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Modulation of CD8+ T Cell Response to Antigen by the Levels of Self MHC Class I

Fabio R. Santori,2 Ivica Arsov,2,3 and Stanislav Vukmanović4

The response of H-Y-specific TCR-transgenic CD8+ T cells to Ag is characterized by poor proliferation, cytolytic activity, and IFN-γ secretion. IFN-γ secretion, but not cytotoxic function, can be rescued by the B7.1 molecule, suggesting that costimulation can selectively enhance some, but not all, effector CD8+ T cell responses. Although the H-Y epitope binds H-2Db relatively less well than some other epitopes, it can induce potent CTL responses in nontransgenic mice, suggesting that the observed poor responsiveness of transgenic CD8+ T cells cannot be ascribed to the epitope itself. Previously reported reactivity of this TCR to H-2Aβ is also not the cause of the poor responsiveness of the H-Y-specific CD8+ T cells, as H-Y-specific CD8+ T cells obtained from genetic backgrounds lacking H-2Aβ also responded poorly. Rather, reducing the levels of H-2K class I molecules by breeding the mice to (C57BL/6 × B10.D2)F1, or TAP1−/− backgrounds partially restored cytotoxic activity and enhanced proliferative responses. These findings demonstrate that the self MHC class I gene dosage may regulate the extent of CD8+ T cell responsiveness to Ag. The Journal of Immunology, 2001, 166: 5416–5421.

Recognition of self peptide/MHC complexes in the thymus determines whether immature T cells live and mature into immunocompetent T cells. Immature CD4+CD8+ thymocytes differentiate into mature CD4+CD8− or CD4−CD8+ T cells as a result of the low affinity interactions of their TCRs with self peptide/MHC complexes, whereas recognition with relatively high affinity leads to negative selection (1). Successful maturation in the thymus leads ultimately to the emigration of mature T cells to the periphery, where continued low affinity recognition of self peptide/MHC complexes is required for their long term survival (1). Relatively high affinity interaction with the peripheral self ligands not shared by the thymus can result in peripheral tolerance (2). Tolerance to self can take various shapes and forms, ranging from physical deletion of T cells, modulation of cell surface expression of recognition/signaling molecules (3–5), complete functional incapacitation of T cells (6, 7), raising the activation thresholds in T cells (8), or selective inactivation of some aspects of T cell responses (9).

There are two principal effector mechanisms used by effector CD8+ T cells: direct cytotoxicity and cytokine secretion. Most of the direct cytotoxic action is mediated by the release of the cytolytic enzymes perforin and granzymes (10–12), with a minor contribution by the Fas-Fas ligand interaction (10–12). The second effector mechanism of CD8+ T cells is the secretion of lymphokines, such as IFN-γ, IL-3, TNF-α, and GM-CSF. Induction of cytokine secretion and cytolysis may follow divergent TCR signaling pathways, as suggested by the variety of ways in which one effector response is induced in the absence of the other (9, 13–20). Signaling requirements appear less stringent for the induction of cytolyis than for the cytokine responses, as reducing the epitope density first affects lymphokine secretion (15, 16). In addition to reduced epitope density, cytotoxicity in the absence of lymphokine secretion can be obtained if CD8+ T cells are stimulated with an altered Ag (9, 14) or if the Ag is provided in the absence of costimulatory signals (13). All these examples suggest that cytotoxic activity is a more sensitive effector function than cytokine secretion and that cytokine secretion responses require relatively stronger stimulation. However, there are examples of lymphokine responses in the absence of cytolytic function (18–20). We here explore the basis of inverted effector responses of H-Y-specific TCR-transgenic CD8+ T cells (21). We find that tolerance to self MHC class I is responsible for the generally poor responsiveness of these cells to Ag and that costimulation can selectively rescue some aspects of effector CD8+ T cell responses, such as IFN-γ secretion.

