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Thymocyte Sensitivity and Supramolecular Activation Cluster Formation Are Developmentally Regulated: A Partial Role for Sialylation[1]

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TCR reactivity is tuned during thymic development. Immature thymocytes respond to low-affinity self-ligands resulting in positive selection. Following differentiation, T cells no longer respond to low-affinity ligands, but respond well to high-affinity (foreign) ligands. We show in this study that this response includes integrin activation, supramolecular activation cluster formation, Ca^{2+} flux, and CD69 expression. Because glycosylation patterns are known to change during T cell development, we tested whether alterations in sialylation influence CD8 T cell sensitivity to low affinity TCR ligands. Using neuraminidase treatment or genetic deficiency in the ST3Gal-I sialyltransferase, we show that desialylation of mature CD8 T cells enhances their sensitivity to low-affinity ligands, although these treatments do not completely recapitulate the dynamic range of immature T cells. These studies identify sialylation as one of the factors that regulate CD8 T cell tuning during development. ***The Journal of Immunology,*** **2003, 171: 4512–4520.**

The TCR is unique among cell surface receptors in that its ligand is a fixed MHC molecule noncovalently bound to a peptide. A functional TCR must recognize MHC molecules carrying foreign peptides generated from pathogens but not self-peptides. To produce a repertoire of T cells capable of recognizing the plethora of pathogen peptides, each T cell generates a unique TCR via a semirandom process of somatic recombination. Once this TCR is successfully expressed on the surface of the T cell, it is screened in the thymus (1). A thymocyte expressing a TCR that responds strongly to self-peptide/MHC is eliminated via apoptosis, thus avoiding autoimmune responses, while thymocytes expressing a TCR that cannot interact with a self-peptide/MHC are also eliminated, via a default pathway of apoptosis. However, thymocytes expressing a TCR that weakly interacts with self-peptide/MHC survive and differentiate into mature T cells, which are released from the thymus to patrol the periphery. As the T cell matures, it loses responsiveness to low-affinity ligands. Surprisingly, this loss of sensitivity is selective for low-affinity ligands, as the response to high-affinity ligands is not tempered (2, 3). We refer to this selective loss of sensitivity for low-affinity ligands as the tuning or developmental attenuation of the T cell dynamic range.

One potential explanation for the decreased sensitivity of mature T cells would be that peripheral APCs and the accompanying cytokines differ from those in the thymus, which could elicit different TCR responses due to secondary signaling from costimulatory receptors and/or cytokine receptors (4). However, experiments controlling the APC and external environment indicate that extrinsic factors cannot fully account for the decreased dynamic range (2, 3, 5). Therefore TCR signaling must be influenced by developmentally or continually regulated gene expression within the T cell. These regulated genes could affect signal transduction in many ways including changing the levels of downstream signaling factors such as kinases or phosphatases (3, 6), altering accessibility to these factors via changes in cytoskeletal or adaptor proteins (7) or redefining the cell surface landscape by altering coreceptors (5, 8–10) or adhesion molecules (4). To explain tuning, however, these genes must specifically reduce sensitivity to low-affinity ligands while maintaining sensitivity to high-affinity ligands. Conceptually, this could be accomplished by differentially affecting kinetics of receptor binding (11) or by actively tuning the threshold required for activation (6).

It has long been known from studies with lectins that T cell maturation is accompanied by increased sialylation of cell surface molecules (12, 13). One of the enzymes responsible for this is the sialyltransferase ST3Gal-I, which is up-regulated as thymocytes are positively selected and catalyzes the addition of sialic acid to the core 1 O-linked glycan, Galβ1–3GalNAc-Ser/Thr (Ref. 14 and references therein). Sialylation was recently shown to result in a decreased ability of the CD8 coreceptor to bind class I MHC molecules (8, 9). Given this, we were interested in determining whether surface sialylation reduces the TCR sensitivity to low-affinity self-ligands.

In this report we systematically looked at four responses of the T cell to low- and high-affinity peptide/MHC stimulation. We compared the ability of thymocytes and mature T cells to form conjugates, to form an ordered synapse, up-regulate CD69, and flux calcium. We found that mature T cells had a reduced response to low-affinity stimulation in all cases. This was true even for supramolecular activation cluster (SMAC)[3] formation, which was

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[3] Abbreviations used in this paper: SMAC, supramolecular activation cluster; tg, transgenic; LN, lymph node; β_2m, β_2-microglobulin; PKC, protein kinase C; RAG, recombination-activating gene.

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quite robust in thymocytes. To understand the mechanism of developmental attenuation, we explored the role of sialylation. Using enzymatic and genetic means of altering sialylation, we present evidence that sialylation of O-linked glycans contributes to T cell tuning.

Materials and Methods

Mice and peptides

OT-I is a C57BL/6 TCR transgenic (tg) strain, expressing a receptor specific for peptide OVA (OVAp)/K^b (15). TAP-$1^{-/-}$ mice on a C57BL/6 background, β_2-microglobulin (β_2m)$^{-/-}$ mice on a mixed B6/129 background, and bm8 mice were maintained in our colony at the University of Minnesota (Minneapolis, MN). Congenic (CD45.1-Ptprc a) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Recombination-activating gene (RAG)$^{-/-}$ C57BL/6 mice were purchased from Taconic, Germantown, NY. ST3Gal-I$^{-/-}$ mice were generated as described (14) and were a generous gift of J. Marth (University of California, San Diego, CA). OT-I mice were crossed to the ST3Gal-I$^{-/-}$ mice. Peptides OVAp (SIINFEKL), G4 (SIIGFEKL), E1 (EIINFEKL), and P815p (HIYEFPQL) were synthesized by Research Genetics (Huntsville, AL).

Cell preparations

Thymi, lymph nodes (LNs), and spleens were harvested, and single cell suspensions were prepared in RPMI 1640 (10% FCS). Splenocytes were briefly treated to remove RBC and then washed in medium containing serum and resuspended at 2×10^7 cells/ml. In some experiments thymocytes and LN cells were labeled with 1 mM CFSE in serum-free medium for 10 min at 37°C, washed, and resuspended in medium containing serum at 2×10^7 cells/ml. In some experiments LN $CD8^+$ cells were enriched by panning two times for 30 min at room temperature on plates coated with goat anti-mouse IgG or by depletion using MACs magnetic separation columns and magnetic beads coated with anti-MHC class II Abs per manufacturer's instructions (Miltenyi Biotec, Auburn, CA). To collect thymic stromal cells, E15.5 fetal thymi from CD45.1 pregnant females were harvested and whole organs were cultured as described (16) in medium containing 1.35 mM deoxyguanosine for 5 days. Thymic lobes were digested 15 min at 37°C in 1 ml Ca^{2+}/Mg^{2+}-free PBS with trypsin (0.25%)/EDTA (0.02%), washed, and resuspended in RPMI 1640 (10% FCS).

