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CD8 T-Cell Ability to Exert Immunodomination Correlates with T-Cell Receptor: Epitope Association Rate

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ABSTRACT

When presented alone, H7^a and HY antigens elicit CD8 T-cell responses of similar amplitude, but H7^a totally abrogates the response to HY when both antigens are presented on the same antigen-presenting cell. We found that H7^a- and HY-specific T-cell precursors had similar frequencies in nonimmune mice and expressed similar levels of CD5. The H7^a-specific CD8 T-cell repertoire harvested at the time of primary response showed highly restricted T-cell receptor (TCR) diversity. Furthermore, T cells specific for H7^a and HY expressed equivalent levels of CD8 and TCR and displayed similar tetramer decay rates. The key difference was that anti-H7^a T cells exhibited a much more rapid TCR:epitope on-rate than anti-HY T cells. Coupled with evidence that primed CD8 T cells limit the duration of antigen presentation by killing or inactivating antigen-presenting cells, our data support a novel and simple model for immunodomination: the main feature of T cells that exert immunodomination is that, compared with other T cells, they are functionally primed after a shorter duration of antigen presentation.

KEY WORDS

Immunodominant determinants • CD8 T cells • Antigen presentation

INTRODUCTION

It is relatively easy to elicit CD8 T-cell responses against an epitope presented alone on professional antigen-presenting cells (APCs), provided that antigen-reactive T cells are present in the T-cell repertoire. However, under most circumstances, such as with infection and transplantation, immune responses are triggered by APCs that present a multitude of nonself epitopes. In this case, CD8 T cells respond to only a few immunodominant epitopes and neglect other potentially immunogenic peptides. Restriction of CD8 T-cell responses to a few selected epitopes has been termed *immunodominance*, a central and robust feature of immune responses [1-8]. At face value, restricting cytotoxic T lymphocyte (CTL) responses to immunodominant epitopes can be viewed as a low-risk, high-efficiency strategy for the immune system: limiting the diversity of the immune response minimizes the potential for autoimmune recognition, and focusing on the best epitopes confers good chances to rapidly eliminate pathogens [9,10].

The immunodominance hierarchy is largely dictated by the immunodomination process whereby dominant epitopes abrogate responses to nondominant epitopes [11]. Immunodomination results from cross-competition between T cells specific for different epitopes [12]. Studies based on adoptive transfer of graded numbers of CD8 T cells specific for various influenza peptides showed that the capacity to exert (and to resist) immunodomination varies considerably with the specificity of CD8 T cells and is a cause rather than an effect of the immunodominance hierarchy [11]. In most models, immunodomination results from competition for APC resources among responding CD8 T cells, because it disappears when competing epitopes are presented on different APCs

or when APCs are present in large excess [3,13-17]. Accordingly, immunodomination is mitigated under conditions in which antigen presentation is not limiting, such as lymphocytic choriomeningitis virus (LCMV) infection [12,18].

The mechanisms of competition for APC resources that lead to immunodomination remain ill defined. Many models suggest that the crux of immunodomination is APC killing [19,20]. However, at least under certain conditions, immunodomination can be driven by means other than APC destruction [12]. One attractive possibility would be that early interactions with CTLs specific for immunodominant epitopes lead to functional exhaustion or inactivation of APCs. Indeed, after activation, dendritic cells produce interleukin (IL)–12 only transiently and rapidly become functionally exhausted [21]. It is interesting to note that secretion of interferon (IFN)-γ, which is rapidly produced at the CTL/APC interface, is instrumental in suppressing responses to nondominant epitopes [17,22]. Whether IFN-γ entails APC demise or inactivation remains to be determined. Thus, although our mechanistic understanding remains incomplete, the dominant paradigm holds that CTLs exert immunodomination by physically or functionally impairing the ability of APCs to present antigen, thereby preventing priming against nondominant epitopes [3,19,20]. Restricting the duration of antigen presentation through APC physical or functional deletion provides an efficient feedback mechanism with pervasive consequences on the size, diversity, and duration of CD8 T-cell responses [20]. Of note, the abilities to exert and to resist immunodomination are directly related [11,13,23]. Thus, whereas cell-surface expression of the $H7^a$ minor histocompatibility antigen (MiHA) prevents priming against many MiHAs present on C57BL/6 cells, $H7^a$-specific CTLs consistently resist immunodomination by CTLs specific for a myriad of other MiHAs [13,16,23]. In contrast, the HY MiHA elicits a CTL response only when presented alone. Although expression of other MiHAs does not modify expression of HY at the APC surface, it totally abrogates the HY-specific CTL response [16,24]. Two fundamental and related inferences can be drawn from these data. First, compared with other CTLs, those that exert immunodomination lead to more rapid APC deletion or inactivation. Second, that swift termination of antigen presentation affects only CTLs specific for nondominant epitopes means that these CTLs require a longer duration of antigen presentation than CTLs specific for dominant epitopes.

To elucidate the mechanisms of immunodomination, we performed a comprehensive set of studies on CD8 T cells specific for 2 models of antigens presented by $H2D^b$: $H7^a$ (also known as $B6^{dom1}$) and HY. We thought that selecting these antigens would maximize the sensitivity of our studies because, as mentioned previously, $H7^a$ and HY lie at opposite ends of the immunodomination scale (that is, in their ability to exert and resist immunodomination). Thus, when presented alone, these antigens elicit CTL responses of similar amplitude, but $H7^a$ totally abrogates the response to HY when both antigens are presented on the same APC [13,16]. This cannot be explained by differences in epitope stability, because the half-lives of $H7^a/D^b$ and HY/D^b complexes are similar (8 hours) [23]. We focused herein on the following parameters: T-cell frequency in the preimmune repertoire, T-cell receptor (TCR) diversity of antigen-reactive T cells, and T-cell interactions with self as well as with their cognate antigen. We report that the salient difference between CTLs specific for $H7^a$ and HY is that the former display much more rapid tetramer binding than the latter. Our data allow for the development of a model in which the result of cross-competition between CTLs specific for different epitopes depends on the TCR:epitope on-rate of competitors. This paradigm makes distinct and testable predictions and integrates numerous reports concerning immunodomination.

