Title
Migration and glycosylation in T cell development

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Publication Date
2004

Peer reviewed|Thesis/dissertation

MIGRATION & GLYCOSYLATION IN T CELL DEVELOPMENT

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I would like to thank my advisor Nigel Killeen for his encouragement and guidance. Without his support and dedication, this work would not have been possible. It has been rewarding and fun to work with past and present members of the Killeen lab. Thank you all-Bryan, Kurt, Linda, Qi, Julie, Sean, Arin, Kevin, Mark, Joong, Vinh, DongJi, Yan, the J's (Jie W., Jie F., Jay D., Jay W.), Fernando, Rosie, Erin, and Ayako. I also want to thank the members of my committee, Jason Cyster and Lewis Lanier, for their advice and for critical reading of this thesis. To my friends, Brenda, Gretchen, Joy, Lucie, Bernice, Robin, Chris, and Charles-thank you for keeping me sane. To my family, Mom, Dad, and my brother Anson, thank you for your unconditional love and support.

Migration and Glycosylation in T cell Development

Joanne Kwan

Abstract

Thymocyte survival, selection, and differentiation depend on their interactions with thymic stromal cells. After positive selection, thymocytes migrate from the cortex to the medulla in the thymus. The factors involved in guiding migration during this developmental step are poorly understood, but evidence suggests that chemokines and their receptors are required. The chemokine receptor, CCR7, is expressed at this step concurrent with positive selection and movement into the medulla. In addition to upregulation of CCR7, positively selected thymocytes induce expression of ST3Gal-1 sialyltransferase (ST3Gal-1), an enzyme that attaches sialic acid onto core 1 O-glycans. Sialylation of glycoproteins can modify their function and thereby alter thymocyte development. The significance of downregulation of ST3Gal-1 in double-positive thymocytes and its upregulation in single-positive thymocytes is not clear.

We show that premature expression of CCR7, the receptor for the chemokines CCL19 and CCL21, promotes the migration of thymocytes into the thymic medulla. Premature entry of thymocytes into the medulla is associated with defects in the development of CD4 and CD8 single-positive thymocytes. Specifically premature

expression of CCR7 impaired the development of CD4 single-positive thymocytes and caused a two-fold reduction in their numbers. Impaired selection of CD8 single-positive thymocytes was revealed in MHC I-restricted TCR transgenic mice. These studies demonstrate the involvement of CCR7 in thymocyte positioning and the importance of positioning for proper T cell development.

We have also studied the consequences of aberrant expression of ST3Gal-1 throughout T cell development. We characterized mice in which transgenic double-positive thymocytes have equivalent levels of sialylation as normal single-positive thymocytes. T cell development was not obviously altered in these mice. In contrast to studies that show sialylation negatively regulates the interaction of CD8 with MHC I, we find that increased sialylation of CD8 does not alter the ability of CD8 to bind to MHC I. The precise mechanism by which sialylation affects T cell development and CD8 function has yet to be determined.

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Chapter 1: Introduction

The majority of T cells are αβ T cells, i.e. T cells that express αβ T cell receptors (TCRs). The development of these cells in the thymus proceeds through an ordered series of differentiation events and gives rise to mature T cells that bear a diverse repertoire of TCRs (1). Thymocytes at various stages of development are positioned in different regions of the thymus. Consequently, during the course of their development, thymocytes interact with different thymic stromal cells. These stromal cells provide signals and factors such as cytokines and chemokines necessary for the survival, differentiation, proliferation, and selection of thymocytes. A key step in thymocyte development is the transition from the $CD4^+CD8^+$ double-positive (DP) stage to the $CD4^+$ or $CD8^+$ single-positive (SP) stage, which is accompanied by thymocyte migration from the cortex to the medulla. One well-described change on thymocytes at this transition is a modification of their cell surface glycosylation state, specifically the addition of sialic acid onto core 1-O glycans (2, 3). The mechanisms that direct the differentiation and migration of thymocytes and the significance of changing glycosylation patterns are the subject of this thesis.

Early T cell development

Development of αβ T cells in the thymus is a continuous process that begins between day 10 and day 12 of fetal life in the mouse (1). Waves of hematopoietic stem cells, originating from the yolk sac and paraaortic foci and then the fetal liver in the

embryo and from the bone marrow in the adult, populate the thymus (1, 4). The generation or export of prothymocytes from hematopoietic tissues and their gated import into the thymus is synchronized and regulated by the limited number of niches available in the thymus (5-7).

The thymus is divided into two main compartments: an outer cortex and an inner medulla (Figure 1). Distinct cell types are found in the medulla and the cortex. Stromal cells, such as cortical epithelial cells and macrophages, are found in the cortex (4). In the medulla, epithelial cells, macrophages, and Hassall's corpuscles (small, highly keratinized onion-like structures with no known function) are found. Medullary epithelial cells have been reported to originate from a precursor cell distinct from ones that give rise to cortical epithelial cells (8). Bone marrow-derived dendritic cells are primarily found at the cortical medullary junction and in the medulla (9). The various cell types found in the thymus likely promote different steps in thymocyte development and their roles will be discussed later in this introduction (4).

Progenitor cells enter the thymus from venules located near the junction of the cortical and medullary compartments (10, 11). These progenitor cells can give rise to NK cells, B cells, dendritic cells, αβ T cells, and γδ T cells, i.e. T cells that express γδ TCRs (1). Early T cell progenitors (called triple-negative cells because they lack expression of the TCR and the coreceptor molecules CD4 and CD8) migrate outwards into the cortex towards the subcapsular zone. During this period, they undergo a series of differentiation events and lose the ability to give rise to non-T cell lineages (8, 12, 13). Interactions of

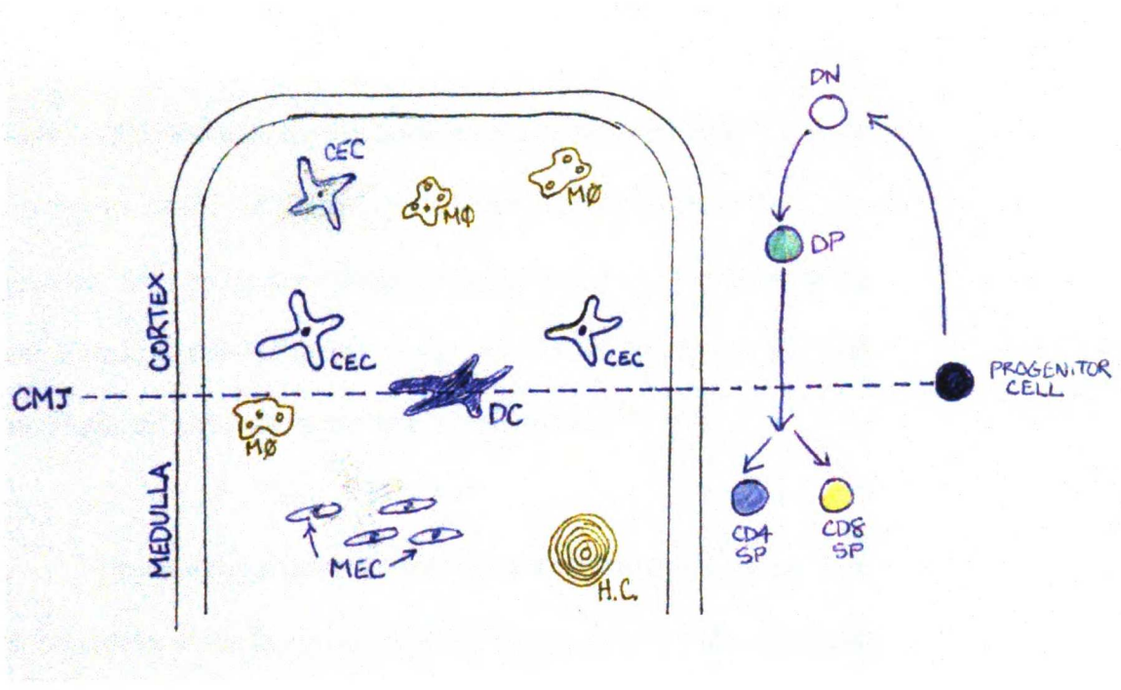


Figure 1. Diagram of thymic architecture

CMJ: corticomedullary junction
DN: $CD4^{-}CD8^{-}$ double-negative thymocyte
DP: $CD4^{+}CD8^{+}$ double-positive thymocyte
SP: single-positive thymocyte
MØ: macrophage
CEC: cortical epithelial cell
DC: dendritic cell
MEC: medullary epithelial cell
HC: Hassall's corpuscle

Notch family members with ligands (such as Delta) expressed on stromal components influence commitment to the T cell lineage, as well as commitment to either the αβ or γδ lineage (8). Evidence for the involvement of Notch in early T cell development initially came from studies of heterozygous Notch mutants in which the γδ T cell lineage was favored (14). Subsequent studies showed that early thymocyte development is blocked in conditional Notch-1-deficient mice, and Notch-1 overexpression suppresses B cell development and promotes T cell development (15, 16).

Positioning of the earliest T cell precursors in the subcapsular zone coincides with upregulation of the *RAG-1* and *RAG-2* gene products and rearrangements of their *TCRγ, TCRδ,* and *TCRβ* loci (8, 17). It is not clear what induces upregulation of RAG-1 and RAG-2, but the cytokine IL-7 has been implicated in this step (18, 19). IL-7 and the cytokine stem cell factor also likely influence other steps of survival and differentiation of immature thymocytes (20).

If a thymocyte assembles a functional γδ TCR on its surface, it proceeds along the pathway to become a mature γδ T cell. A thymocyte that productively assembles a β chain expresses a pre-TCR composed of the rearranged TCRβ chain and a surrogate α chain termed pre-Tα (20). These thymocytes receive signals through their pre-TCR that rescue them from apoptosis, trigger proliferation, induce differentiation to the DP stage which is characterized by co-expression of CD4 and CD8, and promote migration towards the medulla (11, 12, 21, 22).

Some components downstream of the pre-TCR include the src family of protein tyrosine kinases, specifically $p56^{lck}$ (23, 24), the tyrosine kinases ZAP-70 and Syk (25), and components of the ras signaling pathway (22, 26). The finding that molecules downstream of αβTCR signaling are also downstream of pre-TCR signaling is not surprising since both the αβTCR and the pre-TCR assemble with CD3 and ζ components (27). A notable difference in signals mediated by the pre-TCR and αβ TCR is that the pre-TCR signal promotes the proliferation of thymocytes, resulting in a 100- to 500- fold expansion of cells (13, 28). This pool of thymocytes increases the numbers of cells that have productively rearranged their TCRβ chain, maximizes the chances of assembling a functional α chain paired to a particular β chain, and contributes to a diverse TCR repertoire.

Positive selection

After DP cells have stopped dividing, they re-express the *RAG* gene products and initiate rearrangement of the TCRα locus and attempt to express a mature αβ TCR (1). At this stage of development, cells undergo positive selection (1, 29-31). The process of positive selection is important because it selects for T cells that express TCRs that can recognize self-expressed MHC (1). This process is mediated by interactions between TCRs on thymocytes and MHC ligands on thymic stroma (29). Thymocytes that express TCRs that do not have a minimum reactivity to self-MHC do not receive a positively selecting signal and die by neglect. Thymocytes that express TCRs that recognize self-MHC with intermediate affinity get positively selected and survive. Thymocytes that

express TCRs that interact with too high an affinity to self-MHC die by negative selection. The signals that mediate selection and models of selection will be discussed later in this introduction.

Positively selected thymocytes upregulate expression of various molecules including the TCR and the early activation marker CD69 (32), and downregulate RAG (32, 33), which prevents further arrangements of the α locus. Positive selection also coincides with changes in thymic localization; positively selected thymocytes migrate out of the cortex into the medulla (17).

Positive selection has been primarily associated with the thymic cortex, and cortical cells, such as cortical epithelial cells, express MHC molecules that influence selection (1, 34). Evidence that cortical stromal cells mediate selection have come from studies with MHC II-deficient mice, which have defects in $CD4^+$ T cell development. Expression of MHC II in thymic cortical cells rescues the development of $CD4^+$ T cells, but expression of MHC II in medullary cells fails to rescue development of those cells (35, 36). Other studies have provided support that cortical epithelial cells are required for positive-selection (17). For example, cortical epithelial cells are necessary and sufficient for the positive selection and development of $CD4^+$ T cells in reaggregate fetal thymic organ culture systems (37). In contrast, most studies have shown that medullary epithelium and antigen presenting cells of hematopoietic origin are unable to support efficient positive selection (17, 38-40).

Negative selection and differentiation in the medulla

Negative selection is the process by which thymocytes with too high an affinity for peptide/self-MHC ligands are deleted (1, 17). This process of central tolerance, which occurs in the thymus, eliminates cells expressing potentially auto-reactive T cell receptors. Negative selection can occur throughout thymocyte development and is mediated by different cells than those involved in positive selection (30, 41, 42). Bone marrow-derived dendritic cells and macrophages found at the cortico-medullary junction or in the medulla can induce deletion of thymocytes and clearance of apoptotic cells (17, 43). Dendritic cells are particularly efficient in mediating negative selection because they express high levels of costimulation molecules (44).

After positive selection, thymocytes migrate into the medulla where they undergo additional differentiation events as well as negative selection (45). The final steps of T cell differentiation include upregulation of CD62L, Qa-2, and β7 integrin and downregulation of CD69 and HSA (46, 47). There is also evidence that some thymocytes undergo cell division just before they exit the thymus, but the importance of this expansion is not known (48, 49). Mature thymocytes leave the thymus through either blood or lymphatic vessels located at the cortico-medullary junction (50).

The medulla is densely packed with antigen presenting cells and medullary epithelial cells, which can promote negative selection (8, 17, 43). Additional evidence that medullary epithelial cells are involved in negative selection has come from the

observation that some of these cells can express tissue specific antigens (51). Loss of the transcription factor AIRE (autoimmune regulator) results in autoimmune disorders, such as the human autoimmune polyendocrinopathy type I syndrome (52). This transcription factor regulates the expression of tissue specific proteins in medullary epithelial cells, as loss of AIRE function leads to a reduction of ectopic gene expression in these cells (52). AIRE-deficient mice also have defects in negative selection of a transgenic TCR specific for an ectopic pancreatic antigen (53). Recently, lymphotoxin signaling, which was previously found to play a role in cytokine and chemokine expression, has also been shown to regulate AIRE expression (54). These studies indicate that AIRE is involved in central tolerance by shaping the T cell repertoire and inducing deletion or anergy of autoreactive T cells in the medulla.

Models of selection

What determines the opposing outcomes of positive and negative selection is a major question in T cell development. The differential avidity model postulates that the overall strength of the interactions between thymocytes and thymic stroma determines their fate. High avidity interactions lead to negative selection and low avidity interactions lead to positive selection (30, 31, 55-57). Support for this model has come from studies in which the same peptide can support positive and negative selection (58). Low doses of a peptide can induce positive selection whereas high doses of the same peptide can induce negative selection (57, 58). Accessory molecules, which augment TCR signaling, such as the coreceptors CD4 and CD8, and costimulatory molecules, such

as ICAM and CD40, also influence the outcome of selection (17, 59). Other studies have suggested that selection outcome depends on the TCR kinetics of binding (60). The affinity of the TCR with its ligands and its disassociation rate is critical (61). For example, low affinity interactions are required for the maturation of $CD8^+$ T cells, as shown in studies of the TCR affinities required for positive and negative selection in peptide add-back experiments with mice expressing TCRs of known specificity (62, 63). A recent study has shown that the selection of $CD4^+CD25^+$ regulatory T cells requires a TCR with high affinity (64). Thus, a range of TCR binding affinities can promote the positive or negative selection of T cells.

Another model is the differential signaling model, which states that the stimuli that give rise to positive and negative selection are qualitatively different (65). The fact that cortical epithelial cells, but not medullary cells, can support positive selection indicates that unique signals provided by cortical epithelial cells are required for positive selection (17). Another possible model is that the stimuli that induce negative or positive selection differ in their ability to trigger conformational changes on TCRs, but there is limited evidence for these structural changes (65, 66).

Positive selection mediated by the mature αβTCR induces the differentiation of thymocytes, but unlike the signals mediated by the pre-TCR, signals mediated by the αβTCR are not associated with promoting cell division (1). The signals that mediate positive and negative selection are not well understood. $p56^{lck}$ and ZAP-70 are both necessary for selection as demonstrated by studies of mice deficient in these proteins (23,

67). Evidence has accumulated that differential signals mediate positive and negative selection. For example, studies have shown that signals mediated by the ras and calcineurin pathways are required for positive selection but they are not required for negative selection (68-70).

Coreceptors CD4 and CD8

Two major types of αβ T cells can be identified based on expression of the coreceptors CD4 and CD8 (71). Expression of CD4 is associated with helper T cells and expression of CD8 is associated with cytotoxic T cells. These cells differ in the types of antigen and MHC they detect as well as their regulatory and effector functions (71).

CD4 is a 55kD type I glycoprotein with four extracellular Ig homology domains (D1-D4) (Figure 2) (72, 73). Some studies have shown that CD4 can exist as a dimer (74-77). CD4 contacts the α3 and β2 domains on MHC II via its D1 and D2 domains (78, 79). Although CD4 binding to MHC II on its own is of low affinity, it can cooperate with the TCR to enhance the overall avidity of TCR-MHC interactions (80-83). The ability of CD4 to recruit $p56^{lck}$ to the TCR-MHC interface enhances TCR signaling (82, 84-90)

The structure of the coreceptor CD8 is quite different from CD4, but like CD4, it enhances TCR signaling by binding to non-polymorphic regions of MHC and recruiting $p56^{lck}$ to the TCR-MHC interface (Figure 2) (85, 86, 91-97). CD8 is encoded by two

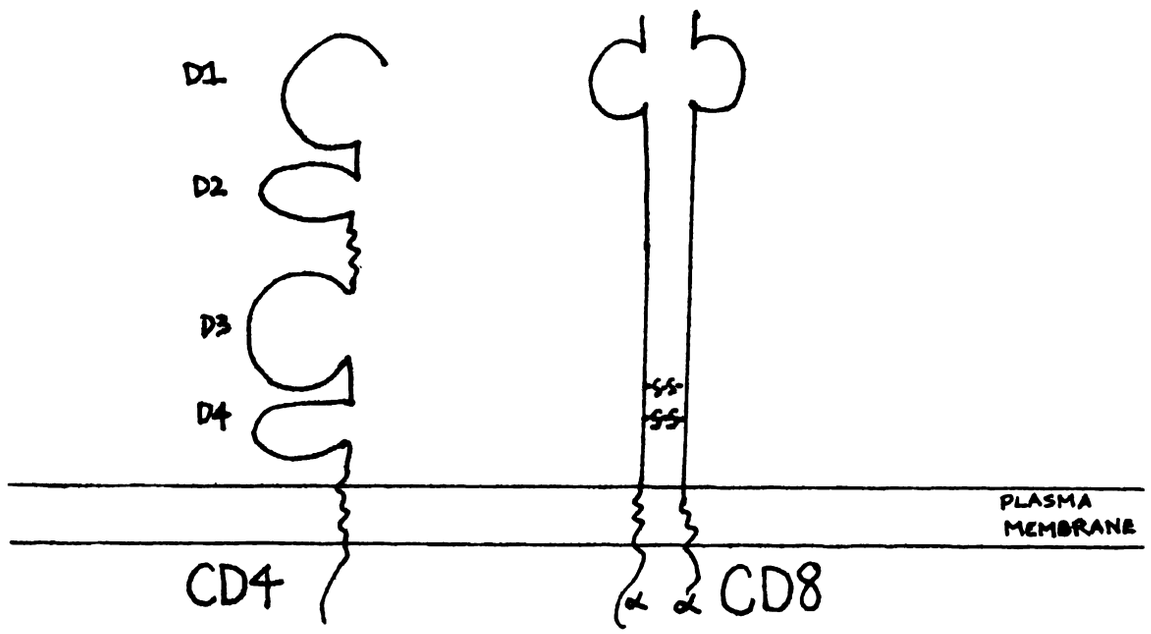


Figure 2. Schematic diagrams of CD4 and CD8 coreceptors

genes α and β and is expressed as disulfide-linked CD8αβ heterodimers and CD8αα homodimers (98-103). An alternatively spliced form of CD8α, α', which lacks the cytoplasmic domain, is expressed in mouse thymocytes (104). The *CD8α* gene also undergoes alternative splicing in humans (105, 106). In contrast to mouse CD8α', which lacks the cytoplasmic domain, human CD8α encodes a secreted form that lacks the transmembrane domain (105, 106). Surface expression of CD8β is dependent on CD8α as CDβ is retained and degraded in the endoplasmic reticulum in the absence of CD8α (99, 107, 108).

