1996

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Published August 1, 1996

Brief Definitive Report

Identification of CD8 as a Peanut Agglutinin (PNA) Receptor Molecule on Immature Thymocytes

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Summary

Differentiation of most T lymphocytes occurs within the thymus and is characterized by variable expression of CD4/CD8 coreceptor molecules, increased surface density of T cell antigen receptor (TCR) αβ proteins, and decreased expression of glycan chains recognized by the galactose-specific lectin peanut agglutinin (PNA). Although appreciated for several decades that PNA agglutination is useful for the physical separation of immature and mature thymocyte subpopulations, the identity of specific PNA-binding glycoproteins expressed on immature thymocytes remains to be determined. In the current report, we studied the expression of PNA-specific glycans on immature and mature T cells and used lectin affinity chromatography and immunoprecipitation techniques to characterize PNA-binding glycoproteins on thymocytes. Our data demonstrate that PNA-specific glycans are localized on a relatively small subset of thymocyte surface proteins, several of which were specifically identified, including CD43, CD45, and suprisingly, CD8 molecules. CD8α and CD8α′ proteins bound to PNA in the absence of CD8β expression showing that O-glycans on CD8β glycoproteins are not necessary for PNA binding and that glycosylation of CD8α and CD8α′ proteins proceeds effectively in the absence of CD8β. Finally, we demonstrate that PNA binding of CD8 is developmentally regulated by sialic acid addition as CD8 proteins from mature T cells bound to PNA only after sialidase treatment. These studies identify CD8 as a PNA receptor molecule on immature thymocytes and show that PNA binding of CD8 on immature and mature T cells is developmentally regulated by sialic acid modification.

Most T cells of the immune system differentiate within the thymus by a highly ordered process referred to as thymic selection (1, 2). The maturation status of thymocyte subpopulations is most often evaluated by their variable expression of CD4/CD8 coreceptor molecules and surface density of αβ TCR (T cell antigen receptor) proteins. By these criteria, three major thymocyte subpopulations are identified within the thymus which exemplify the progession of thymocytes along the CD4/CD8 developmental pathway: (*a*) immature $CD4^-CD8^-$ (double-negative) thymocytes, which express no/low surface density of αβ TCR; (*b*) immature $CD4^+CD8^+$ (double-positive) thymocytes, which express no/low surface density of αβ TCR; and (*c*) mature $CD4^+CD8^-$ and $CD4^-CD8^+$ (single positive) thymocytes, both of which express high surface density of αβ TCR (1, 2). The predominant thymocyte subpopulation is the immature $CD4^+CD8^+$ thymocyte subset, which accounts for ~85% of T cells within the thymus (1, 2).

The characterization of immune cell subsets has been greatly facilitated by the use of lectins, which recognize specific glycan residues attached to lipid molecules or polypeptide chains (3, 4). Approximately two decades ago, Reisner et al. noted that immature and mature thymocyte subpopulations were easily separated based on their differential agglutination with the galactose-binding lectin peanut agglutinin (PNA), specific for Galβ1,3GalNAc residues attached in O-linkage to proteins (5, 6). Expression of PNA-specific glycans is quantitatively regulated during T cell development with immature $CD4^+CD8^+$ thymocytes expressing significant amounts of PNA-specific glycans on their cell surfaces relative to mature T cells, which express little, if any, surface PNA-specific glycans (4, 5).

Glycoproteins bearing O-linked Galβ1,3GalNAc glycans can be modified by numerous glycosyltransferases localized in the Golgi complex, including Galβ1,3GalNAc α2,3 O-linked sialyltransferase (ST) and GalNAc α2,6 ST, which transfer sialic acid (SA) to Gal and GalNAc residues, respectively, (7). Surface density of PNA-specific glycans on developing thymocytes is inversely correlated with the expression of α2,3 O-linked ST (7), suggesting that decreased expression of PNA-specific glycans on mature thymocytes results from increased sialylation of O-linked glycoproteins which "mask" their recognition by PNA (4, 7).

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The functional significance of the developmentally regulated expression of PNA-specific glycans on T lymphocytes is unknown, but has been suggested to play a role in signal transduction during thymic selection (7) and the retention of immature thymocytes within the thymic cortex, preventing their premature escape into the periphery (4, 7).

