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Immunodominance of H60 Is Caused by an Abnormally High Precursor T Cell Pool Directed against Its Unique Minor Histocompatibility Antigen Peptide

Eun Young Choi,[1,4] Gregory J. Christianson,[1] Yoshitaka Yoshimura,[2] Thomas J. Sproule,[1] Nadja Jung,[1] Sebastian Joyce,[2] and Derry C. Roopenian[1,3]

[1]The Jackson Laboratory
600 Main Street
Bar Harbor, Maine 04609

[2]Department of Microbiology and Immunology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

Summary

The H60 minor histocompatibility (H) antigen peptide is derived from a glycoprotein that serves as a ligand for the stimulatory NKG2D receptor. We show that this peptide is remarkably immunodominant in that it competes effectively with MHC alloantigens, is efficiently crosspresented by host antigen-presenting cells (APCs), and readily elicits naive CD8 T cell responses in vitro. H60 immunodominance is neither a consequence of NKG2D engagement nor competition among minor H antigens on APCs. Instead, H60 immunodominance is a consequence of an abnormally high naive precursor frequency of H60 peptide reactive CD8 T cells. Understanding why the H60 peptide is so immunogenic has important implications in tissue transplantation and vaccine design.

Introduction

Tissues are rejected when transplanted onto genetically different individuals because of allogeneic differences at histocompatibility (H) antigens that evoke CD4 and CD8 T cell responses. Considerable effort is dedicated to matching major histocompatibility complex (MHC) alloantigens because they are generally considered to be the most significant barriers to successful transplantation. However, despite the fact that they are known transplantation barriers, H antigens encoded outside of the MHC, the so-called minor H antigens, have only recently attracted serious consideration.

Both MHC class I alloantigens and minor H antigens are comprised of self-peptide/MHC class I complexes arising from processed fragments of normal endogenous proteins loaded onto MHC class I molecules. The former arises from natural genetic variation in the MHC molecule itself, resulting in a quantum change in the assortment of processed self-peptides that bind each MHC class I molecule. In contrast, each minor H antigen is a consequence of the presentation of a genetically variant self-peptide by an invariant MHC class I molecule (Simpson and Roopenian, 1997). In a transplantation setting, the recipient of MHC-disparate tissue is thus confronted with thousands of novel MHC-presented peptides resulting in the activation and expansion of a large number of clonally diverse CD8 T cells (Martin and Dyer, 1993; Pimsler and Forman, 1978). In contrast, the recipient of MHC-matched but minor H antigen-disparate tissue confronts only a single altered peptide ligand per minor H antigen, resulting in the expansion of a few clonally restricted T cells (Teh et al., 1982; Theobald and Bunjes, 1993), a situation similar to that which occurs in response to viral peptide epitopes.

Immunodominance is a poorly understood property that allows immune responses to certain peptide epitopes to prevail over others. Proposed mechanisms for this phenomenon can be lumped into two categories. The first is properties inherent to the peptide and its precursor protein such as selective peptide processing, peptide binding avidity to MHC proteins, and/or high peptide/MHC number on antigen-presenting cells (APCs). The second is T cell based, and includes elusive qualitative and quantitative differences in the antigen-specific T cell repertoires (Chen et al., 2000; Yewdell and Bennink, 1999). Regardless of the "upstream" mechanisms involved, the expression of the immunodominance phenotype requires successful access to antigen and costimulation on APCs, and many studies have reported that successful competition for antigen on the APC is necessary for the immunodominance trait (Grufman et al., 1999; Kedl et al., 2000, 2002; Perreault et al., 1998). Overall, however, the key determinants of immunodominance remain to be firmly established.

H60 is an unusual mouse minor H antigen. While many minor H antigens are alloantigenic because of discrete amino acid changes in a core MHC-presented self-peptide, alloantigenicity of H60 is because responder mice, such as C57BL/6 (B6), fail to express the protein from which the H60 peptide derives (Malarkannan et al., 1998). Moreover, the H60 glycoprotein is a member of the retinoic acid early inducible (Rae1) gene family. These genes are orthologs of the human ULBP gene family and paralogs of the MHC I gene family (Cerwenka et al., 2000; Diefenbach et al., 2000). Their glycoproteins serve as ligands for the stimulatory natural killer (NK) receptor NKG2D and, among the mouse Rae1 family, H60 has the highest affinity for NKG2D (Carayannopoulos et al., 2002). NKG2D is expressed by NK cells and activated CD8 T cells (Jamieson et al., 2002), and its engagement on CD8 T cells results in signals transmitted through the same KAP/DAP2 adaptor molecules used for the CD28 costimulatory cascade, thus linking innate with adaptive immunity (Jamieson et al., 2002). Ectopic expression of H60 and the RAEs in tumor cell lines results in rejection mediated by NK and/or CD8 T cells (Diefenbach et al., 2001), and induced expression in carcinomas results in protection mediated by cutaneous T cells expressing $\gamma\delta$TCRs (Girardi et al., 2001). The CD8 T cell response to a single H60-derived octamer peptide (LTFNYRNL) complexed with the H-2K^b MHC class I immunodominates over other known minor H antigens both in spleen cell immunization protocols (Choi et al., 2001) and in graft-versus-host disease (Choi et al., 2002).

[3]Correspondence: dcr@jax.org
[4]Present address: Department of Pathology, Seoul National University College of Medicine, Seoul 110-799 Korea.

Remarkably, this same octamer peptide partially inhibits NKG2D from binding the H60 glycoprotein, suggesting that it encompasses a critical NKG2D binding site (Cerwenka et al., 2002). However, the relationship between H60 as a dominant minor H antigen and H60 as a ligand for NKG2D remains poorly understood. The goal of our studies was therefore to thoroughly examine the phenomenon of H60 immunodominance.

Results

Up to One-Half of the CD8 CTL Response Is Directed against Immunodominant Minor H Antigens in a Haplo-MHC Mismatched Situation

To examine whether minor H antigens are subordinate to MHC alloantigens, C57BL/6 (B6: H-2^b) female mice were immunized, and their T cells were challenged in mixed leukocyte culture (MLC) with MHC-matched but background-disparate BALB.B (H-2^b) or haplo-MHC-mismatched and background-disparate (B6 x BALB/c: H-2^{dxb}) F1 (CB6F1) male spleen cells. To quantify the responses to allo-MHC and minor H antigens, resting CD8 T cells from the MLC were tested for intracellular IFN-γ production after a 6 hr restimulation with CD8 T cell-depleted stimulator spleen cells. As expected, most (80%) of the B6 CD8 T cells primed and boosted with BALB.B cells produced IFN-γ in response to H-2^b-restricted BALB.B-derived minor H antigens (Figure 1A). Surprisingly, 30% of the CD8 T cells stimulated by CB6F1 male cells produced IFN-γ in response to H-2^b-restricted BALB.B minor H antigens (Figure 1A), while 35% and 42% of the cells produced IFN-γ in response to BALB/c and B10.D2 alloantigens (both H-2^d), respectively (Figure 1A). To determine the cytotoxic T lymphocyte (CTL) specificity, cells from the MLCs were also evaluated for their ability to lyse minor H antigen and MHC disparate target cells. A similar level of specific lytic activity was detected against MHC-matched but minor H-disparate BALB.B targets (48%) with MHC-disparate but background-matched B10.D2 targets and both minor H and MHC alloantigen-disparate BALB/c targets, 50% and 53%, respectively (Figure 1B). Since H60 dominates over other known minor H antigens in MHC-matched spleen cell priming protocols and graft-versus-host disease (Choi et al., 2002, 2001; Malarkannan et al., 2000, 1998), specific lysis directed against H60-congenic target cells was also examined. High levels of H60-specific lysis were detected, while negligible lysis was detected against H13- and HY-bearing target cells (Figure 1B), even though BALB.B males are known to be disparate from B6 females at these antigens. These results indicated that minor H antigens competed surprisingly well with MHC alloantigens when presented on the same APCs and that CTLs directed against H60 are major participants in this process.

