S. CHRISTINE HENDERSON-PACKTOR

Role of Ly-6A.2 Expression in Regulating Growth of T Cells in the Thymus and Periphery
(Under the Direction of ANIL K. BAMEZAI)

We demonstrate that Ly-6A.2 expression on T cells inhibits their growth, both in the thymus and periphery. The growth inhibitory effects observed in the thymus occur when Ly-6A.2 is expressed ectopically on developing cells that normally do not express this protein. Diminished amount of LAT in the $CD4^-CD8^-$ double negative (DN) subset, enhanced apoptosis of the $CD4^+CD8^+$ double positive (DP) cells and altered selected TCR-V_β repertoire is observed in Ly-6A.2 dysregulated thymocytes. These results also suggest the importance of regulated expression of Ly-6A.2 on developing T cells and also provides clues related to the mechanism underlying the thymic block. Ly-6A.2 exerts antigen-specific growth inhibitory effects on $CD4^+$ peripheral T cells when precociously over-expressed on the cell surface. CD4 T expressing dysregulated Ly-6A.2 cells exhibited reduced Ca^{2+} flux upon stimulation, indicating membrane proximal events were altered. A major significance of these observations is that Ly-6A.2 might regulate clonal expansion of $CD4^+$ T cells induced by a foreign antigen. Surprisingly, Ly-6A.2 over-expression on $CD4^+$ T cells generates a Th2 promoting factor, IL-4, and may therefore play a role in Th2 differentiation.

The chance observation that ectopic expression of Ly-6A.2 in MHC I and II-deficient mice rescued the $CD4^+$ T cell population was intriguing and provided a golden opportunity to examine the nature of the T cell repertoire that exists prior to positive and negative selection. We were surprised to note that the pre-selected T cell repertoire

exhibited considerable reactivity to MHC. These observations might explain the phenonmenon of allogenic reactivity by mature $CD4^+$ T cells.

INDEX WORDS: Ly-6A.2, Thymus, Development, Activation, Inhibition, Linker for Activation of T cells (LAT), $p56^{lck}$, TCR, TCR-V_β, Major-Histocompatibility (MHC), CD4, CD8, Cytokine, Interleukin-4 (IL-4), Transgenic (Tg)

ROLE OF LY-6A.2 EXPRESSION IN REGULATING GROWTH OF T CELLS IN THE THYMUS AND PERIPHERY

by

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DEDICATION

For my best-friend, Barry. I thank you for your continual and un-conditional loving support, all of the lunches you made (1,354 complete with motivational notes), all the hugs at the end of the day, the "western blot" song, and all the late nights you spent with me in the lab (so I wouldn't be alone). By taking interest in my work, you've always asked the right questions: "Did your experiment work?" "What's does the data tell you?" "Is there anything I can do to help?" And through this endeavor, which has brought out the best and worst of me, you enthusiastically agreed to add "husband" to the long list of brilliant adjectives that can be used to describe you. I am extremely grateful for your friendship, which helped me to persevere in completion of this degree.

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INTRODUCTION AND LITERATURE REVIEW

T Lymphocyte Development and Maturation

The thymus is a unique organ dedicated to shaping the T cell repertoire through processes of selection and lineage differentiation. Bone marrow-derived precursor cells entering the thymus undergo tightly regulated developmental progressions, which ensure production of functionally distinct helper and cytolytic subclasses of T cells that have sufficient T cell receptor (TCR) diversity, are self-MHC restricted, and can discriminate between foreign and self peptide they encounter in the periphery (1). The dynamic processes involving development and maturation of T cells in the thymus are poorly understood. Inherent to these successions are the ability of progenitor cells to respond to both intrinsic and extrinsic cues as they move through the thymic microenvironment. As a result of these interactions, the biological endpoints for developing thymocytes are differentiation, apoptosis and lymphocyte survival (2).

Thymus

The thymus is a bi-lobed organ encapsulated by a thick outer layer of connective tissue and a thin inner layer directly overlying the cortex (3). Branching fibroblast and type-1 collagen-rich trabeculae penetrate the cortex, an outer part of the thymus, and terminate at the cortico-medullary junction, thereby lobulating the thymus and providing a structural link to the inner medulla (4). The thymus constitutes three cell types: cortical epithelium, Ulex europeus agglutinin $^{+}$ (UEA) medullary epithelium, and bone marrow-derived hematopoetic cells- which include B cells, macrophages, and dendritic cells (5). Immediately lining the subcapsule and trabeculae is a basement membrane supporting a specialized flattened type I epithelium, which creates a barrier between the external and internal thymic environments (6). Although cortical and medullary capillaries are impermeable to blood-born material, entry of exogenous antigen does occur via the transcapsular route in the cortex. Thus, any circulating antigen has access to the thymic microenvironment.

While the basic thymic architecture is contained within an epithelial framework, two important components of the thymic microenvironment, epithelial cell-free regions (ECF) and the extracellular matrix (ECM) must also be considered (7). The ECF correspond to sites deficient in epithelium presenting MHC molecules, but are not devoid of stromal cells as they contain reticular fibroblasts and macrophages. The ECF are variable in size and can extend from the subcapsular region of the cortex into the deep cortico-medullary junction. The cortical macrophages housed in these areas are characterized by numerous lysosomes containing remnants of phagocytosed lymphocytes, which failed to complete development. Generally, the ECM consists of an integrated complex of multiple collagens, reticulin fibers, glycosaminoglycans, and

glycoproteins including laminin (LN) and fibronectin (FN) (8, 9). These proteins are usually immediately adjacent to or surround epithelial cells, and function to regulate many thymocyte-stromal cell interactions.

Thymic Precursor Cells

T cells in the fetal and adult thymus are a heterogeneous mixture of cells at different stages of development. It has long been established that thymocytes begin to alternate expression of cell surface antigens at distinct developmental checkpoints (10, 11). Extensive analysis of these cell surface markers has resulted in a molecular map of T cell development in the thymus. T cells arise from common lymphoid progenitor cells that seed the thymus from bone marrow. Development of T cells in the fetal thymus entails migration of early progenitor cells into the subcapsular region of a non-vascularized thymic rudiment (12-15). In contrast, bone marrow-derived progenitor cells can enter the adult thymus, which is highly vascularized, at the cortico/medullary junction (16-18). Once inside the thymus, progenitors that commit to the T cell lineage migrate into the subcapsular region of the outer cortex where the precursor cells interact with thymic stromal elements resulting in their proliferation and differentiation (19, 20). This migration and expansion is in part mediated by two key growth/cytokine receptors, c-kit and interleukin-7 receptor (IL-7R), which are expressed on pro-thymocytes. The ligands binding these receptors, stem cell factor and IL-7, are provided by epithelial stroma (21). Also expressed on these pro-thymocytes are high levels of Ly-6A/E (Sca-1), which has a uniquely regulated pattern of expression during thymocyte development. Murine stem cells do not express other major lineage- specific differentiation antigens and therefore Ly-6A.2 is one of the earliest differentiation antigens expressed on

thymocyte precursor cells (22). These earliest progenitors that have committed to the T cell lineage do not express CD3, CD4 or CD8 surface antigens and are referred to as the triple negative (TN) subset (23, 24). As these thymocytes move to the inner cortex they upregulate expression of CD4 and CD8 co-receptors and functional TCR/CD3 complexes. These double positive (DP) cells ultimately cross the cortico-medullary junction to migrate into the medulla where differentiated CD4 or CD8 single positive (SP) thymocytes exit the thymus and enter the peripheral circulation (25).

Trafficking of developing T cells in the thymus

An understanding of the basic architecture of the thymus, its cellular components and vasculature, is central to any discussion of thymocyte trafficking. However, little is known about the molecular cues that guide thymocytes to interact with stromal cells and other types of cells that deliver key inductive signals for development. Best described are the roles of ECM in regulating thymocyte migration. ECM effects are multi-factorial: for example they support the growth and function of thymocytes and epithelial cells; enhance cell-cell interactions including migration; and may bind soluble cytokines to provide locally high concentrations (26, 27). Thymocyte subsets differentially express ECM receptors, including CD44, β1 chain integrins, VLA-4, VLA-5 and VLA-6 (7, 28). Blocking FN receptors VLA-5 and VLA-6 inhibits differentiation of immature TN cells on thymic epithelial cells (29). Additionally, antibodies against FN cause down-regulation of the TCR/CD3 complex, thereby preventing thymocytes from continuing development (7). These and other studies demonstrate these interactions are critical for proper thymocyte development.

It has been only within the past decade that chemokines, chemokine receptors and adhesion molecules have been identified, that regulate the trafficking of mature T cells into the lymph node and spleen (30). As thymocytes have also been shown to respond to chemokines in a highly specific manner, it is believed that chemokines direct thymocyte trafficking and association with important stromal elements during development. Chemokines are basic polypeptides of about 100 amino acids, usually containing four cysteine residues linked by intra-molecular disulfide bonds. Four subfamilies are defined according to the spacing of two NH_2-terminal cysteines, with most chemokines falling into the CXC or CC class (31). Chemokines stimulate migration of responding cells by activating pertussis toxin-sensitive $G_{i\alpha}$ protein-coupled receptors. Several chemokines have been identified to play a role in thymocyte trafficking. SDF-1 is a CXC chemokine implicated in the homing of progenitors to the thymus and direction of recent immigrants to the outer cortex after arrival (32). Once seeded in the thymus these progenitors begin to migrate under the guidance of c-kit and IL-7R (35). Thymocytes also receive migratory signals from MIP-1α and lymphotactin as they progress from the TN to DP developmental stage (33, 34). Double positive thymocytes show a strong response to thymus-expressed chemokine (TECK) in chemotaxis assays, which may help drive thymocyte trafficking through the medulla before they exit to the periphery (36). Several other chemokine receptors have been identified, however, their function remains to be characterized (35).

Although regulated trafficking of thymocytes through the cortex into the medulla is critical for development of thymocytes, studies have shown that thymic organogenesis is a complex process that depends on mutually inductive thymocyte-thymic epithelial cell

(TEC) interactions to generate a functional environment capable of supporting T cell differentiation (37). This conclusion is based upon several observations using mouse systems in which thymocyte development is blocked at different stages of maturation. Mutations that inhibit development of thymocytes early within the TN subset result in a thymus that is extremely hypoplastic, contains atypically arranged cortical TEC and lacks an organized medulla (38, 39). In contrast, an organized cortex is present in recombination activating gene (RAG)-$1^{-/-}$ RAG-$2^{-/-}$ deficient and severe combined immunodeficiency (SCID) mice, which sustain a developmental block within the TN subset as thymocytes transition to DP cell stage (40). Thus, it is suggested that interactions between TEC progenitors and migrating thymocytes are required for development and organization of the thymic microenvironment.

Development of DN Thymocytes

As with most general outlines, the above mapping of thymocyte subsets can be further sub-divided into even more distinct stages of thymocyte maturation. Early TN thymocyte development can be broken down into discrete subsets characterized by differential expression of many cell surface proteins, including CD44 and CD25 surface antigens (Pgp-1 and IL-2 receptor, respectively) (reviewed in 2 and 41). The most immature thymocytes are $CD44^{+}CD25^{-}$, express high levels of c-kit and SCA-1 (Ly-6A.2) and low level expression of Thy-1 and heat stable antigen (HSA). Upon migration through the thymic cortex, the next developmental subset is characterized by up-regulated expression of CD25, HSA, Thy-1, and IL-7-receptor α. Down-regulation of CD44 and c-kit defines the transition of $CD44^{+}CD25^{+}$ to the next developmental cell stage, $CD44^{-}$

$CD25^+$, in which a successfully rearranged TCR_β joins the pre-T_α on the cell surface to form the pre-TCR. Thymocytes that have passed developmental checkpoints thus far reach the final TN stage marked by down-regulation of CD25. Upon expression of $TCR_{\alpha\beta}$ in conjunction with CD3 on the cell surface, late stage $CD44^-CD25^+$ TN thymocytes continue developmental processes by inducing expression of both CD4 and CD8 co-receptor molecules on the cell surface, thus transitioning into the DP stage of development.

The transition of TN into DP subset is not a trivial progression and the major event marking it deserves further consideration. The selective expansion of $CD44^-CD25^+$ TN cells, their differentiation into DP cells and the inhibition of further TCR_β rearrangement (i.e. allelic exclusion) are all dependent on signals delivered through the pre-TCR (42). The first component of the pre-TCR, a monomorphic surrogate α chain (pT_α) associated with CD3 chains, is expressed on the cell surface as early as the $CD44^-CD25^+$ TN subset. It is at this stage that $CD44^-CD25^+$TN thymocytes undergo somatic V-D-J rearrangement of their TCR_β genes (43). Once the final arrangement has been made, further TCR_β rearrangement is inhibited to ensure that each thymocyte expresses only one, clonotypic TCR. Once the final TCR_β product is expressed on the surface, it forms a transient heterodimer with the pT_α (44). The presence of this dynamic duo and its derived signals determine the fate of thymocytes as they transition to the DP stage. Proper signaling ensures that CD25 is down regulated, TCR_β locus rearrangements are inhibited and TCR_α rearrangements are induced. Transition into the DP stage is complete when thymocytes express a stable and complete $TCR_{\alpha\beta}$ heterodimer associated with the CD3 complex on the cell surface (45).

The importance of pre-TCR signaling on thymocyte development has been well documented. Mice with targeted disruption of genes encoding components critical for the generation and assembly of the pre-TCR including: RAG, $pT_{\alpha,}$ CD3 $_{\varepsilon,}$ $CD3_{\gamma,}$ $CD3_{\zeta, or}$ TCR_{β}- exhibit severe defects in T cell development (46-51). For example, experiments using mice with deletion of RAG-1/RAG-2 or with mutated TCR_{β} genes demonstrate that somatic TCR_{β} gene rearrangement is critical for this early phase of T cell development (47, 52). A predominantly $CD44^{-}CD25^{+}$TN population and an observed 60-fold reduction in thymus size suggests development is blocked at the final stage of TN development, thereby preventing further progression of these thymocytes to the DP subset. Rescue of these phenotypes requires introduction of rearranged TCR-α and -β transgenes, which not only promotes differentiation to the DP stage but also restores the thymus to almost normal size (52). These studies indicate that rearrangement of TCR_{α} and TCR_{β} loci and expression of a functional $TCR_{\alpha\beta}$ are necessary for transitioning of thymocytes into the DP subset.

Signal transduction emanating from the pre-TCR requires participation of several membrane-proximal signaling molecules, many of which have been identified to be critical components of thymocyte maturation within this early subset. The best characterized of these signaling molecules is the src family tyrosine kinase, $p56^{lck}$ (Lck). Lck phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMS) of CD3 chains. Signaling through the pre-TCR requires phosphorylation events induced by this Src-family tyrosine kinase. In mice, targeted disruption of the Lck gene, or transgenic expression of catalytically inactive Lck under the control of the Lck proximal promoter (which drives transgene expression in DN and

DP thymocytes), interferes with both cellular expansion and allelic exclusion at the TCR_{β} chain gene locus during the transition from the DN to the DP stage (54, 55). Thus, Lck is indispensable for initiating signaling to facilitate up-regulation of intracellular and cell surface molecules required for continual development past the DN stage.

Analyses of molecules downstream from Lck have revealed Linker for Activation of T cells (LAT) to be a component critical for the continual progression of thymocytes within the DN subset. LAT is an integral membrane protein further anchored by palmitoylation, which targets it to the glycolipid-enriched microdomains (GEMs) (56). The importance of LAT in T cell development has best been demonstrated using LAT-deficient mice, which exhibit a profound block in thymocyte development (57). Neither DP nor SP thymocytes are detected and thymocyte development appears to be blocked at the $CD44^{-}CD25^{+}$ TN stage. Additionally, no peripheral T cells are detected in these mice, further demonstrating the importance of LAT in the development of T cells. Although the regulatory effects of LAT signaling have not been clearly defined in the thymus, studies examining peripheral T cell activation have shown that participation of LAT in TCR-induced signal transduction is required for full activation (58, 59). Thus, it is conceivable that alterations in production and activation of LAT could inhibit proliferation of DP thymocytes undergoing selection and differentiation.

Proteins differentially expressed at stage-specific checkpoints may also modify TCR-transduced intracellular signaling. One well-described molecule known to regulate thymocyte development during different cell stages is Notch. Experiments suggest Notch signaling plays a critical role during the commitment of progenitor cells to the T cell lineage (60). Inducible deletion of Notch-1 in newborn mice results in a severe block

early in thymocyte development at the $CD44^{+}CD25^{-}$ TN stage. Phenotypic analysis based on expression of cell surface markers showed these progenitors were not committed to the T cell lineage. The majority of cells in this subset expressed B cell markers and phenotypically resembled immature bone marrow B cells. Additional support for involvement of Notch at this stage of development is provided by analysis of thymocytes from mammalian hairy and Enhancer of split homolog 1 (HES-1) $^{-/-}$ mice (61). Expressed in thymocytes and stromal cells, HES is known to be regulated by Notch signaling. $HES^{-/-}$ mice that live past gestation have a very small thymus that is devoid of mature thymocytes. In contrast, B cell lineage is un-altered in these animals. The observation that a deficiency of Notch-1 or its downstream moderator, HES-1 causes a block in early thymocyte development (but not other hematopoietic lineages) supports the hypothesis that Notch signaling is necessary for lymphoid progenitor cells to commit to the T cell lineage.

In addition to its role in T cell lineage commitment, Notch-1 has also been implicated in later stages of thymocyte development, consistent with its expression of thymocytes throughout their maturation. Following commitment to the T cell lineage, developing T cells differentiate into the αβ or γδ lineage. Analysis of T cell development in transgenic mice expressing the constitutively active intracellular domain of Notch-1 has provided evidence supporting a role for Notch signaling in αβ vs. γδ differentiation (62). Although the number of γδ T cells in these mice was normal, a higher percentage of them expressed markers of the αβ T cell lineage. Additionally, constitutive activation of Notch signaling overcomes the block in αβ T cell development in $TCR^{\beta-/-}$ mice (62, 63), but not in RAG-$1^{-/-}$ mice (64). The majority of DP thymocytes that developed in the

$TCR^{\beta-/-}$ Notch-Intracellular (NotchIC) mice had in-frame rearrangements of their genes for TCR γ and δ, suggesting that their development is dependent upon expression of a functional γδ TCR (62, 65). Based on these observations, a model was proposed suggesting that during normal development, thymocytes receiving a signal through the pre-TCR also receive a Notch-1 signal that directs them to the αβ T cell lineage (66).

More recently, a member of the hedgehog (Hh) family of secreted proteins, Sonic hedgehog (Shh), has been shown to play a major part in regulating the differentiation of the DN subset into the DP cell stage (380). The Hh signaling pathway is active during early thymocyte development (380). Shh is produced by the thymic epithelium, and its receptor molecules Ptc and Smo are expressed by DN thymocytes (378-380). In thymus explants, anti-Shh antibody can increase differentiation from DN to DP cell and Shh can arrest thymocyte development at the DN stage (380). Thus, termination of Hh signaling seems to be necessary for differentiation from DN to DP cells (380). Additionally, this suggests that Hh signaling up-regulates Smo in DN thymocytes but conversely, pre-TCR signaling down-regulates Smo expression in DN cells, suggesting a direct link between the pre-TCR and Hh signaling pathways (380). Together these findings suggest that Hh signaling plays an important part in thymocyte development by regulating the progression from DN to DP cell and by modulating pre-TCR signaling.

