# Modulation of MHC Binding by Lateral Association of TCR and Coreceptor

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ABSTRACT The structure of a T cell receptor (TCR) and its affinity for cognate antigen are fixed, but T cells regulate binding sensitivity through changes in lateral membrane organization. TCR microclusters formed upon antigen engagement participate in downstream signaling. Microclusters are also found 3–4 days after activation, leading to enhanced antigen binding upon rechallenge. However, others have found an almost complete loss of antigen binding four days after T cell activation, when TCR clusters are present. To resolve these contradictory results, we compared binding of soluble MHC-Ig dimers by transgenic T cells stimulated with a high (100 $\mu$M) or low (100 fM) dose of cognate antigen. Cells activated by a high dose of peptide bound sixfold lower amounts of CD8-dependent ligand $K^b$-SIY than cells activated by a low dose of MHC/peptide. In contrast, both cell populations bound a CD8-independent ligand $L^d$-QL9 equally well. Consistent with the differences between binding of CD8-dependent and CD8-independent peptide/MHC, Förster resonance energy transfer (FRET) measurements of molecular proximity reported little nanoscale association of TCR with CD8 (16 FRET units) compared to their association on cells stimulated by low antigen dose (62 FRET units). Loss of binding induced by changes in lateral organization of TCR and CD8 may serve as a regulatory mechanism to avoid excessive inflammation and immunopathology in response to aggressive infection.

## INTRODUCTION

T cells face the extraordinary challenge of finding as few as 1–10 cognate peptides presented in the context of major histocompatibility complex (MHC) (1,2), masked by thousands of irrelevant but structurally similar peptide/MHCs on the opposing membrane. In addition, they must respond to complex environmental stimuli which heighten or dampen the response. As a result, T cells regulate the lateral organization of surface receptors to achieve optimal ligand recognition and activate downstream signaling cascades after binding (3,4).

Antigen recognition by naïve T cells induces lateral reorganization of membrane proteins on several length scales (5). The coreceptor CD8, which binds an invariant region of the polymorphic MHC (6,7), mediates the association of T cell receptor (TCR) with Src-family tyrosine kinases to initiate downstream signaling (8). Robust activation subsequently induces the formation of TCR and adhesion structures, including bulls-eye-shaped immune synapses (5,9) and TCR microclusters hundreds of nanometers in diameter (10), which function to generate, sustain, and terminate TCR signals (11,12).

TCR clusters can also be observed days after the initial antigen recognition event, long after the antigen has been cleared (13,14). In activated T cells, TCR clustering has been reported to lead to enhanced binding of soluble peptide-MHC (15). These studies stand in contrast with observations that activated T cells may also lose the ability to bind cognate soluble MHC (16–20). Changes in the CD8 coreceptor (20) have been implicated in loss of binding, but the precise signals regulating binding behavior are poorly understood. Furthermore, the contradiction between observations of enhanced binding and complete loss of binding after activation has not been explained.

Here, we resolve this contradiction and show that changes in nanoscale colocalization of TCR and CD8 determine changes in binding after antigen stimulation. Primary stimulation with a high dose but not low dose of antigen leads to a transient spatial segregation of TCR and CD8. This spatial segregation can impair CD8-dependent MHC binding antigen depending on antigen dose during primary stimulation. Thus, despite having identical TCR, activated T cells can have different MHC binding properties due to changes in membrane organization of TCR and CD8.

## MATERIALS AND METHODS

### Mice and reagents

2C TCR $Rag^{-/-}$ transgenic mice were maintained as heterozygotes by breeding on a C57/BL6 background. PME TCR/$Thy1^a$ Rag−/− transgenic mice were a gift from Nicholas Restive (National Institutes of Health (NIH), Bethesda, MD) and maintained as homozygotes. Balb/C mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained according to Johns Hopkins University's Institutional Review Board. Peptides SIY (SIYRYYGL), SIIN (SIINFEKL), QL9 (QLSPFPFDL), mCMV (YPHFMPTNL), GP100 (KVPRNQDWL), and ASN (ASNENMETH) were purchased from Genscript (Piscataway, NJ). Fluorescently labeled monoclonal antibodies were purchased from BioLegend (San Diego, CA). MHC monomers were obtained from the NIH Tetramer Facility (Bethesda, MD).

