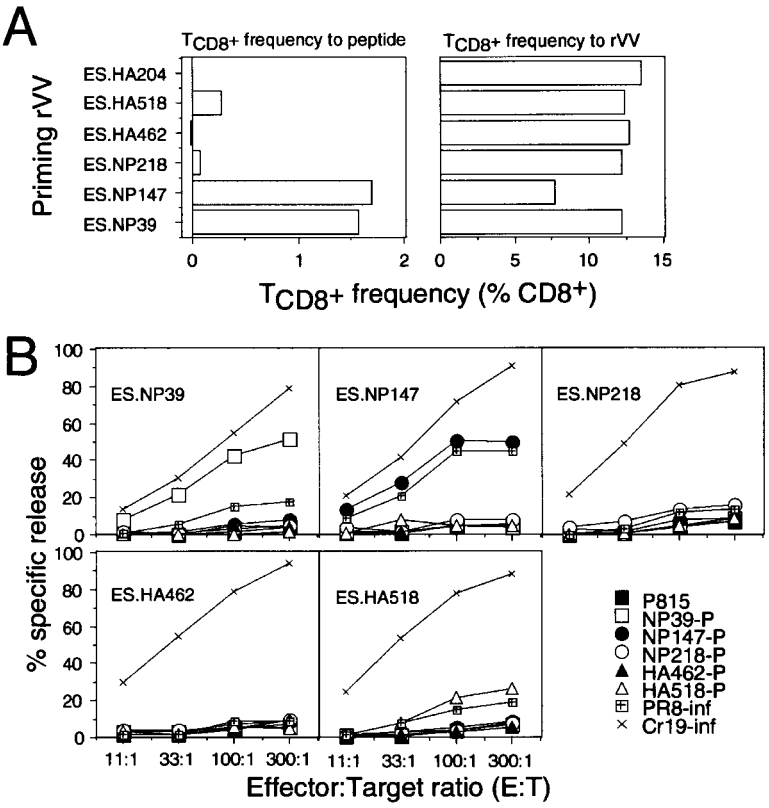

Figure 4. Quantitation of $pT_{CD8}+$ Responses to VV-ES-X Infection

(A) BALB/c mice were infected by rVVs expressing the ER-targeted peptide indicated, and 7 days later splenocytes were prepared and the number of cells responding to P815 cells sensitized with the peptide indicated was enumerated by ICS. Corresponding background values ranging from 0.75% to 1.45% obtained by stimulating with cells not exposed to peptide were subtracted from each data point. An rVV ("ES-HA_{204}") expressing a K^d binding peptide from a unrelated influenza virus was included as a control.

(B) A sample of the splenocytes used in (A) were assayed for lytic activity against either virus-infected or peptide-pulsed ^{51}Cr-labeled P815 cells at the E:T ratio indicated.

peptides closely reflected the numbers of cells determined by ICS (Figure 5C). These findings demonstrate that $mT_{CD8}+$ to different determinants can exhibit major qualitative differences in their ability to exert immunodomination.

Effect of Passively Transferred $T_{CD8}+$ on the Immunodominance Hierarchy

We next examined the relationship between the ID status of determinants and the abilities of their corresponding $mT_{CD8}+$ to persist in vivo and exert immunodomination. To level the playing field, we passively transferred equal numbers (2×10^6 cells) of $mT_{CD8}+$ that had been generated by multiple rounds of in vitro stimulation with individual peptides. $T_{CD8}+$ lines specific for each peptide demonstrated similar peptide dose responses (data not shown). ICS revealed that >95% of the cells present in these cultures were specific for their cognate determinants (data not shown). Two weeks after the final restimulation, cells were transferred into naive BALB/c mice, either separately or all five lines together.

Ten to twelve weeks later, the survival of functional transferred $T_{CD8}+$ in mice was measured by ICS (Figure 6A). This ranged from 0.02% to 0.1% of the total number of $T_{CD8}+$ recovered from spleens. The recovery of SDD-specific $T_{CD8}+$ was similar or better than the recovery of $NP_{147-155}$-specific $T_{CD8}+$ following individual transfer. Indeed, following multiple transfer, the recovery of $NP_{147-155}$-specific $T_{CD8}+$ was the least efficient of the five lines.