MATERIALS AND METHODS

Mice

C57BL/10Snj (B10), B10.C-*H7*^{b}(47N)/SN ($B10.H7^b$), and BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were bred in the Guy-Bernier Research Center and maintained in specific pathogen–free conditions according to the standards of the Canadian Council on Animal Care.

Peptides

$H7^a$ (KAPDNRETL) and $HY_{(Uty)}$ (WMHHNMDLI) peptides were synthesized by the Sheldon Biotechnology Center (Montreal, QC, Canada). Purity, as determined by reverse-phase high-performance liquid chromatography and mass spectrometric analysis, was greater than 97%.

Antibodies, Major Histocompatibility Complex Class I Tetramers, and Flow Cytometry

Cell suspensions were stained with the following antibodies from BD Biosciences Pharmingen (San Diego, CA): anti-CD8 (53-6.7), anti-TCRαβ (H57-597), anti-Vα2 (B20.1), anti-Vα3.2 (RR3-16), anti-Vα8.3 (B21.14), anti-$V\alpha11.1/11.2^{b,d}$ (RR8-1), anti-Vβ2 (B20.6), anti-Vβ3 (KJ25), anti-Vβ4 (KT4), anti-Vβ5.1/5.2 (MR9-4), anti-Vβ6 (RR4-7), anti-Vβ7 (TCR310), anti-Vβ8.1/8.2 (MR5-2), anti-Vβ9 (MR10-2), anti-$V\beta10^b$ (B21.5), anti-Vβ11 (RR3-15), anti-Vβ12 (MR11-1), anti-Vβ13 (MR12-3), anti-Vβ14 (14-2), anti-$V\beta17^a$ (KJ23), anti-$H2D^b$ (28-14-8), and anti-CD5 (53-7.3). $H7^a/D^b$ and $H7^b/D^b$ tetramers were obtained from the Canadian

Network for Vaccines and Immunotherapies Tetramer Core Facility (Montreal, QC, Canada). $HY_{(Uty)}/D^b$ and LCMV gp33-41/D^b tetramers were obtained from Beckman Coulter Immunomics (San Diego, CA). All acquisitions were made on a FACSCalibur flow cytometer by using CellQuest software (BD Biosciences, Mountain View, CA).

Tetramer Binding Assays

Tetramer association assays (TCR-peptide:major histocompatibility complex [pepMHC] on-rate) were performed as described previously [25,26]. Anti-$H7^a$ and -HY CTLs were obtained after priming of B10.$H7^b$ female mice by intraperitoneal injection of 2×10^7 splenocytes from B10 and B10.$H7^b$ male donors, respectively. On day 14 after priming, spleen cells were stained by anti-CD8 antibody and graded concentrations of tetramers ranging from 3.5 to 270 nmol/L. After incubation, cells were washed extensively, fixed in 1% paraformaldehyde, stained on ice for 45 minutes, and analyzed by flow cytometry. Tetramer decay assays (TCR-pepMHC off-rate) were performed as described [26,27]. Briefly, in vivo–primed spleen cells were stained with saturating concentrations of anti-CD8 antibody and $H7^a/D^b$ or $HY_{(Uty)}/D^b$ tetramers for 45 minutes. Cells were washed, cultured for various periods of time at 25°C in the presence of saturating amounts of purified anti-D^b antibody, fixed in 1% paraformaldehyde, and analyzed by flow cytometry. The tetramer-specific total fluorescence was determined according to Savage et al., [27] normalized to the percentage of the total fluorescence at the initial time point, and plotted on a logarithmic scale.

Single-Cell Reverse Transcriptase-Polymerase Chain Reaction and TCR Sequencing

B10.$H7^b$ mice were primed by intraperitoneal injection of 2×10^7 B10 spleen cells. On day 14 after priming, single immune ($CD8^+V\beta8^+V\alpha8^+H7^a/D^b$ tetramer$^+$) T cells were sorted directly in a 96-well polymerase chain reaction (PCR) plate containing 20 μL of complementary DNA (cDNA) reaction mix by using a FACSVantage SE with Diva Option (BD Biosciences, San Jose, CA). The cDNA reaction mix contained 1× cDNA buffer, 1.0 μL of Sensiscript Reverse Transcriptase, 0.5 mmol/L each deoxynucleotide triphosphate, 1 μmol/L oligo-dT primers, and 10 U ribonuclease inhibitor (Qiagen Inc., Mississauga, ONT, Canada). After sorting, plates were frozen, thawed, and incubated at 37°C for 60 minutes to perform the reverse-transcriptase reaction, followed by 5 minutes at 93°C to stop the reverse-transcriptase activity. The Vβ8 or Vα8 transcripts were amplified from 2.5 μL of cDNA by nested PCR in a 25-μL amplification reaction with *Taq* polymerase (Invitrogen, Burlington, ON, Canada), by using oligonucleotides designed by Baker et al. [28] The first-round reaction was performed with the external primers: Vβ8-ext (TGGGAACAAAACACATGGAGGC)/Cβ-ext (CTATAATTGCTCTCTTGTAGG) and Vα8-ext (CTGTGATGCTGAACTGCACC)/Cα3′-ext (TCAACTGGACCACAGCCTCAG). The second round was performed with a 2.5-μL aliquot from the first round with internal primers: Vβ8-int (ATGTACTGGTATCGGCAGGAC)/Cβ100-int (AAGCCCCTGGCCAAGCACAC) and Vα8-int (GCCACTCTCCATAAGAGCAG)/CαR-int (TAGGTGGCGGTGGTCTCTTTG). For each round, PCR conditions were 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, followed by a final extension cycle of 72°C for 10 minutes. PCR products were resolved on a 2% agarose gel, purified on QIAquick Gel extraction spin columns (Qiagen), and sequenced at the Analysis and Synthesis Facility of Laval University (Quebec, QC, Canada).