The CD8α and β polypeptides have similar structures; each polypeptide has one globular N-terminal Ig-like domain attached to a stalk region separating it from its transmembrane and cytoplasmic domains (95, 96). The cytoplasmic domain of CD8α can associate with $p56^{lck}$ (109). The cytoplasmic domain of CD8β cannot bind to $p56^{lck}$ but studies indicate that it can enhance TCR signaling through its ability to bring CD8αβ heterodimers to lipid rafts and TCR complexes (110).

The maturation of T lymphocytes is dependent on expression of the coreceptors as T cell development is impaired in mice that are deficient in coreceptor expression (111-114). This defect is incomplete in CD4-deficient mice in which a population of CD4 lineage cells can develop (112, 115). CD8β is also not completely required for development (116, 117). Additional studies have shown that interactions of coreceptors and MHC are required for efficient positive and negative selection (111, 112, 118-121). For instance, a mutation of the α3 domain of MHC I, which is recognized by CD8,

causes defects in CD8 T cell development (92, 122, 123). Analogous studies have demonstrated the importance of CD4-MHC II interactions for CD4 development (124).

Lineage commitment

Positive selection coincides with lineage commitment in which thymocytes downregulate CD4 or CD8 molecules and develop into either CD8 lineage T cells or CD4 lineage T cells, respectively (125-128). During this process of lineage commitment, thymocytes, which recognize MHC I ligands, downregulate CD4 expression and develop into $CD8^+$ T cells, while thymocytes, which recognize MHC II ligands, downregulate CD8 expression and develop into $CD4^+$ T cells (129).

Several models have been proposed to explain how DP thymocytes become committed to the CD4 or CD8 lineage (129-131). The instructive model proposes that perception of the type of MHC recognized by the TCR directs differentiation into one of the two pathways. The stochastic model postulates that DP thymocytes initially randomly downregulate expression of one of the coreceptors and only thymocytes that express TCRs and coreceptors with matching MHC specificities survive.

There has been support for both these models (1, 131). For example, a kinetic analysis of selection of CD4 and CD8 lineage thymocytes supports the instructive model (132). The stochastic model predicts that constitutive expression of a coreceptor should rescue the development of those thymocytes that have chosen to downregulate the

coreceptor that matched its TCR specificity. The demonstration that T cells with mismatched coreceptors can be found under certain experimental conditions contradicts the instructive model and supports the stochastic model (56, 128, 131, 133, 134). One caveat is that the results from these studies have been conflicting and high expression of the coreceptors is required to rescue development of those cells (56, 128, 133).

"Strength of signaling" has been also shown to influence lineage commitment, i.e. stronger TCR signals promote CD4 differentiation and weaker TCR signals promote CD8 differentiation (131). Support for this mechanism has come from the observation that $p56^{lck}$ preferentially associates with CD4, rather than CD8, so that TCR/CD4 coengagement induces stronger signals than TCR/CD8 coengagment (85, 86).

Recent evidence has emerged suggesting that it is the "duration of signaling" and not necessarily the "strength of signal" that influences lineage commitment (131, 135-138). In one study, the duration of coreceptor signaling was shown to influence lineage commitment (135). In other experiments, DP thymocytes have been shown to downregulate *CD8* gene expression and persistent TCR signaling is required for CD4 differentiation and not CD8 differentiation (136-138).

Notch proteins may also be involved in CD4 and CD8 lineage commitment (139). These proteins are important in lineage decisions in many developmental systems, such as vulval development in *C. elegans* and embryonic neurogenesis in *D. melanogaster,* as well as cell fate decisions in T cells such as the $\alpha\beta$–$\gamma\delta$ decision and Th1-Th2

differentiation (14, 140). Expression of activated Notch1 leads to an increase of CD8 lineage T cells at the expense of $CD4^+$ T cells, suggesting that Notch signaling enhances differentiation to the CD8 lineage (141). However, the precise role of Notch in lineage commitment is difficult to interpret because Notch is also involved in thymocyte survival and not only lineage choice (137, 142, 143).

Chemokines

During their development, thymocytes migrate extensively within and between thymic microenvironments, and are located in specific regions within the thymus during particular stages of development (1, 17). Positioning of thymocytes, in particular thymic regions, and therefore near particular stromal cells, is likely important for their development. For example, the selection of thymocytes may be impaired if they are not positioned near cortical epithelial cells that mediate positive selection (35, 36). The mechanisms that direct the migration and positioning of developing thymocytes are poorly understood.

Members of the chemokine family of proteins likely direct thymocyte migration. These molecules are primarily small (8-14 kD) basic proteins (144, 145). They are divided into four classes (CXC, CC, CX_3C, and C), which are defined by the structures of their conserved cysteine motifs. Chemokines bind to seven transmembrane G-coupled protein receptors and induce the directional migration of different classes of cells in the immune system. The role of chemokines is not limited to chemotaxis; they also function

in angiogenesis, proliferation, Th1/Th2 differentiation, integrin activation and adhesion, and cytokine production (144, 145).

Evidence that chemokines influence thymocyte migration includes their expression patterns within the thymus and their ability to mediate chemotaxis of various thymocyte subsets (see citations in Table 1 which summarizes some information on chemokines: their receptors, expression patterns, and possible roles in thymocyte development). Chemokines likely influence multiple steps of thymic development and migration including migration into the cortex, migration within and between compartments, and migration from the thymus into the periphery.

The entry of immature T cells into the cortex may be dependent on CXCR4. In mixed chimeras with a majority of wildtype cells, CXCR4-deficient cells fail to enter the cortex and remain near the cortico-medullary junction (147). These CXCR4-deficient cells also fail to develop past the DN stage. These results suggest that CXCR4-dependent positioning is involved in early thymocyte development.

Trafficking from the cortex to the medulla is associated with positive selection. Various chemokine receptors are upregulated by DP thymocytes after positive selection, i.e. in $CD69^{hi}$ or $CD3^{hi}$ DP thymocytes. These receptors include CCR7, the receptor for CCL19 (ELC) and CCL21 (SLC), CCR4, the receptor for CCL22 (MDC) and CCL17 (TARC), CCR8, the receptor for CCL1 (TCA-3), and CCR9, the receptor for CCL25 (TECK) (152-155, 160-162, 169). Expression data such as these raise the possibility that

Chemokine	Chemokine receptor	Expression pattern of chemokine	Expression pattern of chemokine receptor	Possible function	References
CXCL12 (SDF-1)	CXCR4	CMJ, cortex	DN	Movement into cortex	(146-151)
CCL19 (ELC)	CCR7	CMJ, medulla	$CD3^{hi}CD69^{hi}$DP SP	Movement into the medulla Thymic exit	(152-154)
CCL21 (SLC)	CCR7	medulla	$CD3^{hi}CD69^{hi}$DP SP	Movement into the medulla	(150, 152, 153)
CCL22 (MDC) CCL17 (TARC)	CCR4	medulla	$CD69^{hi}$ DP CD4 SP	Movement into the medulla	(153, 155-158)
CCL25 (TECK)	CCR9	cortex & medulla	DP CD8 SP γδ T cell	Movement in the medulla	(150, 153, 159-167)
CCL1 (TCA-3)	CCR8	not determined	DN DP CD4SP	Movement into medulla	(168-170)
CXCL10 (IP-10) CXCL9 (MIG), CXCL11 (I-TAC)	CXCR3	medulla	CD8 SP γδ T cell, NK cells $CD3^{lo}$ DP	Movement in medulla	(171)

Table 1. Expression and possible functions of chemokines in mouse T cell development are summarized in the table. CMJ: cortico-medullary junction. CXCR3 and its ligands have been characterized in human thymuses.

chemokines and their receptors may be essential for guiding cells from one thymic compartment to another which is coincident with developmental transitions. Despite these data, however, mice deficient in the expression of CCR7, CCR4, CCR8, or CCR9 do not have obvious defects at this stage of development in the thymus, although positioning has not been studied in detail in these mice (156, 166, 170, 172). It is likely that some element of redundancy is involved, as the chemokines and their receptors have overlapping expression patterns.

The chemokine CCL25 (TECK) and its receptor CCR9 may also influence the trafficking of thymocytes at other stages of development. For example, some studies have suggested that TECK recruits prothymocytes to the thymus (150, 159). Another study has shown that CCR9 is upregulated after pre-TCR signaling, suggestive of a role for CCR9 in DP thymocyte movement in the cortex (165).

There is also evidence that CD4 and CD8 SP thymocytes express different chemokine receptors and respond to different chemokines, although it is not clear how differential chemokine receptor expression and migration responses affect their development. For example, higher expression of CCR9 by CD8 SP T cells suggests that CCR9 is involved in the development or selective migration of $CD8^+$ T cells (163). In contrast to CD8 SP thymocytes, CD4 SP thymocytes have been reported to migrate in response to MDC, the ligand for CCR4, and express higher levels of the receptors CCR8. The receptors, CCR4 and CCR8, may play analogous roles in $CD4^+$ T cells as CCR9 does in $CD8^+$ T cells (158, 169).

A number of studies have shown that pertussis toxin, which inhibits $G\alpha_i$ mediated signaling, potentially disrupts the migration of thymocytes into the medulla and inhibits the exit of mature cells from the thymus to the periphery (173, 174). Because many chemokines signal through pertussis toxin sensitive G-protein coupled receptors, these studies have been used to implicate chemokines in medullary migration and thymic exit.

Consistent with the pertussis toxin experiments, one study has provided evidence that the chemokine receptor CCR7 and its ligands may influence thymic emigration (152). In this study, it was shown that treatment of fetal thymic organ cultures with CCL19 could promote the emigration of mature T cells out of the thymus. Neonatal CCR7-deficient mice and mice treated with a CCL19 antagonist showed decreased emigration from the thymus to the spleen. However, as this defect was not found in adult CCR7-deficient mice, the CCR7 contribution is not essential and may be restricted to early development or cooperates with other factors in thymic emigration.

Studies with an immunosuppressant drug FTY720, which is structurally similar to sphingosine-1-phosphate (S1P), have implicated members of the S1P receptor family in mediating thymic emigration (175-180). These G-protein coupled receptors signal through $G\alpha_i$. In these studies, mice treated with FTY720 show decreased numbers of peripheral blood lymphocytes due to a block in emigration from lymphoid organs. Phosphorylated FTY720 binds and acts on four of the five known S1P receptors. Recent studies have shown that $S1P_1$ (Edg1) affects thymic emigration (179, 180). Mice

deficient in $S1P_1$ lack peripheral T cells due to a block in the ability of mature SP thymocytes to leave the thymus. FTY720 appears to downregulate cell surface expression of $S1P_1$ (180). Therefore, expression of $S1P_1$ in SP thymocytes is crucial for their thymic emigration.

Glycosylation in T cell development

Glycosylation changes in T cells regulate aspects of their development, survival, and differentiation (3, 181, 182). These carbohydrate modifications are mediated by Golgi enzymes, such as glycosyltransferases and glycosidases, which add or remove carbohydrate residues, respectively. An N-glycan is formed by linking carbohydrates onto asparagine residues of proteins (consensus sequence is NXS or T, X cannot be proline). An O-glycan is formed on serine or threonine residues typically by the addition of N-acetylgalactosamine (GalNAc). Certain sialyltransferases in the Golgi can attach sialic acids onto Galβ1-3GalNAc-Ser/Thr, an early O-glycan core subtype called the core 1-O glycan. Other modifications on core 1 O-glycans include the addition of GlcNAc to form core 2 O-glycans (Figure 3).

The importance of glycosylation in regulating T cell function has been demonstrated in several studies (3, 181). For example, studies of mice deficient for *Mgat-5*, the gene that encodes the N-glycosylation enzyme, β1,6 N-acetylglucosaminyltransferase V (GlcNAcT-V) revealed a role for N-glycosylation in TCR activation (183). These studies showed that TCR signaling is enhanced in the

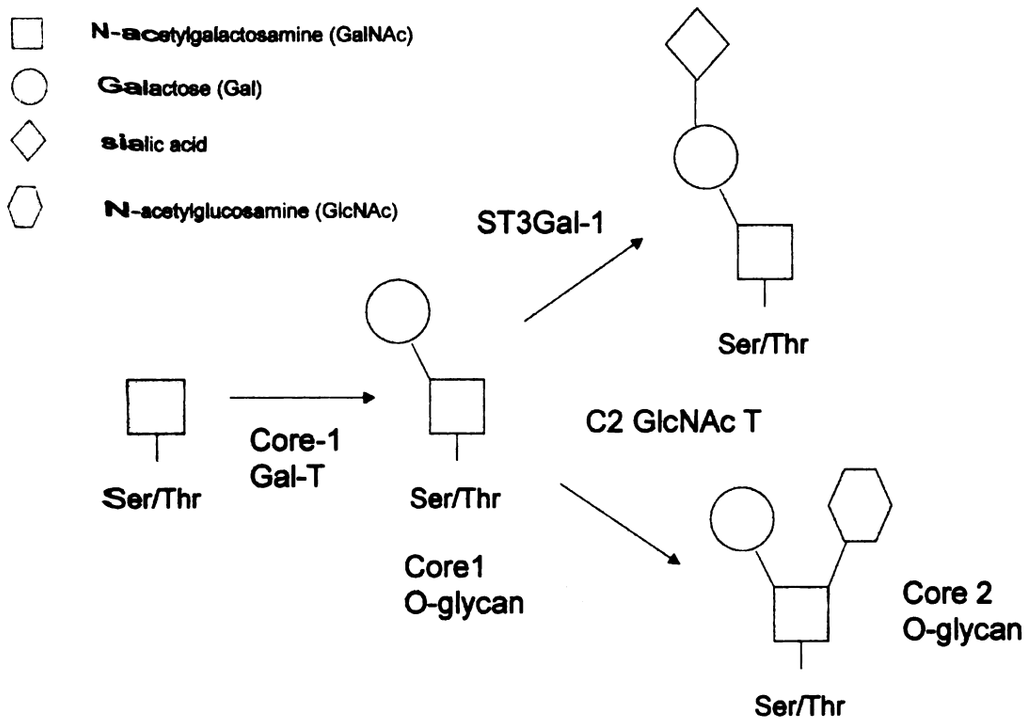


Figure 3. O-linked glycosylation pathways

Core-1 Gal-T: Core 1 Galactose transferase
Core-2 GlcNAc T: Core 2 N-acetylglucosamine transferase
ST3Gal-1: ST3Gal-1 sialyltransferase

absence of *Mgat-5* and led to a model in which the binding of galectin-3 to GlcNAcT-V dependent structures forms a lattice that restricts TCR clustering.

Another example of glycosylation affecting protein function has been shown in studies of agrin, a 400kD heavily glycosylated heparan sulfate proteoglycan. After T cell activation, an activated form of agrin is secreted (184). Glycosylation influences the formation of the activated form of agrin and changes the ability of agrin to associate with lipid rafts and enhance T cell signaling.

A highly conserved change in glycosylation is observed at the cortical-medullary boundary in the thymuses of vertebrates, including lizards, chickens, mice, rats, and humans (181). Staining with the plant lectin peanut agglutinin (PNA) is higher on cortical DP thymocytes than medullary SP thymocytes (2, 185, 186). This difference in PNA staining between DP thymocytes and SP thymocytes is regulated by a sialyltransferase, β-galactosidase α (2,3) sialyltransferase (ST3Gal-1). This enzyme attaches sialic acid onto Galβ1-3GalNAc-Ser/Thr core 1 O-glycans in an α2-3 linkage to generate Siaα2-3Galβ1-3GalNAc-Ser/Thr (2, 181). ST3Gal-1 expression is downregulated in DP thymocytes and upregulated in SP thymocytes. As a result, PNA, which binds more efficiently to unsialylated core 1 O-glycans, stains DP thymocytes at higher levels than SP thymocytes. The selective downregulation of ST3Gal-1 at the DP stage raises the possibility that hyposialylation of DP glycoproteins may be important for T cell development

Major ST3Gal-1 substrates include the receptor-like protein tyrosine phosphatase CD45, the mucin-like molecule CD43, and the coreceptor CD8 (3, 187-189). Glycosylation and sialylation patterns change on these molecules and presumably other substrates during T cell development and activation, and in some cases can affect their function (3). For instance, sialylation increases the negative charge on proteins leading to conformational changes and disruption of protein-protein interactions (190, 191).

CD45 is a phosphatase that removes inhibitory phosphates on tyrosine residues of src family kinases $p56^{lck}$ and $p59^{fyn}$ and enhances TCR signaling. T cells express various isoforms of CD45, which differ in size, charge, extent of glycosylation, and the ability to dimerize. CD45RO isoforms dimerize more efficiently than more heavily glycosylated isoforms such as CD45RA (192, 193). Removal of sialic acids or inhibition of O-glycosylation on CD45RA isoforms enhances their dimerization. Therefore, glycosylation and sialylation influence the ability of CD45 to dimerize and can alter TCR signaling.

CD43, another major substrate of ST3Gal-1, is expressed at high levels on T cells. During T cell activation, core 1 O-glycans on CD43 become desialylated and core 2 O-glycans are induced (194). Although CD43 has been implicated in regulating T cell responses, apoptosis, and cell adhesion, it appears to be dispensible for normal T cell development (194-197). The significance of glycosylation and sialylation on CD43 is not clear.

Glycosylation and sialylation patterns also change on CD8 during T cell development and activation, and may regulate CD8 function (198, 199). Studies of noncognate MHC I tetramer binding (i.e. non-TCR dependent) on mouse thymocytes have shown that sialylation influences the ability of CD8 to interact with MHC I tetramers (198, 199). DP thymocytes bind to MHC I tetramers more efficiently than SP thymocytes and mature T cells. Neuraminidase treatment, which cleaves sialic acids on oligosaccharides, partially enhances binding to MHC I tetramers. These results suggest that downregulation of ST3Gal-1 in DP thymocytes enhances the ability of CD8 to bind to MHC I. Conversely, upregulation of ST3Gal-1 on SP thymocytes, which increases sialylation levels on cell surface proteins, would inhibit CD8 function.

ST3Gal-1-deficient thymuses have an altered TCR repertoire and defects in mature $CD8^+$ T cell development (194, 199). These defects in $CD8^+$ T cell development are consistent with defects in selection, but it is not clear which glycoproteins are responsible for the abnormalities. The number of peripheral $CD8^+$ T cells was also reduced in ST3Gal-1-deficient mice (194). The survival of naïve and memory $CD8^+$ T cells is dependent on ST3Gal-1 function, although the mechanism for the elimination of these $CD8^+$ T cells is unknown. Additional work to determine the importance of regulated expression of ST3Gal-1 will clarify its role in T cell development and function.

Objectives of this thesis

Thymic development is a compartmentalized process with thymocytes positioned in particular areas of the thymus. The factors that influence the positioning and the relationship between positioning and development are poorly understood. Positioning of thymocytes is likely important for proper development since interactions of thymocytes with stromal cells mediate their survival, selection, and differentiation. The work described in this thesis shows that CCR7, a chemokine receptor which is upregulated after positive selection, promotes thymocyte migration from the cortex to the medulla and disrupts the thymic architecture. Premature migration is associated with defects in positive selection of both CD4 and CD8 SP thymocytes. These studies show the importance of positioning for development.

We have also studied the consequences of aberrant expression of ST3 Gal-1 sialyltransferase in DP thymocytes. Expression of ST3Gal-1 is normally downregulated in DP thymocytes and upregulated in SP thymocytes. We have found that T cell development is not affected by increased sialylation in DP thymocytes. Increased expression of ST3Gal-1 in DP thymocytes also does not affect CD8-MHC I interactions. These results indicate that increased sialylation in thymocytes does not change their development or alter CD8 function.

Chapter 2: CCR7 Directs the Migration of Thymocytes into the Thymic Medulla

Abstract

Developing thymocytes migrate from the cortex to the medulla of the thymus as a consequence of positive selection. This migration is likely to be essential for tolerance because it allows the developing cells to move into an environment that is optimal for negative selection. Guidance mechanisms that draw positively selected thymocytes into the medulla have not been clarified, but several studies have implicated chemokines in the process. CCR7, the receptor for the medullary chemokines CCL19 and CCL21, is induced on thymocytes during their positive selection. Here we show that premature expression of CCR7 repositions $CD4^+CD8^+$ double-positive cells into the medulla of transgenic mice. This repositioning of the thymocytes is accompanied by impairment of their development. The data show the involvement of CCR7 in medullary migration and emphasize the importance of proper thymocyte positioning for efficient T cell development.