H60 Dominates In Vivo and Is Efficiently Presented by Crosspriming

To assess directly whether the CD8 T cell response against H60 can be generated directly in vivo in the presence of an MHC disparity, female B6 mice were immunized with CB6F1 male spleen cells, and their peripheral blood leukocytes (PBLs) were analyzed 7 days after immunization for specificity by flow cytometry using H60 peptide (H60p) tetramers. Approximately 4% of the total blood CD8 T cells from the immunized mice stained with H60p tetramers, and all of these cells expressed the CD11a activation marker (Figure 2A). These results showed that substantial expansion of H60-specific CD8 T cells occurred in vivo as a consequence of CB6F1 immunization.

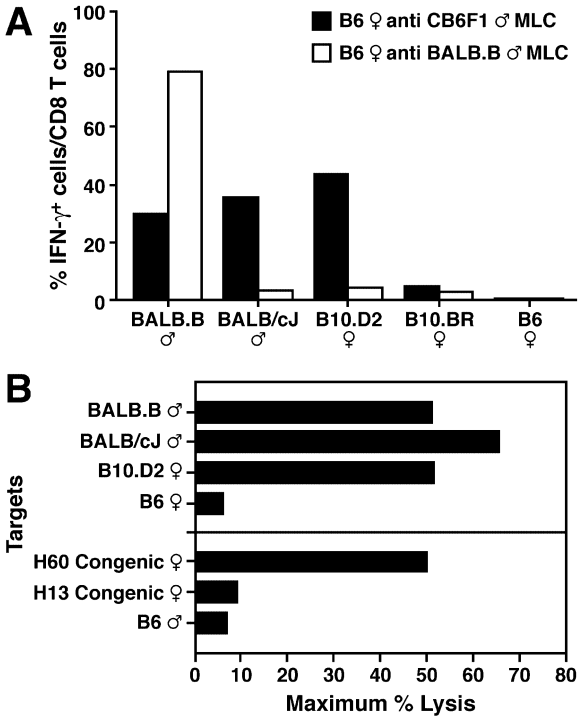


Figure 1. Minor H Antigen Responses Compete Successfully with the Allogeneic MHC Response

(A) Female B6 mice (H-2^b) were immunized in vivo, and their T cells were challenged in MLC with haplo-matched CB6F1 male or BALB.B male splenocytes. Resting cells from the MLCs were restimulated with the indicated CD8 cell-depleted stimulator splenocytes and analyzed for intracellular IFN-γ production. BALB.B (H-2^b: MHC-matched but minor H antigen-disparate), BALB/c (H-2^d: MHC- and minor H antigen-disparate) and B10.D2 (H-2^d: MHC-disparate but minor H antigen-matched) mice.

(B) B6 anti-CB6F1 MLC effector cells were tested in a cell-mediated lympholysis (CML) assay against Con-A lymphoblasts from the indicated mouse strains, including H60-congenic and H13-congenic mice. Maximum values of % specific lysis at 25:1 effector to target cell ratio are shown.

Unexpectedly, a substantial expansion of H60p tetramer$^+$ CD8 T cells ($\sim$7%) was detected after immunization with fully incompatible BALB/c splenocytes (Figure 2A), but in no case did tetrameric analysis show appreciable expansion of CD8 T cells specific for subordinate minor H antigens, H13 and HY (data not shown). The occurrence of H60p tetramer$^+$ CD8 T cells in BALB/c-immunized mice was not a consequence of a cross-reactive immune response to H-2^d alloantigens, because H60p tetramer$^+$ CD8 T cells were never detected in B6 mice immunized with B10.D2 spleen cells (which are of H-2^d type and fail to express the H60 protein) (Figure 2A) and H60-specific CTLs failed to lyse B10.D2 target cells (Figure 2B). Furthermore, the H60p tetramer$^+$ CD8

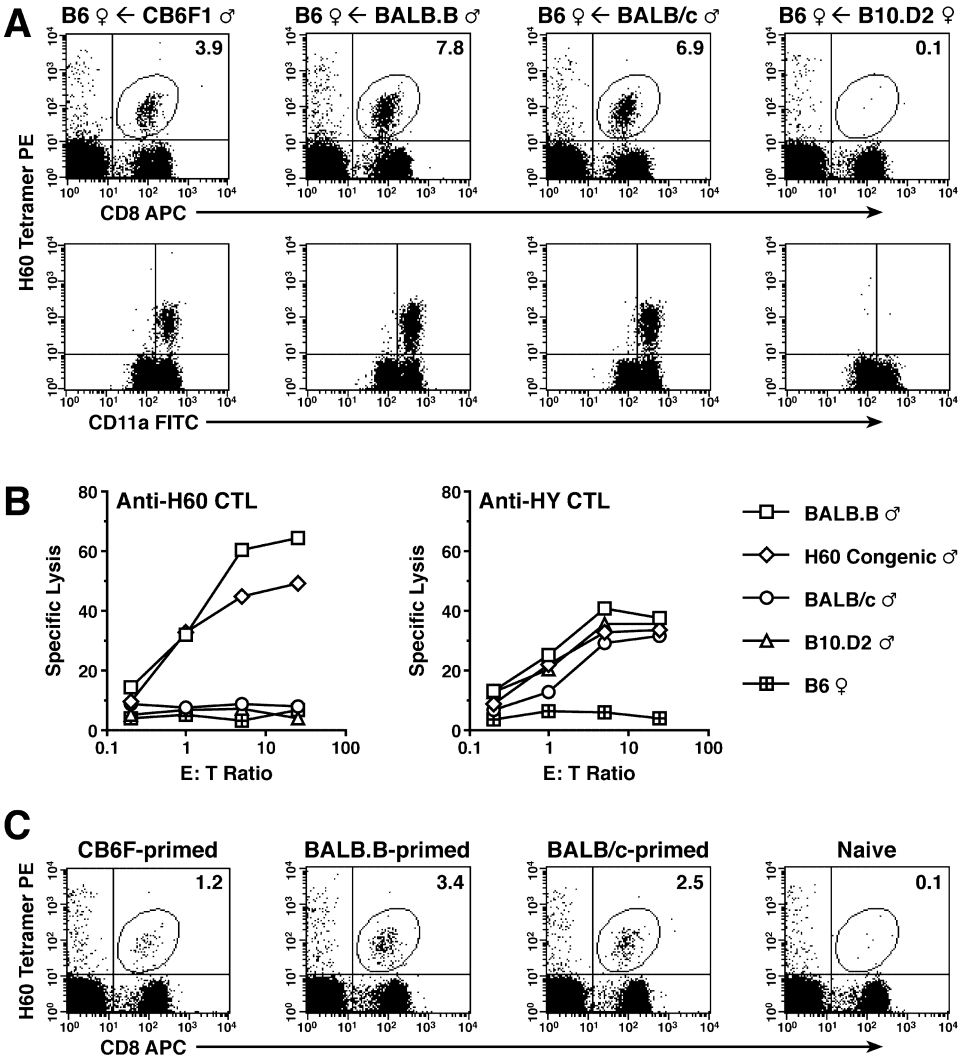


Figure 2. H60-Specific CD8 T Cells Are Readily Generated In Vivo during the Allo-MHC Response via Indirect Presentation