Materials and Methods

Mice

C57BL/6 (B6)3 and H-Y TCR-transgenic mice on a B10.D2 or B6, recombinase-activating gene-2−/− background were purchased from Taconic Farms (Germantown, NY). B10.A(2R) and TAP1−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were used at 6–8 wk of age. B6 H-Y TCR-transgenic mice were backcrossed for two generations to B10.A(2R), or for one generation with TAP1−/− mice. B10.D2 H-Y TCR-transgenic mice were crossed once with B6 mice to obtain the F1 generation. All matings were performed in the animal facility of the Skirball Institute of Biomolecular Medicine at New York University Medical Center. Mice were screened by immunofluorescence of peripheral blood cells.

3 Abbreviation used in this paper: B6, C57BL/6.
Peptides

Synthetic peptides corresponding to aa 245–253 (WMHHNNMDLI) of the Uty gene product (22) or aa 738–746 (KCSRNRQYLM) of the Smcy gene (23, 24) as well as influenza A/PR8/34 NP366–374 (ASNENMETM) (25) were purchased from Research Genetics (Huntsville, AL).

Cell lines

The derivation and maintenance of the H-Y TCR-transgenic CD8\(^+\) T cell line, designated HYCD8, were previously described (20). The anti-H-Y-specific CD8\(^+\) T cell line (designated FB.1) from a nontransgenic female B6 mouse was obtained using previously described protocol (26) and was maintained by weekly restimulations with irradiated spleen cells from B6 male mice in the presence of 5% rat Con A supernatant. EL-4 cells transfected with B7.2 (27) as well as control cells transfected with expression vector alone were provided by Dr. Yang Liu (Ohio State University, Columbus, OH). The male lymphoblastoid cell line 13a and its H-Y Ag loss variant 13a-575 (28) were provided by Dr. Derry Roopenian (The Jackson Laboratory).

RMA-S stabilization assay

To analyze binding affinity of peptides for H-2D\(^b\), RMA-S cells (5 \(\times\) 10\(^6\)) were incubated overnight at room temperature and then pulsed with different concentrations of peptides in a final volume of 200 \(\mu\)l for 1 h at 37°C. After extensive washing, cells were stained with anti-H-2D\(^b\) Ab (KH95; BD Pharmingen, San Diego, CA), washed twice, incubated with FITC-conjugated goat-anti mouse Ig (Southern Biotechnology Associates, Birmingham, AL) and then analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Peptide immunization

Peptides were emulsified in TiterMax adjuvant (Vaxcel, Norcross, GA) according to the manufacturer’s recommendation, and a total of 50 \(\mu\)g peptide in this mixture was injected s.c. into one footpad of metofane-anesthetized animals, as described by Dyal et al. (29). Seven days after immunization, spleen cells from immunized animals were restimulated in 25-cm\(^2\) tissue culture flasks (Falcon; BD Biosciences) at 3 \(\times\) 10\(^6\) of responder cells/flask with 2 \(\times\) 10\(^5\) peptide-coated irradiated spleen cells that have been pulsed with 100 \(\mu\)g of peptide/spleen in RPMI 1640 medium (Life Technologies, Grand Island, NY) for 1 h at 37°C, and then washed three times in RPMI 1640. Cultures were incubated for 7 days at 37°C in 5% CO\(_2\), in a total volume of 10 ml of RPMI 1640 supplemented with 10% FCS. After 7 days, cells were washed and used as effector cells.