Introduction

Small $CD4^+CD8^+$ (double-positive) cells are the most numerous hematopoietic cell-type in the thymus (1). These short-lived cells are packed densely into the cortex of the thymus where they will either die by apoptosis or receive signals that induce them to develop further. The few cells that differentiate into $CD4^+CD8^-$ or $CD4^-CD8^+$ (single-positive) cells subsequently complete their development in the thymic medulla. Both the cortex and the medulla are comprised of complex networks of epithelial, mesenchymal and hematopoietic cells (8, 200). The division of the thymus into cortical and medullary regions is perhaps its most striking histological characteristic, yet comparatively little is known about how these regions are formed, or how T cell precursors are induced to move between them during their development.

Blood-borne hematopoietic precursor cells enter the thymus from vessels that are located deep in its cortex near the boundary with the medulla (10, 11). The cells proliferate on entry and gradually migrate towards the outer regions of the cortex. As they move, they eventually cease dividing, express the RAG-1 and RAG-2 proteins and undergo VDJ rearrangements at the *Tcrβ*, *Tcrγ* and/or *Tcrδ* loci. Cells that complete productive *Tcrβ* rearrangements can express TCRβ proteins and will display these on their surfaces in precursor-T cell receptor (pre-TCR) complexes (1). Signals from the pre-TCRs then induce the cells to reenter the cell cycle, and ultimately to reverse their migration back towards the medulla while they differentiate into double-positive cells (10–12). After a rapid series of five or more divisions, the cells again cease proliferating

and re-express RAG-1 and RAG-2 prior to rearranging the *Tcrα* locus. Productive rearrangements this time permit the expression of αβ TCR complexes, which are capable of delivering signals to the cells when they engage peptide/MHC complexes (1).

Although the majority of double-positive cells die because they fail to receive the correct form of signal from the αβ TCRs they express, a minor fraction of them receive signals that allow for differentiation into single-positive cells. These cells migrate into the medulla as they downregulate RAG-1 and RAG-2, and complete the changes in gene expression that define the CD4 or CD8 lineages (1). Interestingly, single-positive cells can reside in the medulla for two-to-three weeks – a period of time that may be essential for the completion of negative selection (45). At the end of this time, medullary single-positive cells leave the thymus, at least in part via the blood (152).

Chemokines are small diffusible or matrix-associated proteins that transmit chemotactic signals into cells through pertussis-toxin-sensitive G protein-coupled receptors (144, 145). Just as they have proved essential for lymphocyte migration in secondary lymphoid tissue, there are several indications that chemokines are important for the migration of cells within and from the thymus. Most notably, a number of studies have shown that pertussis toxin can impede the migration of single-positive cells into the medulla and prevent the exit of mature cells from the thymus to the periphery (173, 174). There are also thymic abnormalities in mice lacking $G\alpha_I$ (201, 202). In addition to these functional observations, the expression patterns of chemokines and their receptors are highly suggestive of potential roles in guiding cells in the thymus. Thus, passage through

the TCRβ and TCRα developmental checkpoints is associated with the upregulation of distinct chemokine receptors, the ligands for which are regionally localized in the thymus. For example, pre-TCR signaling leads to increased CCR9 expression, which would allow the cells to migrate towards CCL25 (thymus-expressed chemokine) (161, 162, 165, 166). Similarly, CCR4, CCR7 and CCR8 are all upregulated during positive-selection, and would permit migration towards medullary CCL22 (macrophage-derived chemokine) or CCL17 (thymus and activation-regulated chemokine), CCL19 (EBI1-ligand chemokine, macrophage inflammatory protein-3β, and Exodus-3)/21 (secondary lymphoid tissue chemokine, 6-C-kine, Exodus-2, and thymus derived chemotactic agent 4), and CCL1 (T cell activation gene-3) respectively (150, 152, 153, 169, 203).

Notwithstanding their suggestive expression patterns, however, evidence for the involvement of specific chemokines in thymocyte migration *in vivo* has so far been limited. Mutant mice deficient in the expression of individual chemokines or their receptors generally show well defined cortical and medullary regions with little evidence for defects in the positioning of thymocytes in them (145). An exception to this is the recent demonstration that thymus-specific deletion of CXCR4 can impair the migration of double-negative thymocytes into the cortex (147). Interestingly, the defect in cortical migration is associated with impaired thymocyte development, suggesting that proper positioning may be essential for cells to receive the developmental cues they would normally require. Nonetheless, thymocytes can still complete their development without CXCR4, albeit at reduced efficiency compared to wild-type cells (147-149). Similarly, mice lacking CCR7 support largely normal thymocyte development, yet these mice show

a defect in the emigration of mature cells from the neonatal thymus (152). Although it remains to be tested, an attractive hypothesis to reconcile the pertussis toxin, expression pattern, and genetic data is that multiple chemokines and their receptors are involved in positioning thymocytes, and that there is at least some redundancy in the positioning process at different thymocyte developmental stages.

As one means to examine the specific roles of CCR7 in thymocyte migration, we generated transgenic mice that express CCR7 prematurely. CCR7 is normally induced during positive selection, so it seemed reasonable that this receptor might be important for directing the migration of cells from the cortex to the medulla in response to the medullary chemokines CCL19 and CCL21 (153, 154). Our expectation was that premature expression of CCR7 might be sufficient to cause a redistribution of double-positive thymocytes and perhaps result in their inappropriate entrance into the medulla before positive selection had occurred. Here we report the results of these experiments and identify CCR7 as a participant in the directed migration of thymocytes that accompanies positive selection.

Materials and Methods

Mice, antibodies, and flow cytometry

A cDNA encoding a Flag-tagged form of mouse CCR7, kindly provided by Drs. Vu Ngo and Jason Cyster (University of California, San Francisco; the FLAG epitope is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (154), was cloned into untranslated sequence in the second exon of a mouse CD4 minigene that lacks the intronic transcriptional silencer (204). The resulting construct was linearized and injected into C57BL/6xDBA/2 F2 embryos. Founder transgenic CCR7 transgenic mice were identified by PCR, Southern blot and flow cytometry, and these were then crossed to C57BL/6 (B6) mice for 5-6 generations. HY-specific B6.2.16 αβ TCR transgenic mice and *plt/plt* mice were kindly provided by Dr. Ellen Robey (UC Berkeley) and Dr. Jason Cyster (UCSF), respectively. CD45 congenic (B6.Ly5.2) mice were obtained from the National Cancer Institute Developmental Therapeutics Program. All experiments were performed according to protocols approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Antibodies were purchased from BD Biosciences (CD4, CD8, CD3ε, CD69, Ly5.1), Caltag Laboratories (CD4, CD8), and Sigma-Aldrich (M2-Flag). FITC-conjugated peanut agglutinin was purchased from Vector Laboratories. The CCL19-Fc protein and human LFA3-Fc have been described (205). The T3.70 antibody specific for

the B6.2.16 TCR (206) was purified from hybridoma supernatant fluid by protein G affinity chromatography (Amersham).

For flow cytometry, single cell suspensions were prepared from thymuses, spleens, and lymph nodes using cell strainers (Falcon) and PBS containing BSA (0.3%w/v) and 1.54 mM NaN_3. The cells were washed two or more times and then incubated with antibodies for 30 minutes on ice. The cells were washed a further two times before analysis or prior to incubation with secondary reagents.

Chemotaxis assays

Chemotaxis assays were performed in RPMI 1640 containing 10% fetal bovine serum as described (153). 10 x 10^6 thymocytes in 0.1 ml of medium were placed in each of the upper compartments of transwell chambers containing polycarbonate membrane (5 μm pore size, Corning Costar Corp). Variable concentrations of chemokines (CCL19 or CCL21, R&D Systems) in 0.5 ml of medium were placed in the bottom wells of the chambers. After 3 hours incubation at 37°C, cells were recovered from the bottom chambers, counted and analyzed by flow cytometry.

Immunohistochemistry and immunofluorescence

Paraffin sections (3-5 μm) were stained with hematoxylin and eosin and prepared by Biopathological Sciences Medical Corporation. Frozen sections (6 μm) were fixed

with acetone, blocked with 0.1% BSA in TBS and stained with biotin-labeled PNA (Vector Laboratories), MTS10 (BD Pharmingen), or CDR1 (ATCC). Horseradish Peroxidase (HRP)-labeled streptavidin (Jackson ImmunoResearch Laboratories), HRP conjugated anti–rat IgM (Southern Biotech), or HRP-conjugated and alkaline phosphatase-conjugated anti-rat IgG antibodies (Jackson Laboratories) and 3,3'-diamino-benzidine (Sigma-Aldrich) or Fast red (Sigma Aldrich) were subsequently added. After washing with Tris buffered saline and water, sections were counterstained with hematoxylin Gill's Formulation #1 (Fisher Scientific).

For immunofluorescence, frozen sections (6 μm) were blocked with 5% dry milk in PBS and stained with FITC-labeled PNA (Vector Laboratories), biotin-labeled anti-Ly5.1 antibody (BD Biosciences) and Cy3-labeled streptavidin (Jackson ImmunoResearch Laboratories) in 0.1%BSA/1% normal mouse serum/PBS. Sections were examined with a Leica DML fluorescence microscope or a Carl Zeiss LSM 510 confocal microscope.

Cell transfers

Bone marrow cells were collected from wildtype B6.Ly5.2/Cr and Ly5.1-expressing CCR7 transgenic or non-transgenic littermates using Iscove's modified Dulbecco's medium containing 15% fetal calf serum. The CCR7 transgenic or non-transgenic cells were mixed with B6.Ly5.2 cells in various ratios ranging from 1:19 to 1 : 1 and a total of 5 x10^6 cells were injected into each lateral tail vein of lethally irradiated

(2 x 550 rads, 3 hours apart) 6-7 week old B6.Ly5.2 mice. The recipients received water containing 1.1% neomycin (Sigma-Aldrich) and 850 U/ml polymixin B sulfate (Paddock Laboratories) for 6 weeks.

BrdU labeling of thymocytes

8 week old mice received one intraperitoneal injection of 1.8 mg of BrdU (Sigma) dissolved in 200 µl water and were thereafter provided with BrdU (0.8mg/ml) and 1% glucose in their drinking water for up to 8 days. Thymuses were harvested at various time points and stained for BrdU incorporation. BrdU detection was performed as previously described, with some modifications (207). Briefly, 2 x 10^6 thymocytes were stained with antibodies specific for cell surface antigens. After washing twice with PBS, the cells were fixed for 30 minutes in 2% paraformaldehyde and subsequently permeabilized with 0.1% NP-40 for 30 minutes. After this step, the cells were pelleted, incubated with 50 KU of DNAse I (Sigma-Aldrich) for 30 minutes at 25°C, washed with PBS, and then stained with FITC-conjugated anti-BrdU antibody (BD Biosciences) for 30 minutes.

Intrathymic injections

5-6 week old mice were anesthetized with avertin (tribromoethanol, Sigma-Aldrich). An incision was made in the sternum to reveal the thymus and 20 µl of a FITC (Molecular Probes) solution (1 mg/ml) in PBS was injected into each thymic lobe. Spleen and mesenteric lymph node cells from mice with equivalent levels of FITC

labeling in the thymus were analyzed by flow cytometry 16 hours after injection for the presence of FITC-labeled recent thymic emigrants.

Results

Expression and function of CCR7 on thymocytes from transgenic mice

To express CCR7 on immature thymocytes, we generated transgenic mice in which expression of the chemokine receptor was controlled by regulatory elements from the mouse *Cd4* gene (204). A cDNA encoding a FLAG-tagged form of CCR7 (154) was embedded in a *Cd4* minigene construct that lacked the transcriptional silencer normally present in the first intron of the *Cd4* gene. CCR7 expression on cells in these mice could be detected either with an antibody specific for the FLAG epitope, or with a CCL19-Fc fusion protein (205). As shown in Figure 4A and 4B, all transgenic thymocyte subsets expressed CCR7, whereas the endogenous protein was expressed on post-positive selection double-positive thymocytes (i.e. $CD69^{hi}CD4^{+}CD8^{+}$ cells), $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ single-positive thymocytes. Consistent with the expression pattern, both immature and mature thymocytes from the transgenic mice migrated toward a source of CCL19 or CCL21 in transwell chemotaxis assays (Figure 4C) with double-negative cells demonstrating slightly enhanced migration relative to double-positive cells. In contrast, only the mature cells from non-transgenic mice demonstrated CCL19- or CCL21-dependent chemotaxis. These data showed that expression of the transgene-derived CCR7 was sufficient to render immature thymocytes responsive to chemokines that would normally only induce the migration of $CD69^{hi}CD4^{+}CD8^{+}$ double-positive cells and mature single-positive cells (153, 154).

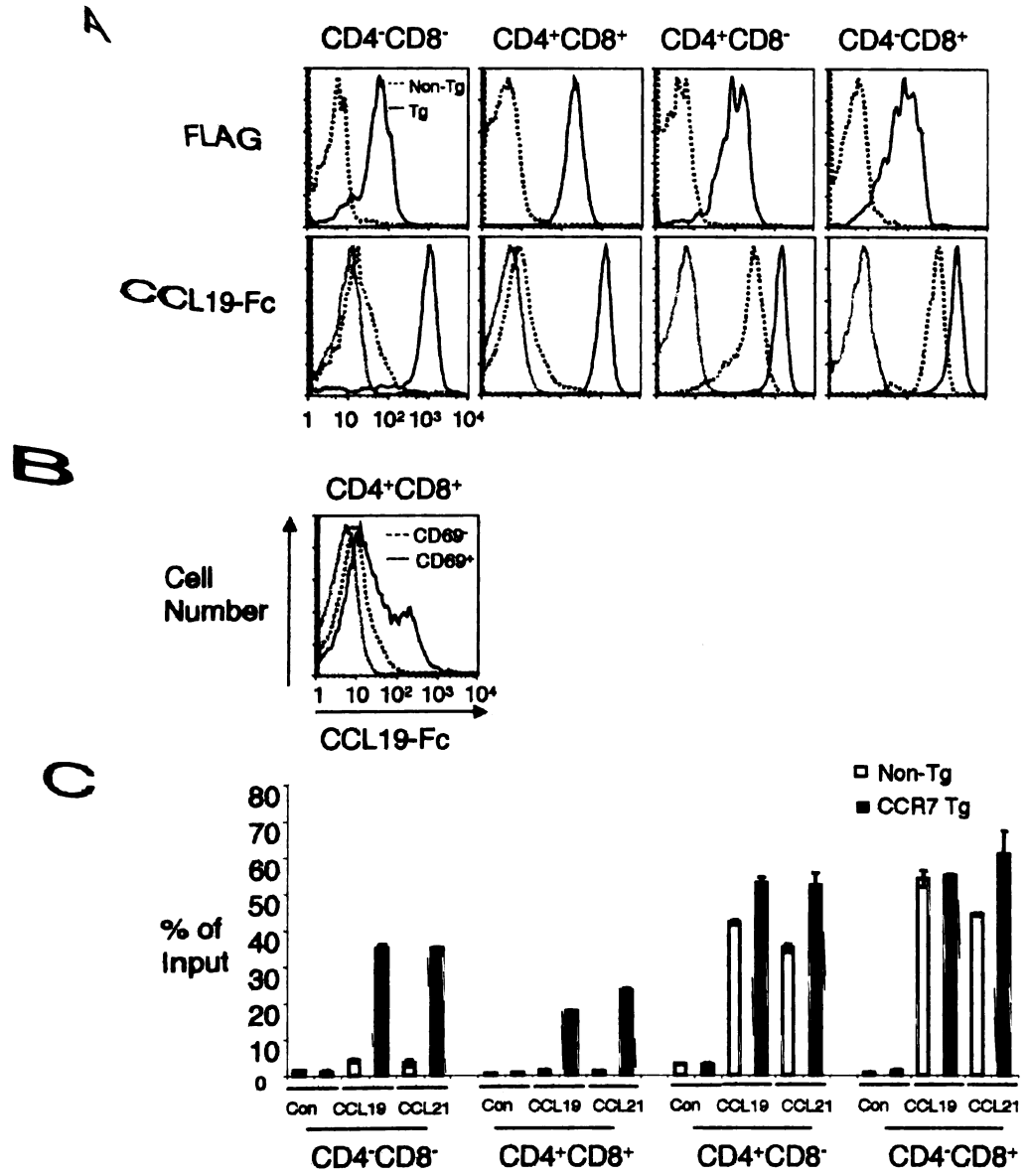
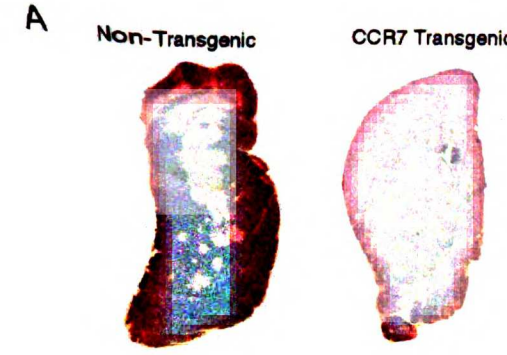


Figure 4. Expression and function of CCR7 on thymocytes from transgenic mice (*A*) Thymocytes from CCR7 transgenic (solid line) and non-transgenic littermate mice (dashed line) were stained with an antibody specific for the FLAG epitope (upper panel) that detects the transgene-encoded protein, or a CCL19-Fc fusion protein (lower panel) that detects both transgene-encoded and endogenous CCR7. The shaded curve shows control staining with human LFA3-Fc. (*B*) The histogram shows binding of CCL19-Fc to $CD69^+CD4^+CD8^+$ (solid line) or $CD69^-CD4^+CD8^+$ (dashed line) cells. The shaded curve shows control staining with human LFA3-Fc. (*C*) CCR7 transgenic and non-transgenic thymocytes were placed in the upper compartments of transwell chambers containing 500 ng/ml CCL19, 500 ng/ml CCL21 or no chemokine in the lower compartment. Cells that had migrated into the lower chamber were detected by flow cytometry after 3 hours. The data shown are representative of 3 independent experiments.

CCR7 expression induces the movement of double-positive cells into the thymic medulla

The cortical and medullary regions of wild-type mice can be readily distinguished from one another in fixed sections of thymus tissue stained with hematoxylin and eosin. In the representative image shown in Figure 5A, the medulla can be seen as a large central and mostly contiguous lightly stained area. Such medullary areas were more difficult to identify in sections of CCR7 transgenic thymuses, which typically showed much smaller islands of weak staining amidst an expanded area of dense staining. Specifically, compared to wild-type thymuses, the CCR7 transgenic mice had a 4-fold increase in areas of weak staining, which together accounted for 2 to 3-fold less surface area (Figure 5B).

The reduction in the weakly stained areas visible in hematoxylin/eosin stained sections could represent either a compression of the medullary space, or an invasion of the medulla by double-positive thymocytes brought on by CCR7-dependent chemotaxis. To resolve between these possibilities, we stained serial sections of thymuses from the two types of mice with two monoclonal antibodies that distinguish cortical from medullary epithelium. We also stained the sections with peanut agglutinin (PNA), a lectin that binds to desialylated O-linked glycans, which are much more abundant on double-positive than on single-positive thymocytes. As shown in Figure 5C, CCR7 transgenic and wild-type thymuses contained similar masses of cells that stained with MTS 10, an antibody which is specific for medullary epithelium (208). These same medullary masses could be identified in the serial sections by their absence of staining



B

	Number of Low Density Regions	Surface Area of Low Density Regions (% of Total Surface Area)
Non-Tg	11±2	20.7±1.7
CCR7 Tg	43±5	7.6±2.4

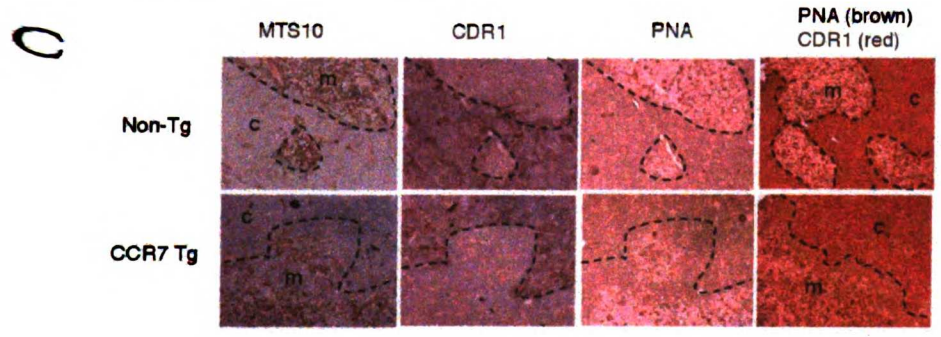


Figure 5. Abnormal architecture of CCR7 transgenic thymuses
(*A*) Paraffin sections of CCR7 transgenic and non-transgenic thymuses were stained with hematoxylin and eosin. Images were taken at 4x magnification. The data shown are representative of sections from 3 or more tissue blocks. (*B*) The table shows the mean number of low-density regions (*i.e.*, weakly stained regions) and the total surface area they occupied in thymic sections. Means were calculated from the analysis of 8 sections in each case. The difference in surface area of low density regions was determined to be significant using the Student's *t* test ($p<0.0001$). (*C*) Adjacent sections of CCR7 transgenic and nontransgenic thymuses were stained with MTS10, CDR1, or PNA (brown) and counterstained with hematoxylin. Alternatively, different sections (far right) were double-stained with PNA (brown) and CDR1 (red). Note that in addition to staining cortical double-positive cells, PNA also normally stains elements of the medullary stroma, but these stromal elements can be readily discriminated because of their large size and the fact that they stain more intensely than the thymocytes. The dashed lines correspond to the medullary and cortical regions defined by CDR1 staining. m: medulla; c: cortex. Images were taken at 50x magnification.

with CDR1, an antibody which binds to cortical epithelium (209). Strikingly, whereas PNA+ cells were largely excluded from the wild-type medullary areas identified by absence of CDR1, they were clearly present in the transgenic equivalents. This presence of CCR7 transgenic (but not nontransgenic) PNA+ cells in the medullae was apparent both from the analysis of serial sections, and also from images of double-stained sections (Figure 5C, far right) in which the brown wash of PNA-stained cells characteristically extended well beyond the red CDR1+ cortical areas. These data therefore indicate that the transgenic thymuses have similar areas of medullary epithelium to those of wild-type mice, with the striking difference being that PNA+ double-positive cells are present in the former but not the latter. Thus, the premature expression of CCR7 leads to invasion of the medulla by double-positive cells.

