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Antigen-Specific Modulation of an Immune Response by In Vivo Administration of Soluble MHC Class I Tetramers

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Soluble MHC/peptide tetramers that can directly bind the TCR allow the direct visualization and quantitation of Ag-specific T cells in vitro and in vivo. We used HY-Db tetramers to assess the numbers of HY-reactive CD8⁺ T cells in HYTCR-transgenic mice and in naive, wild-type C57BL/6 (B6) mice. As expected, tetramer staining showed the majority of T cells were male-specific CD8⁺ T cells in female HY-TCR mice. Staining of B6 mice showed a small population of male-specific CD8⁺ T cells in female mice. The effect of administration of soluble MHC class I tetramers on CD8⁺ T cell activation in vivo was unknown. Injection of HY-Db tetramer in vivo effectively primed female mice for a more rapid proliferative response to both HY peptide and male splenocytes. Furthermore, wild-type B6 female mice injected with a single dose of HY-Db tetramer rejected B6 male skin grafts more rapidly than female littermates treated with irrelevant tetramer. In contrast, multiple doses of HY-Db tetramer did not further decrease graft survival. Rather, female B6 mice injected with multiple doses of HY-Db tetramer rejected male skin grafts more slowly than mice primed with a single injection of tetramer or irradiated male spleen cells, suggesting clonal exhaustion or anergy. Our data highlight the ability of soluble MHC tetramers to identify scarce alloreactive T cell populations and the use of such tetramers to directly modulate an Ag-specific T cell response in vivo. The Journal of Immunology, 2001, 167: 3708–3714.

Materials and Methods

Mice

HY TCRαβ-transgenic mice (15) that carry a transgene specific for male HY Ag were obtained from the National Institute of Arthritis and Infectious Diseases via Taconic Farms (C57BL6, TgN(TcrHY); Germantown, NY). These mice are referred to as HY-TCR mice. Normal B6 mice were purchased from (Charles River Breeding Laboratories, Wilmington, MA). All animals used in this study were maintained under specific pathogen-free conditions in the American Association of Laboratory Animal Care-accredited University of North Carolina, Department of Laboratory Animal Medicine Facilities, and were routinely used at 8 wk of age.
peritoneal cavity. CD11b+ cells were purified by negative selection (Miltenyi Biotec, Auburn, CA).

Collins, CO). Two- and three-color staining was performed using standard methods. List ed CD8+ cells.

FIGURE 1. Peptide-specific staining of CD8\textsuperscript{bush} (male-reactive) T cells in the HY-TCRβ-transgenic mouse strain. Splenocytes from naive 8-wk-old female-transgenic mice were stained with CD8-FITC and either PE-labeled irrelevant gp33-D\textsuperscript{b} tetramer (A) or HY-D\textsuperscript{b} tetramer (B). Numbers shown in the analysis gates are percentages of total CD8\textsuperscript{+} cells.

Peptides

HY peptide (KCSRNRQYL) (14) and gp33 peptide (KAVYNFATM) were synthesized by the University of North Carolina Microchemical Facility, purified by HPLC, and tested for purity by mass spectroscopy.

Tetramer preparation and injection

Recombinant protein was prepared as previously described by Wang et al. (16). For in vivo tetramer injection experiments, HY-D\textsuperscript{b} was prepared in sterile PBS and 30 \( \mu \)g/mouse in 150 \( \mu \)l was injected directly into the peritoneal cavity.

Flow cytometric analysis

The directly conjugated anti-mouse Abs used for cell surface staining in this study were anti-CD8 (53-6.7), anti-B220 (RA3-6B2), and anti-MHC class II (I-A\textsuperscript{b}, 25-9-17) purchased from BD PharMingen (San Diego, CA). Two- and three-color staining was performed using standard methods. List mode data were collected on a FACScan (BD Biosciences, Mountain View, CA) and analyzed using Summit software (Cytomation, Ft. Collins, CO).

Purification of CD8\textsuperscript{+} TCR-transgenic T cells from spleen

Cell suspensions were prepared from the spleens of TCR-transgenic mice. Cells were incubated at 37\(^\circ\)C for 1 h in tissue culture dishes (Nunc, Naperville, IL) to eliminate adherent cells before purification. CD8\textsuperscript{+} T cells were negatively selected by depletion of CD4\textsuperscript{+}, MHC class II\textsuperscript{+}, and CD11b\textsuperscript{+} cells (16) using the MACS magnetic separation system according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA).