Limiting Dilution Assays of CTL Precursors

Limiting numbers of responder spleen cells from unprimed animals were cultured in 96-well round-bottomed plates with constant numbers (2.5×10^5) of irradiated stimulator spleen cells pulsed with 20 μmol/L $H7^a$ or $HY_{(Uty)}$ peptide for 40 minutes at 37°C. Cell cultures were made in Iscove medium with fetal bovine serum (10%), L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), IL-2 (20 U/mL), IL-4 (10 ng/mL), and IL-7 (10 ng/mL). On day 7 and 14, 50% of the medium was replaced by fresh medium containing IL-2, IL-4, IL-7, and 20 μmol/L of the respective peptide. Tetramer labeling was performed on day 21, and detection of tetramer-positive cells was performed as described [29]. Briefly, cells were washed and incubated at room temperature for 30 minutes with specific and irrelevant (LCMV gp33-41/D^b) tetramers. The analysis procedure is depicted in Figure 1A and was aimed at gating out those among CD8 T cells that emit a higher-than-background fluorescence at 670 nm when excited at 488 nm (autofluorescence) or those that bind an irrelevant tetramer. According to Poisson statistics, the tetramer-labeled clusters in the positive microcultures (less than 10% of the wells) each represent a single clone. Wells were scored positive when the percentage of cells specifically labeled by tetramers containing the peptide used for stimulation was more than 3 SD over mean results for negative controls (ie, male cells stimulated by HY and B10 cells stimulated by $H7^a$).

In some experiments, analyses of precursor frequencies were performed on subsets of CD8 T cells. In this case, unprimed spleen cells were depleted with

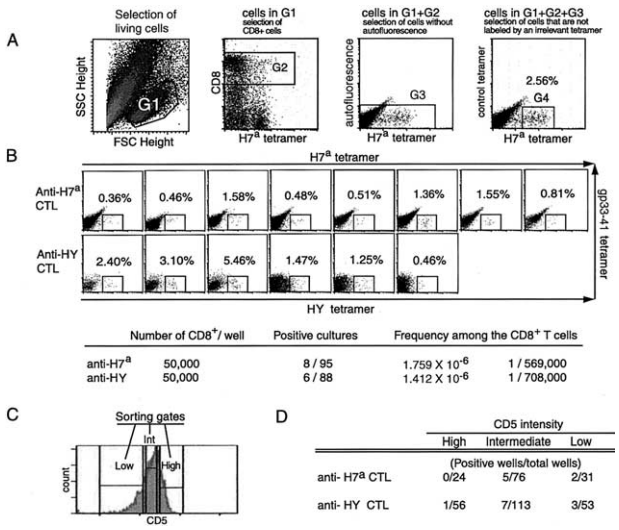

Figure 1. Anti-H7^a and anti-HY CD8 T-cell precursors exhibit similar frequencies and CD5 expression levels in naive B10.H7^b mice. A, Procedure for the analysis of lymphocytes labeled by H7^a or HY tetramers. Autofluorescent cells and those that bind an irrelevant tetramer are gated out. B, One hundred eighty-three microcultures were set up with splenocyte suspension adjusted to 5×10^4 CD8 T cells per well. These cells were stimulated with HY or H7^a as described in Materials and Methods. Plots show tetramer labeling of CD8 T cells in the positive anti-H7^a (top row) and anti-HY (bottom row) wells. C, Gating procedure used to separate CD5^{lo}, CD5^{int}, and CD5^{hi} CD8 T-cell subsets. Cells from these 3 subsets were plated in different wells. D, Frequency of positive microcultures obtained from CD5^{lo}, CD5^{int}, and CD5^{hi} CD8 T cells. Culture conditions and cell gating procedures were similar to those used in the estimation of precursor frequency (A and B).

anti-pan B (B220) and anti-CD4 (L3T4) beads (Dynal Biotech, Brown Deer, WI) and stained with antibodies against CD8 and CD5. The low, intermediate, and high CD5 subsets of CD8 T cells were sorted, and 5×10^4 CD8 T cells were cultured in 96-well round-bottomed plates with 2.5×10^5 stimulator spleen cells (pulsed with 20 μmol/L of peptide) and 1×10^5 autologous irradiated spleen cells as feeders.

RESULTS

The Diversity of the Repertoire of H7^a-Specific CD8 T Cells Is Extremely Limited

Significant insight into the bases of immunodomination may be gained from studies on MiHAs because, with their tens of thousands of proteins, mammalian cells represent the most complex entity to which CTLs can be confronted in the antigenic universe [30]. Studies based on 4 molecularly defined BALB.B MiHAs recognized by C57BL/6 cells provided evidence that the diversity of the CD8 T-cell TCR repertoire may be correlated with immunodominance. Thus, BALB.B H28 and H60 MiHAs are immunodominant, and C57BL/6 responder mice carry null alleles [30-32]. In contrast, H13 and H47 are not immunodominant, and B6 mice share closely matched allelic analogs with BALB.B mice [33-35]. It was therefore proposed that in the absence of a self analog peptide, the complete lack of negative selection should provide a more diversified peripheral repertoire [32]. However, recent biochemical definition of the model immunodominant H7^a MiHA (KAPDNRETL, corresponding to amino acids 770 to 778 of the SIMP/STT3-B protein) and its allelic product (H7^b: KAPDNRDTL) appears at face value difficult to reconcile with this paradigm [36]. Because the 2 allelic H7 MiHAs differ by a single methylene group, it is difficult to imagine how they might elicit diversified arrays of TCR clonotypes. We therefore deemed it important to directly assess the diversity of the anti-H7^a TCR repertoire.

As a prelude to detailed studies of the T-cell repertoire, it was essential to validate the specificity of the H7^a/D^b tetramers used to identify and isolate H7^a-reactive CD8 T cells. Splenocytes from B10.H7^b mice primed against B10 cells and B10 mice primed against B10.H7^b cells were stained with H7^a/D^b and H7^b/D^b tetramers. Even though H7^a and H7^b differ by a single CH2 group [36], T cells bound only the allelic product against which they were primed and not the self allelic product (Figure 2). Thus, H7^a/D^b tetramers used in this work showed exquisite specificity.