The function of transferred $T_{CD8}+$ in vivo was examined by measuring ICS (Figure 6B) and lysis (Figure 6C) ex vivo 7 days after challenging mice with PR8. As observed following vaccination with the various VV-ESX viruses, $NP_{147-155}$-specific $T_{CD8}+$ completely inhibited responses to all of the SDDs, and each of the SDD-specific $T_{CD8}+$ lines partially inhibited the primary response to $NP_{147-155}$. Accounting for $pT_{CD8}+$ responses, NP_{39-47}-specific $T_{CD8}+$ again demonstrated both the greatest specific expansion and the least effect on $NP_{147-155}$-specific $pT_{CD8}+$ responses. This is similar to the properties of $mT_{CD8}+$ induced by VV-$ESNP_{39-47}$. $HA_{518-526}$-specific $T_{CD8}+$ demonstrated the smallest specific expansion but still managed to exert the greatest suppressive effect on $NP_{147-155}$-specific responses. Curiously, $HA_{462-470}$-specific $T_{CD8}+$ exerted greater relative inhibition on $HA_{518-526}$-specific $T_{CD8}+$ than on $T_{CD8}+$ specific for the other determinants. This selective inhibition was observed in several additional experiments, but not completely consistently.

The lytic activity of $T_{CD8}+$ on target cells sensitized with the different peptides closely reflected the numbers of cells determined by ICS, with the exception of $NP_{218-226}$-specific $T_{CD8}+$, which demonstrated lower activity than predicted.

We further explored asymmetries in immunodomination by transferring a greater number of $mT_{CD8}+$ (7×10^6 cells) prior to PR8 infection and ex vivo ICS on day 7 post infection (panel D). As expected, $NP_{147-155}$-specific $T_{CD8}+$ still completely suppressed responses to the subdominant determinants. Increasing the number of $HA_{518-526}$-specific $T_{CD8}+$ resulted in complete suppression of NP_{39-47}-specific $T_{CD8}+$ and reduced yet did not completely suppress $NP_{147-155}$-specific $T_{CD8}+$.

These experiments indicate that $T_{CD8}+$ specific for IDDs and SDDs can have a similar capacity to persist