Proliferation, and CTL and IFN-\(\gamma\) secretion assays

Spleen cells from H-Y-specific TCR-transgenic mice (5 \(\times\) 10\(^6\)/well) were incubated in round-bottom 96-well plates with the test peptide at the desired concentration. Cultures were maintained in RPMI 1640 medium supplemented with 5 \(\times\) 10\(^{-5}\) M 2-ME, 1 mM sodium pyruvate (Life Technologies), 0.1 mM nonessential amino acid solution (Life Technologies), 0.1 mM nonessential amino acid solution (Life Technologies), and 10% FCS (PM-10 medium) for 72 h. Each microculture was then pulsed with 0.5 \(\mu\)Ci of [\(\text{3H}\)]thymidine (ICN Biomedicals, Costa Mesa, CA) indicating that IFN-\(\gamma\) release, too, may be missing when H-Y TCR-transgenic CD8\(^+\) T cells and indicating that IFN-\(\gamma\) release, too, may be missing when H-Y TCR-transgenic CD8\(^+\) T cells are stimulated with nonprofessional APCs. As expected, IFN-\(\gamma\) secretion was undetectable even in the presence of a high concentration of Smcy\(_{738–746}\) added to EL4 stimulator cells (Fig. 2B). In contrast, when Smcy\(_{738–746}\)-expressing EL4 cells (27) were used as stimulators significant IFN-\(\gamma\) secretion was observed with the wide range of antigenic peptide concentration (Fig. 2B). To directly address whether costimulation selectively enhances IFN-\(\gamma\) secretion or perhaps Ag is not due to either a signaling or a cytolytic machinery defect (20). In fact, these findings raised the possibility that the apparent absence of cytotoxic responses to Ag could be due to technical aspects of the experimental systems used, where Ag and APCs were not fully defined. The absence of obvious lysis of LPS-induced blast cells, for example, could be explained by a potential restricted Ag distribution. Ag expression by a very small subset of male LPS blast cells could lead to a very small experimental \(\text{51Cr}\) release. To address this question, we first used cloned male lymphoblastoid tumor 13a as a target. The lysis of 13a cells by non-transgenic H-Y-specific CD8\(^+\) cells, FB.1, confirms the expression of the male Ag by 13a cells (Fig. 1). However, TCR-transgenic cells did not demonstrate significant lysis against 13a cells (Fig. 1).

Recently, two peptides have been identified as male-specific epitopes presented to H-Y-specific CD8\(^+\) T cells by H-2D\(^b\). One (WMHHNNMDLI) is derived from the product of the Uty gene (22), and the other (KCSRNRQYLM) from the product of the Smcy gene (23, 24), both located on the Y chromosome. Transgenic CD8\(^+\) cells recognize the latter, whereas most of the other cell lines/clones recognize the former epitope, including the FB.1 cell line (data not shown). Both Smcy and Uty genes were identified by Southern blotting in 13a cells (28), but it is theoretically possible that Smcy is not expressed or that the epitope Smcy\(_{738–746}\) is not processed and presented in 13a cells. Therefore, we assessed the cytotoxic response of transgenic CD8\(^+\) T cells to synthetic Smcy\(_{738–746}\) (KCSRNRQYLM) epitope and determined in a conclusive manner that the poor cytolytic response of H-Y TCR-transgenic CD8\(^+\) T cells was not due to technical reasons, but, rather, to a specific inability of transgenic cells to respond by cytolysis (Fig. 2A).

**Costimulation rescues IFN-\(\gamma\), but not the cytotoxic response of H-Y TCR-transgenic CD8\(^+\) cells to cognate Ag**

Abs to costimulatory molecule B7.2 inhibited the IFN-\(\gamma\)-response to male LPS blasts (20), suggesting that costimulation is essential for induction of cytokine secretion by transgenic CD8\(^+\) T cells and indicating that IFN-\(\gamma\)-release, too, may be missing when H-Y TCR-transgenic CD8\(^+\) cells are stimulated with nonprofessional APCs. As expected, IFN-\(\gamma\) secretion was undetectable even in the presence of a high concentration of Smcy\(_{738–746}\) added to EL4 stimulator cells (Fig. 2B). In contrast, when Smcy\(_{738–746}\)-expressing EL4 cells (27) were used as stimulators significant IFN-\(\gamma\) secretion was observed with the wide range of antigenic peptide concentration (Fig. 2B). To directly address whether costimulation selectively enhances IFN-\(\gamma\) secretion or perhaps
whether Smcy 738–746 can stimulate efficiently CD8 T cell response and that the poor responsiveness of TCR-transgenic mouse to a range of Ag concentrations to proliferative responses of CD8 T cells to a range of Ag concentrations to proliferative responses of CD8 T cells carrying another MHC class I-restricted TCR, OT-I (34). Proliferation of H-Y-specific CD8 T cells was much lower and required higher Smcy738–746 Peptide concentrations to achieve a maximal response relative to the proliferative response of OT-I CD8 T cells to OVA257–264 (Fig. 5). These findings demonstrate that H-Y TCR-transgenic CD8 T cells may be qualified as generally poor responders.