Stage-specific differential expression of Ly-6A.2 is also critical for thymocyte development. Ly-6A.2 belongs to a multi-gene family, which encodes for several proteins that are expressed differentially on cells of hematopoietic origin (reviewed in 67). One of the earliest lineage markers of thymic progenitors, GPI-anchored Ly-6A.2 can be detected on pluripotent hematopoietic stem cells migrating towards the thymus

and continues to be expressed until thymocytes reach the DP stage of maturation. Strict regulation ensures that DN thymocytes turn off expression of Ly-6A.2 before progressing to the DP subset. Following selection and differentiation processes, Ly-6A.2 is re-expressed on mature, single positive thymocytes as they begin to move out of the thymus and is detected on most $CD4^+$ cells and approximately 40% of $CD8^+$ cells in the periphery. The loss of Ly-6A.2 expression at the double positive stage is critical for thymocyte selection and differentiation processes. Constitutive transgenic expression of Ly-6A.2 on developing thymocytes results in a marked impairment in thymocyte maturation (68). Thymocytes in Ly-6A.2 transgenic animals are developmentally arrested at the double negative stage, which corresponds to the time point at which Ly-6A.2 is normally turned off. Both $CD4^+CD8^+$ and mature subsets appear in Ly-6A.2 transgenic thymi, however their numbers are reduced by >90% (68). The mechanism for this block is unknown.

Development of DP thymocytes

The $CD4^+CD8^+$ DP thymocytes are still immature and account for ~80% of the total population of thymic cells (71). These cells express a functional cell-surface $TCR_{\alpha\beta}$ heterodimer, which is required for participation of thymocytes in positive and negative selection events that shape the TCR repertoire. Although transitioning through the DN cell stage does not require ligation of TCR to MHC-presenting self-peptide, interactions between TCR-MHC are critical for the selection of DP thymocytes. Conceptually, selection of the TCR repertoire is confusing because it seems contradictory in nature. In order to maintain self-tolerance, T cells that recognize “self” proteins are deleted during

the process of negative selection. However, some recognition of "self" is required for positive selection, which permits maturation of $CD4^+$ or $CD8^+$ SP T cells. Based on these criteria, it would seem that since the condition of self-recognition is required for both negative and positive selection, then all thymocytes bearing a functional TCR would be marked for death. Additionally, there are other mechanistic questions: what cell types mediate positive and negative selection; what is the nature of the MHC/peptide complexes and other adhesion/signaling molecules that mediate selection; and what are the intracellular signals generated during these processes?

Nature of the T Cell Repertoire Prior to Thymic Selection

Rearrangement of the $TCR_{\alpha\beta}$ genes gives rise to 10^9 distinct receptors that can potentially recognize all self and foreign antigens (1). This vast TCR repertoire of immature thymocytes is largely due to V-D-J gene recombination. Because V-D-J recombination generates a random assortment of TCRs, the immature $CD4^+CD8^+$ DP population of thymocytes will express TCRs with all possible specificities. While germline V, D, and J regions of the combining site can be selected over evolutionary time, CDR3 residues affected by N-nucleotide addition or nuclease trimming cannot be evolutionarily selected. Since T cells are focused on the recognition of antigen through MHC presentation, it is plausible that the germline elements of the TCR would co-evolve with MHC molecules to promote effective and more precise ligation of TCR to MHC/ peptide complexes. Evidence for an evolutionary fit between TCR germline segments and MHC molecules initially came from *in vitro* examination of TCRs isolated from developing T cells of MHC-deficient mice (69). In this fetal thymic organ culture

(FTOC) system, where no selection by MHC molecules can bias the TCR, a substantial fraction of the individual TCRs isolated in the form of immortalized hybridomas showed functional reactivity to a random panel of MHC-bearing presenting cells. The results suggest that a minimum of 5.7% of the pre-selected repertoire is potentially subject to negative selection. Consistent with these findings are observations from an *in vivo* model in which ectopic expression of Ly-6A.2 resulted in maturation of CD4 SP cells in absence the of MHC I and II (70). Approximately 12-15% of the T-T hybridomas generated from Ly-6A.2 Tg^{+} MHC^{-} mice reacted with different MHC molecules. This was similar to the 10-12% of hybrids from normal mice that showed this reactivity. These results would suggest that allo-reactivity is inherent in the pre-selected repertoire, and is not the consequence of selection of a receptor that reacts with self-MHC/peptide in the thymus. Together, they suggest that the reactivity of the TCRs generated may not be as diverse and random as originally thought. Undoubtedly these receptors are very diverse but show intrinsic reactivity to MHC molecules.

Positive and Negative Selection

The distinguishing feature of the T cell lineage from other types of lymphocytes is the absolute requirement for TCR genes to be rearranged in a developmentally ordered manner (2). Thymocytes that do not generate and express a functional gene rearrangement cannot interact with MHC molecules and will be incapable of completing maturation. DP cells that do express a functional TCR face continual testing of the TCR repertoire as they migrate through the cortex and medulla. Those lacking specificity for self-ligands (self-MHC + self-peptide) in the thymus fail to receive signals required for

survival, and die by a process termed “death by neglect”. Thymocytes that have a relatively high avidity for thymic ligands receive signals that result in their deletion. On the other hand, thymocytes expressing TCRs that have a lower avidity for thymic ligands receive quantitatively or qualitatively distinct signals that allow for their survival and eventual development to mature T cells. This is the basis for positive and negative selection, in which the outcome is maturation of functional SP $CD4^+$ or $CD8^+$ T lymphocytes that have sufficient TCR diversity, are self-MHC restricted and can discriminate between foreign and self-peptide (72).

Positive selection is a process by which thymocytes expressing TCRs with low avidity to self-MHC molecules complexed with peptide are permitted to survive. Those thymocytes that lack the ability to bind self-MHC die. Weak recognition of MHC/self-peptide occurs when the avidity of the TCR interaction is low (73, 74). Although other molecules contribute to overall avidity, the affinity of a TCR to selecting MHC/self-peptide ligand and the number of TCR-MHC/self-peptide ligand interacting at the time of recognition primarily dictate the outcome of selection. Thus, the fate of the thymocyte depends upon the number of TCRs expressed, the number of MHC/self-peptide ligands presented by thymic stromal cells it contacts, and the affinity of each TCR for these selecting ligands. This process eliminates all non-self MHC-restricted T cells that would be in-capable of recognizing and responding to antigen being presented in the periphery. The outcome of positive selection is the generation of self MHC-restricted as well as potentially auto-reactive T cells.

During negative selection, clones of thymocytes expressing TCRs that bind with high affinity to MHC-self peptide ligands are either eliminated (clonal deletion) or

inactivated (clonal anergy) (75). The basis for negative selection is that approximately 97% of thymocytes that "audition" for selection die in the thymus. This leaves only 3-5% that continue the maturation process and go onto become CD4 or CD8 SP cells (76). There have been many studies to determine the impact of negative selection on the T cell repertoire. One approach has been to examine T cells that have been positively selected on diverse MHC/peptide ligands, but have not been negatively selected by bone marrow-derived APCs (78). In this study, transgenic expression of I-A^b MHC II was limited to thymic epithelial cells. Bone marrow-derived donor cells did not express I-A^b and therefore could not mediate deletion of thymocytes expressing TCRs reactive to this ligand. Limiting dilution analysis showed that 5% of selected cells mounted an allogeneic and strong syngeneic proliferative response to APC presenting I-A^b MHC II/peptide ligands. A similar study was conducted using a chimeric model in which host animals expressing MHC I and II were reconstituted with bone marrow that did not express these molecules (78). The results of this study showed there was a two-fold increase in the numbers of $CD4^+$ and $CD8^+$ T cells generated in the absence of negatively selecting bone marrow-derived cells. From these experiments it was estimated that in a normal selecting environment 50-60% of positively selected thymocytes undergo deletion. Other models have studied selection of thymocytes by a single ligand and examined the frequency of cells reacting to syngeneic MHC. Although it is believed that the repertoire selected by a single ligand is different from the repertoire generated by diverse MHC/peptide interactions, these studies suggest a range of 65-75% of the repertoire is reactive to self-MHC/peptide (79). Finally, experiments by Zerrahn *et al*., in which MHC reactivity of the T cell repertoire prior to positive and negative selection was

examined, suggested that a minimum of 5%-30% of the repertoire may be negatively selected in a normal mouse (69). Collectively, these studies have examined the broad T cell repertoire produced by different types of MHC/peptide interaction and suggest that a range of 5%-75% of cells that undergo positive selection are also eligible for negative selection.

The generation of TCR transgenic mice has been instrumental in clarifying some of the events surrounding selection. Analysis of mice expressing defined transgenic TCRs specific for MHC I or MHC II clearly showed that thymocytes were skewed towards the $CD8^+$SP or $CD4^+$SP lineage, respectively (80, 81). Self-reactive transgenic thymocytes were clonally deleted, supporting earlier studies that suggested this may be a mechanism for thymic self-tolerance. Although these findings set down the basic elements of thymocyte development, it became necessary to identify the other molecules and signaling events involved in order to understand the full complexity of thymocyte selection and differentiation.

Selection: Role of MHC Complexes

Perhaps the best-characterized molecules that exert the most influence on thymocyte development are major histocompatibility complexes (MHC) presented by thymic stroma. Thymocytes are incapable of recognizing soluble antigen with their TCRs and require its presentation by MHC molecules. The major histocompatibility complex is a collection of highly polymorphic genes arrayed within a long continuous stretch of DNA on chromosome 6 in humans and on chromosome 17 in mice (82). The MHC is referred to as the HLA complex in humans and as the H-2 complex in mice.

MHC molecules are integral membrane glycoproteins and their structures have been well studied at the crystallographic level. On their top surface, all of these molecules exhibit a deep cleft in which peptides are bound. A β-pleated sheet flanked by two α-helices make up the binding groove (83). The cleft is lined with many amino acid residues that vary among alleles, which could influence the specificity of peptide conformation. Although MHC I and MHC II molecules are structurally similar, there are important differences to consider.

MHC class I consists of a polymorphic heavy chain and a second non-polymorphic subunit, β2-microglobulin (β2-m) (84). In mice, MHC class I is encoded by the K, D, and L regions of the H-2 complex. The peptide-binding site of MHC class I consists of a deep groove in which a peptide of 8-11 amino acids can reside. Endogenous peptides are derived from cytosolic and nuclear proteins, which are degraded by large ATP-dependent proteasomes. The degraded products are translocated into the endoplasmic reticulum (ER) by a heterodimeric peptide transporter consisting of two proteins, TAP 1 and TAP 2 (85). The peptides may be further trimmed in the lumen of the ER to be of the proper length to bind MHC class I. As peptides are being prepared, newly synthesized class I heavy chain and β2-m are deposited in the ER and assembled into a heterodimer that binds the peptide (86). The resulting MHC I/peptide complex is transported through the Golgi to the cell surface for presentation to TCRs capable of interacting with MHC I/peptide complexes.

MHC class II molecules are encoded by IA and IE regions and exist as an αβ dimer with a peptide groove similar to that formed by MHC I. The notable distinction from MHC I is the ends of the groove appear to be open-ended, which allows for an

overhang of amino and carboxyl termini of bound peptides of 12-19 amino acids in length (87, 88). The αβ chains that make up MHC II are assembled in the ER where they associate with the invariant chain (Ii). The binding of the Ii, which has a sorting sequence located in the cytoplasmic tail, induces efficient transport of MHC II molecules from the ER to the trans-Golgi-reticulum (TGR) (89). At this point, the molecules are sorted to the endocytic route where the Ii is degraded, and MHC II molecules will contact internalized and degraded proteins (90). It was originally thought that MHC II molecules predominantly present peptides from exogenous antigens, but more recent studies have shown several peptides to be derived from other MHC or MHC-associated molecules, from the Ii chain, retroviral protein or transferrin receptor. In addition to these endogenous peptides, foreign peptides are also found (82). Thus, MHC II molecules predominantly present peptides derived from proteins occurring in, or directed to, the endosomal/lysosomal compartment of the cell.

During thymic maturation, progenitors trafficking through the thymus encounter both MHC I and MHC II molecules. These interactions occur first in the thymic cortex as progenitors begin to undergo maturation processes, and are continued in the medulla where thymocytes complete selective and differentiation events. Immunohistochemical staining and Brd-U labeling of thymocytes have given some insight as to the location of maturing subsets as they migrate through the thymus. It was reported that the most immature progenitors begin migration from the subcapsular layer of the cortex where primarily MHC I molecules are presented (91-93). Since these early progenitors do not express a TCR it was suggested that interaction between thymocytes and stromal elements in this region of the cortex are MHC-independent. As $CD44^{+}CD25^{-}$ TN cells

move through cortical epithelium, presenting both MHC I and II, they begin maturation processes that enable them to transition to the DP stage of development (94, 95). The cortical epithelium is arranged in a highly interconnected network of elongated cells, which form a web through which thymocytes spend a mean resident time of 3-5 days "crawling" towards the medulla. During this time thymocytes are continually interacting and responding to developmental cues from the thymic environment. The passage of thymocytes into the medulla requires interaction with a high concentration of bone marrow-derived dendritic cells and macrophages strongly staining for MHC I and MHC II at the cortico-medullary junction (96, 97). The medullary epithelial network is more tightly organized, with much shorter cytoplasmic processes than in the cortex (98). Expression of MHC molecules in the medulla differs both quantitatively and qualitatively from that in the cortex: the medullary epithelial cells all express MHC I but have varied expression of MHC II (10). Furthermore, there are non-polymorphic MHC II molecules (H-2O), expressed on bone marrow derived cells and medullary epithelium (90, 99). The most abundant cell type are reticulated cells with a dendritic morphology, which may play an active role in deletion of potentially auto-reactive cells. The passage of thymocytes into the medulla, and subsequent completion of development, requires interaction of progenitors with these cells.

Selection: Role of Self-Peptides

The earliest studies set out to examine how MHC/peptide ligands presented in thymocyte development alter directly the mature TCR repertoire (100-105). Nikolic-Zugic and Bevan used mutants of H-2K^b to examine T cell responses to ovalbumin

peptide. Using a series of bone marrow chimeras, they demonstrated a direct correlation between the ability of the MHC molecule to present ovalbumin and the ability of the same MHC molecule to select a TCR that could respond to the ovalbumin peptide (106). Further analysis of peptide-specific thymocyte selection became possible with the generation of gene-targeted mice lacking molecules associated with MHC I expression. For example, disruption of TAP genes prevents peptide transfer and results in an unstable MHC I molecule on the surface. Unstable MHC I surface expression is also found in the absence of β_2m. Studies have shown that development of mature $CD8^+$ SP thymocytes was dramatically reduced in β_2m or TAP-deficient mice (107, 108). This maturation was arrested due to a lack of positively selecting MHC I/peptide ligands. Addition of exogenous β_2m and mixtures of peptides to β_2m-deficient thymic lobes *in vitro* restored positive selection (109). The ability of researchers to manipulate further the system, such that only one or two MHC/self-peptide complex is available for TCR ligation, allowed the demonstration that although this single ligand was capable of supporting selection, the most diverse TCR repertoire requires a mixture of random peptides (110). Thus, the peptide bound to a selecting MHC molecule can alter the ability of this ligand to mediate either positive or negative selection. Additionally, these studies suggested that the peptides present during thymic selection have an impact in selecting the final TCR repertoire.

Selection: Role of Superantigens

Viral superantigens (SAg) are proteins encoded by the 3' long terminal repeat (LTR) of mouse mammary tumor viruses (Mtvs) (111, 112). These can be exogenous,

transmitted via maternal milk, or endogenous, as proviral integrations into the mouse genome, that are transmitted vertically in a Mendelian fashion. A number of different sites of integration of endogenous Mtvs have been reported in various inbred mouse strains. An open reading frame (ORF), within the long terminal repeat (LTR) of Mtv, encodes a type 2 integral membrane glycoprotein. The ORF products are expressed in association with MHC class II molecules attached at the cell surface and have an affinity for certain TCR-V_β chains such that $CD4^+8^+$ DP thymocytes expressing these TCR-V_βs undergo programmed cell death in mice carrying the appropriate endogenous or exogenous Mtvs (113). Mtv integrations have been characterized in several mouse strains and deletion of specific TCR-V_βs associated with expression of each has been described. For example, mice of the Balb-c ($H\text{-}2^d$) genetic background express Mtvs 6, 8, and 9, which are associated with deletion of TCR-V_β 3, 5, 11, and 17 (114). Examination of T cell selection by Mtvs has demonstrated that Mtv-mediated deletion of DP thymocytes constitutes a measurable part of negative repertoire selection of the T cell repertoire.

Selection: Epithelial versus Bone Marrow Derived Cells

Although an understanding of signal transduction is central to understanding how thymocytes respond to maturation cues, the role of thymic stroma, which presents selecting MHC/peptide ligands, must also be considered. There is evidence to suggest that positive and negative selection occur on different cell types. The tissue requirements for positive selection are more clearly defined. The development of MHC-deficient mice, in which transgenic expression of MHC I and MHC II molecules is limited to certain cell

types in specific areas of the thymus, has helped to establish that cortical epithelium is uniquely capable of supporting and directing positive selection (115). Additionally, these studies demonstrate medullary epithelium, total bone marrow, and dendritic cells are incapable of mediating this process. The exact tissue requirements for negative selection have been more difficult to elucidate. Use of the K14 mice in which I-A^b MHC II is transgenically expressed only on cortical epithelium demonstrated the inability of this tissue type to mediate negative selection (116). In this system neither medullary epithelium nor bone marrow-derived APC express MHC II molecules, thus all selection processes would have to take place on cortical epithelium. The $CD4^+$ SP cells selected to mature in these mice were autoreactive when tested *in vitro*, thus demonstrating this model was not capable of supporting negative selection. These findings are consistent with data presented by researchers using the rel-B system (117). This transcription factor is required for the development of thymic medullary epithelium and dendritic cells. In rel-$B^{-/-}$ animals, T cell development occurs only through thymocyte interaction with cortical epithelium. Crossbreeding rel-$B^{-/-}$ to B10.D2 mice, which undergo mtv/I-E-mediated clonal deletion of TCR $V_\beta 5$ and 11, results in very poor negative selection of these TCRs, further demonstrating cortical epithelium was not efficient at directing negative selection. Due to the large numbers of APCs that encompass the cortico-medullary junction and medulla, it has been speculated a majority of clonal deletion occurs between self-reactive thymocytes and hematopoetic APCs in this region. Hybridization studies performed *in situ* by Surh and Sprent showed these regions to be a site of active apoptosis, which supports the notion that MHC expressed on medullary

epithelium and bone marrow derived APCs in this region direct clonal deletion of potentially autoreactive thymocytes (118).

Selection Models

The mechanisms that distinguish positive and negative selection have not been clearly defined. Although it is understood that TCR-MHC/peptide ligation drives selection, the exact role of the peptide is not clear. It is the nature of the selecting ligand that is at issue; either qualitatively distinct peptides drive positive and negative selection, or the collective affinity for each TCR-MHC/peptide complex (avidity) affects fate of thymocytes. This debate has lead to the generation of two popular models for thymocyte selection: the qualitative/peptide model and the quantitative/avidity model.

The qualitative model proposes that qualitatively different peptides promote positive and negative selection (119-121). The outcome of selection relates exclusively to the peptide ligand being presented. Positive selection is achieved when unique, peptide-derived signals are delivered through the TCR. This model has been primarily supported by studies demonstrating that non-stimulatory antagonist peptides could promote positive selection. Using the OT-1 TCR transgenic mouse, which is specific for ovalbumin presented on H-2K^{b}, researchers were able to show that antagonist peptides were capable of supporting positive selection, while stimulatory agonist peptides promoted clonal deletion (122, 123). The qualitative model was also supported by studies showing that interactions with altered peptide ligands produced very distinct intracellular signaling, including altered TCR/CD3ζ-chain phosphorylation and a lack of

ZAP-70 activity (124, 125). These studies demonstrated the qualitatively different signals induced by qualitatively different peptides directly affect thymocyte fate.