(A) Tetrameric analysis of PBLs from immunized B6 female mice. Pooled PBLs from five female B6 mice immunized with spleen cells from CB6F1 male, BALB.B male, BALB/c male, or B10.D2 female mice were analyzed by flow cytometry using PE-conjugated H60p tetramers, anti-CD8 mAb-APC, and anti-CD11a mAb-FITC 7 days after immunization. % of tetramer$^+$ CD11a^{high} CD8 T cells are indicated. No appreciable tetramer staining was observed using H13p or HYp tetramers (data not shown).

(B) Anti-H60 CTLs do not recognize or crossreact with H-2^d or H-2^d + H60. H60p-specific CTL line, SP/H60, was tested for lytic activity against BALB.B, B10.D2, BALB/c, H60-congenic, and syngeneic splenic lymphoblasts. HY-specific CTL line (CTL-10) known to show crossreactivity to H-2^d alloantigens was included as a positive control.

(C) B6 mice were immunized with spleen cells from CB6F1, BALB.B, or BALB/c mice, and 30 days later the immunized mice along with naive B6 mice were grafted with skin from H60-congenic females. Ten days later, PBL CD8 T cells from the grafted mice were assessed for H60p tetramer$^+$ cells.

T cells generated in response to BALB/c cells were not a consequence of CD8 T cells recognizing the native H60 glycoprotein or H60-derived peptides bound to H-2^d because H60-specific CTLs did not lyse BALB/c targets (Figure 2B) nor did the cells raised by BALB.B stimulation produce IFN-γ in response to BALB/c alloantigens (Figure 1A). Therefore, the expansion of H60p tetramer$^+$ CD8 T cells in BALB/c-immunized mice is best explained by specific T cell recognition of H60p that was efficiently crosspresented by B6 host APCs.

We then examined whether the H60-specific CD8 T cells generated while undergoing an MHC allo-response persisted as memory cells capable of eliciting a recall response. Thirty days after female B6 mice were immunized with BALB/c, CB6F1, or BALB.B splenocytes, the frequency of persisting H60p tetramer$^+$ cells was 0.1$\sim$0.2% of the total CD8 T cell in blood (data not shown). Tail skins from H60-congenic female mice were then grafted onto these immunized mice and onto control naive B6 mice. On day 10 following skin grafting, the frequency of H60p tetramer$^+$ CD8 T cells in PBLs had increased considerably compared with PBLs from grafted naive controls: 3.4% in BALB.B-, 2.5% in BALB/c-, and 1.2% for CB6F1-immunized mice versus 0.1% for naive control (Figure 2C), and these frequencies paralleled the burst sizes of the primary responses (Fig-

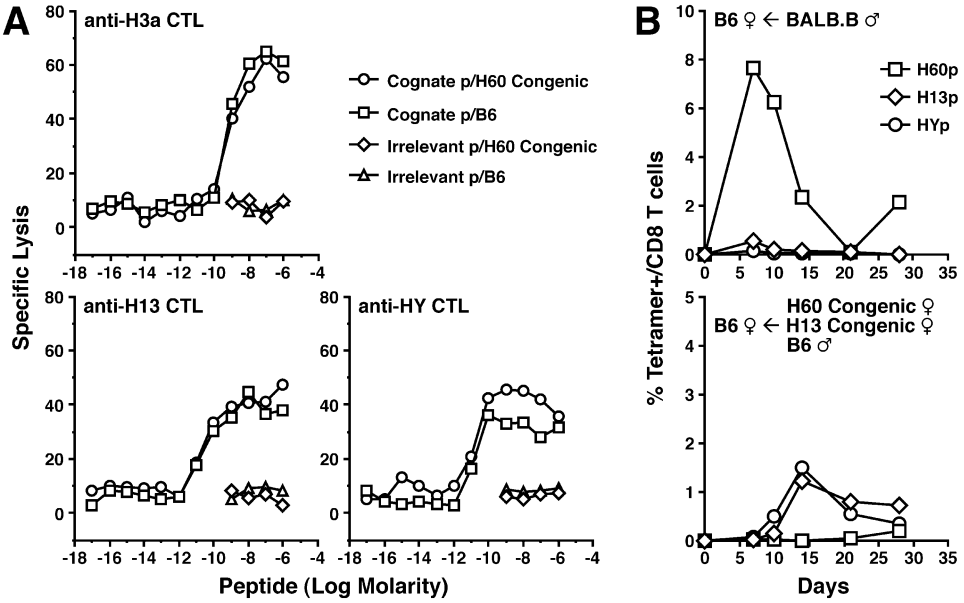


Figure 3. Physiological Expression of the H60 Protein Fails to Augment CTL Activation or Deliver Help

(A) H-2D^b-restricted specific CTL lines for H3a, H13, and HY were tested for cytolytic activity against splenic lymphoblast targets that do (H60-congenic female splenocytes) or do not (B6 female splenocytes) express H60 protein. The targets were loaded with varied concentrations of cognate peptide (H3a, H13, or HY) or irrelevant peptide (F5NP).

(B) Female B6 mice were immunized with male BALB.B splenocytes (induces CD4 help) or a mixture of splenocytes from female H60-congenic (no CD4 help) and female H13-congenic (induces CD4 help due to H3b minor H antigen) and B6 male (induces CD4 help) mice. PBLs from the immunized mice were serially sampled for staining with H60p, H13p, and HYp tetramers.

ure 2A). These results suggested that H60-specific CD8 T cells competed successfully with those directed against MHC alloantigens under conditions requiring indirect presentation and were fully capable of establishing memory and then undergoing a recall response to H60 in the context of self-MHC (K^b) molecules.

Physiological Expression of the H60 Glycoprotein Does Not Enhance the Immunostimulatory Capacity of pMHC Ligands

It has been suggested that H60 immunodominance could be a consequence of the H60 glycoprotein acting as an immunostimulatory ligand via its interaction with NKG2D (Cerwenka et al., 2002). To determine whether the H60 glycoprotein increases the generic sensitivity of TCR triggering, we compared the ability of D^b-restricted CTL lines (all of which express NKG2D as assessed by RT-PCR; data not shown) to lyse H60-negative (B6) and H60-positive (H60-congenic) T cell lymphoblast target cells incubated with limiting concentrations of cognate peptide ligands. Regardless of whether the target cells were H60-positive or not, the peptide concentrations required to achieve specific cytolysis were virtually superimposable (Figure 3A), indicating that physiological expression of H60 does not reduce the threshold of TCR triggering.

