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A Role for CD8 in the Developmental Tuning of Antigen Recognition and CD3 Conformational Change

Diana Gil, Adam G. Schrum, Mark A. Daniels, and Ed Palmer

TCR engagement by peptide-MHC class I (pMHC) ligands induces a conformational change (ΔC) in CD3 (CD3Δc) that contributes to T cell signaling. We found that when this interaction took place between primary T lineage cells and APCs, the CD8 coreceptor was required to generate CD3Δc. Interestingly, neither enhancement of Ag binding strength nor Src kinase signaling explained this coreceptor activity. Furthermore, Ag-induced CD3Δc was developmentally attenuated by the increase in sialylation that accompanies T cell maturation and limits CD8 activity. Thus, both weak and strong ligands induced CD3Δc in preselection thymocytes, but only strong ligands were effective in mature T cells. We propose that CD8 participation in the TCR/pMHC interaction can physically regulate CD3Δc induction by “translating” productive Ag encounter from the TCR to the CD3 complex. This suggests one mechanism by which the developmentally regulated variation in CD8 sialylation may contribute to the developmental tuning of T cell sensitivity. The Journal of Immunology, 2008, 180: 3900–3909.

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In this study, we considered the impact of the CD8 coreceptor on cognate Ag recognition as measured by CD3\(^\gamma\) induction in primary T lineage cells of different maturation states. The results from this study shed light on how CD3\(^\gamma\) is induced, how CD3\(^\gamma\) reflects ligand discrimination, and how developmental tuning directly affects Ag recognition.

**Materials and Methods**

**DNA constructs and mice**

The construct pGEX-4T1-GST-SH3.1 (SH3.1 derived from human Nck-1) was provided by Dr. R. Geha (Children’s Hospital, Harvard Medical School, Boston, MA). OT-I and OT-I β\(_2\)-microglobulin-deficient (β\(_2\m^-/–\)) mice were bred and maintained on C57BL/6 background and used between 6 and 12 wk of age. Animal procedures were performed in accordance with the guidelines and regulations of the University Hospital-Basel and the Canton of Basel-Stadt (Basel, Switzerland).

**Abs, peptides, and other reagents**

Rabbit anti-CD3\(^\gamma\) antisera s448 was used to detect mature TCR/CD3 complexes by Western blotting as previously described (30). Monoclonal anti-CD3\(^\gamma\) (H146) was used to immunoprecipitate the TCR/CD3 complex (31). Abs from Biolegend included anti-H-2K\(^b\)/H-2Db (28.8-6), anti-Thy1.2 (53-2.1), anti-CD4 (RM4-5), anti-CD8\(^\alpha\) (53.6.7), anti-CD8\(^\beta\) (53.5.8), anti-CD69 (H12F3), rat IgG isotype control (R35-38), anti-TCR\(^\beta\) (B20.1), and anti-CD3-PE (2C11). Abs from Upstate Biotechnology included mouse anti-phosphotyrosine (4G10) and rabbit anti-linker for activation of T cell (anti-LAT). The peptides pFARL (SSIEFARL), pVSV (RGYVYQGL), pE1 (EIINFEKL), pG4 (SIIGFEKL), pQ7 (SIINFEQL), pQ4H7 (SIIQFEHL), pT4 (SIITFEKL), pQ4 (SIQFEKL), pKB (SINFEDiaminobutyrate-L), and pOVA (SIINFEKL) were synthesized as previously described (32). Other reagents included the Src kinase inhibitor PP2 (Calbiochem), protein G-Sepharose (GE Biosciences), type II neuraminidase from Vibrio cholerae and the detergent Brij 58 (Sigma-Aldrich), streptavidin (Molecular Probes), and the peanut lectin agglutinin (PNA)-FITC (Vector Laboratories).

**Thymocyte and mature T cell stimulation**

Without performing further purification procedures, thymi and lymph nodes from OT-I mice described were used as source of thymocytes (>95% pure) and mature T cells (50–65% pure), respectively. T2-K\(^b\) wild-type (WT) cells or T2-K\(^b\) MUT (H2-K\(^b\) molecule mutant at 227 residue) cells (33), provided by T. Potter (National Jewish Medical and Research Institute, Denver, CO).
Center, Denver, CO), which express the WT or the point mutation D227K within H-2K\textsubscript{b}, were used as APCs in all experiments. APCs were cultured with 2 \textmu M exogenous peptide (3 h, 37°C), washed, verified for equal peptide loading by staining with anti-H-2K\textsubscript{b} mAb 28.8-6, and cocultured with thymocytes or mature T cells (30 min, 37°C, 1:1 ratio). In some experiments, thymocytes were stimulated with 10 \textmu g/ml soluble Ab in RPMI 1640 medium supplemented with 10% FBS (15 min, 37°C). Where indicated, PP2 treatment (20 \textmu M) was performed as previously described (8).

