Examination of HY Response: T Cell Expansion, Immunodominance, and Cross-Priming Revealed by HY Tetrramer Analysis

Maggie Millrain, Phillip Chandler, Francesco Dazzi, Diane Scott, Elizabeth Simpson and P. Julian Dyson

*J Immunol* 2001; 167:3756-3764; doi: 10.4049/jimmunol.167.7.3756
http://www.jimmunol.org/content/167/7/3756
Examination of HY Response: T Cell Expansion, Immunodominance, and Cross-Priming Revealed by HY Tetramer Analysis

Maggie Millrain,* Phillip Chandler,* Francesco Dazzi, † Diane Scott,* Elizabeth Simpson,* and P. Julian Dyson1*

We have applied MHC class I tetramers representing the two H2b MHC class I-restricted epitopes of the mouse male-specific minor transplantation Ag, HY, to directly determine the extent of expansion and immunodominance within the CD8+ T cell compartment following exposure to male tissue. Immunization with male bone marrow (BM), spleen, dendritic cells (DCs) and by skin graft led to rapid expansion of both specificities occupying up to >20% of the CD8+ T cell pool. At a high dose, whole BM or spleen were found to be more effective at stimulating the response than BM-derived DCs. In vivo, immunodominance within the responding cell population was only observed following chronic Ag stimulation, whereas epitope immunodominance was established rapidly following in vitro restimulation. Peptide affinity for the restricting MHC molecule was greater for the immunodominant epitope, suggesting that this might be a factor in the emergence of immunodominance. Using tetramers, we were able to directly visualize the cross-primed CD8+ HY response, but we did not find it to be the principal route for MHC class I presentation. Immunization with female spleen or DCs coated with the full complement of defined HY peptides, including the Aβ-restricted CD4+ Th cell determinant, failed to induce tetramer-reactive cells. The Journal of Immunology, 2001, 167: 3756–3764.

In MHC-matched human transplantation and in animal models, immune responses to minor histocompatibility (H)2 Ags can be responsible for host-vs-graft (HvG) rejection and graft-vs-host (GVH) disease. Recently, the molecular identity of a number of human and mouse minor H Ags has been elucidated (reviewed in Refs. 1 and 2). As indicated from the detailed genetic analysis of minor H Ags, polymorphic polypeptides can give rise, following processing, to unique peptides that associate with MHC class I and II molecules. Failure of a peptide to bind to a MHC class I or II molecule and clonally delete reactive thymocytes during T cell ontogeny can permit T cell reactivity to the MHC-binding allelic version present on the transplanted tissue. Alternatively, MHC association and presentation of polymorphic peptide residues for TCR contact can permit a T cell response to a related allelic product. Further mechanisms, such as null alleles of minor H Ags and differential processing of allelic products, will contribute to the complexity of the surface array of MHC-associated peptides. Collectively, these mechanisms operate to define T cell tolerance and reactivity to endogenous polymorphic proteins.

Molecular dissection of minor H antigenic loci has revealed the presence of both MHC class I- and class II-restricted epitopes, indicating a potential requirement for cooperation between the CD4+ and CD8+ T cell subsets to generate effective minor H-specific responses (3). Indeed, presentation of a single MHC class I-restricted self epitope in the absence of CD4+ T cell help or depletion of either the CD4+ or the CD8+ recipient T cell subset can lead to the induction of nonresponsiveness (4, 5). Recently, the mechanism of CD4+ T cell help has been defined as operating through the presenting dendritic cell (DC), equipping the APC to deliver activating signals to subsequently encountered CD8+ T cells (6–8). The HY minor H Ag is particularly amenable to analysis because all the epitopes are encoded by genes on the Y chromosome, and in mice, these map to the Sxr deletion interval (∆Sxr) (9). In inbred mouse strains, females grafted with syngeneic male skin can respond only to products of these genes. Some of these Y chromosome genes have X chromosome homologues encoding related peptides. It is the processing and selection of X and Y chromosome-encoded peptides by MHC Ag-presenting molecules that, in part, determines the magnitude of the HY response. In mice, responsiveness to HY varies widely between strains; females of H2b haplotype strains are strong responders capable of rejecting primary syngeneic male skin grafts. In contrast, females of some H2b strains can reject syngeneic male grafts following immunization, whereas females of most H2d strains are nonresponders to HY (10). The basis for low reactivity to HY in non-H2b strains is not entirely clear because F1 females with one H2b parent can reject male skin grafts of the non-H2b parent. Furthermore, after footpad (FP) immunization with syngeneic male cells, some low responder strains can generate CD4+ and CD8+ HY-specific T cells (11).

Recently, MHC class I- and II-restricted HY peptide epitopes of the H2b haplotype have been identified (12–14). The two MHC class I-restricted peptides, WMHHNLMDI and KCSRNRQYL, originate, respectively, from the Uty and Smcy genes and associate with the H2-D8 molecule. Some evidence suggests that the WMHHNLMDI peptide is immunodominant because T cells with specificity for H2-D8/KCSRNRQYL are less commonly
isolated than those with the alternate specificity (15). Further, transgenic mice expressing a TCR specific for the KCSNRQYL epitope fail to reject skin grafts, suggesting that this receptor may have poor reactivity for its ligand (16). TCR-transgenic CD8+ T cells from this strain also have an apparent defect in cytotoxicity in response to male targets, although this can be overcome by provision of an elevated density of the cognate peptide (17, 18).