### Cells

Cells used were obtained from homogenized mouse spleens after depletion of red blood cells by hypotonic lysis. For mixed lymphocyte reaction,

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$10 \times 10^6$/mL 2C splenocytes were activated by coculture with $18 \times 10^6$/mL irradiated Balb/C splenocytes as allogeneic stimulator cells for 4–7 days in complete RPMI media supplemented with T cell factor, a cytokine cocktail harvested from human plasma (21). For peptide activation, $10 \times 10^6$/mL 2C splenocytes were activated by incubation with cognate peptide at the indicated concentration in complete RPMI plus T cell factor.

## Preparation of MHC-Ig dimers

Soluble MHC-Ig dimers, $K^b$-Ig and $L^d$-Ig, were labeled fluorescently and loaded with peptide as described previously (22). Unless otherwise indicated, $K^b$-SIY and $L^d$-QL9 refer to soluble MHC-Ig dimer reagent of the corresponding allele loaded with the indicated peptide. Labeling with Alexa-488 or Alexa-657 succimidyl ester (Molecular Probes, Eugene, OR) was performed at pH 7.4 and labeled protein was purified by dialysis with a 50-kDa filter. Protein concentration was determined after labeling by size-exclusion high-performance liquid chromatography. The efficiency of fluorophore labeling was calculated by measuring absorbance at 280 nm and fluorophore emission wavelength for the labeled proteins. Typically, approximately one dye molecule was attached per MHC-Ig molecule. Alexa $K^b$-Ig molecules were loaded with peptide by stripping at alkaline condition (pH 11.5), and then refolded in the presence of 40-fold excess peptide. $L^d$-Ig molecules were stripped under mildly acidic conditions (pH 6.5) and refolded in the presence of 40-fold molar excess peptide and twofold molar excess of human $\beta_2$-microglobulin (23).

## MHC-Ig dimer binding assay

MHC-Ig dimer binding assays were performed as previously described in Fahmy et al. (24). Briefly, $CD8^+$ T cells were incubated at 4°C at a concentration of $10^7$cells/mL in FACS wash buffer (phosphate-buffered saline (PBS) + 2% fetal calf serum (FCS) + 0.05% sodium azide). A quantity of 15-$\mu$L aliquots of cells was mixed with varying concentrations of peptide-loaded, fluorescently labeled MHC-Ig dimers for 60–90 min. Without any washing, cells were analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using FlowJo software (Treestar, Ashland, OR). The mean channel fluorescence was a measure of the amount of MHC-Ig dimer bound on cells. A noncognate dimer ($K^b$-SIIN or $L^d$-MCMV) was used to account for nonspecific binding which was subtracted from the total binding to yield specific MHC-Ig binding. The specific binding was normalized to the plateau of the binding isotherm and plotted against the peptide MHC-Ig dimer concentration.

## Confocal microscopy

For confocal microscopy, T cells were stained with Alexa Fluor 488-labeled H57 anti-mouse TCR antibody and Alex Fluor 647-labeled 53.6.7 anti-mouse CD8$\alpha$ (Molecular Probes, Eugene, OR) at a 1:100 dilution for 30 min on ice. Samples were washed and fixed immediately with 2% paraformaldehyde. Images were acquired on an LSM 510 META laser scanning confocal microscope (Zeiss, Oberkochen, Germany) at 100× magnification at the Johns Hopkins School of Medicine Microscopy Facility.