Several studies have questioned the interpretation of these results. For example, antagonist peptides have been shown to induce clonal deletion, inhibit negative selection, or inhibit T cell development (126-128). Additionally, Ignatowicz *et al*. demonstrated that mature T cell function was not antagonized by the same positively selecting ligand (79). Furthermore, virus-mediated delivery of defined peptides in an *in vivo* model did not show a correlation between antagonist activity and positive selection (129). Collectively these studies demonstrate positive selection is not strictly associated with presentation of antagonist peptide ligands. Also called into question is the concept that antagonist peptide ligands deliver unique signals through the TCR. Using a panel of altered peptide ligands, ranging from agonist to antagonist, researchers showed a gradient of intracellular signals could be produced (129-131). Upon examination of phosphorylation profiles, researchers found that they could reproduce phosphorylation patterns observed in antagonist stimulation by using a low concentration of agonist peptide. Thus, the altered peptide ligands used in this study were shown to induce quantitatively but not qualitatively different intracellular signals.

The quantitative/ avidity model postulates that cell fate is determined by cumulative thymocyte interactions that are governed, in part, by avidity (132-134). These interactions are "quantitated" and thymocyte fate is determined via thresholds that define positive and negative selection. The avidity model proposes that positive selection is the result of low avidity thymocyte interactions, whereas high avidity interactions elicit negative selection. Furthermore, molecules that affect thymocyte-stromal cell interaction

are addressed and become a part of the big picture. It is suggested that co-receptors and adhesion molecules influence the duration or strength of TCR signaling and therefore make an impact on thymocyte fate. Thus, key to the avidity model are TCR affinity for MHC/peptide and the overall avidity of the TCR for these ligands expressed on thymic stromal cells. In this model, multiple TCR-MHC/peptide interactions are integrated to form a signaling gradient by which cell fate is determined (135). Either numerous weak TCR/MHC interactions or a few strong TCR-MHC/peptide interactions would meet the criteria for positive selection. Stronger signals would be needed to reach the tolerizing threshold. In the past, the main argument against the avidity model was that T cells selected on agonist ligands were essentially non-functional when stimulated *in vitro* (136). However, several groups have demonstrated that functional T cells can be generated in the presence of an agonist peptide in both MHC I and MHC II models (137-140). These studies showed thymocytes selected on weak peptide agonists required increased ligand concentrations in order to elicit a functional T cell response.

The use of TCR transgenic mouse lines bred into different MHC-deficient backgrounds has been used extensively to generate evidence to support the idea that quantitative/avidity interactions governed thymocyte selection. Using these models, two separate groups were able to induce positive selection using low concentrations of a strong peptide agonist whereas clonal deletion occurred when using high concentrations of that same peptide (141, 142). Similar results were reported by investigators using both MHC I and MHC II models in which the they were able to control and quantitate the level of cell surface expression of a single MHC/peptide ligand (143-145). Positive selection occurred when expression of a single MHC/peptide ligand was low, yet high

cell surface expression of the same MHC/peptide ligand resulted in negative selection. Additionally, using the ova-specific TCR transgenic mouse model, direct affinity measurements conducted by Alam, *et al*. demonstrated that positively selecting ligands had a significantly lower affinity for transgenic TCR than negatively selecting ligands (146). Taken together, these studies indicate that different concentrations of the same peptide/ligand can mediate both positive and negative selection, which is in support of the quantitative/avidity model.

Although debate on these models is ongoing, mapping the signaling events following initial TCR-MHC engagement may help to define the mechanisms that drive positive and negative selection. Interactions between TCR and MHC/peptide ligands during the selection process have been shown to result in internalization of TCR (147, 148). Studies have shown that strongly stimulating MHC/peptide ligands known to induce negative selection also promote maximal TCR internalization. Conversely, weaker ligands that promote positive selection were unable to trigger these maximal levels of TCR internalization, which resulted in prolonged interaction (149-151). It is hypothesized that maintaining contact with the selecting MHC/peptide ligand allows for continual stimulation through the TCR/CD3 complex. This maintained stimulation could provide survival signaling otherwise not received when "communication" through the TCR is shut down quickly as in negative selection (152). In order to better understand signaling involved, exhaustive research has been directed towards generating a molecular sequence of proximal to distal signaling events emanating from the TCR/CD3 complex.

Lineage Commitment

Positive and negative selection are not the only developmental processes thrust upon DP thymocytes. These cells must also undergo lineage commitment, a process that drives differentiation of DP thymocytes into $CD4^+$ or $CD8^+$ SP T cells. As with positive and negative selection, the mechanisms are not well understood. It is generally accepted that during thymic selection, DP thymocytes that recognize and co-engage MHC I with their TCR and CD8 co-receptor down-regulate CD4 and progress towards the CD8 T-cytotoxic lineage. Conversely, selection of thymocytes on MHC II results in down-regulation of the CD8 co-receptor and maturation toward the CD4 T-helper subset (153-156). In an attempt to explain the correlation between MHC specificity and co-receptor expression, two lineage commitment models were initially proposed: "The Stochastic" and "Instructional Models" for lineage commitment.

The stochastic model proposes that commitment to the CD4 or CD8 lineage occurs independently of TCR specificity. Differentiation occurs as the thymocyte randomly (stochastically) shuts down either its CD4 or CD8 co-receptor. The thymocyte is "tested" before leaving the thymus to make sure the proper co-receptor for TCR specificity is being displayed (157-159). Since MHC I recognition by specific TCR requires co-engagement of CD8 and MHC II recognition by specific TCR requires co-engagement of CD4, only cells with the appropriately matched TCR and co-receptor are allowed to complete maturation processes.

The instructive model proposes that a distinct signal is provided to precursor $CD4^+CD8^+$ DP cells by co-engagement of the TCR and either CD4 or CD8. It is hypothesized that the "instructive" signal is given through the co-receptor that is engaged with MHC (160). The different signals drive the cell into one or the other developmental

lineage, with concomitant extinction of expression of the inappropriate co-receptor gene. Thus, co-engagement of MHC I-specific TCR and CD8 co-receptor results in "instructive" signaling which down-regulates expression of CD4. The result of this interaction is the generation of a $CD8^{+}$SP T cell. Conversely, recognition and co-engagement of a MHC II-specific TCR and CD4 promotes a program to turn off CD8 co-receptor expression, thereby producing a $CD4^{+}$SP T cell.

Attempts to distinguish between these models have mainly used mice bearing TCR or co-receptor transgenes and/or lacking expression of either MHC or co-receptor molecules. In a stochastic/selection model, the transgenic expression of a co-receptor whose MHC specificity matches that of the TCR should rescue thymocytes expressing an incorrect match. Indeed, it has been shown by two separate groups that an MHC I-specific TCR expressing a CD4 co-receptor and an MHC II- specific TCR expressing a CD8 co-receptor can be rescued by constitutive transgenic expression of CD8 or CD4, respectively (161-163). Additionally, the existence of transitional subsets $CD4^{+}CD8^{lo}$ in MHC II-deficient mice and $CD4^{lo}CD8^{+}$ in MHC I-deficient subsets fueled the belief that lineage commitment was stochastic (164, 165). However, recent studies have offered data to challenge this proposed model. Data emerged to show rescue of T cells with mismatched MHC recognition and co-receptor expression was inefficient when transgene-encoded co-receptors were expressed at physiological levels. Co-receptor re-expression assays and thymocyte transfer experiments demonstrated that the $CD4^{lo}CD8^{+}$transitional subset contained precursors of both the CD4 and CD8 lineage (166, 167). These results suggested that an instructive model might be correct. Finally, the instructive model receives its strongest support from experiments showing that

swapping the cytoplasmic tails of the CD4 and CD8 proteins alters the phenotype of maturing T cells with a fixed transgenic TCR specificity (168, 207). When expressed in a transgenic mouse model, this chimera directed the differentiation of T cells with MHC class I -specific TCRs into CD4 lineage.

More recent data has led some investigators to propose a modified instruction model that emphasizes a quantitative rather than qualitative effect of conjoined TCR/co-receptor signaling on cell fate. This is based on experiments in which cross-linking thymocyte TCRs with antibodies resulted in the generation of CD4 but not CD8 T cells (171, 172). It is presumed that the aggregation caused by cross-linking induced stronger signaling conducive to CD4 maturation. Conversely, cross-linking with modified antibodies that did not cause aggregation resulted in maturation of CD8 T cells, presumably because a weaker signal was produced (173). This model has been further refined in recent years by ascribing the discrimination between the CD4 and CD8 co-receptors contributions to a quantitative difference in the signals provided by these molecules. Collectively, these experiments have led to the hypothesis that the CD4 co-receptor contributes to stronger signaling and therefore the generation of CD4 T cells, while the weaker signal delivered by the CD8 co-receptor directs maturation of CD8 T cells. Thus, the focus has been to find the mediator of signaling that brings about such distinct SP T cell subsets.

In an attempt to reconcile both the instructive and selective/stochastic models a new idea has been proposed. An influential series of studies involving the examination of cells believed to be intermediates in the differentiation of DP cells into mature CD4 or CD8 SP cells has been the basis for this new model. A key assumption made while

analyzing phenotypic surface markers on thymocytes has been that maturation from the double positive stage to $CD4^+8^-$ or $CD8^+4^-$ SP lineages occurs in a simple manner with either co-receptor gradually down regulating to null expression. However, recent experiments have shown that passage from the DP to the SP subsets does not simply involve shutting off one or the other co-receptor (167). Using co-receptor re-expression assays, investigators have shown that $CD4^+CD8^{lo}$ thymocytes contain subpopulations of cells able to re-express only either CD4 or CD8, consistent with their having undergone lineage commitment. In MHC I-deficient mice, only cells re-expressing CD4 molecules were found among $CD4^+$ $CD8^{lo}$ thymocytes. Remarkably, thymocytes cultured from MHC I and MHC II-deficient mice re-expressed only CD4 molecules. Investigators concluded that thymocyte commitment to the CD8 lineage required MHC I-dependent instructional signals, whereas thymocyte commitment to the CD4 lineage was MHC-independent and would occur by default. Thus, a new model of asymmetric signaling requirements, which draws from both the stochastic/selective and instructive models for thymocyte differentiation, was proposed.

Building upon the idea of asymmetric signaling was a report from Lucas and Germain, which suggested an even more complex regulation of CD4/CD8 co-receptor expression during thymocyte differentiation (174). These investigators demonstrated double positive thymocytes undergoing selection initially shut down both co-receptors. These CD4/CD8 "dull" cells then re-express CD4 or CD8 molecules in an asymmetric fashion: the rate of CD4 re-expression exceeds that of CD8 by 2- to 4-fold thus giving rise to a $CD4^+CD8^{lo}TCR^{int}$ population. At this stage the cells activate a lineage-specific differentiation program that results in selective suppression of CD4 or CD8 synthesis.

Thymocytes whose TCRs engage MHC II molecules continue along the $CD4^+$ $CD8^-$ TCR^{hi} single positive pathway and completely shut off CD8 transcription, whereas thymocytes whose TCRs engage MHC I molecules continue up-regulation of CD8 co-receptor genes while turning off CD4 transcription. This new understanding of the complex relationships between co-receptor expression and thymocyte differentiation invalidates arguments in favor of stochastic/selective model that were based upon analyses of the transitional cells in MHC-deficient mice. In contrast, they imply that enhanced survival is the first event accompanying TCR engagement in the thymus, preceding lineage commitment. Although this model could not distinguish between instructive vs. selective/stochastic models, it does argue against a default pathway for the CD4 population. Additionally, these findings provide a means of isolating purified populations of cells for identifying the selective mechanisms that influence lineage commitment.

Thymocyte Selection and Lineage Commitment: Cell Signaling

Several membrane-proximal signaling molecules have been identified to be critical components for the further development of DP thymocytes. The earliest molecule to move into the TCR/CD3 complex is the src family tyrosine kinase, $_p56^{lck}$ (Lck). Lck phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) of TCR-associated ζ chains as well as CD3 ε, γ, δ, chains (175). Lck is important in early (TN to DP) and late (DP to SP) transitional stages of thymocyte maturation (154). Signaling through the pre-TCR requires phosphorylation events induced by this Src-family tyrosine kinase. In mice, targeted disruption of the Lck gene,

or transgenic expression of catalytically inactive Lck under the control of the Lck proximal promoter (which drives transgene expression in DN and DP thymocytes), interferes with both cellular expansion and allelic exclusion at the TCR_{β} chain gene locus during the transition from the DN to the DP stage. Use of the Lck distal promoter, which is active mainly in mature thymocytes, has enabled researchers to circumvent early developmental arrest observed with the proximal promoter, thereby allowing them to study the role of Lck in the maturation of DP into SP thymocyte (177). In these mice the overall number of SP thymocytes is reduced, indicating that positive selection is inhibited. Once phosphorylated by Lck, ITAMs recruit ZAP-70 to the activated receptor through a high affinity interaction, which involves both src homology 2 (SH2) domains of ZAP-70 (178). This facilitates subsequent phosphorylation and activation of ZAP-70 by Lck. Studies have shown that ZAP-70 is crucial for both positive and negative selection (179). In mice deficient in ZAP-70 or carrying a mutation that inactivates ZAP-70 kinase function there is an arrest at the DP to SP stage of development. Only over-expression of syk, a related kinase, can restore thymocyte development and maturation of functional CD4 and CD8 T cells (179, 180). One of the identified substrates of ZAP-70 is the adaptor protein LAT (linker for activation of T cells) (181). LAT is an integral membrane protein further anchored by palmitoylation, which targets it to the gylcolipid-enriched microdomains (GEMs). Palmitoylation and partition to the GEMs has been shown to be essential for phosphorylation of LAT and for T cell activation. The importance of LAT in thymocyte development has been best demonstrated using LAT-deficient cell lines and mice. LAT deficient T cell lines are defective in calcium mobilization, Ras activation and IL-2 gene expression (182). $LAT^{-/-}$ mice have a

profound block in thymic development (178, 183). Neither DP nor SP thymocytes are detected and thymocyte development appears to be blocked at the $CD44^{-}CD25^{+}$ TN stage. No peripheral T cells are detected in these mice. The intracellular domain of LAT contains a number of tyrosine residues, which upon phosphorylation are capable of associating with a number of molecules including Grb-2, Grap, Gads, PLCγ-1, phosphatidyγl inositol (PI)-3 kinase, SLP-76 and Cbl (184, 185). Activation of PLC results in the hydrolysis of phosphoinositol-bi-phosphate (PIP2) into inositol tri-phosphate (IP3) and diacylglycerol (DAG) (186). These molecules are instrumental in stimulating the release of intracellular Ca^{+} and activation of the calcineurin/calmodulin pathway. Signaling emanating from this pathways leads to the translocation of nuclear factor of activated T cells (NF-AT) into the cell nucleus, where it activates AP-1 to transactivate a variety of genes (187). The signaling cascade described above has taken years to map, and new members are continually being added to this scaffolding. The specific interactions that dictate whether or not a thymocyte completes maturation and developmental processes will most likely come from understanding how these molecules and pathways intertwine.

Role of Accessory Molecules in Thymocyte Selection

The TCR repertoire generated is sufficiently diverse to respond to a wide array of foreign peptide presented on self-MHC. In the periphery, it is estimated that MHC molecules present 10^{3}-10^{4} different self-peptides on an average of 10^{5}-10^{6} MHC molecules on the cell surface (188). However, thymic epithelial cells express approximately 10-fold lower MHC levels than those in peripheral tissues (189). It is

clear that interactions between thymocyte TCRs and MHC/peptide ligands play an important role in selection and generation of a diverse TCR repertoire. However, if thymocytes require prolonged contact in order to receive and generate signaling necessary for development, it would seem implausible that such a limited number of MHC/peptide interactions would be sufficient to mediate this interaction. Thus, the contribution of surface molecules that may modify TCR-transduced intracellular signaling and subsequently influence thymocyte development has been the focus of many studies.

In regards to positive and negative selection the obvious molecules known to affect T cell survival and stimulation in the periphery have been examined. However, much of the data are conflicting. The tumor necrosis factor receptor (TNFR) family member Fas (CD95) has been implicated in clonal deletion, but only when abnormally high concentrations of antigen are used in the selection system (190, 191). Systems testing other TNFR members and their downstream moderators, including CD40, CD30 and CPP3 are also inconclusive (192-195). Additionally, the B7-1/2 interactions with CD28, which are reported to enhance T cell signaling in the periphery, do not appear to be a requirement for thymocyte maturation (196-198). It is possible that these surface receptors trigger signals that overlap with signaling cascades emanating from the TCR, thus modifying signaling pathways. However, in general the significance and contributions of these molecules in thymocyte selection remain unclear.

The most convincing candidates for directly influencing maturation are the CD4 and CD8 co-receptor molecules. Initially believed to only provide stability to the TCR-MHC complex, these molecules have recently been shown to play a role in augmenting

signal transduction via the TCR. There are data to suggest levels of signaling though Lck is linked to lineage commitment (199-206). Lck associates non-covalently with the ITAMS on cytoplasmic tails of CD4 and CD8 and becomes catalytically active when these co-receptors are cross-linked (203, 205). It has been demonstrated by Weist, et al. that there is a higher association of Lck with CD4 versus CD8 (200). Examination of DP cells showed 25-50% of surface CD4 but only 2% of CD8 are associated with Lck. From these observations it is hypothesized that the amount of Lck associated with each co-receptor as it is brought into the TCR/CD3 complex plays a key role in lineage commitment (202-204).

Support for this model comes from a number of studies utilizing many different experimental approaches. Using the AND-MHC II-specific TCR system, Matachek et al. showed thymocytes lacking a CD4 co-receptor differentiated into CD8 SP cells (206). Conversely, altering the cytoplasmic tail of CD8 co-receptor in such a way to promote Lck association results in the generation of CD4 SP cells (207). In another approach, Yasutomo *et al*. proposed that the duration of antigen receptor signaling determined the CD4 versus CD8 lineage fate (208). In this study, an *in vitro* culture system was used to examine the outcome of the interaction between TCR and MHC II molecule lacking a CD4 binding site. As expected, TCRs interacting with normal MHC II resulted in differentiation of CD4, whereas interaction with mutant MHC II resulted in differentiation of CD8 cells. Finally, using transgenic mice expressing constitutively active or dominant-negative forms of Lck, Hernandez-Hoyas *et al*. showed that increase in Lck activity can induce MHC I-specific thymocytes to differentiate into CD4, whereas diminished levels of Lck induced MHC II-specific thymocytes to differentiate into the

CD8 lineage (209). These studies support an instructive model that emphasizes a quantitative rather than qualitative effect of conjoined TCR-coreceptor signaling on cell fate.

In addition to a role in commitment of precursor cells to the T cell lineage and differentiation of αβ versus γδ thymocytes, there is evidence to suggest that Notch signaling influences differentiation of thymocytes to the CD8 lineage (210). Investigators studying NotchIC transgenic mice noticed these mice had an approximately 10-fold increase in the number of $CD8^+$ SP thymocytes and a slight decrease in the number of $CD4^+$ SP thymocytes. Brd-U labeling confirmed a 3-fold increase in the rate of production of $CD8^+$ thymocytes, and a 5-fold decrease in the rate of production of $CD4^+$ thymocytes. On the basis of these results, it was proposed that Notch-1 signaling functions to regulate the $CD4^+$ versus the $CD8^+$ cell fate choice during normal T cell development. According to this model, DP thymocytes that are selected on MHC class I also receive a Notch-1 signal that directs them to the $CD8^+$ lineage whereas DP thymocytes that interact with MHC class II do not receive a Notch-1 signal and develop along the $CD4^+$ lineage. Support for this model comes from TCR transgenic experimental models in which Notch is constitutively expressed (210). In the presence of constitutive Notch-1 signaling, DP thymocytes expressing a MHC II-specific TCR mature into the CD8 lineage. Additionally, transgenic expression of NotchIC in MHC class I deficient mice, where no maturation of CD8 thymocytes should occur, resulted in the rescue of the $CD8^+$SP subset. Interestingly, this rescue was not observed in mice deficient for both MHC I and II suggesting that signaling through TCR-MHC interactions

prior to lineage commitment was required for survival, independent of TCR-MHC specificity.

