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Molecules and Cells

A Single-Amino-Acid Variant of the H60 CD8 Epitope Generates Specific Immunity with Diverse TCR Recruitment

Su Jeong Ryu, Ji Yeong Jeon, Jun Chang[1], Thomas J. Sproule[2], Derry C. Roopenian[2], and Eun Young Choi*

TCR of CD8 T cells recognizes peptides of 8-9 amino acids in length (epitope) complexed with MHC class I. Peptide ligands differing from an epitope by one or two amino acids are thought to modulate the immune response specific to that epitope. H60 is a minor histocompatibility antigen for which the specific CD8 T-cell response dominates during alloresponse after MHC-matched allogeneic transplantation. In the present study, we developed a transgenic mouse (designated H60H Tg) expressing a variant of H60, designated H60H, in which the arginine residue at position 4 of the H60 epitope sequence (LTFNYRNL) is replaced by a histidine residue (LTFHYRNL). Immunization of female C57BL/6 mice with splenocytes from male H60H Tg induced a CD8 T cell primary response and memory response after re-challenge. The response was CD4 help-dependent, demonstrating the potency of H60H as a cellular antigen. The response induced by the H60H cellular antigen was comparable to that induced by H60 in its peak magnitude and overall immune kinetics. H60H challenge recruited broadly diverse TCRs to the specific response, shaping a TCR repertoire different from that of the natural H60 epitope. However, some of the TCRs did overlap between the H60H- and H60-specific CD8 T cells, suggesting that H60H might modulate the H60-specific response. These results may provide a basis for the modulation of the H60-specific CD8 T-cell response.

INTRODUCTION

Minor histocompatibility antigens are peptide fragments naturally processed from polymorphic proteins that generate epitope presented by MHC I and II (Roopenian et al., 2002). After allogeneic transplantation, allogeneic minor H antigens (minor histocompatibility peptide/MHC complexes) are recognized as foreign antigens by CD4 and CD8 T cells, resulting in graft rejection or graft-versus-host disease (GVHD). In particular, in MHC-matched allogeneic transplantation, mismatches in the donor and recipient minor histocompatibility antigens (MiHAs) increase the risk of transplantation failure.

In the case of multiple MiHA mismatches, such as in the B6 anti-BALB.B setting, the specific CD8 T-cell response to the MiHA H60 dominates over the responses specific to other MiHAs (Choi et al., 2001; 2002a; 2011). H60, which is expressed only in hematopoietic cells (Malarkannan et al., 1998) is considered a target antigen for modeling tumor therapy that enhances the graft-versus-leukemia (GVL) effect while suppressing GVHD.

The H60-specific CD8 T-cell response is CD4-dependent both in its induction of the primary response and in the expansion of its memory cells (Jung and Choi, 2007; Ryu et al., 2009). Immunization of female C57BL/6 (B6) mice with male H60 congenic (H60C) splenocytes induces the H60-specific CD8 T-cell response with the help of CD4 T cells that recognize a Y-chromosome-derived HY-Dby epitope. A second immunization leads to a strong memory expansion of the H60-specific CD8 T cells. The help is mediated via an interaction between CD40 and CD40L and is provided through cognate recognition of antigen-presenting cells (APCs) by CD4 and CD8 T cells (Ryu et al., 2009).

The H60-specific response recruits diverse Vβ families, including Vβ4, Vβ5, Vβ8, and Vβ11 (Choi et al., 2001). Spectratyping analysis has demonstrated the inclusion of various spectratypes within each of the recruited Vβ families, and clonotyping analysis has demonstrated that the H60-specific T-cell receptor (TCR) repertoire includes a wide range of TCRs (Choi et al., 2009). Further analysis through characterization of H60-specific cytotoxic T lymphocyte clones established *in vitro* has demonstrated that the repertoire of H60-specific CD8 T cells encompasses T cells with various levels of cytotoxic activity, proliferative ability, and avidity for the H60 peptide/H-2K^b complex (Jeon et al., 2011). The large repertoire of T cells recruited to the H60-specific response has been ascribed to the presence of their precursors at a high frequency in the naïve T cell pool, which is a proposed mechanism for the dominancy of the H60-specific CD8 T-cell response (Choi et al., 2002b).

The TCRs of CD8 T cells recognize peptides 8 or 9 amino acids in length bound in the groove of MHC class I proteins. Because no more than three amino acids of the MHC-bound peptide directly interact with the TCR, T cells are highly cross-

Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul 110-799, Korea, [1]Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Korea, [2]The Jackson Laboratory, Bar Harbor, ME, USA
*Correspondence: eycii@snu.ac.kr

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reactive, responding to more than one epitope (Borghans and De Boer, 1998; Mason, 1998; Wilson et al., 2004; Wucherpfennig et al., 2007). On the other hand, each TCR is specific for only a small fraction of possible peptides and possesses a different affinity or avidity for given agonist and partial agonist peptides. Partial agonists usually differ from agonist peptides in the identities of only one or two amino acid residues, have a decreased avidity for the given TCR, and are required in higher concentrations to induce T-cell proliferation and cytokine production (Kersh and Allen, 1996). Such peptide variants (known as "altered peptide ligands") are thought to be involved in positive selection during thymic development (Ashton-Rickardt et al., 1994; Sebzda et al., 1994) and might regulate peripheral responses by modulating the TCR interaction with agonist peptides.

The B6 mouse strain does not express H60. The corresponding lack of negative thymic selection of T cells with high affinity for H60 epitope has been hypothesized to be a basis for the high precursor frequency (Choi et al., 2002b), but this hypothesis currently lacks direct supporting evidence. In addition, no investigations into positive thymic selection of H60-specific CD8 T cells using altered peptide ligands that mimic self-protein have yet been reported.