As an additional means to visualize whether premature CCR7 expression could induce double-positive thymocytes to migrate into the medulla, we performed adoptive transfer experiments that would allow for the relative locations of transgenic and non-transgenic cells to be compared within individual thymuses. Lethally irradiated B6.Ly5.2 mice were reconstituted with B6.Ly5.2 bone-marrow cells mixed with variable percentages of transgenic or non-transgenic Ly5.1$^+$ bone-marrow cells. The recipients were then aged for six weeks to allow for repopulation of the thymus with cells from the injected bone marrow. Sections of thymuses from the aged chimeras were stained with PNA to detect double-positive cells, together with an antibody specific for Ly5.1 to distinguish between the transgenic and non-transgenic cells. Representative images from chimeras containing 5% Ly5.1 marked transgenic or non-transgenic cells are shown in

Figure 6. In marked contrast to their non-transgenic counterparts, the transgenic PNA^+ immature cells were preferentially localized to the medulla and the cortico-medullary junction of the thymus. These experiments, therefore, provide additional evidence that CCR7 expression is sufficient to relocate immature cells from the cortex to the medulla, and suggest that the normal induction of CCR7 during positive selection is important for the migration of positively-selected cells into the medulla.

Premature expression of CCR7 impairs the development of single-positive thymocytes

The presence of double-positive cells in the thymic medulla raised the possibility that early expression of CCR7 might affect the development of thymocytes. This seemed reasonable given the demonstrated importance of cortical epithelium in positive selection and the medulla in negative selection (1). Thus, inappropriate apposition of double-positive thymocytes with medullary epithelium might be expected either to impair positive selection or perhaps accentuate negative selection.

Using flow cytometry, we saw no effect of premature CCR7 expression on early thymocyte development (as assessed by CD44 and CD25 expression on double-negative cells) and there was no obvious decrease in the average number of double-positive cells present in the transgenic thymuses. We also found that double-positive cells were not enriched for annexin V^+ cells relative to wild-type controls. Thus, the mislocalization of cells had little detectable impact on development prior to positive selection, and it likewise did not appear to induce an increase in apoptosis of double-positive cells. There

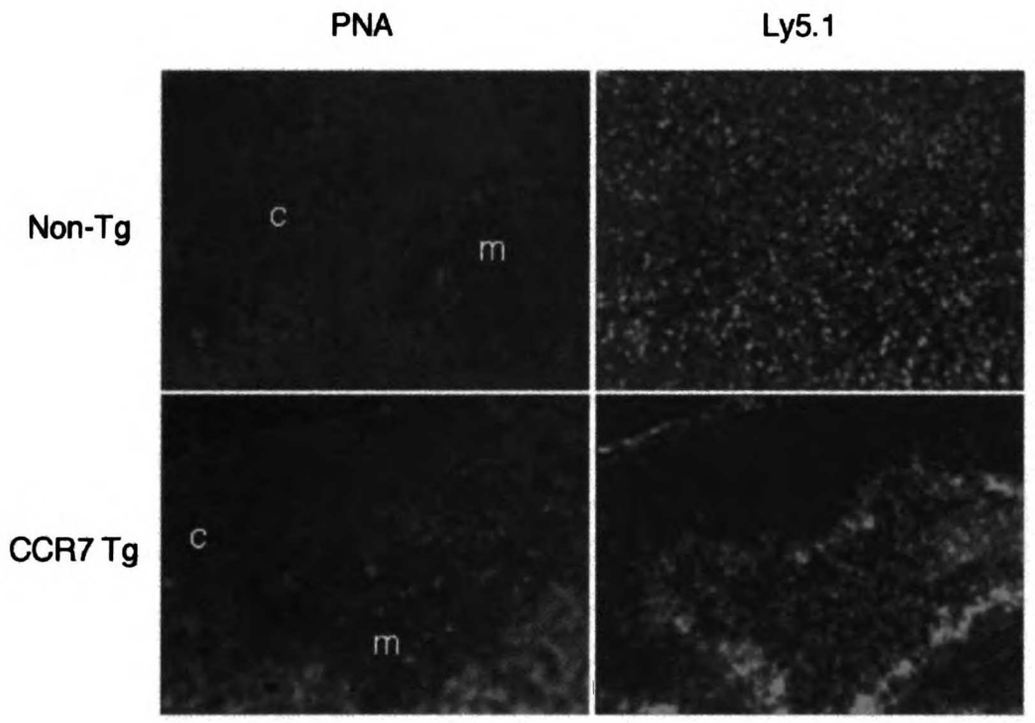


Figure 6. CCR7 expression induces movement of double-positive cells into the thymic medulla

Lethally-irradiated mice were reconstituted six weeks before analysis with mixtures of wild-type and allotype-marked ($Ly5.1^+$) transgenic or non-transgenic cells. Thymic sections were stained with PNA and an antibody specific for Ly5.1. Representative sections from chimeras reconstituted with 95% $Ly5.1^-$ and 5% $Ly5.1^+$ cells are shown at 50x magnification. c: cortex; m: medulla.

was, however, an obvious effect of the transgene on the representation of $CD4^+CD8^-$ single-positive cells in the thymus. Most notably, we observed a two-fold reduction in the percentage of $CD4^+CD8^-$ cells relative to other cells in the thymus (Figure 7A and 7B). Since the total cellularity of the thymus was unchanged by the presence of the transgene, there was a commensurate decrease in the absolute number of the $CD4^+CD8^-$ cells per thymus. Reduced numbers of single-positive cells were apparent in the fetal and neonatal transgenic thymuses, and this developmental effect was also reflected in the reduced numbers of $CD4^+$ T cells that were present in the lymph nodes and spleens of young adult transgenic mice (Figure 8 and data not shown). Despite the developmental abnormalities, however, the $CD4^+$ T cells that emerged from the transgenic thymuses retained the capacity to mediate immune responses, as shown by a test of delayed type hypersensitivity (Figure 9 and data not shown).

The frequency of $CD4^-CD8^+$ cells was not obviously different in transgenic compared to non-transgenic mice. We, therefore, crossed the CCR7 transgenic mouse to a mouse expressing an MHC class I-restricted TCR transgene so that selection of the $CD4^-CD8^+$ T cells could be examined with greater sensitivity. The TCR transgenic mouse we chose for this purpose encoded the well-characterized B6.2.16 $\alpha\beta$ TCR specific for the H-$2D^b$-restricted H-Y antigen from the *Smcy* gene (210). Most thymocytes expressing this TCR are deleted at the double-negative stage of development or just after it in male H-2^b thymuses (211), but they are efficiently selected into the CD8 lineage in female thymuses (206). We observed no effect of premature CCR7 expression on the deletion of H-Y-specific thymocytes (data not shown), but we consistently

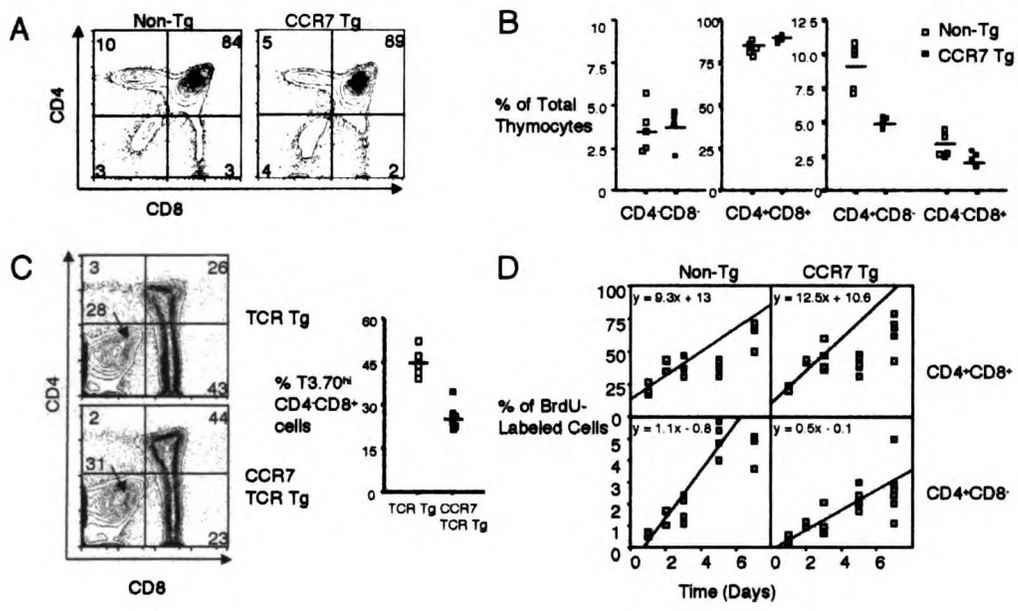


Figure 7. Premature expression of CCR7 impairs the development of single-positive thymocytes

(*A*) Representative flow cytometric data showing decreased numbers of $CD4^+CD8^-$ thymocytes in a CCR7 transgenic mouse compared to a nontransgenic control mouse. (*B*) Scatter plots showing the relative percentages of thymocyte subsets identified by CD4 and CD8 expression from 7 week-old mice. The absolute numbers of thymocytes for transgenic and non-transgenic mice were 89±11 x 10^6 and 82±17 x 10^6 respectively. The difference in percentages of $CD4^+$ $CD8^-$ cells was determined to be significant using the Student's *t* test ($p<0.002$). (*C*) Impaired selection of clonotype-expressing $CD4^-CD8^+$ thymocytes in mice transgenic for both CCR7 and the H-Y-specific B6.2.16 αβ TCR. The contour plots show the expression of CD4 and CD8 on thymocytes expressing high levels of the αβ TCR clonotype identified with the T3.70 antibody. The scatter plot shows the frequency of $T3.70^{hi}CD4^-CD8^+$ cells in the two types of TCR transgenic thymuses. The percentages of $CD4^-CD8^+$ T cells were found to be significantly different using the Student's *t* test ($p<0.001$). (*D*) The kinetics of formation of $CD4^+CD8^-$ cells in CCR7 transgenic mice were examined by continuous *in vivo* bromo-deoxyuridine labeling followed by flow cytometry. The relative frequencies of bromo-deoxyuridine labeled cells are plotted for $CD4^+CD8^+$ (upper panel) and $CD4^+CD8^-$ (lower panel) cells. In each case, the curves were generated using three data points, and the slopes were estimated using the Ordinary Least Square Method. The slopes for formation of $CD4^+CD8^-$ cells (lower panel), were significantly different when compared using an F test ($p<0.05$).

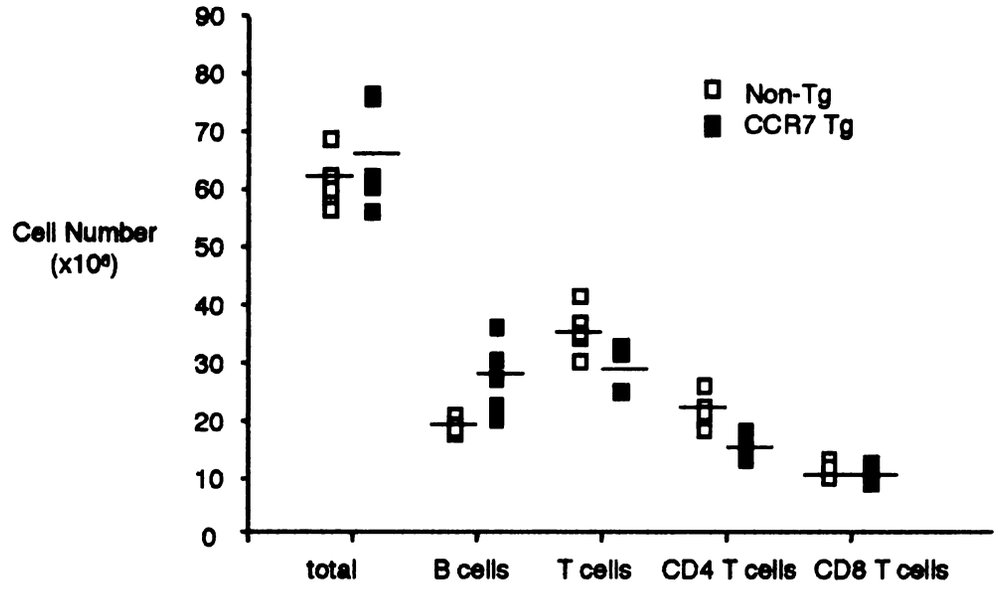


Figure 8. Numbers of total splenocytes, B220$^+$ B cells, CD3$^+$ T cells, CD4$^+$, and CD8$^+$ T cells were enumerated

The total numbers of splenocytes from non-transgenic and CCR7 transgenic mice were equivalent. Increased numbers of B cells and decreased numbers of CD4$^+$ T cells were found in transgenic mice, and the ratio of CD4/CD8 (1.4±0.1) in transgenic mice was decreased compared to non-transgenic mice (1.9±0.1). 5 mice (7 weeks old) were analyzed per group

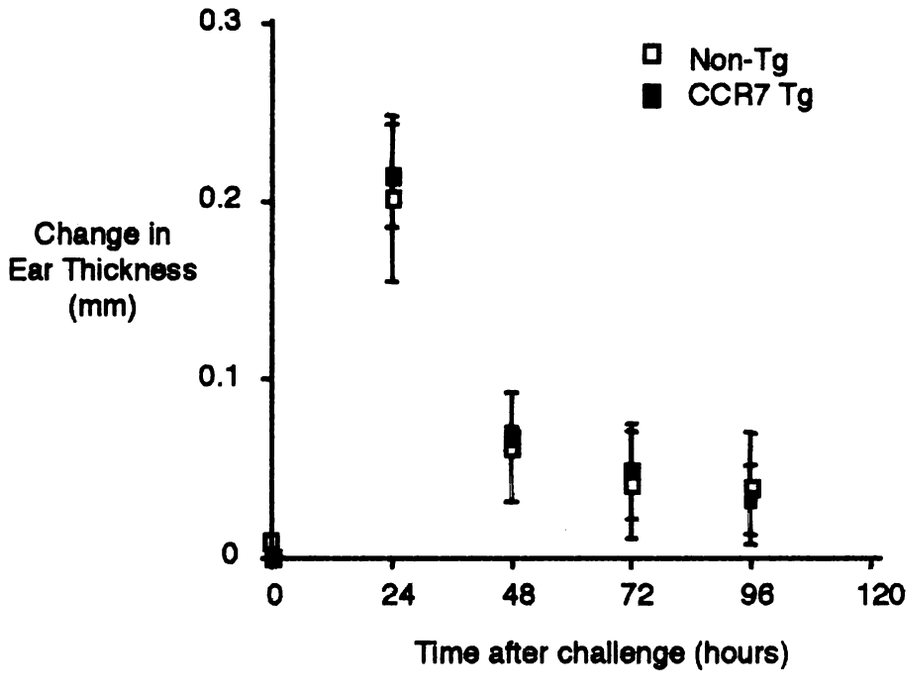


Figure 9. Transgenic T cells mediate delayed type-hypersensitivity (DTH) response to oxazolone (4-ethoxymethylene-2 phenyl-2-oxazolin-5-one)
DTH response was quantitated with a standard ear swelling assay. Mice were sensitized by the epicutaneous application of 50 μl of 1% oxazolone in 4:1 acetone/sesame oil onto their shaved left flanks on two consecutive days. On the 5th day after initial sensitization, mice were challenged by the application of 10 μl of 1% oxazolone in 4:1 acetone/sesame oil (20 μl total) on each side of their left ears. The change in ear thickness was calculated by subtracting the baseline ear thickness measured before challenge from the ear thickness measured at various times after challenge. Maximal swelling was obtained at 24 hours.

observed a reduced frequency of $CD4^-CD8^+$ single-positive cells in the double-transgenic female thymuses. More strikingly, we found that compared to TCR transgenic mice lacking the CCR7 transgene, the double-transgenic thymuses contained fewer $CD4^-CD8^+$ cells that stained brightly with the T3.70 anti-TCR clonotype monoclonal antibody (Figure 7C). This phenotype suggests that premature expression of CCR7 compromised the selection of clonotype-bearing cells such that cells employing endogenously encoded TCRα chains were at a selective advantage. Cumulatively, the data indicate that, as seen for $CD4^+CD8^-$ cells, the presence of the CCR7 transgene, and the mislocalization it induced, interfered with the positive selection of $CD4^-CD8^+$ thymocytes.

In an attempt to clarify whether the impaired development of $CD4^+CD8^-$ single-positive thymocytes in CCR7 transgenic mice was due to a decrease in their rate of formation, the kinetics of thymocyte development were examined by tracing BrdU incorporation following continuous BrdU oral administration in their drinking water. In the thymus, BrdU is incorporated into rapidly proliferating double-negative cells, with little incorporation after the cells reach the double-positive stage of development where they largely, but not completely, cease dividing (48, 212). Thus, the rate of appearance of the BrdU label in single-positive cells is a useful index of the rate of formation of these cells from double-positive precursors. As shown in Figure 7D, the kinetics of double-positive thymocyte labeling were similar in CCR7 transgenic and non-transgenic mice. In contrast, $CD4^+CD8^-$ single-positive cells in CCR7 transgenic mice showed a reduced rate of BrdU labeling compared to $CD4^+CD8^-$ single-positive cells from non-transgenic mice. Consistent with the analysis of steady-state thymocyte frequencies, this result

suggests that premature expression of CCR7 causes a decrease in the fraction of cells that commits to the CD4 lineage during positive selection.

Chemokine ligands of CCR7 direct impaired selection of CD4+CD8- cells in CCR7 transgenic mice

The data shown in Figures 5 and 6, indicate that premature CCR7 expression causes the redistribution of immature thymocytes towards the thymic medulla. To show that the developmental effects associated with this redistribution were dependent on interactions between CCR7 and its chemokine ligands CCL19 and CCL21, we crossed the CCR7 transgenic mice to *plt/plt* mice that show impaired expression of these chemokines (213, 214). The *plt* mutation is a deletion of a large genomic region that contains the gene encoding CCL19 and one of two linked genes encoding CCL21. Mice homozygous for the *plt* mutation lack expression of CCL19 and show much reduced expression of CCL21 (214-216). As shown in Figure 10A, the effect of premature CCR7 expression on T cell development was effectively suppressed in *plt/plt* mice. In addition, PNA-positive thymocytes were no longer present in the *plt/plt* CCR7 transgenic medullae and the thymuses had normal cortical and medullary areas (Figure 10B). These data indicated that, as expected, the positioning and developmental effects of the transgene were dependent on CCL19/21.