Surface staining with PE-MHC tetramers

K\textsuperscript{b} WT and K\textsuperscript{b} MUT D227K PE-labeled tetramers were produced following standard refolding, biotinylation, and tetramerization protocols. K\textsuperscript{b} plasmids were gifts of P. Holman and S. Jameson (University of Minnesota, Minneapolis, MN) (34). Tetramers were purified via gel filtration and FPLC just before use. For surface staining of OT-I cells with the tetramers, 0.5 \times 10^6 thymocytes, or mature T cells were incubated with the corresponding peptide tetramer (50 nM) together with mAbs that do not inhibit tetramer binding, anti-CD8-PE, anti-CD4-PerCP (data not shown and 35). Mean fluorescence intensity (MFI) and SEM are indicated.

CD3 pull-down (CD3-PD) assay and Western blots

The CD3-PD assay was previously described and proven as a means to detect CD3\textsubscript{c} (7). Briefly, cells were lysed in 0.3% Brij 58 isotonic buffer, and postnuclear fractions were obtained. Samples were precleared by incubation with GST-beads (1 h, 4°C) before specific pull-down with GST-SH3.1 beads (4 –12 h, 4°C), followed by reducing SDS-PAGE (13%), polyvinylidene difluoride transfer, and Western blotting for CD3\textsubscript{c} (s448). Where indicated, immunoprecipitations were performed with anti-CD3\textsubscript{c} (H146)/protein G-Sepharose beads, subjected to reducing SDS-PAGE (13%), transferred to nitrocelulose membranes, and blotted as indicated.

**FIGURE 2.** Disruption of CD8/pMHC interaction inhibits the induction of CD3\textsubscript{c} in preselection DP thymocytes. A, OT-I DP thymocytes from OT-I \beta\textsubscript{m}\textsuperscript{−/−} RAG2\textsuperscript{−/−} mice were incubated with either rat IgG control (■) or the anti-CD8\beta blocking mAb, 53.5.8 (□) (30 min, 37°C), and stained with K\textsuperscript{b} WT/pOVA or K\textsuperscript{b} MUT/pOVA tetramers (45 min on ice) before analysis by flow cytometry. Tetramer identity is indicated on the x-axis, and the quantity of surface bound tetramers gated on CD4\textsuperscript{+} CD8\textsuperscript{−} DP thymocytes is represented as MFI on the y-axis. The fold difference in tetramer binding is indicated. B, The CD3-PD assay was performed on C57Bl/6 thymocytes stimulated with the following Abs: IgG control, anti-CD8\beta (53.5.8), 2C11 (anti-CD3\alpha), or anti-CD8\beta+ 2C11 (top). A fraction of the postnuclear lysate of each sample was used to determine the total CD3 content by Western blot of CD3\textsubscript{c} (bottom), C–E, OT-I DP thymocytes from OT-I \beta\textsubscript{m}\textsuperscript{−/−} RAG2\textsuperscript{−/−} mice were either pretreated with control rat IgG (C), anti-CD8\beta blocking mAb 53.5.8 (D), or control rat IgG plus PP2 Src kinase inhibitor (30 min, 37°C) (E). Then thymocytes were cocultured with T2-K\textsuperscript{b} WT APCs loaded with pFARL, pQ7, pT4, pOVA (2 \textmu M, 30 min, 37°C). Cells were lysed and subjected to either the CD3-PD assay (upper panels), or anti-CD3\textsubscript{c} immunoprecipitation and Western blotting with anti-CD3\textsubscript{c} (upper and lower panels) or anti-phosphotyrosine (p-p-Tyr) (middle panels). TCR down-regulation (F) and CD69 up-regulation (G) were analyzed by flow cytometry. Peptides are represented on the x-axis and surface TCR expression (F) on the y-axis as a percentage of the expression level gated on Thy1.2\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{−} DP thymocytes cocultured with the null peptide pFARL. G, CD69 expression is represented on the y-axis as a percentage of the maximum induction on Thy1.2\textsuperscript{+} CD4\textsuperscript{+} CD8\textsuperscript{−} DP thymocytes cotultured with peptide pOVA.
Early T cell response monitored by flow cytometry

