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The Sialyltransferase ST3Gal-I Is Not Required for Regulation of CD8-Class I MHC Binding during T Cell Development

Charly Kao, Michelle M. Sandau, Mark A. Daniels, and Stephen C. Jameson

The CD8 coreceptor plays a crucial role in thymocyte and T cell sensitivity by binding to class I MHC and recruiting downstream signaling molecules to the TCR. Previous studies reported considerable changes in TCR-independent CD8/class I MHC binding (i.e., CD8 noncognate interactions) during T cell development, changes that correlated with altered glycosylation of surface molecules. In particular, expression of the sialyltransferase ST3Gal-I has been proposed as a critical factor regulating the attenuation of CD8 avidity during the double-positive to CD8 single-positive progression. This hypothesis is strengthened by the fact that ST3Gal-I−/− animals show a profound disruption of CD8 T cell homeostasis. In contrast to this model, however, we report in this study that ST3Gal-I deficiency had no detectable impact on CD8 noncognate binding to multimeric peptide/MHC class I ligands at any stage of thymocyte development. We also found that the susceptibility to CD8-induced cell death is not markedly influenced by ST3Gal-I deficiency. Thus, the profound effects of ST3Gal-I on CD8 T cell survival evidently do not involve a role for this enzyme in controlling CD8-class I binding. The Journal of Immunology, 2006, 176: 7421–7430.

The coreceptor and adhesion molecule, CD8, has a critical role during T cell development in the thymus and response to Ag by CTLs in the periphery (1). It is typically expressed as a CD8αβ heterodimer on conventional TCRαβ-expressing T cells, although CD8α homodimers are used instead by important subsets of specialized T cells, NK cells, and dendritic cells (2–4). The α- and β-chains of CD8 have similar architectures; however, they share only limited homology (<20% amino acid identity) to each other (1, 5). The N-terminal Ig head domain makes direct contact with the class I MHC molecule (6), while the hinge and stalk regions connecting the head to the transmembrane portion contain numerous Ser/Thr residues that anchor glycan appendages (7–9). CD8α has a cytoplasmic cysteine motif that binds to the Src kinase, p56lck, while the CD8β tail contains the amino acid sequence CVR that can be palmitoylated and regulate association into lipid rafts (10–12). Thus, CD8αβ functions at multiple levels to regulate T cell signaling. It serves as a coreceptor through coordinate (i.e., cognate) binding with TCR to specific class I pMHC ligands (13–15), and its intracellular domains function in signal transduction (16). In addition, CD8 can bind class I MHC independent of the TCR. This noncognate MHC class I binding highlights the role of CD8 as an adhesion molecule and was shown to be up-regulated in activated CTLs (17–19). In addition, our group and others have shown that CD8 noncognate binding is efficient on immature thymocytes, at the CD4+CD8+ double-positive (DP) stage, but that this property is diminished as thymocytes complete thymic maturation to the CD8 single-positive (SP) pool (20, 21).

Developing thymocytes undergo positive and negative selection to generate a T cell pool that is MHC restricted, but not overtly autoreactive (22). A critical checkpoint occurs at the DP stage, in which they test the ability of the rearranging TCRαβ to pair effectively with TCRβ and cooperate with the appropriate coreceptor to engage self MHC. Prepositive selection DP cells are acutely sensitive to low affinity TCR ligands, despite expressing ~10-fold lower levels of TCR compared with mature cells (23–25). This loss in sensitivity as the cells mature presumably limits their potential autoreactivity toward self peptide/MHC ligands in the periphery (22). A longstanding goal has been to determine the molecular basis for this change in sensitivity.

Thymocyte maturation has also been associated with altered glycosylation, specifically changes in sialylation of surface molecules (26–28), including CD8 itself (29–32). Altered sialylation has typically been revealed by changes in binding to carbohydrate-binding proteins (i.e., lectins) and Abs specific for particular glycoforms. One commonly used plant lectin, peanut agglutinin (PNA), shows much more avid binding to immature compared with mature thymocytes (20, 21, 27, 28, 30, 33, 34). PNA recognizes unsialylated, but not sialylated, core-1 glycosylated oligosaccharides (PNAlow phenotype) observed on mature thymocytes (20, 21). However, in contrast, a PNAhigh phenotype observed on immature thymocytes (20, 21, 27) suggests loss of this enzyme leads to selective disregulation of maturation of mature SPs (20, 27). ST3Gal-I-deficient mice were found to have a dramatic survival defect for CD8 T cells in the periphery, suggesting loss of this enzyme leads to selective regulation of mature CD8 T cell homeostasis (27). Furthermore, a report by Moody et al. (20) showed that a fraction of the mature CD8 SPs lacking ST3Gal-I exhibited higher noncognate binding similar to that of DP thymocytes. Those results correlate well with data showing...
that neuraminidase-mediated stripping of surface sialic acid residues leads to enhanced CD8 noncognate binding (20, 21). Together, these data have led to the popular model that ST3Gal-I up-regulation is responsible for the loss in noncognate CD8-class I binding that accompanies thymocyte maturation.