To further characterize the immune response to HY in the context of the strong responder H2b haplotype, we have produced tetrameric H2-Dd complexed with the two peptides. Tetrameric MHC class I/peptide complexes have been used extensively to detect and analyze Ag-specific T cells during viral infections and for the detection of tumor-specific and autoactive T cells (19–26). They have also been applied in one study for the detection of minor H-specific cells potentially involved in GvH disease following bone marrow (BM) transplantation (27). We were interested in comparing the efficacy of different cell populations for the stimulation of epitope-specific HY responses and in determining the extent of clonal expansion in HvG responses in comparison to those seen during acute viral infection. We reasoned that tetramers would also be able to detect cross-primed CD8+ T cells if this mode of Ag presentation was effective for the HY Ag.

Materials and Methods

MHC class I tetramers

Soluble MHC/peptide tetramers were produced using a modified version of that described by Altman et al. (19). Recombinant MHC class I H2-Dd H chain containing a C-terminal substrate peptide (biotin-specific peptide) for BirA-dependent biotinylation was expressed in BL21 E. coli. Protein expression was induced at mid-log growth phase with 0.5 mM isopropyl-β-D-thiogalactosidase (Sigma, St. Louis, MO). After 5 h, cells were harvested and lysed by overnight freeze/thaw (−70°C). H chain protein was purified from inclusion bodies and denatured in 8 M urea. Human β2-microglobulin (β2m) was prepared in the same way.

H chain and β2m were refolded in 1 liter of arginine buffer (100 mM Tris (pH 8), 400 mM l-arginine, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 2 mM EDTA) with the HY peptides WMMH NMLDI or KCSNRQYL at a molar ratio of 1:2:10, respectively, for 48 h at 4°C. Refolded material was concentrated in a Na2S2O5-prepressurized stir cell to −5 ml and was purified by gel filtration on a Sephadex G-75 column (Amersham Pharmacia Biotech, Piscataway, NJ). The complex was biotinylated using BirA (Avidity, Denver, CO) at 12 h for 25°C (reaction conditions: 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM biotin D, 5 mM ATP, and 5 mM MgCl2). Biotinylated complexes were purified by gel filtration as described previously, followed by Mono Q ion exchange (Amersham Pharmacia Biotech). Tetramers were prepared by mixing the biotinylated complex with PE-labeled ExtrAvidin-R-PE conjugate (Sigma) at a molar ratio of 4:1.

Cell culture

Cell lines and clones were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FCS (Labtech International, Sussex, U.K.). 100 U/mL penicillin and streptomycin, 50 mM 2-ME, 2 mM glucose, and 10 mM HEPES in 2-mL Linbro 24-well plates (ICN Pharmaceuticals, Costa Mesa, CA) at 37°C in 5% CO2. Bulk cultures from mouse spleen were restimulated every 10 days with 5 × 106 irradiated syngeneic splenic APCs and 20 IU rIL-2/ml. The HY Dβ1/υ-specific T cell clone CTI-10 (28) was restimulated every 14–21 days. RMA-S is a transporter-deficient H2b thymoma cell line.

Cytotoxicity assays

Fresh or cultured cells were assayed 5 days following stimulation with Ag and 20 1U/ml rIL-2. RMA-S target cells were labeled with 31Cr alone or in the presence of 1 mM peptide for 1 h at 37°C, washed three times, and plated at 106 cells/well in round-bottom 96-well plates. Effector cells were incubated with the target cells at E:T ratios of 10:1, 3:1, and 1:1. After 4 h, 100 μl of supernatant was collected and 31Cr release was measured using a gamma counter. Results were calculated at an E:T ratio of 10:1. The percentage of lysis was calculated from the formula 100 × (E − M)/(T − M), where E is the experimental release, M is the spontaneous release in the presence of medium alone, and T is the maximum release in the presence of 5% Triton X-100.

Flow cytometry

Spleen cell suspensions were depleted of B cells with sheep anti-mouse Ig G (Dynal, Wirral, U.K.). Aliquots of 106 cells were stained in 50 μl of PBS containing 2% FCS (FACS buffer) with 1 μl of HY Dd tetramer for 10 min at room temperature, and then with FITC- or PerCP-labeled anti-CD8 Ab (BD Pharmingen, San Diego, CA) for 15 min at 4°C, followed by two washes in FACS buffer. Ag-specific cells were phenotyped with FITC-labeled anti-CD69, -CD44, -CD45RB, and -CD62l ligand Abs (BD Pharmingen). DCs were phenotyped with FITC-labeled anti-CD1c and -B7-1 and biotinylated anti-H2-A2 and -B7-1; biotinylated Abs were detected with streptavidin-PerCP (BD Pharmingen). To detect apoptosis, cells were first stained with tetramer as previously described, stimulated with HY peptide-pulsed APCs, and stained with FITC-conjugated annexin V (BD Pharmingen). Samples were acquired on either a FACSscan or FACS-Calibur instrument (BD Biosciences, San Jose, CA). Data were analyzed using CellQuest software (BD Biosciences).