Differential expression patterns of Ly-6 family proteins make them candidate molecules for mediating development of T cells. As previously described, expression of Ly-6A.2 is highly regulated on developing thymocytes. Ly-6A.2 is expressed on progenitors immigrating into the thymus, and continues to be detected until thymocytes transition into the DP subset. Following selection and lineage commitment, thymocytes re-express Ly-6A.2 on SP T cells, as they migrate into the periphery (68). Another Ly-6 family member, Ly-6d (Thb), shows a reciprocal pattern of expression to Ly-6A.2 during T cell development (211). This molecule is expressed on a fraction of $CD4^-CD8^-$ and a majority of $CD4^+CD8^+$ thymocytes. Its expression is down regulated on a subset of $CD4^+CD8^+$ thymocytes that have received initial signals for thymic selection but have not yet down-regulated the CD4 and CD8 co-receptors, and remains extinguished on all the mature CD4 and CD8 T cells in the thymus and periphery. More importantly, interaction of TCR/coreceptor with the self-MHC-peptide contributes to the down-regulation of ThB expression on developing thymocytes. Finally, during T cell development in the thymus, the expression of thymic shared antigen-1 (TSA-1)/stem cell antigen-2 (Sca-2), a GPI-anchored differentiation antigen, is also developmentally regulated. The expression level of TSA-1 is the highest in most immature $CD4^-CD8^-$ thymocytes, high in $CD4^+CD8^+$ thymocytes, but barely detectable in mature $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes and peripheral T cells.

Regulated expression of Ly-6A.2 during thymocyte development has been shown to be critical for proper thymocyte maturation. Continued expression of Ly-6A.2 results

in developmental arrest within the DN cell stage. The loss in cellularity of >90% of this subset has led researchers to conclude that disruption of regulated expression somehow alters signaling mechanisms necessary for survival and developmental progression. It is interesting that the effect of Ly-6A.2 dysregulation is observed at such a critical stage of selective development and differentiation. Although the mechanism for this block is not defined, there are two possibilities to explore. Given the recently described adhesive properties of Ly-6A.2 (212), it is speculated that the expression of mouse Ly-6 proteins during thymic development allows the cells to adhere to a specific region in the thymus (211). Loss of expression would result in de-adhesion, allowing the cells to move towards the medulla, where they would receive signals necessary to continue along their developmental path. Alternatively, continual ligand binding could result in greater overall avidity of TCR-MHC interactions, thus leading to prolonged interaction and altered signaling through the TCR. Alterations in the signaling cascade could enhance negative selection of these thymocytes, which would account for such a great loss in DP cellularity.

The consequences of Ly-A.2-ligand binding are unknown, as ligands for Ly-6 proteins have only recently been identified. A recently identified ligand for Ly-6d has been described as a 9kd protein, with similarity to mouse Notch (213). The sequence homology to Notch indicates a possible role for Ly6d-L in multiple physiological processes, including thymocyte development and lineage commitment. However, the functional consequences of interaction of this and other Ly-6 proteins with their ligand(s) remain unknown. A candidate ligand for Ly-6A.2 has also been described (212). This protein was detected on cells primarily localized around the blood vessels in the cortex.

The consequences of Ly-6A.2 interaction with its candidate ligand have not been studied, however, addition of mAb against this ligand to thymocyte cultures results in abrogation of cell aggregation, indicating that Ly-6A.2-ligand binding enhances cell-cell contact. Whether or not this enhanced interaction contributes to thymocyte developmental signaling remains to be discovered.

Although our understanding of thymocyte development continues to improve, the molecular mechanisms that govern maturation, selection and differentiation processes remain unresolved. Evidence suggests that molecules other than classical TCR-MHC/peptide interactions are instrumental in shaping the T cell repertoire. If overall affinity indeed governs successful selection, then flexibility is achieved not only by variation in peptide presented, but also by co-ligation of accessory molecules expressed by developing thymocytes and stromal elements. Compounding this process is the necessity for differentially expressed molecules, including Ly-6A.2, to bind their receptors at the right time and in the right place. Altering normal expression of highly regulated molecules may provide insight into how they function to promote normal thymic maturation.

T Lymphocyte Activation

Anatomy of Lymphocyte Activation

Upon completion of selection and differentiation $CD4^+$ and $CD8^+$ SP thymocytes exit the medulla and migrate to the peripheral lymphoid organs. In order to optimize the

cellular interactions necessary for the recognition and activation phases of specific immune responses, the majority of lymphocytes, mononuclear phagocytes, and other accessory cells are localized in anatomically defined tissues or organs, where foreign antigens are transported and concentrated. The peripheral lymphoid organs, which include the lymph nodes and spleen, are the sites where lymphocyte responses to foreign antigens are initiated and develop.

Lymph nodes are encapsulated bean-shaped structures containing a reticular network packed with different cell types, including lymphocytes, macrophages and dendritic cells (214). Clustered at the junctions of the lymphatic vessels, lymph nodes are the first organized lymphoid structure to encounter antigens that enter the tissue spaces. As lymph moves through a node, a network of lymphocytes trap any particulate antigen that is brought in with the lymph. Additionally, dendritic cells and other APCs from the skin can sample antigen and bring it into the lymph node for presentation to naïve T cells (215). The afferent vessels leading into each node out number the efferent vessels, which slow the rate of lymph flow, thereby allowing prolonged sampling of antigen. Thus, the overall architecture of a lymph node provides an ideal microenvironment for lymphocytes to effectively encounter and respond to trapped antigens.

Morphologically, a lymph node can be divided into three roughly concentric regions: the cortex, paracortex, and medulla; each of which provides a distinct microenvironment. The cortex is the outermost layer and contains lymphocytes (mostly B cells) and macrophages arranged in primary follicles (216). Following antigenic challenge, the primary follicles enlarge into secondary follicles, each containing a germinal center (217). Intense B cell activation and differentiation into plasma and

memory B cells occurs in the germinal center of lymph nodes. Beneath the cortex is the paracortex, which is populated with CD4 intermingled with a sparse population ofCD8 T cells, and also dendritic cells that have migrated from tissues into the node. These dendritic cells express high levels of MHC II, which present antigen to CD4 T cells (218). The medulla is the innermost layer and is more sparsely populated with lymphocytes, but many of these are plasma cells actively secreting antibodies.

As antigen is carried into a lymph node, it is trapped, processed, and presented by MHC II ondendritic cells in the cortex and paracortex, resulting in CD4 T cell and B cell activation (219). Once activated, these cells migrate to the primary follicles of the cortex. Within a primary follicle, cellular interactions among follicular dendritic cells, B cells and T cells take place, leading to the development of a secondary follicle with a central germinal center (220). Follicular dendritic cells trap antigen complexed with antibody and retain the antigen-antibody complexes on the membrane for long periods of time. Antigen trapped on the membrane of these cells is believed to be especially effective in activating B cells. The activated B cells rapidly divide and either differentiate into plasma and memory B cells or die by programmed cell death (221, 222). Plasma cells leave the germinal center and migrate to the medulla where they secrete large quantities of antibody (223). Interdigitating dendritic cells, which are also abundant in the T cell areas, as well as other accessory cells, are located along the connective tissue scaffold of the node and present antigens they have picked up to naïve cells (224). The activated T cells undergo rapid proliferation, migrate out of the lymph node, and home to the site of infection (225). Thus, the lymph nodes are the sites where T cell responses to lymph-borne protein antigens are initiated.

The spleen is a secondary lymphoid organ situated high in the abdominal cavity. Unlike lymph nodes, which are specialized to "trap" localized antigen from regional lymph, the spleen is adapted to filtering blood and trapping blood-borne antigens, and thus can respond to systemic infections (226, 227). The spleen is surrounded by a capsule that sends a number of trabecular projections into the interior to form a compartmentalized structure. The compartments are of two types, red pulp and white pulp, which are separated by a diffuse marginal zone (228). The splenic red pulp consists of a network of macrophages and numerous erythrocytes. The splenic white pulp surrounds the arteries, forming a periarteriolar lymphoid sheath (PALS) populated primarily by T cells (229). The marginal zone, located peripheral to the PALS, is rich in B cells organized into primary lymphoid follicles (230). Upon antigenic challenge, these primary follicles develop into characteristic secondary follicles containing germinal centers like those found in lymph nodes.

T Lymphocyte Activation

The initial requirement for antigen-induced stimulation of T lymphocytes is ligation of TCR to MHC presenting antigenic peptide. Most nucleated cells present between 10^4-10^6 surface MHC I, and those cells expressing MHC II either constitutively or as a result of cytokine induction express similar amounts (231). It is estimated that as many as 1000-2000 different self-peptides represented at greater than 10 copies per cell are displayed on an APC (232). In contrast, as few as 10^2 copies of foreign pathogen proteins processed during an infection are accumulated on the APC cell surface for presentation to T cells (233). It has been shown for memory cells and effector T cells that as few as 10-100 copies of antigenic peptide are needed to evoke effector function

(234-236). In terms of percentage, only 0.01%-0.1% of a specific ligand, in a virtual sea of self peptide-ligands, is needed for a T cell to respond with activation. Given the unfavorably skewed density on the APC surface, it is remarkable that T cells can discriminate foreign from self-peptide. Although this process of recognition and response is not clear, it is speculated that overall affinity plays a key role. The trend is for less potent ligands (antagonists) to have a lower affinity and a faster dissociation-rate (237). Agonist or partial agonists exhibit a higher affinity for binding, and thus are engaged for longer intervals (238-242). The relationship between strength of binding and duration of interaction was first formalized by McKeithan as "kinetic proofreading" (243). The number and half life of TCR-MHC complexes appear to determine the overall level of intracellular signals achieved and subsequent extent of T cell activation. Thus, the longer the association of TCR-MHC/peptide, the better the chances of completing a signaling cascade or biochemical process. Any interaction lasting a shorter time would attenuate downstream signaling (244). Because of the branching nature of the signaling cascade within a T cell, some signals would be more affected than others, giving rise to different ratios of activated signaling molecules. There have been variations on this kinetic proofreading theme to include the contributions of accessory molecules in enhancing the overall affinity of interaction, but it remains the basic model for translating how differences in TCR-MHC/peptide ligand affinity change the functional effect of the T cell (238, 240, 245). Extensive mapping of proximal to distal signal transduction and differentiating distinct from overlapping pathways is key to testing this theory.

Optimal T cell activation requires signaling through the TCR upon binding to MHC/peptide on the APC (and CD4 and CD8 co-receptors), and a second antigen-

independent signal called co-stimulation (246, 247). Engagement of the TCR in the absence of co-stimulation results in T cell inactivation through either anergy or apoptosis. CD28, the prototypic T cell co-stimulatory molecule, is constitutively expressed on naïve cells and binds to inducible B7-1/B7-2 presented by the APC (248). CD28-mediated co-stimulation influences multiple aspects of T-cell physiology, resulting in enhanced T cell response (249). For example, CD28 ligation enhances the magnitude of and duration of the T cell response, induces the anti-apoptotic gene BCLXL, increases cytokine secretion (particularly IL-2), enhances cell adhesion, facilitates re-organization of the T cell plasma membrane, and supports germinal center formation (250, 251). The importance of the role of CD28 on T cell activation is shown by several experiments *in vitro* and *in vivo*, which collectively demonstrate that absence of CD28 function results in reduced ability to respond to antigen (248, 250). Contributions toward understanding the mechanisms underlying this process come from data presented by Lanzavecchia's group, which provided evidence that co-stimulatory signals derived from CD28 function to lower the activation threshold of TCRs, thus making T cells more sensitive to antigenic stimulation (252). These and other studies suggest CD28 acts to amplify and increase potency of signaling through the TCR, thus decreasing the number of TCRs that need to be engaged in order to elicit a T cell response.

The first biochemical changes known to occur following TCR engagement involve the activation of src-family kinases, most notably Lck (253-255). The enzymatically activated Lck begins to add phosphates to the tyrosines within ITAMs present on the two TCR- ζ chains as well as CD3 δ, γ, and ε (256, 257). It is generally believed that ITAMs play the primary role in downstream signaling through the

recruitment of syk-family kinase, ZAP-70 (261). This kinase has tandem SH2 domains, which have been shown to interact with high avidity to phosphorylated tyrosines in a single ζ-chain ITAM (260). Once activated, ZAP-70 phosphorylates LAT, which is a major substrate critical for complete T cell activation (261). LAT and other adapter proteins are speculated to function as scaffold proteins, which act as sites of integration of signals arising from several biochemical pathways. Thus, scaffold proteins provide an opportunity for controlling signals sent to downstream effectors. LAT serves as an important adapter for interaction with SLP-76, Grb-2, PI-3 kinase, PLC and other downstream signaling molecules and adapters that mediate more distal steps in signal transduction including stimulating the release of intracellular Ca^{+} and activation of the calcineurin/calmodulin pathway (263-266). These signaling events in the membrane are initiated at the T cell –APC contact site. Imaging studies of the immunological synapse (IS) have shown a defined structure and organization of supramolecular activation clusters (SMACs), which form within a few minutes of TCR-MHC interaction (267-270). TCR-MHC complexes and membrane associated Lck, Fyn, and PKCθ are clustered in the center of this junction. After an initial period of LFA clustering, a ring of ICAM-bound LFA molecules and cytoskeletal protein talin surrounds the central cluster (271-272). Except for transient movement in and out of the SMACs, CD45 –tyrosine phosphatase is excluded from these regions (273-274). The final stages of IS formation is characterized by the stabilization of the cSMAC by at least 60 TCR-MHC/peptide-ligand interactions (271). Once this occurs, the IS can be maintained for greater than 1 hour, which is correlated with full T cell activation. The molecular basis for IS organization has remained elusive, though recent experiments have suggested that ligand engagement of

co-stimulators CD28, CD2, LFA-1 and GPI-anchored CD48 facilitate receptor recruited sorting and cytoskeletal interaction at the TCR contact cap (271-276). Further enhancing our understanding of this process is the recent characterization of clustering of lipid raft domains, which function to facilitate processive and sustained signal transduction (277).

Recent developments in cell biology have confirmed the plasma membrane is not homogenous, rather it is composed of laterally associated lipid rafts enriched in cholesterol, sphingolipids and proteins that segregate into relatively ordered domains (278-279). Src family members, lapidated heterotrimeric and small G proteins, some transmembrane and palmitoylated proteins including LAT, CD44, CD4, and CD8α are specifically enriched within these domains. Additionally, preferential association of GPI-anchored molecules, which include Thy1, Ly-6, CD59, and CD48 has been described (261, 280-283). Because these glycolipid-enriched microdomains (GEMS) are enriched with src family and other signal transducers, it has been proposed that GEMs function as pre-formed platforms for signal transduction (284, 285). In this model, as engagement and activation of the TCR complexes within the GEMs increases, ITAM-rich domains of the TCR/CD3 are brought in close association with these signaling proteins (287). This preferential segregation enhances Lck hyper-phosphorylation of the ITAM-rich TCRζ chain, which initiates downstream signaling. The signaling cascade is further enhanced as several other molecules are recruited to the GEMs, including ZAP-70, PLC-γ1, members of the Grb2/Gads/Grap adapter family, PI-3K, SLP-76, Vav, and LAT (261, 286). Signaling emanating from these pathways leads to the translocation of nuclear factor of activated T cells (NF-AT) into the cell nucleus, where it activates AP-1 to transactivate a variety of genes characteristic of T cell activation (287, 288). Thus, raft

dynamics during activation may profoundly influence net activity of src family kinases and the initiation and maintenance of TCR signals.

Consistent with this conjecture are data that demonstrate partitioning of phosphorylated TCR-ζ to GEMS following stimulation through the TCR is critical for proper antigen-induced T cell activation (289). Perturbation of the structural integrity of lipid rafts by depleting or sequestering cholesterol prevents TCR-induced phosphorylation of TCR-ζ and downstream protein tyrosine kinases, which inhibit Ca^{+} flux. Mutation of a motif in the TCR, which prevents increased TCR-ζ/GEM association also prevents TCR-ζ phosphorylation and impairs sustained extracellular signal-regulated kinases 1 and 2 (ERK-1 and –2) (289, 290). Cumulatively, these and other findings are consistent with a model in which TCR engagement enhances the association of TCR-ζ with src family and adapter proteins in the GEMs, thus facilitating initiation of downstream protein tyrosine phosphorylation and T cell activation.

Co-Stimulation

Characterizing the underlying mechanism of co-stimulation for "fine tuning" signal transduction is a complex process. A number of observations suggest it is unlikely that all co-stimulator molecules function identically in facilitating T cell activation. For instance, while LFA-1 and CD2 facilitate T cell activation by increasing the number of TCRs engaged over time, other molecules act to either strengthen the initial signal or lower the activating threshold (291, 292). Other members of the CD28 family have been identified, and their role in the "two-signal" model of T cell activation is just beginning to be understood. Best characterized is inducible co-stimulator (ICOS), which was

identified as a unique molecule expressed on T cells following activation (292, 294). The ligand for ICOS is also a member of the B7 family, B7 homolog (B7h), and is expressed on B cells, splenic and peritoneal macrophages, and some but not all dendritic cells. ICOS-B7h ligation enhances T cell proliferation and influences effector function. The observation that ICOS-mediated co-stimulation does not induce IL-2 production but increases secretion of IL-4, IL-5, IL-10, IFNγ, and TNFα suggests ICOS functions primarily to induce T cell effector function (293-295).

GPI-linked proteins have also been implicated in functioning as accessory molecules to facilitate T cell activation, though the molecular basis of their participation remains largely uncharacterized. The CD2:CD48 ligand pair is among the best-studied accessory molecule:ligand pairs (296, 297). Most examinations of CD2:CD48 interactions have focused on T cell CD2 binding to APC CD48, likely because in humans CD2 expression is limited to T cells. However, in murine immune systems CD2 is also expressed on APCs, including B cells, macrophages, and some dendritic cells (298-300). Because CD48 is expressed on T cells, binding to CD2 expressed on APCs is speculated to facilitate adhesion and TCR contact. Moreover, because CD48 is a GPI-linked molecule concentrated within lipid rafts, it is reasoned that CD48/TCR co-engagement may additionally facilitate TCR-induced cytoskeletal rearrangement and signal transduction by recruiting associated lipid rafts to the TCR activation cap (301). This was initially demonstrated *in vitro* by Moran and Miceli, who showed that co-engagement of CD48 contributed to TCR signal transduction through its association with lipid rafts specialized in facilitating TCR ζ chain tyrosine phosphorylation and downstream signal transduction (302). Additional studies have provided evidence that

CD48 ligation enhances actin cytoskeleton-mediated re-organization of the TCR contact cap and F-actin reorganization, thus constructing a platform that facilitates TCR engagement and processive and sustained signal transduction (302).

Clustering by mAb of the GPI-anchored protein Thy-1 (CD90) also leads to T cell activation, as demonstrated by proliferation and IL-2 secretion (303, 305). Although it is unknown how Thy-1 participates in cell signaling, interplay between this protein and the TCR/CD3 complex is suspected, since a requirement for a functional complex in Thy-1 mediated activation has been reported in several studies (303-305). Additionally, Thy-1/CD3 co-engagement has recently been shown to promote TCR signaling and enhanced tyrosine phosphorylation of the lipid raft-associate LAT, resulting in the recruitment and activation of downstream effector molecules (306). From these studies it is speculated that association of the highly abundant Thy-1 molecule and its putative ligand could help in recruiting signaling molecules into a contact cap and/or in maintaining the TCR/CD3 components on the cell surface by preventing their internalization (306). In this manner, the threshold number of TCRs required for effective activation would be lowered and successful T cell activation could occur more readily.