Despite the widespread use of PNA lectins in the distinction of immature and mature thymocyte subpopulations, virtually nothing is known regarding the identity of polypeptides modified by PNA-specific glycans on developing T cells. Although previous studies have reported the isolation of PNA-binding glycoproteins from both human and mouse thymocytes (8–12), the identity of specific PNA-binding glycoproteins on thymocytes remains to be established. Because CD43 and CD45 molecules are highly expressed on thymocytes and migrate with similar mobility as proteins recognized by PNA, it has been suggested that CD43 and CD45 are the predominant PNA-binding glycoproteins expressed on immature thymocytes (9–12). Relatedly, the comigration of PNA-binding molecules with Thy-1 proteins led to the proposition that Thy-1 was a major PNA-binding glycoprotein on thymocytes (9, 10); subsequent studies demonstrated, however, that Thy-1 proteins do not contain O-linked glycans (13), making the interaction of Thy-1 with PNA somewhat enigmatic.

In the current report we studied the expression of PNA-specific glycans on developing thymocytes. Approximately 8–10 PNA-binding glycoproteins were isolated from thymocytes using lectin affinity chromatography and immunoprecipitation techniques, several of which were specifically identified, including CD43, CD45, and, unexpectedly, CD8. Moreover, we show that CD8α, CD8α′ proteins bind to PNA in the absence of CD8β and that PNA binding of CD8 is developmentally regulated on immature and mature T cells by sialic acid modification.

Materials and Methods

Animals and Cell Preparation. C57BL/6 (B6) mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). CD8α deficient (CD8−/−) mice (H-2^b haplotype; reference 14) were kindly provided by Drs. Tak Mak (Ontario Cancer Institute, Toronto) and Dinah Singer (NIH, Bethesda, MD); CD8β-deficient (CD8β−/−) mice (H-2^b haplotype; reference 15) were kindly provided by Drs. Tak Mak (Ontario Cancer Institute, Toronto) and Alfred Singer (NIH, Bethesda, MD). $CD4^+$ $CD8^+$ thymocytes, dexamethasone-resistant thymocytes, and splenic T cells were isolated as previously reported (16).

Reagents and Antibodies. Immobilized and fluorescent-labeled lectins were purchased from EY Laboratories (San Diego, CA) and Sigma Chemical Co. (St. Louis, MO). The following mAbs were used in this study: M1/9.3.4 anti-CD45 mAb (17); S7 anti-CD43 mAb (18); (PharMingen, San Diego, CA); 53-6.7 anti-CD8α mAb, (PharMingen); and 53-5.8 anti-CD8β mAb (PharMingen). Cells were analyzed on FACSTAR® and FACSCAN® (Becton Dickinson, Mountain View, CA) flow cytometers. All fluorescence data were collected using logarithmic amplification on 50,000 viable cells as determined by forward light scatter intensity or propidium iodide exclusion.

Surface biotin-labeling, Lectin Affinity Chromatography, Immunoprecipitation, and Gel Electrophoresis. Cells were washed 2× in cell wash buffer (PBS containing 1 mM $MgCl_2$ and 100 μM $CaCl_2$), resuspended in wash buffer containing 1 mg/ml sulfo-NHS biotin (Pierce Chem. Co., Rockford, IL), and incubated at 4°C for 30 min. Labeling was terminated by washing in buffer containing 25 mM lysine. Cells were solubilized in 1% NP-40 lysis buffer and lysates were precipitated with lectins conjugated to agarose beads (EY Laboratories) for 3 h at 4°C according to established methods (19). Immunoprecipitation and gel electrophoresis was performed as previously described (16). Biotin-labeled proteins were visualized by chemiluminescence, performed according to manufacturer's instructions (Renaissance Chemiluminescence Reagent; Dupont, NEN, Boston, MA).

Sialidase Digestion. Cells were washed 2× in RPMI medium without FCS, resuspended in RPMI at a density of 1×10^6 cells/ml, and incubated at 37°C for 30 min in the presence of 10–20 mU of sialidase (Vibrio cholerae); (Boehringer Mannheim, Indianapolis, IN). Sialidase treatment was terminated by washing cells 2× in RPMI containing 10% FCS. Viability was identical in untreated and sialidase treated groups (data not shown).