CSFE labeling

Cells were washed and resuspended at a concentration of 10^6/ml in PBS. CSFE (Molecular Probes, Eugene, OR) (29) was added at a final concentration of 5 mM, and cells were incubated at 37°C for 10–15 min. Cells were washed three times before restimulation with male APCs.

Stabilization of H2-Dd on RMA-S

RMA-S cells (106) were incubated with serial 10-fold dilutions of either Dβ1/υ/WMMHNMLDI or Dβ1/υ/KCSNRQYL in 200 μl of RPMI 1640 at 26°C overnight. After washing, they were incubated at 37°C for 1 h to allow the decay of empty class I molecules and were then stained with FITC-labeled B22.249 (anti-H2-Dd).

Immunization and skin grafting

Female 4- to 5-wk-old C57BL/6 mice (Ola, Bicester, U.K.) were used as responders. The β2m−/− (C57BL/6 background) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type or β2m-deficient male C57BL/6 tissues were used as immunogen. DCs were prepared from BM cultured with GM-CSF for 7 days. To promote maturation, cultures were passaged 24 h before harvesting (30). The majority of cells in these cultures were CD11c positive, and of these ~90% were MHC class II and -B7-1 positive, but only a minority were B7-2 positive (data not shown). For the time-course experiments, 106 DCs were injected s.c. into FPs. Grafting of tail skin was conducted using the method of Billingham et al. (31). Other immunizations were conducted with 5 × 10^6 DCs or single-cell suspensions of fresh BM or spleen. For peptide immunizations, cells were incubated with the appropriate peptides (10 μM) at 37°C for 90 min, washed twice with PBS, and 5 × 10^6 cells were injected i.p. in 200 μl of PBS.

ELISPOT

MultiScreen-IP plates (96-well; Millipore, Bedford, MA) were coated with 5 μg/ml anti-mouse IFN-γ mAb (BD Pharmingen), blocked with RPMI 1640–10% FCS, and washed with PBS. Peptide-pulsed stimulator cells were prepared by incubating 10^7 irradiated female spleen cells in 10 μM peptide for 2 h at 37°C. Peptide-pulsed or unpulsed spleen cells (2 × 10^6) were added to the wells with 10^6 B cell-depleted responder spleen cells to give a 200-μl final volume. After a 24-h incubation, cells were water-lysed, and the plates were washed 6 times with PBS and 0.1% Tween 20. A total of 100 μl 20 ng/ml biotinylated anti-mouse IFN-γ mAb in PBS and 1% BSA was added and incubated overnight at 4°C. Plates were washed as described above, and 1 μg of streptavidin-alkaline phosphatase (DyNaBeads) was added in 100 μl of PBS and 1% BSA. After 1 h at room temperature, the plates were washed four times as described above and two times with 0.1 M Tris (pH 7). Plates were developed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Sigma).

Results

HY/Dd tetramers

The specificity of the H2-Dd tetramers was confirmed by staining Ag-specific T cells. The Dβ1/υ/WMMHNMLDI tetramer preparation stained virtually 100% of the T cell clone CTI-10 (28), which is specific for the Dβ1/υ/WMMHNMLDI complex, whereas this T cell clone was not stained by the Dβ1/υ/KCSNRQYL tetramer (Fig. 1).
HY responses in mice immunized with male dendritic cells or skin graft

A time-course experiment was undertaken to establish the kinetics and extent of HY D\(^\beta\)/KCSRNRQYL T cell expansion in female H2\(^b\) mice following immunization with male DCs. DCs were produced by 7 days of GM-CSF stimulation of BM. Female recipients were immunized in the FP with 10\(^6\) male or female DCs and spleen cells analyzed at 7, 14, 21, 35, and 76 days for tetramer-positive CD8\(^+\) cells. Exposure to female skin grafts or DCs did not lead to expansion of the D\(^\beta\)/HY tetramer-reactive populations, which appeared as a background of \(\sim 0.1\%\) of the splenic CD8\(^+\) T cell population in both untreated mice and those given female cells (Fig. 2, A and B). Males are tolerant to HY principally through central thymic deletion of reactive cells; we did not observe any difference in D\(^\beta\)/HY tetramer-positive cell frequencies in CD8\(^-\)-single-positive thymocytes/spleen cells between male and female (data not shown). This is expected, because the frequency of HY-specific CD8\(^+\) T cells in naive females was anticipated to be very low.