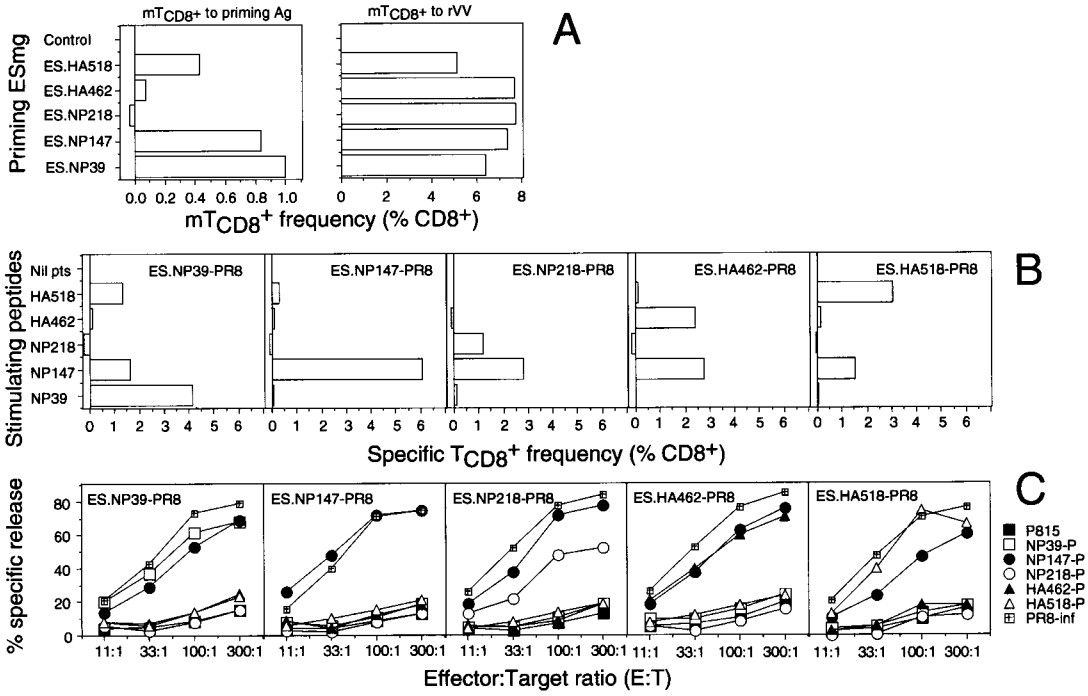

Figure 5. Quantitation of $mT_{CD8}+$ Responses to VV-ES-X Infection

(A) BALB/c mice infected for 6 weeks previously with the indicated rVV and splenic $mT_{CD8}+$ were quantitated by ICS. Corresponding background values ranging from 0.27% to 0.45% obtained by stimulating with cells not exposed to peptide were subtracted from each data point.

(B) BALB/c mice infected with the indicated rVV 35 days previously were infected with PR8, and 7 days later specific splenic $T_{CD8}+$ were quantitated by ICS. Corresponding background values ranging from 0.39% to 0.52% obtained by stimulating with cells not exposed to peptide were subtracted from each data point.

(C) A sample of the splenocytes used in (B) were assayed for lytic activity against either virus-infected or peptide-pulsed ^{51}Cr-labeled P815 cells at the E:T ratio indicated.

in a functional state in vivo and also reinforce the conclusion that the capacity for immunodomination varies considerably with the specificity of $T_{CD8}+$, suggesting that domination is a cause rather than an effect of the immunodominance hierarchy.

Discussion

Hierarchy Rules

Our analysis of immunodominance in the K^d-restricted response to PR8 in BALB/c mice demonstrates, first, that determinants are recognized in a hierarchy that is highly stable between individual animals and, second, that the hierarchy is based on multiple factors. We adopt the nomenclature used to describe animal hierarchies and refer to the position of determinants using Greek characters α, β, etc., based on the prevalence of responding $T_{CD8}+$ cells.

ϵ-*determinant* $HA_{462-470}$ makes a bold statement, as it is the determinant most abundantly expressed by PR8-infected cells yet is the least immunogenic following PR8 infection. In conjunction with the failure of ES-$HA_{462-470}$ to enhance immunogenicity despite producing even greater numbers of complexes on infected APCs, this emphatically points to deficiencies in responding $T_{CD8}+$ as the cause of low immunogenicity. This is not due, however, to an absolute inability of $T_{CD8}+$ to recognize $HA_{462-470}$–K^d complexes, since highly sensitive $T_{CD8}+$ clearly exist and can utilize at least five different Vβ gene products (Vβ2, 4, 8.1/8.2, 9, 11, or 12). Further, the $T_{CD8}+$ that are elicited are not silenced or deleted more rapidly than $T_{CD8}+$ to other SDDs or the IDD, as indicted by transfer experiments and the persistence of $mT_{CD8}+$. This points to either a very low absolute number of precursors and/or some mechanism that limits expansion of the precursors. Low numbers of precursors could result from an intrinsic difficulty in producing high-affinity TCRs given the genes available for the task or difficulties in producing such TCRs that do not cross-react with self-antigens with too high an affinity.

δ-*determinant* $NP_{218-226}$ has the lowest affinity for K^d, stemming at least in part from possessing the highest off rate. The low affinity correlates with its poor rescue of K^d molecules when targeted to the ER of T2-K^d cells (correcting a lamentable error in our original publication describing VV-ES-$NP_{218-226}$ [Deng et al., 1997]). Assuming that TAP-transported peptides exhibit a similarly low affinity, this is probably the major factor in the relatively low number of complexes produced by infected APCs. $pT_{CD8}+$ responses were not enhanced by immunization with VV-ES-$NP_{218-226}$ (although $mT_{CD8}+$ were effectively elicited). Although ES-$NP_{218-226}$ only marginally increases K^d expression in T2-K^d cells, it is presented much more rapidly than full-length NP expressed either by VV or PR8 (Deng et al., 1997), which indicates that ER targeting does enhance the generation of complexes. This suggests that the limitations in responding $T_{CD8}+$ are a major factor in the poor primary response to $NP_{218-226}$. It is