Results

Surface Expression of PNA-specific Glycans on Immature and Mature T Cell Subpopulations. Initially, we examined the surface density of PNA-specific glycans on various T cell subpopulations by staining with fluorescein (FITC)-labeled PNA. Significant amounts of PNA-specific glycans were expressed on thymocytes (Fig. 1, 1st column, top panel), the vast majority of which were localized on the immature $CD4^+CD8^+$ thymocyte subpopulation (Fig. 1, *1st column, 2nd panel*), as expected. In contrast, low levels of PNA-specific glycans were present on mature, dexamethasone-resistant thymocytes and mature splenic T cells in the periphery (Fig. 1, *1st column, 3rd and 4th panels*, respectively). Sialidase treat-

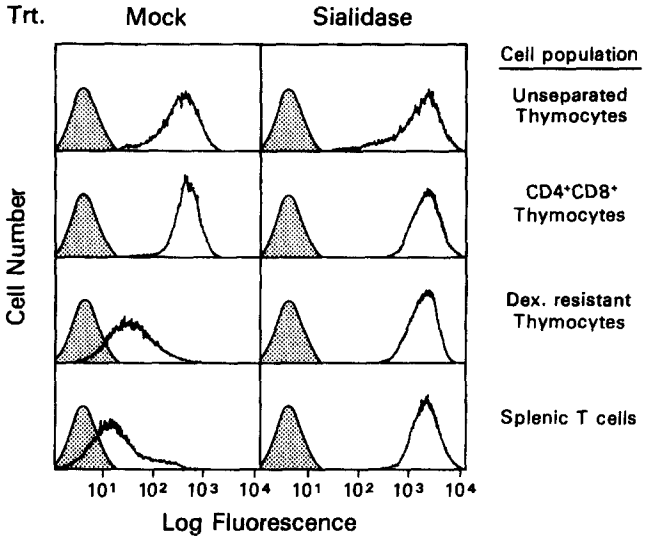

Figure 1. Surface expression of PNA-specific glycans on immature and mature T cells. Mock-treated and sialidase-treated T cells were stained with FITC-conjugated PNA. One color histograms also include negative control staining (*shaded curve*).

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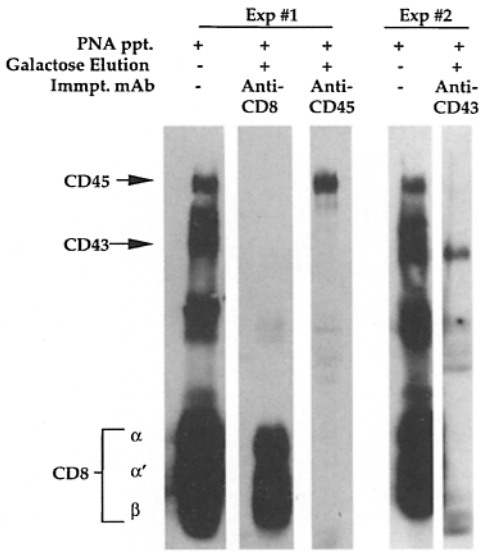

Figure 2. Identification of specific PNA-binding proteins on immature thymocytes. Thymocytes were biotin labeled, solubilized in 1% NP-40, and lysates precipitated with PNA affinity matrices. Where indicated, PNA-bound material was eluted by addition of 200 mM galactose. Galactose eluates were precipitated with indicated mAbs specific for immune receptor proteins. Precipitates were analyzed on one-dimensional 10% SDS-PAGE gels under reducing conditions and visualized by chemiluminescence techniques. The positions of CD8, CD43, and CD45 proteins are indicated; addition bands in anti-CD43 precipitates represent nonspecific proteins which were also present in control (no lysate) precipitates (data not shown).