B10.H7^b mice were primed with 2×10^7 B10 cells, and their splenocytes were harvested on day 14, when approximately 5% of CD8 T cells were H7^a specific [16]. H7^a tetramer$^+$ CD8 T cells were stained with a panel of 14 anti-Vβ and 4 anti-Vα antibodies. The TCR Vβ repertoire of anti–anti-H7^a T cells was dramatically skewed because, in most mice (7 of 8), tetramer$^+$ CD8 T cells used a single Vβ element, in most cases Vβ8 (Figure 3). Although the panel of available anti-Vα antibodies is relatively restricted, our data strongly suggest that TCR Vα use is also skewed because in each mouse no more than 1 Vα element was detected on tetramer$^+$ CD8 T cells.

To analyze more precisely the diversity of the H7^a-specific repertoire, we focused on tetramer$^+$ CD8 T cells bearing the TCR Vα and TCR Vβ elements most frequently detected in our flow cytometry analyses: Vα8 and Vβ8. The third complementarity-determining regions (CDR3) of the TCRα and β chains have a primary role in recognition of the MHC-bound peptide antigen and are thus responsible for most of the specificity of TCR interactions. Single-cell PCR can estimate T-cell frequency accurately, because it is not affected by skewed PCR amplification or different TCR messenger RNA expressions in individual T cells [37]. Thus, our experimental strategy involved single-cell sorting of tetramer$^+$/Vα8$^+$/Vβ8$^+$ CD8 T cells, reverse transcriptase-PCR amplification, and sequencing of CDR3 loops of the TCRα and β chains [28,38]. These single-

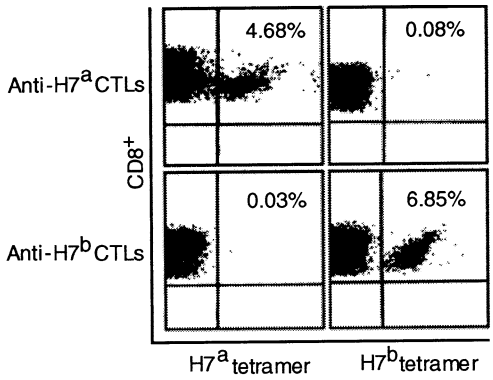

Figure 2. H7^a (KAPDNRETL) and its allelic peptide H7^b (KAPDNRDTL) are not cross-reactive at the TCR level. Splenocytes from B10.H7^b mice primed against B10 cells (anti-H7^a) and from B10 mice primed against B10.H7^b (anti-H7^b) were stained with H7^a and H7^b tetramers.

cell analyses were performed in 4 mice in which more than 80% of tetramer$^+$ CD8 T cells were stained by anti-Vα8 and -Vβ8 antibodies. CDR3 lengths were designated from the amino acid sequences, whereas J-element use was determined after comparison with known genomic sequences [39-42]. Analysis of 65 TCRβ transcripts from 4 mice showed that the diversity of the TCRβ chains was strikingly limited, and conserved features were clearly identified (Table 1). Practically all TCRβ chains from sorted tetramer$^+$/Vα8$^+$/Vβ8$^+$ CD8 T cells used Vβ8.1 and Jβ2.1 segments. In 94% of the cases (61/65), the CDR3β region had a length of 12 amino acids with conserved charged residues: aspartic (D) or glutamic (E) acid at P2, arginine (R) at P3, and glutamic acid (E) at P11. The TCRβ chains showed low diversity in each individual mouse and were remarkably similar among mice. Overall, 94% of the CDR3β segments from the 4 mice harbored the following 12–amino acid consensus sequence: S(D/E)R(T/A)GGXNYAEQ. Analysis of 47 TCRα transcripts from the same tetramer$^+$/Vα8$^+$/Vβ8$^+$ CD8 T-cell samples confirmed the limited diversity of the H7^a-specific repertoire (Table 1). Two types of dominant TCRα chains were found: Vα8.3-S(G/A)SSNTNKV-Jα27 in 2 mice and Vα8.3-GTGGYKV-Jα10 in the other 2. We therefore conclude that the H7^a-specific CD8 T-cell repertoire harvested at the time of primary response shows highly restricted TCR diversity.

Anti-H7^a and -HY CD8 T-Cell Precursors Have Similar Frequencies

Because the role of precursor T-cell frequency in immunodomination is controversial [5,11,43,44], we deemed it necessary to assess the frequency of H7^a- and HY-specific CD8 T cells in naive mice. The frequency of antigen-specific CD8 T cells in naive mice is typically approximately 10^{-5} to 10^{-6} and is thus below the detection limit of standard tetramer-staining methods [43,45]. Indeed, the proportion of spleen CD8 T cells stained by H7^a/D^b tetramers was not higher in B10.H7^b mice than in our negative controls: H7^a-positive mice (B10) and H2D^b-negative mice (BALB/c; data not shown). Likewise, the proportion of CD8 T cells stained by HY/D^b tetramers was similar in male and female B10.H7^b mice (data not shown). Thus, the frequency of H7^a- and HY-specific T cells is less than 10^{-3}. To evaluate precursor frequencies, we therefore used a very sensitive method developed by Karanikas et al [29]. This method involves in vitro culture of T cells with peptide-coated stimulating cells under limiting dilution conditions in cytokine-supplemented milieu. On day 21, T cells from B10.H7^b female mice stimulated with HY- or H7^a-coated cells were labeled with anti-CD8 antibody and the cognate tetramer. Cells that were autofluorescent or that were labeled by an irrelevant tetramer (LCMV gp33-41/D^b) were gated out (Figure 1A). As a result of the limiting dilution, each positive microculture represents a single clone. Because in vitro peptide-driven CTL expansion systems may allow expansion of CTLs exhibiting relatively low affinity for