Neuraminidase treatment

Cells were prepared as previously described and incubated at 37°C at a concentration of 2×10^6 cells/ml in RPMI in the presence or absence of 1 μl (0.016 U) type II neuraminidase from *Vibrio cholerae* (Sigma-Aldrich, St. Louis, MO) per million cells for 25–45 min. This enzyme cleaves α2,3, α2,6, and α2,8 sialic acid linkages, and the dose used was determined (by titration) as sufficient to induce maximal PNA staining of mature thymocytes. Cells were washed two times in RPMI 1640 (10% FCS).

Stimulations

Splenocytes or stromal cells were pulsed with 1 μM peptide for 30 min at 37°C, washed, and resuspended at 2×10^7 cells/ml. Thymocytes and LN cells were mixed with peptide pulsed APC at a 1:1 or 1:2 ratio in round-bottom 96-well plates and lightly pelleted by centrifugation ($150 \times g$ for 5 s). For tetramer stimulation, K^b/β_2m/peptide tetramers were prepared as described (9). Cells at 2×10^7 cells/ml were mixed with 10 μg/ml tetramer and incubated for 3 h at 37°C. Cells to be analyzed by microscopy were incubated for 30 min at 37ºC, 5% CO_2 and then pelleted ($150 \times g$ for 5 min) and fixed by resuspension in 1% paraformaldehyde for 15 min at room temperature. Cells analyzed by flow cytometry were not fixed.

Conjugate assay

To distinguish responders from APC, thymocytes or LN cells were either labeled with CFSE (1 μM for 10 min at 37°C) or CD45.1 congenic splenocytes were used as APC. After stimulation cells were fixed in 1% paraformaldehyde for 15 min at room temperature. Cells were stained with APC-CD8, CyChrome-CD4, PE-B220, and FITC-CD45.2 (or CFSE) and analyzed by flow cytometry. Conjugates were calculated as the percentage of $CD8^+$/$CD45.2^+$ (or $CFSE^+$) events that were also $B220^+$.

Integrin blocking

Cells were prepared as previously described and immediately before mixing effectors with APCs, azide-free blocking Abs to β7, αL, and α4 (BD PharMingen, Mountain View, CA) were added at a final concentration of 7.5 μg/ml.

Flow cytometry

Surface staining was done with FITC-PNA (Vector Laboratories, Burlingame, CA), FITC-CD43 (clone S7), PE-anti-B220, PE-anti-CD69, FITC-anti-CD45.2, PE-anti-CD45.1, PE-anti-HSA, PE-anti-CD44, PE-anti-CD25, CyChrome or PerCP anti-CD4, and/or APC-anti-CD8α (BD PharMingen) in PBS containing 2% FCS and 0.2% NaN_3. Cells were analyzed on a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ) using CellQuest (BD Biosciences) and FlowJo (TreeStar, San Carlos, CA) software.

Calcium flux

Calcium flux was measured as described (17). Briefly, T cells were treated with or without neuraminidase, loaded with Indo-1-AM at 37°C, washed, and resuspended in medium at 37°C and analyzed on a FACSDiva instrument. After establishing a baseline flux for 1 min, T cells were added to a pellet of peptide-pulsed APC (1:1 APC–T cell ratio), briefly pulsed and resuspended and immediately analyzed for an additional 6 min. The resulting flux is shown as the median of the ratio FL4/FL5 for the population tested.

Microscopy

After fixation, cells were permeabilized in PBS containing 0.1% saponin, 2% FCS, and 0.2% NaN_3. Staining for protein kinase C (PKC)θ was done in permeabilization buffer sequentially with a polyclonal rabbit anti-PKCθ (Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated goat anti-rabbit IgG (BD PharMingen), and streptavidin-conjugated Alexa568 (Molecular Probes, Eugene, OR) for 30 min each on ice. Cells were mounted on ProbeOn Plus Microscope slides (Fisher, Pittsburgh, PA) in glycerol containing 0.2 M Tris, Mowial 40–88 (Aldrich, Milwaukee, WI) and 5% 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich) and set with nail polish. Z-stack images were collected using a Bio-Rad MRC 1024 confocal unit (Hercules, CA) mounted on an Olympus Provix AX70 microscope. Three-dimensional images of the synapse were analyzed using National Institutes of Health Image Software (Washington, DC) and Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). Scoring of PKCθ polarization and CD45/PKCθ bulls-eye patterning was done in a blind fashion.

Results

Thymocytes, but not mature CD8 T cells, form conjugates with APCs presenting low-affinity TCR ligand

Efficient TCR signaling requires conjugate formation with an APC (18). We determined the extent of conjugate formation between immature thymocytes or mature CD8 T cells expressing the transgenic OT-I TCR and APC presenting a range of peptides. Thymocytes were obtained from TAP-$1^{-/-}$ OT-I mice ensuring that the majority of cells had not yet been exposed to self-peptide/MHC ligands, whereas mature CD8 T cells were enriched from LNs of OT-I mice. Splenocytes from wild-type C57BL/6 mice were pulsed with a variety of peptides and were incubated with the OT-I thymocytes and T cells. T cell–APC conjugates were measured using a flow cytometry based assay. Approximately 40–50% of both thymocytes and mature T cells formed conjugates with APC presenting the high-affinity peptide, OVAp (Fig. 1*A*). Conjugate formation occurred rapidly, within minutes of mixing, and reached maximal conjugation at around 30 min (data not shown), and this time point was used for additional experiments. In response to APC presenting the low-affinity peptide, G4, mature T cells did not form conjugates, whereas ~25% of thymocytes were paired with an APC (Fig. 1*A*). These results show that thymocytes respond more efficiently than mature CD8 T cells in terms of forming APC conjugates.