In addition, a recent report by Grebe et al. showed that DP thymocytes were exclusively sensitive to apoptosis induced by CD8 cross-linkage, including CD8 engagement by noncognate class I MHC ligands (38). This susceptibility to cell death was lost as thymocytes matured to the CD8 SP stage, and it was proposed that regulation of CD8 noncognate binding by altered sialylation may be responsible for these changes in mature thymocyte sensitivity (38). In keeping with this last point, we and others showed that both enzymatic desialylation and ST3Gal-I deficiency can enhance sensitivity of mature T cells to low affinity peptide/MHC ligands (39, 40). Furthermore, neuraminidase treatment can promote induction of apoptosis following T cell activation through the TCR (41). Together, these data indicate that sialylation, and in particular sialylation by ST3Gal-I, is important for regulating CD8-class I interactions and CD8 T cell survival.

However, it was unclear from the studies of Moody et al. why only a fraction of the ST3Gal-I–/– CD8 SPs, and not all of them, showed increased CD8 noncognate binding. In addition, we recently found that PNAhigh effector CD8 T cells show poor binding to noncognate MHC class I multimers (42), further putting into question the correlation between core-1 desialylation and noncognate CD8 binding. Thus, we sought to re-examine the role of ST3Gal-I in regulating CD8 avidity and its effects on T cell sensitivity.

In this study, we demonstrate that ST3Gal-I is not required for regulating the changes in CD8 noncognate binding observed during thymocyte development. Furthermore, PNA binding (a reflection of core-1 O-glycan sialylation) does not correlate with noncognate binding, indicating that the sialylation status of this glycan is not predictive for CD8-class I interactions. Our data indicate that the interpretation of previous findings might have been complicated by differential CD8 noncognate binding exhibited by mature vs immature CD8 SP thymocytes. We also show that ST3Gal-I deficiency has a minimal impact on T cell susceptibility to CD8-induced cell death by anti-CD8 Abs or MHC class I ligands. Alternative mechanisms for the CD8 homeostasis defect in ST3Gal-I–/– mice, as well as the potential roles of other glycosyltransferases in regulating CD8/MHC class I binding, are discussed.

Materials and Methods

**Mice and cells**

C57BL/6 mice were obtained from The Jackson Laboratory. ST3Gal-I-deficient mice (27) were provided by J. Marth (University of California, San Diego, CA), then backcrossed more than five generations to C57BL/6. The 2C TCR transgenic animals (43) were provided by L. Pease (Mayo Clinic, Rochester, MN) and M. Mescher (University of Minnesota, Minneapolis, MN), and were maintained on a normal B6 and an ST3Gal-I–/– background. The ST3Gal-I knockout and wild-type alleles were identified via PCR on tail DNA, using the following primers (sequences provided by J. Marth): ST3Gal-I forward, 5′-TCC CAA AGT GAC CCT GTG TCT CTC C-3′; knockout ST3Gal-I reverse, 5′-ATT TGA AGA CAC AGG TGA TGT CCA-3′; ST3Gal-I forward, 5′-GGG TAG CCG GAT ATC AAT TCG AG-3′. The accuracy of typing ST3Gal-I–/– animals was confirmed in several experiments by demonstration of increased PNA binding on mature ST3Gal-I–/– T cells compared with wild-type and ST3Gal-I+/+ littermates (data not shown). Additional mice were obtained from the Consortium for Functional Glycomics via Scripps Clinic (La Jolla, CA). All mice were maintained under specific pathogen-free conditions. Thy- mocytes and major lymph nodes were isolated, and single cell suspensions were made by passing over a metal mesh screen immersed in HBSS (In- vitrogen Life Technologies), filtered, and then pelleted and resuspended in FACS buffer (PBS, 1% FCS, 0.02–0.2% sodium azide) or RP10 medium (RP1640 from Invitrogen Life Technologies, supplemented with 10% FCS, 5 mM HEPES, 50 U of penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin sulfate, 50 μM 2-ME, and 2 mM glutamine). Cells were used for subsequent experiments without further purification.