NKG2D engagement converges with the CD28 signaling cascade (Jamieson et al., 2002). H60 immunodominance might thus be a consequence of costimulatory signals delivered to responding H60p-specific CD8 T cells via NKG2D engagement with native H60 protein on APCs. To test this possibility, we serially analyzed PBLs from B6 female mice immunized with a mixture of splenocytes from B6 male and H13-congenic female mice (both of which differ from B6 female mice by CD4 T cell stimulatory helper determinants; data not shown) and female H60-congenic mice (which lack a CD4 T cell helper determinant; data not shown). The rationale was that if the H60 glycoprotein induced costimulation/help, it would support the generation of H60p-specific CTLs in a CD4 help-deprived situation. However, H60p tetramer$^+$ CD8 T cells were not detected in the mice immunized with this splenocyte mixture, while H13p and HYp tetramer$^+$ CD8 T cells (deliberately provided with cognate CD4 T cell help) were readily detected (maximum frequency of 1$\sim$1.5% of total CD8 T cells on day 14; Figure 3B, lower panel). In further support of the inability of H60 to induce immune responses autonomously, B6 mice (n = 10) showed no signs of rejecting tail skin allografts from sex-matched B6.C-H60-congenic mice, at least up to 100 days. H60 immunodominance was therefore entirely dependent on help from a CD4 T cell response generated by recognition of antigens on the same APC. Taken together, the results suggest that H60 immunodominance is independent of functions associated with NKG2D engaging its H60 glycoprotein ligand.

H60 Immunodominance Occurs Even When There Is No Competition In Vivo for Donor APCs

H60 immunodominance over other minor H antigens could be determined by antigenic competition on APCs. To test this possibility, B6 female mice were immunized with spleen cells from BALB.B male or with a mixture of splenocytes from B6 male, H13-congenic male, and

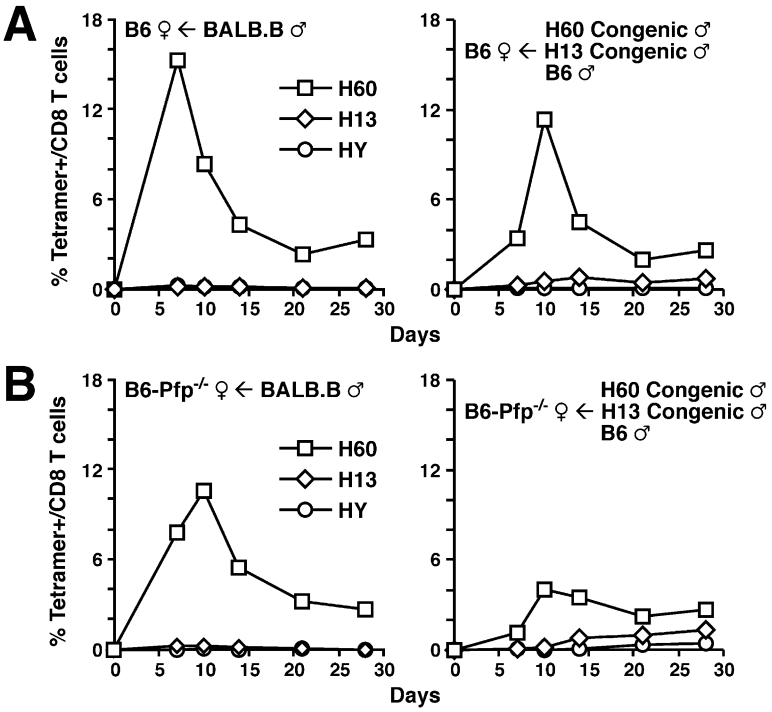


Figure 4. The anti-H60p Response Dominates Regardless of Whether Antigens Are Presented Separately or Together on Donor APCs

(A) B6 mice were immunized with BALB.B spleen cells or with mixture of H60-congenic male, H13-congenic male, and B6 male splenocytes (all induce CD4 help). PBLs pooled from five immunized mice were stained with H60p, H13p, and HYp tetramers. (B) Pfp-deficient B6 mice were immunized and analyzed in the same way.

H60-congenic male mice. (Male APCs were used to ensure a CD4 T cell stimulus.) As expected (Choi et al., 2001), immunization of mice with BALB.B male cells resulted in a substantial expansion of H60p tetramer$^+$ CD8 T cells but not H13p or HYp tetramer$^+$ cells (Figure 4A, left panel). Immunization with cells presenting the three antigens separately reduced the level of H60 immunodominance only slightly in that a small but significant percentage of H13p tetramer$^+$ cells was detected (Figure 4A, right panel). These results suggested that H60 immunodominance was slightly relaxed when the minor H antigens were presented on separate donor APCs, but not enough to account for the immunodominant H60 phenotype.

We then examined whether prolonged survival of donor APCs would influence the immunodominance hierarchy. Perforin-deficient (Pfp$^{-/-}$) mice generated 20$\sim$25-fold reduced CTL activity against minor H antigens (data not shown), which should extend the survival of donor APCs by reducing cytotoxic cell-mediated destruction. Perforin-deficient B6 (B6-Pfp$^{-/-}$) female mice immunized with BALB.B male cells showed a slightly reduced magnitude of their CD8 response to H60p, but there was no concomitant increase in the H13p or HYp responses (compare Figures 4A and 4B, left panels), indicating that prolonged survival of donor APCs did not influence the dominance hierarchy. Moreover, the magnitude of the H60 response was reduced $\sim$3-fold in B6-Pfp$^{-/-}$ mice immunized with a mixture of APCs presenting minor H antigens separately as compared with B6-wild-type mice immunized with the same cells (Figures 4A and 4B, right panels), and the CD8 T cell responses against H13 and HY antigens were unchanged. These results suggested that donor APC destruction by host cytolytic effectors enhances H60 immunodominance, but it does so more efficiently when there is no competition for antigens on donor APCs. Thus, destruction by cytotoxic effectors enhances H60 immunodominance, but not by a mechanism involving antigenic competition on APCs

H60 Generates a Primary CTL Response In Vitro While Other Minor H Antigens Do Not

Conventional wisdom holds that it is not possible to generate minor H antigen-specific CD8 T cells from mixed leukocyte culture (MLC) of naive T cells (Bevan, 1975; Gordon et al., 1975). To attempt to elicit specific CD8 T cells from naive mice, responder spleen cells from naive B6 female mice were cultured in primary (1°) MLC with irradiated stimulator spleen cells from H60-congenic female, H13-congenic female, or B6 male mice. Eight days after 1° MLC (which included supplementation with rIL-2 to insure help), $\sim$6% of the CD8 T cells (range 6$\sim$12% in seven experiments) generated in response to H60-congenic stimulators were H60p tetramer$^+$, while MLCs against H13 and HY stimulators yielded insignificant frequencies of the respective tetramer$^+$ cells (Figure 5A). Since in vitro stimulation of T cells in IL-2 drives cell differentiation into short-lived effector cells (Manjunath et al., 2001; Weninger et al., 2001), we could not rule out the possibility that H13- and HY-specific CD8 T cells might have gone through an undetected clonal burst followed by premature activation-induced cell death. Since IL-15 drives CD8 T cell differentiation into the long-lived memory phenotype, we therefore examined whether H60 would dominate after 1° MLC supplemented with IL-15. Only H60p tetramer$^+$ CD8 T cells were generated, although the percentage was reduced to $\sim$2% (Figures 5B and 5C). Moreover, a high frequency of H60 tetramer$^+$ CD8 T cells was also observed after 1° MLC of naive CD4$^-$CD8$^+$-enriched thymocytes, indicating that H60 immunodominance was