Interestingly, we noted that the "background" level of conjugation seen in response to APC presenting self-peptides (none) or a control peptide (P815p) was consistently higher in thymocytes (5–10%) than mature T cells (1–2%) (Fig. 1*A*). To test whether this increased basal level of conjugation seen in thymocytes was mediated by TCR interaction with endogenous peptide/MHC ligands,

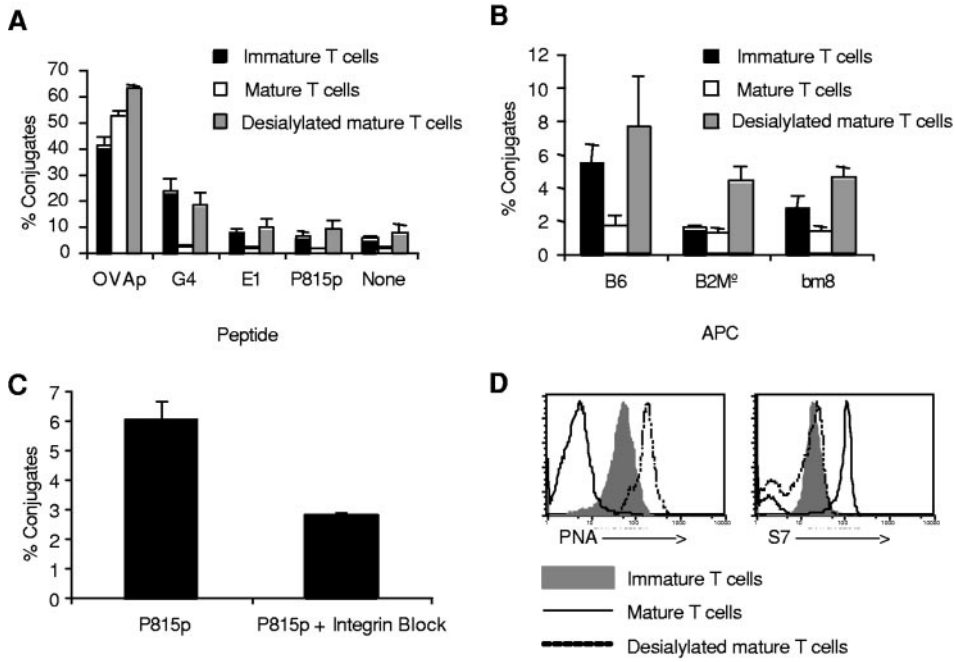

FIGURE 1. Thymocytes and desialylated mature CD8 T cells form conjugates with APC presenting low-affinity ligand. *A*, CFSE labeled OT-I TAP$^{-/-}$ thymocytes or OT-I RAG$^{-/-}$ LN cells ± neuraminidase treatment were stimulated with unlabelled B6 splenocytes pulsed with 1 μM OVAp, G4, E1, P815p, or no additional exogenous peptide for 30 min. Cells were fixed and stained for CD8 and the B cell marker, B220. Graph shows the percentage of CFSE^{+}/CD8^{+} events that were also B220^{+}, indicating a T cell-B cell conjugate. *B*, Same as *A*, except splenocytes were from B6, β_2m$^{\circ}$, or bm8 mice and were not pulsed with peptide. *C*, Blocking Abs to integrins β7, αL, and α4 (7.5 μg/ml) were added to wells with OT-I TAP$^{-/-}$ thymocytes and P815p peptide-pulsed B6 splenocytes. The percentage of conjugates was measured as in *A*. *D*, Cells were treated with or without neuraminidase and stained with PNA or the S7 Ab.

we used APC that either lacked MHC class I (β_2m-deficient mice) or had normal levels of an MHC class I allele (bm8) that cannot present selecting self-peptide ligands to the OT-I TCR (19). In both cases, basal levels of conjugation were decreased to the level seen on mature T cells (Fig. 1*B*). These data demonstrate that the higher basal level of conjugation achieved by thymocytes is mediated by endogenous peptide/MHC self-ligands. Preincubating thymocytes with blocking Abs to β7, α4, and αL integrins reduced this basal level of conjugation to that observed in mature T cells (Fig. 1*C*). This suggests that thymocyte interaction with low-affinity self-ligands results in TCR-mediated integrin activation (20), and may explain the stable association observed in reaggregate cultures between thymocytes and stromal cells of the appropriate MHC haplotype (21).

Other surface molecules besides the TCR and integrins could also play a role in conjugate formation. Our in vitro conjugate assay used APC that express the CD28 ligands, B7.1 and B7.2. These ligands are not present on cortical epithelial cells, which are a significant APC for immature thymocytes in vivo (22). To assess the role of CD28 in conjugate formation we used blocking Abs to B7.1 and B7.2 and found they had a negligible effect on the percentage of conjugates formed (data not shown). This finding is consistent with recent work by Bromley et al. (23), which supports the idea that CD28 does not have an adhesive function.

To test whether CD8 T cell–APC conjugation was reduced by the increased sialylation that occurs as T cells mature, we pretreated mature T cells with *V. cholerae* neuraminidase, an enzyme which cleaves multiple sialic acid linkages. The effect of neuraminidase on mature T cells can be measured by binding with the lectin PNA, which binds the unsialylated core-1 moiety of O-glycans (24), and by the S7 Ab, which recognizes sialylated forms of CD43 (25). Using these reagents, we found that neuraminidase treatment of mature T cells reduced O-glycan surface sialylation below that of thymocytes, while reducing the level of sialylated CD43 to the same level as thymocytes (Fig. 1*D*). We found that desialylated mature T cells, unlike sialylated mature T cells, formed conjugates with APC presenting low-affinity ligand (Fig. 1*A*). The level of conjugate formation between desialylated mature T cells and APC was similar to the level seen with thymocytes. Immature thymocytes also responded to neuraminidase treatment, increasing their ability to form conjugates, especially to low-affinity ligands (data not shown).

Desialylated mature CD8 T cells also displayed a higher basal level of conjugate formation, similar to thymocytes (Fig. 1*A*). However, when tested with the β_2m-deficient or bm8 APC, the percentage of conjugates did not decrease to the level seen with untreated mature T cells (Fig. 1*B*). This suggests that the increased basal conjugate formation seen with neuraminidase was due to pleiotropic effects as well as enhanced TCR-mediated integrin activation. Overall, surface sialylation contributes to the reduced ability of mature CD8 T cells to form conjugates with APC.

Both thymocytes and mature CD8 T cells form SMACs

Mature T cells segregate certain proteins within the T cell–APC synapse in a "bulls-eye" pattern termed SMAC, and it has been argued that such segregation is critical for full T cell activation (26). Consistent with this interpretation, a SMAC is observed after stimulation with high-affinity, but not low-affinity ligands (27, 28). Because a thymocyte response to high- and low-affinity ligands differs from mature T cells, we were interested in testing SMAC formation in thymocytes. To do this we analyzed the location of PKCθ and CD45, two well-studied markers of the central and peripheral SMAC, respectively (26), in thymocyte-APC conjugates using confocal microscopy.