Early responses of thymocytes and mature T cells were monitored by flow cytometry analysis of TCR down-regulation and CD69 up-regulation. For TCR down-regulation, cells were stimulated for 30 min as indicated and stained with anti-CD8-allophycocyanin, anti-CD4-PerCP, anti-Thy1.2-FITC, and anti-Vα2-PE. For CD69 expression, cells were stimulated for 20 h as indicated and stained with anti-CD8-allophycocyanin, anti-CD4-PerCP, anti-Vα2-FITC, and anti-CD69-PE. Staining took place for 30–45 min on ice, and washed cells were analyzed on a BD FACSCalibur flow cytometer calibrated with RCP-30-5A beads (Spherotech). MFI and SEM are indicated.

Neuraminidase treatment

Thymocytes or mature T cells were incubated with or without neuraminidase (0.007 U/10⁶ cells) in RPMI 1640 medium at a density of 30 × 10⁶ cells/ml (45 min, 37°C), followed by addition of 10% FBS to quench enzymatic activity. Washed cells were either analyzed by flow cytometry or stimulated by APC as described.

FIGURE 3. Involvement of CD8 in CD3Δc induction by cognate ligand in a role beyond affinity. The relative affinity for the OT-I TCR of variant peptides when loaded either in Kb WT or Kb MUT molecules is: Kb WT/pFARL < Kb WT/pG4 < Kb WT/pQ7 < Kb WT/pQ4H7 < Kb MUT/pOVA < Kb WT/pOVA. Preselection OT-I DP thymocytes were cocultured with these peptide-loaded APCs and the CD3-PD assay was performed (top). A fraction of the postnuclear lysate of each sample was used to determine the total CD3 content by Western blot of CD3ζ (bottom).

FIGURE 4. Peptide presentation by T2-Kb D227K MUT APCs inhibits CD3Δc induction in preselection DP thymocytes. Preselection OT-I DP thymocytes were cocultured with T2-Kb WT (A) or T2-Kb MUT (B) APC preloaded with pFARL, pG4, pQ7, pQ4H7, pQ4, or pOVA, and the CD3-PD assay was performed (top). Total CD3ζ content in the postnuclear lysates is also shown (bottom). C and D, Samples from A and B were immunoprecipitated with anti-CD3ζ and associated proteins were detected by Western blot. Linker for activation of T cell (LAT) association with CD3ζ was inducible by either T2-Kb WT (C) or T2-Kb MUT (D) APC (upper panels), and some of this LAT was phosphorylated when blotted with anti-phosphotyrosine (α-p-Tyr) (middle panels). Total CD3ζ content in the postnuclear lysates is also shown (lower panels). TCR down-regulation (E) and CD69 up-regulation (F) were analyzed by flow cytometry for the experiments shown in A and B. Peptides are listed on the x-axis, whereas surface TCR expression (E) is represented on the y-axis as a percentage of the expression level on DP thymocytes cocultured with the null peptide pFARL. F, CD69 expression is represented on the y-axis as a percentage of the maximum induction on DP thymocytes cocultured with peptide pOVA.
Results