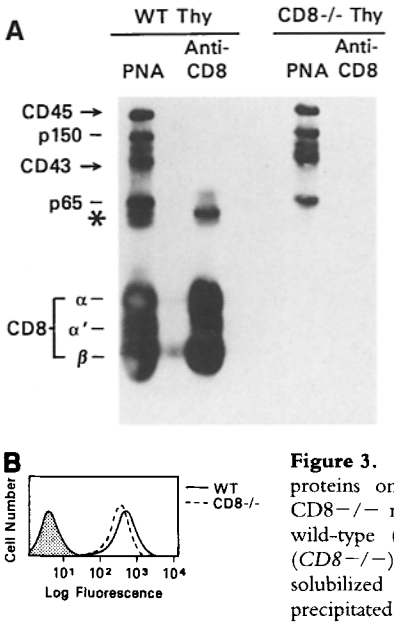

Figure 3. Expression of PNA-binding proteins on thymocytes of WT and CD8−/− mice. (*A*) Thymocytes from wild-type (*WT*) and CD8α-deficient (*CD8−/−*) mice were biotin labeled, solubilized in 1% NP-40, and lysates precipitated with PNA affinity matrices and anti-CD8 mAb. Precipitates were analyzed on one-dimensional 10% SDS-PAGE gels under reducing conditions and visualized by chemiluminescence techniques. The positions of CD8, CD43, and CD45 proteins are indicated; * denotes an unknown 55-kD protein that coprecipitates with CD8; unidentified proteins present in PNA precipitates migrating at ~65 and 150 kD are denoted p65 and p150, respectively. (*B*) WT thymocytes (*solid line*) and CD8α-deficient (*CD8−/−*) thymocytes (*dashed line*) were stained with FITC-conjugated PNA. One color histograms also include negative control staining (*shaded curve*).

ment significantly increased expression of PNA-specific glycans on mature T cells to similar levels observed on sialidase-treated $CD4^+CD8^+$ thymocytes, which also expressed higher levels of PNA-specific glycans after sialidase treatment (Fig. 1, 2nd column). Taken together, these data demonstrate that expression of PNA-specific glycans is quantitatively regulated during T cell development with immature $CD4^+CD8^+$ thymocytes expressing high surface density of PNA-specific glycans relative to mature dexamethasone-resistant thymocytes and splenic T cells. Furthermore, these data show that differential expression of PNA-specific glycans on various T cell subpopulations is regulated by posttranslational modification of galactose-containing glycans by sialic acid addition.

Identification of Specific PNA-binding Glycoproteins on Immature Thymocytes. To identify specific PNA-binding glycoproteins expressed on immature thymocytes, cells were surface-labeled, solubilized in 1% NP-40, and lysates precipitated with a PNA affinity matrix. As shown in Fig. 2, ~8–10 molecular species were visualized in PNA precipitates of thymocyte lysates, with molecular masses ranging from ~25–200 kD, (Fig. 2). Several proteins precipitated by PNA matrices migrated with similar mobility as immune receptor proteins precipitated by specific mAb, including CD45 proteins, CD43 proteins, and CD8 proteins (Fig. 2). PNA precipitation of unseparated thymocyte lysates and purified $CD4^+CD8^+$ thymocytes was identical (data not shown), in agreement with our previous findings in Fig. 1 that the vast majority of thymocyte PNA receptors are localized on the $CD4^+CD8^+$ subpopulation. Binding of thymocyte surface proteins to PNA matrices was specific in that the vast majority of thymocyte molecules precipitated by PNA were not isolated on lectin matrices specific for sialic acid-containing glycans and splenic T cell surface proteins were not bound by PNA (data not shown). To confirm the identity of individual PNA-binding glycoproteins on thymocytes, glycoproteins bound to PNA matrices were eluted with 200 mM galactose and precipitated with mAbs specific for various immune receptor proteins. As demonstrated, CD8, CD43, and CD45 proteins were effectively recaptured from galactose eluates of PNA precipitates by immunoprecipitation with specific mAb (Fig. 2). These studies directly identify CD8, CD43, and CD45 molecules as PNA-binding glycoproteins expressed on thymocytes. The identity of other thymocyte PNA-binding glycoproteins remains to be established, but as determined by precipitation/recapture experiments do not represent Thy-1, TCR, CD4, CD28, or major histocompatibility class I proteins (W. Wu and K.P. Kearse, unpublished observations).