Preparation of irradiated splenocytes

Splenocytes were prepared from male B6 mice and resuspended at 2 \( \times \) 10\(^6\) cells/ml in RPMI 1640 medium. Cells were then irradiated by exposure to 3000 rad emitted from a GammaCell 40 Cesium-137 source (Atomic Energy of Canada Limited, Ottawa, Canada).

Proliferation assay

Purified CD8\textsuperscript{+} T cells (4 \( \times \) 10\(^3\)/well) were stimulated with Ags or tetramer at different concentrations in 200 \( \mu \)l of complete RPMI 1640 in 96-well flat-bottom plates. The cultures were incubated for 48 h, and 1 \( \mu \)Ci of \([\text{H}]\text{thymidine was added to each well for the final 10 h of culture. Cells were harvested using a multiple sample harvester (Otto Hiller, Madison, WI), and incorporation of \([\text{H}]\text{thymidine was measured by scintillation counting using a Beckman LS5000 counter (Pal Alto, CA). All data represent the average counts per minute of triplicate determinations. All proliferation experiments were repeated at least three times.}

Tail grafting

Tail grafting was performed as previously described (17). Each female recipient mouse received a male allograft and a female isograft as a control. Glass tubes were placed over the grafted area for 3 days to prevent removal of the graft by the mouse. Grafts that failed to vascularize properly with apparent rejection at 3 days were classed as “technical failures” and removed from the analysis. Remaining grafts were scored daily. Fully intact grafts were scored as 100% and when <30% of the graft remained, it was considered rejected.

Results

HY-D\textsuperscript{b} tetramer identifies HY-reactive T cells in naive C57BL/6 (B6) mice

To investigate the ability of HY-D\textsuperscript{b}-reactive T cells to stain with PE-labeled HY-D\textsuperscript{b} tetramer, we used the HY-TCR-transgenic mouse strain (14). Fig. 1 illustrates peptide-specific staining of CD8\textsuperscript{bush} (male-reactive) T cells with HY-D\textsuperscript{b} tetramer in female HY-TCR splenocytes. To demonstrate the ability of MHC class HY-D\textsuperscript{b} tetramers to identify the presence of HY-reactive T cells in naive, unmanipulated nontransgenic mice, splenocytes from 8-wk-old female B6 mice were stained with PE-conjugated labeled HY-D\textsuperscript{b} and counterstained according to the figure legend. Fig. 2, a and b, shows that HY-D\textsuperscript{b} tetramer staining revealed the presence of a surprisingly large population of HY-specific CD8\textsuperscript{+} T cells, which represent 1.5–2.0% of splenic CD8\textsuperscript{+} T cells. A control D\textsuperscript{b} tetramer, assembled with lymphocytic choriomeningitis virus (LCMV\textsuperscript{V}) gp33 peptide stained <0.6% of CD8\textsuperscript{+} T cells. Furthermore, after adoptive (i.v.) transfer of irradiated male splenocytes into female B6 mice, the HY-D\textsuperscript{b} tetramer staining population had expanded ~2.5 fold at 5 days after transfer (Fig. 2d). Control female B6 mice receiving irradiated female splenocytes did not expand the HY-reactive CD8\textsuperscript{+} T cells (Fig. 2c). Fig. 2e illustrates the summary of the data (three mice per group). There was significant (Student’s t test, \( p < 0.005 \)) priming with male but not with female splenocytes. These observations demonstrate that naive female B6 have detectable male-specific T cells in the spleen.

CD8-dependent in vitro stimulation of HY-reactive T cells by HY-D\textsuperscript{b} tetramer

In our previous studies (16), using soluble MHC class I tetramer alone to stimulate naive CD8\textsuperscript{+} T cells into effector cells, we showed that the response to LCMV in vitro was blocked by addition of Abs to the TCR coreceptor CD8. Since the LCMV-transgenic TCR has a relatively high affinity to the immunodominant gp33 peptide (18) we wanted to be certain that the same argument pertained to the relatively low-affinity HY-TCR (our unpublished observations). Here, we stimulated purified CD8\textsuperscript{+} HY-TCR T cells with HY-D\textsuperscript{b} tetramer in the presence of 20 \( \mu \)g/ml anti-CD8 Ab (53-6.7) or control Ig. This concentration of anti-CD8 Ab was found to completely block HY-TCR T cell proliferation in dose-response experiments (data not shown). As in our LCMV study, treatment with anti-CD8 Ab blocked the T cell proliferative response to HY-D\textsuperscript{b} tetramer (Fig. 3), suggesting an important role for the CD8 coreceptor in the activation of HY-TCR T cells by specific HY-D\textsuperscript{b} tetramer.