When bound to the MHC class I H-2K^b molecule, the natural H60 epitope LTFNYRNL recruits diverse TCRs to the specific response. Experiments using altered H60 peptide ligands might be helpful in understanding the thymic selection process for H60-specific CD8 T cells and in controlling the specific response. In addition, the effect of a single amino acid substitution in the H60 epitope on the composition of the TCR repertoire is unknown. To address these issues, we generated a transgenic mouse expressing a single-amino-acid variant of H60, designated H60H, and analyzed its immunogenicity and the composition of the TCR repertoire it recruits to the specific CD8 T-cell response. Because position 4 (p4) of the H60 epitope is thought to be important for the TCR interaction (Boesteanu et al., 1998; Garcia et al., 1996; Strausbauch et al., 1998), we elected to replace the naturally occurring arginine residue at p4 with histidine, thereby creating the H60 variant H60H, based on peptide screening with a cytotoxicity assay (manuscript in preparation). Using splenocytes from H60H transgenic (H60H Tg) mice, we induced the CD8 T-cell response in B6 mice and compared the immune kinetics and the diversity of the responding TCRs with those for the natural H60 epitope (hereafter, H60N). Our results demonstrate that H60H could be used as an immunogenic epitope that recruits diverse TCRs to the specific response. In addition, we identified some overlap in TCR usage between CD8 T cells responding to H60H vs. H60N stimulation, suggesting that the H60H Tg mouse will be useful for understanding the selection process and control of CD8 T cells specific for H60.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice from Jackson Laboratory (USA) were used for immunization. Transgenic mice expressing the H60H variant (H60H Tg mice) were obtained by microinjecting DNA into the fertilized eggs of B6 mice. The injected DNA contained a complete but altered, FLAG-tagged, H60 cDNA sequence under control of the chicken β-actin promoter and a cytomegalovirus enhancer; the altered sequence encoded an asparagine (N) → histidine (H) substitution at p4 of the epitope sequence. The H60H Tg mice were maintained at Jackson Laboratory or at the Center for Animal Resource Development of Seoul National University College of Medicine in Korea, as were B6.C-*H60^C*/DCR mice and H60 transgenic (H60N Tg) mice expressing H60 protein under the control of the chicken β-actin promoter (Russell et al., 2011)

Immunization

To induce a specific CD8 T-cell response, splenocytes (2×10^7 cells) from H60 congenic (H60C), H60N Tg, or H60H Tg mice were injected intraperitoneally (i.p.) into female B6 mice. A secondary response was induced by i.p. injection of the splenocytes into B6 mice 30-50 days after their initial immunization

Antibodies and flow cytometry

For flow cytometry, peripheral blood lymphocytes (PBLs) remaining after red blood cell lysis or splenocytes were stained with H60H- or H60N-tetramer-PE in addition to FITC-conjugated CD11a monoclonal antibody (mAb) (M17/4; eBioscience, USA) and APC-conjugated anti-mouse CD8 mAb (53-6.7; eBioscience). Vβ analysis was performed by staining splenocytes with PE-conjugated H60H or H60N tetramers, APC-conjugated anti-mouse CD8 mAb, and FITC-conjugated anti-Vβ mAbs. The antibodies used for Vβ typing were purchased from BD Pharmingen (USA) as follows: Vβ2 (B06), Vβ3.1 (KJ25), Vβ4 (KT4), Vβ5.1/5.2 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8,1/8.2 (MR5-2), Vβ8.3 (1B3.3), Vβ9 (MR10-2), Vβ10 (B21.5), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (14-2), and Vβ17 (KJ23).

Purification of H60 specific CD8 T cells and clonotyping

CD8 T cells were purified from splenocytes of primed mice using a CD8 T-cell isolation Kit (Miltenyi BioTec Inc., USA). H60H- or H60N-tetramer-binding cells were re-sorted from the purified CD8 T cells. Isolated H60H- or H60N-tetramer-binding CD8 T cells confirmed to be at least 85% pure by flow cytometry were then used for total RNA extraction. The total RNA was processed for cDNA synthesis using Cβ1 primer (5′-TAG CCATCACCACCAGTGTACTG-3′), and the synthesized cDNA was amplified by PCR using Cβ2 3′ primer (5′-GTGTACTG ACAAGCACAGCATACA-3′) and the Vβ-specific 5′ primers described in our previous report (Choi et al., 2009). The PCR products were cloned into pGEM-T easy vector (Promega, USA), and the resulting constructs were transformed into *E. coli* strain DH10B using electro-transformation. Plasmid DNAs with inserts were prepared from the transformants, and the CDR3 sequence of the TCR Vβ regions was determined.

RESULTS

Generation of a transgenic mouse ubiquitously expressing the H60H variant of epitope H60

To generate a transgenic mouse expressing a single-amino-acid variant of H60 (H60H) in a B6 background, a DNA fragment containing an altered, FLAG-tagged, full-length H60H cDNA sequence under control of the chicken β-actin promoter was microinjected into B6 mouse eggs. The altered cDNA encoded an asparagine (N) → histidine (H) substitution at p4 of the CD8 epitope, changing the sequence from LTFNYLN (H60N) to LTFHYLN (H60H). Three lines of transgenic mice with PBLs positive for genomic DNA by PCR screening were obtained. One line was selected for further use because it yielded an H60H reverse transcription (RT)-PCR band comparable in density to the H60N band obtained from H60C mouse PBLs (data not shown). The splenocytes from this line, designated H60H Tg, were used as cell-based immunogens for the induction of the H60H-specific CD8 T-cell response via i.p.