**Self MHC molecules affect responsiveness of H-Y TCR-transgenic CD8 T cells to Ag**

The data presented in Fig. 4 argue that the poor response of transgenic CD8 T cells cannot be ascribed to any stimulatory failure of Smcy738–746 peptide, whereas stimulation of the cytotoxic response with anti-CD3 Abs (20) eliminates the defect in transgenic CD8 T cells. Therefore, the key to the poor responsiveness of H-Y-specific transgenic CD8 T cells has to lie in a specific interaction of this receptor with H-Y Ag (such as low affinity of the receptor for the epitope/MHC complex) and/or elevated threshold effects.

*Smcy* 738–746 stimulates potent cytolytic responses in wild-type mice

Poor cytolytic responses of H-Y-specific CD8 T cells could be due to defective antigenic stimulation. For example, the Smcy738–746 peptide might bind poorly to H-2D<sup>e</sup>, or TCRs with high affinity/avidity for Smcy738–746/H-2D<sup>e</sup> complex might be absent from the repertoire due to negative selection by a similar self peptide (perhaps derived from the Smcx homologue). To address these issues, we first compared the affinity of binding of Smcy738–746, Uty245–253 (22), or NP366–374 (25) to H-2D<sup>e</sup> using an RMA-S stabilization assay. Although high concentrations of Smcy738–746 induced maximal up-regulation of the H-2D<sup>e</sup> comparable to the other two peptides, the concentration of Smcy738–746 required to induce 50% maximal up-regulation were higher, indicating somewhat lower affinity of binding to H-2D<sup>e</sup> (Fig. 3). To determine whether this lower affinity for the H-2D<sup>e</sup> might explain the low responsiveness of H-Y-specific CD8<sup>+</sup> T cells, we examined whether Smcy738–746 can stimulate efficiently CD8<sup>+</sup> T cells other than those from the TCR-transgenic mouse. To test for the presence of Smcy738–746/H-2D<sup>e</sup>-reactive CD8<sup>+</sup> T cells, mice were immunized and restimulated with this peptide. CD8<sup>+</sup> T cell effectors generated in this manner efficiently lysed EL-4 cells pulsed with Smcy738–746 (Fig. 4). Thus, these experiments demonstrate that the Smcy738–746 peptide can induce a specific cytotoxic response and that the poor responsiveness of TCR-transgenic CD8<sup>+</sup> T cells cannot be ascribed to the poor immunogenicity of the epitope.
of activation of these cells. Given that the cells produced IFN-γ in response to a relatively low peptide concentration in conjunction with costimulation (Fig. 2B), we reasoned that physical aspects of Ag recognition by this TCR may not be the reason for hyporesponsiveness, although the actual physical parameters remain to be determined. Therefore, we decided to first examine whether interactions with self MHC molecules might have raised the activation threshold of H-Y-specific CD8+ cells.

We have previously demonstrated a cross-reactivity of the H-Y TCR for self MHC class I (35), and interactions of MHC class I-restricted receptors with MHC class II ligands were reported to reduce the responsiveness of CD8+ cells to class I-restricted ligand (8). MHC class I molecules could also influence the responsiveness of H-Y-specific CD8+ cells. The candidate peptides might involve an Smcx homologue of the Smcy antigenic peptide or perhaps self peptides with antagonist activity. All self MHC molecules can be totally removed/replaced with the exception of H-2Dβ, which has to be present for positive selection of H-Y-specific CD8+ cells to occur (31). In this case we can only reduce the levels of H-2Dβ by half. To test whether dilution of self MHC might impact the responsiveness of H-Y-specific CD8+ cells we compared the Smcy738–746-induced proliferative and cytotoxic responses of H-Y-specific CD8+ cells from B6 and F1(B6 × B10.D2) backgrounds. The magnitude of proliferative responses to Ag was about 3-fold higher in F1 than in the B6 background, although not as high as in the OT-I mice (Fig. 5). In addition, the proliferative response of these cells was reduced by 50% (Fig. 6). In the presence of 1 μM Smcy738–746 peptide and used as effector cells at the indicated E:T cell ratios. 5419The Journal of Immunology