T cell sensitivity to undergo CD3Δc decreases upon thymic selection

To assess whether the sensitivity of Ag recognition may be developmentally regulated, we examined the ability of T lineage cells to undergo CD3Δc when presented with pMHC ligands. The following three cell maturation states were tested: preselection, CD4+CD8+ double positive (DP) thymocytes from OT-I β2m−/− RAG2−/− mice, which lack pMHC presentation (36); postselection thymocytes, DP and CD4−CD8+ single positive thymocytes from OT-I β2m+/+ mice, which express pMHC ligands that mediate positive selection of the OT-I TCR (37); and mature, CD8+ T cells from OT-I β2m+/+ mice. Cells were cocultured with T2-Kb WT APCs preloaded with the following OVA variant peptides of increasing affinity for the OT-I TCR: pFARL (null), pQ7 (positive selector/antagonist), pT4 (mixed agonist/antagonist), pQ4, pKB, or pOVA (negative selectors/agonists) (8, 38–40). The induction of CD3Δc was assessed by the CD3-PD assay, which detects the exposure of a cryptic polyproline site in CD3e upon productive TCR engagement by pMHC ligands, allowing the SH3.1 domain of Nck to bind and capture TCR/CD3 complexes, as described in Materials and Methods (7, 8). Like TCR down-regulation, the measurement of CD3Δc relies on the accumulation of engaged TCRs, which increases with the time of T cell to APC contact. Therefore, although induction of CD3Δc has been shown to occur immediately upon TCR engagement (7, 8), thymocytes were stimulated during 30 min to ensure maximal CD3Δc detection in the CD3-PD assay, as reported previously (8). We found that preselection DP thymocytes underwent CD3Δc when presented with both weak and strong pMHC ligands, but postselection thymocytes and mature T cells were productively engaged only by negative selectors/agonists (Fig. 1, A–C). In this and other experiments, including more variant peptides (data not shown), postselection thymocytes and mature T cells never induced CD3Δc when engaged by positive selector/antagonist ligands. These data demonstrate that the ability of weak (i.e., positive selecting) ligands to induce CD3Δc is substantially reduced after T lineage cells have undergone positive selection. This reduction in sensitivity cannot be explained by the expression level of either TCR or CD8 present under negative selection DP thymocytes or mature T cells when presented with agonist ligands in mature T cells (Fig. 1, D and E).

Disruption of CD8/pMHC interaction inhibits the induction of CD3Δc in preselection DP thymocytes

Because the TCR-independent nognocognitive interaction of CD8 with MHC is known to decrease after positive selection (26, 27, 41, 42), we studied whether CD8 plays a role in CD3Δc induction. Preselection DP thymocytes from OT-I β2m−/− RAG2−/− mice were incubated with the anti-CD8β mAb 53.5.8 (27, 34) and stained with either pOVA-loaded H-2Kb WT tetramers (Kb WT/pOVA) or H-2Kb D227K mutant tetramers, defective in CD8 binding (Kb MUT/pOVA) (33). Both the blocking mAb and the tetramer mutation inhibited pMHC binding to the OT-I TCR (Fig. 2A). We confirmed that blocking with anti-CD8β mAb neither autonomously induces nor inhibits the CD3-Δc-PD assay; i.e., treatment with anti-CD8β mAb alone or together with 2C11 (anti-CD3e mAb) had no effect on CD3Δc detection in thymocytes (Fig. 2B).

To study CD3Δc induction when CD8/pMHC interaction is blocked, T2-Kb WT APCs were preloaded with OVA variant peptides and cocultured with preselection OT-I DP thymocytes that had been preincubated either with control Ig, anti-CD8β, or the Src kinase inhibitor, PP2. As expected, all cognate peptides induced CD3Δc in control thymocytes (Fig. 2C, upper) even when Src kinase activity was inhibited (Fig. 2E, upper). However, CD8 blockade dramatically inhibited CD3Δc induction by all peptides (Fig. 2D, upper). Similar to Src kinase inhibition, CD8 blockade inhibited CD3Δc phosphorylation (Fig. 2, C–E, middle panel), TCR down-regulation (Fig. 2F), and CD69 up-regulation (Fig. 2G), consistent with the idea that CD8 normally contributes to Src kinase delivery and activation during cognate pMHC recognition (43, 44). We conclude that CD8/MHC class I interaction during cognate pMHC stimulation of preselection OT-I thymocytes is required for CD3Δc induction, whereas Src kinase activity is not.

FIGURE 5. Induction of CD3Δc by agonist ligands in mature T cells requires CD8. A, Mature CD8+ T cells from OT-I β2m+/+ lymph nodes were incubated with either rat IgG control (□) or the anti-CD8β blocking mAb, 53.5.8 (■) or the anti-CD8β blocking mAb, 53.5.8 (□) (30 min, 37°C), and stained with Kb WT/pOVA or Kb MUT/pOVA tetramers (45 min on ice) before analysis by flow cytometry. Tetramer identity is indicated on the x-axis, and the quantity of surface bound tetramers gated on CD8+ cells is represented as MFI on the y-axis. The fold difference in tetramer binding is indicated. B–D, Mature CD8+ T cells from OT-I β2m+/+ lymph node were either pretreated with control rat IgG (B and D) or anti-CD8β blocking mAb 53.5.8 (30 min, 37°C) (C). T cells were then cocultured with T2-Kb WT (B and C) or T2-Kb MUT (D) APCs loaded with pFARL, pQ7, pT4, pQ4, pKB, or pOVA (2 μM, 30 min, 37°C). Cells were lysed and subjected to the CD3-PD assay (top panels). Total CD3Δc content in the postnuclear lysates is also shown (bottom panels).
Involvement of CD8 in CD3Δc induction by cognate ligand has a role beyond affinity

