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Cutting Edge: Negative Selection of Immature Thymocytes by a Few Peptide-MHC Complexes: Differential Sensitivity of Immature and Mature T Cells

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The process of negative selection of thymocytes is highly sensitive to the dose of ligand (1–7). Early studies demonstrated that negative selection was more sensitive than activation of mature T cells (1–4, 7, 8). The conclusions were based solely on the concentrations of ligand that were effective in these experimental systems.

A direct quantitation of the density of ligand required for negative selection may be very useful for understanding the natural history of autoreactive T cells. To do this, we have taken advantage of a TCR transgenic mouse specific for an epitope of hen egg white lysozyme (HEL),3 3A9 (9). First, we know the precise HEL tag of a TCR transgenic mouse specific for an epitope of hen egg white lysozyme that is required for the deletion or activation of 3A9 TCR transgenic thymocytes. Deletion of immature double positive thymocytes was very sensitive, taking place with approximately three peptide-MHC complexes per APC. However, the activation of mature CD4+ thymocytes required 100-fold more complexes per APC. Therefore, a “biochemical margin of safety” exists at the level of the APC. To be activated, autoreactive T cells in peripheral lymphoid tissues require a relatively high level of peptide-MHC complexes. The Journal of Immunology, 1999, 162: 3117–3120.

We quantitated the number of peptide-class II MHC complexes required to affect the deletion or activation of 3A9 TCR transgenic thymocytes. Deletion of immature double positive thymocytes was very sensitive, taking place with approximately three peptide-MHC complexes per APC. However, the activation of mature CD4+ thymocytes required 100-fold more complexes per APC. Therefore, a “biochemical margin of safety” exists at the level of the APC. To be activated, autoreactive T cells in peripheral lymphoid tissues require a relatively high level of peptide-MHC complexes.

Mice

The 3A9 mice used in this study were obtained from Dr. Mark Davis (Stanford University School of Medicine, Stanford, CA) (9). They were back-crossed to B10.BR (H-2b) and maintained in the Washington University mouse facility. Mice were screened by FACS analysis of PBLs with anti-CD4 FITC (GK1.5) and biotinylated anti-V-β8 (MR5-2; PharMingen, San Diego, CA).

Antibodies

Anti-CD4, anti-CD69, and anti-V-β8 were purchased from PharMingen. Anti-CD8 quantum red and propidium iodide (PI) were purchased from Sigma (St. Louis, MO).

Activation determined by CD69 induction

The APCs used in these studies were M12.C3.F6 (14) and murine B lymphoma cells transfected with I-Ak. These APCs were incubated for 20 h with titrating concentrations of 48–61 peptide (5 × 10^4 in 100 µl/well in 96-well trays) and subsequently washed three times before the addition of thymocytes. Thymocytes and APCs were then incubated together for an additional 20 h. Next, cells were stained with anti-CD4 FITC, anti-CD8 quantum red, and anti-CD69 biotin, followed by NeutrAvidin-phycoerythrin (Southern Biotechnology Associates, Birmingham, AL). PI at 300 µg/ml was added to samples immediately before analysis on the FACSScan (Becton Dickinson, Mountain View, CA). Lymphocytes were gated by forward and side scatter, and cells incorporating PI (dead cells) were excluded from the analysis. The geometric mean fluorescence (MF) of FL-2 (CD69) was then calculated for the CD4+CD8− T cells. The maximum CD69 up-regulation was calculated as follows: 100 × (X – min/max – min), where X is the MF for a given Ag concentration, min is the MF of T cells incubated with APCs but no Ag, and max equals the maximum MF observed.
In vitro deletion

In vitro deletion was performed as described by Iwabuchi et al. (15), with the following modifications: APCs were prepared as described above. Thymocytes from 6–8-wk-old 3A9 mice were added to the wells at a ratio of 10 thymocytes to one APC (usually about $7.5 \times 10^3$ thymocytes/well). Thymocytes and APCs were incubated for 20 h and then resuspended; a sample was counted by trypan blue exclusion on a hemocytometer. The percentage of CD4+CD8- PI-negative thymocytes (non-FL-3bright) was subsequently examined.