**Peptides and MHC multimers**

The peptides OVA (SIINFEKL), SIY (SIYRYYGL), A6 (SIYRAGL), and VP10 (STLNNFNWL) were synthesized by Research Genetics or In- vitrogen Life Technologies. β2-microglobulin/peptide complexes were made, as described previously (15), using procedures adapted from those developed by Busch et al. and Altman et al. (44, 45). Mutant D227K Kb DNA constructs were created using site-directed mutagenesis, as described previously (46). Biotinylated Kb multimers were concentrated to 1 mg/ml and stored in 25–100 μl aliquots at −80°C. Multimers were prepared by mixing with regular grade streptavidin-PE (Molecular Probes) at an 8:1 molar ratio (1:1 mass ratio) and incubating at room temperature for 1–2 h before staining. The mass concentration of multimers was assumed to be 2× the mass concentration of streptavidin-PE (e.g., 0.5 mg/ml streptavidin-PE yields ≈1 mg/ml multimers). Typically, multimer staining was performed at 4°C for at least 1 h in FACS buffer (PBS, 1% FCS, 0.02–0.2% azide) in the presence of the indicated anti-CD8 Abs, as described in detail previously (15). In some cases (where specified), multimer staining was performed at 4°C for 2 h in RP10 medium (RP1640, 10% FCS with no azide), and stained for surface markers (including CD8) following washes; this staining protocol enhances weak CD8 noncognate multimer staining (42).

**Neuraminidase treatment**

Cells were treated with neuraminidase, as previously described (21, 42). Briefly, cells were prepared as above, washed one to two times in HBSS, and resuspended at 1–2×106/ml in HBSS. Following preincubation at 37°C for −15 min, neuraminidase (0.0095 U/1–2 million cells) from Vibrio cholerae (Sigma-Aldrich) was added, and the treated cells were incubated at 37°C for 20–40 min. Cells were washed one to two times with RP10 before staining with the indicated multimers. Controls were processed in the same way without addition of neuraminidase.

**Abs, PNA, and flow cytometry**

T cells (4–10×106) were stained with Abs for 15–30 min on ice in FACS buffer (PBS, 1% FCS, 0.02–0.2% sodium azide). CT-CD8α was from Caltag Laboratories. All other Abs were purchased from eBioscience or BD Pharmingen. Abs for flow cytometry were used at 1–2 μg/ml, except where noted in the death induction assays. FITC-conjugated PNA was purchased from Vector Laboratories and used at 0.5 μg/ml. Cells were costained with PNA and the appropriate Abs for 15 min in FACS buffer. Flow cytometry was performed using a BD Biosciences FACSCalibur or LSRII, and data were analyzed using FlowJo (TreeStar) software.

**T cell death induction assay**

Procedures for inducing T cell death were adapted from those developed by Grebe et al. (38). Cell death was induced via cross-linking of surface molecules using biotinylated Abs or MHC class I monomers and streptavidin-coated beads (5-μm-diameter latex beads from Spherotech). Assays were performed in 96-well plates. For Ab cross-linking, 1×106 thymocytes or lymph node T cells were mixed with 1×106 beads and prewarmed to 37°C in 100 μl of RP10, then mixed with 100 μl of diluted biotinylated Ab (prewarmed) in RP10 for 5–10 min in a 37°C water bath. Ab concentrations are given in the figure legends. Cells were spun down immediately (~300 × g for 5 min at 4°C) and washed in FACS buffer, then stained for 15 min with flow cytometric Abs and washed again in FACS buffer. Cell death was then measured using the annexin-V staining kit (BD Pharmingen). FITC-conjugated annexin-V was diluted 1:100 in 1× staining buffer, and the cells were stained in 100 μl for 15 min on ice, then diluted with 200 μl of 1× staining buffer and analyzed by flow cytometry. Streptavidin beads were coated with biotinylated MHC class I monomers, as follows: beads were incubated with 500 ng of MHC class I monomers/1×106 beads for ~2 h with constant mixing at room temperature, then washed and resuspended in RP10 to a final concentration of 10×106/ml. Beads were prewarmed to 37°C, added to T cells (1×106 beads to 1×106 cells, 200 μl total volume), spun down together briefly (1–2 min at room temperature), and incubated in 37°C water bath for 10 min. Pellet was then resuspended, and sample was then incubated for another 20 min at 37°C. Cells were then stained with flow cytometric Abs and annexin-V, as described above.
Results

Analysis of maturing thymocytes reveals differences in CD8 noncognate binding by mature and immature CD8 SP thymocytes

The report by Moody et al. (20), documenting an increase in CD8 noncognate binding by a subset of ST3Gal-I−/− CD8 SP thymocytes, suggested a key role for ST3Gal-I in regulating CD8-class I interactions during thymic development. However, these authors also reported that the ST3Gal-I−/− CD8 SP thymocyte pool includes an overrepresentation of immature CD8 SP cells (CD8 ISP). CD8 ISP cells are at an intermediate stage between DN and DP compartments, and thus represent a population of preselection thymocytes (47–50). Hence, it was possible that the increased CD8 noncognate binding reported by Moody et al. related to the increased prevalence of CD8 ISP cells that they observed. Because there has been no analysis of CD8 noncognate binding on this population, we initially studied noncognate binding patterns on CD8 ISP and other thymocyte subsets in normal B6 mice.