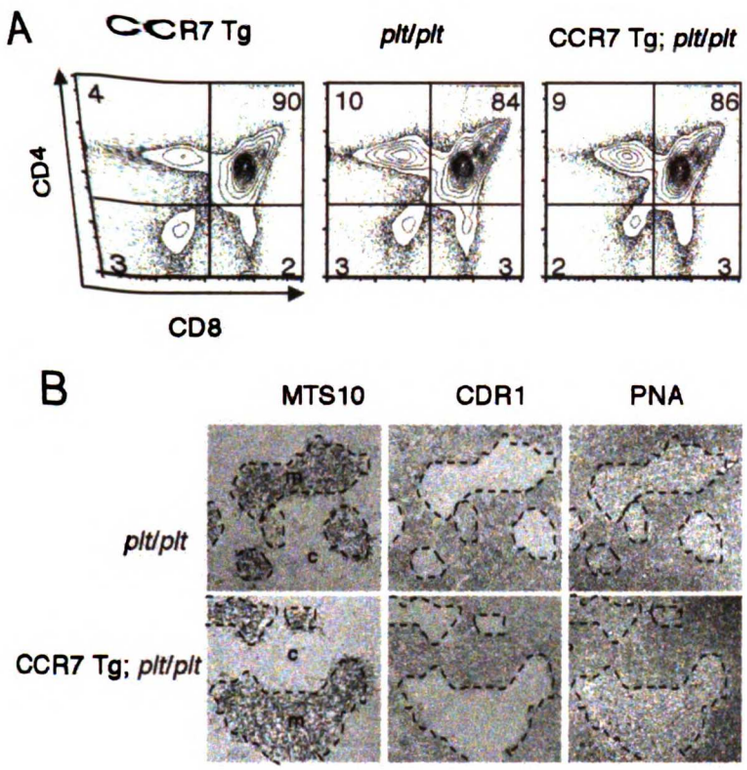


Figure 10. Chemokine ligands of CCR7 direct impaired selection of $CD4^+CD8^-$ cells in CCR7 transgenic mice

CCR7 transgenic mice were crossed with *plt/plt* mice that show reduced or absent expression of CCL21 and CCL19 respectively. (*A*) Thymocytes from the mice were analyzed by flow cytometry using antibodies specific for CD4 and CD8. (*B*) Adjacent sections of *plt/plt* and CCR7 transgenic; *plt/plt* thymuses were stained with MTS10, CDR1, or PNA in brown and counterstained with hematoxylin. Images were taken at 50x magnification.

Effect of transgenic CCR7 on the exit of T cells from the thymus and their accumulation in the spleens of newborn mice

The most abundant source of CCL19 and CCL21 in the thymus is medullary epithelial cells, but there is also some expression of CCL19 on thymic endothelial cells. This latter site of CCL19 expression has recently been implicated as being important for the directed emigration of thymocytes from the medulla at the completion of their development (152). Support for this notion came principally from the finding that neonatal mice lacking CCR7 expression showed delayed accumulation of recent thymic emigrants in their spleens compared to wild-type littermates. Furthermore, CCL19, but not CCL21, could induce the migration of cells when it was applied to thymic organ cultures. Because of these findings, it was important to test whether premature exit of cells from the thymus might be associated with the developmental effects caused by transgenic CCR7 expression.

Two assays were used to quantify recent thymic emigrants in the CCR7 transgenic mice. In one, a solution of FITC was injected directly into the thymuses of transgenic and non-transgenic mice, and the numbers of FITC-labeled emigrant cells in secondary lymphoid organs were then determined by flow cytometry the following day. In the second, thymocyte and splenic T cell numbers were counted in neonatal mice. As shown in Figures 11A and 11B, neither assay showed any evidence of an increase in thymic emigration as a consequence of the expression of the transgene. In fact, in both

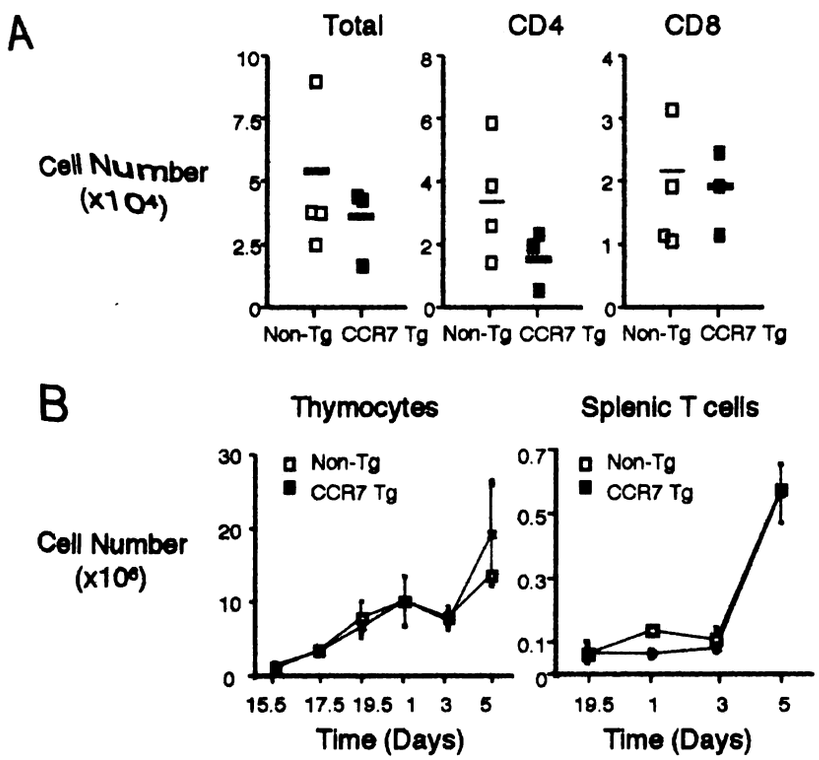


Figure 11. Effect of transgenic CCR7 on the exit of T cells from the thymus and their accumulation in the spleens of newborn mice
(*A*) A solution containing FITC was injected into the thymuses of CCR7 transgenic and non-transgenic mice. One day later, FITC+ recent thymic emigrants (all $FITC^+$ cells, or the $CD4^+$ or $CD8^+$ fractions thereof) were enumerated in the spleens of the injected mice. (*B*) The graphs show the numbers of thymocytes or splenic T cells in embryonic and newborn transgenic or non-transgenic mice.

cases, there was reduced accumulation of $CD4^+$ cells in the transgenic mice reflective of the developmental effect described above.

Finally, we also examined neonatal and adult blood and lymphoid tissues for the presence of elevated numbers of double-positive cells, but again could find no evidence that the transgene was inducing early exit of these cells from the thymus (data not shown).

Discussion

In this study, we have shown that CCR7 can promote the migration of double-positive cells from the cortex to the medulla of the thymus. This role was apparent both from the steady-state analysis of CCR7-transgenic thymuses, and from the positioning of the transgenic thymocytes in radiation chimeras. Inappropriate localization of double-positive thymocytes reduced the efficiency with which single-positive cells were formed indicating that the correct positioning of thymocytes is essential for their development. The data suggest that regulating responsiveness to medullary chemokines is a crucial component of the normal development of thymocytes and highlight CCR7 as a participant in cortical to medullary thymocyte migration.

CCR7 binds to CCL19 and CCL21, both of which are encoded by a small cluster of genes on mouse chromosome 4. In the thymus, CCL19 is expressed by medullary epithelial cells, CD11c$^+$ dendritic cells, and CD31$^+$ endothelial cells (152). The expression of CCL21 overlaps with that of CCL19, but its range of action may be somewhat more constrained because unlike CCL19 it has a charged carboxy-terminal extension that allows for binding to the extracellular matrix (217-219). We have shown that the effect of the CCR7 transgene on thymocyte development is lost in mice that are homozygous for the *plt* mutation in which both CCL19 and CCL21 expression is impaired. Although this observation makes clear that the transgenic phenotype is ligand-dependent, it leaves open the question of whether CCR7-directed migration into the medulla might normally be dependent on both or just one of the two chemokines.

plt/plt mice have clearly defined cortical and medullary regions in the thymus, and they support overtly normal development of thymocytes. Similar statements apply to mice that lack CCR7 (152, 172). Such observations could be used to infer an absence of a role for CCR7 in medullary translocation and to call into question the conclusions of our study. It remains true, however, that positive selection in normal mice results in upregulation of CCR7, and as we show here, the acquisition of CCR7 is sufficient to move double-positive cells into the medulla. Other chemokine receptors are also upregulated during positive selection raising the possibility that the migration of cells into the medulla could be a brought about by the combined influence of multiple redundant mechanisms. Possibilities in this respect would include CCR4 and CCR9. CCR4 may be an especially attractive candidate because like CCR7 its expression is markedly induced as a consequence of positive selection. Moreover, CCL22, one of the ligands for CCR4 is also expressed in the medulla, and this ligand is an efficacious attractant for cells undergoing positive selection (*i.e.*, $CD69^+$ cells) (153, 155, 165, 220). CCR9 is expressed by double-positive cells, but its greatest increase in expression may occur following the pre-TCR signal (161, 162, 165). The ligand for CCR9, CCL25 is found both in the cortex and the medulla and thymocytes show apparently equivalent migration toward it both before and immediately after positive selection (153, 161, 162). Mice lacking CCR4 (156) or CCR9 (160, 166) have been generated, but neither mouse has so far been described as showing a defect in thymocyte positioning, or in the formation of cortical or medullary areas. To date, mice showing defective responses to combinations of chemokines (*e.g.*, mice lacking both CCR4 and CCR7) have not been generated, but such

mice are of obvious importance for discerning the nature of the redundancy in the migration mechanism. If CCR4 and CCR7 are the major contributors to medullary migration, then the loss of both receptors would presumably have a marked effect on migration and development.

In addition to its capacity to guide cells towards the medulla, CCR7 and its ligands are apparently also involved in directing cells out of the thymus. Neonatal mice lacking CCR7, or mice treated with a CCL19 antagonist show decreased emigration of cells from the thymus to the spleen, although in neither case is emigration completely impaired (152). Thus, as just argued for migration to the medulla, the dependency of thymocytes on CCR7-directed chemotaxis for emigration is not complete and other factors are also likely to be involved in this process. Consistent with this, we found that transgenic expression of CCR7 on double-positive cells and mature single-positive cells was insufficient to direct them out of the thymus, and we saw no detectable increase in thymocyte emigration in the transgenic mice using two separate assays. Emigration from the thymus could involve both lymphatic and blood vessels, and the mechanisms that regulate it are largely unclear. Since CCR7 is expressed from an early point after positive selection yet single-positive cells can reside in the medulla for two weeks or more (45), additional signals may be required to prime cells for exit and these signals may only be received after the cells have resided in the medulla for a substantial length of time.

The migration path of thymocytes is circuitous involving movement from the medulla to the outer cortex followed by reentry into the medulla after αβ TCR selection

(10-12). As mentioned in the introduction, recent data implicate CXCR4 in the first step of this path, *i.e.*, the guidance of progenitor cells into the cortex (147). When in competition with wild-type thymocytes, CXCR4-deficient cells failed to enter the cortex and their development was arrested at the double-negative stage. In a similarly competitive situation, CCR7-transgenic cells did not show the same type of developmental block despite the fact that they were found concentrated in the medulla and showed little if any penetration into the cortex. Although the intrathymic migration phenotype of CXCR4-deficient and CCR7-transgenic cells appears to be superficially similar, additional experiments will be required to determine if this is in fact the case. It remains possible, for example, that CXCR4 expression on progenitor thymocytes allows for them to move towards CXCL12-secreting cells close to the medullary boundary, and that limited penetration into the cortex of this sort is sufficient for development to proceed.

The thymic medulla is rich in cell types that are effective in inducing negative selection. Double-positive cells are especially sensitive to apoptosis in part because of their reduced expression of proteins, such as Bcl-2, that would protect them against programmed cell death (1). It seemed possible, therefore, that by causing double-positive cells to move into the medulla prematurely, there would be an increase in apoptosis because of the induced proximity of sensitive cells to antigen presenting cells replete with costimulatory ligands. Although we did observe a defect in thymocyte development in the transgenic mice, we found no evidence that this was due to increased cell death. Thus, the overall cellularity of the thymus was not decreased and the only observable

defects were those in the rate of formation of $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive cells. Cortical epithelial cells are distinctive in their capacity to promote the positive selection of cells (8), and it could be that less of the transgenic cells are able to interact efficiently with these cells because of premature migration towards medullary stromal elements. Alternatively, the apposition of the cells next to medullary components may in fact suppress signals that are necessary for positive selection. Whatever the basis of the effect, the data make clear that normal thymocyte development hinges on the proper regulation of cortico-medullary migration and retention of cells in the cortex until positive selection signals have been received.

Patterning of the thymus during and after embryogenesis is a dynamic process that can be reinitiated in response to thymocyte depletion and that can also be modeled *in vitro* in reaggregate organ culture systems. The formation of the cortex and medulla in both contexts is crucially dependent on the presence of thymocytes (8). There is, for instance, a lack of recognizable medullary or cortical areas in mice that lack intrathymic T cell precursors (*e.g.*, mice deficient in both *c-kit* and γ_c expression, or mice that overexpress human CD3ε) (221, 222). Such thymuses are extremely small in size and contain seemingly disorganized aggregations of epithelial cells. RAG-1- or RAG-2-deficient mice that lack double-positive thymocytes have larger thymuses, but these are still much smaller than wild-type thymuses. Although cortical epithelium forms in these thymuses, they contain hypoplastic and disorganized medullae (223, 224). TCRα-deficient thymuses are also devoid of properly organized medullae, despite the presence of abundant cortical epithelium (225). Cumulatively, observations of this sort show that

the formation of the medulla is dependent on the presence of mature single-positive cells, whereas the cortex can be organized in response to the presence of double-negative thymocytes.

Data presented in this chapter show that the medulla could remain organized despite the presence of invading double-positive cells. Thus, double-positive cells do not appear to recruit cortical epithelial cells to their vicinity, nor do they appear to suppress the formation of the medulla. Recent data indicate that lymphotoxin β receptor on single-positive thymocytes and the NFκB-inducing kinase (Nik) in the medullary epithelial cells are essential for proper organization of the medulla (226). These findings support a model for the development of the medulla that depends on inducing signals from single-positive cells, but is not dependent on the exclusion of double-positive cells.

Through their capacity to bring stromal and thymocyte components together, it is sensible to consider a role for chemokines in the organizing process that forms cortical and medullary regions. We show here that chemokine receptors can drive associations between thymocytes and medullary cells, consistent with the involvement of chemokines in selection and perhaps cortico-medullary patterning. Transgenic gain-of-function systems, such as the one employed here, may provide further insight into the signals that are important for formation of the thymic medulla.

Chapter 3: Effect of Transgenic Expression of ST3Gal-1 sialyltransferase on T cell Development and CD8 Function

Abstract

Glycosylation patterns change during thymocyte development. One such modification is the addition of sialic acid on core 1 O-glycans, a process mediated by ST3Gal-1 sialyltransferase. This enzyme is selectively downregulated in immature $CD4^+CD8^+$ double positive thymocytes and upregulated in single positive thymocytes. To explore the significance of ST3Gal-1 sialyltransferase downregulation and the consequences of increased cell surface sialylation in double-positive thymocytes, we generated transgenic mice that express ST3Gal-1 sialyltransferase in all thymocytes. We examined a line in which the level of sialylation on transgenic double-positive thymocytes was equivalent to that found on normal single-positive thymocytes. Sialylation has been implicated in decreasing the ability of the coreceptor CD8 to bind to class I MHC. Interactions of CD8 with MHC I influence thymic development. We found that increased sialylation of CD8 did not inhibit CD8-class I MHC interactions on double-positive thymocytes. Thymic development was also not obviously altered in these mice. These results indicate that ST3Gal-1 downregulation in double-positive thymocytes is not essential for their development or CD8 function.

Introduction

Glycosylation patterns change extensively during T cell development, differentiation, and activation, and there is increasing evidence of their importance in regulating protein function in the immune system (3, 181). One highly conserved example in chickens, mice, and humans, occurs as $CD4^+CD8^+$ double-positive (DP) cells develop into $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) cells and move from the cortex into the medulla. Cortical DP thymocytes express the Galβ1-3GalNAc-Ser/Thr core 1 O-glycan, which is recognized by the plant lectin peanut agglutinin (PNA) (2, 185, 186). Positive selection induces expression of ST3Gal-1 sialyltransferase (ST3Gal-1). This enzyme transfers sialic acid onto core 1 O-glycans to generate Siaα2-3Galβ1-3GalNAc-Ser/Thr (2). The addition of sialic acid onto core 1 O-glycans masks the PNA binding epitope and results in the loss of PNA staining of medullary SP thymocytes. Thus, developmentally regulated differential expression of ST3Gal-1 accounts for the long-recognized difference in PNA binding to DP and SP thymocytes.

The selective down-regulation of ST3Gal-1 in DP thymocytes and upregulation in SP thymocytes raises the possibility that ST3Gal-1 is involved in the differential regulation of protein function in DP and SP thymocytes, and is important in thymocyte development. Sialylation increases the negative charge associated with proteins and could influence protein conformation and protein-protein interactions. Some of the major ST3Gal-1 substrates are the co-receptor CD8, the mucin-like molecule CD43, and the receptor-like protein tyrosine phosphatase CD45 (3, 187-189). Glycosylation and

sialylation changes on these molecules can affect their function. For instance, CD45 dimerization regulates its activity through formation of an inhibitory wedge in the catalytic site, and glycosylation and sialylation on the extracellular domains of CD45 influence the extent of dimerization (192, 193).

Recent data have revealed an important contribution of sialylation to CD8 function. CD8 exists as several forms on mature T cells including CD8αβ heterodimers and CD8αα dimers (96, 99, 100, 103, 187). Interactions of CD8 with major histocompatibility class I molecules (MHC I) influence T cell selection and lineage commitment (117, 119, 122). Regulation of the ability of CD8 to bind to MHC I in a TCR-independent manner, i.e. through noncognate interactions, is affected by sialic acid addition (198, 199). DP thymocytes bind to MHC I tetramers more efficiently than SP thymocytes, and neuraminidase treatment of SP thymocytes partially enhances binding to MHC I tetramers. These results suggest that downregulation of ST3Gal-1 expression in DP thymocytes would enhance the ability of CD8 to bind to MHC I tetramers. Conversely, upregulation of ST3Gal-1 expression in SP thymocytes, which increases sialylation levels on cell surface proteins, would inhibit CD8 function.

The phenotype of ST3Gal-1-deficient mice suggests that ST3Gal-1 expression in SP thymocytes influences T cell development (194, 199). These mice have an altered TCR repertoire with a reduction of mature SP thymocytes and a deficiency of peripheral $CD8^+$ T cells. The survival of naïve and memory $CD8^+$ T cells is also dependent on ST3Gal-1 function although the mechanism that eliminates these $CD8^+$ T cells is not

clear. These studies indicate that ST3Gal-1 expression is important in SP thymocytes, but they do not address the importance of ST3Gal-1 downregulation in DP thymocytes on T cell development.

As a way to study the importance of downregulation of ST3Gal-1 expression on T cell development and CD8 function, we generated transgenic mice that express ST3Gal-1 in all thymocytes, including DP thymocytes, which do not normally express the enzyme. Our expectation was that expression of ST3Gal-1 would increase the amount of sialylation on core 1-O glycans on the surface of DP thymocytes, and that the addition of sialic acid to proteins that are normally not sialylated could alter T cell development. We characterized a line of mice in which the level of sialylation on DP thymocytes was increased to a level equivalent to that found on SP thymocytes. We found that transgenic ST3Gal-1 expression did not alter T cell development in the thymus. Biochemical characterization of CD8 indicated that it was more sialylated, but increased sialylation of CD8 did not inhibit CD8-MHC I interactions on DP thymocytes. These unexpected results demonstrate that increasing the level of sialylation is not sufficient to alter T cell development or change CD8 function.

Materials and Methods ST3

Mice, antibodies, and flow cytometry

A cDNA encoding a Flag-tagged form of mouse ST3Gal-1 sialyltransferase was cloned into the vector p1017 containing the lck proximal promoter (227, 228). The resulting construct was linearized and injected into C57BL/6xDBA/2 F2 embryos. Founder transgenic ST3 Gal-1-transgenic mice were identified by Southern blot and PCR. These were then crossed to C57BL/6 mice for 5-6 generations and crossed to TCR transgenic (H-Y and W15αβ) strains. CD8α-deficient mice were obtained from Jackson Laboratories. All experiments were performed according to protocols approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

Antibodies (CD4, CD8 (clones 53-6.7 and 53-5.8), CD3ε) were purchased from BD Biosciences. Biotin-conjugated and FITC-conjugated peanut agglutinin was purchased from Vector Laboratories. The T3.70 antibody specific for the B6.2.16 TCR was purified from hybridoma supernatant fluid by Protein G affinity chromatography (Amersham). The LACK/I-A^d multimer reagent that stains LACK-specific T cells has been described (229, 230). The tetrameric form of K^b with the OVA peptide (SIINFEKL) was purchased from Immunomics. Staining was perfomed as described (3). 200,000 cells in 110µl were stained with K^b/OVA at various concentrations. Neuraminidase treatment of thymocytes was performed as described (198). Briefly, 2 $x10^6$ cells per ml in RPMI 1640 were incubated with 0.016 U type II neuraminidase from

Vibrio cholorae (Sigma Aldrich) per million cells for 30 minutes at 37°C and then washed 3 times with RPMI 1640 containing 10% fetal calf serum.