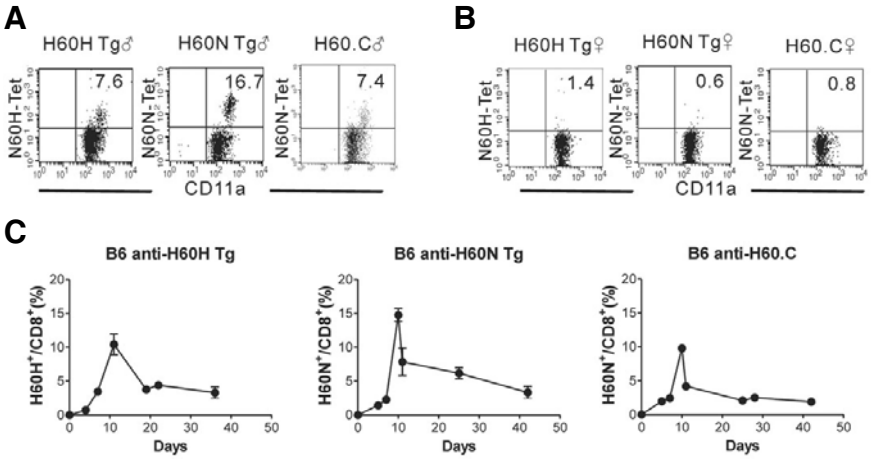

Fig. 1. Flow cytometric evaluation of the H60H-specific CD8 T-cell response. Female B6 mice were i.p. injected with splenocytes from male (A) or female (B) H60H Tg, H60N Tg, or H60C mice. PBLs taken from the immunized mice on day 10 post-immunization were stained with H60H or H60N tetramer-PE in combination with APC-conjugated anti-CD8 mAb and FITC-conjugated anti-CD11a mAb for flow cytometry. Representative flow cytometry data obtained after gating on CD8 cells are shown, with percentages of H60H or H60N tetramer-binding CD8 T cells noted. (C) Female B6 mice were i.p. injected with splenocytes from male H60H Tg (left), H60N Tg (center), or H60C (right) mice. PBLs were taken from the immunized mice at the indicated number of days post-immunization, stained, and analyzed by flow cytometry as in (A) and (B) for the percentages of H60H or H60N tetramer-binding CD8 T cells. Data shown are representative of at least three independent experiments.

injection into B6 mice.

To examine the expression and immunogenicity of the H60H transgenic protein, we immunized five female B6 mice with splenocytes from a male H60H Tg mouse and assessed them for the development of an H60H-specific CD8 T-cell response. As controls, female B6 mice were immunized with splenocytes from male H60C mice or from male H60N Tg mice, which express the native H60 protein under control of the β-actin promoter (Russell et al., 2011). PBLs periodically collected from the immunized B6 mice of each group were stained with H60N or H60H tetramers and analyzed by flow cytometry to detect H60N- or H60H-reactive CD8 T cells (Fig. 1). The flow cytometry results confirmed that H60H tetramer-binding CD8 T cells were present in the blood of B6 mice immunized with male H60H Tg mouse splenocytes. The peak frequency was observed on day 10 post-immunization, when 8% to 14% of the CD8 T cells were H60H tetramer-binding cells (Fig. 1A). The peak values were very similar to those obtained for H60N tetramer-binding CD8 T cells detected in the blood of B6 mice immunized with male H60N Tg or H60C mouse splenocytes. The overall kinetics of the H60H-specific CD8 T-cell response was similar to that of the H60-specific response induced by immunization with male H60N Tg or H60C mouse splenocytes (Fig. 1C). These observations confirmed the expression of the H60H transgene and ability of the LTFHYLN variant peptide to act as an epitope, inducing a readily detectable level of CD8 T-cell response.

H60H or H60N tetramer-binding CD8 T cells were not detected in the blood from female B6 mice immunized with splenocytes from female H60H Tg, H60N Tg, or H60C splenocytes (Fig. 1B). In this case, the lack of an H60H- or H60N-specific CD8 T-cell response was attributable to the lack of a CD4 T-cell response, which requires the recognition of the HY-Dby epitope originating from the male H60H splenocyte. This help-dependence is characteristic of the CD8 T-cell response to a non-inflammatory cellular antigen. Therefore, in subsequent experiments, only splenocytes originating from male mice were used for immunization.

These results demonstrated that splenocytes from H60H Tg mice provide a new cell-based CD8 epitope. Furthermore, they showed that the H60H epitope is as immunogenic as the natural H60N epitope originating on splenocytes of H60N Tg or H60C mice.

Induction of an H60H-specific CD8 T-cell memory response and detection of the cross-reactive population

We next examined whether i.p. injection of H60H Tg splenocytes would induce a memory response in female B6 mice previously challenged with the same splenocytes. Using flow cytometry, we examined the PBLs of H60H Tg splenocyte-immunized mice after re-challenge with H60H Tg splenocytes (Fig. 2A) to determine the kinetics of the memory response (Fig. 2B). We compared this kinetics with that of the H60-specific memory response induced by secondary challenge with H60N Tg or H60C mouse splenocytes. A comparative plot of the frequencies of peripheral blood CD8 T cells positive for H60H or H60N tetramer staining over time demonstrated that re-challenge with splenocytes from H60H Tg mice induced a specific memory CD8 T-cell response (Fig. 2B, left panel), just as re-challenge with splenocytes from H60N Tg or H60C mice induced a specific memory response (Fig. 2B, middle and right panels). The peak frequencies (50-70% of CD8 T cells in blood) were observed on day 7 post-secondary immunization and were 5 times higher than the peak frequencies after primary challenge in all three cases (Figs. 2A and 2B).