Further enhancing our understanding of GPI-induced signaling are reports characterizing the mechanisms underlying the ability of CD55 to enhance stimulation upon co-ligation with the TCR/CD3 complex. Crosslinking studies have revealed as TCR/CD3 complexes are brought into close proximity of CD55, TCRζ chains are quickly phosphorylated and downstream signaling is quickly initiated (307). These studies also described the failure of crosslinking CD55 alone to induce phosphorylation of PLCγ1, elevation of the intracellular Ca^{+} concentrations, and IL-2 production (307). Conversely,

independent crosslinking was sufficient to induce phosphorylation of tyrosine residues on TCRζ, Lck, and ZAP-70, which led to speculation that although proximal signaling appeared to be intact, continuation of the signaling to distal cascades required engagement of both CD55 and TCR/CD3 (307, 308). The importance of CD55-mediated phosphorylation of the TCRζ chain was further demonstrated upon deletion of the cytoplasmic domain of TCRζ in Jurkat cells (307). In this system, signaling was severely impaired, and rescue was observed only after transfection of the TCRζ cytoplasmic tail into the cells. Thus, although it has been well documented that some GPI-anchored molecules require co-ligation with the TCR/CD3 complex in order to function in T cell activation, this study was among the first to provide an insight on the mechanism underlying this interdependence.

Regulation of T Lymphocyte Activation and Proliferation

T cell activation is followed by a marked proliferative response, which is enhanced by IL-2 cytokine production. This clonal expansion is mediated primarily by an autocrine growth pathway, in which the responding T cell secretes its own growth-promoting cytokines and also expresses cell surface receptors for these cytokines (reviewed in 309). Clonal expansion is instrumental in generating a large number of T lymphocytes expressing antigen-specific TCRs. Once differentiated, these effector cells are capable of participating in effector functions such as cytotoxicity or secretion of activating cytokines. While clonal expansion initiates a primary response to antigen, un-checked proliferation could lead to a breakdown in maintaining self-tolerance.

There is a growing appreciation for the concept that lymphocytes are regulated by inhibitory as well as activating signals. A candidate molecule for this type of regulation is cytotoxic T lymphocyte antigen-5 (CTLA-4) (310). Although CTLA-4 displays common features of the CD28 family, it is unique in that it delivers an inhibitory signal to temper the immune response (311). CTLA-4 is a molecule expressed by T lymphocytes and has a markedly higher affinity for shared ligands B7-1/B7-2 compared with CD28 (312). Unlike CD28, which is constitutively expressed on the surface of the T cell, CTLA-4 is not detectable on the surface of resting T cells, rather it is quickly up-regulated upon TCR-CD28 engagement (313). In contrast to CD28, *in vivo* and *in vitro* studies have shown that CTLA-4/B7 ligation transmits signals that inhibit T cell activation, progression through the cell cycle and IL-2 synthesis (310). Conversely, blocking CTLA-4 *in vivo* enhances antigen-specific and anti-parasitic responses, tumor rejection and autoimmune disease (310, 314). Furthermore, animals deficient in CTLA-4 expression develop lymphoproliferative disorders and die shortly after birth (311). A number of mechanisms have been proposed to explain the mechanism of CTLA-4 function, including competition with CD28 for ligand binding, induction of immunosuppressive cytokine secretion, sequestration of signaling proteins and overall reduced phosphorylation levels (311). However controversial, it is clear from these studies that CTLA-4 is an important inhibitory CD28 family member, and that it functions to inhibit TCR and CD28-mediated signal transduction in a T cell response.

CD28 family member PD-1, which is expressed on T cells, B cells and monocytes following activation, has been shown to mediate an inhibitory signal. However the exact role of PD-1 in T cell responses is just beginning to be elucidated (315-317). PD-1-PD-

L interactions have been demonstrated *in vivo* and *in vitro* to negatively regulate autoreactive CD8 and B cells in lymphoid and non-lymphoid tissues, suggesting that this molecule plays an important role in peripheral tolerance (318, 319). In light of the ongoing characterization of CD28-family members, it seems that regulation of T cell activation by co-stimulation is more complex than originally envisioned. Based on what is already known, it appears that CD28-mediated co-stimulation in conjunction with TCR engagement by MHC-peptide complexes on the surface of an APC would initiate T cell activation, thereby inducing expression of ICOS CTLA-4, and PD-1. ICOS-mediated co-stimulation could then act to further expand the T cell population and influence effector differentiation. Up-regulation of CTLA-4 would result in competition with CD28 for B7 ligands, with the potential to inhibit T cell activation early in the response owing to its competitive advantage for ligand binding.

Antibodies against several members of the tumor necrosis factor receptor (TNF-R) family have been shown to augment T cell proliferative responses. 4-1BB is expressed primarily on activated CD4 and CD8 T cells during primary stimulation. This molecule is one of the few co-stimulatory receptors that can stimulate high-level IL-2 production by resting T cells in the absence of CD28 signaling (320, 321). Antibodies against 4-1BB have been shown to prevent activation-induced death of previously activated T cells (322). It has been demonstrated that after prior stimulation and rest, CD4 T cells proliferated negligibly to stimulation with anti-CD3 plus anti-CD28 alone but showed enhanced proliferative responses to anti-CD3, anti-CD28 and anti-4-1BB (323). Based on these observations, it is suggested that 4-1BB may function during later stages of response, to sustain T cell activation after CD28 is down-regulated. Like 4-

1BB, OX40 expression is limited to activated T cells (324). The role of OX40 in T cell activation has been studied in several autoimmune disease models, which demonstrate upon activation this protein stimulates proliferation and IL-2 production (325, 326). Additionally, OX40-OX40L interaction has been shown to promote production of IL-4 and differentiation of CD4 T cells to a Th2 phenotype (327). Upon activation, CD4 T cells express CD40 ligand (CD40L), which specifically binds to a constitutively expressed CD40 receptor presented by several types of APCs, including B cells, macrophages, and dendritic cells (328). CD40/CD40L interaction has been shown to significantly enhance up-regulation of both B7-1 and B7-2 molecules, which enhance the ability of APCs to activate responding T cells (329). CD40-mediated production of inflammatory cytokines, such as TNF-α further matures APCs for optimal antigen presentation by way of inhibiting MHC II turnover rates and by increasing the level and stability of MHC/peptide complexes on the surface of the APC (330). IL-12 secreted by the APC after CD40 ligation acts on expanding CD4 and CD8 T cell populations via the IL-12 receptor, inducing the production of IFN-γ and polarizing the response to the Th-1 phenotype (331). Thus, CD40-mediated induction of co-stimulatory and inflammatory elements allows for the maturation of APCs, resulting in optimal CD4 and CD8 T cell expansion and survival as well as effector maturation and Th-1 differentiation.

Regulation of an antigen-induced T cell response may also include the participation of Ly-6A.2. Ly-6A.2 belongs to a multi-gene family, which encodes for several proteins that are expressed differentially on cells of hematopoietic origin. Although the precise function of Ly-6A.2 is unknown, investigations into the role of this protein during T cell stimulation have offered data, which support activation of T cell

signaling (reviewed in 67). Upon T cell activation, Ly-6A.2 is up-regulated on the cell surface. This increased expression may also be induced by IFN-αβ, IFN-γ, or IFN-γ synergistically with tumor necrosis factor (TNF) (370, 371, 372). Maximal induction of Ly-6A.2 by these cytokines has been shown to induce a dramatic 25-fold enhancement of proliferation (373). This high sensitivity to IFN led to speculation that Ly-6A.2 may play a role in T cell activation.

Ly-6 molecules have been shown to function in activation by inducing T cell hybridomas or normal T cells to proliferate, which is accompanied by IL-2 production. Experiments using antibodies directed against Ly-6 epitopes have been instrumental in mapping the requirements for stimulation through this protein. Antibody cross-linking of Ly-6A.2 alone does not induce activation of T cells or T-hybridomas (374). However, cross-linking in conjunction of phorbol myristate acetate (PMA), which triggers the TCR signaling pathway via protein kinase C (PKC), stimulates mitogenesis in T cells (67). Studies further demonstrated that the ability of anti-Ly-6A/E mAb (D7) to inhibit IL-2 production by T hybridomas is dependent on the nature of the TCR activating signal because IL-2 production is not inhibited when T hybridomas are stimulated with antigen or immobilized anti-CD3 (374). Similar to its ability to enhance anti-Ly-6A/E-induced activation of T cells, IFNγ enhanced the D7-induced inhibition of IL-2 production by alloantigen-activated normal T cells (374). Collectively these studies demonstrate that activation of T cells through Ly-6A.2 requires co-stimulation through the TCR/CD3 complex.

The interdependence of TCR and Ly-6 on T cell activation has also been shown by experiments using T cells and T cell hybridomas defective in their ability to present

Ly-6A.2 on the cell surface. Mutations to transcription of Ly-6A.2 or defects in the ability to biosynthesize the GPI-linkage inhibit antigen-stimulated, TCR-mediated activation of T cell hybridomas (375). The use of anti-sense oligonucleotides complementary to the 5' end of the mRNA encoding the Ly-6A protein to block expression of this molecule in T cells resulted in the inability to re-stimulate *in vitro*, antigen-primed T cells (376). It also blocked the activation of normal spleen cells by concanavalin A (Con A), monoclonal antibody to CD3, and mAb to Ly-6 (376). Additionally, antisense Ly-6 RNA expressed in a stably transfected antigen-specific T cell clone resulted in inhibition of responses to antigen, anti-TCR cross-linking and Con A (377). Furthermore, substantial reduction of Ly-6A resulted in reduction of TCR expression, which mRNA analysis indicated was specific for the TCR_{β} chain (377). These data establish that Ly-6 expression is required for TCR-mediated T cell activation. Although there is no evidence of a physical link between Ly-6A.2 and the TCR, these data support speculation that co-ligation of both receptors is important and necessary for T cell activation.

In contrast to a stimulatory role, recent studies using T cells expressing high levels of Ly-6A.2 (100-200 fold higher than naïve $CD4^+$ T cells) suggest this protein exerts a signal to regulate proliferation (334). $CD4^+$T cells that express high levels of Ly-6A.2 proliferate 7-8 fold less than non-transgenic T cells when stimulated by antigen. Additionally, this inhibition of growth appears to be mediated through interactions with a ligand present on an APC. Support for the notion that Ly-6A.2 is a negative regulator of T cell growth comes from data generated from mice deficient of Ly-6A.2. Cross-linking the TCR/CD3 complex on T cells from Ly-6A.$2^{-/-}$ mice resulted in a hyper-proliferative T

cell response when compared to wild-type controls (335). These observations led to speculation that the role of Ly-6A.2 was to slow down the immune response of these cells by preventing over-proliferation. Additionally, a number of published studies have suggested growth inhibitory roles for GPI-anchored proteins, including those from the Ly-6 family (336). It is important to remember that these studies have all relied heavily upon the use of antibodies to induce stimulation of T cells and T-T hybridomas (67). Although conflicting, these data collectively suggest Ly-6A.2 does participate in T cell signaling and that the mechanism is somehow conjoined with TCR signal transduction. The role of Ly-6A.2 in an antigen-stimulated model has not been studied and may provide more relevant data, which may resolve the inconsistencies in these reports.

The mechanisms by which molecules expressed on the cell surface regulate T lymphocyte activation are largely unknown. It is plausible that regulation is mediated by interaction with intracellular components known to down modulate signaling. Given the importance of Lck activity and of phosphorylation of TCR/CD3 ITAMs for induction of signaling through the TCR, it is not surprising that a number of inhibitory molecules target these interactions. Csk is a tyrosine kinase that preferentially binds to src-family kinases, including Lck (337, 338). Upon T cell activation and recruitment of Lck to ITAMs, Csk is found to localize to these complexes where it catalyzes the phosphorylation of Lck Y505, rendering it inactive (339). Another mechanism of inhibition involves tyrosine phosphatase, CD45. Although initially believed to function as a positive regulator of TCR signaling by removing Csk-added phosphates from Lck, CD45 has recently been shown to participate in negative regulation by de-phosphorylating ITAMs on the TCRζ chain (337, 340). Cbl is yet another molecule

identified to participate in inhibiting TCR signaling by specifically binding to activated auto-phosphorylated ZAP-70 via its negative regulatory phosphorylation site (341). The c-cbl$^{-/-}$ mouse develops spontaneous autoimmunity, and mutation of c-cbl uncouples T cell proliferation and IL-2 production from the requirement for CD28 co-stimulation (342). Although all of these mechanisms for signal attenuation are important components of T cell regulation, little is known about the pathways that activate these molecules. It is speculated that they participate in feedback regulation that is linked to the initiation of TCR signaling, but how they are controlled remains to be characterized.

T Lymphocyte Differentiation

Effector T cells undergoing an immune response show varying patterns of cytokine production. Since cytokines are important mediators of T cell-dependent immune responses to protein antigens, the types of cytokines produced during a response determine the nature and effectiveness of that response (343). Two distinct populations of CD4 T cells can be distinguished on the basis of their secreted cytokines (344). The T-helper 1 (Th1) subset principally secretes IFNγ, lymphotoxin (LT), and IL-2. These cytokines stimulate antibody production from B cells, proliferation and differentiation of CD4 and CD8 T cells, and increased killing and phagocytosis by neutrophils and macrophages (reviewed in 344). The characteristic cytokines secreted by T-helper 2 (Th2) cells are IL-4, IL-5, IL-10 and TGF-β. Effector functions of these cytokines include suppression of macrophage activation, enhanced mast cell de-granulation and eosinophil activation, and production of neutralizing IgG antibodies by B cells (344, 345). The principal effector function of Th1 cells is in phagocyte-mediated defense

against infections (346). Th2 cells are the mediators of allergic reactions and defense against helminthic and arthropod infections. In addition, Th2 cells produce cytokines that antagonize the actions of IFNγ and suppress macrophage activation. Therefore, Th2 cells are proposed to be capable of functioning as physiological regulators of immune responses by inhibiting potentially injurious Th1 responses.

The differentiation of naïve CD4 T cells into Th1 or Th2 cells is influenced by cytokines produced early in response to the agent that triggers the immune reaction. Intracellular bacteria, viruses and some parasites stimulate natural killer cells to produce IFNγ, which in turn acts on macrophages to induce IL-12 secretion (344). IL-12 binds to receptors on antigen-stimulated CD4 cells and promotes their differentiation into the Th1 subset (348). In turn, IFNγ produced by Th1 cells inhibits proliferation of Th2 cells, thus promoting Th1 dominance (347). The differentiation of Th2 cells is stimulated primarily by IL-4, which is initially secreted in small amounts by antigen-activated T cells. If the antigen is persistent and present at high concentrations, the production of IL-4 increases and a progressive accumulation of Th2 cells is generated (344).

While many established T cell clones completely fit Th1 and Th2 profiles without overlapping patterns of IL4 and IFNγ expression, it is important to consider that under physiological conditions, populations rather than individual cells define the response. In an immune response it is possible for a site of immunological activity to have a varied mixture of responding cell secreting a heterologous combination of cytokines. In cases such as this, components distinct from cytokine production come into play in directing differentiation of T-helper subsets. Cytokine, chemokine and adhesion receptors, as well as differential expression of transcription factors, are all contributing factors in

maintaining certain patterns of cytokine production (344). For example, if IL-4 and IL-12 are present in cultures of naïve cells at the time of antigenic stimulation, T cells lose expression of the IL-12β receptor and begin expressing and responding to IL-4 in an autocrine fashion (345, 346, 349). Examination of transcription factors up-regulated in these cells reveals that transcriptional activation GATA-3 and cMaf by STAT-6 are instrumental in facilitating this switch (350, 351). Conversely, if high levels of IFNγ are present in addition to IL-4, STAT-1 and STAT-4 induced transcription of ERM and T-bet allow the IFNγ and IL-12β receptors to be retained, resulting in production of both IFNγ and IL-4 (352). Thus, although positive feedback in an autocrine fashion is a driving force for maintaining a Th1 or Th2 response, these studies demonstrate that in conditions of mixed Th1/Th2 stimulation, it is possible for disruption of the feedback loop to occur.

In addition to cytokines, the nature, dose and route of administration of antigen, as well as the amount represented on the surface of the interacting APC influence the process of T cell differentiation (353). Studies using altered peptides at varying concentrations have shown that low level stimulation of CD4 cells favors only differentiation of uncommitted IL-2 producing cells (353). A moderate to high level of stimulation favors differentiation of Th2-like cells, which produce IL-4 and IL-5, whereas a very high level of stimulation favors Th1-like cells which produce IFNγ (353, 358). How other accessory molecules expressed on naïve or activated T cells participate in T cell differentiation is not entirely clear. It is hypothesized that signaling from these molecules is integrated with the dose and affinity of antigen to regulate differentiation by increasing or decreasing the strength of signal (353). Thus, differentiation towards committed Th1 or Th2 subsets is not only dependent upon the antigen, but also requires

involvement of specific accessory/co-stimulatory molecules. In support of this model are data which demonstrates that interaction of LFA-1 with ICAM-1 (360, 361) or 2, CD28 with B7-1 (362, 363), and CD40 with CD40-Ligand (364) supports Th1 differentiation . Conversely, interaction of CD28 with B7-2, CD4 with MHC II, and OX-40 with OX-40-Ligand promotes Th2 differentiation.

Inducible co-stimulators have also been implicated in mediating Th-1 versus Th-2 differentiation. Up-regulation of ICOS on activated human T cells has been shown to increase secretion of Th-2 type cytokines IL-4, IL-5 IL-10, IFNγ, and TNF (365-367). CTLA-4 is also implicated in this pathway, as naïve T cells lacking this molecule differentiate into the Th2 subset (368). Induced up-regulation of SLAM following T cell activation results in induction of IFNγ by activated T cells, including those of the Th2 subset (369). Although the ability of inducible GPI-anchored molecules to mediate Th1 and Th2 differentiation has not been documented, given the signaling capabilities of these proteins, it seems plausible that they might influence differentiation of T-helper effector cells. A candidate molecule for this type of regulation is Ly-6A.2. As previously described, Ly-6A.2 is up- regulated upon T cell activation. Since this up-regulation is inducible by IFNγ, it is possible that Ly-6A.2 is involved in an autocrine feedback mechanism in response to T cell activation. Maximal expression of Ly-6A.2 could induce regulatory signals into the cell, which could promote continual expression of IFNγ and commitment to the Th1 subset. Alternatively, if Ly-6a.2 is involved in arresting proliferation, it may do so by altering production of cytokines from proliferative to those associated with suppressing the immune response. In this scenario, abrogation of Th1-

type cytokines, and induction of those associated with a Th2 response would be facilitated by Ly-6A.2 signaling.

It is generally accepted that the decision of a T cell to "sense and respond" with proliferation and effector function, rather than to "sense and not respond" either through anergy or apoptosis is initially dependent upon TCR-MHC/peptide interactions. Accessory molecules, along with differentially expressed surface molecules contribute to this process, mainly by altering intracellular signaling cascades. Although molecular mapping of proximal to distal cascades is ongoing, it is clear that early tyrosine phosphorylation events are critical components distinguishing between T cell activation and quiescence.

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CHAPTER 2

$CD4^+$ T CELLS MATURE IN THE ABSENCE OF MHC CLASS I AND CLASS II EXPRESSION IN LY-6A.2 TRANSGENIC MICE[1]

[1]Henderson, S.C., A. Berezovskaya, A. English, D. Palliser, K. L. Rock and A. Bamezai. 1998. Journal of Immunology. 161: 175-182. Reprinted here with permission of publisher

ABSTRACT

The TCRs expressed on T lymphocytes recognize foreign peptides bound to MHC molecules. This reactivity is the basis of specific immune response to the foreign antigen. How such specificities are generated in the thymus is still being debated. Signals generated through TCR upon interaction with self MHC-peptide complexes are critical for maturation of the $CD4^+$ helper and $CD8^+$ cytotoxic subsets. We have observed maturation of $CD4^+$ but not $CD8^+$ T cells in Ly-6A.2 transgenic MHC-null mice. Since there can be no interactions with MHC molecules in these mice, these $CD4^+$ cells must express the T cell repertoire that exists before positive and negative selection. Interestingly, despite an absence of selection by MHC molecules, the $CD4^+$ cells that mature recognize MHC molecules at a frequency as high as in $CD4^+$ cells in normal mice. These results demonstrate that: 1) the germline sequences encoding TCRs are biased toward reactivity to MHC molecules; and 2) $CD4^+$ cells as opposed to $CD8^+$ cells have distinct lineage commitment signals. These results also suggest that signals originating from Ly-6 can promote or substitute for signals generated from TCR that are required for positive selection. Moreover, this animal model offers a system to study T cell development in the thymus that can provide insights into mechanisms of lineage commitment in developing T cells.