For flow cytometry, single cell suspensions were prepared from thymuses, spleens, and lymph nodes using cell strainers (Falcon) and PBS containing BSA (0.3%w/v) and 1.54 mM NaN_3. The cells were washed two or more times and then incubated with antibodies for 30 minutes on ice. The cells were washed a further two times before analysis or prior to incubation with secondary reagents.

Immunohistochemistry

Paraffin sections (3-5 μm) were stained with hematoxylin and eosin and prepared by Biopathological Sciences Medical Corporation (South San Francisco, CA). Frozen sections (6 μm) were fixed with acetone, blocked with 0.1% BSA in TBS, stained with biotin-labeled PNA (Vector Laboratories), and subsequently with horseradish peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories). After washing with TBS and water, sections were counterstained with hematoxylin Gill's Formulation #1 (Fisher Scientific). Images were taken at 50x magnification.

T cell stimulation assay

Lymph nodes from non-transgenic and transgenic mice were harvested, disaggregated, and cultured in RPMI 1640/10% fetal calf serum on plates that had been

pre-coated with 2 μg/ml anti-CD3 antibody (2C11) for 24 hours and subsequently washed with PBS. Cells were harvested at various time points and the expression of cell surface molecules was determined by flow cytometry.

T cell isolation

T cells were purified using the MACS system (Miltenyi). Briefly, total thymocytes were resuspended in 1 ml of PBS/2%BSA/2mM EDTA per 10^8 cells and incubated with 40 μl of anti-CD4 microbeads (Miltenyi) for 30 minutes and then applied to LS columns (Miltenyi) to isolate DP and CD8 SP thymocytes. CD8 SP thymocytes were collected in three washes of the column, and DP thymocytes were flushed off the column with a plunger. To enrich peripheral $CD8^+$ T cells, pooled lymph nodes and spleen cells were incubated with anti-CD4, anti-B220, anti-MHC II microbeads (Miltenyi), and applied to LS columns. Three washes were collected to isolate $CD8^+$ T cells.

Biotinylation, Immunoprecipitation, SDS-PAGE and Immunoblotting

T cells were biotinylated in PBS buffer containing EZ-link sulfo-NHS-biotin (Pierce) at a concentration of 100 μg/ml per 5 x 10^6 cells for 30 minutes at 4°C. This concentration of EZ-link sulfo-NHS-biotin was found to be optimal for cell surface biotinylation from titration experiments using it from 50ng/ml to 2 mg/ml. Over-biotinylation using high concentrations of EZ-link sulfo-NHS-biotin reduced the ability

to discriminate differences in the mobilities of some proteins in SDS-PAGE analysis. Labeling was terminated by 2 washes in lysine containing RPMI. For some samples, neuraminidase treatment was performed as described (199). Cells were washed in PBS and lysed in 20 mM Tris-HCL pH 7.5 containing 1% NP-40, 150 mM NaCl, 2.5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml approtinin, and 200 μg/ml phenylmethylsulfonyl fluoride. The lysates were clarified by centrifugation and precleared with protein G- and protein A- agarose for 30 minutes at 4°C. The precleared lysate was incubated with anti-CD8α antibody (clone 53-6.7) and protein G-/protein A- agarose overnight at 4°C. Precipitates were washed 3 times in lysis buffer and eluted in nonreducing or reducing SDS sample buffer.

For 1D SDS-PAGE analysis, samples were run on 12.5% reducing gels. For 2D analysis, samples were run on 10% nonreducing SDS-PAGE gels in the first dimension. Individual lanes were excised, soaked in reducing sample buffer containing 7.5% glycerol, 60 mM Tris, pH 6.7, 2% SDS, and 0.6 M β-2 mercaptoethanol for 30 minutes, and then placed onto 12.5% SDS-PAGE gels (Bio-RAD) for electrophoresis in the second dimension. Proteins were transferred onto Immobilon P membrane (Millipore). Blots were blocked in Tris buffered saline/0.05% Tween-20/5% BSA for 1 hour, washed once with TBS/0.05% Tween-20, incubated with HRP-conjugated streptavidin (Jackson ImmunoResearch) for 1 hour, and washed 3 times with TBS/0.05% Tween-20. Biotin-labeled proteins were visualized by the addition of ECL chemiluminescent reagents (Amersham) and autoradiography.

Results

Expression of ST3Gal-1 on thymocytes from transgenic mice

To express ST3Gal-1 on all thymocytes, including DP thymocytes, which do not normally express ST3Gal-1, we generated transgenic mice in which expression of the enzyme was controlled by regulatory elements from the proximal promoter of the mouse *lck* gene (227, 228). PNA staining was then used to study the levels of sialylation on core 1 O-glycans induced by ST3Gal-1 on developing thymocytes from the transgenic mice. Low levels of PNA staining indicate high levels of sialylation and reflect high levels of ST3Gal-1 expression. As shown in the shaded histogram in Figure 12A, PNA staining of non-transgenic thymocytes was increased on DP thymocytes compared to CD4 SP and CD8 SP thymocytes, consistent with the normal downregulation of ST3Gal-1 expression in DP thymocytes.

In contrast, PNA staining was reduced on all thymocyte subsets in transgenic mice derived from two founders (6225 and 6205). As shown in Figure 12A, thymocytes from line 6225 displayed a greater reduction in PNA staining than thymocytes from line 6205. Of particular significance, the level of PNA staining on 6225 DP thymocytes was equivalent or slightly reduced compared to the level on non-transgenic CD8 SP thymocytes (Figure 12B). Line 6225 was therefore chosen for further study because their DP thymocytes displayed a sialylation level that was similar to that of normal non-transgenic SP thymocytes.

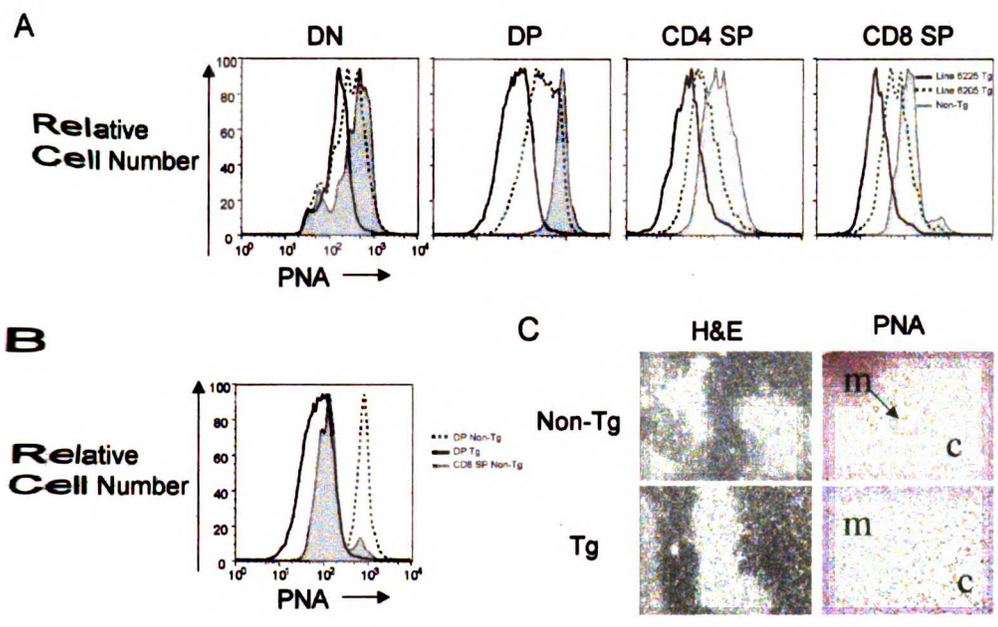


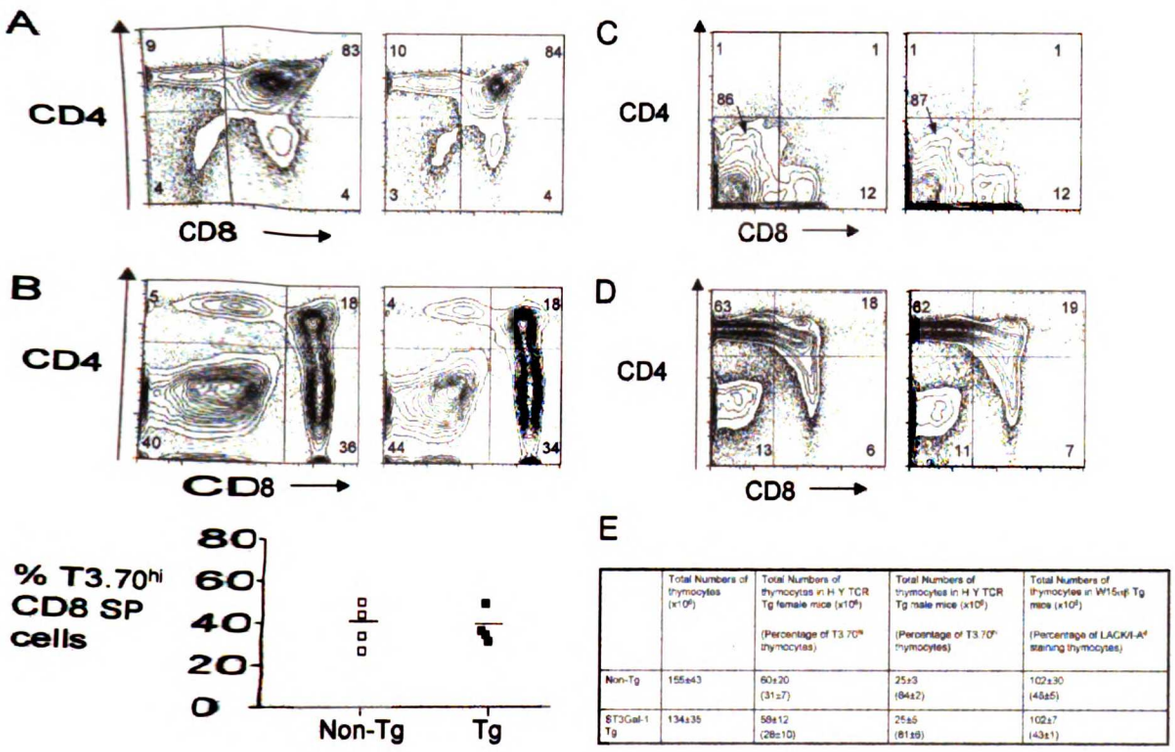
Figure 12. Expression of ST3Gal-1 in the thymus of transgenic mice
A, Thymocytes from ST3Gal-1 transgenic and non-transgenic littermates were stained with CD4 and CD8 antibodies to identify blasting $CD4^-CD8^-$ DN (identified by FSC), $CD4^+CD8^+$ DP, $CD4^+CD8^-$ SP, and $CD4^-CD8^+$ SP thymocytes and with PNA to examine the level of sialylation on core 1-O glycans. The histograms show the level of PNA staining on thymic subsets from 2 transgenic ST3Gal-1 lines (lines 6205 and 6225) and from non-transgenic mice. B, The histogram shows the level of PNA staining on DP thymocytes from transgenic and non-transgenic mice and on CD8 SP thymocytes from non-transgenic mice. C, Paraffin sections of ST3 Gal-1 transgenic (line 6225) and non-transgenic thymuses were stained with hematoxylin and eosin. Images were taken at 50x magnification and converted to greyscale. (left panels) Frozen sections were stained with PNA (brown) and counterstained with hematoxylin. Images were taken at 100x. c: cortex; m: medulla.

Transgenic thymuses were sectioned and stained with hematoxylin and eosin to identify cortical and medullary regions. As shown in the left panels of Figure 12C, transgenic thymuses exhibited normal cortical and medullary regions. In normal non-transgenic thymuses, cortical areas display more desialylated core 1 O-glycans and consequently have higher levels of PNA staining than medullary areas. As shown in the right panels of Figure 12C, non-transgenic and transgenic thymic sections were stained concurrently with PNA. PNA staining of cortical areas in transgenic thymuses was lower than that seen in non-transgenic thymuses reflecting the increased levels of sialylation in transgenic mice.

ST3Gal-1 transgenic mice have normal T cell development

Increased expression of ST3Gal-1 expression in DP thymocytes did not obviously alter thymocyte development. Using flow cytometry, transgenic expression of ST3 Gal-1 did not affect early thymocyte development as assayed by expression of CD25 and CD44 in DN thymocytes (data not shown). Normal proportions of $CD4^+$ and $CD8^+$ T cells developed in ST3Gal-1 transgenic mice and the expression of cell surface molecules, including CD3, TCRβ, Qa-2, HSA, CD69, CD5, and CD62L, was not altered (Figure 13A and data not shown).

To determine if transgenic expression of ST3Gal-1 could alter thymocyte selection, we bred MHC class I- and MHC class II-restricted TCR transgenic (H-Y and W15αβ respectively) mice to the ST3Gal-1 transgenic mice to examine the efficiency of



	Total Numbers of thymocytes ($x10^6$)	Total Numbers of thymocytes in H-Y TCR Tg female mice ($x10^6$) (Percentage of T3.70^{hi} thymocytes)	Total Numbers of thymocytes in H-Y TCR Tg male mice ($x10^6$) (Percentage of T3.70^{hi} thymocytes)	Total Numbers of thymocytes in W15αβ Tg mice ($x10^6$) (Percentage of LACK/I-A^d staining thymocytes)
Non-Tg	155±43	60±20 (31±7)	25±3 (84±2)	102±30 (48±5)
ST3Gal-1 Tg	134±35	58±12 (28±10)	25±6 (81±9)	102±7 (43±1)

Figure 13. Normal thymocyte development in ST3 Gal-1 transgenic mice
A, Representative flow cytometric analysis of thymuses stained with CD4 and CD8 antibodies. B & C, Positive and negative selection of clonotype-expressing $CD4^-CD8^+$ SP thymocytes in mice transgenic for the H-Y-specific B6.2.16 αβ TCR. The contour plots show the expression of CD4 and CD8 on thymocytes expressing high levels of the αβ TCR clonotype identified with the T3.70 antibody in female (B) and male (C) mice. The scatter plot shows the frequency of $T3.70^{hi}CD4^-8^+$ SP cells in female mice. D, Selection of $CD4^+CD8^-$ SP thymocytes in mice transgenic for the LACK-specific W15αβ TCR. The contour plots show the expression of CD4 and CD8 on thymocytes expressing high levels of the αβ TCR identified with a LACK/I-A^d multimer. E, The total numbers of thymocytes for each group are given in the table. The percentages of TCR transgenic expressing cells are given in parentheses.

thymocyte selection. The H-Y TCR transgene encodes the B6.2.16 TCRαβ specific for the H-2D^{b}-restricted H-Y antigen from the *Smcy* gene (210). Thymocytes expressing this receptor are selected into the CD8 lineage in female mice (211). Most thymocytes expressing this receptor are deleted in male mice (126). As shown in Figure 13B, expression of ST3Gal-1 did not affect the selection of CD8 thymocytes in female mice that stained brightly with the T3.70 anti-TCR clonotype mAb. Total thymocytes numbers and T3.70^{hi} thymocyte numbers were similar. Spleens and lymph nodes from transgenic and non-transgenic littermates had similar numbers of CD4^{+} cells, CD8^{+} T cells, and T3.70^{hi} T cells (data not shown). Efficient deletion of H-Y specific thymocytes occurred in non-transgenic and transgenic male mice indicating that transgenic ST3Gal-1 expression also did not affect negative selection (Figure 13C). Transgenic and non-transgenic mice had similar numbers of total and T3.70^{hi} T cells in peripheral tissues (data not shown).

The W15αβ TCR transgene encodes a TCR specific for the I-A^{d}-restricted LACK antigen and thymocytes expressing this receptor are efficiently selected into the CD4 lineage (230). We did not observe any difference in positive selection of W15αβ TCR expressing thymocytes identified by staining with a fluorescent LACK/I-A^{d} multimer (Figure 13D) (229, 230). Total thymocyte cell numbers and percentages of LACK/I-A^{d} staining cells were similar, and there was no difference in the numbers of CD4^{+} T cells selected in transgenic mice compared to non-transgenic mice.

Normal activation responses in peripheral ST3Gal-1 transgenic mice

Expression of ST3Gal-1 was controlled by regulatory elements from the proximal promoter of the mouse *lck* gene. Although this promoter has been reported to be inactive in peripheral T cells, in some mouse lines, it stays active (227, 228, 231). As shown in Figure 14A, PNA staining of line 6225 $CD8^+$ T cells from lymph nodes was decreased compared to non-transgenic $CD8^+$ T cells indicating that there was peripheral expression of the ST3Gal-1 transgene. Using flow cytometric analysis, we found that the expression of CD4, CD8, CD3, CD44, and B220 markers was not altered in transgenic spleen and lymph nodes (data not shown). These results indicate that transgenic expression ST3Gal-1 did not obviously alter the numbers of peripheral T cells.

ST3Gal-1 transgenic and non-transgenic lymph node cells were stimulated with anti-CD3 antibodies to determine if ST3Gal-1 expression altered the activation responses of peripheral $CD8^+$ T cells. Expression of the activation marker CD69 on $CD8^+$ T cells after CD3 stimulation was monitored by flow cytometry. As shown in Figure 14B, ST3Gal-1 transgenic $CD8^+$ T cells responded normally and upregulated CD69 after CD3 stimulation. Thus, transgenic expression of ST3Gal-1 does not interfere with TCR mediated signaling responses assayed by CD69 induction.