Previous studies demonstrated that by 30 min PKCθ segregates to the center of the SMAC whereas CD45 is found mainly in the periphery in mature T cells stimulated with high-affinity peptides (27, 29–31). In agreement with the previous studies, we saw that PKCθ was recruited to the center of the synapse with CD45 distributed in a ring on the periphery in mature T cells stimulated with the high-affinity (OVAp), but not low-affinity (G4 & E1) ligands (Fig. 2, *A* and *B*). Surprisingly, thymocytes recruited PKCθ and formed a CD45 ring in response to both the high- and low-affinity ligands, again demonstrating a broader dynamic range of TCR response in thymocytes. To see whether PKCθ polarization to the synapse was evidence of SMAC formation we looked at CD45 and

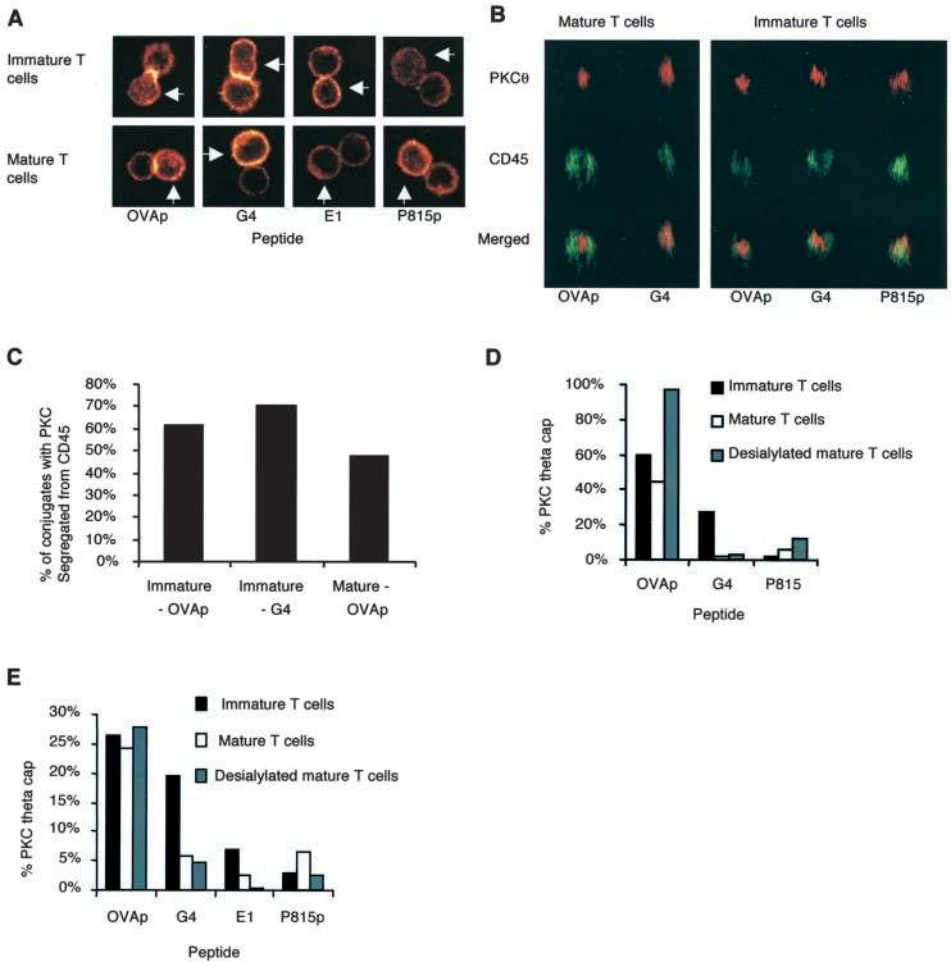

FIGURE 2. Thymocytes stimulated with high-affinity and low-affinity peptides form SMACs whereas mature CD8 T cells only form SMACs in response to high-affinity peptides. *A*, Thymocytes or LN cells (arrows) ± neuraminidase were mixed with CD45.1 congenic B6 splenocytes pulsed with OVAp, G4, E1, or P815p peptides and were incubated together for 30 min. Cells were fixed, permeabilized, and stained for PKCθ (red) and CD45.2 (data not shown). Cell pairs were selected for imaging if they contained an OT-I cell (CD45.2$^+$) paired with an APC (CD45.1$^+$). Images are representative of each condition. *B*, Computer generated orthogonal slices through the synapse in both the PKCθ (red) and CD45.2 (green) channels were overlaid. Images are representative of each condition. *C*, Cell pairs scoring positive for PKCθ polarization were analyzed for presence of CD45 in a ring around a central accumulation of PKCθ, indicating a SMAC, $n > 20$ for all conditions. *D*, Cell pairs from *A* were scored for PKCθ polarization, $n > 70$ for all conditions. *E*, Same as *D*, except splenocyte APC were replaced with thymic stromal cells from deoxyguanosine-treated fetal thymi.

PKCθ distribution within the synapse. Roughly 60% of all conjugates with evident PKCθ polarization displayed protein segregation where a ring of CD45 surrounded PKCθ (Fig. 2*C*). Of all cell conjugates analyzed, ~50% of both thymocytes and mature T cell–OVAp/APC conjugates recruited PKCθ to the synapse (Fig. 2*D*). Mature T cells did not polarize PKCθ in response to G4/APC while 27% of immature T cell–G4/APC conjugates did polarize PKCθ (Fig. 2*D*).

To test whether surface sialylation levels affected the ability to form a SMAC we pretreated mature CD8 T cells with neuraminidase and looked at the location of PKCθ and CD45 in T cell–APC conjugates. Although the percentage of conjugates formed by desialylated mature T cells with both high-affinity and low-affinity peptide-pulsed APC increased (Fig. 1*A*), the ability of desialylated mature T cells to recruit PKCθ to the synapse only increased for high-affinity OVAp/APC and remained at baseline in response to low-affinity G4/APC (Fig. 2*D*). Because the APC may also affect SMAC formation we repeated the experiment using thymic stromal cells instead of splenocytes as APC. Similar patterns of PKCθ polarization in response to low-affinity ligands were observed with thymic stromal APCs, in that immature but not mature OT-I cells polarized PKCθ when exposed to G4/APC, and desialylation of mature T cells did not restore the level of polarization (Fig. 2*E*). These results show that even though desialylation enhances conjugate formation, it does not enhance SMAC formation.