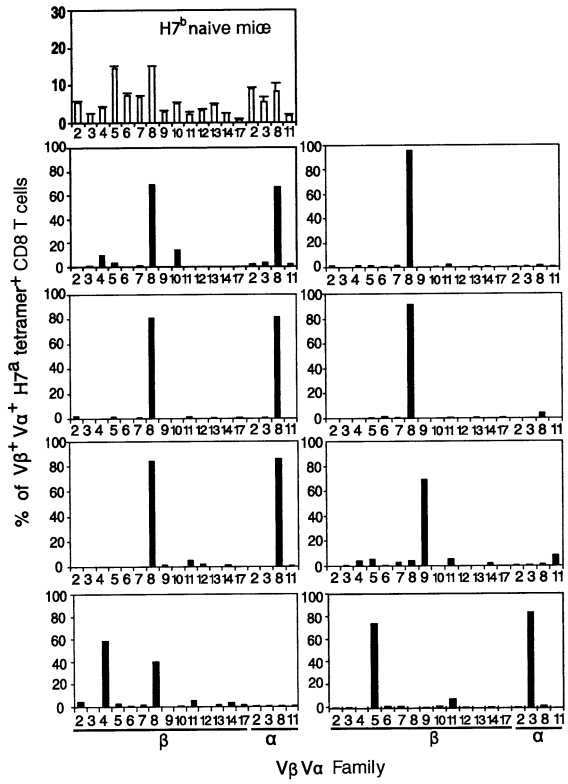

Figure 3. H7^a-tetramer$^+$ CD8 T cells use mainly TCR Vα8$^+$ and Vβ8$^+$ chains. Splenocytes from B10.H7^b mice primed with B10 splenocytes were stained on day 14 with antibodies against various TCR Vα and Vβ chains. Nonimmune B10.H7^b mice were used as controls. Results from nonimmune B10.H7^b control mice (n = 5) are presented as the mean + SD. Results from mice primed against H7^a are presented individually for the 8 mice tested.

Table 1. *Amino Acid and Nucleotide Sequences of the TCRα and β Chain Hypervariable Regions of H7[a] Tetramer[+]/Vα8[+]/Vβ8[+]/CD8 T Cells**

Mouse	TCRβ V Gene	CDR3 Sequence 1	2	3	4	5	6	7	8	9	10	11	12				TCRβ D/J	Occurrence
Mouse 1	8.1	C	A	S	12	S	D	R	T	G	G	A	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	7/14
		TGT	GCC	AGC		AGT	GAC	AGG	ACT	GGG	GGG	GCA	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.1	C	A	S	12	S	D	R	A	G	G	V	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	5/14
		TGT	GCC	AGC		AGT	GAT	CGG	CCT	GGG	GGG	GIT	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.1	C	A	S	12	S	D	R	T	G	G	V	N	Y	A	E	Q	F	F	G	D2.1/J2.1	1/14
		TGT	GCC	AGC		AGT	GAT	CGT	ACT	GGG	GGG	GTG	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.1	C	A	S	8	S	E	R	T	S	Y	E	Q					Y	F	G	D2.1/J 2.6	1/14
		TGT	GCC	AGC		AGT	GAG	AGG	ACT	TCC	TAT	GAA	CAG					TAC	TTC	GGA		
Mouse 2	8.1	C	A	S	12	S	D	R	T	G	G	V	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	12/14
		TGT	GCC	AGC		AGT	GAT	CGT	ACT	GGG	GGG	GTG	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.1	C	A	S	12	S	D	T	T	G	G	E	Y	Y	A	E	Q	F	F	G	D1.1/J 2.1	1/14
		TGT	GCC	AGC		AGT	GAT	ACT	ACA	GGG	GGG	GAA	TAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.3	C	A	S	12	S	G	D	G	G	G	Y	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	1/14
		TGT	GCC	AGC		AGT	GAT	CGT	ACT	GGG	GGG	GTG	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
Mouse 3	8.1	C	A	S	12	S	E	R	T	G	G	P	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	16/16
		TGT	GCC	AGC		AGT	GAG	AGG	ACT	GGG	GGG	GCA	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
Mouse 4	8.1	C	A	S	12	S	E	R	T	G	G	P	N	Y	A	E	Q	F	F	G	D2.1/J 2.1	18/21
		TGT	GCC	AGC		AGT	GAA	CGG	ACT	GGG	GGG	CCT	AAC	TAT	GCT	GAG	CAG	TTC	TTC	GGA		
	8.1	C	A	S	11	R	P	S	G	G	A	N	Y	A	E	Q		F	F	G	D2.1/J 2.1	3/21
		TGT	GCC	AGC		AGA	CCC	TCT	GGG	GGG	GCT	AAC	TAT	GCT	GAG	CAG		TTC	TTC	GGA		
Consensus	**β CDR3**				**S**	D/E		**R**	T/A		**G**	**G**	**X**	**N**	**Y**	**A**	**E**		**Q**			
					AGT	**GA**T/A		**CGT**	A/G**CT**		**GGG**	**GGG**		**AAC**	**TAT**	**GCT**	**GAG**		**CAG**			

Mouse	TCRα V Gene	CDR3 Sequence 1	2	3	4	5	6	7	8	9				TCRα J	Occurrence
Mouse 1	8.3	C	A	L	9	S	G	S	S	N	T	N	K	V				V	F	G	27	5/6
		TGT	GCT	CTG		AGT	CGA	TCT	TCC	AAT	ACC	AAC	AAA	GTC				GTC	TTT	GGA		
	8.3	C	A	L	4	S	D	R	I									F	F	G	24	1/6
		TGT	GCT	CTG		AGC	GAC	AGA	ATC									TTC	TTT	GGT		
Mouse 2	8.3	C	A	L	9	S	A	S	S	N	T	N	K	V				V	F	G	27	10/10
		TGT	GCT	CTG		AGT	GCC	TCT	TCC	AAT	ACC	AAC	AAA	GTC				GTC	TTT	GGA		
Mouse 3	8.3	C	A	L	7	G	T	G	G	Y	K	V						V	F	G	10	13/13
		TGT	GCT	CTG		GGG	ACT	GGA	GGC	TAT	AAA	GTG						GTC	TTT	GGA		
Mouse 4	8.3	C	A	L	7	G	T	G	G	Y	K	V						V	F	G	10	18/18
		TGT	GCT	CTG		GGG	ACT	GGA	GCC	TAT	AAA	GTG						GTC	TTT	GGA		