The gating strategy used to identify relevant thymocyte subsets is shown in Fig. 1, A–C. By using CD69 and TCR expression levels, we separated preselection (TCRlow CD69low) and postpositively sialylated at core-1 ligands correlated well with thymocyte maturation, with the pivotal step being between pre- and postselection DP thymocytes. Interestingly, our findings argue against PNA binding as being a useful marker for distinguishing the developmental stage at which CD8 noncognate binding changes: PNA binding is only moderately increased in postselection DP population (Fig. 1B), but multimer binding on postselection DPs is just as low as on mature CD8 SPs (Fig. 2, B and C). These data suggest that the mechanism regulating CD8 noncognate binding occurs immediately after positive selection and before the loss of PNA binding.

We next assessed noncognate CD8 binding by staining with class I MHC multimers in the presence of 53.6.7, an anti-CD8α Ab that enhances CD8/MHC binding (15). As expected, DP thymocytes show considerably higher noncognate binding than CD8 SP thymocytes and naive peripheral (lymph node) CD8+ T cells (Fig. 2A), consistent with previous observations (20, 21). We note that CD8 SP thymocytes and peripheral cells showed noticeably better noncognate binding above background than our earlier report (21) because of improvements in the methods used for preparation and storage of multimers (see Materials and Methods and Ref. 42).

Upon analysis of thymocyte subsets within the DP and CD8 SP pools, however, we noticed significant changes in CD8 noncognate binding. Although the bulk of DP thymocytes and the preselection DP pool exhibits efficient CD8 noncognate binding, postselection (TCRhigh, CD69high) DP cells showed poor noncognate binding, similar to mature CD8 SP cells (Fig. 2B). Within the CD8 SP compartment, we observed that mature and semimature CD8 SP cells showed poor noncognate binding, while CD8 ISP bound noncognate multimers similar to DP cells (Fig. 2C). Thus, the capacity of thymocyte populations to efficiently bind noncognate class I ligands correlated well with thymocyte maturation, with the pivotal step being between pre- and postselection DP thymocytes.

ST3Gal-I deficiency does not alter the pattern of CD8 noncognate binding by thymocytes

Building on the results described above, we next examined CD8 noncognate binding on thymocyte subsets in ST3Gal-I-deficient mice.
animals (provided by J. Marth). As reported previously (20, 27, 39), we find that these animals show normal representation of major thymocyte populations, but have a severe reduction in peripheral CD8\(^+\) T cells (Fig. 3, A and B). Also, as expected, PNA binding to all thymic and peripheral ST3Gal-I/−/− T cells was higher than on wild-type cells (Fig. 3 C). However, in distinction with the report by Moody et al. (20), we did not observe an increase in the frequency of CD8\(^+\) T cells in ST3Gal-I/−/− thymi (Fig. 3C). The reason for this discrepancy is currently unclear (see Discussion).

Moody et al. (20) had reported an increase in the noncognate binding exhibited by ST3Gal-I/−/− CD8 SP thymocytes. Surprisingly, however, we were unable to confirm this finding in our experiments, observing very similar patterns of CD8 noncognate binding on all thymocyte subsets from ST3Gal-I/−/− and control (wild-type or ST3Gal-I/−/−) thymocytes (Fig. 4A and data not shown). Analysis of relevant thymocyte subsets (mature and immature CD8 SP cells, and pre- and postselection DP thymocytes) and of the few peripheral CD8\(^+\) T cells in ST3Gal-I/−/− animals showed the expected changes in CD8 noncognate binding with thymocyte maturation, and did not reveal any substantial difference in multimer binding by the ST3Gal-I/−/− vs wild-type populations (Fig. 4B). These studies described used K\(\beta\)-based multimers, but we observed similar results using D\(\beta\)/MT389 multimers.
that ST3Gal-I-deficient thymocytes exhibit normal developmental regulation of noncognate binding.