3 Abbreviation used in this paper: LCMV, lymphocytic choriomeningitis virus.
Direct injection of HY-D<sup>b</sup> tetramer into female normal B6 mice primes for accelerated rejection of male B6 skin grafts

We have previously shown that soluble MHC class I tetramer alone is sufficient for in vitro activation and differentiation into effector cells of naive CD8<sup>+</sup>T cells from transgenic HY-TCR mice (16). We wished to determine whether direct injection of HY-D<sup>b</sup> into female B6 mice would result in functional priming of a specific immune response in vivo, measured here by accelerated rejection of male skin grafts. One group of mice was injected with 1 X 10<sup>7</sup> male splenocytes as a positive control. Twenty days later groups of naive female mice were treated with either 30 µg/mouse HY-D<sup>b</sup> tetramer or PBS (unprimed control). Three days after HY-D<sup>b</sup> injection, we grafted tail skin grafts from unmanipulated male B6 donors onto all the female recipients. Fig. 4<sup>a</sup> depicts the data from a representative (one of three) experiment. A single dose of HY-D<sup>b</sup> tetramer to female B6 mice caused significant priming and earlier rejection of graft tissue (Wilcoxon rank order, p = 0.0001). Indeed, this response had identical kinetics to that of male splenocyte-primed female mice, demonstrating that tetramer injection had primed normal female B6 recipients. This also provides further evidence that the HY (KCSRNRQYL) peptide is an important epitope recognized in the anti-male response by female mice in vivo.

Direct injection of HY-D<sup>b</sup> tetramer into HY-TCR mice causes T cell activation, expansion, and priming

To further examine the effects of tetramer injection into female mice, 30 µg HY-D<sup>b</sup> or irrelevant gp33-D<sup>b</sup> tetramer was injected i.p. into female HY-TCR mice. CD8<sup>+</sup> T cells were purified from the spleens of HY-TCR female mice harvested 72 h after tetramer injection and restimulated in vitro with HY-D<sup>b</sup> tetramer or irrelevant gp33-D<sup>b</sup> tetramer. Fig. 4<sup>b</sup> shows that CD8<sup>+</sup> T cells purified from tetramer-injected female mice responded in an Ag-specific manner, with an increased proliferative response to HY-D<sup>b</sup> and not to gp33-D<sup>b</sup>. Additionally, splenocytes from transgenic mice treated with HY-D<sup>b</sup> exhibited an increased proliferative response to HY-D<sup>b</sup> tetramer than gp33-D<sup>b</sup>-treated littermates. It was apparent that in mice 48 h after HY-D<sup>b</sup> tetramer injection, very few cells stained with either anti-CD8 or anti-V<sub>β</sub>8.2 but had a phenotype rejection of male skin grafts. One group of mice was injected with 1 X 10<sup>7</sup> male splenocytes as a positive control. Twenty days later groups of naive female mice were treated with either 30 µg/mouse HY-D<sup>b</sup> tetramer or PBS (unprimed control). Three days after HY-D<sup>b</sup> injection, we grafted tail skin grafts from unmanipulated male B6 donors onto all the female recipients. Fig. 4<sup>a</sup> depicts the data from a representative (one of three) experiment. A single dose of HY-D<sup>b</sup> tetramer to female B6 mice caused significant priming and earlier rejection of graft tissue (Wilcoxon rank order, p = 0.0001). Indeed, this response had identical kinetics to that of male splenocyte-primed female mice, demonstrating that tetramer injection had primed normal female B6 recipients. This also provides further evidence that the HY (KCSRNRQYL) peptide is an important epitope recognized in the anti-male response by female mice in vivo.

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consistent with T cell activation (data not shown). We believe that this represents down-regulation of TCR and CD8, as observed in other studies with peptide stimulation (19, 20). These data demonstrate that administration of HY-Db tetramer in vivo has the ability to prime transgenic mice to HY peptide.