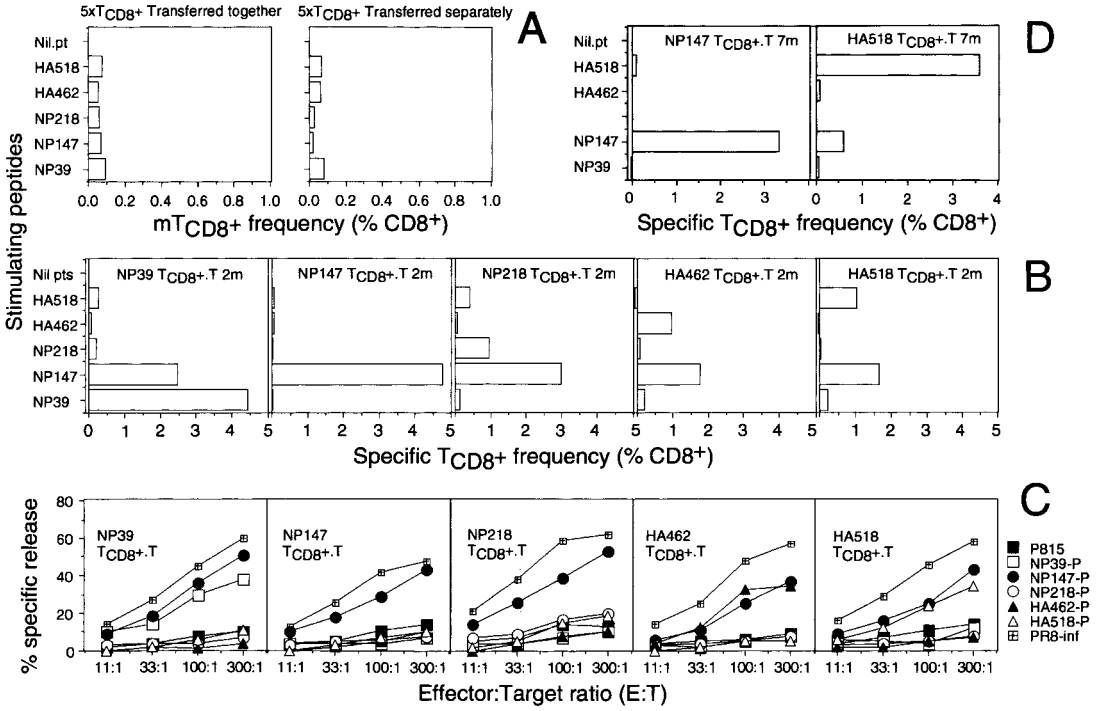

Figure 6. Functional Properties of Passively Transferred Short-Term $T_{CD8}+$ Lines

(A) Naive BALB/c mice received by i.v. injection either 2×10^6 cells of a single $T_{CD8}+$ line specific for the peptide indicated (right panel) or a mixture of all 5 $T_{CD8}+$ (2×10^6 of each line). 10–12 weeks later, the number of responding splenic $T_{CD8}+$ was determined by ICS. Corresponding background values ranging from 0.23% to 0.33% obtained by stimulating with cells not exposed to peptide were subtracted from each data point.

(B) BALB/c mice receiving $T_{CD8}+$ specific for the indicated determinant 10–12 weeks previously were infected with PR8, and 7 days later the number of responding splenic $T_{CD8}+$ was determined by ICS. Corresponding background values ranging from 0.56% to 1.07% obtained by stimulating with cells not exposed to peptide were subtracted from each data point.

(C) A sample of the splenocytes used in (B) were assayed for lytic activity against either virus infected or peptide pulsed ^{51}Cr-labeled P815 cells at the E:T ratio indicated.