INTRODUCTION

The thymus is a unique organ dedicated to the development and maturation of T cells. This is the generative lymphoid organ in which central tolerance is established. Selection of T cells in the thymus is controlled by three major factors: 1) the initial T cell repertoire, which is generated by the rearrangements of V-D-J and V-J gene segments; 2) the nature and concentration of self peptides present in the thymus; and 3) the functional consequence of stimulating developing T cells, i.e., growth stimulation (positive selection) or cell death (negative selection). A body of data indicates the role of self peptides in thymic selection (1, 2). The nature of the T cell repertoire that exists before positive and negative selection is not known. An understanding of this initial T cell repertoire will provide clues to our understanding of the underlying mechanisms of thymic selection.

The immature T cells that differentiate into $CD4^+$ helper cells express MHC class II-restricted receptors, whereas those that differentiate into $CD8^+$ cytotoxic T cells express MHC class I-restricted T cells. It is generally thought that recognition of the MHC class I molecule by both the TCR and CD8 molecules will provide appropriate signals giving rise to $CD8^+$ mature T cells. Likewise, recognition of the class II molecule by TCRs in conjunction with the CD4 molecule will give rise to CD4 helper T cells. The signals that arise from the coreceptor alone are not sufficient for lineage commitment, since cells do not mature in TCR- mutant mice (3). What remains unclear is how the signal delivered by the TCR alone or in conjunction with each of the co-receptors can

give rise to two distinct T cell subsets. It also remains unclear at what stage of development the thymocytes commit to CD8 or CD4 lineage and what, if any, additional signals are required for lineage commitment to CD4 and CD8 T cells.

A striking property of the mature T cell repertoire is the high frequency of reactivity to allogeneic MHC molecules (4, 5). Approximately 1 to 10% of mature T cells are alloreactive, and this repertoire overlaps with the Ag-specific T cell repertoire. It has been hypothesized that alloreactive T cells recognize MHC molecules independently of peptides (6). More recently, it was proposed that much of this reactivity is peptide dependent but may not be peptide specific (7). In addition, there are some examples of alloreactive T cells that co-recognize specific peptides complexed with allo-MHC (8). It is unclear whether alloreactivity is a consequence of selecting TCRs that recognize self MHC and therefore may be more likely to cross-react with allogenic MHC molecule or if the initial TCR repertoire has an inherent reactivity to the MHC molecule. It is hypothesized that the initial TCR is biased in its reactivity to MHC molecules (9). Some recent studies in which a c-ovalbumin peptide covalently linked to I-Ab was expressed in transgenic mice observed a high frequency of alloreactive T cells (10). More recently, an in vitro system was developed in which maturation of CD4+ cells was induced in fetal thymic organ culture (FTOC)5 with anti-TCR + anti-CD4 Abs (11). Furthermore, T-T hybridomas were generated from the CD4+ thymocytes that mature in FTOC from MHC class I- and II-deficient mice in the presence of anti-TCR + anti-CD4 Abs. Similar frequencies of MHC reactivity were observed in the preselected repertoire and in the mature T cell repertoire in the thymus (11).

We were able to examine the initial T cell repertoire directly based on a chance observation in a Ly-6A.2 transgenic mouse. We have previously generated transgenic mice with the Ly-6A.2 gene under the control of the human CD2 enhancer. The Ly-6A.2 transgene is highly expressed on all T cells in the thymus. This dysregulated expression of Ly-6A.2 causes a substantial but incomplete block in T cell development, which occurs at the stage when Ly-6A.2 expression is normally turned off (12). In the present study, we crossed the Ly-6A.2 transgene with mice that lack MHC class I and class II molecules. In control (nontransgenic) MHC-deficient mice, mature CD4 and CD8 T cells failed to develop as expected, due to an absence of MHC-dependent positive selection (13, 14). Surprisingly, we found that only CD4 T cells, but not CD8 T cells, mature in the absence of MHC molecules in the Ly-6A.2 transgenic mice. The $CD4^+CD8^-$ T cells that develop in the absence of MHC in Tg^+MHC^- mice are similar to the $CD4^+$ cells that mature in normal mice and represent the initial T cell repertoire that exists before positive and negative selection. In this report, we describe the characteristics of the $CD4^+$ cells that mature in the absence of MHC in Ly-6A.2 transgenic mice, and we also determine the reactivity of these cells to MHC molecules to provide direct evidence of MHC reactivity of unselected T cells in an adult mouse. More importantly, these studies indicate that overexpression of Ly-6A.2 can initiate signaling into developing T cells that normally use their Ag-specific ß TCR.

MATERIALS AND METHODS

Transgenic mice. The Ly-6A.2 transgenic and MHC class I x II-deficient mice that were used in this study have been previously described (13). The mice expressing the Ly-6A.2 transgene were backcrossed to MHCclass I- x II- mice for six to eight generations.

Flow cytometry. Cells were stained for immunofluorescence as described previously (15). One x 10^6 thymocytes, Tris-NH4Cl-treated spleen cells or lymphocytes purified from peripheral blood were incubated with various Abs followed by appropriate fluorochrome-conjugated second-step reagents. Cells (5 or 10 x 10^3) were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Reagents used for this analysis were: phycoerythrin-conjugated anti-CD4 (YTS 191.1.2, Life Technologies), FITC-conjugated anti-CD8 (53-6.7, Life Technologies), FITC-anti-heat-stable Ag (HSA; PharMingen, San Diego, CA), biotin anti-Vß, biotin-anti-CD44, biotin-anti-CD40 ligand, biotin-anti-Ly-6A/E, biotin-anti-TCR-ß (PharMingen), anti-Ly-6A.2 (3E7) (15), anti-Ly-6A.2 (3A7) (16), streptavidin-Red 613 and streptavidin-Red 670 (Life Technologies, Arlington Heights, IL), and goat anti-rat IgG-FITC (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Anti-IAd/b/q (M5/114) and anti-class I (M1/42) were obtained from American Type Culture Collection, Manassas, VA.

T-T hybridomas. T cells from spleen were cultured with anti-CD3 Abs, and after 2 days of incubation the blasts were separated by Ficoll-Hypaque separation. The CD4+ blast cells were obtained by panning on anti-mouse Ig-coated plates after incubating the cells with anti-CD4 (GK1.5). Purification of cells by panning usually gave 80 to 90% pure cell population in our hands. The CD4+ cells were fused to BW5147 ß-negative (17), and

hypoxanthine-aminopterin-thymidine (HAT)-resistant cells were selected per standard protocols. Plating efficiency was >80%.

Cell culture. Microcultures were set up as previously described (18) in 96-well flat-bottom plates (Corning Glass, Corning, New York) in a final volume of 200 µl of culture medium consisting of RPMI 1640 supplemented with 20 mM HEPES, 2 mM L-glutamine, 1mM nonessential amino acids (Irvine Scientific, Irvine, CA), 10% heat-inactivated FCS (Sigma Chemicals, St. Louis, MO), 0.25 µg/ml of fungibact (Life Technologies), and 5 x 10-5 M 2-ME; or in some experiments, cultures were set up in 24-well plates (Corning Glass) in a final volume of 1 ml in the presence or absence of PMA and calcium ionophore (Sigma Chemicals). The precise culture conditions are given in the applicable figure legend. Reactivity to MHC was examined by culturing 5 x 105 or 1 x 105 hybrid cells with 5 x 105 gamma-irradiated splenic stimulator cells in a 200-µl culture medium. After 24 h, 100 µl from these cultures was harvested and evaluated for IL-2 content by incubating it with the IL-2-dependent cell line HT-2.

RESULTS

Appearance of $CD4^+CD8^-$ T cells in the thymus of Ly-6A.2Tg$^+$MHC$^-$ mice. Ly-6A.2Tg+ mice were bred with MHC class I- and class II- double deficient mice (in which the Aß gene and ß2-microglobulin were disrupted by homologous recombination), and their transgenic progeny were backcrossed with MHC I- x II- mutant mice for several generations. The progeny obtained from these breedings that lacked the expression of MHC class I and II molecules, with or without expression of the Ly-6A.2 transgene, were

used for analysis. As shown in Figure 1C, the total number of cells recovered from the thymi of Tg^+MHC^- mice was similar to the number of cells observed in Tg^+MHC^+ mice and is markedly reduced compared with Tg^-MHC^+ mice. The Tg^+MHC^- animals also have a reduction in the development of T cells beyond the $CD4^-CD8^-$ stage as compared with Tg^+MHC^+ mice. These observations indicate that interactions of TCRs with MHC molecules at the $CD4^+CD8^+$ cell stage does not contribute to the block in T cell development in Ly-6A.2 transgenic mice. To further determine the phenotype of the thymocytes that mature in Tg^+MHC^- thymus, we examined the expression of CD4 and CD8 molecules. Interestingly, in multiple experiments, 17 to 38% of the thymocytes in Tg^+MHC^- thymi were $CD4^+$ cells that had down-regulated the expression of the CD8 coreceptor (Fig. 1A and B). In contrast, $CD4^+CD8^-$ thymocytes were almost completely absent (<0.5%) in the Tg^-MHC^- littermates, as reported previously (13, 14). Approximately 10 to 15% of the thymocytes were $CD4^+CD8^-$ in Tg^-MHC^+ mice, which is similar to the 7 to 10% of these cells found in Tg^+MHC^+ mice (Ref. 12 and Fig. 1A). Furthermore, the $CD4^+CD8^-$ cells that appear in the Tg^+MHC^- thymus are different from the transitional cells described in MHC class II- mice, because these latter cells only partially down-regulate their CD8 coreceptor (19, 20). It is noteworthy that expression of the Ly-6A.2 transgene had no effect on the maturation of $CD4^-CD8^+$ T cells in Ly-6A.2 Tg^+MHC^- mice (Fig. 1A and B). These data indicate that development and maturation of $CD4^+CD8^-$ T cells are selectively supported in the thymus of Ly-6A.2 transgenic mice in the absence of cell surface MHC molecules.

Up-regulation of TCR on $CD4^+CD8^-$ thymocytes in Tg^+MHC^- mice. In the normal thymus, mature T cells up-regulate their expression of TCR after being positively

selected. Therefore, to determine whether the $CD4^+CD8^-$ thymocytes in Ly-6A.2 Tg^+MHC^- mice are phenotypically mature, we examined their expression of TCR. Thymocytes were stained with fluorochrome-conjugated mAbs directed against CD4, CD8, and TCR molecules in three-color immunofluorescence. As shown in Figure 1B, the $CD4^+$ thymocytes that appear in Tg^+MHC^- thymi have up-regulated the surface expression of TCR. Furthermore, this increase in the expression of TCR is similar to that seen in $CD4^+$ thymocytes from normal mice. Very few cells showing TCRintermediate/high were observed in Tg-MHC- mice, which is consistent with other published reports (Fig. 1A and 13). Our results indicate that TCR expression in $CD4^+CD8^-$ cells from normal mice was similar to that in cells that mature in Ly-6A.2 transgenic mice in the absence of MHC.

Down-regulation of HSA on $CD4^+CD8^-TCR^{high}$ thymocytes in Tg^+MHC^- mice. Another molecule expressed on the cell surface of thymocytes in which down-regulation correlates with cells undergoing positive selection is the HSA. This molecule is highly expressed on $CD4^+CD8^+$TCRlow/intermediate thymocytes, but its expression is low on the majority of TCRhigh thymocytes that are also either $CD4^+CD8^-$ or $CD4^-CD8^+$ (21, 22). Therefore, we examined the expression of HSA in normal (Tg^-MHC^+), Tg^-MHC^-, and Tg^+MHC^- mice by staining with anti-TCR-ß and anti-HSA Abs. Figure 2 shows that the expression of HSA is down-regulated in TCR^{high} cells in the Tg^+MHC^- thymus. This reduction in HSA expression was similar to that observed in TCR^{high} cells in the normal (MHC^+) thymus (Fig. 2). Moreover, the high level of HSA expression on $CD4^+CD8^+$ thymocytes from Tg^+MHC^-, Tg^-MHC^- animals is comparable. These results suggest that the Ly-6A.2 overexpressed on $CD4^+CD8^+$ thymocytes delivers a signal(s) that is MHC+

peptide independent but results in the selection of $CD4^+CD8^-TCR^{high}HSA^{low}$ mature thymocytes.

The $CD4^+CD8^-TCR^{high}$ T cells from Ly6A.2 Tg^+MHC^- thymus are present in peripheral lymphoid organs. We next examined whether the positively selected $CD4^+CD8^-$ cells are exported from the thymus and populate the peripheral lymphoid organs. We analyzed the spleen and lymph nodes of 4- to 8-wk-old mice for the expression of CD4 and CD8 molecules. Figure 3A shows that $CD4^+CD8^-$ T cells account for 50.2% (range, 35–60% in different experiments) of cells in lymph nodes of Tg^+MHC^- mice and for 49.6% (range, 50–70%) of lymph node cells in normal MHC+ mice. In contrast, this subpopulation made up only 8.4% (range, 4–8%) of lymph node cells in mice lacking MHC class I and II molecules. Moreover, the level of expression of CD4 on lymph node T cells from Tg^+MHC^- and control (Tg^-MHC^+) mice was comparable but higher than that observed in Tg^-MHC^- mice. The level of expression of the TCR on $CD4^+CD8^-$ cells from Tg^+MHC^- lymph node and spleen was also similar to that on $CD4^+CD8^-$ cells in normal mice (data not shown). These results indicate that the T cells that mature in Tg^+MHC^- mice migrate to the periphery and accumulate in lymph nodes (Fig. 3A) as well as in the spleen (data not shown). In contrast, very few $CD4^-CD8^+$ cells were detected in the spleen and lymph nodes of Tg^+MHC^- and Tg^-MHC^- mice, which is consistent with the observation that there are few of these cells in the thymi of mice lacking MHC molecules. These observations reinforce our conclusion that overexpression of Ly-6A.2 results in the maturation of $CD4^+$ and not $CD8^+$ cells in the absence of MHC molecules.

Expression of CD44 on exported $CD4^+CD8^-$ T cells in Tg^+MHC^- mice. It has been reported previously that a majority of mature naive, $CD4^+CD8^-$ lymph node T cells from normal mice express low levels of the CD44 molecule (14). Therefore, we examined the expression of CD44 molecules on $CD4^+$ cells in lymph nodes obtained from Tg^+MHC^- mice. Figure 3B shows that in these mice a majority of $CD4^+$ lymph node cells were $CD44^{low}$. Their expression of CD44 molecules is similar to that on $CD4^+$ T cells from lymph nodes of normal mice. In contrast, the small number of $CD4^+CD8^-$ cells in class I and II double-deficient mice express high levels of CD44 (Fig. 3), as reported earlier (23). These results indicate that $CD4^+CD8^-$ cells in Tg^+MHC^- mice have a mature phenotype.

The $CD4^+CD8^-TCR^{high}$ cells from Ly-6A.2 Tg^+MHC^- mice up-regulate the CD40 ligand upon stimulation. $CD4^+CD8^-$ T cells that are selected by MHC class II molecules help B cell function in normal mice. The helper activity is mediated in part by the interaction of the CD40 molecule (expressed on B cells) with the CD40 ligand (expressed on activated $CD4^+CD8^-$ T cells) (24). Since the Ly-6A.2 transgene supports positive selection of $CD4^+CD8^-$ cells, we next sought to investigate whether these lymphocytes cells had the ability to express the ligand for CD40. The CD40 ligand is expressed preferentially on $CD4^+CD8^-$ T cells on stimulation with PMA and calcium ionophore (23). Lymphoid cells from the spleen (Fig.4) and lymph nodes (data not shown) of Tg^+MHC^- mice were exposed to the combination of PMA and calcium ionophore for 4 to 5 h and analyzed for the expression of CD4, CD8, and CD40 ligand. Figure 4 shows that a majority of $CD4^+CD8^-$ (Fig. 4B) and not $CD4^-CD8^+$ (Fig. 4A) splenic cells from Ly-6A.2 transgenic MHC- mice expressed the CD40 ligand. Similar

expression was also observed on $CD4^+CD8^-$ cells from the spleen (Fig. 4B) and lymph nodes (data not shown) of normal mice. In contrast, the CD40 ligand was not up-regulated on the small number of $CD4^+CD8^-$ cells that are observed in Tg^-MHC^- lymph nodes. These results indicate that the $CD4^+CD8^-$ cells that selectively mature in the Ly-6A.2 transgenic and MHC^- thymus respond to stimulation in a manner similar to normal $CD4^+CD8^-$ Th cells and that they are functionally mature.

The $CD4^+CD8^-TCR^{high}$ cells from Tg^+MHC^- mice are immunoresponsive. Another property of mature T cells selected in the normal thymus is their responsiveness to stimulation through the TCR/CD3 complex by proliferation. In contrast, immature $CD4^+CD8^+$ thymocytes do not proliferate under similar conditions (25). To determine whether the Tg^+ $CD4^+CD8^-$ thymocytes that have matured in the absence of MHC molecules are immunoresponsive, we examined whether they responded to stimulation with anti-CD3 Abs. As shown in Figure 5, thymocytes from Tg^+MHC^- mice proliferated under these culture conditions. Similar results were obtained when Con A was used as mitogen (data not shown). The magnitude of these responses was even greater than that observed with Tg^-MHC^+ thymocytes (data not shown). In contrast, proliferation was not observed in cultures with Tg^-MHC^- thymocytes (Fig. 5). These results further indicate that T cells that mature in Ly-6A.2 transgenic mice in the absence of MHC molecules are functionally competent.

$V_ß$ repertoire of $CD4^+$ T cells in Ly-$6Tg^+MHC^-$ mice represents the unselected T cell repertoire. A body of data indicates that negative and positive selection in the thymus occurs at the $CD4^+CD8^+$ cell stage, and therefore the $V_ß$ repertoire of most $CD4^+CD8^+$ cells is an unselected one. To examine whether the $V_ß$ repertoire is unselected, we

compared the $V_ß$ repertoires of $CD4^+$ that mature in Ly-6A.2Tg^+MHC^- mice with the double-positive (DP) thymocytes from the same mouse. Figure 6 shows that these two repertoires are similar. Moreover, this $V_ß$ repertoire is also similar to one observed in DP thymocytes from the Tg^-MHC^- mice. This later observation supports the idea that the CD4+ cells that mature in Ly-6Tg^+ mice in the absence of MHC is unaltered. Analysis of the $V_ß$ (Vß3, -5, -8, -9, -10, and -11) repertoire in the CD4+ and $CD4^+CD8^+$ subsets in the C57BL/6 parent mice indicated no differences except positive selection of $V_ß8$ $CD4^+$ T cells (data not shown). The absence of clonal deletion may be due to the lack of I-E expression in C57BL/6, which is known to present MTV-8, -9, and -17, resulting in efficient deletion of specific $V_ß$-expressing T cells (26, 27). The fact that all of the $V_ß$ tested are represented in the T cell repertoire in Ly-6A.2Tg^+MHC^- mice indicates that transgene expression does not skew the T cell repertoire as has been observed with MHC class I-selected NK1.1+ cells (28).