To examine the ability of the in vivo-primed T cells to respond to natural HY Ag, we stimulated them with B6 male spleen cells. Fig. 4 illustrates the restimulation response of tetramer-treated CD8\(^{\text{H11001}}\) T cells to irradiated male B6 splenocytes. Again, CD8\(^{\text{H11001}}\) T cells from tetramer-treated female mice responded much more vigorously than cells from gp33-Db-treated controls. The lack of an apparent response of CD8\(^{\text{H11001}}\) T cells from gp33-Db-treated female mice to male splenocytes is due to the short assay time (48 h), typical of primed T cells. Unprimed HY-TCR shows a peak response at 72 h at this cell density. These data demonstrate that soluble HY-Db tetramer can prime the female HY-TCR mouse to respond to normally processed male Ag as well as HY-Db tetramer.

In vivo administration of multiple doses of HY-Db tetramer resulted in nonresponsive CD8\(^{\text{H9252}}\) T cells

In studies of peptide-induced T cell nonresponsiveness, multiple doses of Ag often had to be administered to observe Ag-specific tolerance (21–23). We wanted to examine whether the priming observed with a single tetramer dose could be changed to exhaustion using multiple tetramer doses.

We injected one, two, or three doses of tetramer or PBS (i.p.) into female HY-TCR mice, each dose separated by 2-day intervals. CD8\(^{+}\) T cells were purified from the spleens and stimulated in vitro with HY-Db tetramer, HY peptide, or irrelevant gp33. Fig. 5 summarizes these data. Mice injected with a single dose of relevant HY-Db tetramer (all PBS-treated groups exhibited high responses to HY-Db tetramer and HY peptide, but no response to gp33 peptide, as in untreated mice; data not shown) exhibited a primed response to HY peptide and tetramer (Fig. 5a). In contrast, if two injections were administered, then the proliferative response to peptide and tetramer is greatly diminished (Fig. 5b) and after three injections becomes undetectable (Fig. 5c).

Flow cytometric examination of the splenocytes in the tetramer-injected mice showed a marked dose-dependent difference in their levels of CD8 expression compared with other cell surface markers (CD69, CD62L, CD44, CD25, V\(^{\text{H9252}}\)8.2; data not shown). Fig. 6a shows that in HY-TCR mice treated with three doses of PBS, the majority of HY-Db tetramer staining cells reside in the CD8\(^{\text{H9252}}\) compartment. However, with increasing tetramer doses, the majority of tetramer-reactive CD8\(^{\text{H9252}}\) T cells are CD8\(^{\text{H9252}}\). This is a result of a loss of tetramer-reactive CD8\(^{\text{H9252}}\) T cells and an increase in CD8\(^{\text{H9252}}\) numbers. Importantly, if the same experiment was repeated in a group of naive B6 female mice, a very similar pattern of tetramer and CD8 staining emerged (Fig. 6b), an increase in CD8\(^{\text{H9252}}\)tetramer\(^{+}\) cells and a decrease in CD8\(^{\text{H9252}}\)tetramer\(^{-}\) cells. This suggests that sustained T cell unresponsiveness associated with multiple tetramer doses is due to sustained decreased expression levels of CD8. Treatment with anti-CD8 Ab blocked an in vitro T cell proliferative response to HY-Db tetramer (data not shown), further confirming an important role for...
Results are expressed as counts per minute of \[^{3}H\]thymidine incorporation. The CD8\^{hi}/H11001, two (\(^{1}\)a, two (\(^{1}\)b), or three (\(^{1}\)c) doses of 30 \(\mu g\) HY-D\(^{b}\) separated by 2-day intervals. Spleens were harvested 72 h after final tetramer injection, and CD8\(^{+}\) T cells were purified by negative selection. The CD8\(^{+}\) T cells were then stimulated in vitro with HY-D\(^{b}\), HY peptide, or irrelevant gp33-D\(^{b}\) tetramer at various concentrations for 72 h.

FIGURE 5. In vivo administration of a multiple doses of HY-D\(^{b}\) tetramer results in anergic CD8\(^{\text{low}}\) T cells. To examine the functional effect of altered levels of CD8 expression after tetramer treatment, female HY-TCR mice were injected with either one (\(^{1}\)a), two (\(^{1}\)b), or three (\(^{1}\)c) doses of 30 \(\mu g\) HY-D\(^{b}\) separated by 2-day intervals. Spleens were harvested 72 h after final tetramer injection, and CD8\(^{+}\) T cells were purified by negative selection. The CD8\(^{+}\) T cells were then stimulated in vitro with HY-D\(^{b}\), HY peptide, or irrelevant gp33-D\(^{b}\) tetramer at various concentrations for 72 h. Results are expressed as counts per minute of \[^{1}\text{H}\]thymidine incorporation (\(\pm\)SEM).