TCR stimulation or anti-CD3 stimulation leads to alterations in glycosylation of membrane proteins on $CD8^+$ T cells (194, 232, 233). Naïve peripheral $CD8^+$ T cells are highly sialylated (as detected with PNA) and lack core 2 O-glycans on CD43 and CD45 (as detected with the 1B11 antibody). Within 24 to 48 hours after activation core 1 O-

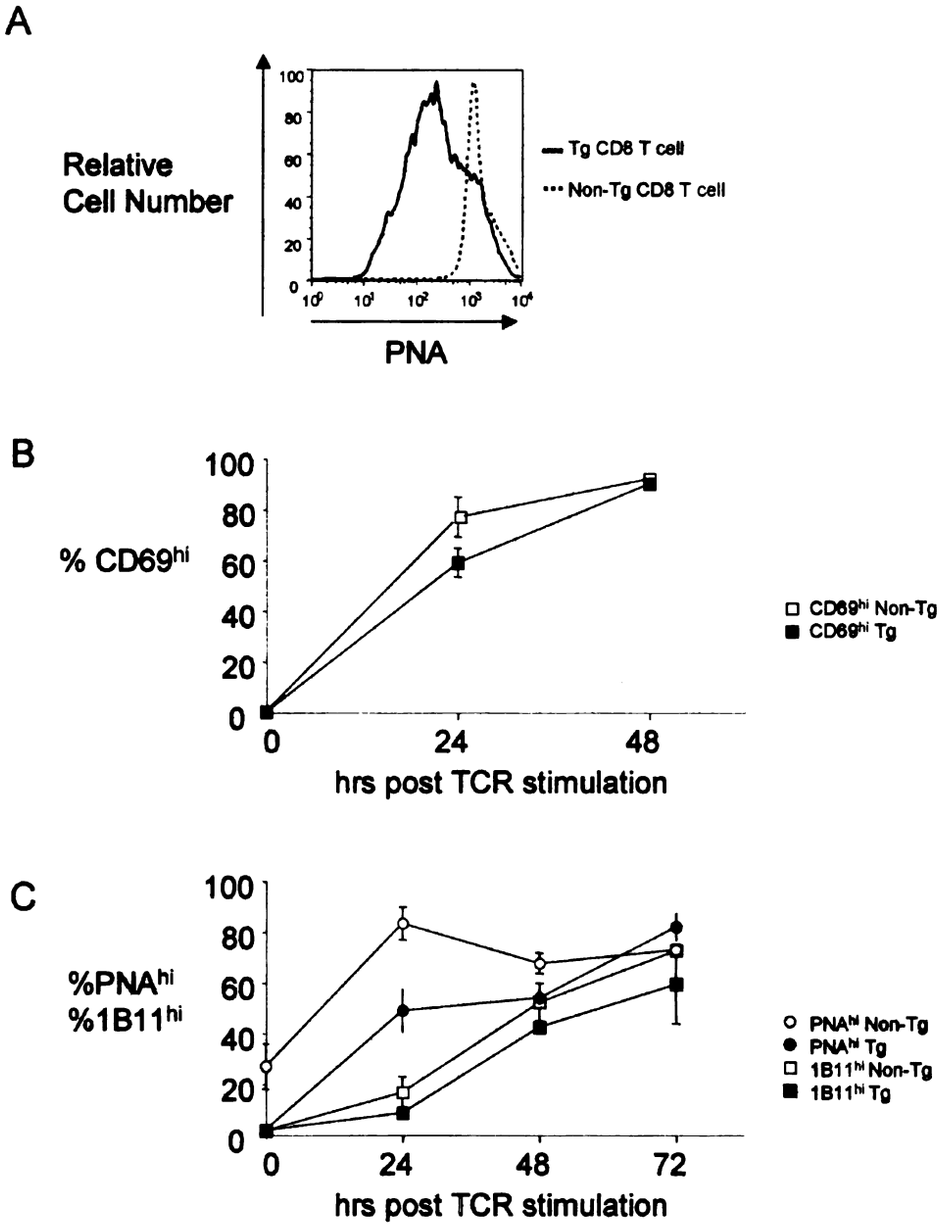


Figure 14. Immune responses and induction of PNA and IB11 staining after CD3 stimulation of CD8$^+$ T cells

A, PNA staining of transgenic lymph node CD8$^+$ T cells is decreased compared to non-transgenic CD8 T cells. B, Upregulation of CD69 on CD8 T cells after CD3 stimulation. Lymph node cells were cultured on plates pre-coated with 2 μg/ml of anti-CD3 antibody. Cells were removed at various times after stimulation and stained with CD8 and CD69-specific antibodies, and analyzed by flow cytometry. B, Modification of cell surface O-glycans from PNA^{lo}1B11^{lo} to PNA^{hi}1B11^{hi} on CD8$^+$ T cells after CD3 stimulation. As in C, lymph node cells were cultured on plates pre-coated with 2 μg/ml of anti-CD3 antibody. Cells were removed at various times after stimulation and stained with PNA, CD8 and 1B11 antibodies, and analyzed by flow cytometry.

glycans become desialylated and core 2 O-glycans on CD43 are induced yielding a $PNA^{hi}1B11^{hi}$ phenotype. Desialylation results from downregulation of ST3Gal-1 and potentially the upregulation of sialidases in activated $CD8^+$ T cells (194). As shown in Figure 14C, ST3Gal-1 transgenic $CD8^+$ T cells initially had lower levels of PNA staining than non-transgenic $CD8^+$ T cells, indicating that they carried more desialylated core 1-O glycans. After 48 hours, ST3Gal-1 transgenic and non-transgenic $CD8^+$ T cells had a similar $PNA^{hi}1B11^{hi}$ phenotype indicating that ST3Gal-1 transgenic $CD8^+$ T cells became desialylated and induced core 2 O-glycans similar to non-transgenic $CD8^+$ T cells. Increased levels of sialylation were not maintained in activated peripheral T cells perhaps due to upregulation of sialidases and/or downregulation of the ST3Gal-1 transgene in activated $CD8^+$ T cells (194, 234).

Increased sialylation and alteration of CD8 in ST3Gal-1 transgenic mice

The decreased level of PNA staining in transgenic mice suggest that the overall level of sialylated core 1 O-glycans in transgenic DP thymocytes is similar to that found in CD8 SP thymocytes. To confirm that there was increased sialylation on ST3Gal-1 substrates in transgenic mice, we examined the mobility of CD8, a substrate of ST3Gal-1, in SDS-polyacrylamide gels. For this purpose, sorted populations of thymocytes and peripheral $CD8^+$ T cells were surface labeled with EZ link sulfo-NHS-biotin. The sorted populations of cells were then lysed, and CD8 proteins were immunoprecipitated with anti-CD8α antibody (53-6.7), separated on SDS-PAGE gels, transferred to PVDF, and probed with strepatividin-horseradish peroxidase to identify biotinylated proteins.

As shown in Figure 15A, CD8α and CD8β polypeptides were separable under reducing conditions. Two bands corresponding to the CD8α and CD8α' polypeptides (an alternatively spliced form of CD8) ran at approximately 37 and 33 kDa respectively. CD8β polypeptides ran as a heterogeneous set of bands spanning the 27 to 32kDa size range. Aggregates of CD8 molecules, which run as dimers, resolved at approximately 75kDa. None of these bands were detected in immunoprecipitates from CD8α-deficient thymocyte lysate (lane 4), demonstrating the specificity of the immunoprecipitation conditions. Comparison of immunoprecipitates from transgenic DP thymocyte lysate (lanes 2 and 4) with immunoprecipitates from non-transgenic DP thymocyte lysate (lanes 1 and 3) revealed that the mobilities of transgenic CD8β and CD8α were significantly reduced in transgenic thymocytes. There was also a similar reduction in the mobility of the 75kD CD8 aggregates in immunoprecipitates from transgenic DP thymocyte lysates (lanes 2 and 4) compared with immunoprecipitates from non-transgenic DP thymocyte lysates (lanes 1 and 3). Furthermore, comparison of immunoprecipitates of SP thymocyte lysates from non-transgenic (lane 6) and transgenic mice (lane 7) revealed that transgenic ST3Gal-1 expression also reduced the mobility of CD8β in SP thymocytes which was consistent with the reduction in PNA staining in transgenic SP cells seen in Figure 12A.

CD8β from immunoprecipitates of non-transgenic SP thymocytes (lane 6) and non-transgenic peripheral $CD8^+$ T cells (lane 8) displayed less heterogeneity and reduced mobility compared to CD8β from immunoprecipitates of non-transgenic DP thymocytes (lane 1 and 3). The changes reflect the maturation of CD8, including the addition of

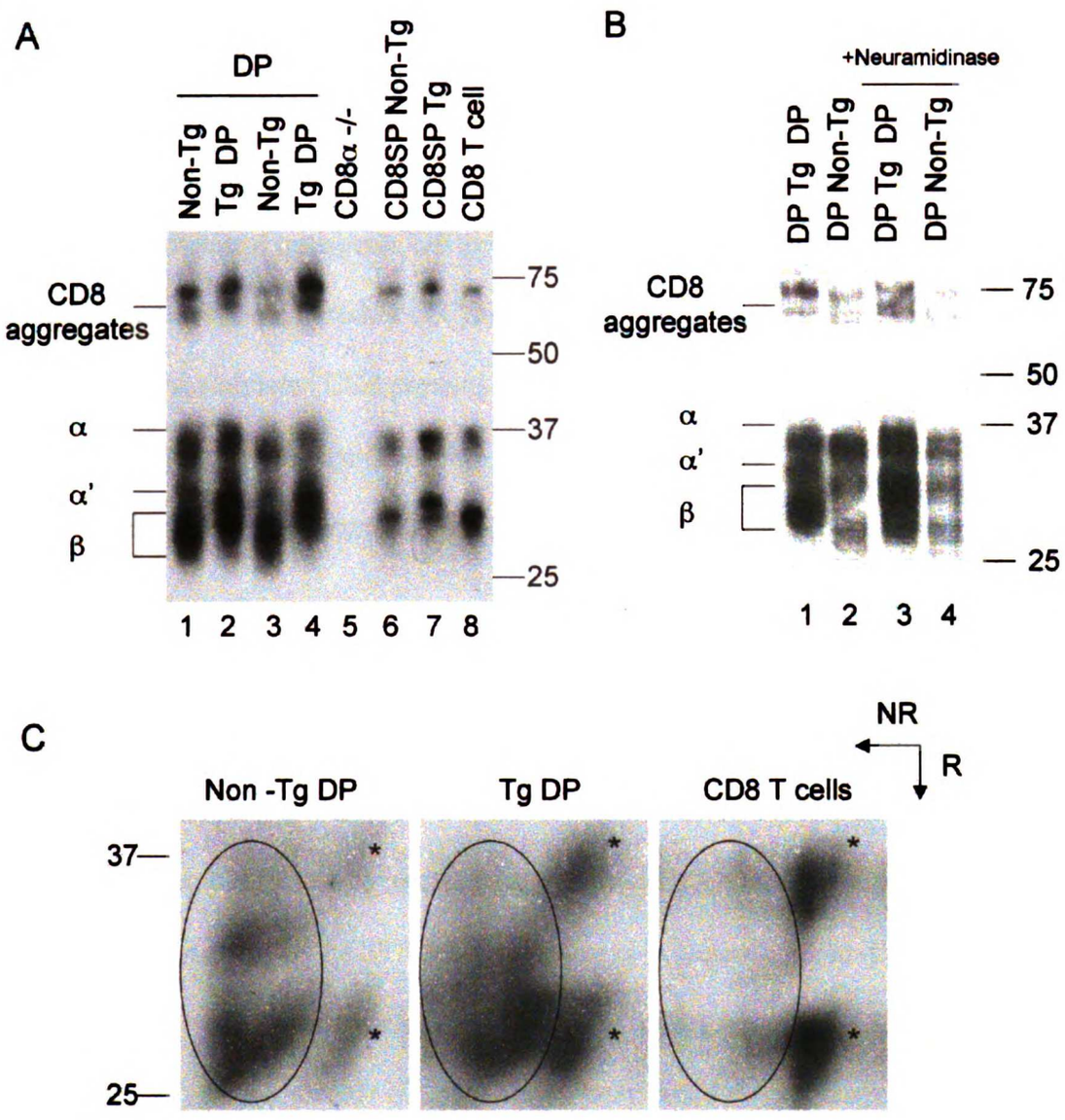


Figure 15. Glycosylation of CD8 proteins in thymocytes from ST3Gal-1 transgenic mice

A, CD8 proteins immunoprecipitated with anti-CD8α mononclonal antibody (53.6.7) from lysates of cell-surface-biotinylated thymocytes and peripheral $CD8^+$ T cells were resolved on 12.5% SDS polyacrylamide gels followed by Western blotting with HRP conjugated Streptividin. B, Comparison of CD8 proteins immunoprecipitated from untreated and neuraminidase treated thymocytes and $CD8^+$ T cells. C, 2D nonreducing/reducing gel analysis of surface-labeled CD8 proteins immunoprecipitated from lysates of sorted DP thymocytes and peripheral $CD8^+$ T cells. CD8 heterodimers (α37kD, β30kD) are labeled with asterisks *. At least 2 sets of CD8 heterodimers (α37kD, β29kD and α'33kD, β28kD)) are circled.

sialic acid, as DP thymocytes develop into SP thymocytes. The mobilities of CD8α and CD8β from immunoprecipitates of transgenic DP thymocyte lysate (lane 2 and 4) were similar to the mobilities of CD8α and CD8β from immunoprecipitates of SP thymocyte and peripheral CD8 T cell lysate (lane 6 and 8), although CD8β proteins from immunoprecipitates of transgenic DP thymocyte lysate were more heterogeneous. From this type of analysis, the mobility of CD8 proteins in transgenic cells were similar to that observed for the sialylated CD8 proteins found in SP thymocytes and peripheral $CD8^+$ T cells. These data suggest that CD8 from transgenic cells is sialylated at a similar level to CD8 from normal non-transgenic SP thymocytes and peripheral $CD8^+$ T cells.

Neuraminidase treatment, which cleaves sialic acid residues from oligosaccharides on the cell surface, increased the mobility of CD8 polypeptides from transgenic DP thymocyte immunoprecipitates (compare lanes 1 and lane 3, Figure 15B). The ability of neuraminidase to increase the mobility of CD8 in transgenic thymocytes indicates that the effect of the ST3Gal-1 transgene is to generate glycosylation changes that can be reversed by neuraminidase and supports the argument that CD8 in transgenic thymocytes is hyper-sialylated. Neuraminidase treatment did not remove all the differences in the mobility of CD8 polypeptides between transgenic and non-transgenic DP thymocytes (compare lanes 2, 5, and 6), perhaps because some sialic acid residues were inaccessible and there was incomplete desialylation.

Two-dimensional non-reducing/reducing SDS-PAGE analysis can improve the resolution of CD8 polypeptides. As shown in Figure 15C, CD8 heterogeneity is reduced

in $CD8^+$ peripheral T cells compared to DP thymocytes. In DP thymocytes, at least 3 sets of CD8 heterodimers (α37kD, β30kD, which are labeled with asterisks and α37kD,β29kD and α'33kD,β28kD, which are circled) can be identified. In peripheral $CD8^+$ T cells, the major bands are CD8α which runs at 37kD, and CD8β which runs at 30kD. These forms likely correspond to the sialylated forms of CD8. In transgenic DP thymocytes the heterogeneity of CD8 polypeptides was similar to that found in non-transgenic DP thymocytes, but the pattern of CD8 spots was changed. Transgenic DP thymocytes had an increased amount of CD8α which runs at 37kD and CD8β which runs at 30kD (labeled with asterisks) and decreased amounts of the smaller less sialylated forms of CD8 (circled). Therefore, consistent with the 1D analysis, a significant portion of the CD8 expressed by transgenic DP thymocytes is similar to the sialylated form of CD8 found in peripheral $CD8^+$ T cells.

Noncognate tetramer binding is not altered in ST3Gal-1 transgenic mice

Sialylation has been implicated in negatively regulating the ability of CD8 to bind to MHC I tetramers in a noncognate, i.e. non-TCR dependent, manner during thymocyte development (198, 199). This regulation of MHC I binding is depicted in the shaded histogram in Figure 16A in which normal non-transgenic thymocytes were stained with CD4 and CD8α (53-6.7) antibody, and K^b/OVA MHC class I tetramer at 4.5 μg/ml. DP thymocytes, which do not express ST3Gal-1, bind the K^b/OVA tetramer at higher levels than CD8 SP thymocytes, which upregulate ST3Gal-1. CD4 SP thymocytes do not bind the K^b/OVA tetramer.

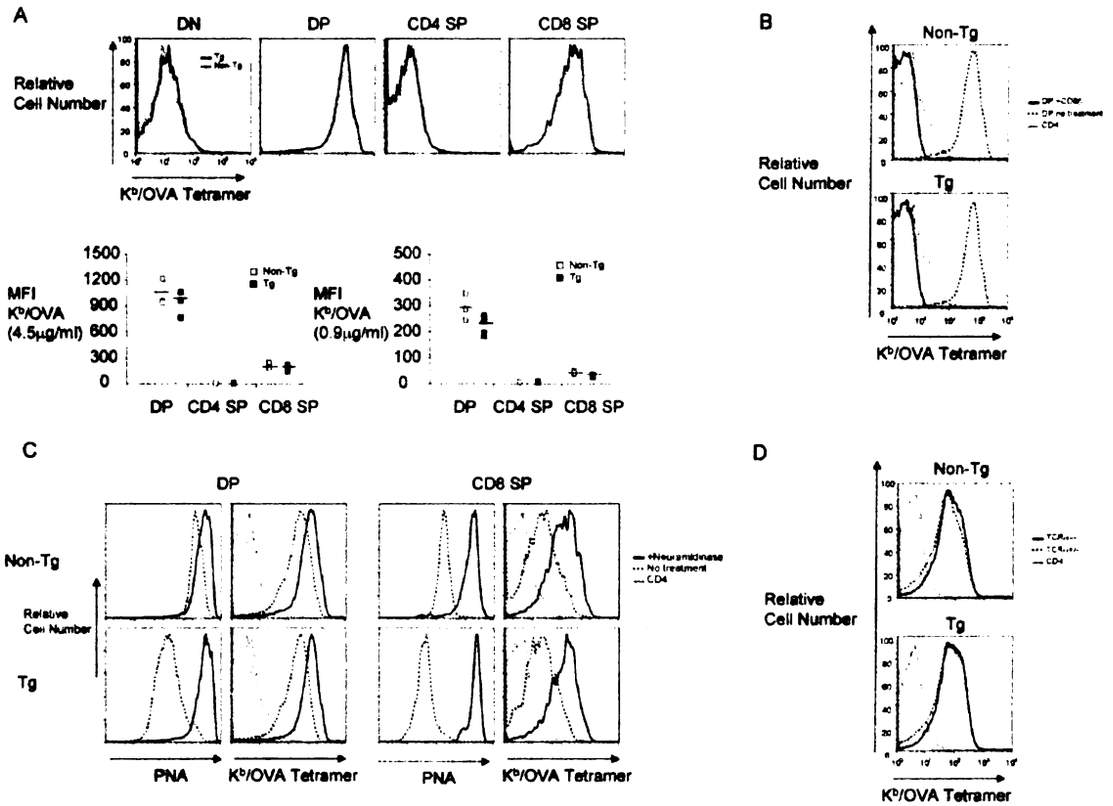


Figure 16. Noncognate tetramer binding on thymocytes

A, Thymocytes from ST3 Gal-1 transgenic and non-transgenic littermates were stained with CD4, CD8 (53.6.7), and K^b/OVA tetramer. The scatter plots show MFI of K^b/OVA tetramer staining from 2 independent experiments with 4.5 μg/ml (bottom right graph) and 0.9 μg/ml (bottom left graph) K^b/OVA tetramer. B, Preincubation of thymocytes with saturating concentrations of anti-CD8β monoclonal antibody (53-5.8) blocked K^b/OVA tetramer binding on DP thymocytes. C, Neuraminidase treatment of thymocytes induced PNA and K^b/OVA tetramer binding on thymocytes. D, K^b/OVA tetramer binding on DP thymocytes was TCR independent. For B, C, D, tetramer staining on CD4 SP thymocytes was used as a negative control.

Given that ST3Gal-1 expression inhibits the binding the MHC I to CD8, increased sialylation of core 1-O glycans in DP thymocytes of ST3-Gal-1 transgenic mice should reduce noncognate tetramer binding. When transgenic thymocytes were stained with K^b/OVA tetramer, noncognate tetramer binding on transgenic DP thymocytes was not significantly affected compared to that on non-transgenic DP thymocytes, despite the increased sialylation on CD8 in transgenic DP thymocytes. Although the levels of PNA staining and sialylation on transgenic DP thymocytes and non-transgenic CD8 SP thymocytes were roughly equivalent, the level of tetramer binding on transgenic DP thymocytes remained 5-fold higher than on CD8 SP thymocytes. These data indicate that increasing the levels of sialylation of core 1 O-glycans on the cell surface is not sufficient to inhibit noncognate tetramer binding by CD8.

As shown in Figure 16B, pre-incubation of thymocytes with anti-CD8β antibody 53-5.8 blocked noncognate tetramer binding on both transgenic and non-transgenic DP thymocytes consistent with other studies (198. Neuraminidase treatment increased the level of PNA staining and noncognate tetramer binding on both DP thymocytes and CD8 SP thymocytes consistent with previous reports that the addition of sialic acid inhibits tetramer binding (Figure 16C). Although neuraminidase treatment induced roughly equivalent PNA staining on CD8 SP and DP thymocytes, CD8 SP thymocytes bound MHC I tetramers at a lower level than DP thymocytes. Consistent with a previous report comparing tetramer binding on DP thymocytes from B6 and TCR?-deficient mice (Daniels, 2001 #81, 199), we found that the presence of TCR on DP thymocytes did not affect noncognate tetramer binding (Figure 16D).

Discussion

In this study, we describe the consequences of transgenic expression of ST3Gal-1 on T cell development. We have characterized a line of transgenic mice in which the level of sialylation in transgenic DP thymocytes was equivalent to that observed in normal SP thymocytes. Increased sialylation in DP thymocytes did not significantly affect thymic development and did not alter the selection of T cells expressing MHC I-restricted and MHC II-restricted TCRs. Peripheral T cells also appeared normal. Biochemical analysis indicated that CD8 proteins in transgenic DP thymocytes were more sialylated than normal DP thymocytes. However, although sialylation is implicated in negatively regulating CD8 function, increased sialylation of CD8 did not significantly affect the binding of MHC I tetramers on DP thymocytes.