Our initial studies had indicated a role of sialylation in regulating CD8 noncognate binding through enzymatic desialylation using neuraminidase. Given the data described above, suggesting loss of noncognate binding with maturation of ST3Gal-I−/− CD8 T cells, we next tested whether neuraminidase treatment would also enhance noncognate binding by ST3Gal-I−/− thymocytes. As expected (20, 21), neuraminidase treatment enhanced noncognate class I multimer binding to wild-type CD8 SP thymocytes and (to a lesser extent) DP thymocytes (Fig. 5A). Interestingly, we observed very similar augmentation of noncognate multimer staining with desialylation of ST3Gal-I−/− thymocytes (Fig. 5A). Hence, despite the fact that mature ST3Gal-I−/− thymocytes are already partially desialylated compared with wild-type cells (as demonstrated by PNA binding; Fig. 3C), neuraminidase treatment was still capable of enhancing noncognate multimer association by the mutant CD8 SP thymocytes.

FIGURE 4. Developmental regulation of CD8 noncognate binding is not altered in ST3Gal-I−/− mice. Thymocytes and lymph node (LN) T cells from ST3Gal-I−/− and wild-type animals were stained with 10 μg/ml noncognate MHC class I multimers. A, Noncognate (OVA/Kb) multimer staining on major thymocyte populations. Some samples were stained in the presence of the blocking anti-CD8 Ab CT-CD8 (as indicated), but in all other samples the anti-CD8 Ab 53.6.7 was included in staining. B, Wild-type and ST3Gal-I−/− thymocytes were stained with the noncognate multimer SIY/Kb (in the presence of the anti-CD8 Ab 53.6.7). Histograms show overlaid multimer staining of the indicated thymocyte subsets. The numbers represent the mean fluorescent intensity (MFI) for SIY/Kb multimer staining on the B6 (upper bold number) and ST3Gal-I−/− (lower number). C, Wild-type and ST3Gal-I−/− thymocytes were stained in RP10 medium (without azide) with 10 μg/ml noncognate multimer OVA/Kb in the absence of CD8 Abs. Following multimer staining and washes, the cells were stained for surface markers to identify thymocyte subsets (see Materials and Methods). The data in all panels are representative of at least three experiments.

FIGURE 5. ST3Gal-I−/− thymocytes show enhanced CD8 noncognate binding following desialylation and exhibit normal cognate multimer staining. A, Thymocytes from ST3Gal-I−/− and wild-type animals were treated with neuraminidase (or mock treated) and then stained with 10 μg/ml OVA/Kb MHC class I multimers in the presence of anti-CD8a Ab 53.6.7. B, Thymocytes from 2C and 2C ST3Gal-I−/− animals were stained with cognate multimers (SIY/Kb and A6/Kb) or noncognate multimers (OVA/Kb) in the presence of CD8 Abs. The data are representative of at least two independent experiments.
We also examined whether cognate interactions (involving the TCR ± CD8) would be influenced by ST3Gal-I deficiency by breeding the 2C TCR transgene onto the ST3Gal-I/Ki background. Similar to non-TCR transgenic ST3Gal-I/Ki (27) and OT-I TCR transgenic ST3Gal-I/Ki animals (39), ST3Gal-I/Ki 2C mice showed decreased representation of peripheral CD8 T cell numbers and increased PNA binding to CD8 SP thymocytes, compared with wild-type 2C transgenic controls (data not shown). The 2C system offers the opportunity to study cognate ligands with different dependence on CD8: although binding of SIY/Kb multimers to 2C T cells is partially independent of CD8, binding of A6/Kb multimers absolutely requires CD8 (42, 46). As expected (46), multimer staining of 2C CD8 SP thymocytes was more intense with SIY/Kb multimers compared with the low affinity A6/Kb multimers (Fig. 5B). This staining pattern was also observed for ST3Gal-I/Ki 2C T cells, however, indicating that cognate multimer staining is not altered by loss of the ST3Gal-I sialyltransferase (Fig. 5B). Similar observations were made for wild-type and ST3Gal-I/Ki 2C T cells, however, indicating that cognate multimer staining is not altered by loss of the ST3Gal-I sialyltransferase (data not shown). These data suggest that cognate multimer staining is not impacted by ST3Gal-I deficiency.

ST3Gal-I deficiency does not alter the sensitivity of thymocytes or T cells to CD8-mediated apoptosis

ST3Gal-I-deficient animals have a profound defect in CD8 T cell homeostasis, as exemplified by the activated/memory phenotype of the few peripheral CD8" cells in ST3Gal-I-deficient animals and their high susceptibility to induction of apoptosis (27). Interestingly, a recent report (38) showed that CD8 cross-linking (via anti-CD8 Abs or class I MHC molecules immobilized on beads) induces apoptosis in preselection (TCRlow) DP thymocytes, but not in postselection (TCRhigh) DPs, CD8 SP thymocytes, or CD8" peripheral T cells. Thus, as was proposed by Grebe et al. (38), expression of ST3Gal-I may be involved in diminishing the susceptibility of maturing thymocytes to CD8-mediated cell death. Despite our results indicating that ST3Gal-I deficiency does not impact the extent of CD8 noncognate binding, it was still possible that ST3Gal-I modulated the T cell’s response to CD8 engagement, and hence may affect susceptibility to CD8-mediated apoptosis.