FIGURE 5. Proliferative responses of OT-I- and H-Y TCR-transgenic spleen cells to their respective cognate Ags. A total of 5 × 10^5 well of OT-I, H-Y (B6), or H-Y (F1(B6 × B10. D2)) TCR-transgenic cells were stimulated for 48 h with the indicated concentration of cognate Ag (Smcy738–746 peptide for H-Y cells and OVA257–264 for OT-I cells). Cells were pulsed with [3H]thymidine for 16 h, and incorporation was measured by scintillation counting. The assay was performed under identical conditions for all responder mice. Shown are the mean and SD of triplicate cultures.

FIGURE 6. Ag-specific cytotoxic activity of H-Y-specific CD8+ cells from various genetic backgrounds. H-Y-specific TCR-transgenic cells from the indicated backgrounds were stimulated for 5 days in the presence of 1 μM Smcy738–746 peptide and used as effector cells at the indicated E:T cell ratios. 35Cr-labeled EL4 cells were used as target cells in the absence or the presence of 1 μM Smcy738–746.

Discussion

We demonstrate that the strength of CD8+ T cell responses to Ag can be regulated by levels of self MHC class I molecules. H-Y-specific CD8+ T cells are poorly cytolytic and do not produce detectable amounts of IFN-γ in response to Ag unless costimulatory signals are provided that selectively rescue IFN-γ secretion. Under identical culture conditions, proliferation to the respective cognate Ag of H-Y-specific transgenic CD8+ T cells is severalfold lower than that of OT-I-transgenic CD8+ T cells. The poor responsiveness to Ag is not due to intrinsic properties of the antigenic epitope, as it can induce potent cytolytic responses in non-transgenic mice. Reducing the levels of self MHC class I molecules leads to enhanced proliferative responses and recovery of cytolytic activity of H-Y-specific CD8+ T cells.

In addition to MHC/peptide recognition, resting CD8+ T cells must be provided with one or more costimulatory signal(s) to be fully activated (38). In contrast to the priming of naive T cells, the

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The effector function of CD8+ T cells was shown to be independent of costimulatory signals (33), in line with the expression of B7 molecules restricted on professional APCs (39) and the cell type-independent ability and requirement of lysis by CD8+ cells. However, costimulation-dependent cytolysis by CD8+ cells has previously been reported (27, 40). Our results concur with these reports, supporting the idea that under certain circumstances costimulation may be required to trigger effector CD8+ T cell responses. One characteristic in common is that in all these cases antigenic stimulation was suboptimal, either because altered epitope was used (27), self Ag was used (40), or some form of tolerance to self is involved (present study). Thus, costimulation may help trigger the CD8+ T cell effector functions in response to relatively weak stimulation.

Another implication of the present study may be that costimulation selectively enhances the signals that result in lymphokine secretion. Induction of cytolytic CD8+ T cell responses in the absence of cytokine secretion (9, 13–17) or, less frequently, the reverse phenotype (18, 19) suggest that signaling events controlling granule exocytosis might be distinct. Three major signaling pathways were implicated in selective regulation of granule exocytosis in CD8+ T lymphocytes. These involve protein kinase C (17, 41), mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 (42, 43), and phosphoinositol 3-kinase (44). In contrast, calcineurin is, in general, involved in cytokine secretion (45), but not in perforin/granzyme-mediated cytolysis (46), suggesting the selective involvement of calcineurin in cytokine responses. IFN-γ secretion was shown to be controlled by activation of mitogen-activated protein kinase p38 (47), but the potential role of this activation pathway in the induction of granule exocytosis has not yet been addressed. It remains to be determined how costimulation signaling pathways contribute selectively to particular effector functions such as IFN-γ secretion, and why cytolysis in some circumstances is affected by costimulation (27, 40) and in others apparently not affected (present study). These differences underscore the caution required when interpreting complex relationships among costimulation, various avidities of antigen stimulation, and lymphocyte effector responses induced.