CD8 coreceptor in the activation of HY-TCR T cells by specific HY-D\(^{b}\) tetramer.

Direct injection of multiple HY-D\(^{b}\) doses into female wild-type B6 mice enhanced survival of male skin grafts

Experiments with B6 females (Fig. 4c) suggested that a single dose of HY-D\(^{b}\) tetramer induced priming to HY peptide and earlier rejection of male skin grafts. However, the above data demonstrated that multiple doses of specific HY-D\(^{b}\) tetramer ablated an in vitro proliferative response to HY Ag. We therefore asked whether multiple tetramer exposures would change rejection of male skin grafts. One group of B6 female mice was injected with \(1 \times 10^7\) male splenocytes as a positive control. Twenty days after priming this group, three groups (six mice) of naive female mice were injected with one, two, or three doses of 30 \(\mu g/mouse\) HY-D\(^{b}\) tetramer or PBS. Three days after tetramer administration, tail skin grafts from naive male B6 donors were grafted onto all the B6 female recipients. Fig. 7 illustrates the data from a representative experiment; a single dose of HY-D\(^{b}\) tetramer to female B6 mice caused priming and rapid rejection of skin grafts. However, two or three doses of tetramer reversed this effect; grafts on mice receiving three doses of tetramer surviving significantly (Wilcoxon rank order, \(p < 0.001\)) longer than the control unprimed mice. Indeed, 25% of treated mice showed long-term graft survival. Thus, although there is at least one other defined HY epitope (15, 24, 25), induction of unresponsiveness to the KCSRNRQYL epitope resulted in prolonged graft survival.

Discussion

The results presented in this study demonstrate the dual ability of soluble MHC class I tetramers to directly visualize HY Ag-specific T cells in vivo without any immune manipulation and to directly stimulate HY Ag-specific T cells in vitro and in vivo. HY-D\(^{b}\) tetramer was used to measure the numbers of HY-reactive CD8\(^{+}\) T cells in naive, wild-type female B6 mice. Male-reactive CD8\(^{+}\) T cells were apparent after specific tetramer staining and expressed high levels of CD8 coreceptor. In female wild-type B6 mice, these cells represent \(\sim 1.5-2.0\%\) of the splenic CD8\(^{+}\) T cell population, the first time such an estimate has been made. This is consistent with the strong in vivo response to HY Ag.

We have previously demonstrated that for naive CD8\(^{+}\) T cells from female HY-TCR mice, soluble MHC class I tetramer alone (signal 1) is sufficient for activation and differentiation into effector cells (16). Two other studies have used tetramers to stimulate T cell clones or hybrids (26, 27). Soluble MHC class I tetramers have also been used to stimulate calcium flux in Ag-specific T cell clones (27), and some evidence exists that MHC class I monomers were able to activate CD8\(^{+}\) T cells in vivo (28). Here, we describe the use of soluble MHC class I tetramers to induce and modulate an Ag-specific immune response in vivo. Injection of the HY-D\(^{b}\) tetramer (but not the irrelevant D\(^{b}\) tetramer) into female HY-TCR mice induced a very rapid but transient activation, resulting in

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cells that were nonresponsive to both male Ag and anti-CD3 Abs, starting number (29). Transfer of female B6 CD8+ T cells down-modulates CD8 expression on CD8+ T cells (30). Earlier studies have postulated that immune recognition of HLA class I tetramer and show that tetramer injection can inactivate T cells that recognize them, again possibly mediated by the mucosal immune system (30).

FIGURE 7. Multiple injections of HY-Db tetramers into female normal B6 mice enhanced survival of male skin grafts. One group of B6 female mice was injected with 1 × 107 male splenocytes as a positive, male-primed control group. Twenty days after the priming of this group, three other groups of six naive female mice were injected with one, two, or three doses of 30 μg/mouse HY-Db tetramer or PBS (unprimed control). Three days after tetramer administration, skin grafts from naive male B6 donors were grafted onto the B6 female recipients. Numbers in parentheses, number of mice that were included in the statistical analyses after rejection of technical failures.

Acknowledgments

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References

receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell 65:293.


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