We wished to determine whether CD8 helps induce CD3Δc by enhancing the binding strength/affinity of TCR/pMHC interaction. The relative affinity of various Kb WT or Kb MUT/peptide complexes for the OT-I TCR is: Kb WT/pFARL < Kb WT/pQ4 < Kb WT/pQ7 < Kb WT/pQ4H7 < Kb MUT/pOVA < Kb WT/pOVA (Fig. 3) (40, 45–47). When preselection OT-I DP thymocytes were stimulated by T2-Kb WT APC preloaded with pFARL, pQ7, pQ4H7, or pOVA (2 μM, 30 min, 37°C). The induction of CD3Δc was detected using the CD3-PD assay (top). Total CD3ζ content in the post-nuclear lysates is also shown (bottom). TCR down-regulation (E) and CD69 up-regulation (F) were analyzed by flow cytometry for the experiments shown in B–D. Peptides are listed on the x-axis, whereas surface TCR expression (E) is represented on the y-axis as a percentage of the expression level gated on Thy1+ CD4+ CD8+ DP thymocytes cultured with the null peptide pFARL. F, CD69 expression is represented on the y-axis as a percentage of the maximum induction on Thy1+ CD4+ CD8+ DP thymocytes cultured with peptide pOVA.

Induction of CD3Δc by agonist ligands in mature T cells requires CD8

Lymph node CD8+ cells from OT-I β2m+/+ mice were incubated with anti-CD8β mAb, and stained with either Kb WT or Kb MUT/
pOVA tetramers. As observed with preselection thymocytes, both the blocking mAb and the H-2Kb D227K mutation inhibited binding to the OT-I TCR on mature T cells (Fig. 5A). To study CD3ζ induction when CD8/pMHC-I interaction is blocked, mature CD8+/H11001 T cells that had been preincubated with either mock-treated (B) or treated with neuraminidase (C and D). These thymocytes were cocultured with T2-Kb WT (B and C) APC or T2-Kb MUT (D) APC preloaded with pFARL, pQ7, pQ4H7, or pOVA (2 μM, 30 min, 37°C). The induction of CD3ζ was detected using the CD3-PD assay (top panels). Total CD3ζ content in the postnuclear lysates is also shown (bottom panels). TCR down-regulation (E) and CD69 up-regulation (F) were analyzed by flow cytometry for the experiments shown in B–D. Peptides are listed on the x-axis, whereas surface TCR expression (E) is represented on the y-axis as a percentage of the expression level gated on Thy1+ CD4+CD8+ DP thymocytes cocultured with the null peptide pFARL. F, CD69 expression is represented on the y-axis as a percentage of the maximum induction on Thy1+ CD4+CD8+ thymocytes cocultured with peptide pOVA.