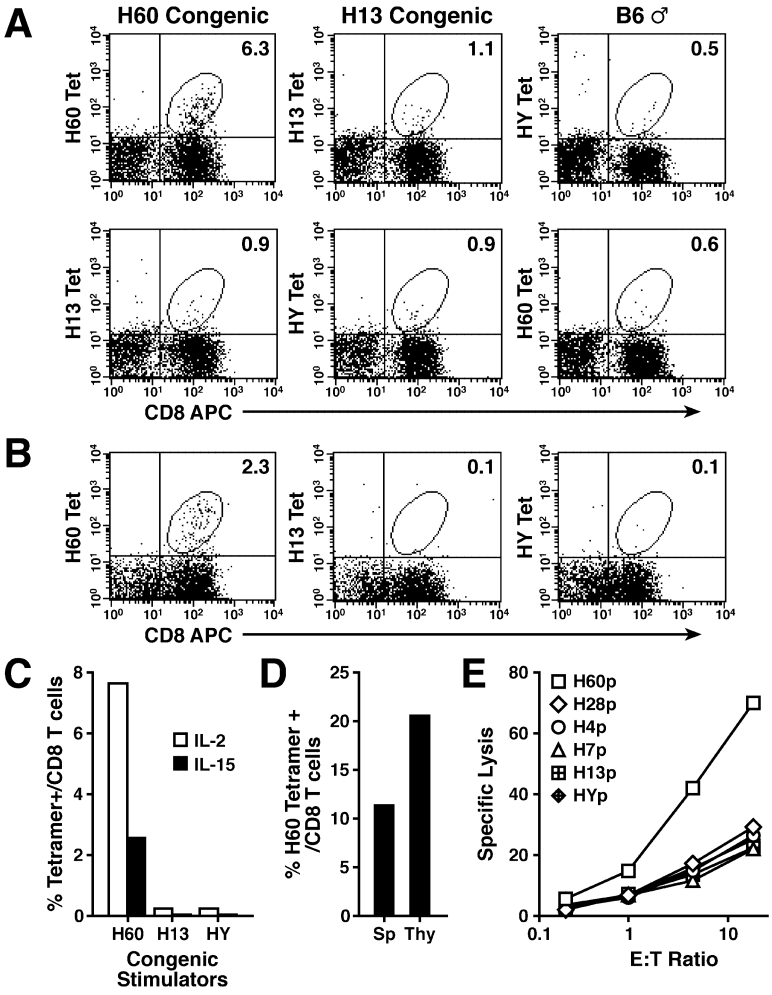


Figure 5. H60 Is the Only Minor H Antigen that Induces Specific T Cells In Vitro from the Naive Pool

(A) Naive splenocytes of B6 female mice were subjected to 1° MLCs with irradiated H60-congenic female, H13-congenic female, or B6 male spleen cells. rIL-2 (50 U/ml) was included at the initiation of culture and supplemented again on day 5. On day 8 of the culture, the CD8 T cells were assessed for H60p, H13p, and HYp tetramer staining.

(B and C) Similar primary MLCs were harvested on day 4, washed, and split into cultures containing rIL-15 (20 ng/ml) (B) or IL-2 (50 U/ml) for 4 additional days (compiled in [C]).

(D) CD8 T cell-enriched thymocytes and splenocytes from naive B6 female mice were cultured in MLC with H60-congenic stimulators and tested as in (A) for H60 tetramer staining. No significant H13 tetramer staining was observed with parallel analysis with H13-congenic stimulators (data not shown).

(E) CML analysis using cells from 1° MLC of naive B6 splenocytes and irradiated stimulators from various congenic mice. Target cells were T2-K^b or T2-D^b pulsed with the appropriate minor H antigen peptides. Only H60p targets showed lysis above irrelevant peptide-pulsed control values (data not shown).

a consequence of thymic selection rather than peripheral expansion events (Figure 5D).

To determine whether a 1° MLC would lead to the generation of CTLs directed against minor H antigens, including H4, H7, and H28, which, like H60, are considered immunodominant in certain experimental settings (Eden et al., 1999; Malarkannan et al., 1998; Nevala and Wettstein, 1996), we stimulated naive B6 female cells in 1° MLC (with added rIL-2) in separate cultures with H60-, H28-, H7-, H4-, or H13-congenic stimulator spleen cells and assessed the specific lytic activity generated. Substantial lytic activity against H60p was generated by stimulation with H60-congenic cells, while only residual, nonspecific NK-like cytotoxicity was detected after stimulation with cells from other minor H-congenic cells (Figure 5E). The combined results suggest that among an array of minor H antigens, all of which were expressed in MLC at physiological levels, H60 was the only one capable of generating appreciable numbers of CTLs in vitro from the naive precursor pool.

To address whether H60 immunodominance of primary response was dependent on expression of the native H60 glycoprotein, we cultured naive B6 female cells in 1° MLC with H60p-, H13p-, or HYp-pulsed irradiated syngeneic spleen cells (Figure 6A). We then analyzed the resulting cultures several days later for tetramer staining and for specific cytolysis. Considerable amplification of H60p tetramer staining CD8 T cells occurred at both physiological and superphysiological peptide levels (Figure 6A). In contrast, H13p tetramer staining CD8 T cells showed minimal expansion and only at superphysiological peptide levels, and no significant HYp tetramer staining cells were detected at any peptide concentration tested. Specific cytolytic activity correlated with the tetramer data (Figure 6B). Stimulation with H60p, in the absence of the native H60 glycoprotein, was therefore sufficient to recapitulate the immunodominance phenomenon. Moreover, H60 immunodominance occurred even when the need for crosspresentation was obviated by direct peptide pulsing of naive B6 responder cells (Figure 6C), indicating that H60 immunodominance was not dependent on crosspresentation.

CD8 T Cells Respond to the H60 Minor H Peptide with High Frequency

The ability of naive T cells to generate a response to H60p in vitro led us to speculate that H60 immunodominance was a consequence of a high number of precursor T cells. We were not able to detect a significant number of H60p tetramer$^+$ CD8 T cells in lymphoid tissues from naive mice (data not shown), suggesting that the frequency of naive cells falls below flow cytometric resolu-

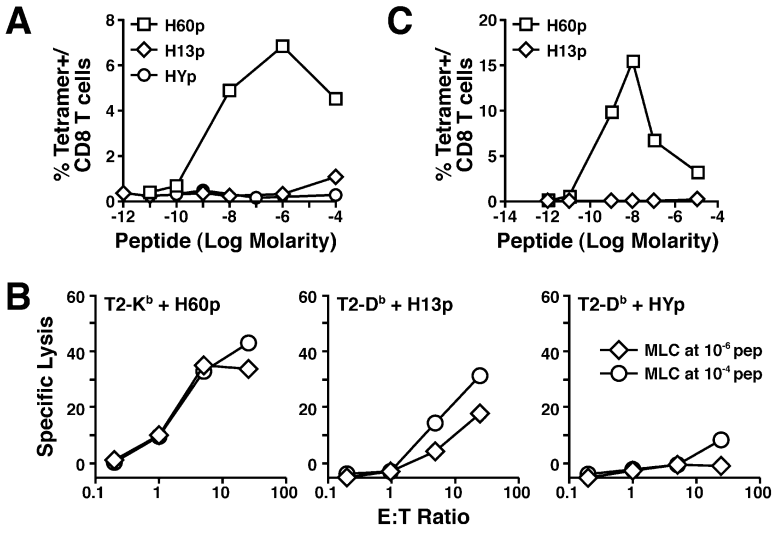


Figure 6. The H60 Peptide Readily Induces Specific CD8 T Cells from the Naive Pool

(A) Tetrameric analysis of naive female B6 splenocyte responder CD8 T cells after culture independently with syngeneic irradiated splenocytes pulsed with various concentrations of H60p, H13p, or HYp. Cultures were supplemented with rIL-2.