We performed T cell apoptosis assays based on those described by Grebe et al. (38). In initial experiments, we stimulated thymocytes with Abs conjugated to cell-sized beads, followed by incubation at 37°C. Cells were then labeled with fluorescent Abs for flow cytometric gating and stained with annexin-V to assess induction of apoptosis. Anti-CD8β Abs were capable of inducing apoptosis in CD8-expressing thymocyte populations (Fig. 6), but not in CD4 SP thymocytes (data not shown). At relatively high doses of the anti-CD8 Ab, we observed some cell death in all the CD8"populations (Fig. 6); however, with titration of the Ab, we reproducibly observed heightened apoptotic sensitivity of the preselection (TCRlow) DP pool compared with subsequent maturation steps. Importantly, Ab engagement using anti-CD45 failed to induce death in any of the thymocyte populations (Fig. 6), a result that is significant because it suggests that apoptosis is not a general outcome of cross-linking any heavily O-glycosylated surface molecule. Upon examination of thymocyte subsets, we determined that postselection DPs were considerably more resistant to CD8-mediated death than preselection DPs (Fig. 6), which suggests that the protective mechanism involved is initiated shortly after positive selection. CD8 ligation appears to have little effect on CD8 ISPs; however, we found that this population showed considerable variability in the levels of basal apoptosis (even when cultured...
with beads alone; data not shown), complicating the analysis (Fig. 6).

Next, we tested the susceptibility of ST3Gal-I−/− thymocytes to cell death induced in the same way. As shown in Fig. 7A, we were unable to detect a difference in susceptibility to anti-CD8-mediated apoptosis in ST3Gal-I−/− compared with wild-type thymocytes and mature T cells. Hence, even populations that would normally express ST3Gal-I (posselective DPs, CD8 SPs, and peripheral CD8+ T cells) were unaffected in their susceptibility to Ab-induced apoptosis.

The high affinity of anti-CD8 Abs makes it difficult to interpret the physiological relevance of these findings. Hence, we also studied induction of thymocyte death using MHC class I ligands displayed on cell-sized microspheres. As expected based on the report by Potter and colleagues (38), noncognate MHC class I ligands could induce cell death on thymic subsets similar to that seen with lower doses of CD8 Abs (compare Figs. 6 and 7), in that TCRlow DP thymocytes were exquisitely sensitive to CD8 noncognate ligand-induced apoptosis, while TCRhigh postpositive selection DP thymocytes and mature CD8 T cells were much less sensitive to death induction (B6 cells in Fig. 7B). CD8 ISP cells seemed mostly resistant to apoptosis induction, although, as discussed above, interpretation of these data was made difficult by the highly variable level of spontaneous apoptosis in the ISP pool (Figs. 6 and 7 and data not shown). Introduction of the D227K mutation into the noncognate class I MHC molecule prevented induction of DP thymocyte apoptosis, as expected based on previous studies (38). In our hands, D227K mutant Kb molecules are unable to participate in CD8-dependent multimer binding (42, 46) or support CD8-mediated adhesion to noncognate peptide/MHC ligands (our unpublished observations), and hence are appropriate negative control in these assays. Importantly, ST3Gal-I−/− thymocytes and mature T cells showed a very similar pattern of susceptibility to noncognate class I MHC-mediated apoptosis (Fig. 7B). Thus, even in response to this more physiological stimulus, we were unable to detect changes in susceptibility due to ablation of the ST3Gal-I gene.

**Discussion**

Previous work has suggested that the CD8 avidity for MHC class I is dramatically altered during T cell development, and these changes have been correlated with the appearance of particular modifications of glycan adducts on surface glycoproteins (31, 54, 55). Specifically, the removal of terminal sialic acid adducts was shown to dramatically enhance TCR-independent binding of CD8 to MHC class I (i.e., noncognate CD8 binding) (20, 21). Desialylation was also shown to enhance T cell sensitivity to lower affinity TCR ligands (39, 40). Furthermore, based on the data from Moody et al. (20), it was proposed that sialylation of core-1 O-glycans catalyzed by ST3Gal-I was a critical element in the developmental regulation of CD8-class I binding. ST3Gal-I deficiency leads to a profound defect in peripheral CD8 T cell homeostasis (27, 39) (Fig. 3), and our previous studies also indicated ST3Gal-I−/− TCR transgenic T cells had enhanced reactivity to low affinity cognate ligands (39). However, in contrast to these expectations, we report in this study that ST3Gal-I deficiency showed no discernible effect on the pattern of CD8 noncognate binding during thymic development, and did not impact susceptibility of thymocytes or mature T cells to CD8-induced apoptosis.