**FIGURE 7.** Neuraminidase treatment of postselection thymocytes re-establishes preselection sensitivity for cognate ligand recognition and CD3ζ induction. A, Surface PNA staining (MFI) of CD8+ Kb WT/pOVA tetramer plus thymocytes from OT-I β2m−/− mice that were either mock-treated (B) or treated with neuraminidase (C and D). These thymocytes were cocultured with T2-Kb WT (B and C) APC or T2-Kb MUT (D) APC preloaded with pFARL, pQ7, pQ4H7, or pOVA (2 μM, 30 min, 37°C). The induction of CD3ζ was detected using the CD3-PD assay (top panels). Total CD3ζ content in the postnuclear lysates is also shown (bottom panels). TCR down-regulation (E) and CD69 up-regulation (F) were analyzed by flow cytometry for the experiments shown in B–D. Peptides are listed on the x-axis, whereas surface TCR expression (E) is represented on the y-axis as a percentage of the expression level gated on Thy1+ CD4+CD8+ DP thymocytes cocultured with the null peptide pFARL. F, CD69 expression is represented on the y-axis as a percentage of the maximum induction on Thy1+ CD4+CD8+ thymocytes cocultured with peptide pOVA.
surface sialylation on the sensitivity of mature T cells, lymph node T cells from OT-I β2m+/+ mice were either untreated or treated with the enzyme neuraminidase. As expected, neuraminidase treatment resulted in increased PNA staining, which binds unsialylated O-linked core-1 glycans (25) (Fig. 6A), and increased the binding of K8 WT/pQ7 and K8 WT/OVA tetramers in a CD8-dependent fashion (data not shown). Mature T cells were cocultured with T2-K′ WT APCs preloaded with null (pFARL), antagonist (pG4, pQ7), or agonist (pOVA) peptides in the presence or absence of anti-CD8β. Control T cells displayed the expected low sensitivity for CD3Δc induction, responding only to pOVA (Fig. 6B). However, neuraminidase treatment of T cells restored CD3Δc induction by the weak ligands (Fig. 6C) by a mechanism dependent on CD8 (Fig. 6D). Neuraminidase treatment also enhanced functional responsiveness, as measured by TCR down-regulation (Fig. 6E) and CD69 up-regulation (Fig. 6F). Similar experiments were performed with postselection thymocytes from OT-I β2m+/+ mice. Likewise, the induction of CD3Δc was restored and functional responsiveness to the positive selectors pQ7 and pQ4H7 was enhanced in a CD8-dependent manner after neuraminidase treatment in postselection thymocytes (Fig. 7). Neuraminidase treatment did not enhance CD3Δc induction in preselection DP thymocytes (data not shown), as these cells express low basal levels of sialic acid (48) and maximal sensitivity for CD3Δc induction (Fig. 1). We conclude that the level of membrane sialylation on postselection thymocytes and mature T cells limits the sensitivity of cognate Ag recognition to agonist ligands by a mechanism dependent on CD8.

Discussion

We found that the induction of CD3Δc by cognate pMHC ligands for the OT-I TCR required CD8/TCR/pMHC interaction (Figs. 2–5). CD8 was required, whereas Src kinase activity was not (Fig. 2). Moreover, a high affinity for the OT-I TCR did not overcome the CD8 requirement for CD3Δc induction (Fig. 3). Previous studies have not resolved whether the CD3Δc is induced by an upstream conformational change in the αβ TCR heterodimer (TCRΔc). The majority of bound TCR/pMHC crystal structures reveal “induced-fit” TCRΔc that do not extend outside of the ligand binding interface and do not involve observable rearrangements of the TCR constant regions (49). We propose a model where CD8 physically induces and/or stabilizes CD3Δc during cognate TCR/pMHC interaction, “translating” productive Ag encounter from the TCR to the TCR complex (Fig. 8). Importantly, this model does not require the αβ TCR to directly transmit a stable conformational change to the TCR complex, consistent with the vast majority of TCR/pMHC crystal structures.

We used the OT-I TCR to describe how αβ TCR and CD3 can communicate through CD8. However, because CD3Δc induction is required for optimal signaling (7, 10), we suspect that coreceptor-independent T cells (50–52) may induce CD3Δc by an alternative mechanism. For example, the LC13 αβ TCR is specific for a viral peptide presented by HLA-B8 (53) and is CD8-independent (54). The crystal structure of this ligand-receptor pair provides the only case to date of an allosteric conformational change in the extracellular constant region of the TCR α-chain where CD3ɛ likely associates (55). Other recent experiments showed that the class I-restricted “T1” TCR, previously described as functionally independent of CD8 (56), can induce CD3Δc in response to a strong pMHC ligand in hybridomas lacking CD8 expression (10). However, because most pMHC-restricted T cell responses are CD8-dependent (52), we suspect that CD8 usually plays a major role inducing CD3Δc.

CD3Δc seems to clearly reflect productive TCR engagement, but whether CD3Δc also plays a role in discerning ligand potency has been less clear. We studied the sensitivity of T lineage cells to undergo CD3Δc and found that, although weak/positive selecting ligands induced CD3Δc in preselection DP thymocytes, these weak ligands failed to induce CD3Δc once thymocytes had begun positive selection (Figs. 1 and 7B). Though first observable in postselection thymocytes, this reduced sensitivity to CD3Δc induction was further maintained in mature peripheral T cells (Figs. 1, 5B, and 6B), and correlated with a decreased cellular response to weak ligands (Figs. 6, E and F, and 7, E and F).