(D) As in (B), but mice received 7×10^6 of $NP_{147\text{-}155}$- or $HA_{518\text{-}526}$-specific $T_{CD8}+$.

also possible, however, that the primary factor is the relatively high off rate of the peptide.

Another complicating factor in the poor immunogenicity of $NP_{218\text{-}226}$ is the presence of a Cys residue: to reduce the complexity and increase the reproducibility of the assays, we included reducing agents whenever using Cys containing peptides. We previously demonstrated that $T_{CD8}+$ specific for cysteinylated $NP_{218\text{-}226}$ are elicited by PR8 infection (Chen et al., 1999), and the contribution of these $T_{CD8}+$ to anti-PR8 responses remains to be established, as does their possible immunodominating effects on $T_{CD8}+$ specific for unmodified $NP_{218\text{-}226}$.

γ-determinant $NP_{39\text{-}47}$ beautifully illustrates the contribution that antigen processing can play in immunodominance: it has the highest affinity for K^d and is perfectly capable of eliciting a vigorous $T_{CD8}+$ response when expressed at sufficiently high levels. Indeed, in the context of the ER-targeted determinants, $ESNP_{39\text{-}47}$ moves to the top of the immunodominance hierarchy. Its status as the γ-determinant in the context of PR8 infection clearly reflects a deficiency in antigen processing. Given its very low levels of expression on infected cells, the magnitude and diversity of the response induced by PR8 infection indicates that $T_{CD8}+$ eagerly await its appearance.

β-determinant $HA_{518\text{-}526}$ is clearly dominated by $NP_{147\text{-}155}$. This cannot be attributed to inefficient antigen processing by virus-infected cells (assuming that APCs in vivo process antigens similarly to P815 cells) nor to gross deficiencies in the $T_{CD8}+$ repertoire elicited by the determinant, which is highly diverse. In contrast to $NP_{39\text{-}47}$, overexpression using VV-$ESHA_{518\text{-}526}$ did not improve the response, suggesting an intrinsic limit in the capacity of $HA_{518\text{-}526}$-specific $T_{CD8}+$ to expand. This may be a less extreme example of the mechanism that limits responses to $HA_{462\text{-}470}$.

α-determinant $NP_{147\text{-}155}$ also makes a strong statement, as it demonstrates that an α determinant need not be the most abundant nor exhibit the slowest off rate. Given its relatively low affinity for K^d, its processing and delivery to the ER may be somewhat better than the average class I ligand, since a reasonable number of complexes are created by PR8-infected cells (800 per cell). The efficiency of producing the determinant does not appear to limit its immunogenicity in the context of PR8, since VV-ES-$NPNP_{147\text{-}155}$ elicits less $NP_{147\text{-}155}$-specific $T_{CD8}+$ than does PR8 (on the other hand, as discussed below, there are limitations in comparing between the immunogenicities of different viral vectors). The secret to $NP_{147\text{-}155}$'s α status clearly lies in the capacity of $T_{CD8}+$ to respond to

the peptide. This is not a "jackpot" phenomenon, in which a single abundant $T_{CD8}+$ clone or highly related family of clones recognize the determinant (Bousso et al., 1999). Clearly, $T_{CD8}+$ of high sensitivity bearing different Vβ chains are present in the responding population. Others have observed that responses to α-determinants can be highly diverse, and it has been shown in both human and mouse systems that upon elimination of a dominant $T_{CD8}+$ clonotype, others can take their place (this forms a parallel hierarchy in the $T_{CD8}+$ response) (Burrows et al., 1995; Jones et al., 1997; Wallace et al., 1999). Responses to α-determinants represent the other side of the looking glass relative to the SDD-specific $T_{CD8}+$, requiring the opposite sorts of explanations, i.e., high number of $NP_{147\text{-}155}$-specific precursors, increased proliferative capacity, and enhanced ability to exert immunodomination.

Conclusions

These findings illustrate how numerous factors can combine to establish an immunodominance hierarchy. Deeper insight into immunodominance hinges on better understanding the properties of responding $T_{CD8}+$, with an emphasis on discerning the number of precursors in naive animals, the relationship between $T_{CD8}+$ sensitivity and TCR avidity for cognate class I–peptide complexes, and the differences among $T_{CD8}+$ clones in their proliferative capacities, antiviral activities, and suppressive effects on other responding $T_{CD8}+$ (discussed further below).