In female H2\(^b\) mice receiving male DCs, D\(^\beta\)/WMHHNMDLI- and D\(^\beta\)/KCSRNRQYL-specific CD8\(^+\) T cell populations were undetectable at day 7 (data not shown) but were clearly present as distinct populations at day 14 and at subsequent time points. Representative spleen cell staining profiles at day 14 from two females immunized by primary male skin graft or by male DC are shown in Fig. 2, C–F. Three points emerge from the accumulated data on ex vivo staining of spleen cells following DC immunization (Fig. 3A). Firstly, the proportion of either HY-specific CD8\(^+\) T cell cohort generally does not expand above 1% of the total splenic CD8\(^+\) population. Secondly, there is variation between individual responders both in the size and skew of the two cohorts with no clear immunodominance. Thirdly, the response is maintained at approximately the same level throughout the observation period (76 days).

HY responses in DC-immunized mice rejecting male skin grafts

Primary syngeneic male skin grafts are rejected by female H2\(^b\) mice with median survival time of \(\sim 35\) days in comparison with the faster response (approximate median survival time of 15 days) following immunization (9). To establish whether immunization with male DCs and its influence on the speed of graft rejection would be reflected in an increase in the D\(^\beta\)/HY-specific CD8\(^+\) T cell cohorts detected, female recipients were given male or female skin grafts 2 wk after male DC immunization, leading to graft rejection at around days 13–15. Fig. 3B shows the appearance of the HY-specific cohorts detected during the second-set skin-graft-rejection response in a series of recipients. Firstly, as was seen following DC immunization alone, variation between individual responders is observed in both the magnitude and fine specificity of the response; however, no evidence of significant skew toward the immunodominant epitope was observed. Secondly, the magnitude of the response is, in many cases, above that seen following DC immunization alone. However, the peak expansion size seen for either specificity is \(\sim 2\%\) of the total splenic CD8\(^+\) T cell subset.

HY responses in mice chronically exposed to male tissue

Because no clear evidence of epitope immunodominance emerged in the above experiments using standard immunization procedures, we used a protocol designed to more extensively stimulate the anti-HY immune response and promote clonal selection, which might reveal epitope dominance. Female H2\(^b\) recipients were immunized with male spleen cells, and 18 days later, the mice were sublethally irradiated (400 rad) and reconstituted with male BM. Five weeks later, male skin grafts were applied. Recipients that rejected both their BM and skin graft, and hence showed evidence of a vigorous response to HY, were analyzed with tetramers 5–13 days following rejection of skin (Fig. 3C). This chronic stimulation led to considerably larger expansions of HY-specific CD8\(^+\) T cells; interestingly, the majority of mice (9 of 10) showed marked
preferential expansion of the immunodominant D\textsuperscript{d}/Ut\textsuperscript{y}-specific cells (3- to 18-fold) over the alternate specificity.

**Influence of cell type and route of immunization**

DCs are considered to be critical APCs for the initiation of an immune response. Ags that are present within other cell types can access host DCs via endocytosis and can be processed and presented to T cells via the indirect or cross-priming route (33). We have directly compared the efficacy of male spleen, BM, and DCs to stimulate a class I-restricted HY response. Injection of 5 \times 10^6 male BM cells led to small (0.5%) cohorts of tetramer-positive cells (data not shown). Furthermore, DCs were inferior to spleen or BM (Fig. 4A). BM was the most effective immunizing tissue, leading to 6, 10, and 23% of the CD8\textsuperscript{+} T cells 14 days later; surprisingly, DCs were inferior to spleen or BM (Fig. 4A). BM was the most effective immunizing tissue, leading to 6, 10, and 23% of the CD8\textsuperscript{+} compartment becoming HY specific in three recipients (D\textsuperscript{d}/Sm\textsuperscript{yc} and D\textsuperscript{d}/U\textsuperscript{ty} populations). The efficacy of spleen and BM is dependent on the cell dose used because DC immunizations of 5 \times 10^6 and 5 \times 10^7 promoted similar expansions of tetramer-positive cells, whereas spleen or BM cell immunizations of 5 \times 10^6 led to small (<0.5%) cohorts of tetramer-positive cells (data not shown).

To look at the influence of route of Ag delivery on immunization efficiency, male BM was injected i.p., i.v., or into the FP. Fig. 4B shows representative data from each group of three recipients. The i.v. and i.p. routes gave similar levels of cell expansion, whereas FP immunization was markedly less effective. Functional CD8\textsuperscript{+} cytotoxic T cell responses to minor H Ags require help provided through activation of CD4\textsuperscript{+} T cells. To confirm that help is necessary for CD8\textsuperscript{+} T cell expansion in vivo, we used BALB/c (H2\textsuperscript{b}-K\textsuperscript{d}, -A\textsuperscript{b}, and -D\textsuperscript{b}) mice that lack the HY-presenting class II molecule A\textsuperscript{b} and are HY nonresponders. Female BALB/c recipients receiving male BALB/c BM did not show expansion of either HY CD8\textsuperscript{+} T cell population (data not shown), although they did display the appropriate MHC class I peptide complexes.