During our investigation of the H60H-specific memory response, we noticed that some CD8 T cells in blood from H60H splenocyte re-challenged mice were positive for H60N tetramer staining (4.1% of peripheral blood CD8 T cells; Fig. 2A, lower left panel). The reverse was also true: H60H tetramer-binding CD8 T cells were detected in up to 3% of the peripheral blood CD8 T cells from H60N splenocyte re-challenged mice (Fig. 2A, upper middle panel). These observations implied that some CD8 T cells from the H60H- or H60-specific responses were cross-reactive but were not detected until after expansion during the memory response. This finding suggested overlap in the TCR usage of CD8 T cells responding to H60H and H60N.

Diverse Vβ families respond to H60H stimulation

The ready detectability of H60H tetramer-binding CD8 T cells in blood during the H60H-specific primary response suggested that either a broad repertoire of T cells with diverse TCRs or a narrow repertoire of T cells with repeated TCRs was involved in the response. Therefore, we examined whether the TCR reper-

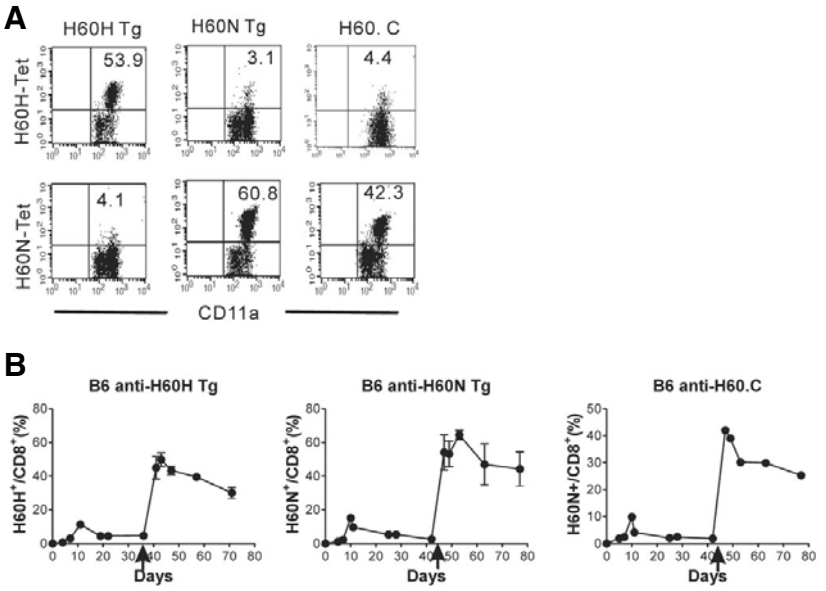

Fig. 2. Flow cytometric evaluation of the secondary response to H60H challenge. Female B6 mice were immunized with splenocytes from male H60H Tg (left panels), H60N Tg (center panels), or H60C (right panels) mice and re-challenged 30-50 days later to induce a secondary response. PBLs taken from the immunized mice were stained with H60H or H60N tetramer-PE in combination with APC-conjugated anti-CD8 mAb and FITC-conjugated anti-CD11a mAb for flow cytometric analysis. (A) Representative flow cytometry data for PBLs taken from the immunized mice on day 7 post-secondary immunization are shown, with percentages of tetramer-binding CD8 T cells noted. (B) PBLs were taken from the immunized mice at the indicated days after primary challenge, stained, and analyzed by flow cytometry for the percentages of tetramer-binding CD8 T cells as in (A). The day of re-challenge is indicated by an arrow. Data shown are representative of at least three independent experiments.