Why do our CD8 noncognate binding data differ from that reported by Moody and colleagues? Perhaps the most likely explanation concerns the noncognate binding properties of the CD8 ISP pool. Moody et al. reported an increased frequency of CD8 ISPs in ST3Gal-I−/− thymi, indicated by elevated percentages of CD69low, CD24high, Qa2low, and CD5low CD8 SPs compared with

![FIGURE 7. ST3Gal-I−/− deficiency has a minimal effect on CD8-induced apoptosis in thymocytes and mature T cells.](http://www.jimmunol.org/). Cells were cultured with A, streptavidin beads ± 50 ng/ml 53–5.8 Ab for 5 min at 37°C, or B, beads coated with MHC class I ligands (VP10/Kb or SIY/Kb D227K, as indicated) for 30 min at 37°C. Annexin-V staining on the indicated T cell subsets from ST3Gal-I−/− and wild-type groups is shown. Additional experiments confirmed that the induction of cell death by the class I MHC ligands was not peptide specific. The data are representative of at least two experiments.
wild-type animals (20). Importantly, our studies suggest that CD8 ISPs show higher levels of CD8 noncognate binding compared with mature CD8 thymocytes (Fig. 2). In agreement with this interpretation, Moody et al. (20) reported that only a fraction of ST3Gal-I−/− CD8 SPs showed efficient noncognate binding: they found that 23–26% of CD8 SP thymocyte T cells showed enhanced noncognate binding, which corresponds roughly to the increase in immature CD8 ISPs they saw in ST3Gal-I−/− thymi (~14–33%). Unfortunately, those investigators did not determine directly whether the cells exhibiting enhanced noncognate binding were mature or immature CD8 SPs. Because CD8 ISP cells precede the DP stage, it is perhaps not surprising that their noncognate binding more closely resembles the preselection DP thymocytes. In contrast to the report by Moody et al., we found ST3Gal-I−/− thymi to possess normal percentages of mature and immature CD8 SP populations (Fig. 3), and this potentially would explain why we were unable to detect differences between ST3Gal-I−/− and wild type in CD8 noncognate binding by CD8 SP cells (Figs. 3 and 4). At present, we can only speculate on why we do not observe the overrepresentation of CD8 ISP in ST3Gal-I animals: one possible answer could be the extent to which the ST3Gal-I−/− allele was backcrossed to C57BL/6 background (from the original mixed B6/129 background) in our colony vs that used by Moody et al., but further investigations will be required to test this idea.

If ST3Gal-I is dispensable in the developmental regulation of CD8 noncognate binding, which sialyltransferases do regulate this process? Previous data indicated that desialylation using the broad-spectrum neuraminidase from V. cholerae was able to augment CD8 noncognate binding on CD8 SP thymocytes (20, 21). Moreover, neuraminidase treatment had a similar effect on ST3Gal-I−/− CD8 T cells (Fig. 5A), suggesting that sialylation at other sites may be involved in controlling noncognate binding. In this context, it is important to note that thymic development involves changes in expression of the substrates for sialyltransferases: this feature is well exemplified by the observation that ST6Gal-I is expressed throughout human T cell development, but its activity is only detected in mature T cells, evidently due to changes in expression of the preferred CD45 isoform substrates (56, 57). ST6Gal-I−/− animals have been generated (58); however, our preliminary studies on thymocytes from these mice do not suggest a change in CD8 noncognate binding (data not shown). Alternatively, it was possible that the impact of ST3Gal-I deficiency is partially masked by compensatory changes in glycosylation: for example, ST3Gal-I−/− animals were reported to exhibit heightened addition of core-2 O-glycans (27), which might potentially diminish CD8 noncognate binding in mature CD8 T cells. However, developmental regulation of CD8 noncognate binding appears normal in mice deficient for C2GnT (the enzyme chiefly responsible for initiating the core-2 branch in lymphocytes (59)) and in ST3Gal-I/C2GnT double-deficient mice (data not shown), arguing against that model. It is also currently unclear whether regulated CD8-class I binding in thymocyte development depends on sialylation at specific residues, or whether overall level of sialylation (and thus the cell’s net charge) is the key feature. On this point, we would stress that ST3Gal-I is the primary enzyme responsible for core-1 O-glycan sialylation in T cells (31), and as such contributes a sizeable fraction of the cell surface sialic acid. Indeed, our analysis confirms that mature ST3Gal-I−/− T cells exhibit markedly increased PNA binding, reflective of reduced sialylation at the Galβ1-3GalNAc-Ser/Thr (core-1 O-glycan) residues. Furthermore, previous biochemical studies have indicated that ST3Gal-I plays a role in the sialylation of CD8 itself, during thymocyte development (20, 27, 32). Thus, we would need to conclude that, although ST3Gal-I plays a key role in directing core-1 O-glycan sialylation, other sialyltransferases are important in controlling CD8/MHC class I noncognate binding.