Binding of CD8 Proteins to PNA Affinity Matrices. Because of the importance of CD8 molecules in immune function, the binding of thymocyte CD8 proteins to PNA matrices was evaluated in greater detail. CD8 molecules are ex-

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pressed on immature thymocytes primarily as disulfide-linked dimers of CD8α-CD8β and CD8α′-CD8β proteins (27); CD8α′ is a splice variant of CD8α, lacking the cytoplasmic tail (20). To confirm our results on precipitation of CD8 by PNA affinity matrices, we examined expression of PNA-binding glycoproteins on thymocytes from mice containing a targeted disruption of the CD8α gene; in the absence of CD8α expression CD8β proteins fail to egress the ER, and thus are not expressed on the cell surface (14, 20). As is evident, CD8 proteins were present in PNA precipitates of wild-type (WT) thymocytes but not CD8-deficient (CD8−/−) thymocytes (Fig. 3, *A*). In contrast, equivalent amounts of remaining PNA-binding proteins were present in both lysates, including a 65- and 150-kD protein (designated p65 and p150, respectively) and CD43, CD45 molecules (Fig. 3, *A*). Interestingly, an unidentified protein of 55kD (*) was present in anti-CD8 precipitates of WT lysates, which was also visible in PNA precipitates of WT lysates, but not CD8−/− lysates (Fig. 3, *A*). Expression of PNA-specific glycans was decreased on CD8−/− thymocytes relative to WT thymocytes (Fig. 3, *B*), showing that CD8 proteins (and/or molecules coexpressed with CD8) significantly contribute to the total surface density of PNA-specific glycans expressed on immature thymocytes.

Recent studies by Casabo et al. have shown that CD8β proteins are differentially glycosylated on immature and mature T cells, with O-linked glycans on thymocyte CD8β proteins being hyposialylated relative to CD8β proteins expressed on mature T cells (21). To determine if CD8α, CD8α′ proteins bound to PNA effectively in the absence of CD8β, experiments were performed using thymocytes from mice containing a targeted disruption of the CD8β gene (15); CD8α proteins are efficiently expressed

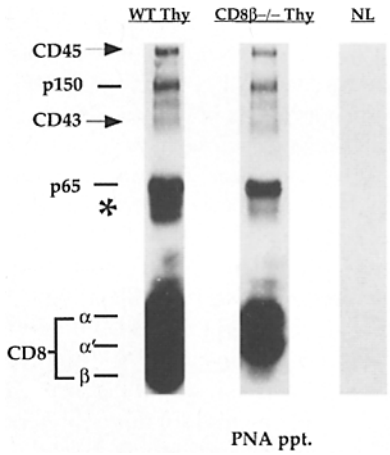

Figure 4. CD8α and CD8α′ proteins bind to PNA in the absence of CD8β. Thymocytes from wild-type (*WT*) and CD8β deficient (*CD8β*−/−) mice were biotin labeled, solubilized in 1% NP-40, and lysates precipitated with PNA affinity matrices. Precipitates were analyzed on one-dimensional 10% SDS-PAGE gels under reducing conditions and visualized by chemiluminescence techniques. The positions of PNA-binding proteins are indicated as in Fig. 3 *A*.

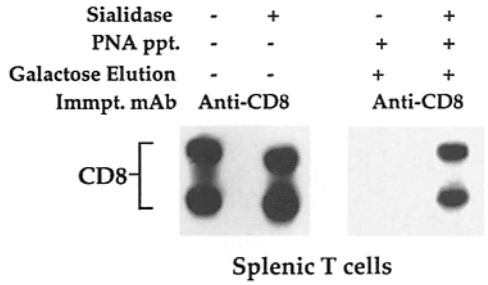

Figure 5. PNA binding of CD8 is developmentally regulation by sialic acid modification. Splenic T cells were biotinylated, mock treated or digested with sialidase, solubilized in 1% NP-40, and lysates precipitated with anti-CD8 mAb or PNA. PNA-bound material was eluted by addition of 200 mM galactose and eluates precipitated with anti-CD8 mAb. Precipitates were analyzed on one-dimensional 13% SDS-PAGE gels under reducing conditions and visualized by chemiluminescence techniques. The positions of CD8 proteins are indicated.

on the cell surface in the absence of partner CD8β proteins as disulfide-linked homo/heterodimers of CD8α and CD8α′ (15, 20). As shown in Fig. 4, CD8 molecules on CD8β−/− thymocytes were efficiently captured by PNA precipitation, as were remaining PNA-binding proteins (Fig. 4). These data show that CD8α, CD8α′ proteins bear PNA-binding O-linked glycans and that glycosylation and processing of CD8α, CD8α′ proteins occurs efficiently in the absence of CD8β expression. Interestingly, fewer 55* kD proteins were present in PNA precipitates of CD8β−/− thymocytes relative to WT thymocytes (Fig. 4), a finding which was also observed in anti-CD8α precipitates of WT and CD8β−/− thymocyte lysates (W. Wu and K.P. Kearse, unpublished observations). The significance of this finding is unclear, but suggests that expression of PNA-binding 55*-kD proteins on thymocytes may be linked to expression of CD8β gene products.