*Lymphocytes were obtained from the spleen of individual B10.H7[b] mice 14 days after priming against H7[a] cells and were then processed for single-cell CDR3 analysis as described in Materials and Methods.

the target peptide, further studies are needed to evaluate the functional avidity of our in vitro–grown T-cell clones. Nevertheless, under these conditions, the frequencies of HY- and $H7^a$-specific CD8 T cells in nonimmunized $B10.H7^b$ female mice were approximately 1.4×10^{-6} and 1.7×10^{-6}, respectively (Figure 1B). These frequencies are consistent with those of other antigen-specific cells [43,45] and show that the immunodomination of $H7^a$ over HY cannot be ascribed to a higher frequency of $H7^a$-specific T cells in the preimmune repertoire.

Lack of Correlation between Immunodominance and Levels of CD5 Expression on Preimmune T Cells

Models proposed so far have posited a priori that immunodomination should be regulated by only 2 factors: T-cell precursor frequency and TCR interactions with the cognate antigen. We hypothesized that a third factor might play a key role: CTL interactions with self pepMHC as opposed as to foreign antigens. This idea stems from the demonstration that TCR engagement by self pepMHC actively sustains the naive T cells in an optimal state of sensitivity by sustaining partial phosphorylation of TCRζ and TCR clustering. The strength of TCR interactions with self pepMHC differs among TCR clonotypes and can be estimated by using 2 parameters: the level of TCRζ chain phosphorylation and expression of CD5 levels [46-49]. There is no means to assess TCRζ chain phosphorylation of T cells specific for nominal antigen (eg, $H7^a$ or HY) in the preimmune repertoire of non–TCR-transgenic wild-type mice. Nevertheless, we reasoned that we should be able to determine whether T cells specific for a given antigen are enriched among $CD5^{lo}$ or $CD5^{hi}$ T-cell subsets by estimating the frequency of antigen-specific T cells in sorted populations of CD8 T cells expressing different levels of CD5. We therefore used CD5 expression on CD8 T cells as a surrogate marker for the strength of TCR interactions with self pepMHC.

Spleen CD8 T cells from $B10.H7^b$ mice were electronically sorted into 3 subsets: $CD5^{lo}$, $CD5^{int}$, and $CD5^{hi}$ T cells (Figure 1C). Sorted cells were cultured with peptide-coated stimulating cells under limiting conditions, as in previously described experiments on the estimation of CD8 T-cell precursor frequencies. The proportion of positive cultures was 7 of 131 for $H7^a$-specific T cells and 11 of 222 for HY-specific T cells. The salient finding is that the distribution of positive wells among $CD5^{lo}$, $CD5^{int}$, and $CD5^{hi}$ T cells was similar for $H7^a$ and HY (Figure 1D). In both cases, antigen-specific CD8 T-cell precursors were mainly found in the $CD5^{lo/int}$ as opposed to the $CD5^{hi}$ subset (Figure 1). These data unveil no relation between immunodominance and CD5 expression. They thus provide indirect evidence against the idea that the strength of TCR interactions with self pepMHC shapes the immunodomination hierarchy.

Kinetics of TCR:Epitope Interactions

T-cell structural avidity, determined by the direct binding affinities of multiple cell-bound TCR molecules for pepMHC, is most commonly measured by staining with pepMHC multimers [26,27,50-52]. The results of tetramer association (on-rate) and tetramer decay (off-rate) assays closely correspond to the K_{on} and K_{off} rates, respectively, of the interaction between the soluble TCR and immobilized pepMHC [25,27]. Thus, we next examined the structural avidity of interactions between $H7^a$- and HY-specific T cells and their specific ligand on day 14 after in vivo priming, that is, at the time of primary response. In contrast with other studies [53], our experiments were conducted on freshly harvested spleen CD8 T cells without in vitro restimulation because in vitro culture may favor preferential expansion of selected T-cell clonotypes. Splenocytes from each mouse were tested individually (not pooled), and a single batch of $H7^a/D^b$ and HY/D^b tetramers was used in these experiments.

We first compared the tetramer staining decay (TCR:pepMHC off-rate) of $H7^a$- and HY-specific CD8 T cells. Of note, anti-$H7^a$ and -HY $tetramer^+$ cells expressed equivalent levels of CD8 and TCR (Figure 4A). Noticeably, the mean tetramer decay rate was not slower for $H7^a$-specific relative to HY-specific T cells (Figure 4B and C). Consistent with a previous report [27], the level of animal-to-animal variation (the standard variation of decay plots) after primary immunization was significant in each group. A key point is that the TCR:pepMHC off-rate showed no less intragroup variation for $H7^a$-specific relative to HY-specific T cells (Figure 4C). This means that there was no enrichment for T cells with a slower TCR dissociation rate among anti-$H7^a$ compared with anti-HY T cells.

To estimate the TCR:pepMHC on-rate, T cells harvested on day 14 after primary immunization were incubated with increasing concentrations of tetramers. $H7^a$-specific T cells showed a much higher tetramer binding rate than HY-specific T cells (Figure 4D). Thus, tetramer concentrations required to reach 50% and 75% normalized fluorescence were 3.0 and 7.5 nmol/L for anti-$H7^a$ T cells versus 30.0 and 130.0 nmol/L for anti-HY T cells ($P < .001$; Student t test). Thus, at the time of primary response, the TCR:pepMHC off-rate is similar for anti-$H7^a$ and -HY T cells, whereas the on-rate is much more rapid in the case of $H7^a$-specific T cells.