Regulation of ST3Gal-1 expression in thymocyte development had led us to hypothesize that ST3Gal-1 downregulation in DP thymocytes was important to generate glycoprotein substrates, such as CD43, CD45, and CD8, in a desialylated state. Sialylation, which adds negative charge to proteins could alter their conformations, influence carbohydrate dependent interactions, and affect thymocyte development. For example, sialylation inhibits the capacity of CD8 to bind to MHC class I, which could affect thymocyte selection (198, 199). TCR and coreceptor (CD4 or CD8) interactions with peptide/MHC complexes influence thymic development (119-122). At the DP stage of development, normal thymocytes express desialylated forms of CD8. The downregulation of ST3Gal-1 in normal DP thymocytes may be important to enhance the

interactions of CD8 with MHC and assist T cells in achieving the threshold of TCR signaling that is necessary for positive selection. In principle, therefore, the increased sialylation in ST3Gal-1 transgenic thymuses could decrease the binding affinity between CD8 and MHC I and might cause defects in positive selection. Such defects were not observed in ST3Gal-1 transgenic mice.

In contrast to the lack of developmental defects in ST3Gal-1 transgenic thymuses, ST3Gal-1-deficient thymuses have an altered TCR repertoire and defects in mature $CD8^+$ T cell development (194, 199). These results are not necessarily inconsistent because the consequences of aberrant expression of ST3Gal-1 can differ from the consequences of loss of expression. Although the defects in $CD8^+$ T cell development in ST3Gal-1-deficient mice cannot be directly attributed to changes on a particular glycoprotein, alteration of CD8 function may explain the defects in development. Normally, molecules such as CD8 are sialylated in SP thymocytes. Loss of sialylation in SP thymocytes would enhance the ability of CD8 to interact with MHC I and could cause increased negative selection of CD8 SP thymocytes.

The CD8 coreceptor is encoded by two genes α and β and is expressed as CDαβ and CD8αα dimers (103). Both CD8αα and CD8αβ dimers bind to the α3 domain of MHC I, but may contribute to TCR signaling differently (86, 94-96, 110, 117, 235). The α and β polypeptides have similar extracellular structures (95, 96). Each polypeptide has one globular N-terminal Ig-like domain attached to a stalk region, and putative O-glycosylation sites have been identified on both α and β stalk regions (236). As yet, only

developmental regulation of sialylation of CD8β has been described (187). It is not clear whether sialylation changes on CD8α occur during development and how that influences the conformation of CD8.

Glycosylation is thought to hold the stalk regions of CD8 in extended conformations and therefore promote interactions of the globular heads with each other and with the α3 domain of MHC I (96, 190, 191, 237). One possible effect of sialylation is that the sialic acids add negative charge and bulk to the stalk regions, disrupting the interactions of the stalk regions, and leading to reorientation of the globular heads and reduced binding of CD8 to MHC I. The negative charges could also reduce the ability of CD8 to cluster on the cell surface and inhibit interactions of multivalent MHC I tetramers with CD8.

The CD8 molecules in transgenic DP thymocytes is similar to those found in CD8 SP T cells, indicating that sialylated forms of CD8 are enriched in transgenic DP thymocytes. Increased sialylation of CD8 was not sufficient to inhibit CD8's ability to bind to MHC I. This discrepancy between our results and reports that sialylation inhibits CD8 MHC I interactions can have several possible explanations.

One possibility is that sialylation alters the kinetics of binding of MHC I to CD8. In the absence of sialylation, the conformation of CD8 is optimal for binding to MHC I. In transgenic thymocytes, increased sialylation of CD8 could alter its ability to cluster or change its orientation, and inhibit the efficiency of binding to MHC I, but not necessarily

change the overall level of MHC I binding after long incubations. Kinetic analysis of MHC I tetramer binding on transgenic thymocytes would resolve this issue.

Another possibility is that critical O-glycan residues on CD8 required for inhibition of noncognate tetramer interactions remain unmodified in the transgenic mice. We have shown that transgenic expression of ST3Gal-1 increased the overall level of sialylation on the cell surface. Biochemical analysis indicates that CD8 from transgenic thymocytes had decreased mobility compared to non-transgenic thymocytes, and had similar mobility as sialylated CD8 isolated from SP thymocytes and peripheral $CD8^+$ T cells. Two-dimensional gel analysis shows that the sialylated forms of CD8 were enriched in transgenic thymocytes. Although this type of analysis is suggestive that CD8 from transgenic mice is more sialylated, more precise characterization of the structure of CD8 is required to show that the same residues are modified in transgenic DP thymocytes and in normal SP thymocytes. Characterization of the glycosylation changes on CD8 will help identify the contribution of O-glycans to CD8 function. In fact, recent studies using mass spectrometry have identified O-glycosylated residues in the CD8β stalk region that may influence CD8's ligand binding ability (238, 239).

Multiple enzymatic modifications are involved in the formation of carbohydrate modifications on proteins. Glycoproteins go through successive steps of glycan addition and remodeling by glycosyltransferases and glycosidases as they travel through the Golgi. Sialyltransferases are located in trans-Golgi compartments that act distally from glycosyltransferases found cis and medial Golgi (182). Differential expression of

enzymes and cofactors involved in glycosylation of CD8 in DP thymocytes and SP thymocytes could influence whether transgenic ST3Gal-1 expression would sialylate CD8 in DP thymocytes sufficiently to mimic the CD8 found in SP thymocytes.

The importance of sialylation in SP thymocytes has been demonstrated in ST3Gal-1-deficient mice. Although some of these developmental effects can be attributed to an alteration of CD8 function, other glycoproteins likely influence T cell development. Analysis of the changes induced on these substrates and a comparison of their functions in ST3Gal-1-deficient and transgenic mice will clarify the role of sialylation in thymocyte development.

Chapter 4: Discussion

Thymocyte development is intimately linked with movement within the thymus and specific interactions with a variety of stromal cell types. The mechanisms that regulate and coordinate thymocyte development and positioning are poorly understood. As thymocytes develop from the double-positive (DP) stage to the single-positive (SP) stage, they move from the cortex to the medulla. There is also a concurrent decrease in their ability to bind the lectin, peanut agglutinin (PNA), which binds desialylated core 1-O glycans at this step.

In one study, we have shown the involvement of CCR7, a receptor for the chemokines CCL19 and CCL21, in promoting the migration of thymocytes into the medulla. Premature entry of thymocytes into the medulla is accompanied by impaired development of mature T cells. In a separate study, we have examined the consequences of aberrantly expressing ST3Gal-1 sialyltransferase (ST3Gal-1), the enzyme that attaches sialic acid onto core 1 O-glycans, in DP thymocytes. We have found that expression of ST3Gal-1 in DP thymocytes does not obviously alter thymocyte development or affect CD8 coreceptor function.

Thymic compartmentalization

The thymus is divided into two main compartments, an outer cortex and an inner medulla. Thymocytes at different developmental stages are spatially organized within the

thymus, and critical processes in thymocyte development therefore occur in different places (1, 17). For example, positive selection occurs in the cortex and negative selection primarily occurs at the cortico-medullary junction and in the medulla. There are several possible reasons for separating these processes spatially and temporally. First, it may be more efficient to select initially for the ability of T cells to recognize self MHC and then to subsequently delete or anergize potentially autoreactive T cells that bind to self-MHC ligands with high affinity, rather than having positive and negative selection occur simultaneously. Second, spatial separation of these processes may allow for thymic stromal cells to have specialized functions, e.g. cortical epithelial cells mediate positive selection and medullary epithelial cells mediate negative selection. Third, multiple checkpoints are crucial to ensuring that only cells that express TCRs that are potentially useful later in life survive. Multiple checkpoints allow for further refinement of the processes of central tolerance and maximize the variability of the repertoire. Early in the evolution of the adaptive immune system, positive selection and negative selection may have been quite simple processes. However, evolutionary refinements, such as induction of tissue-specific gene expression in medullary epithelial cells, could have been introduced. If negative selection was not physically separated from positive selection, then these refinements to selection could be detrimental and lead to autoreactivity in the periphery. Finally, spatial separation allows thymocytes to change gene expression and respond differently to selection cues, depending on their thymic microenvironment, e.g. a cell upregulates *bcl-2* after positive selection and thus protect itself from deletion.

Studies with 2-photon microscopy have shown that thymocyte movement within the thymus is dynamic; thymocytes actively crawl and interact with stromal cells (240, 241). Different types of thymocyte movement have been described; some cells move randomly while others appear to move in a directed manner. These latter cells may be responding to cues such as chemokine gradients during different developmental stages. The guided movement of thymocytes indicates that directional cues must exist. For instance, it has been estimated that an average thymocyte would have moved 1000 μm from its starting point in a 4 day period, if it displayed a completely random walk (241). This is inconsistent with observations that thymocytes at specific stages of development are found in specific regions and require interactions with particular cells to develop. Directional cues, such as chemokines, are needed to direct efficient thymocyte movement and organize the thymus.

Role of CCR7 in thymic development

CCR7 is normally induced after positive selection. Its ligands CCL19 and CCL21 are expressed in overlapping stromal cell types, including epithelial cells, dendritic cells, and endothelial cells in the medulla and at the cortical medullary junction (152). Consistent with this expression pattern, transfers of CCR7-transgenic thymocytes resulted in accumulation of DP thymocytes at the corticomedullary junction and in the medulla. These data suggest that under normal circumstances CCR7 may be involved in inducing the migration of positively selected thymocytes into the medulla or retention of those cells in the medulla.

CCR7 may be involved in promoting thymocyte emigration. Neonatal mice lacking CCR7 or treated with a CCL19 antagonist show increased thymic numbers and decreased accumulation of T cells in the spleen (152). This defect in thymic emigration is not complete and is not seen in adult CCR7-deficient mice, suggesting that CCR7 plays a secondary or cooperative role in thymic emigration. Consistent with this idea, transgenic expression of CCR7 was not sufficient to induce thymic emigration. Other factors are likely involved in this process. In support of this possibility, a recent study has shown that a sphingosine-1-phosphate receptor $S1P_1$ directs thymic emigration (180). Mice deficient in $S1P_1$ do not have peripheral T cells because mature thymocytes cannot exit the thymus, resulting in accumulation of mature $CD62L^{hi}$ SP thymocytes in the thymus. This block in thymic exit indicates that $S1P_1$ is probably a major determinant in promoting thymic emigration with CCR7 as a secondary factor.

An alternative interpretation of these data is that CCR7 is involved in retaining mature thymocytes in the medulla. After positive selection, thymocytes migrate into the medulla where they reside for a period of up to two weeks, which may be important for their further maturation and negative selection (12, 224). A gradient of CCL19 and CCL21 may act to induce positively selected thymocytes to migrate into the medulla and then to retain mature thymocytes in the medulla or at the corticomedullary junction. After thymocytes have upregulated $S1P_1$, they can then be exported from the thymus.

Premature CCR7 expression caused defects in thymocyte selection and impaired development of both CD4 and CD8 SP T cells. Early removal of DP thymocytes from the cortex may disrupt their positive selection. Alternatively, premature entry into the medulla may result in increased negative selection. These results show that regulation of chemokines and their receptors is crucial for thymic organization, and positioning is important for proper development.

Crosstalk between thymic stromal cells and thymocytes is important for the development of both the thymic cortex and medulla. Mouse mutants that exhibit a block in early T cell development (e.g. mice with deficient *c-kit* and γ_c expression) have disorganized cortical epithelium, and mice that lack DP and mature SP thymocytes (e.g. RAG-1- and RAG-2- deficient mice) have small medullary areas (221-224). These results suggest that the presence of immature DN thymocytes is necessary to form a cortex and mature SP thymocytes are required to form a medulla. In our studies, we show that premature migration of DP thymocytes into the medulla does not recruit cortical epithelial cells into the medulla and does not suppress its formation. Thus, while formation of the medulla is dependent on SP thymocytes, it is not inhibited by DP thymocytes.

The Roles of CCL19 & CCL21

The phenotype of the CCR7 transgene on thymocyte development is rescued in *plt/plt* mice with deficiencies in both CCL19 and CCL21, indicating that interaction of

CCR7 with its ligands is required for the phenotype (215, 216). Efforts are underway to establish whether medullary localization of CCR7 transgenic cells is dependent on expression of its ligands in radioresistant stromal or hematopoietic cells by asking whether CCR7 transgenic cells could induce migration into the medulla in radiation bone marrow chimeras, in which the ligands were absent in either hematopoietic cells or radioresistant stromal cells. Studies have shown that radiation resistant stromal cells in peripheral lymphoid tissues express CCL19 and CCL21 and that hematopoietic components express low levels of CCL19 and CCL21 (215). Therefore, it seems likely that the CCR7 thymic phenotype is dependent on stromal expression of its ligands.

The individual contributions of CCL19 and CCL21 to medullary localization are not known. Additional experiments, such as transfers of transgenic cells into CCL19-deficient mice, will clarify whether one or both ligands are required for medullary localization.

Other chemokines may promote medullary migration

Positive selection induces the expression of a number of chemokine receptors (CCR4, CCR8, and CCR9) in addition to CCR7 (150, 153, 160-162, 165, 169, 170). There is no definitive evidence that these receptors are involved in promoting medullary migration other than circumstantial expression and *in vitro* migration data. As discussed in Chapter 2, CCR4 is an attractive candidate potentially involved in medullary migration because it is highly induced after positive selection in $CD69^{hi}$ DP thymocytes and its

ligand CCL22 (MDC) efficiently attracts $CD69^{hi}$ DP thymocytes in *in vitro* migration assays (153).

Although the receptors CCR4, CCR8, and CCR9 are upregulated at positive selection, their individual roles in thymocyte positioning have not been well studied. One possibility is that these receptors cooperate with CCR7 to induce migration into the medulla (Figure 17A). Studies of thymocyte positioning in mice deficient for these receptors or in mice deficient for multiple chemokine receptors will clarify their possibly redundant functions. These receptors may also coordinate regulation of medullary migration. For example, CCR7 may be involved in moving thymocytes to the cortico-medullary junction where its ligands are expressed at high levels and then another receptor such as CCR4 could promote migration further into the medulla (Figure 17B). Another possibility is that some of these receptors are involved in moving thymocytes closer to certain stromal cells that promote their negative selection or differentiation in the medulla (Figure 17C). Lastly, CCR7 could function to retain thymocytes in the medulla and expression of $S1P_1$ in mature thymocytes could promote their emigration from the thymus (Figure 17D). The possibility that CCR7 is involved in actively promoting thymic emigration, however, is unlikely, as we have shown that expression of CCR7 does not induce thymic exit.

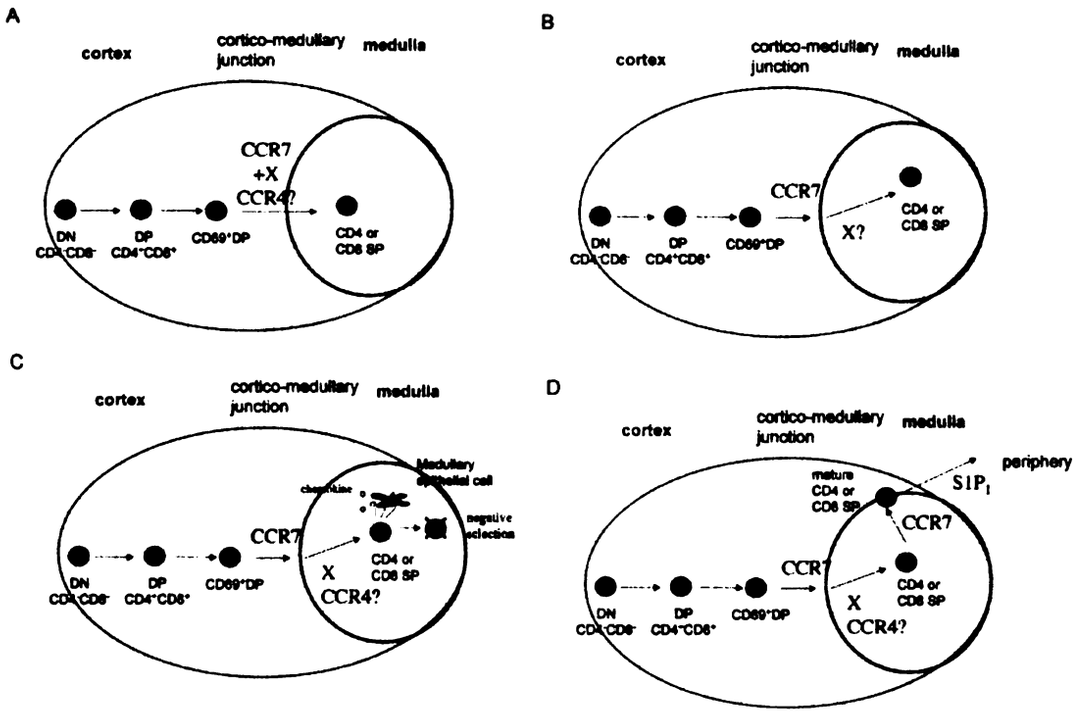


Figure 17, Models for how CCR7 and other chemokine receptors regulate migration into the medulla

A, CCR7 and chemokine receptor X, perhaps CCR4, cooperate to promote migration into the medulla. B, Coordinate regulation of migration into the medulla. CCR7 promotes migration to the corticomedullary junction and another chemokine receptor, perhaps CCR4, promotes further migration into the medulla. C, A chemokine receptor promotes migration to specific stromal cells in the medulla (e.g. medullary epithelial cells that mediate negative selection). D, CCR7 is involved in the retention of thymocytes in the medulla and $S1P_1$ promotes thymic emigration.

Glycosylation

Increased expression of ST3Gal-1 in DP thymocytes does not significantly alter thymocyte development or inhibit CD8 function in our studies. These results are not entirely consistent with previous studies that show that ST3Gal-1 deficient mice have altered thymic development or that sialylation inhibits CD8-MHC I interactions (198, 199).

Additional experiments to clarify these potentially conflicting results are needed. These studies include performing a kinetic analysis of MHC I tetramer binding on transgenic and non-transgenic thymocytes to determine whether sialylation changes binding kinetics. Mass spectrometry, to study the glycosylation changes on glycoproteins such as CD8, would identify the residues that are modified by glycosylation and sialylation in SP thymocytes and transgenic DP thymocytes. It would also reveal whether there is equivalent glycosylation and sialylation on specific proteins in transgenic DP compared to SP thymocytes.

Studies to characterize the effect of increased sialylation on other glycoprotein substrates such as CD45 could also be performed. We have treated ST3Gal-1 transgenic and non-transgenic thymocytes with a crosslinker (sulfo-EGS) to study homodimerization of CD45 on thymocytes. The results were not informative because thymocytes express a heterogeneous mix of CD45 isoforms and it was difficult to quantify the extent of homodimerization (data not shown). We have also examined the

ability of thymocytes to mobilize calcium in response to CD3 stimulation to determine whether increased sialylation affected TCR signaling. There was no difference in the ability of DP thymocytes, CD8 SP thymocytes, or CD4 SP thymocytes to mobilize calcium (data not shown).

Our hypothesis was that expression of ST3Gal-1 and increased sialylation in DP thymocytes, which do not normally express the enzyme, should cause defects in T cell selection and development. As yet, no impairment of T cell development has been observed. Additional experiments to find an effect in ST3Gal-1 transgenic mice remain. For instance, mixed chimeras of ST3Gal-1 transgenic and non-transgenic cells could reveal developmental defects in a situation where ST3Gal-1 transgenic cells have to compete with normal non-transgenic cells. A study has shown that the CD8 population in CD4-deficient mice is contaminated by MHC II-restricted T cells (242). The duration of signaling model would predict that in the absence of persistent signaling by CD4, T cells should develop into the CD8 lineage (131). However, the mechanisms that influence the development of MHC II-restricted CD8 T cells in CD4-deficient mice are not known. One possibility is that noncognate interactions of CD8 with MHC I could compensate for the lack of CD4 and lead to the development of these MHC II-restricted CD8 T cells. Analysis of ST3Gal-1-transgenic; CD4-deficient mice could reveal whether noncognate interactions of CD8 with MHC I are important for the selection and development of those cells.

Conclusions

These studies describe our efforts to understand how the thymus is organized. We have found that the chemokine receptor CCR7 is involved in thymocyte positioning. Additional experiments to characterize its ligands and to understand how CCR7 and other factors organize the thymus will be important. Our studies with ST3Gal-1 show that despite its very striking expression pattern in the thymus, transgenic mice, which express ST3Gal-1 aberrantly in DP thymocytes, had normal thymocyte development. The ability of CD8 to bind to MHC I in DP thymocytes was also unaffected by increased sialylation. These studies are in contrast to studies of ST3Gal-1-deficient mice in which lack of ST3Gal-1 in SP thymocytes led to defects in CD8 SP thymocyte development. Desialylation of CD8 in SP thymocytes increased its ability to bind MHC I. Our studies show that the effect of sialylation on CD8 function and T cell development may be spatially or temporally regulated. The precise role of sialylation on CD8 function remains to be established.

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