The fact that both positive and negative selecting ligands induce CD3Δc in preselection thymocytes rules out CD3Δc induction as the universal read-out of ligand affinity. However, another possibility is that the duration of CD3Δc could play a decisive role in this regard. We speculate that the length of the CD8/TCR/pMHC interaction (57–59) might determine the duration of CD3Δc and the extent to which the cell signaling machinery is activated. This idea is consistent with the finding that CD4 (60) and CD8 (61) activity can determine the strength of signal delivered by pMHC ligands. The latter study showed that the kinetics of CD8 association with the OT-I TCR predicted the strength of ligand better than the affinity of the TCR/pMHC interaction itself (61). Future experiments will need to address whether coreceptors impact ligand discrimination by controlling the duration of CD3Δc.

The attenuation of CD3Δc induction by weak ligands following positive selection establishes an interesting relationship between productive TCR engagement and the developmental tuning of T cell sensitivity. Following positive selection, CD3Δc induction and productive Ag recognition are confined to agonist ligands. This

![FIGURE 8. Model of developmental tuning of T cell Ag recognition mediated by changes in the sialylation state of CD8.](http://www.jimmunol.org/DownloadedFrom/3907)
limitation is reversed by desialylation with neuraminidase (Figs. 6 and 7). Although desialylation may affect other surface molecules relevant to TCR/CD3 signaling (like CD45 (48)), the direct effect we observe on cognate Ag recognition provides at least a partial explanation for how sialylation contributes to the regulation of T cell sensitivity via CD8 (Figs. 6 and 7). This observation is supported by previous work, which showed that the TCR-independent noncognate CD8/MHC binding characteristic of preselection thymocytes was down-regulated by sialylation after positive selection (26, 27). The regulation of ligand sensitivity (developmental tuning) is controlled on several levels, including the regulation of CD8 expression (62) and the regulation of phosphatase activity (19–24). The change in sialylation and subsequent regulation of CD3Δc is one proximal aspect of this process. How CD8 sialylation contributes to an alteration in ligand binding and an alteration in signal initiation is currently under investigation.

Our results reconcile previous discrepancies regarding the efficiency with which positive selection pMHC ligands induce CD3Δc in thymocytes. Previous work from our laboratory demonstrated that positive and negative selecting ligands similarly induce CD3Δc in preselection (βm m−/−) thymocytes (8). However, Risueno et al. (13) demonstrated an increased number of thymocytes displaying CD3Δc in negative-selecting male H-Y mice when compared with positive-selecting female H-Y mice, arguing that the efficiency of detecting CD3Δc is increased under negative selection. The fact that postselection thymocytes from βm m−/−/H9004c animals were used in their studies may partially explain the lower detection of CD3Δc in positive selecting H-Y mice. The increased levels of surface sialylation on the thymocytes in these βm m−/−/H9004c mice would minimize their reactivity to positive selecting ligands, as we observed with OT-I postselection thymocytes (Figs. 1 and 7). Furthermore, it is not clear whether the H-Y negative selecting peptide is expressed on more thymic APCs compared with the positive-selecting peptide. For these reasons, positively selected thymocytes with a detectable CD3Δc may be difficult to visualize in βm m−/−/H9004c mice. However, when bona fide preselection thymocytes from βm m+/−/H9004c mice are stimulated with positive selection ligands in vitro or in vivo, CD3Δc is efficiently induced (8) (Figs. 1–4).

In conclusion, we tested the idea that changes in the glycosylation pattern of developing T cells influences Ag recognition via CD3Δc induction. When enzymatically desialylated with neuraminidase, mature T cells and postselection thymocytes recovered CD3Δc induction by weak cognate stimuli (Figs. 6B and 7B). As expected, this treatment also enhanced responses to weak stimulation in mature T cells and postselection thymocytes (Figs. 6, E and F, and 7, E and F). Moreover, the positive effect of the neuraminidase treatment on CD3Δc induction and T cell responses was always dependent on the integrity of CD8/MHC interaction (Figs. 6D and 7D). These observations support the idea that CD8 sialylation regulates the developmental tuning of cognate Ag recognition and CD3Δc induction (Fig. 8). Throughout T cell maturation, the induction of CD3Δc is a feature of productively triggered TCRs, likely involved in a basic mechanism to enable signaling activity. Future experiments will focus on quantifying the interplay between coreceptors, TCR, pMHC, and the induction of CD3Δc.

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Disclosures

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References