(B) CML assay of effector cells from 10^{-4} and 10^{-6} M peptide doses from (A) against peptide-loaded T2-K^b or T2-D^b targets. Nonspecific lysis against VSVp- or F5NPp-loaded T2 targets was subtracted.

(C) Tetrameric analysis of CD8 T cells from cultures of naive female B6 splenocytes directly pulsed with H60p or H13p. Cultures were supplemented with rIL-2.

tion. To determine their precursor frequencies, conventional limiting dilution analysis was performed using B and NK cell-depleted spleen cells from naive female B6 mice as responders and syngeneic spleen cells irradiated and pulsed with H60p, H13p, or HYp as stimulator cells. The minimal CTL precursor frequency (CTLpf) specifically reactive with H60p ranged between 1/11,140 to 1/23,600 (four determinations), while the anti-H13p and HYp CTLpfs were extrapolated to be at least 16-fold lower (two determinations) (Figures 7A and 7C). These results suggested that precursor frequency is sufficient to explain H60 immunodominance. For comparison, the H60p CTLpf was approximately 10∼20-fold lower than that directed against H-2K^d alloantigens (1/890∼1/1,500; Figures 7A and 7C).

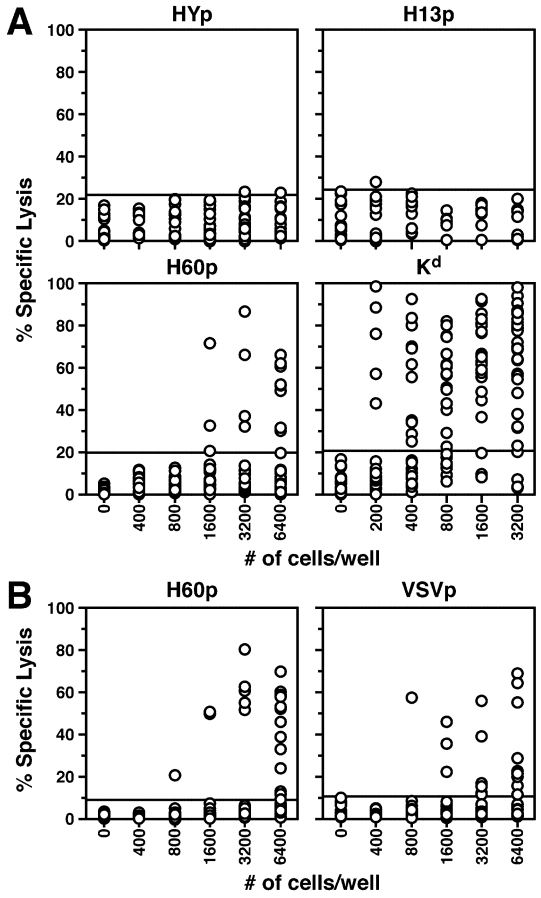
Importantly, the CTLpf dominant for the K^b-restricted VSV peptide (Ono et al., 1998; Van Bleek and Nathenson, 1990) was only slightly lower compared with the anti-H60p CTLpf (Figures 7B and 7C), indicating that immune responses against these two dominant antigens can be induced at a similar intensity. To determine whether other dominant antigens induced high T cell precursor frequency, we cultured naive B6 spleen cells with synthetic immunogenic viral peptide epitopes, including several known to immunodominate after viral infection (Belz et al., 2000). Epitope-specific CTLs were elicited in vitro from naive T cells stimulated with VSVp and PB1-F2$_{62\text{-}70}$, but at reproducibly lower levels compared with H60. No activity was detected against the other epitopes (Figure 7D). This finding is consistent with a high frequency of CTLp directed against H60p, VSVp, and PB1-F2$_{62\text{-}70}$, but not against the other peptides tested, at least under the experimental conditions used.

Discussion

Our studies show that the immune response directed against H60 is atypical of minor H antigens in that it competes well with an allo-MHC response and induces naive T cells to generate a potent CD8 T cell response in vitro. The expression of H60 immunodominance was entirely dependent on cognate help from CD4 T cells, and physiological expression of the native H60 glycoprotein provided neither help nor other activation signals that supported CTL activation. Moreover, H60p in the absence of the H60 glycoprotein was sufficient to recapitulate H60 immunodominance in vitro, suggesting that it is an inherent property of the H60 peptide itself. Interestingly, peptide blocking and mutation studies have suggested that NKG2D interacts with the H60 glycoprotein at a site that includes the immunodominant H60p sequence (Cerwenka et al., 2002). However, considering our inability to link the anti-H60p T cell response with the native H60 glycoprotein, it is unclear how this observation could explain our results. Thus, while there is strong evidence supporting immune enhancing function when H60 is overexpressed in tumor cells (Diefenbach et al., 2000), we were unable to detect any role for the H60 protein other than to provide a processed minor H peptide.

Are there properties of the H60 minor H peptide and its presentation on APCs that could engender immunodominance? H60p is not unusual in its MHC binding avidity, and the estimated number of H60p/K^b complexes on tumor cells is between 5 and 15 copies/cell (Malarkannan et al., 1998), values that are similar to or less than those found for more subordinate minor H epitopes (Eden et al., 1999; Mendoza et al., 1997).

Many explanations for immunodominance center around competition by T cells for their cognate ligands on APCs (Grufman et al., 1999; Kedl et al., 2000, 2002; Perreault et al., 1998). Competition on donor cell APCs played a negligible role in H60 immunodominance. However, accessibility of H60 to recipient APCs facilitated the elaboration of H60p immunodominance in that MHC-disparate BALB/c cells efficiently crossprimed mice for an H60p-specific response, not for an HYp- or H13p-specific response. Furthermore, Pfp-intact mice were more efficiently primed to H60p than were Pfp-deficient mice, suggesting that donor cell lysis may enhance the supply of H60p to recipient APCs. These results indicate that crosspriming (Bevan, 1975) via indirect presentation (Auchincloss and Sultan, 1996) operates efficiently for H60p but not for minor H antigens such as HY (Millrain



C

Stimulating Ag		Reciprocal of frequency	r^2
HYp	n = 2	–	–
H13p	n = 2	–	–
H60p		11,148	0.98
		23,640	0.94
		19,342	0.99
		13,550	0.93
VSVp		18,315	0.99
		49,751	0.86
K^d		1,515	0.97
		891	0.95