Mechanistic Issues

Cytokine/Lysis Disconnect

In several situations, we noted discrepancies between the lytic activity of $T_{CD8}+$ specific for a given determinant and the number of activated cells identified by ICS. This is consistent with the idea that activated $T_{CD8}+$ can vary in their lytic potential and that this occurs in a determinant-specific manner. This raises a number of questions for future investigation, including the cause for the decreased lytic activity, how this is regulated on a determinant level, and the advantages conferred to $T_{CD8}+$ by independent regulation of lysis and cytokine release.

Selective Immunodomination

Immunodomination, which is a feature of both primary and memory $T_{CD8}+$ responses, is poorly understood at the mechanistic level. In the present study, we provide several clues to the puzzle of immunodomination.

First, despite a vigorous response, $mT_{CD8}+$ specific for $NP_{39\text{-}47}$ (particularly those elicited by immunization with VV-ES-$NP_{39\text{-}47}$) exhibited low immunodomination of the other determinants. By contrast, lower numbers of $mT_{CD8}+$ to any of the other determinants completely suppressed $NP_{39\text{-}47}$-specific responses. It seems likely that this is related to the inefficient generation of $NP_{39\text{-}47}$ and is consistent with poor presentation by afferent and efferent APCs resulting in delayed clearance of virus and decreased interference with presentation to $pT_{CD8}+$, either of which could interfere with immunodomination. The former has been suggested to account for immunodomination in responses of H-2^d mice to lymphocytic choriomeningitis virus (LCMV) (Weidt et al., 1998).

Second, $NP_{147\text{-}155}$-specific $mT_{CD8}+$ were both most effective at immunodomination and most capable of resisting the domination by other $mT_{CD8}+$. This phenomenon probably lies at the heart of the immunodominance of $NP_{147\text{-}155}$ in primary responses. The ability of $NP_{147\text{-}155}$ to withstand the suppressive effects of preexisting $T_{CD8}+$ to other determinants strongly suggests that the immunodominance of $NP_{147\text{-}155}$ is not simply due to a greater number of responding cells that stochastically result in more rapid response. One explanation is that $NP_{147\text{-}155}$–K^d complexes have an inherent ability to optimally trigger cognate TCRs—inherent in deference to the observation that the response to $NP_{147\text{-}155}$ is highly diverse. Alternatively, the presentation of $NP_{147\text{-}155}$ in vivo may be far more efficient than its presentation in vitro, e.g., $NP_{147\text{-}155}$ may be selectively presented at high efficiency by cross-priming.

Third, transferred $HA_{462\text{-}470}$ $T_{CD8}+$ more effectively blocked responses to $HA_{518\text{-}526}$ than to NP determinants following PR8 infection. Admittedly, the sample size is minimal (and the effect was not observed in every experiment), but this finding suggests that under some circumstances immunodomination can be protein specific. Since it is difficult to imagine how this could happen if afferent APCs are presenting endogenously synthesized antigens, this finding points to a cross-priming mechanism in which APCs present HA but not NP. The capacity of HA as a sialic acid receptor lends some plausibility to this scenario, since it may enable HA to be selectively acquired by cross-priming APCs.