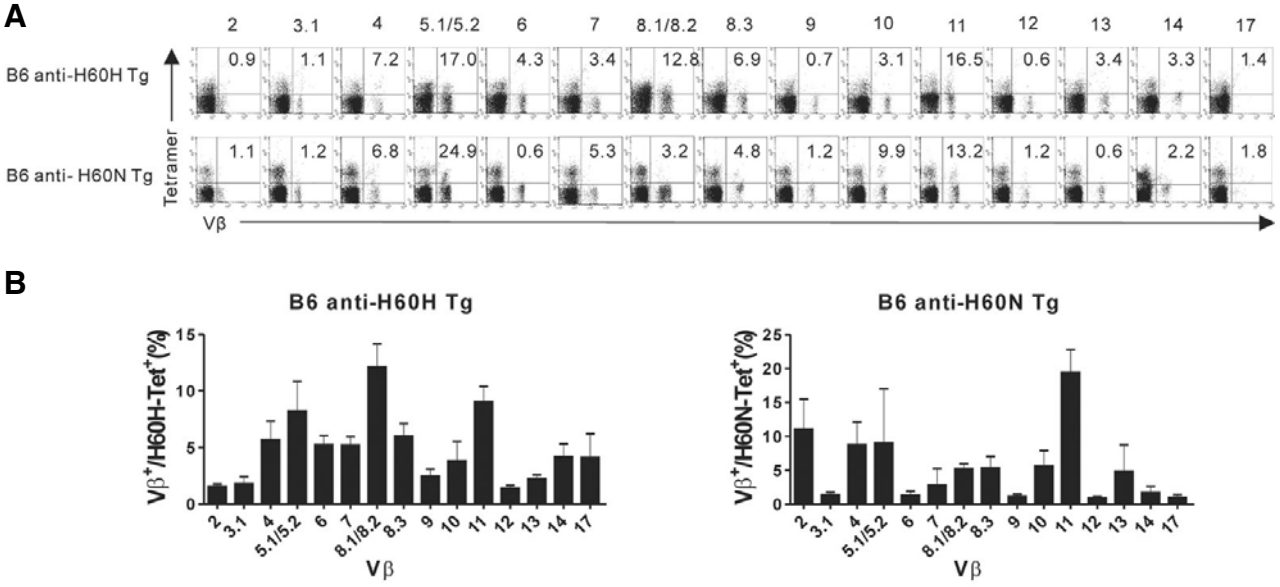

Fig. 3. Vβ analysis of CD8 T cells involved in the H60H-specific response. (A) Ten days after female B6 mice were immunized with splenocytes from male H60H Tg (upper panels) or H60C (lower panels) mice, their splenocytes were harvested, stained with FITC-conjugated Vβ antibodies in combination with APC-conjugated anti-CD8 mAb and PE-conjugated H60H (upper panels) or H60 (lower panels) tetramer-PE, and subjected to flow cytometry. Representative flow cytometry data are shown. Numbers above panels indicate the particular Vβ region analyzed, and numbers within panels indicate the percentages of H60H tetramer-binding (upper panels) or H60N tetramer-binding (lower panels) CD8 T cells positive for the indicated Vβ region. (B) Mean percentages of H60H (left) or H60N (right) tetramer-binding CD8 T cells that were also positive for the indicated Vβ regions. Each data point shown represents the mean ± S.E.M. for 3 immunized mice.

toire participating in the H60H-specific response was as wide as that participating in the response to the natural H60 epitope. In particular, our detection of a minor population of cross-reactive CD8 T cells (Fig. 2A) prompted us to search for overlap in TCR usage for the two responses.

First, we investigated the usage of TCR Vβ regions. Splenocytes from female B6 mice challenged with H60H Tg splenocytes were prepared on day 10 post-immunization, and stained with H60H tetramers and antibodies against specific Vβ regions and CD8 for flow cytometry analysis. Splenocytes from female B6 mice immunized with H60N Tg splenocytes were similarly prepared and stained with H60N tetramers and Vβ and CD8 antibodies for comparison. The flow cytometry analysis showed that the H60H tetramer-positive cells included various Vβ families (Fig. 3A, upper row) and that the diversity of Vβ families participating in the H60H-specific and H60N-specific CD8 T-cell responses was comparable (Fig. 3A, compare both rows). Plotting of the frequencies of occurrence of each Vβ family in the

Table 1. Clonotype analysis of some Vβ regions participating in H60H- or H60N-specific CD8 T-cell responses

Vβ region	B6 anti-H60H		B6 anti-H60N	
	In-frame CDR3 sequences	Unique CDR3 sequences (% of total)	In-frame CDR3 sequences	Unique CDR3 sequences (% of total)
Vβ4	78	17 (21.8%)	48	7 (14.6%)
Vβ8.3	105	36 (34.2%)	38	15 (39.5%)
Vβ11	43	19 (44.2%)	75	22 (29.3%)