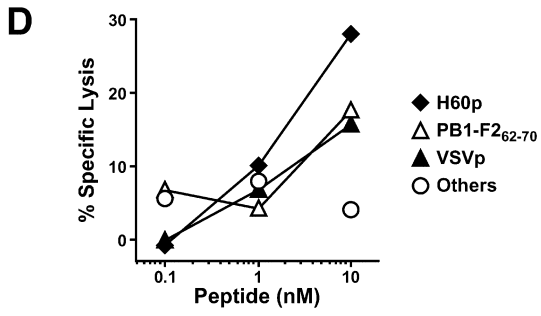


Figure 7. CTLpf Analysis of Naive T Cells

(A and B) Limiting numbers of B and NK cell-depleted spleen cells from naive B6 female mice were cultured in micro-MLC with splenocytes loaded with 50 μM of peptide or with allo-H-2K^d (B6-NOD-H2^{g7}) splenocytes. On day 7–8, each microwell was split and assayed for cytolysis of the cognate target and an irrelevant control target. Representative data of % specific cytolysis of individual microwells determined after subtraction of irrelevant target lysis are shown. Indicated cognate targets (irrelevant targets): HYp-pulsed T2-D^b (F5NPp-pulsed T2-D^b); H13p-pulsed T2-D^b (F5NPp-pulsed T2-D^b); H60p-pulsed T2-K^b (VSVp-pulsed T2-K^b); P1.HTR (RMA); and VSVp-pulsed T2-K^b (H60p-pulsed T2-K^b).

(C) Estimates of antigen-specific CTLpf for each mouse analyzed. CTLpfs of anti-HYp and anti-H13p fell below the limit of resolution of the assays (n = 2, two determinations).

(D) Naive B6 mouse splenocytes were pulsed with the indicated concentrations of synthetic peptides, cultured as in Figure 5C, and tested in a CML against T2-K^b and T2-D^b targets pulsed with 100 nM cognate and noncognate peptide. Values shown are the maximum mean lysis ± SD at E/T 25:1 of target cells pulsed with the same peptide used in the MLC minus lysis against targets pulsed with the noncognate peptide. Others, maximum lysis after pulsing with PA$_{224-233}$, PB1$_{703-711}$, PB2$_{198-206}$, NS2$_{114-121}$, M1$_{128-135}$, or H13p. The data are representative of two independent experiments.

et al., 2001). However, it is unlikely that differential efficiency of crosspriming is the explanation because H60 immmunodominance was recapitulated when naive responder cells were directly pulsed with minor H peptides. More likely, the ability of H60 to be detected by crosspriming is a manifestation of the overall magnitude of the immune response against this antigen.

Our results suggest that H60 immunodominance is an inherent property of the naive precursor pool. This is manifested as an abnormally high frequency of H60p-specific CD8 T cells, which enables naive T cells to generate a robust T cell response in vitro. However, bolstering the frequency of anti-H13 T cells by preimmunization did not reverse H13's subordinance to H60 (Choi et al., 2001), indicating that numbers alone are not sufficient to confer immunodominance. Quantitative and qualitative variation in the naive precursor pool are likely to be determined differences in the extent to which positive or negative thymic selection occurs (Correia-Neves et al., 2001; Vidal et al., 1996). If self-peptides have sufficient structural similarity to foreign peptides, they could act as partial agonists during negative thymic selection, thus reducing the size and avidity of the precursor pool and resulting in subordination of immune response to the foreign epitope (Choi et al., 2001). The great majority of identified autosomal and mitochondrally encoded minor H antigens arise as a consequence of allelic variation within a self-MHC bound peptide and thus have allelic partial agonists that are likely to negatively select TCRs with high avidity for the foreign minor H antigen counterpart. Human *SMCY*-encoded HY antigens and mouse *Smcy*- and *Uty*-encoded HY antigens possess highly sequence divergent "pseudoallelic" *Smcx* and *Utx* X chromosome counterparts (Greenfield et al., 1996; Meadows et al., 1997; Scott et al., 1995; Wang et al., 1995); the human HY antigens are immunodominant (Mutis et al., 1999), but for unknown reasons the mouse HYs are not. H60, and H28, are immunodominant and alloantigenic because B6 mice fail to express the *H60* and *H28* genes and thus lack any allelic counterpart (Malarkannan et al., 2000, 1998). There is an unusually high frequency of thymic and peripheral CD8$^+$ precursor T cells specific for the dominant melan-A/MART-1 tumor antigen (Pittet et al., 1999; Zippelius et al., 2002). Melan-A/MART-1 expression is normally limited to melanocytes (Pittet et al., 1999). The dominant

MAGE-A, *BAGE*, and *GAGE* antigens are normally expressed only in testes germ cells (van der Bruggen, 2000). Immunodominance could therefore be dictated by the extent to which their naive repertoire is unencumbered by negative thymic selection.

However, it is perplexing that among an assortment of viral peptides tested, only the dominant VSVp epitope and the subordinate epitope PB1-F2$_{61-70}$ evoked a robust response from naive T cells (Figure 7E). While the positive results with VSVp are consistent with a robust precursor repertoire, it is not known why reportedly dominant peptide epitopes, such as PA$_{224-233}$ (Belz et al., 2000), repeatedly failed to induce a primary response. Natively processed PB1-F2$_{61-70}$ is usually subdominant because it is processed inefficiently by immunoproteasomes (Chen et al., 2001); our results suggest that its place in the hierarchy shifts upward when its expression is not constrained by inefficient antigen processing. Thus, while immunodominance is multifactoral (Yewdell and Bennink, 1999), our overall studies document considerable variation in the naive precursor repertoire and predict that this variation is an important first step in establishing the immunodominance hierarchy.

The alloantigenic complexity when tissues are transplanted between genetically nonidentical individuals has the potential to be enormous, certainly much more than immune responses to viruses and other more genomically constrained organisms. The fact that the B6 anti-H60p response prevails to the extent that it can account for almost 7% of the allogeneic CD8 T cell response to BALB/c alloantigens (Figure 2) is remarkable given that H60p is only one of potentially thousands of novel peptides naturally presented by MHC proteins (Luckey et al., 2001). Conversely, this high frequency also suggests that the diversity of the alloreactive T cell peptides functionally capable of inducing significant clonal bursts might be much more limited than would be expected by peptide diversity alone. From this enormous antigenic complexity, H60 emerged as being superdominant. Understanding why has considerable implications for tissue transplantation and vaccine design.

Experimental Procedures

Mice, Immunization, and Skin Grafting

Standard C57BL/6J (B6), C.B10-H2^b/LiMcdJ (BALB.B: *H-2^b*), (B6/J X BALB/cByJ) F1 (*H-2^{bxd}*), B10.D2/SnJ (*H-2^d*), B10.BR/SgSn (*H-2^k*), BALB/cByJ (*H-2^d*), and B6-background Perforin-deficient (*Pfp^{Tm1Sdz}*) mice were obtained from the JRS production facility of The Jackson Laboratory. Congenic strains B6.C-*H60^c*/Dcr (Choi et al., 2001), B10.CE-*H13$^bA^w$*(30NX)/Sn, B10.129-*H46$^bH47^b$*(21M)/Sn, B6.C-*H28^c*/By, B10.C-*H7^c*(47N)/Sn, and B6.NOD-H2^{g7} mice (kindly provided by David Serreze, The Jackson Laboratory) were bred and maintained in The Jackson Laboratory Research Colonies. All mice were 8 to 14 weeks of age. For immunization, female B6 mice were injected intraperitoneally with 2×10^7 nonirradiated spleen cells.