Implications for Vaccines

Our findings indicate that following a single i.p. immunization with influenza virus there is less bias among K^d-restricted $T_{CD8}+$ toward the IDD among $mT_{CD8}+$ than $pT_{CD8}+$. Similarly, studying responses to a Sendai virus determinant that is presented by both K^b and D^b, Cole et al. (1997) found that $pT_{CD8}+$ populations are more skewed toward K^b-restricted $T_{CD8}+$ than $mT_{CD8}+$ populations. By contrast, Busch et al. (1998) found that for K^d-restricted *Listeria monocytogenes*, the ratios of $pT_{CD8}+$ and $mT_{CD8}+$ among three determinants were similar. The extent to which this discrepancy relates to differences in the antigens versus the responding $T_{CD8}+$ remains to be determined. Moreover, a critical issue for vaccines is the ability of $mT_{CD8}+$ induced by prior immunization to function following infection with the natural pathogen. Flynn et al. (1998) have examined this issue in their studies of H-2^b-restricted responses to influenza virus. In this system, the IDD is $NP_{366\text{-}374}$ (D^b restricted), and the major SDD is $NS_{114\text{-}121}$ (K^b restricted). Prior i.p. immunization resulted in up to a 10-fold enhanced response to the IDD in cells recovered from the lungs, while the response to the SDD was only slightly enhanced or even reduced. Importantly, the frequency of $mT_{CD8}+$ in the spleen was not an accurate predictor of local $T_{CD8}+$ responses; 4- to 6-fold difference in splenic $mT_{CD8}+$ parlayed in up to a 100-fold difference in $T_{CD8}+$ recovered from actively infected lungs.

These latter findings do not necessarily reflect poorly on the vaccine potential of SDDs. The primary reasons for including SDDs in vaccines are to increase the number of responding $T_{CD8}+$ and to prevent the emergence

of viral escape mutants that fail to express IDDs. The enhanced response to the IDD upon local challenge may be a manifestation of immunodomination in activating $mT_{CD8}+$. Under most circumstances, there is probably no disadvantage in having the response dominated by IDD-specific cells (and this may actually be advantageous in minimizing the chance for autoimmunity [Deng et al., 1997]). Even a minimal response to SDDs may mitigate against the emergence of escape mutants, and in the event of infection with an escape mutant, the presence of SDD-specific $mT_{CD8}+$ will be highly beneficial.

We previously reported that primary responses to $NP_{147-155}$ following infection of mice with a rVV encoding NP are difficult to detect (Restifo et al., 1995). This contrasts markedly with the primary response to PR8. Moreover, we show here that even the massive overexpression of $NP_{147-155}$–K^d complexes resulting from targeting the peptide to the ER fails to match the immune response to PR8. This cannot be attributed to the poor overall immunogenicity of VV, which, in fact, elicits a much more vigorous $T_{CD8}+$ response than PR8. The most likely explanation for the relatively poor immunogenicity of $NP_{147-155}$ in the context of VV is competition for $T_{CD8}+$ responses between VV determinants and $NP_{147-155}$. These findings point to a limitation in the usefulness of recombinant viral vectors that elicit massive $T_{CD8}+$ response to their own antigens.

A further detriment to using VV or similar vectors is that massive responses to VV gene products leave "scars" in the $T_{CD8}+$ memory: they result in the generation of large numbers of long-lived $mT_{CD8}+$ (Sourdive et al., 1998). To the extent that the antigen may reappear, this is no doubt a wise evolutionary strategy, and in the particular case of VV, also probably a wise public health strategy given the recent fears regarding the introduction of variola virus into human populations. On the other hand, given a vector that is not itself closely related immunologically to a potential pathogen (such as avipox vectors), the $mT_{CD8}+$ are at best useless and at worst, a potential source of autoimmunity. The advantage of vaccines based on the immunization of modified forms of the target pathogen is that they focus immune responses on relevant antigens.

Experimental Procedures

Monoclonal Antibodies and Other Reagents

All mAbs were FITC labeled except PE-labeled anti-IFNγ, and all were purchased from PharMingen. The anti-K^d mAb SF1-1.1 was used for all peptide binding and decay assays. All mAbs were used at 1:100 dilution in PBS supplemented with 0.1% BSA. Anti-CD4 mAb culture supernatant from hybridoma GK1.5 and unlabeled anti-B220 antibody (PharMingen) were used to coat M450 Dynal beads (Dynal, Holland) for depleting $CD4^+$ cells and $B220^+$ activated LAK and NK cells.