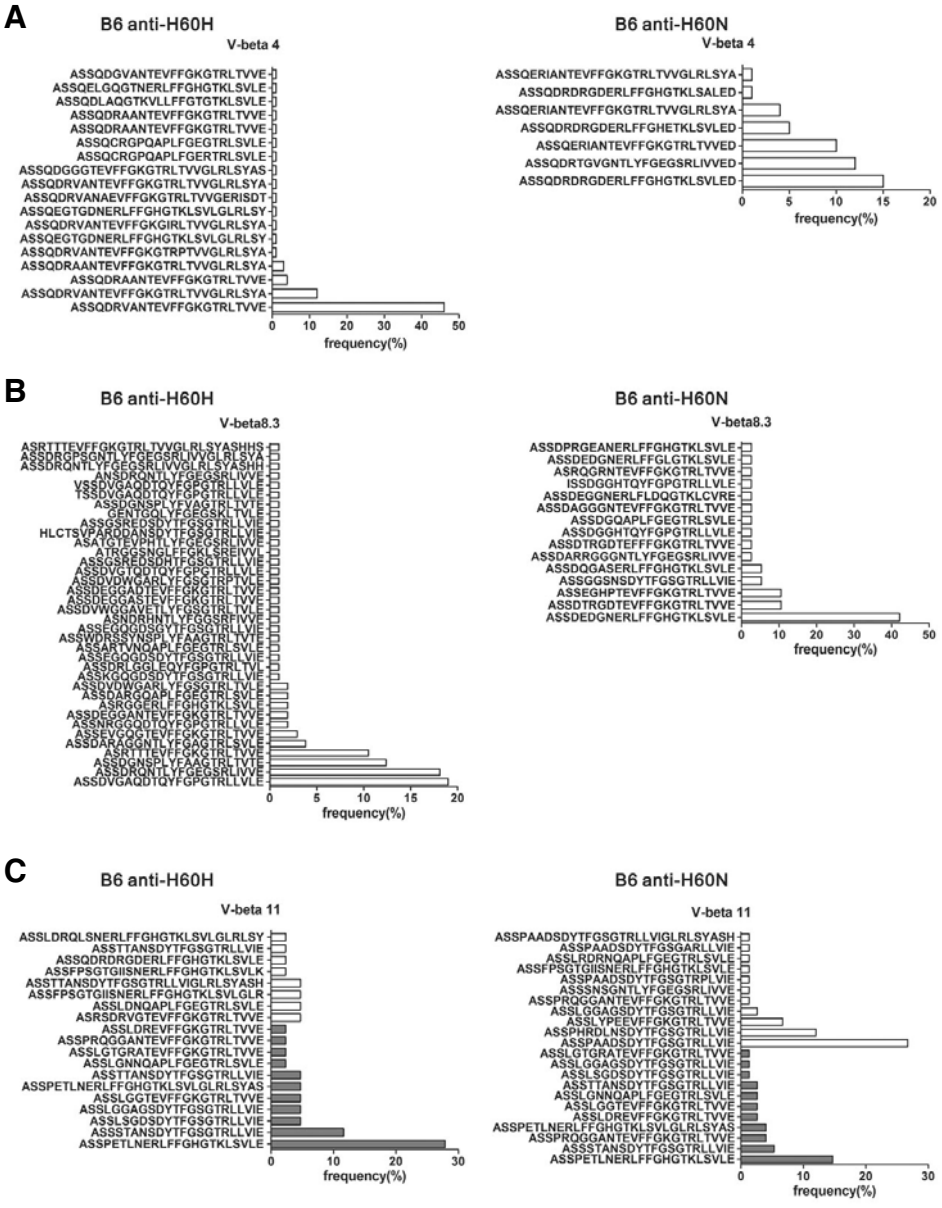

Fig. 4. Clonotypic analysis of H60H-specific CD8 T cells. Ten days after immunization of B6 mice with male H60H Tg or H60N splenocytes, their H60H tetramer-binding (for H60H-immunized mice) or H60N tetramer-binding (for H60N-immunized mice) CD8 T cells were purified by magnetic cell separation. Total RNA extracted from the purified cells was processed for cDNA synthesis, and the synthesized cDNA was amplified by PCR using Cβ2 3′ primer and Vβ4-, Vβ8.3-, or Vβ11-specific 5′ primers (A, B, and C, respectively). The RT-PCR products were subcloned into the pGEM-T vector and sequenced to obtain the predicted amino acid sequences of the Vβ4 (A), Vβ8.3 (B), or Vβ11 (C) CDR3 regions. More than 100 different bacterial transformant colonies were processed for sequencing for each Vβ region. Each unique CDR3 sequence identified in H60H-specific (left panels) or H60N-specific (right panels) CD8 T cells is shown, together with its frequency of occurrence among all the colonies with inframe CDR3 sequences. Gray bars in (C) correspond to those Vβ11 clonotypes found in both H60H-specific and H60N-specific CD8 T cells.

H60H or H60N tetramer-binding CD8 T cells revealed a considerable difference in Vβ usage (Fig. 3B). While Vβ4, Vβ5.1/2, Vβ8, and Vβ11 were dominant families in both the H60H- and H60N-specific CD8 T cells, Vβ6, Vβ7, Vβ14, and Vβ17 were also dominant families in the H60H-specific cells, but Vβ2, Vβ10, and Vβ13 were dominant families in the H60N-specific cells. Since Vβ4, Vβ5.1/5.2, Vβ8, and Vβ11 are frequently used during the H60-specific CD8 T cell response induced by the injection of H60C splenocytes into female B6 mice (Choi et al., 2001), they appear to be commonly used in the CD8 T-cell response to H60-related antigens. Together, our Vβ analysis demonstrates that diverse Vβ families are recruited in the H60H-specific CD8 T-cell response.

Clonotypic diversity of H60H-specific CD8 T cells and minor overlap in TCR usage with H60N-specific CD8 T cells

The observed commonality of certain Vβ families to H60H- and H60N-specific CD8 T cells raised the issue of possible overlaps at the clonotypic level. To compare the CD8 T-cell clonotypes, we sequenced the Vβ CDR3 regions of Vβ4, Vβ8.3, and Vβ11-positive CD8 T cells. Using magnetic cell separation, we purified H60H tetramer-binding CD8 T cells from the splenocytes of H60H Tg splenocyte-immunized mice on day 10 post-immunization and extracted the total RNA. After cDNA synthesis with a Cβ primer, the cDNA was subjected to RT-PCR with a Cβ 3′ primer and Vβ-specific 5′ primers. The RT-PCR products were subcloned into the pGEM-T vector and transformed into *E. coli.*

More than 100 different transformant colonies were picked and processed for sequencing of the Vβ CDR3 region, and all in-frame CDR3 sequences were selected. As a result, unique clonotypes were identified for each Vβ. Of the 78 bacterial colonies with in-frame Vβ4 sequences from H60H-specific CD8 T cells, 17 unique Vβ4 clonotypes were identified (Table 1): the CDR sequence ASSQDRVANTEVFFGKGTRLTVVE was the most frequent clonotype (Fig. 4A). Of the 48 bacterial colonies with in-frame Vβ4 sequences from H60N-specific CD8 T cells, 7 unique Vβ4 clonotypes were identified, but none overlapped with those from H60H-specific CD8 T cells. Similarly, for H60H- and H60-specific CD8 T cells, we identified 36 and 15 unique Vβ8.3 clonotypes, respectively (out of 105 and 38 respective in-frame Vβ8.3 sequences), but none of the clonotypes overlapped between the two responses (Fig. 4B).

In Vβ11 family, we identified 19 and 22 unique clonotypes for the H60H- and H60-specific CD8 T cell responses, respectively (out of 43 and 75 respective in-frame sequences). Eleven of these clonotypes were common to both responses, suggesting that these common clonotypes might be responsible for the observed cross-reactivity between the H60H and H60N epitopes (Fig. 4C). The CDR sequence ASSPETLERLFFGHGTK-LSVE was the most frequently used CDR sequence in H60H-specific CD8 T cells and the second most frequently used CDR sequence in H60-specific CD8 T cells (Fig. 4C).