Measurement of peptide-MHC complexes per APC

The AW3.18 Ab was radioiodinated using the chloramine T method (13). M12.C3.F6 cells were cultured in vitro at $5 \times 10^5$/ml in the presence or absence of the HEL 48–61 peptide for 20 h. Cells were then washed and resuspended in HBSS media containing 1% BSA and 5 mM HEPES buffer at a concentration of 1 or $2 \times 10^5$/ml. The cells were incubated for 2 h with titrating amounts of intact labeled Ab and then centrifuged through oil to separate cell-bound and free Ab. M12.C3.F6 cells incubated without peptide were used to calculate background binding of the Ab (typically <1%). The total number of sites per cell was calculated using Scatchard analysis of the four to six different Ab input concentrations (each was done in triplicate) per experimental peptide concentration; this allowed us to determine the nanograms of Ab bound at saturation and the number of sites per cell at saturation. To extrapolate the number of peptide-MHC complexes, the data were fitted to the following formula (16, 17) as a "user-defined nonlinear regression" with GraphPad Prism Software (GraphPad, San Diego, CA): 
$$
\text{[peptide-MHC]} / \text{[MHC]}_{\text{total}} = (K_a \times [\text{peptide}]_{\text{total}})/(1 + K_a \times [\text{peptide}]_{\text{total}}),
$$

where peptide-MHC equals the number peptide-MHC complexes per APC, [MHC]_{\text{total}} equals the total number of MHC molecules available for binding peptide per APC, $K_a$ is the association constant for peptide-MHC, and [peptide]_{\text{total}} is the concentration of peptide. This formula derived the following apparent values for $K_a$ and MHC_{\text{total}}, $K_a = 272,500$ M$^{-1}$ (SEM = 96,640), and MHC_{\text{total}} = 126,000 (SEM = 22,850). Using these values in the above equation, we were able to determine the number of peptide-MHC complexes per APC at the relevant concentrations of peptide.

Results

We compared in simultaneous culture the activation of CD4 single positive (SP) thymocytes and the deletion of CD4, CD8 double positive (DP) thymocytes. Thymocytes from disrupted 3A9 thymi were incubated for 20 h with APCs pulsed with titrating concentrations of 48–61 peptide overnight and then washed before the addition of the thymocytes. We recovered >85% of the input thymocytes when they had been incubated with APCs that were not pulsed with peptide. Incubating 3A9 thymocytes with APCs pulsed with 10 μM peptide resulted in the recovery of ~50% of input thymocytes. This loss was attributable to the deletion of DP thymocytes, because there was no significant decrease in SP cells (Fig. 1). The extent of deletion was dependent upon the amount of peptide (Fig. 2).

Fig. 1 shows the up-regulation of CD69 on SP thymocytes, whereas Fig. 2 shows the dose-response of the maximal MF expression. Up-regulation of CD69 is a reliable index of T cell activation, as demonstrated by many groups using different T cells (18–21) including 3A9 (9, 22, 23). It is important to note two points. First, we found a comparable dose-response of CD69 up-regulation on SP CD4+ T cells from the thymus, lymph nodes, and spleen. Second, the percentage of maximum CD69 up-regulation was directly comparable in dose-response to the proliferation of peripheral T cells in vitro (data not shown).

Fig. 2 shows the striking difference in the sensitivity between these two responses. Although 100 pM peptide affected 50% maximum deletion, 10 nM peptide was required for a 50% maximum induction of CD69 on the SP cells. Thus, the dose of peptide required for 50% maximum deletion was 100-fold lower than for 50% maximum activation (Fig. 2).

Using $^{125}$I-radioiodabeled AW3.18, the number of peptide-MHC complexes formed ranged from 70,000 to 100,000 for the highest doses of peptide. The lowest directly measurable complexes were ~5000/ACP at $1 \times 10^{-7}$ M peptide (Fig. 3). These directly measured values of peptide occupancy fit the equation (curve shown in Fig. 3) used previously (16, 17) to describe peptide binding to MHC molecules (i.e., apparent association constant and maximum number of sites per cell). From these values, we extrapolated the number of complexes at the peptide concentrations in which we observed deletion and activation. The number of peptide-MHC complexes per APC for activation (10 nM) was ~340 complexes (164–579 at the 95% confidence interval). The number of complexes per APC for deletion (100 pM) was only 3.4 (1.6–5.8 at the 95% confidence interval).

![FIGURE 1](http://www.jimmunol.org/) In vitro deletion of DP and activation of SP thymocytes after 20 h of incubation of thymocytes with peptide-pulsed APCs. There was a specific deletion of DP thymocytes but no change in the SP thymocytes. The SP thymocytes uniformly up-regulated the activation marker CD69. The 10 μM peptide-activated SP thymocytes had a mean CD69 fluorescence of 147, and >90% of the cells had up-regulated CD69; in contrast, the 0.01 μM of peptide-activated SP thymocytes (±47%) had an MF of 75, and 69% of the cells had up-regulated CD69 (data not shown). In this experiment, the recovery of thymocytes was 1.3 × 10^5 (19% SP and 49% DP) in the "no Ag" control and 6.8 × 10^5 (36% SP and 7% DP) in the "10 μM 48–61". These results are representative of seven different experiments.