$T_{CD8}+$ Purification

PR8-primed splenocytes were stimulated by PR8-infected P815 cells for 7 days. The stimulated T cells (more than 95% are $CD8^+$) were positively selected for Vβ8.1/8.2 TCR expression using FITC-labeled Vβ mAb, and the resulting Vβ8.1/8.2 negative population was then further depleted of Vβ6, or Vβ6, Vβ8.3, and Vβ10 expressing cells. anti-FITC antibody-conjugated microbeads and positive selection columns were used (Miltenyi, Germany) for all the enrichment and depletion steps. The purity of selected populations was confirmed by flow cytometry using the entire panel of TCR β chain–specific mAbs for characterization.

Viruses

rVVs and influenza virus used in this study were produced, propagated, and used to infect cells as described previously (Deng et al., 1997).

$T_{CD8}+$ Activation

For in vivo priming, 8- to 10-week-old female BALB/c mice were either injected i.p. with ~600 hemagglutinating units of PR8 or injected i.v. with 10^7 PFU of VV. Splenocytes or cells collected from the peritoneal cavity by PBS-lavage were directly assessed after 3 to 11 days for ICS/lytic activity. Alternatively, at least 3 weeks after priming, splenocytes were cultured for 7 days in vitro with PR8-infected or peptide-pulsed P815 cells and then assessed for ICS/lytic activity. $T_{CD8}+$ were cultured in RP-10 supplemented with 10 U/ml recombinant human interleukin 2 (IL-2). In brief, 3×10^7 splenocytes were stimulated with 6×10^6 to irradiated (220 Gy) P815 cells infected with PR8 or pulsed with peptides. Viable cultured $T_{CD8}+$ were selected by centrifugation through Ficoll-Hypaque and, in some cases, enriched by depleting $B220^+$ and $CD4^+$ cells using M-450 Dynal beads coated with appropriate antibodies.

Generation of $T_{CD8}+$ Lines

After initial 7 day culture, splenocytes were restimulated weekly in 6-well plates (Becton Dickinson) in the presence of 10 U/ml rIL-2 at 5×10^6 responders per well and 1/10~1/20 the number of irradiated P815 stimulator cells. The purity of each $T_{CD8}+$ population used for transfer experiments was determined by ICS upon activation. After reaching >95% purity, $T_{CD8}+$ were restimulated once and then "rested" for 2 weeks before being transferred into mice. Such resting $T_{CD8}+$ were small and proliferated very slowly.

Peptides, Binding Assays, and Flow Cytometry

Peptide binding assays were modified slightly from a previous description (Chen et al., 1999). To examine peptide dissociation, T2-K^d cells were incubated at 26°C for 12 hr and then pulsed with 10 μM peptides for 60 min at 26°C. BFA (5 μg/ml) was added to cells during peptide pulsing to prevent the appearance of newly synthesized class I molecules. Cells were then washed, aliquoted in BFA-containing medium, and transferred to 37°C. Starting at this time point, cells were harvested onto ice, and, following the final time point, the amount of cell surface K^d was determined by mAb staining and analyzed by flow cytometry.

$T_{CD8}+$ Functional Assays

For ICS, following lysis of erythrocytes, splenocytes were resuspended at 10^7/ml in I-10, and 200 μl were added per well to round-bottom 96-well plates. Peptides were added to a concentration of 0.5 μM. In the case of Cys containing peptides, media were supplemented with 200 μM of TCEP to prevent sulfhydryl modification. For established $T_{CD8}+$ lines, peptide-pulsed P815 cells were used. After 2 hr incubation at 37°C, BFA was added and cells were incubated for a further 3 hr to accumulate IFNγ in the ER of activated cells. Cells were then incubated with a fluorescein-conjugated anti-CD8α mAb on ice for 30 min, washed, fixed with 1% paraformaldehyde in PBS at 20°C for 20 min, and then incubated with phycoerythrin-conjugated anti-IFNγ mAb diluted in PBS containing 0.1% saponin. Stained cells were analyzed by flow cytometry gating on the $CD8^+$ cells. ^{51}Cr-release assays were performed as described (Chen et al., 1999). Data are expressed as % specific lysis = ($T_{CD8}+$ induced release - spontaneous release)/(release by detergent - spontaneous release) × 100.

HPLC Fractionation and Quantitation of Viral Peptides

This method has been described by Antón et al. (1997).

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