$CD4^+$ T cells from Tg^+MHC^- mice show high reactivity to many MHC molecules. To determine whether the preselected T cells are predisposed to recognize MHC molecules, we first tested the reactivity of these cells to irradiated spleen cells derived from H-2^b, H-2^k mice. Lymph node cells from Tg^+MHC^- mice show high levels of alloreactivity (response to APC from CBA mice) as well as reactivity to self MHC (I-A^b-expressing APC, the same as in the MHC mutant mice) (Fig. 7). These responses are blocked by the M5/114 (anti-I-A^b, -I-A^d, -I-E^d, -I-E^k) Ab, and no response is elicited by APC from mice that lack MHC molecules (Fig. 7, right panel) nor is this reactivity blocked by irrelevant isotype matched Ab (data not shown). Reactivity of the $CD4^+$ cells that mature independently of MHC was also observed with APC derived from mice of the H-2^d, H-2^s,

and H-2^q haplotype, albeit to varying degrees (data not shown). These data demonstrate that lymph node cells from Ly-6Tg^+MHC^- mice respond to MHC molecules. Similar results were also obtained with T cells from the thymi of these mice (data not shown). These results suggest that the preselected repertoire is evolutionarily biased to recognize MHC molecules.

Alloreactivity of T-T hybrids derived from Ly-6Tg^+MHC^- mice. Figure 7 indicates that CD4+ cells that mature in the absence of MHC molecules in Ly-6A.2 transgenic mice show reactivity to a number of MHC molecules. To test the frequency of this reactivity, we stimulated T cells from Ly-6Tg^+MHC^-, Ly-6Tg^-MHC^+ (C57BL/6) mice with soluble anti-CD3 Ab and purified $CD4^+$ cells and fused them with TCR-ß-negative BW5147 (17). Hybridomas that tested positive for CD4 expression were further tested for reactivity with different MHC. Each hybridoma was stimulated with APCs from three different strains. An average of 12 to 15% of the T cells from Ly-6Tg^+MHC^- mice reacted to each of the MHC-expressing APCs tested (Figure 8). In contrast, 10 to 12% of the T cells from Tg^-MHC^+ mice reacted with two different MHC tested, whereas about 8% of the hybrids from Ly-6A.2Tg^+MHC^- mice reacted with self MHC (I-A^b) and about 4% of the hybrids from the normal mice reacted with self MHC. These results indicate that TCRs on $CD4^+$ cells that mature in the absence of MHC in Ly-6A.2 transgenic mice recognize many MHC molecules tested at high frequencies.

DISCUSSION

The present study was originally undertaken to understand the mechanisms involved in the inhibition of T cell development in Ly-6A.2 transgenic mice. To that end, we conclude that TCR interactions with the MHC molecules do not contribute to this block in development (Figure 1C). These observations are not surprising because we now know that the blockade of maturation in the Ly-6A.2 transgenic mice occurs at a stage of development when the TCR is not expressed (12). However, this was not known when these experiments were initiated. Nevertheless, these experiments led us to the unexpected observation that ectopic expression of Ly-6A.2 molecule on $CD4^+CD8^+$ thymocytes allowed maturation of $CD4^+CD8^-$ cells independently of MHC-peptide complexes. This later observation provides another reason that Ly-6A.2 may be extinguished on $CD4^+CD8^+$ cells. These results raise the possibility that another Ly-6 family member, which is endogenously expressed on CD4+CD8+ cells, might play a role in positive selection.

The $CD4^+CD8^-$ cells that appear in the thymus and periphery of Tg^+MHC^- mice appear to be similar to the $CD4^+$ mature cells by a number of phenotypic criteria. They have down-regulated CD8 and HSA molecules and up-regulated TCR proteins. The $CD4^+CD8^-$ cells that mature in the thymi of Ly-6A.2^+MHC^- mice also express the CD40 ligand on cell stimulation and proliferate upon cross-linking of the TCR/CD3 complex. The proliferative responses of Tg^+MHC^- thymocytes were similar to the response of Tg^+MHC^+ thymocytes (data not shown) and were, in all cases, not less than those observed for thymocytes from normal (Tg^-MHC^+) mice. In contrast, the thymocytes from

the $Tg^{-}MHC^{-}$ do not proliferate after cross-linking of their TCRs, which is consistent with a previous report (14). Furthermore, the $CD4^{+}CD8^{-}$ T cell emigrate from the thymus to peripheral lymphoid organs.

Previous reports have demonstrated that overexpression of CD8 can promote the selection of $CD4^{+}$ cells in the absence of class II molecules (23), and Bcl-2 expression allows maturation of $CD8^{+}$ cells in the absence of a class I molecule (29). More recently, preferential maturation of CD8 cells in Notch 1 transgenic mice was observed (30). The Notch-mediated maturation of $CD8^{+}$ cells was not observed in the absence of both class I and II molecules. The Ly-6 transgenic mouse is the first example that we are aware of in which $CD4^{+}$ cells appear in the absence of both MHC class I and II molecules and therefore provides a unique opportunity to study thymic selection.

Maturation of $CD4^{+}$ cells in the absence of MHC expression is surprising. Our experiments also indicate that $CD4^{+}$ cells do not mature in Ly-6A.2Tg^{+} MHC^{-} mice if the expression is low (fivefold; data not shown). It is possible that this maturation is driven by the interaction of over-expressed Ly-6A.2 on thymocytes with its ligand in the thymus. This notion is consistent with our recent observation that indicates the presence of intrathymic Ly-6A.2 ligand (31). This interaction may either result in signals that mimic the TCR signaling or use the TCR/CD3 complex to signal into the cell. The rationale for this hypothesis is as follows. First, cross-linking Ly-6A.2 proteins on T cells with Abs causes cell activation and secretion of cytokines (16). Second, Ly-6A.2 binds to several key protein tyrosine kinases, e.g., $_{p}56^{lck}$ and $_{p}59^{fyn}$, that are critical for signaling through TCR (32). Third, mutations or antisense oligonucleotides that decrease Ly-6A.2 expression also diminish T cell responsiveness (33, 34, 35). Reciprocally, a loss

of TCR/CD3 expression also results in a lack of immune responsiveness through Ly-6A.2 (36, 37). More recently, Ly-6A null mice have been generated and have altered the proliferative responses of mature T cells (38). In these mice, lack of Ly-6A.2 expression results in higher proliferation of splenic T cells to anti-CD3 Abs, mitogens, and alloantigens. Taken together, these results indicate that Ly-6A.2 is able to regulate signaling negatively or positively through the TCR/CD3 complex. Why Ly-6A.2 expression has opposite effects on signaling through the TCR/CD3 is unclear.

It is interesting to note that signaling through Ly-6A.2 results in the maturation of only $CD4^+$ cells and not $CD8^+$ cells. One possibility is that Ly-6A.2 molecules provide a signal that favors the maturation of $CD4^+$ cells as opposed to $CD8^+$ cells. Alternatively, this signaling may simply allow progression down the default pathway of maturation for the maturation of $CD4^+$ cells (39). This interpretation is consistent with the recent observation that maturation of CD8 cells needs additional lineage-specific signals, which may be delivered by the activated Notch-1 molecule upon its interaction with its ligand (30). We propose that the signals to activate the Notch or its ligand or other CD8 lineage-specific signals are not delivered in Ly-6A.2Tg^+MHC^- mice. We hope that additional experiments with Ly-6A.2Tg^+MHC^- mice will provide insights into the mechanism of lineage commitment.

Since overexpression of Ly-6A.2 results in the maturation of $CD4^+$ cells in the absence of MHC class I and II molecules, it is likely that these cells represent the initial unselected T cell repertoire. This contention is supported by the observation that the V_β repertoire of the $CD4^+$ cells and $CD4^+CD8^+$ cells from the Ly-6A.2Tg^+MHC^- mice are similar. Furthermore, this similarity extends to the repertoire of the $CD4^+CD8^+$ DP cells

from $Tg^{-}MHC^{-}$ mice. It is very unlikely that CD4+ cells selected in the thymus of $Tg^{+}MHC^{-}$ mice are selected by H2-O, an atypical MHC class II molecule, for the following reasons: 1) H-2O is primarily expressed intracellularly (40); 2) Abs to I-A and I-E block the reactivity of these cells to I-A- and I-E-expressing APCs, and these Abs are not known to cross-react with H2-O (L. Karlsson, unpublished observations). Therefore, these results strongly suggest that CD4+ cells are not selected by the H-2Oprotein. It is also unlikely that $CD4^{+}$ cells are selected by the class I heavy chain on the surface of cells in β2-microglobulin knock-out mice, since the reactivity of these cells to MHC is inhibited by anti-class II Abs. Taken together, these observations strongly argue that $CD4^{+}$ cells that mature in the absence of MHC class I and II molecules represent the T cell repertoire that exists before thymic selection.

The phenomenon of alloreactivity is not well understood. Our results suggest that a higher frequency of alloreactive T cells exists before thymic selection than previously predicted. Approximately 12 to 15% of the hybrids from the Ly-6A.$2Tg^{+}MHC^{-}$ mice reacted with different MHC molecules. This is similar to the 10 to 12% of the hybrids from the normal mice that showed this reactivity. These results would suggest that alloreactivity is inherent in the preselected repertoire, and it is not a consequence of selection of the receptor that reacts with self MHC and peptide. These results are consistent with the recent observations that have analyzed the reactivity of $CD4^{+}$ cells that mature in the FTOC of MHC^{-} mice in the presence of a combination of anti-TCR and anti-CD4 Abs (11) or by bi-specific CD3/CD4 Abs (41). The percentage of reactivity to $H\text{-}2^{b}$ was lower than with $H\text{-}2^{d}$ and $H\text{-}2^{k}$ and probably reflects reactivity to only I-A molecules (since I-E is not expressed). Approximately 4% of the hybrids derived from

C57BL/6 mice reacted with APCs from the same mouse strain (Figure 8). This reactivity may reflect T cells that respond to syngeneic APCs, described as a phenomenon of syngeneic MLR (SMLR) (42, 43, 44).

It has become increasingly clear that negative selection plays a crucial role in shaping the T cell repertoire. The degree to which the clonal deletion may impact the T cell repertoire varies from 5 to 50% (13, 44). $CD4^+$ cells that mature in the Ly-6A.2 transgenic mice react with syngeneic stimulators at a higher frequency, indicating that the T cell repertoire in these mice will include T cells that are normally deleted in the the thymus. Analysis of peripheral repertoire in Ly-6A.2Tg^+MHC^- mice will provide clues about the nature of self-reactive T cells that normally do not exit the thymus.

In summary, our results indicate that $CD4^+$ cells that appear in the thymi of Ly-6A.2 transgenic mice in the absence of MHC molecules are phenotypically like mature T cells. These cells represent the preselected T cell repertoire that has high reactivity to MHC molecules. These later results indicate that the initial, preselected T cell repertoire has an evolutionary bias in its reactivity to MHC protein.

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Figure 2.1 Analysis of T cell subsets in the thymus of Ly-6A.2 transgenic MHC- mice. Thymocytes from 4- to 8-wk-old normal (Ly-6A.2Tg^{-}MHC^{+}), Ly-6A.2Tg^{-}MHC^{-}, Ly-6A.2Tg^{+}MHC^{+}, and Ly-6A.2Tg^{+}MHC^{-} mice were examined for expression of CD4, CD8, and TCR-ß as described in Materials and Methods. **A & B**, Shows expression of CD4 and CD8 on different subsets that appear in the thymi of these mice. The number above box R1indicates the percentage of mature CD4^{+}CD8^{-} cells. CD4^{+}CD8^{-} thymocytes (R1 box in upper four panels) were analyzed for the expression of TCR-ß (lower four panels). The y-axis indicates that TCR-ß expression is shown only from a CD4^{+}CD8^{-} subset.

C, T cell subsets in the thymus of Ly-6A.2 transgenic MHC-negative animals. The total number of thymocytes recovered from 4- to 8-wk-old Ly6A.2Tg+MHC+ (n = 3), Ly-6A.2Tg+MHC- (n = 14) and their control littermates, i.e., Tg-MHC+ (n = 9) and Tg-MHC- (n = 12) are shown. At least three animals from each genotype were analyzed for T cell subsets using CD4 and CD8 cell surface molecules as described in Materials and Methods and in the legend to Figure 1. The data are represented as mean ± SEM.

FIGURE 2.1A & B

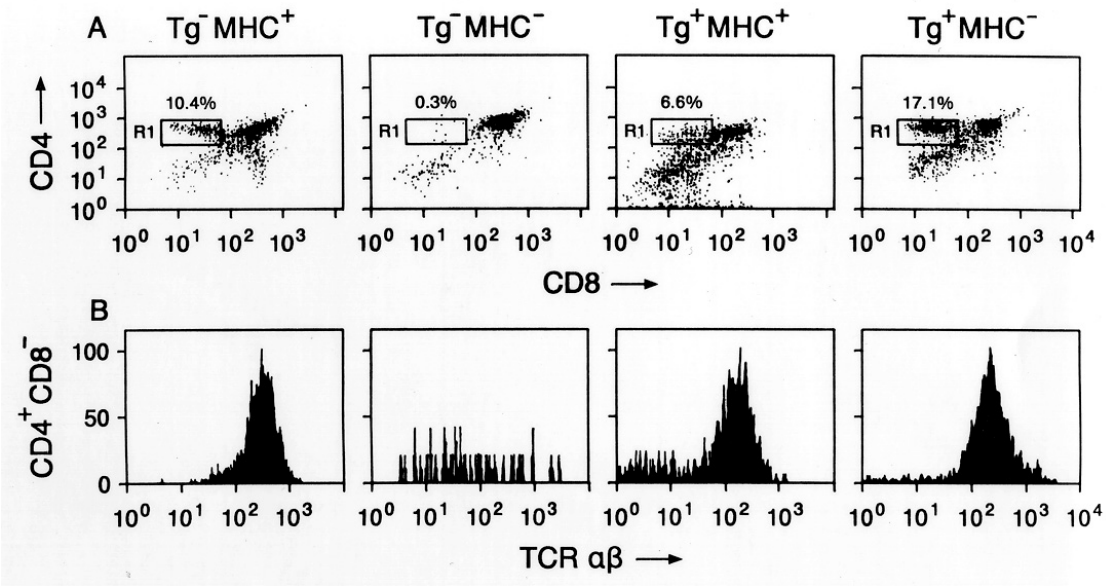

A
Tg⁻MHC⁺
Tg⁻MHC⁻
Tg⁺MHC⁺
Tg⁺MHC⁻
10.4%
0.3%
6.6%
17.1%
R1
CD4
CD8
B
CD4⁺CD8⁻
TCR αβ

FIGURE 2.1C

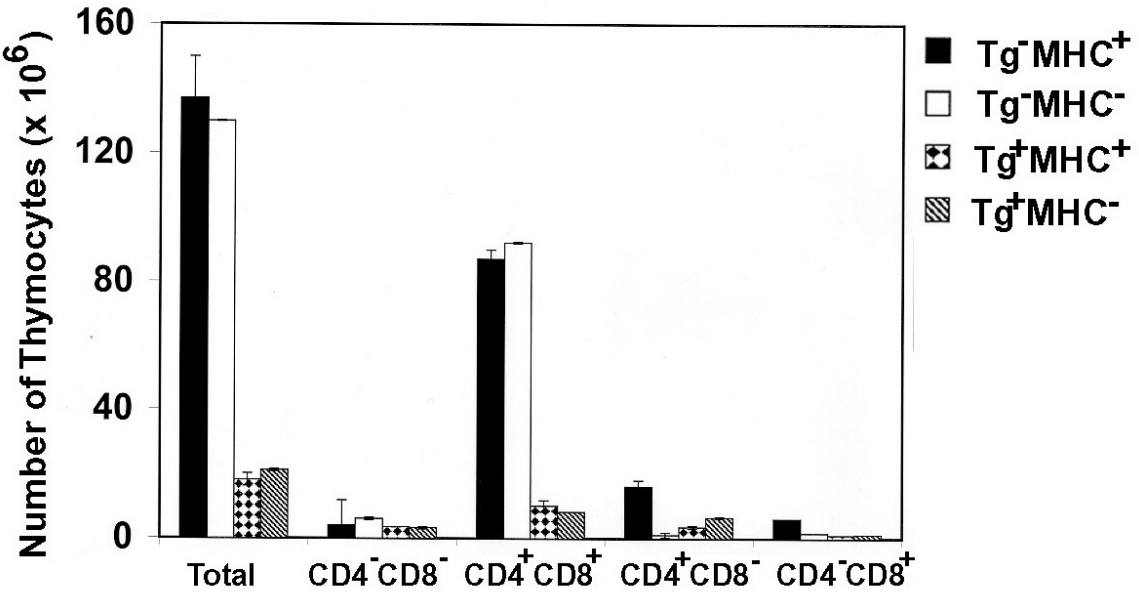

Number of Thymocytes (x 10^6)
160
120
80
40
0
Total
CD4-CD8-
CD4+CD8+
CD4+CD8-
CD4-CD8+
Tg-MHC+
Tg-MHC-
Tg+MHC+
Tg+MHC-

Figure 2.2. Analysis of TCR-ß and HSA expression on thymocytes. T cells from the thymus of 4- to 8-wk-old normal (Ly-6A.2Tg^{-}MHC^{+}), Ly-6A.2Tg^{-}MHC^{-}, and Ly-6A.2Tg^{+}MHC^{-} mice were analyzed for the expression of TCR-ß and HSA.

FIGURE 2.2

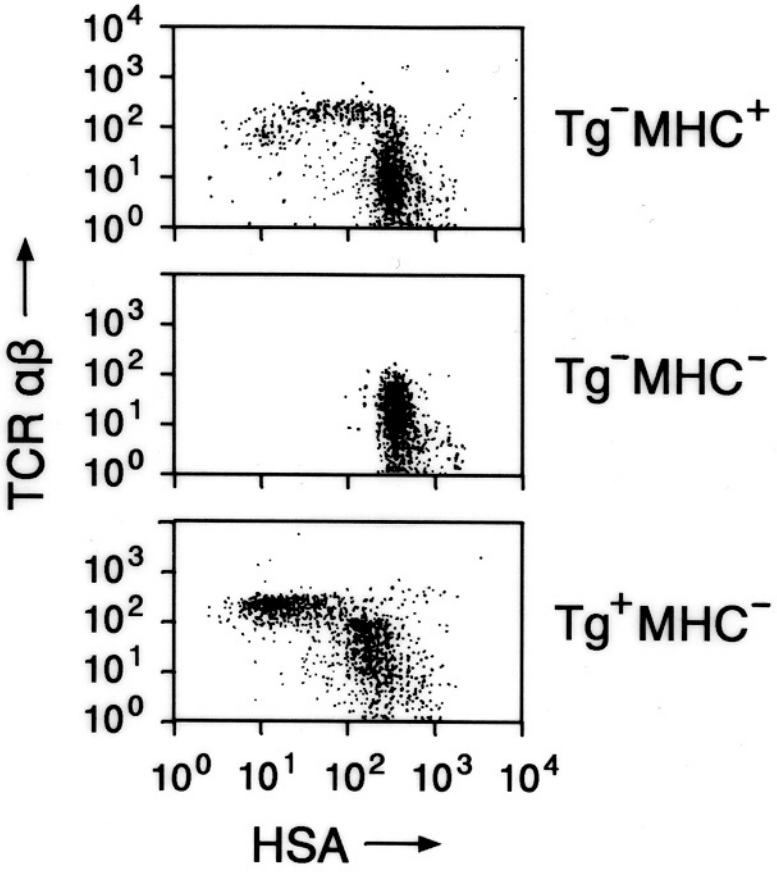

10^4
10^3
10^2
10^1
10^0
Tg^-MHC^+
Tg^-MHC^-
Tg^+MHC^-
TCR αβ
10^0 10^1 10^2 10^3 10^4
HSA

Figure 2.3. Analysis of T cell subsets in the lymph node of Ly-6A.2Tg^{+}MHC^{-} mice. Cells from the lymph nodes of 4- to 8-wk-old normal (Ly-6A.2Tg^{-}MHC^{+}), Ly-6A.2Tg^{-} MHC^{-}, and Ly-6A.2Tg^{+}MHC^{-} mice were analyzed for the expression of CD4, CD8, and CD44 as described in Materials and Methods. A, Shows the expression of CD4 and CD8 on lymph node cells. The numbers above each box represent the percentage of mature CD4^{+}CD8^{-} cells that appear in the lymph node. B, Expression of CD44 on CD4^{+}CD8^{-} (boxed in A) lymph node cells. The number above the bar represents percentage of cells that express higher levels of CD44.