This clonotyping analysis demonstrated that the H60H- and H60N-specific CD8 T cell responses recruited diverse clones within each Vβ family, and that most of the clonotypes were response-specific. However, some Vβ11 clones were shared by H60H- and H60-specific CD8 T cells. These results suggest that a single amino acid variation in epitope sequence can change the array of recruited CD8 T cell clonotypes and that a minor population of clonotypes may be common to response to both the original and altered epitope sequence.

DISCUSSION

In this study, we demonstrated that H60H, a single-amino-acid variant of the natural H60 epitope, induces a CD8 T-cell response as strong as that induced by H60. The TCRs recruited to the H60H-specific and H60-specific CD8 T-cell responses were similarly diverse, suggesting that H60H is an immunodominant artificial protein.

Researchers often use peptide derivatives or variants to modulate immune responses. Some alterations in peptide ligands can affect the Th1 versus Th2 nature of the response: peptides with a high MHC or TCR affinity skew the response towards the Th1 phenotype, whereas peptides with a low MHC or TCR affinity have the opposite effect (Kumar et al., 1995; Malherbe et al., 2000; 2004). Altered peptides have been used to alter the Th2 T-cell response to the immunodominant peptide in an infection model, raising the possibility that sequence derivatives might be used to prevent infection (Jensen et al., 2009). Various sequence derivatives have also been designed for use in therapeutic intervention in experimental autoimmune diseases (Wauben et al., 1992; 1994). Peptide modifications currently used for these purposes include not only changes in sequence but also post-translational modifications (de Haan et al., 2005). Our characterization of a single-amino-acid variant of H60 may provide basic knowledge needed for the development of measures to intervene in H60-related graft rejection or GVHD.

Most studies of altered peptide ligands have focused on the effects of these ligands on the T cell response to the original peptide. We contend that the host immunity conferred by the variant itself has not been fully appreciated. An understanding of the mechanism of this immunity is important in elucidating the mechanism for modulation of the response to the original natural epitope. Therefore, in the present study, we characterized the immunologic response to an altered peptide (H60H) before examining the effect of the altered peptide on the response to the native peptide (H60N). H60H Tg spleen cells were found to provide a cell-based immunogenic epitope that induced a strong immune response in a CD4 help-dependent fashion. The broad diversity of the TCR repertoire recruited in the H60H-specific response suggests a potential for deviation between the H60H- and H60N-specific responses. The partial overlap in TCR usage between H60H- and H60N-specific CD8 T cells increases the probability that H60H could be used as modulator for H60-specific CD8 T-cell responses.

Altered peptide ligands for the OT-1 TCR epitope have been intensively studied for their role in thymic selection (Ashton-Rickardt et al., 1993a; 1993b). A low-affinity peptide variants of Ova CD8 epitope, partial agonists or antagonist, induces positive selection of OT-1-beraing thymocytes. The affinity window for positive versus negative selection is narrow, and small changes can have big effects on the fates of immature thymocytes (Daniels et al., 2006). We do not know at present whether H60H would induce positive thymic selection of H60-specific CD8 T cells. Examination of the affinities of H60H-reactive and H60N-reactive T cells for both the H60N and H60H ligands in association with MHC I will provide a clue as to the role of H60H in thymic selection.

H60 is an exceptionally dominant transplantation antigen. An H60-specific CD8 T-cell response can be detected even in the presence of a response against allo-MHC antigens (Choi et al., 2002b) and is dominant over responses to other minor but dominant H antigens, such as H28, H4, H7, and HY (Choi et al., 2001). The position of the H60H-specific CD8 T-cell response in the immune hierarchy relative to competing responses to MiHAs remains to be determined, as does the influence of H60H expression in the host on the dominance of the H60-specific CD8 T-cell response.

In summary, we have established a transgenic mouse line expressing a peptide mimic of the natural transplantation antigen H60. This system will be helpful in understanding the role of single amino acid variation in thymic selection of H60-reactive CD8 T cells and the mechanism of dominance of the H60-specific CD8 T cell response. In the future, this system might be used to develop a vaccination strategy to relieve GVHD symptoms and graft rejection through modulation of the H60-specific response.

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REFERENCES

Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N., Ploegh, H.L., and Tonegawa, S. (1993a). Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. Cell *73*, 1041-1049.

Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N., Ploegh, H.L., and Tonegawa, S. (1993b). Repertoire-determining role of peptide in the positive selection of CD^{8+} T cells. Immunol. Rev. *135*, 157-182.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell *76*, 651-663.

Boesteanu, A., Brehm, M., Mylin, L.M., Christianson, G.J., Tevethia, S.S., Roopenian, D.C., and Joyce, S. (1998). A molecular basis for how a single TCR interfaces multiple ligands. J. Immunol. *161*, 4719-4727.

Borghans, J.A., and De Boer, R.J. (1998). Crossreactivity of the T-cell receptor. Immunol. Today *19*, 428-429.

Choi, E.Y., Yoshimura, Y., Christianson, G.J., Sproule, T.J., Malarkannan, S., Shastri, N., Joyce, S., and Roopenian, D.C. (2001). Quantitative analysis of the immune response to mouse non-MHC transplantation antigens *in vivo*: the H60 histocompatibility antigen dominates over all others. J. Immunol. *166*, 4370-4379.

Choi, E.Y., Christianson, G.J., Yoshimura, Y., Jung, N., Sproule, T.J., Malarkannan, S., Joyce, S., and Roopenian, D.C. (2002a). Real-time T-cell profiling identifies H60 as a major minor histocompatibility antigen in murine graft-versus-host disease. Blood *100*, 4259-4265.