We next sought to determine whether a more rapid TCR:pepMHC on-rate would correlate with a more rapid T-cell expansion. We therefore immu-

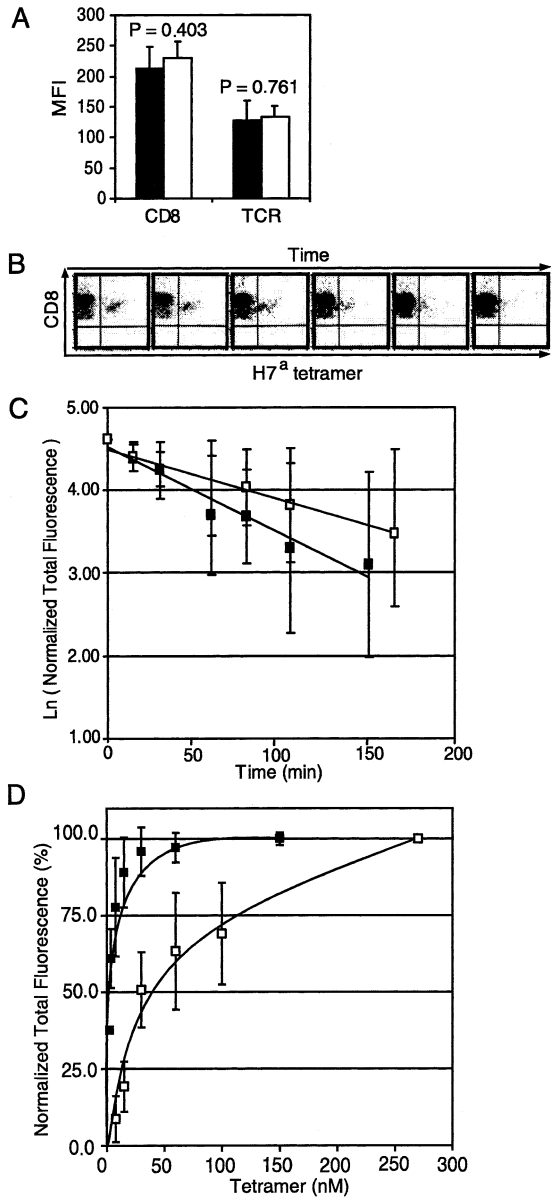

Figure 4. TCR:pepMHC dissociation and association rates for (■) $H7^a$- and (□) HY-specific CD8 T cells harvested at the time of primary response (day 14 after antigen priming). A, CD8 T cells labeled with $H7^a$ and HY tetramers were stained with antibody against CD8 and TCRαβ. B, One representative example of an $H7^a$-tetramer decay plot series. C, Decay plot of the natural logarithm of the normalized total fluorescence versus time (after the addition of anti-D^b monoclonal antibody) for primed splenocytes stained with $H7^a$ or HY tetramers. The rate of tetramer decay was similar for cells labeled with $H7^a$ and HY tetramers ($P = .427$). D, To evaluate the TCR:pepMHC association rate, day 14 splenocytes were incubated with graded concentrations of the relevant tetramer. C and D, Each point represents the mean of 5 to 10 mice tested.

nized B10.$H7^b$ female mice with B10 or B10.$H7^b$ male cells to evaluate the kinetics of CD8 T-cell responses against $H7^a$ and HY, respectively. The number of $tetramer^+$ CD8 T cells was measured from day 5 to 30. The maximum response was defined as 100%, and other responses were plotted as a fraction of that maximum response. The key finding was that the maximum response was reached significantly earlier for $H7^a$-specific T cells than for HY-specific T cells (Figure 5). In addition, contraction of the anti-HY CD8 T-cell population was more rapid than that of anti-$H7^a$ CD8 T cells (cf day 25 in Figure 5). These data support the idea that a more rapid TCR:pepMHC on-rate leads to a faster and more prolonged CD8 T-cell expansion.

DISCUSSION

The Diversity of the TCR Repertoire

The TCR repertoire selected by discrete pepMHC ranges from oligoclonal to extremely diverse [43,54-57]. Our work shows that the TCR repertoire emerging during the primary response to the immunodominant $H7^a$ antigen is oligoclonal. Two points can be made from the very limited heterogeneity of the anti-$H7^a$ repertoire. First, the potential for immunodominance is not directly related to TCR diversity. Second, because the product of the $H7^b$ allele differs from $H7^a$ by a single methylene group, our data support the concept that the degree of homology between the cognate antigen and self peptides has a dominant influence on the diversity of the TCR repertoire [58]. The fact that anti-$H7^a$ CD8 T cells represent approximately 5% of the peripheral T-cell population 14 days after antigen encounter [16], but derive from a small number of clones (Figure 3 and Table 1), indicates that these clones are amplified with remarkable efficiency during the primary response.

The relation between TCR diversity and the efficacy of immune responses is complex. High TCR diversity increases the likelihood that the repertoire

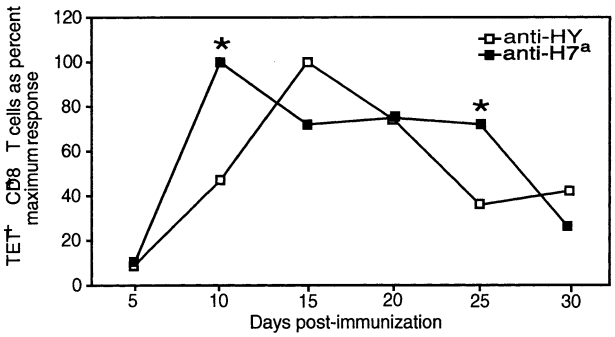

Figure 5. Kinetics of CD8 T-cell responses against $H7^a$ and HY. B10.$H7^b$ female mice were immunized with B10 or B10.$H7^b$ male cells to evaluate the kinetics of CD8 T-cell responses against $H7^a$ and HY, respectively. The number of $tetramer^+$ CD8 T cells in the spleen was measured on day 5, 10, 15, 20, 25, and 30. The maximum response was defined as 100%, and other responses were plotted as a fraction of that maximum response. There were 4 to 7 mice per time point. *$P < .05$; Student t test.