Ut\textsuperscript{y}- and Sm\textsuperscript{yc}-derived peptides bind to and stabilize empty D\textsuperscript{b} molecules on the surface of RMA-S cells efficiently, although at the more physiologically relevant lower peptide concentration range, Ut\textsuperscript{y} stabilizes D\textsuperscript{b} more efficiently (Fig. 5A). To test whether female APCs coated with HY peptides are able to reproduce the immunization process, female spleen cells or DCs were coated with the H2\textsuperscript{b} HY peptide epitope set including the H2-A\textsuperscript{b}-restricted epitope NAGFSNRARSSRSS (14). Cells (5 \times 10^6) were injected i.p., and spleen cell populations were collected after 14 days. Peptide-coated cells failed to induce T cell cohorts detectable by tetramer staining whether the MHC class I-restricted peptides were present individually or together and in the absence or presence of the A\textsuperscript{b} peptide (Fig. 5B and data not shown). Furthermore, tetramer-positive cells were not seen following further in vitro stimulation of spleen cells, confirming the inability of peptide-coated cells to immunize effectively (Fig. 5C).

Cross-priming has been shown to be effective for the generation of helper and cytotoxic responses; indeed, this route has been proposed to be the dominant route for the priming of some responses. To ascertain the relative role of the indirect pathway for HY, we used β\textsubscript{2}m-deficient male spleen cells as immunogen. This material stimulated the expansion of tetramer-positive CD8\textsuperscript{+} T cohorts of both specificities, although the populations were ~10% of those generated by class I-sufficient spleen cells, suggesting that indirect presentation, although clearly operative, is not the principal pathway for this immune response (Fig. 5, E and F). The cross-primed cells expanded further in vitro following exposure to Ag, as seen...
for directly primed cells (data not shown). As observed for the direct pathway of Ag presentation, we did not see immunodominance during this period of the HY immune response, suggesting that both epitopes are presented via the indirect pathway with similar efficacy. To confirm that the HY helper response was not compromised in the absence of MHC class I, an ELISPOT assay was used to look at priming of T cells specific for the class II Aβ-restricted HY epitope and showed the response to be similar with MHC class I-sufficient or -deficient immunogen (Fig. 5D).

**Phenotype of HY-specific CD8⁺ T cells**

Spleen cells from female mice immunized with male DCs or DCs followed by a male skin graft were analyzed ex vivo at day 28 by dual staining with MHC peptide tetramer and Abs specific for markers of activation. The phenotype of both tetramer-positive populations was CD69⁺CD44highCD45RBhighCD69lowCD62li− (data not shown), which is typical of Ag-experienced cells and may represent a recirculating and/or memory pool.

**In vitro cultures**

In all cases in which Ag-specific populations of CD8⁺ splenic T cells were detected ex vivo with either tetramer, expansion of the corresponding population was seen following in vitro stimulation with male APC; importantly, this was not seen with spleen cells from unimmunized mice (data not shown). The correlation between ex vivo detection by tetramer and in vitro expansion is important with regard to the identity of the small populations of CD8⁺ T cells staining with Db/HY tetramers in nonimmunized animals. These cells constitute ~0.1% of the CD8⁺ population but do not form a clustered cohort in the FACS analysis and are distributed throughout the quadrant (Fig. 2, A and B).

In vivo, because immunodominance was apparent only following extensive stimulation with male cells, it was of interest to determine whether immunodominance may be more apparent following in vitro stimulation. Primary splenic cultures from mice immunized with male DCs followed by skin grafting and which, at the time of harvest, contain similar proportions of both HY CD8⁺ T cell specificities were restimulated in vitro. The relative expansion of the two cell populations within the same culture can be assessed during in vitro propagation. In cultures of this type, the Dβ/WMHHNMDDL1-specific population was found to preferentially expand in comparison to the Dβ/KCSRNRQYL population. A representative example is shown in Fig. 6, illustrating the dominance of the Dβ/WMHHNMDDL1-specific population following two in vitro restimulations. The mechanism of in vitro dominance may involve a more rapid expansion of the Dβ/WMHHNMDDL1-specific cells; alternatively, the Dβ/KCSRNRQYL-specific population may be undergoing activation-induced cell death at a greater rate. To investigate these possibilities, T cell cultures containing both specificities were stained with CFSE and cultured for an additional 5 days to determine the relative extent of cell division. Annexin V staining was used to gauge the degree of apoptosis. The result of the CFSE stain indicates that, within these cocultures, the immunodominant Dβ/WMHHNMDDL1-specific population has undergone more cell division than the Dβ/KCSRNRQYL-specific population, consistent with an in vitro growth advantage (Fig. 7, A and C). Furthermore, a larger fraction of Dβ/KCSRNRQYL-specific cells consistently stained with annexin V (Fig. 7, B and D).