Activation of mature CD8 T cells is enhanced by desialylation

The finding that desialylation increased a mature CD8 T cell's ability to form conjugates with APC presenting low-affinity ligands, but did not result in the ability to recruit PKCθ or form a SMAC, lead us to test the effect of desialylation on T cell activation. An early marker of activation for both mature and immature T cells is increased surface expression of CD69 (32), which occurs within 2 h of TCR stimulation. Our lab and others had previously shown that thymocytes up-regulated CD69 in response to both high- and low-affinity stimulation within 3 h, whereas mature T cells only did so in response to high-affinity stimulation (Fig. 3*A* and Refs. 2, 3). To characterize the effect of desialylation on CD8 T cell activation, we pretreated T cells with or without neuraminidase, stimulated with APC presenting varying concentrations of high- and low-affinity peptides for 3 h, and then measured surface CD69 levels using flow cytometry. We found that desialylation of both immature and mature T cells induced up-regulation of CD69 in response to low-affinity ligands, even at low (10 nM) concentrations (Fig. 3*A* and data not shown). In fact, the response of desialylated mature T cells was similar to thymocytes.

Desialylation might affect numerous molecules involved in CD8 T cell–APC interactions. To focus on the TCR/CD8 interaction with peptide/MHC ligand, we stimulated cells with MHC class I tetramers loaded with high- and low-affinity ligands. We found that neuraminidase-treated mature T cells responded to the low-affinity peptide tetramer by increasing CD69 expression (Fig. 3*B*). This is consistent with the fact that CD8β is highly sialylated during development (33, 34), likely contributing to the reduced ability of CD8 to interact nonspecifically with MHC class I (8, 9). The increased interaction between CD8 and noncognate MHC tetramers caused by desialylation, however, was not sufficient to trigger T cell activation, as there was no increase in CD69 up-regulation

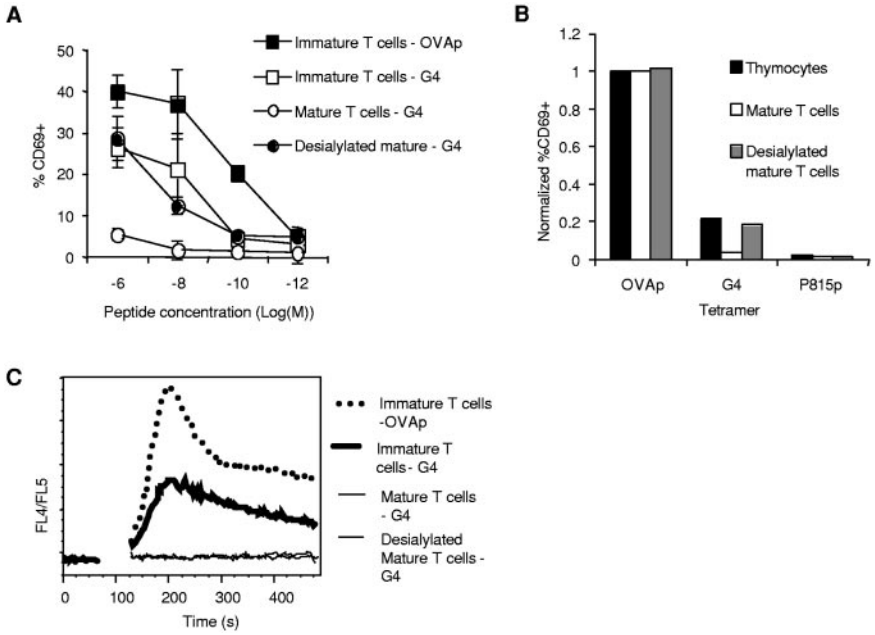

FIGURE 3. Desialylated mature CD8 T cells respond to low-affinity TCR-MHC/peptide stimulation. *A*, OT-I TAP$^{-/-}$ thymocytes or OT-I RAG$^{-/-}$ LN cells ± neuraminidase treatment were incubated for 3 h with congenic (CD45.1) B6 splenocytes loaded with OVAp or G4 at indicated concentrations. Cells were stained for CD8, CD45.2, and CD69 and analyzed by flow cytometry. Graphs show the percentage of CD8$^+$/CD45.2$^+$ T cells that have up-regulated CD69. The data shown are representative of at least four experiments. *B*, Cells ± neuraminidase treatment were stimulated with K^b tetramer loaded with OVAp, G4, or P815p for 3 h then stained for CD69. Thymocyte and LN cell values were normalized to OVAp stimulation without neuraminidase. Normalized values are shown because the mature T cell response to OVAp is significantly greater than the thymocyte response. *C*, OT-I TAP$^{-/-}$ thymocytes or CD8$^+$ OT-I LN T cells ± neuraminidase were loaded with Indo-1-AM and analyzed for calcium flux. After establishing a baseline flux for 1 min, T cells were added to a pellet of peptide-pulsed APC (1:1 APC–T cell ratio), briefly pulsed and resuspended and immediately analyzed for an additional 6 min. The resulting flux is shown as the median of the ratio FL4/FL5 for the population tested. The overlaid plots are normalized for baseline measurements.

on neuraminidase-treated mature T cells when the control peptide, P815p, was loaded on the tetramers (Fig. 3*B*). These results suggest that desialylation of the TCR/coreceptor complex directly affects T cell ability to respond by up-regulation of CD69, although the finding that peptide transfer from tetramer to surface MHC and subsequent T cell–T cell presentation can occur (35, 36) does not allow us to rule out the possibility that the effects of desialylation are mediated through other molecular interactions.

Another TCR-mediated signal is a transient increase in intracellular calcium levels (37). To analyze the effect of surface sialylation on calcium flux we stimulated T cells that had been treated with or without neuraminidase with APC presenting the different peptides and measured calcium flux in responding T cells by flow cytometry. APC presenting high-affinity peptide produced a robust spike in intracellular calcium levels in both thymocytes and mature T cells, whereas APC presenting low-affinity peptide elicited a calcium flux in thymocytes, but not mature T cells (Fig. 3*C*). Unlike CD69 up-regulation, mature T cells treated with neuraminidase did not flux calcium in response to the low-affinity stimulation. Similar results were seen whether MHC-peptide tetramers were used instead of peptide-pulsed APC (data not shown). These results show that desialylation enhances some TCR signals, such as surface expression of CD69 and integrin activation, but not others, such as a calcium flux. This furthermore suggests that a developmental increase in sialylation could contribute to TCR desensitization or tuning.