## kICS

The k-space image correlation spectroscopy (kICS) imaging and analysis were performed as described previously in Kolin et al. (26). T cells were labeled using biotinylated $L^d$-QL9 monomer (NIH Tetramer Facility) at a concentration of 2 $\mu$g/2–5 $\times$ $10^6$ T cells in 100-$\mu$L PBS for 30 min on ice and then washed once with 2-mL PBS, followed by 10-nM streptavidin-coated Quantum Dots 655 (Life Technologies, Carlsbad, CA) in 100-$\mu$L PBS incubated for 30 min at 4°C. Labeled cells were then washed 2× with 2-mL PBS before imaging. Cells were imaged using a Marianas Live Cell Imaging Workstation (3I, Denver, CO) equipped with dual Cascade II 512 electron-multiplying cameras (Photometrics, Tucson, AZ) at the Johns Hopkins University Integrated Imaging Center. A quantity of 150–300 images was obtained at 300-ms intervals.

Each image series was corrected for background intensity values using a top-hat transformation, segmented using the watershed transform, and filtered for immobile components. Degree of aggregation was calculated by dividing mean image intensity by clusters of differentiation, a function of the autocorrelation of fast-Fourier-transformed image series in $k$-space with a custom program written in MATLAB (The MathWorks, Natick, MA) (26).

## FRET

TCR-CD8 Förster resonance energy transfer (FRET) was assessed by flow cytometry (17). Approximately $10^6$ T cells were incubated with a 1:100 dilution of unlabeled or PE-labeled 53.6.7 anti-mouse CD8$\alpha$ as donor and 500-nM uncoupled or Alexa Fluor 647-coupled $L^d$-QL9 (Molecular Probes) in FACS buffer at 4°C for 30 min. Samples were stained with labeled $L^d$-QL9 and anti-CD8 antibody ($E_{both}$), labeled $L^d$-QL9 but unlabeled CD8 ($E_{A647}$), unlabeled $L^d$-QL9 but labeled anti-CD8 ($E_{PE}$), or both reagents unlabeled ($E_{none}$). FRET emission was assessed by flow cytometry on a FACSCalibur (BD Biosciences) without compensation, with FL-3 channel for Alexa Fluor (Molecular Probes) emission without direct laser excitation. FRET efficiency was calculated in FRET units (17,27) as

$$\begin{aligned}\text{FRET unit} = {} & (E3_{\text{both}} - E3_{\text{none}}) \\ & - \left[(E3_{A647} - E3_{\text{none}}) \cdot \left(\frac{E2_{\text{both}}}{E2_{A647}}\right)\right] \\ & - \left[(E3_{PE} - E3_{\text{None}}) \cdot \left(\frac{E\,1_{\text{both}}}{E1_{PE}}\right)\right],\end{aligned}$$

where $E1$ is the fluorescence detected at 580 nm upon excitation at 488 nm, $E2$ is the fluorescence detected at 670 nm upon excitation at 630 nm, and $E3$ is the fluorescence detected at 670 nm upon excitation at 488 nm.

## Intracellular cytokine staining

Four or seven days after primary stimulation, T cell functional activity was assessed by rechallenge with artificial antigen-presenting cells (aAPCs), which were fabricated as described previously in Oelke and Schneck (28) by chemical coupling of MHC dimer and anti-CD28 antibody to Dynal Magnetic Microbeads (Life Technologies).

A quantity of 200,000 T cells was incubated in complete RPMI with the indicated concentration of activator bead for 5 h in a round-bottom 96-well plate in the presence of 0.2 $\mu$L GolgiPlug (BD Biosciences). Cells were washed and fixed using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions, then stained with anti-IL-2 Alexa Fluor 647 (Molecular Probes) and anti-IFN$\gamma$ PE (BioLegend). Cytokine staining was assessed by flow cytometry and frequency of cytokine functional cells was assessed by comparison with an unstimulated control in FlowJo (TreeStar).

# RESULTS

## T cells stimulated with a high dose of peptide transiently lose MHC binding

To evaluate the effects of peptide dose during stimulation on subsequent MHC-Ig binding, splenocytes from a 2C T cell receptor transgenic mouse (specific for peptide SIY