To establish that the populations identified using tetramers represent Ag-specific effectors, an in vitro-propagated spleen-derived line containing both specificities was sorted by FACS into Dβ/WMHHNMDDL1- and Dβ/KCSRNRQYL-specific sublines. In line with the findings discussed above, the Dβ/WMHHNMDDL1-specific

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Visualization of the cross-primed HY response. A, Peptide stabilization of H2-Dβ on RMA-S by HY peptides (Uty, □) and (Smcy, ○). B and C, A total of 5 × 10⁶ HY peptide (Dβ/Uty, Dβ/Smcy, and Aβ HY) coated spleen cells were injected i.p., and B cell-depleted spleen cell populations (14 days after immunization) were stained with anti-CD8 and Dβ/Uty tetramer and analyzed by FACS ex vivo (B) and following in vitro restimulation (C). D, ELISPOT analysis of HY/Aβ-reactive cells in B cell-depleted spleen cell populations from responders in E and F. Presence (+) or absence (−) of HY/Aβ peptide on female APC is indicated. Numbers of ELISPOT reactions per 10⁶ cells are plotted. E and F, A total of 5 × 10⁶ βμ wild-type (E) or -knockout (F) male spleen cells were injected i.p. into female wild-type C57BL/6 recipients. B cell-depleted spleen cell populations (14 days after immunization) were stained with anti-CD8 and Dβ/Uty tetramer and analyzed by FACS. Figures in the upper right quadrants represent percentages of tetramer-positive cells within the CD8⁺ population. Representative examples are shown (range for cross-priming (F), 0.2–0.4%).

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Preferential in vitro expansion of HY-specific CD8⁺ T cells recognizing the immunodominant epitope. Spleen cells from a female immunized with male DCs, which initially had similarly sized cohorts of both MHC class I HY specificities (A and B), were restimulated in vitro. Following one restimulation, HY-specific cells of both specificities expanded (C and D). Following the second restimulation, the DC Dβ/WMHHNMDDL1 (Uty)-specific population expanded to predominate within the culture (E and F). A, C, and E, Stained with anti-CD8 and Dβ/WMHHNMDDL1 (Uty); B, D, and F, stained with anti-CD8 and Dβ/KCSRNRQYL (Smcy). The result is representative of more than five experiments.
line retained a high degree of purity, whereas in the D\(^6\)/KCSRNRQYL-specific CD8\(^+\) T cells had an apparent growth advantage and quickly became a significant population. To remove these contaminating cells, three cycles of depletion with D\(^b\)/WMHHNMDLI monomer-coated dynabeads were used. As shown in Fig. 8A, the cytotoxic effector function of both populations was directed predominantly at the cognate peptide-loaded RMA-S target cells. The low level of cytotoxicity directed at the noncognate peptide-loaded target is attributable to residual T cell populations of the alternate specificity. It was also possible to clone directly from spleens of immunized mice. Although at the time of cloning both specificities were present at similar frequencies, D\(^b\)/KCSRNRQYL clones were under-represented (Expt. 1, 1 of 23; and Expt. 2, 3 of 32), consistent with the more robust in vitro growth of the D\(^b\)/WMHHNMDLI-specific cells. Because all 54 clones stained with one or the other tetramer, we find no evidence for additional antigenic complexity. The poor representation of the Smcy-specific clones suggests that their in vitro endurance relative to the Uty-specific cells is, in part, cell autonomous and not due to coculture. Assessment of the clones by cytotoxicity revealed complete correlation between tetramer stain and function (Fig. 8B). Within the panel of D\(^b\)/WMHHNMDLI-specific clones isolated following a primary HY response, some had markedly weaker staining with tetramer, suggesting that a range of TCR/MHC-peptide affinities are expanded by antigenic stimulation (Fig. 8C), although this was not seen in the clones isolated from animals exposed to male DC and skin graft.

**Discussion**

Definition of the polymorphic self peptides constituting a series of human and mouse MHC class I-associated minor H Ags, combined with the use of MHC/peptide tetramers, allows the dissection of the immune response to minor H Ags at a level not previously possible. The use of tetramers in infectious disease has allowed the identification of Ag-specific cells and has revealed, in some cases, T cell expansions of greater magnitude than anticipated. In infectious mononucleosis, CD8\(^+\) T cells specific for EBV-derived peptides can constitute >40% of the CD8\(^+\) T cell compartment in the acute stage of the disease (20), whereas in the mouse, infection with lymphocytic choriomeningitis virus can lead to an even greater expansion of virus-specific CD8\(^+\) T cells (21, 22). CD8\(^+\) T cell activation in response to viral challenge may be expected to result in larger expansions than in responses to minor H or tumor Ags because the CD8\(^+\) T cell response has evolved to respond to such infectious agents rather than to neootigens on tumor cells or to minor H Ags on tissue grafts. However, studies looking at T cell responses to tumor Ags have detected relatively high frequencies of Ag-specific CD8\(^+\) T cells in tumors of melanoma patients (23), although these may result from chronic stimulation rather than primary expansion. A single report has focused on the definition of anti-minor H responses using tetramers. In a series of BM-transplant recipients undergoing GvH disease, responses to the human minor H Ags HA-1 and to two epitopes of HY have been assessed with tetramers. Minor H Ag-specific CD8\(^+\) T cells were detected in PBMCs from recipients at frequencies up to 10%. However, because elevated frequencies of minor H-specific T cells were seen in some of the donors, these large expansions may have reflected memory responses (27).