In vivo desialylation due to ST3Gal-I deficiency results in increased CD8 T cell sensitivity

If sialylation were a mechanism of tuning, one would predict that disruption of this process would not impact CD8 T cell positive selection per se, but would enhance mature CD8 T cell reactivity possibly leading to increased negative selection and/or altered homeostasis. Recently, Priatel et al. (14) generated mice deficient in the sialyltransferase ST3Gal-I, which is known to be up-regulated during thymocyte development and catalyzes the α2,3 sialylation of the core 1 O-glycan, Galβ1–3GalNAc-Ser/Thr. These mice showed a CD8 T cell-intrinsic homeostasis defect, although it was not clear whether this was due to enhanced self-reactivity, or whether T cell repertoire selection was altered. To explore these issues, we crossed ST3Gal-I$^{-/-}$ mice to OT-I TCR tg mice. Sialic acid levels on core 1 O-glycans on both mature and immature T cells in OT-I/ST3Gal-I$^{-/-}$ mice approached the level seen after neuraminidase treatment, as judged by PNA staining (Fig. 4*A*). This indicates that, as in polyclonal mice, ST3Gal-I is responsible for a large portion of the surface sialylation of these core 1 O-glycans on OT-I T cells.

Introduction of ST3Gal-I deficiency on the OT-I tg background did not detectably alter thymic selection as judged by the similar percentage of CD4 and CD8 expressing cells (Fig. 4*B*), similar cellularity, and equivalent surface expression of HSA, CD69, and the OT-I TCR (data not shown). Nonetheless, these mice did show a reduced number of mature CD8 T cells in peripheral lymphoid organs (Fig. 4*B*), similar to polyclonal mice (14). These results indicate that desialylation due to lack of the ST3 enzyme did not appreciably increase negative selection of OT-I TCR tg thymocytes, yet resulted in reduced survival of peripheral CD8 T cells. In addition, OT-I/ST3Gal-I$^{-/-}$ CD8$^+$ T cells had a CD44^{high}, Ly6C ^{high}, and CD25$^+$ phenotype (Fig. 4*C* and data not shown). Although variable from mouse to mouse, a sizeable fraction of CD8$^+$ cells were CD69$^+$, a phenotype that is typically indicative of an activated cell (Fig. 4*C*), suggesting that CD8 T

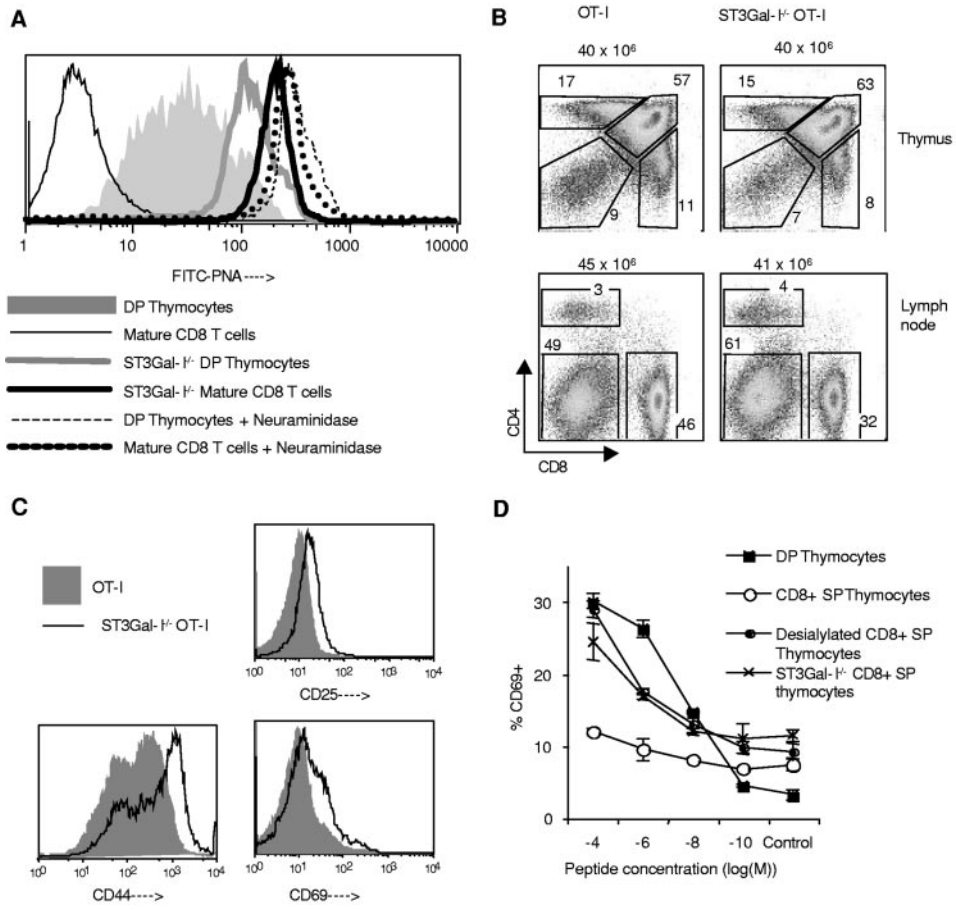

FIGURE 4. ST3Gal-I deficiency increases T cell sensitivity to low-affinity ligands. *A*, OT-I or OT-I/ST3Gal-I$^{-/-}$ T cells ± neuraminidase treatment were stained for PNA. *B*, Thymocytes and LN cells from OT-I and OT-I ST3Gal-I$^{-/-}$ mice were stained for CD4 and CD8 and analyzed by FACs. Plots shown are gated on live cells. Numbers *next* to gates are percentages, numbers *above* plots are total viable cell yield. *C*, OT-I and OT-I/ST3Gal-I$^{-/-}$ LN T cells were stained for CD25, CD44, and CD69. Histograms are gated on live/CD8^{+} cells. *D*, OT-I TAP$^{-/-}$ DP thymocytes, OT-I TAP$^{+/+}$ CD8^{+} single positive thymocytes and OT-I ST3Gal-I$^{-/-}$ CD8^{+} single positive thymocytes were stimulated as in Fig. 3*A*, and stained for CD8, CD4, CD45.2, and CD69. Graph shows percentage of immature double positive TAP$^{-/-}$, mature CD8 single positive TAP$^{+/+}$ or mature CD8 single positive TAP$^{+/+}$ ST3Gal-I$^{-/-}$ thymocytes that have up-regulated CD69.