contains T cells with optimal avidity to recognize immunodominant epitopes and that TCR clonotypes specific for mutated or secondary epitopes are available to prevent escape by mutation [59]. A greater TCR diversity is therefore considered to be beneficial to the host. Remarkably, although we show herein that $H7^a$-specific CD8 T cells display very limited TCR diversity, these T cells are extremely effective in eradicating $H7^a$-positive neoplastic cells [60]. This should not be construed as a deterrent to the putative importance of a diversified T-cell repertoire in general. This apparent paradox rather suggests that in the presence of T cells specific for immunodominant antigens, TCR diversity is dispensable as long as the target epitope shows a low mutation rate. Indeed, in vitro immunoselection studies with anti-$H7^a$ CTLs have shown that $H7^a$ loss was an exceedingly rare event [61]. In line with this, the structure of SIMP/STT3-B, the source protein of $H7^a$, is highly conserved among species and is nearly invariant among human individuals and inbred strains of mice [36,62]. A corollary to data emerging from analyses of anti-$H7^a$ CTL responses is that targeting epitopes with a low mutation frequency may confer an advantage for T-cell immunotherapy.

The Frequency of Antigen-Specific CD8 T Cells in the Preimmune Repertoire and Their Interactions with Self

Because immunodomination results from competition for APC resources, it sounds intuitively plausible that high antigen-specific T-cell precursor frequency in the preimmune repertoire might confer a competitive advantage [44]. Considering the attractiveness of this concept, differential precursor frequencies are commonly invoked as putative contributory factors to immunodomination, although few direct assessments of antigen-specific precursor frequencies have been made in nonimmune animals. Clearly, even though $H7^a$ and HY lie at opposite ends in the immunodomination hierarchy, $H7^a$-specific T cells are not more abundant than HY-specific T cells in naive mice. Even though $H7^a$ is at the summit of the immunodomination hierarchy on C57BL/6 cells, we argue that the unremarkable frequency (10^{-5} to 10^{-6}) of anti-$H7^a$ T cells in the nonimmune repertoire is not idiosyncratic. Indeed, similar precursor frequencies have been observed with T cells specific for immunodominant viral epitopes from LCMV and Epstein-Barr virus [5,45]. Furthermore, 2 studies showed that injection of very large numbers of memory CD8 T cells specific for immunodominated antigen did not enable these T cells to compete more successfully against T cells that recognized dominant epitopes [11,32]. We therefore deduce that, except perhaps in some extreme situations, CD8 T-cell precursor frequency does not impinge on immunodomination.

It has been proposed that one chief role of the background TCR signals induced by self pepMHC is to enhance sensitivity to foreign antigen [46,47]. We therefore tested the novel idea that interactions with self pepMHC might enable some CD8 T cells to react more promptly than others to their cognate antigen and thereby exert immunodomination. Our data on CD5 levels at the surface of naive T cells, used as a surrogate marker for the intensity of TCR tickling in the periphery, provide indirect evidence against our hypothesis.

TCR:Epitope Interaction Kinetics

Data summarized in the Introduction lead us to infer that the crux of immunodomination is speed: compared with other CTLs, those that exert immunodomination are primed after a shorter duration of antigen presentation and swiftly generate effector mechanisms that entail prompt deletion or inactivation of APCs. This paradigm is supported by direct evidence that the duration of antigen presentation required to prime naive T cells varies as a function of T-cell antigen specificity [20,63]. Furthermore, the speed paradigm dovetails well with a recent report that the rapidity with which CD8 T cells initiate IFN-γ synthesis correlates with immunodominance [22]. Moreover, anti-$H7^a$, but not anti-HY, CD8 T cells rapidly eliminate APCs presenting their cognate antigen in vivo [19]. In line with the speed model, we report that the salient differences between anti-$H7^a$ and -HY CD8 T cells are that the former display a much more rapid TCR:pepMHC on-rate (Figure 4) and proliferate more rapidly after antigen priming (Figure 5). Our assumption that the TCR:pepMHC on-rate decisively influences the swiftness of T-cell priming is supported by elegant studies from Kalergis et al [64]. When they examined the effects of point mutations in the CDR3 β-chain of a K^b-restricted TCR, these authors found that in vitro T-cell activation was promoted in a mutant (G97A) that, compared with the wild-type TCR, displayed a more rapid on-rate without prolongation of the off-rate. In contrast, mutants with longer TCR:pepMHC off-rates than the wild-type TCR were less responsive, possibly because of less effective TCR serial engagement [64].

One has to be cautious in extrapolating in vivo the consequences of discrepancies in the in vitro TCR:pepMHC on-rate among T cells with different antigen specificities. In vivo imaging of T-cell interactions with APCs in the lymph nodes has shown that the cascade of events leading to T-cell priming occurs very rapidly. Initial signs of T-cell activation appear during the first 8 hours after the APC encounter, and secretion of cytokines such as IL-2 and IFN-γ begins

during the subsequent 12 hours [65]. Thus, given the fast pace of initial interactions between T cells and APCs, a quick TCR:pepMHC on-rate could confer a decisive advantage among T cells competing for APC resources. As a corollary, immunodomination should not occur when competing CD8 T cells have similar TCR:pepMHC on-rates. This would explain that competition for APC resources does not mold the repertoire of T-cell responses in some models [12,44].

Studies of T cells with other antigen specificities will be needed to evaluate the generality of our model, that is, whether differences in the TCR:pepMHC on-rate are not only sufficient but also necessary to explain immunodomination. One prediction of our model is that an antigen such as $H7^a$ should lose its ability to exert immunodomination over HY, for example, in mice whose T cells specific for the 2 antigens have similar TCR:pepMHC on-rates. Testing this prediction seems demanding but might be achieved by testing the immunodomination potential of TCR-transgenic T cells with the same antigen specificity but different TCR:pepMHC on-rates.

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