![FIGURE 2](http://www.jimmunol.org/) Comparison of the concentration of 48–61 peptide required to induce 50% of the maximal response of both deletion and activation parameters. There is a 100-fold difference in the sensitivity of these two processes. Percent maximal response was calculated as described in Materials and Methods. The data shown are the mean of three experiments. Bars represent the SE.
The thymic blood barrier as well as the competition among proteins for per thymic APC, when considering their levels in the blood and the blood serum proteins would be represented by only a few peptides most dangerous autoreactive T cells. Indeed, we expect that many thymic APCs will eliminate the highest affinity and presumably the line that contributes a strong adhesion/accessory function to the nature and amount of the ligand, and the contribution of adhesion/development (i.e., DP to a naive and to an effector/memory stage, the 

From this perspective, the entire process of a T cell response is a of TCRs interacting with MHC molecules that do not contain the signal may represent an increment above the initial basal process aggregation as a requirement for this sensitive signaling process. This 

In this study, we demonstrate the extreme sensitivity of the negative selection process that takes place on a handful of complexes (approximately three per APC). We do not know the duration of T cell-APC interaction or the number of APCs that need to be contacted. We also have not addressed the stoichiometry of the TCR-peptide-MHC interaction (24); however, we did observe that DP thymocytes down-modulate their TCRs in the first 5 h of culture at doses from 100 nM to 10 μM. Such modulation did not take place at the very low doses that triggered deletion. An analysis of 3A9 TCR expression on thymocytes with clonotypic Ab revealed that the DP thymocytes were heterogeneous, expressing low, intermediate, and high levels of TCR with an average of 3,000 TCRs per cell, whereas the SP thymocytes express a uniformly high level of TCR with an average of 18,000 TCRs per cell (our unpublished data). Therefore, it appears that the deletion signal involved not only a limiting number of peptide-MHC complexes but also a limiting number of TCRs, and that the differential sensitivity is not related to the level of TCR expressed at the surface of the thymocytes. These very low numbers argue against extensive receptor aggregation as a requirement for this sensitive signaling process. This signal may represent an increment above the initial basal process of TCRs interacting with MHC molecules that do not contain the specific peptides, a point that has been argued previously (25, 26). From this perspective, the entire process of a T cell response is a continuum of interactions modulated by the stage of T cell development (i.e., DP to a naive and to an effector/memory stage, the nature and amount of the ligand, and the contribution of adhesion/costimulatory molecules by the APCs). In our case, we use an APC line that contributes a strong adhesion/accessory function to the 3A9 T cell.

The very high sensitivity of the deletion process of the DP thymocytes assures that virtually any level of protein expressed in thymic APCs will eliminate the highest affinity and presumably the most dangerous autoreactive T cells. Indeed, we expect that many blood serum proteins would be represented by only a few peptides per thymic APC, when considering their levels in the blood and the thymic blood barrier as well as the competition among proteins for processing and binding to MHC molecules. Of course, the affinity of the TCR for the complex will be a component that determines the number of peptide-MHC complexes required for a given activation parameter (25, 16). Extrapolating our results to autoreactive T cells having properties akin to the 3A9 T cells, we can argue that a gradient of self-peptide-MHC complexes is unlikely to explain positive and negative selection (6) given the limited number of complexes active in negative selection. We would argue more for positive selection to involve epitopes that are cross-reactive with the natural ligand (27).

Our data also reveal that the self-reactive T cells in the peripheral lymphoid tissues have a substantially higher threshold of activation. This higher threshold is the “margin of safety” to which Yagi and Janeway (2) referred and which we have translated into a “biochemical margin of safety”: Engaging functionally autoreactive T cells that were not centrally purged would require a relatively high efficiency of representation of peptide-MHC complexes on the peripheral APCs (~0.1% occupancy of MHC molecules). APCs varying from 10^5 to 10^6 class II MHC molecules/cell would require 0.03–0.3% occupancy by peptides. (The content in our APCs is similar to dendritic cells; ~10^7/cell.) In a typical chemical isolation of class II bound peptides from APCs, the number of peptides represented below the level required for triggering T cells is considerable. In our experience, a recovery of 500 pmol of peptide isolated from 10^10 APCs would represent 0.3% occupancy, but many peptides are identified with 100- to 100,000-fold less abundance (picomole to femtomole levels) (28–31). Thus, most of the self-peptides bound to MHC would not be found in sufficient quantity to drive a self-reactive T cell response. This margin of safety places the following conditions for the stimulation of autoreactive T cells: that a large pool of self-protein be present, perhaps in a suitable microenvironment; that changes in Ag presentation occur that would preferentially enhance self-Ag presentation; or that events that lower the normal threshold of activation of the T cell take place. Along these lines, activated T cells react to a lower level of peptide-MHC complexes (18, 19), to note is the very high sensitivity of effector CD8 T cells, which can kill targets bearing a few class I MHC-peptide complexes (32).

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**References**