Finally, we compared the PNA binding of CD8 molecules on mature T cells before and after sialidase treatment. As shown in Fig. 5, CD8 molecules from sialidase-treated splenic T cells migrated with slightly increased mobility relative to CD8 from mock treated T cells (Fig. 5), due to removal of sialic acid residues from glycan chains. Most importantly, these data demonstrate that CD8 proteins from sialidase-treated splenic T cells, but not mock treated T cells, were effectively bound by PNA (Fig. 5). No increase in PNA binding of CD8 molecules from immature thymocytes was observed after sialidase treatment (data not shown). Thus, we conclude that PNA binding of CD8 on immature and mature T cells is developmentally regulated by sialic acid addition.

Discussion

The present study has specifically identified several important immune receptor proteins as PNA-binding glycoproteins on immature thymocytes. In agreement with previous suggestions, our data provide direct evidence that

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PNA-binding proteins expressed on immature thymocytes include CD43 and CD45 proteins (8, 10, 11). Unexpectedly, we found that CD8 proteins were among the subset of PNA-binding glycoproteins expressed on developing thymocytes, which most likely represent the lower molecular weight species previously believed to be Thy-1 (9, 10). Recent studies by Casabo et al. demonstrated that CD8β proteins are differentially glycosylated on immature and mature T cells, with O-linked glycans on thymocyte CD8β proteins being hyposialylated relative to CD8β proteins expressed on mature T cells (21). We found that CD8α molecules were efficiently isolated on PNA affinity matrices in the absence of CD8β gene expression, indicating that developmentally regulated PNA-binding of CD8 proteins does not result from variable addition of sialic acid to CD8β proteins on immature versus mature T cells. Although not directly determined in this study, we believe that binding of thymocyte CD8 proteins to PNA matrices is mediated via recognition of CD8α gene products, which like CD8β, contain O-linked glycans (20). However, we have also considered the possibility that CD8 proteins are indirectly bound to PNA affinity matrices via their association with unknown molecules containing O-glycans, that are not efficiently labeled by biotinylation. Experiments designed to address these issues are currently in progress.

The functional significance of regulated expression of PNA-specific glycans on developing T cells is unknown. Current hypotheses suggest that masking/unmasking of galactose-containing glycans on thymocytes regulates their egress from the thymic cortex to the medulla (4) or may be important in regulating signal transduction during thymic selection (7). Galactose-specific lectins have been isolated from thymic epithelium that agglutinate immature thymocytes but not mature thymocytes (22), which could conceivably play a role in intrathymic trafficking of developing thymocytes. More recently, Baum et al. reported that human thymic epithelial cells synthesize endogenous gactose-binding lectins (galectin-1) that bind to oligosaccharides on thymocytes (23), suggesting that interactions between thymocytes and thymic epithelial cells may be regulated by expression of glycans on the thymocyte cell surface. Other potential counterreceptors that may recognize PNA-binding oligosaccharides in vivo include the endogenous lectin CD23, which recognizes Gal-GalNAc saccharides (24). Regarding the role of PNA-specific glycans in thymic signal transduction, the importance of sialic acid containing N-linked glycans in engagement of CD45 proteins by CD22 ligands has been established (25, 26). Thus, it is conceivable that regulated addition of sialic residues to O-linked glycans of CD45 proteins might be of similar significance for their recognition by as yet undetermined ligands within the thymus. Similarly, given the recently appreciated role of CD43 in T cell activation (27, 28) and its extensive modification by O-linked glycans, it is reasonable to speculate that differential processing of O-linked sugars on CD43 proteins may affect delivery of intracellular activation signals to developing thymocytes.

We thank Drs. Richard Hodes, Stephen Shaw, David Wiest, and Robert Haltiwanger for critical reading of the manuscript; Drs. Tak Mak, Dinah Singer, and Alfred Singer for making CD8α−/− and CD8β−/− mice available for our studies; and Dr. Rose Zamoyska for helpful discussion.

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Received for publication 2 February 1996 and in revised form 29 May 1996.

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