In this study, we wished to define the kinetics and extent of T cell expansion in response to primary immunization with male DC and other APC types, following primary- and second-set skin graft rejection and in chronically stimulated anti-HY responses. Responses to the two defined MHC class I-presented HY epitopes of the H2\(^\text{a}\) haplotype have been compared using specific D\(^b\) tetramers. We were also particularly interested in gaining insight into the mechanism underlying the reported immunodominance of the D\(^b\)/WMHHNMDLI epitope over the D\(^b\)/KCSRNRQYL epitope. Following immunization of female mice with male DCs, spleen, or BM, Ag-specific T cells were detectable in spleen with both tetramers from day 14. Interestingly, immunization with primary skin grafts was less effective than DCs in stimulating the MHC class I
HY response, perhaps reflecting the relative numbers of APCs involved in each immunization protocol. Furthermore, immunization with DCs is likely to result in systemic immunization involving multiple lymph nodes, whereas a skin graft is likely to initially prime an immune response only in the draining lymph nodes. Skin grafting subsequent to male DC immunization again led to the appearance of CD8\(^+\) T cells of both specificities. In comparison to DC immunization alone, further immunization by exposure to male grafts led to a small elevation of the Ag-specific CD8\(^+\) T cell frequency, although the responses to the individual epitopes did not exceed 2%. At a high cell number (5 \(\times\) 10\(^6\)), spleen and BM were found to be more efficient than BM-derived DCs at stimulating tetramer-positive T cell expansion. At this high cell number, spleen and BM may contain sufficient DCs to effectively prime the HY response and semiprofessional APC populations to effectively expand the primed cells. Strikingly, none of these immunization protocols led to immunodominance of the Uty epitope over the Smcy epitope. Because skewing was not apparent during primary and secondary HY responses, it is likely that the precursor frequencies of CD8\(^+\) T cells specific for the two class I epitopes do not differ markedly. To test whether immunodominance would arise following persistent Ag exposure, mice were repeatedly immunized with male tissue. In contrast to the conventional immunization protocols, mice immunized with male spleen cells and which subsequently rejected male BM and skin grafts showed a response intensely skewed to the immunodominant epitope. Because skewing was only apparent following chronic antigenic stimulation, it is clear that the typical anti-HY response involves both D\(^b\)-restricted epitopes in similar measure.

These observations make it unlikely that lack of peptide availability or TCR affinity can simply explain the immunodominance that emerges with persistent stimulation; indeed, tetramer staining of the D\(^b\)/Smcy population was as bright as for the D\(^b\)/Uty population. Rather, the evolution of the immune response during continuing stimulation shapes the outcome. It is not clear why, as populations, the D\(^b\)/Smcy-specific cells are ultimately less successful than the D\(^b\)/Uty-specific cells when the immune response is more intensely stimulated. Wolpert et al. (34) and Pion et al. (35) have suggested that immunodominance can emerge due to interference between responding T cells because expression of epitopes on the same APC was required for immunodominance to emerge. Interestingly, as reported here, competition for CTL survival in mixed in vitro culture was also seen for B6 anti-BALB.B minor responses and required coexpression of epitopes on the APC. The T cell cloning experiments described here are consistent with cell survival in vitro being, at least in part, cell autonomous and not dependent on competition or cross-talk between populations. D\(^b\)/Uty is immunorecessive to the B6(dom1) epitope (35); the dramatic difference in cell surface density of the B6(dom1) peptide, present at around 1000 copies/ APC, in comparison to D\(^b\)/Uty, which is present at around 10 copies/ APC, has been proposed, in part, to explain the hierarchy of immunodominance (36). We find that the response to D\(^b\)/Uty is, under chronic stimulation, dominant to that of D\(^b\)/Smcy, suggesting that a high peptide density is not required, at least for the limited immunodominance described here. Relatively small differences in proliferative or survival signals delivered by APCs may cumulatively account for strong skewing during prolonged stimulation. For example, the greater affinity of the D\(^b\)/Uty interaction (Fig. 5) may give this combination a marginal advantage over the immunorecessive epitope combination. It may be important during the early stages of an immune response to use the full range of T cells available for an epitope-specific response, and only when cell numbers have expanded are the less effective cells no longer useful for the response. Consistent with this notion, the clonal analysis (Fig. 8C) detected lower affinity TCRs only in the primary response. A study using tetramers to look at immunodominance within the CD8\(^+\) response to viral epitopes in rhesus monkeys chronically infected with simian/HIV rarely found significant cell populations specific for the immunorecessive epitope (37). It will be of interest to determine how rapidly immunodominance emerges for other chronic immune responses.

However, immunodominance was very apparent during short-term in vitro culture, as the D\(^b\)/WMHHNMDDL-specific T cells rapidly dominated the cultures. The immunodominance we see in vitro can explain the deficit of T cell clones with D\(^b\)/KCSRN RQYL specificity, because these will generally be isolated from cultures expanded in vitro before cloning. Why should immunodominance be more apparent in vitro than in vivo? One possibility is that T cell proliferation is less regulated in the absence of the multiple highly regulated mechanisms that operate in the in vivo microenvironment to self-limit T cell expansion, including Fas/Fas ligand interaction (38), CTLA-4/B7 interaction (39), and IL-2-regulated apoptosis (40). Relatively small differences in access to APC surface due to variation in TCR affinity or half-life may quickly translate to rapid outgrowth of one population in vitro.