cells in these mice were becoming activated in the absence of foreign Ag. Thus, we were interested in testing how ST3Gal-I$^{-/-}$ T cells respond to low-affinity self-Ag. However, because OT-I/ST3Gal-I$^{-/-}$ LN CD8 T cells displayed this dramatic phenotypic and homeostatic alteration, we tested TCR sensitivity using OT-I/ST3Gal-I$^{-/-}$ CD8 single positive thymocytes. Such cells have progressed beyond the positive selection checkpoint, but have not yet left the thymus. The majority of OT-I/ST3Gal-I$^{-/-}$ CD8 single positive thymocytes did not display the activated/memory phenotype seen in peripheral LNs (data not shown). When tested for conjugate formation, OT-I/ST3Gal-I$^{-/-}$ T cells had an increased basal level of conjugate formation, similar to neuraminidase-treated OT-I T cells (data not shown). Significantly, OT-I/ST3Gal-I$^{-/-}$ CD8 single positive thymocytes up-regulated CD69 within 3 h in response to the low-affinity, G4, peptide, similar to the response seen from neuraminidase-treated OT-I CD8 single positive thymocytes (Fig. 4*D*). These results suggest that induced expression of ST3Gal-I during positive selection directly contributes to the reduced sensitivity of mature CD8 T cells to self-Ags. Additionally, it is possible that enhanced TCR reactivity to self-Ag contributes to the reduction in peripheral CD8^{+} T cell numbers in ST3Gal-I$^{-/-}$ mice.

Discussion

Previous studies have shown that thymocytes are uniquely sensitive to low-affinity ligands (2, 3, 5). In agreement with the previous studies we demonstrate that thymocytes could up-regulate CD69 within 3 h and flux calcium within seconds in response to low-affinity ligands, whereas mature T cells did not (Fig. 3). We extended these findings by showing that thymocytes readily formed conjugates with APCs presenting high- and low-affinity peptide/MHC ligands, whereas mature T cells only coupled with APC presenting high-affinity peptide/MHC ligands (Fig. 1*A*). This ability to form conjugates was likely mediated by TCR activation of integrins because it could be blocked by Abs to integrins (Fig. 1*C*). We also found that thymocytes were able to polarize PKCθ and segregate PKCθ from CD45 (Fig. 2*B*). Importantly, we showed that SMAC formation by thymocytes was induced by both high- and low-affinity TCR ligands, while mature CD8 T cells only did so in response to high-affinity ligands (Fig. 3*D*).

The mechanism whereby sensitivity to low-affinity ligands is reduced, while sensitivity to high-affinity ligands is unchanged or even increased as a T cell matures is unknown. In an attempt to uncover the mechanism underlying developmental attenuation of TCR dynamic range we genetically and enzymatically manipulated surface sialylation levels on mature CD8 T cells and then tested the response to low-affinity ligands. Two of four responses tested, conjugate formation (Fig. 1*A*) and CD69 up-regulation (Figs. 3*A* and 4*D*), increased to the level seen in thymocytes, whereas the other two responses, SMAC formation (Fig. 2*D*) and calcium flux (Fig. 3*C*), were not affected. This suggests to us that increased surface sialylation contributes to, but is not the sole factor involved in, developmental attenuation of TCR sensitivity.

SMAC formation in thymocytes

Three recent studies suggested that thymocytes do not form classical SMACs in response to high-affinity stimulation. The first study, by Ebert et al. (7), showed that thymocytes were unable to polarize lipid rafts (38) in response to strong stimulation, whereas a second study by Richie et al. (39) found that CD3ζ and LCK clustered on the periphery of the synapse after high-affinity peptide stimulation but were not recruited to the synapse in response to

low-affinity peptide stimulation. The third study looked at thymocytes settled on a planar bilayer and found that instead of a single bull's-eye SMAC, several small, mobile clusters of MHC were seen surrounded by ICAM (40). Thus, it was somewhat surprising to observe that thymocytes recruited PKCθ and segregated CD45 at a central synapse and did so in response to both high- and low-affinity ligands (Fig. 2*B*). This was observed even when thymic stromal cells were used as APC (Fig. 2*E*).

In agreement with the findings of Ebert et al. (7), we were unable to elicit GM1 ganglioside recruitment to the synapse in thymocytes (data not shown), but it should be noted that our method of stimulation was different (APC vs Ab-coated beads) and we were also unable to detect lipid raft polarization in mature T cell–APC conjugates with high-affinity peptide (data not shown) as had been shown to occur with Ab-coated beads (7, 41). Lipid rafts, characterized by insoluble membrane fractions rich in certain proteins, are important for TCR signaling (42, 43), but to date there have been no conclusive reports regarding whether lipid raft aggregation is necessary for TCR signaling. Indeed, a recent study using sophisticated biochemical techniques for isolating rafts demonstrated that a preassembled, signaling-competent TCR complex constitutively associates with a small fraction of lipid rafts (44), although two studies suggest that lipid rafts are not critical for TCR signaling (45, 46). It is, therefore, possible that both thymocytes and mature T cells can initiate signaling and form a SMAC without a visible lipid raft accumulation at the synapse.

Furthermore, it is currently a topic of debate as to whether or not SMAC formation is necessary for TCR signaling and activation (26, 31, 47–49). It has recently been suggested that the SMAC may function to mediate TCR endocytosis (48) or may be necessary for resetting kinase/phosphatase interactions (31). Whether or not ligation-dependent TCR endocytosis functions to mediate or terminate TCR signal transduction is controversial (50). Our work shows that SMAC formation in response to low-affinity ligand stimulation is selectively attenuated during development (Fig. 2*D*) and this attenuation is not due to increased sialylation levels on the cell surface as it was not restored in neuraminidase-treated mature T cells. Because selective attenuation to low-affinity ligands in terms of CD69 up-regulation (Fig. 3*A*) and conjugate formation (Fig. 1*A*) was abolished by artificially decreasing sialylation levels, it would suggest that these two events are independent of SMAC formation.

The relevance of molecular segregation for positive and negative selection

The significance of SMAC formation in differential signaling between positive and negative selection ligands is currently unclear. We have previously shown that the peptides G4 and E1 are able to positively select OT-I thymocytes in fetal thymic organ culture, whereas OVAp induces negative selection (Ref. 15 and data not shown). However, G4 in particular, is a partial agonist and induces negative selection at high concentrations in organ culture. In most of our assays we used a relatively high concentration of peptide (1 μM), thus it is possible that protein segregation induced in thymocytes by G4 reflects the extent to which G4 is a negative selection ligand. When using the low-affinity E1 peptide there was no detectable SMAC formation (Fig. 2*A* and data not shown). From this perspective our data are consistent with a model whereby positive selection signals are transduced in the absence of an overt SMAC. This provides an interesting potential for differential signaling in thymocytes, because positive selection ligands do not trigger receptor internalization, as negative selection ligands do (Ref. 51 and data not shown), which also correlates with the suggestion that the role of a SMAC is to facilitate receptor internalization (48).