Choi, E.Y., Christianson, G.J., Yoshimura, Y., Sproule, T.J., Jung, N., Joyce, S., and Roopenian, D.C. (2002b). Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. Immunity *17*, 593-603.

Choi, J.H., Ryu, S.J., Jung, K.M., Kim, S., Chang, J., Kim, T.W., and Choi, E.Y. (2009). TCR diversity of H60-specific CD8 T cells during the response evolution and influence of CD4 help. Transplantation *87*, 1609-1616.

Choi, J.H., Yoon, H., Min, C.K., and Choi, E.Y. (2011). Effects of pre-conditioning dose on the immune kinetics and cytokine production in the leukocytes infiltrating GVHD tissues after MHC-matched transplantation. Immune. Netw. *11*, 68-78.

Daniels, M.A., Teixeiro, E., Gill, J., Hausmann, B., Roubaty, D., Holmberg, K., Werlen, G., Hollander, G.A., Gascoigne, N.R., and Palmer, E. (2006). Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature *444*, 724-729.

de Haan, E.C., Moret, E.E., Wagenaar-Hilbers, J.P., Liskamp, R.M., and Wauben, M.H. (2005). Possibilities and limitations in the rational design of modified peptides for T cell mediated immunotherapy. Mol. Immunol. *42*, 365-373.

Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A., Teyton, L., and Wilson, I.A. (1996). An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. Science *274*, 209-219.

Jensen, K.D., Sercarz, E.E., and Gabaglia, C.R. (2009). Altered peptide ligands can modify the Th2 T cell response to the immunodominant 161-175 peptide of LACK (Leishmania homolog for the receptor of activated C kinase). Mol. Immunol. *46*, 366-374.

Jeon, J.Y., Jung, K.M., Chang, J., and Choi, E.Y. (2011). Characterization of CTL clones specific for single antigen, H60 minor histocompatibility antigen. Immune. Netw. *11*, 100-106.

Jung, K.M., and Choi, E.Y. (2007). Role for CD40 and CD40L expression in generating CD8 T cell response to minor histocompatibility antigen, H60. Immune. Netw. *7*, 173-178.

Kersh, G.J., and Allen, P.M. (1996). Essential flexibility in the T-cell recognition of antigen. Nature *380*, 495-498.

Kumar, V., Bhardwaj, V., Soares, L., Alexander, J., Sette, A., and Sercarz, E. (1995). Major histocompatibility complex binding affity of an antigenic determinant is crucial for the differential secretion of interleukin 4/5 or interferon gamma by T cells. Proc. Natl. Acad. Sci. USA *92*, 9510-9514.

Malarkannan, S., Shih, P.P., Eden, P.A., Horng, T., Zuberi, A.R., Christianson, G., Roopenian, D., and Shastri, N. (1998). The molecular and functional characterization of a dominant minor H antigen, H60. J. Immunol. *161*, 3501-3509.

Malherbe, L., Filippi, C., Julia, V., Foucras, G., Moro, M., Appel, H., Wucherpfennig, K., Guery, J.C., and Glaichenhaus, N. (2000). Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major. Immunity *13*, 771-782.

Malherbe, L., Hausl, C., Teyton, L., and McHeyzer-Williams, M.G. (2004). Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. Immunity *21*, 669-679.

Mason, D. (1998). A very high level of crossreactivity is an essential feature of the T-cell receptor. Immunol. Today *19*, 395-404.

Roopenian, D., Choi, E.Y., and Brown, A. (2002). The immunogenoics of minor histocompatibility antigens. Immunol. Rev. *190*, 86-94.

Russell, P.S., Chase, C.M., Madsen, J.C., Hirohashi, T., Cornell, L.D., Sproule, T.J., Colvin, R.B., and Roopenian, D.C. (2011). Coronary artery disease from isolated non-H2-determined incompatibilities in transplanted mouse hearts. Transplantation *91*, 847-852.

Ryu, S., Jung, K., Yoo, H., Kim, T., Kim, S., Chang, J., and Choi, E. (2009). Cognate CD4 help is essential for the reactivation and expansion of CD8 memory T cells directed against the haematopoietic cell-specific dominant minor histocompatibility antigen, H60. Blood *113*, 4273-4280.

Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. Science *263*, 1615-1618.

Strausbauch, M.A., Nevala, W.K., Roopenian, D.C., Stefanski, H.E., and Wettstein, P.J. (1998). Identification of mimotopes for the H4 minor histocompatibility antigen. Int. Immunol. *10*, 421-434.

Wauben, M.H., Boog, C.J., van der Zee, R., Joosten, I., Schlief, A., and van Eden, W. (1992). Disease inhibition by major histocompatibility complex binding peptide analogues of disease-associated epitopes: more than blocking alone. J. Exp. Med. *176*, 667-677.

Wauben, M.H., Joosten, I., Schlief, A., van der Zee, R., Boog, C.J., and van Eden, W. (1994). Inhibition of experimental autoimmune encephalomyelitis by MHC class II binding competitor peptides depends on the relative MHC binding affinity of the disease-inducing peptide. J. Immunol. *152*, 4211-4220.

Wilson, D.B., Wilson, D.H., Schroder, K., Pinilla, C., Blondelle, S., Houghten, R.A., and Garcia, K.C. (2004). Specificity and degeneracy of T cells. Mol. Immunol. *40*, 1047-1055.

Wucherpfennig, K.W., Allen, P.M., Celada, F., Cohen, I.R., De Boer, R., Garcia, K.C., Goldstein, B., Greenspan, R., Hafler, D., Hodgkin, P., et al. (2007). Polyspecificity of T cell and B cell receptor recognition. Semin. Immunol. *19*, 216-224.