The data presented here show that in robust HY responder H2\(^b\) mice, expansions of CD8\(^+\) T cells specific for individual HY epitopes during skin graft rejection or following immunization are substantial, although of smaller magnitude (<10%), than the extremes seen during some acute viral infections. The specific cell density at the graft site may, of course, be higher. It is likely that viral replication leading to an increasing Ag load combined with activation of aspects of innate immunity following virus recognition contributes to the large T cell expansions seen in some acute virus infections. However, chronic stimulation involving multiple male APC types promoted large expansions skewed to the immunodominant epitope. One critical observation was the very extensive HY-specific CD8\(^+\) T cell expansions seen following immunization with BM or spleen, even though the MHC class I-restricted antigenic complexity of HY is very limited. Importantly, spleen and BM populations contain only a small percentage of professional APC types, such as DCs, and yet result in expansions of the same order as in acute viral infection. Indeed, highly enriched BM-derived DC populations were markedly less effective at stimulating CD8\(^+\) T cell expansion; poor stimulation was not due to their rapid disappearance, because BM-derived DCs of the type used here persist for \(~3\) wk in vivo (41).

Cross-priming following cytoplasmic degradation of Ag is an important route for Ag presentation (33) and has been proposed to be of particular relevance in transplantation (42). To test whether the large expansions of HY-specific CD8\(^+\) T cells we see following spleen or BM immunization are stimulated principally through direct or indirect Ag presentation, we used \(\beta\)-m-deficient male spleen cells and found the direct route to predominate. We find the indirect route of Ag presentation to account for \(~10\)% of the response and, hence, to play a minor role in spleen-cell immunization. This may not be surprising because spleen contains professional APCs as well as many semiprofessional APCs. Because HY expression is ubiquitous, we will be able to dissect the role of different cell types and routes of immunization for entry into the indirect pathway. Ag presentation for initiation of an immune response is DC mediated, whereas subsequent expansion involves both semi- and nonprofessional APCs; again, we will be able to dissect the capabilities of different donor cell types to engage in these two stages via the indirect route. These data show that large expansions of CD8\(^+\) T cells are not restricted to acute infection
and have direct relevance to strategies for the development of protective CD8+ immunity through vaccination and the study of HVG responses compromising BM engraftment after transplantation. The role of CD4+ T cell help in minor Ag responses is critical for the development of cytotoxic CD8+ cells (4, 43, 44) and for skin graft rejection (4, 5). In the absence of help, the CD8+ T cell compartment can be rendered tolerant. Female B10.1D (Dp, A1, and Dp) mice do not reject male skin due to lack of the critical A1-restricted helper response. In this strain, we did not see any tetramer-positive cells, suggesting that tolerance does not involve expansion of a regulatory CD8+ population. In view of this, it was not surprising that CD8+ T cell expansions were not seen following the use of MHC class I-restricted HY peptides coated onto female B6 DCs or spleen. However, additional provision of the HY A1-restricted peptide did not lead to tetramer-positive CD8+ T cell expansions, although accelerated graft rejection was observed (data not shown), suggesting some HY-specific CD4+ and/or CD8+ T cell priming. The maturation state of the DCs, density and half-life of the peptide on the cell surface, and the potentially low efficacy of cross-priming with peptide Ag relative to cellular Ag may contribute to the failure to see MHC class I tetramer staining. It will be of interest to directly determine, using MHC class II staining, the efficacy of the HY A1 peptide to expand the CD4+ compartment.

The immune response to MHC class I- and II-presented self peptides is of significant clinical importance in a number of contexts. Responses to minor H Ags following BM transplantation contribute to GvH and HVG responses in both MHC-matched and -mismatched situations, self peptides derived from differentiation Ags or mutant proteins are potential targets for tumor immunotherapy, and inappropriate responses to self peptides can result in autoimmunity. Depending on the context, manipulation of T cell responses to self peptides, leading to diminution or augmentation of a response, represents a powerful strategy for improving clinical outcome. Characterization of the normal course of the MHC class I-restricted response to the model minor H Ag HY provides a useful experimental model for the investigation of the mechanism(s) of action of interventions designed to suppress the immune response to transplanted tissue. This report shows how monitoring of the relevant population(s) can be undertaken both ex vivo and following further in vitro expansion.

Acknowledgments

We thank Dr. Awen Gallimore, Dr. Ann Glithero, and Prof. Tim Elliott for invaluable advice on preparation of tetramers and for the H2-AB and β2m expression plasmids. We thank Dr. Tessa Crompton for the β2m-deficient mice, Dr. Derry Roopenian for the CTL-10 T cell clone, Dr. Alan Bennett for the kind gift of BirA enzyme, and Dr. Hans Stauss for useful discussion and comments on the manuscript.

References