What self ligand could be involved in modulating the antigenic responses of H-Y-specific CD8+ T cells? The data presented here implicate MHC class I molecules, because cytotoxic function was recovered in the TAPI1/+ background (Fig. 6). Furthermore, the influence of MHC class II molecules as well as the K locus of the MHC class I can be eliminated because of the failure of the B10A(2R) background to recover the cytolytic function (Fig. 6). Thus, the tolerogen is either H-2Db itself or, possibly, a nonclassical class I locus downstream of H-2Db. Although the role of class Ib molecules cannot be excluded, given that H-Y TCR-transgenic CD8+ T cells are selected by and recognize Ag restricted by H-2Db, we believe that H-2Db is the more likely candidate. Although the Smcx homologue (KCSSSRQYL) of the Smcy738–746 peptide does not have an anchor at position 5 for binding to H-2Db, it may still bind, as other peptides binding to H-2Db without complete anchors have been identified (48). The presentation of this self peptide could increase the threshold of activation for Smcy738–746-specific T cells. However, we believe that another self peptide is a more likely candidate. We recently discovered an H-2Db-presented self peptide (Ube1X509–517) that can act as an antagonist for Ag-induced in vitro responses of H-Y TCR-transgenic CD8+ T cells. This peptide has both anchors, binds H-2Db relatively well, and is relatively abundant (represents ~3% of the material eluted from the H-2Db). Antagonist peptides expressed in vivo have been shown to induce hyporesponsiveness of T cells (49). Unfortunately, the protein (ubiquitin-conjugating enzyme 1) that is a source of this peptide is essential for cell survival (50), and no gene knockout is available to directly test whether Ube1X509–517 reduces the responsiveness of H-Y TCR-transgenic CD8+ T cells.

Is the phenotype of H-Y-specific transgenic CD8+ cells unique, or are poorly responsive CD8+ part of other immune responses as well? Although cytolysis normally appears easier to trigger (9, 13–17), IFN-γ secretion in the absence of cytolysis has been documented in several instances. For example, CD8+ T cell responses to some endogenous superantigens are characterized by IFN-γ secretion in the absence of cytolysis (18). Also, the response of a CD8+ T cell clone to self peptide was accompanied by IFN-γ production and Fas-mediated killing, but the release of serine esterases was absent (19). Thus, we believe that poorly responsive cells may be a part of any immune response. More sensitive detection of Ag-specific cells by enzyme-linked immunospot assays than by cytolysis assays (51) supports this idea. The reason for their low detectability possibly reflects a disadvantage that these cells may have in growth. Alternatively, it is possible that this type of cell is carried along in many cell lines, but has remained unnoticed because of its requirement for costimulation in responsiveness to Ag. Cloning of cells early, as was the case with the H-Y-specific TCR (32), may allow more frequent isolation of cells with this phenotype. Perhaps the relative frequency of low responder cells with this and/or similar phenotypes relative to the high responder cells may influence epitope immunodominance (52).

Assuming that poorly responsive CD8+ T cells generally arise in immune responses it is not clear what role, if any, these cells could play in protective immunity. First, lymphokine secretion on its own is a major way of controlling the magnitude and other aspects of the immune response. In numerous cases CD8+–mediated cytokine secretion alone (without cytolytic activity) could provide immune protection (53–55). Furthermore, it is possible that these types of cells, although somewhat defective, could, in fact, be advantageous for the immune system. It has been reported that perforin- and Fas-mediated killing by CD8+ T cells may limit their cytokine synthesis and proliferation because of an early destruction of Ag-bearing cells (56). In addition, IFN-γ appears to control the death phase that follows Ag-induced expansion (57). Consequently, CD8+ cells incapable of lysing the target cells, but capable of secreting cytokines, may be more efficient in perpetuating the immune response. This could be quite useful in cases where a long-lasting immune response is required, such as in tumor-specific responses where sustained costimulation may be required for tumor clearance (58). However, these types of cells could be dangerous in cases of autoimmune responses.

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References