Cell Lines

Tumor cell lines T2-D^b, T2-K^b, RMA (H-2^b), and P1.HTR (H-2^d) were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 5% FBS (Hyclone, Logan, UT). The H60^b-specific H-2K^b-restricted CTL line SP/H60, the H13^b-specific H-2D^b-restricted CTL line SP/H13, and the HY (Uty)-specific H-2D^b-restricted CTL clone CTL-10 have been described (Choi et al., 2001). CTL lines were maintained by weekly restimulation with appropriate stimulator cells and 50 U/ml of rIL-2 (Roopenian et al., 1983).

CML Assay

A modified ^{51}Cr release assay has been described (Roopenian et al., 1983). For Concanavlin (Con A)-treated targets, splenocytes from various mice were cultured for 48 hr in the presence of Con A (100 μg/ml). For peptide-loaded targets, ^{51}Cr-labeled T2-D^b and T2-K^b cells or syngeneic and congeneic splenocytes were incubated with 100 nM synthetic peptides for 30 min at 37°C, washed twice with PBS to remove unbound peptide, and then incubated at 37°C with effector cells in V-bottom plates at various E/T ratios. Lysis of target cells was measured as specific cytolysis, based on the level of ^{51}Cr released into the supernatant relative to spontaneous and maximal ^{51}Cr, and is reported as the mean of triplicate wells. Synthetic peptides H60p LTFNYRNL, H13^{b}p SSVIGVWYL, HYp Uty:WMHHNMDLI (Greenfield et al., 1996), H28p ILENFPRL (Malarkannan et al., 2000), H4^{b}p SGIVYIHL (S. Malarkannan and D.C.R., unpublished data), H7^{b}p KAPDNRDTL (S. Malarkannan and D.C.R., unpublished data), VSVp RGYVYQGL (Van Bleek and Nathenson, 1990), and F5Np$_{366-374}$ ASNENMETM (Belz et al., 2000) were produced, chromatography-purified, and mass spectometry-verified by Research Genetics Inc. (Huntsville, AL). PA$_{224-233}$ SSLENFRAYV, PB1-F2$_{62-70}$ LSLRNPILV, PB1$_{703-711}$ SSYRRPVGI, PB2$_{198-206}$ ISPLMVAYM, NS2$_{114-121}$ RTFSFQLI, and M1$_{128-135}$ MGLIYNRM were a kind gift of J. Yewdell (N.I.H.). H60p, H28p, VSVp, PB1$_{703-711}$, NS2$_{114-121}$, PB2$_{198-206}$, and M1$_{128-135}$ are K^b binding peptides. H13p, H4p, H7p, HYp, F5Np$_{366-374}$, PA$_{224-233}$, and PB1-F2$_{62-70}$ are D^b binding peptides.

Intracellular IFN-γ Assay

Modified intracellular IFN-γ staining analysis has been described (Choi et al., 2001). In brief, cells from B6 anti-CB6F1 and anti-BALB.B MLCs were harvested on d11 of MLC. One million responder cells were then cultured with stimulator cells in 96-well round bottom plates (Costar, Cambridge, MA) in DMEM (0.2 ml/well) for 2 hr. The stimulators were RBC- and CD8-depleted splenocytes from various allogeneic and syngeneic mice. After 2 hr restimulation, brefeldin A (Sigma, St. Louis, MO) was added to a final concentration of 10 μg/ml and cells were incubated an additional 4 hr. The cells were then surface-stained with FITC-conjugated anti-CD8 mAb, washed, fixed with 1% paraformaldehyde in PBS, and then incubated with PE-conjugated anti-IFN-γ mAb (PharMingen, San Diego, CA) diluted in 0.1% saponin (Sigma, St. Louis, MO) in PBS. The stained cells were analyzed by flow cytometry with live cell gating.

Flow Cytometry and Cell Purification

RBC-lysed fresh PBLs from immunized mice or cells from MLC were incubated at 4°C for 50 min in staining buffer (1 × PBS with 0.1% BSA and 0.1% sodium azide) containing PE-labeled H60p/K^b, H13^{b}p/D^b, and HY-Utyp/D^b tetramers (Choi et al., 2001) and saturating amounts of APC-conjugated anti-CD8 mAb (PharMingen) and FITC-conjugated CD11a mAbs (M1/70; The Jackson Laboratory). The stained cells were analyzed using a FACSCalibur equipped with CellQuest software (Becton Dickinson, San Diego, CA). Thymic and spleen cells were negatively depleted of CD4$^+$ and B220$^+$ cells using magnetic bead separation methodology (Dynal Biotech, Oslo).

MLC

For MLC of cells from naive female B6 mice, 2.5×10^7 responder splenocytes were cultured with 3.5×10^7 2000 cGY irradiated splenocytes from congenic mice or with B6 splenocytes loaded with H60p, H13p, or HYp. For peptide loading, unless otherwise indicated, splenocytes were incubated with at 500 nM synthetic peptides in medium for 30 min at 37°C and washed twice with PBS to remove unbound peptide. The MLCs were cultured in 10 ml of DMEM-5% FBS medium containing rIL-2 (50 U/ml) for 5 days and fed with additional rIL-2 (50 U/ml), and harvested on d8 of culture. MLC of cells from in vivo immunized mice was similar to that described above except that rIL-2 (50 U/ml) was added after 2–3 days of culture and the cells were harvested on d5–6.

Limiting Dilution and CML Assays

The limiting dilution assay is a modified version of that described by MacDonald et al. (1980). Spleen cells from B6 female mice were depleted of NK- and B cells using magnetic column and were used as responder cells. Varying numbers of the depleted responder cells

(32 microwells/responder cell concentration) were cultured for 5 days in 96-well flat-bottomed microtiter plates (Costar Corp) in the presence of 5×10^5 irradiated stimulator splenocytes in 0.2 ml of DMEM-10% FBS medium supplemented with rIL-2 (50 U/ml). B6 splenocyte stimulator cells were loaded with 50 μM of H60p, H13p, or HYp by incubation for 40 min at 37°C, washed, and then irradiated with 2000 cGY. Irradiated allogeneic B6.NOD-$H2^{g7}$ stimulator splenocytes (disparate from B6 at H-2K) were used where indicated. On d5, each microculture was fed 50 μl of fresh DMEM-FBS containing rIL-2 (200 U/ml). After 4 additional days of culture, 100 μl of each microculture was transferred into two V-bottom microwells and assayed for cytolysis against cognate and irrelevant antigens. T2-K^b or T2-D^b cells loaded with synthetic peptides (100 nM) or syngeneic RMA or allogeneic P1.HTR target cells were added to effector cells to a final volume of 200 μl/well. The plates were incubated for 6 hr at 37°C, 125 μl of supernatant was then harvested, and supernatant radioactivity was determined. Positive wells were defined as wells whose ^{51}Cr-release exceeded the mean spontaneous release (32 replicates) by >3 SD, after subtraction for nonspecific lysis ^{51}Cr-release (targets loaded with irrelevant peptides or syngeneic tumor target cells). CTLpfs were calculated by linear regression and Poisson distribution X^2 analysis according to Taswell (1984).

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