We also found that staining of CD8 SP thymocytes with cognate peptide/MHC multimers (both low and high affinity ligands) was unaffected by ST3Gal-I deficiency (Fig. 5). This contrasts with our previous observation that ST3Gal-I−/− OT-I showed enhanced functional responses to low affinity cognate ligands (39). These data therefore suggest that ST3Gal-I deficiency might have more impact on the biochemical responses to a TCR/CD8 ligand rather than the extent of ligand binding per se.

The reduction in peripheral CD8 cell numbers in ST3Gal-I−/− animals was attributed to increased susceptibility to apoptotic death (27). Given the report by Grebe et al. that CD8 cross-linking could induce apoptosis of immature, but not mature, thymocytes (38), we also investigated whether ST3Gal-I might influence this response. The fact that CD8 noncognate binding was not notably influenced by ST3Gal-I deficiency might argue against this model, but as discussed above for cognate ligands, ST3Gal-I-defective T cells might exhibit altered consequences of CD8 noncognate interactions. Our studies built on the observations by Potter and colleagues (38) suggested a heightened susceptibility of immature DP thymocytes to CD8-mediated apoptosis, a sensitivity that was reduced on postselection DP thymocytes and successive developmental stages (Figs. 6 and 7). However, we saw very similar sensitivity of ST3Gal-I−/− thymocyte populations, implying that the aberrant CD8 homeostasis in ST3Gal-I−/− animals is probably not a consequence of CD8 noncognate binding (Fig. 7). CD8 cross-linking had been shown previously to have little effect on apoptosis in ST3Gal-I−/− T cells (31), but those studies did not involve cross-linking of the CD8 ligand, which Grebe et al. (38) proposed was essential for induction of apoptosis. Hence, the basis for the disregulation of CD8 homeostasis in ST3Gal-I−/− animals is still unclear. We should stress, however, that our studies show that ST3Gal-I−/− mice show a peripheral CD8 T cell defect (39) (Fig. 3), confirming the original description of these animals (27).

Hence, our current data argue that the compromised homeostasis of CD8 T cells in ST3Gal-I−/− animals is probably unrelated to changes in CD8 noncognate binding. As was discussed previously by Priatel et al. (27), ST3Gal-I deficiency may influence engagement or clustering of heavily glycosylated proteins, such as CD43 or CD45, leading to induction of apoptosis, although it is unclear why CD8 T cells would be selectively depleted in this model. Alternatively, changes in the consequences of TCR/CD8 interactions with cognate peptide/MHC ligands might lead to altered maintenance of ST3Gal-I−/− CD8 T cells, although our studies suggest that any such alteration is not manifest at the level of cognate ligand binding (Fig. 5B).

Interestingly, T cell activation also induces changes in O-glycosylation. Activated T cells show increased PNA binding and display core-2 branching, similar to preselection DP thymocytes (60–63). ST3Gal-I activity is slightly reduced in activated T cells, although a novel, undetermined mechanism appears to be responsible for the PNAhigh phenotype on activated cells compared with DP thymocytes (64). Effector T cells are more sensitive to ligand stimulation than naïve T cells (65–68), just as DP thymocytes are more sensitive than mature CD8 (23–25). However, we have shown that activation actually leads to a decrease in noncognate MH C class I binding (42). This is consistent with our finding that core-1 sialylation (and possibly core-2 branching) regulates T cell differentiation independently of changes in ligand avidity.

The developmental regulation of glycosylation and ligand sensitivity has been well documented for decades; however, attempts at making a direct causal correlation remain elusive. Our study of
the sialyltransferase, ST3Gal-I, reveals that the regulation of CD8 signaling by glycan modifications is complex. We showed that the absence of core-1 sialylation due to the lack of ST3Gal-I has negligible impact on CD8/MHC class I binding and susceptibility to CD8-induced cell death. The rapid progress in immunobiology, especially through the creation and study of other specific glycosyltransferase knockouts and use of recombinant sialyltransferases, should allow for a better understanding of the mechanisms regulating this process.

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Disclosures

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