In vitro cultures do not support the full positive selection developmental program. Thus it may be argued that in our experiments thymocytes did not receive a positive selection signal in response to self-ligands. We think this is unlikely because in a two-step experiment where thymocytes were first cultured overnight in suspension, then reaggregated with stromal cells, only those that received the low-affinity peptide stimulation during the overnight culture underwent positive selection in the reaggregated lobe (data not shown). Additionally, when dissociated thymic stromal cells are used as APC instead of splenocytes we obtained similar results (Fig. 2*E*). Given this, we favor the idea that positive selection signaling does not trigger the formation of a SMAC, at least as represented by PKCθ/CD45 separation. However, as previously discussed, the requirement for SMAC formation in TCR signaling is not clear. It is interesting to note that the low-affinity ligand G4 fails to induce protein segregation in mature T cells, despite the fact that it can trigger responses in mature T cells, albeit with significantly delayed kinetics (52). On the other hand, the composition of the SMAC (rather than just its formation) may be crucial for regulating the fate of the thymocyte. For example, different components of the SMAC may determine whether an interaction will result in positive vs negative selection. In accordance with this hypothesis, the study by Richie et al. (39) did not detect significant recruitment of LCK or CD3ζ to the synapse in a positively selecting environment, whereas they mainly remained in the periphery of the synapse in a negatively selecting environment. We are currently pursuing studies to evaluate the composition of the thymocyte SMAC.

The molecular basis of selective attenuation in developing CD8 T cells

At first glance, the observation that TCR surface expression increases ~10-fold as T cells mature (2, 53) is counterintuitive to a model projecting decreased TCR sensitivity to low-affinity ligands. However, Dautigny and Lucas (54) suggest that TCR sensitivity is inversely proportional to the surface density of the TCR due to limiting "competence factors" required for TCR signaling. Too many TCR would cause competition for competence factors, making the rare TCR–peptide/MHC encounter less likely to generate a signal due to lack of these factors. However, this does not explain why mature T cells are more sensitive to agonist stimulation, but less sensitive to low-affinity interactions. In fact, McNeil and Evavold (55) propose that increased receptor levels actually increase T cell sensitivity to low-affinity ligands.

In the current study we have identified developmentally regulated sialylation as one mechanism whereby the TCR response to low-affinity ligands is selectively attenuated during development. It has been known for some time that neuraminidase-treated APC are more effective at inducing a mixed lymphocyte reaction (56, 57) and activating T cells (58). This was assumed to be the result of increased stickiness between cells due to changes in surface charge. To distinguish between desialylation effects that are physiologically relevant and those that reflect a nonspecific stickiness we took several approaches. First, we compared conjugation to APC with self-peptide/MHC vs APC lacking self-peptide/MHC. There was a small increase in conjugation in the absence of MHC (3%), however a significantly larger increase in conjugate levels was seen in desialylated mature T cells in response to the peptide G4 (16%) (Fig. 1, *A* and *B*). Second, we measured the response to soluble peptide/MHC tetramers, which most likely does not depend on cell-cell stickiness, and still saw increased levels of CD69

on neuraminidase-treated mature T cells stimulated with low-affinity ligands (Fig. 3*B*). Third, to further rule out the possibility that our results were due to nonphysiologic desialylation via neuraminidase we obtained the ST3Gal-I null mice. This approach allowed us to look at cells with normal levels of sialylation, except for the sialic acid residues attached by this enzyme. Using these mice we obtained the same results (Fig. 4*D*). Fourth, if the results we saw were mainly due to nonspecific changes in surface charge, we predicted that desialylation of the APC could have the same effect as desialylation of the responder cell. We did not see increased sensitivity to low-affinity ligands when we neuraminidase-treated the APC (data not shown). Finally, we would also note that not all responses were enhanced by desialylation (e.g., the Ca^{2+} flux) arguing against a model in which the sole role for sialylation was to influence cell adhesion.

In this study, we demonstrate that desialylation of the CD8 T cell enhances its ability to respond to low-affinity ligand stimulation (Figs. 1 and 3). Genetic analysis of glycosylation effects on TCR activation comes from recent gene knockout experiments. The MGAT5 gene, which codes for an enzyme responsible for adding complex branches to N-glycans, was knocked out by Demetriou et al. (59). The MGAT5 deficiency had no reported thymic phenotype, but did result in increased TCR sensitivity to stimulation, and the authors suggested the defect was due to an enhanced ability of TCRs to cluster because a lattice of surface glycoproteins was disrupted. Priatel et al. (14) made a T cell–specific knockout of the sialyltransferase ST3Gal-I and also found no thymic phenotype, but saw a large decrease in peripheral $CD8^+$ T cells due to apoptosis, with the remaining CD8 T cells exhibiting a memory phenotype. These findings are consistent with our data showing an increased sensitivity to low-affinity stimulation in OT-I/ST3Gal-$I^{-/-}$ mature T cells. It is possible that peripheral ST3Gal-$I^{-/-}$ T cells are being activated by low-affinity self-peptide/MHC in vivo, causing abortive activation in most of the progeny, whereas the rest convert to "memory phenotype" cells. Because sialylation occurs on several cell surface molecules (60) it will be important to determine which sialylated molecules are affecting the TCR response.

Sialic acid levels do not entirely account for selective attenuation during CD8 T cell development. Sensitivity to low-affinity ligands, as measured by conjugate formation and CD69 up-regulation, can be increased on mature T cells by decreasing surface sialylation (Figs. 1*A*, 3*A*, and 4*D*), yet SMAC formation and calcium flux do not appear to be affected (Figs. 2*D* and 3*C*). Many proteins are developmentally regulated and could account for decreased attenuation of the TCR response. The phosphatase SHP-1 is known to be a negative regulator of TCR response (61) and SHP-1 expression increases as T cells mature (3). Likewise, CD2 and CD5 are up-regulated during T cell development and are also known to attenuate TCR signaling (5, 10, 62). However, the attenuation resulting from increased expression of these three proteins does not seem to be specific for low-affinity ligands, and thus, could explain threshold adjustments, but not narrowing of the dynamic range. Developmental attenuation of the TCR most likely reflects the synergy of sialylation levels and signaling protein expression changes, which are coordinately regulated during maturation and dynamically maintained by continued self-interaction in the periphery.

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