FIGURE 2.3

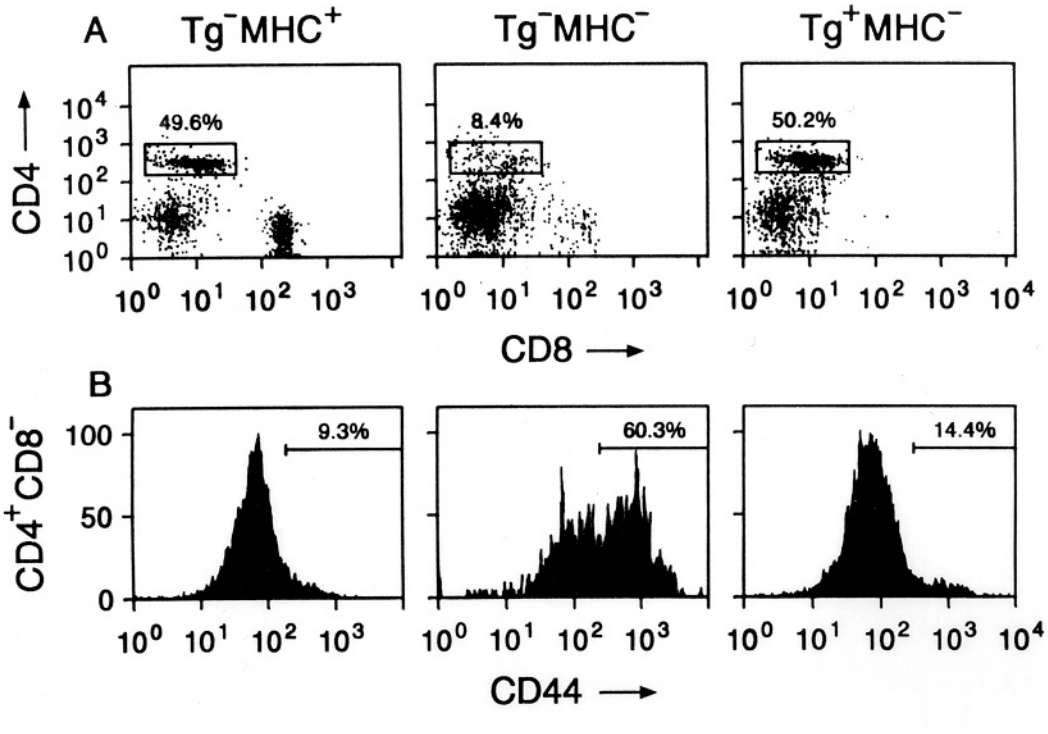


Figure 2.4. Expression of CD40 ligand on spleen cells from Ly-6A.2Tg^{+}MHC^{-} mice. Tris-NH_4Cl-treated splenic cells from 4- to 8-wk-old normal (Ly-6A.2Tg^{-}MHC^{+}), Ly-6A.2Tg^{-}MHC^{-}, and Ly-6A.2Tg^{+}MHC^{-} mice were cultured with a combination of PMA (20 ng/ml) and calcium ionophore (0.25 μg/ml) for 4 to 5 h, then analyzed for the expression of CD4, CD8, and CD40 ligand (gp39) molecules as described in Materials and Methods. The CD4^{-}CD8^{+} (A) and CD4^{+}CD8^{-} (B) cells were gated and examined for the expression of CD40 ligand.

FIGURE 2.4

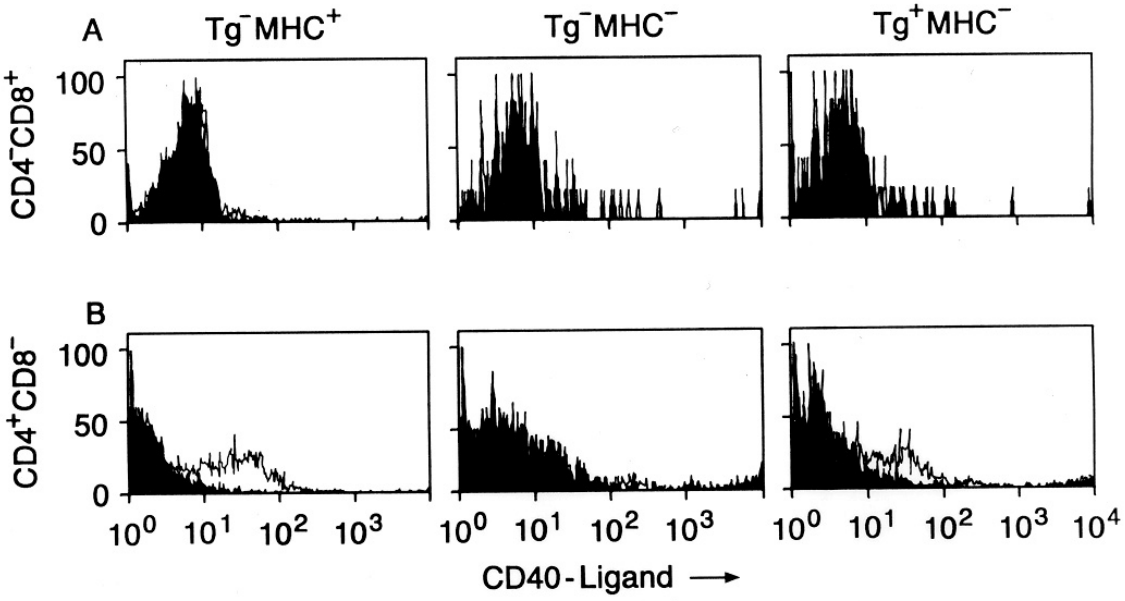

A
Tg⁻MHC⁺
Tg⁻MHC⁻
Tg⁺MHC⁻
CD4⁻CD8⁺
100
50
0
B
CD4⁺CD8⁻
100
50
0
10⁰ 10¹ 10² 10³
10⁰ 10¹ 10² 10³
10⁰ 10¹ 10² 10³ 10⁴
CD40-Ligand

Figure 2.5. Responsiveness of thymocytes from Ly-6A.2Tg^{+}MHC^{-} mice to anti-CD3 stimulation. Microcultures were prepared with 5 x 10^{5} thymocytes from 4- to 8-wk-old Ly-6A.2Tg^{+}MHC^{+}, Ly-6A.2Tg^{-}MHC^{-} (closed squares), Ly-6A.2^{+}MHC^{-} (closed circles) in the presence or absence of anti-CD3 Ab supernatant at dilutions indicated. After 2 days, the proliferation was quantitated by measuring the incorporation of [3H] thymidine. The data displayed are a representative experiment; error bars show intra-assay variation.

FIGURE 2.5

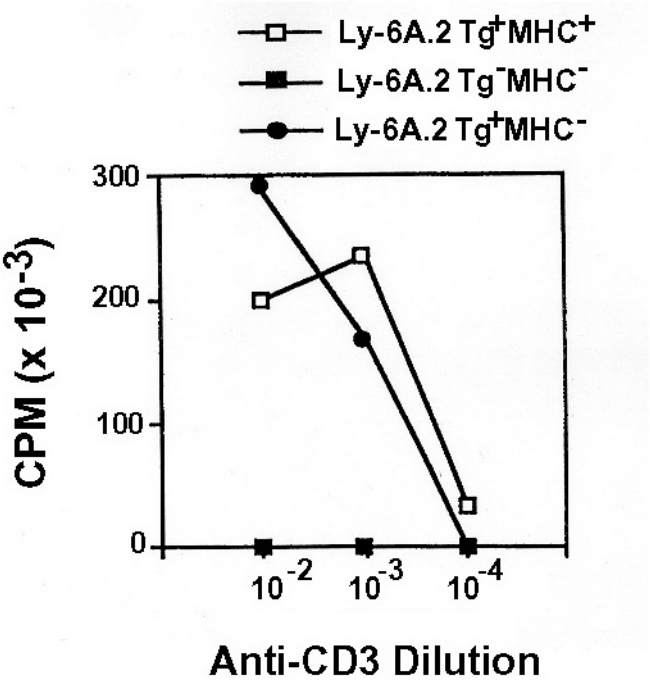

Ly-6A.2 Tg+MHC+
Ly-6A.2 Tg-MHC-
Ly-6A.2 Tg+MHC-
CPM (x 10-3)
300
200
100
0
10-2
10-3
10-4
Anti-CD3 Dilution

Figure 2.6. TCR-$V_ß$ repertoire in Ly-6A.2Tg^+MHC^- mice. Thymocytes from Tg^+MHC^- and Tg^-MHC^- mice were stained with anti-CD4--phycoerythrin, anti-CD8--FITC, and anti-$V_ß$ ($V_ß$3, -5, -8, -9, -10b, and -11)-biotin (PharMingen) followed by streptavidin-Red 613 in a three-color immunofluorescence. Vß expression on gated $CD4^+CD8^+$ (DP) and $CD4^+$ single positive (SP) cells from Ly-6Tg^+MHC^- (three experiments) and on DP cells from Tg^-MHC^- (two experiments) mice is represented.

FIGURE 2.6

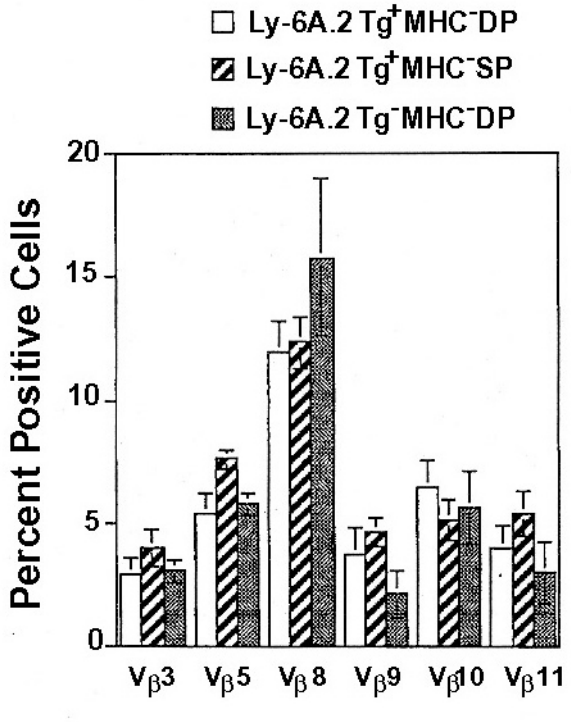

Ly-6A.2 Tg+MHC-DP
Ly-6A.2 Tg+MHC-SP
Ly-6A.2 Tg-MHC-DP
Percent Positive Cells
20
15
10
5
0
Vβ3
Vβ5
Vβ8
Vβ9
Vβ10
Vβ11

Figure 2.7. Reactivity of $CD4^+$ T cells from Tg^+MHC^- mice to MHC molecules. Microcultures with 5 x 10^5 lymph node cells from C57BL/6 (H-2b) or CBA/J (H-2k), or Ly-$6Tg^-$ MHC^- or Ly-$6Tg^+$ MHC^- mice were set up with 2.5 x 10^6 irradiated spleen cells with (white) or without (dark) anti-MHC class II Ab (M5/114). Cells were pulsed with [3H] thymidine and harvested after 5 days of culture. The data are expressed as arithmetic mean cpm of [3H] thymidine incorporated.

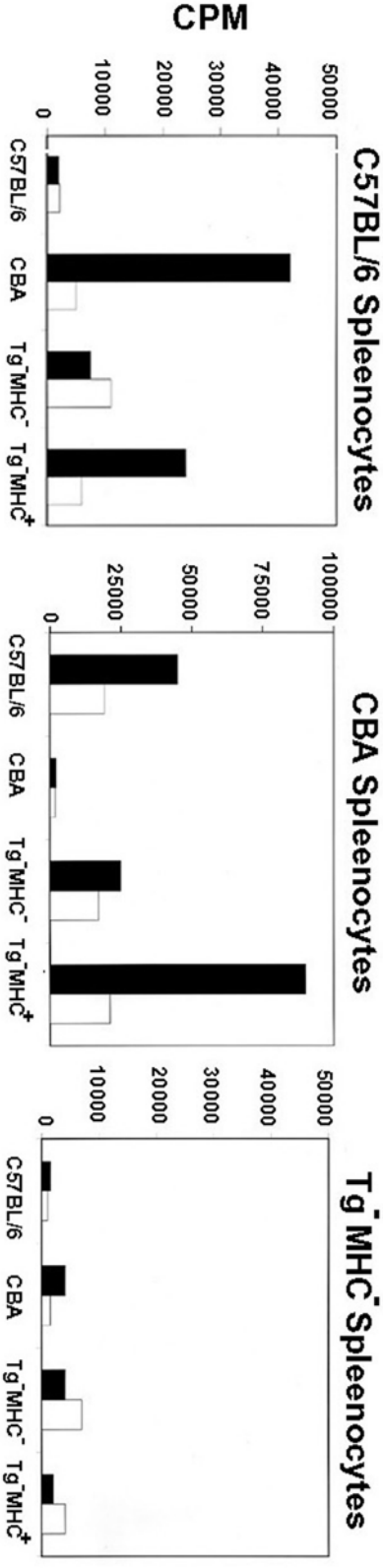
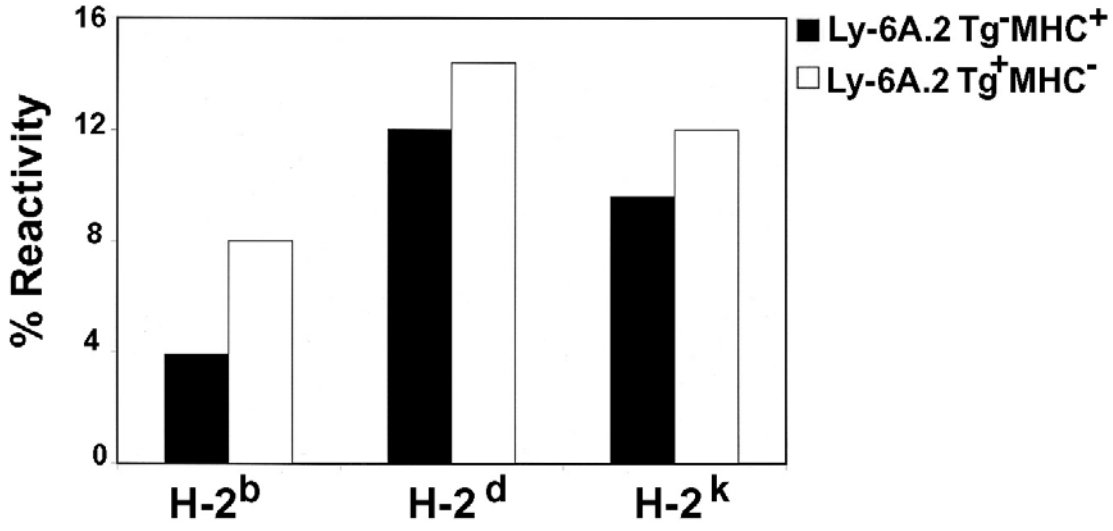

CPM
C57BL/6 Spleenocytes
CBA Spleenocytes
Tg-MHC- Spleenocytes
C57BL/6
CBA
Tg-MHC-
Tg-MHC+

Figure 7

Figure 2.8. T cell hybridomas were generated and analyzed for their response to irradiated spleen cells from C57BL/6 (H-2b), BALB/c (H-2d), and CBA (H-2k) mice as described in Materials and Methods. T-T hybridomas tested were from 3402 or 3841 uncloned wells after the fusion and selection in hypoxanthine-aminopterin-thymidine (HAT) medium. IL-2 content from the cultures was analyzed for its ability to support growth of HT-2 cells. The cultures were pulsed and harvested as described (29). Cultures that gave 10-fold or higher cpm as compared with the background were considered as positively reactive. Background cpm in these cultures was typically <500 cpm.

FIGURE 2.8



CHAPTER 3

UNREGULATED EXPRESSION OF LY-6A.2 ON DEVELOPING THYMOCYTES AFFECTS PRODUCTION OF LAT AND ALTERS THE V_β T CELL REPERTOIRE.

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[1]S. C. Henderson and A. Bamezai

To be re-submitted to *Journal of immunology* after revision.

ABSTRACT

Ly-6A.2 exhibits a regulated pattern of expression on T cells maturing in the thymus. Dysregulated expression of Ly-6A.2 results in developmental arrest of maturing thymocytes within the $CD3^-CD4^-CD8^-$ triple negative (TN) stage where the normal expression of Ly-6A.2 is extinguished. In this study we demonstrate that the block in T cell development in Ly-6A.2 transgenic mice is attributed solely to the bone marrow cells, as the thymic epithelial cells in these mice are normal. In order to further characterize the mechanisms underlying this block, we examined whether cell signaling and/or cell adhesion properties of the Ly-6A.2 molecule influenced the block in the T cell development. Analysis of bone marrow chimeras generated by injecting CFSE-labeled Ly-6A.2 transgenic bone marrow cells into irradiated syngeneic non-transgenic mice revealed normal trafficking of developing T cells from the cortex into the medulla. However, expression of the Ly-6A.2 transgene was significantly reduced on thymocytes that had traversed the cortico-medullary junction to enter the medulla. Two signaling proteins, linker for activation of T cells (LAT) and $p56^{lck}$ (Lck), known to play a critical role in the transition of DN to $CD4^+CD8^+$ double positive (DP) cells were examined. $CD4^-CD8^-$ DN cells from Ly-6A.2 transgenic mice produced diminished amount of LAT protein when compared to the non-transgenic controls. In contrast, dysregulated Ly-6A.2 expression did not alter the production of Lck in these cells. Proper rearrangement of TCR gene loci is critical for the progression of development beyond the DN cell stage. Dysregulated expression of Ly-6A.2 did not suppress endogenous TCR-V_β expression,

which was further supported by the observation that introduction of previously re-arranged $TCR_{\alpha\beta}$ genes did not rescue thymocyte development in Ly-6A.2 transgenic mice. Finally, dysregulated expression of Ly-6A.2 on the DP subset of developing cells enhanced apoptosis in the majority of thymocytes and altered the selected TCR-V_β repertoire by facilitating the rescue of super-antigen deleted TCR-V_β3 but deleting the TCR-V_β8.1, 8.2 expression T cells. Taken together, these observations indicate that the termination of Ly-6A.2 expression and signaling within the $CD4^-CD8^-CD3^-$ subset of developing T cells is an important checkpoint during normal thymic development.

INTRODUCTION

The thymus is a unique organ dedicated to shaping the T cell repertoire through processes of selection and lineage differentiation. Progenitor cells enter the thymus and undergo a series of developmental progressions, which are proposed to ensure production of functionally distinct CD4 and CD8 subclasses of T cells that have sufficient T cell receptor (TCR) diversity, are self-MHC restricted, and tolerant to self antigens highly represented in the thymus (1, 2). The dynamic process involving maturation of pluripotent stem cells to the T cell lineage and subsequent commitment to either CD4 or CD8 differentiation is poorly understood. Inherent to this process is the ability of different cell surface proteins to provide stage specific signals, necessary for proper thymocyte development. Equally important may be the loss of expression of cell signaling/cell adhesion molecules at specific stages of T cell development. It has recently been reported that a member of the hedgehog (Hh) family of secreted proteins, sonic

hedgehog (Shh), plays a major part in regulating the differentiation of the DN subset into the DP cell stage (56).

It has long been established that thymocytes begin to alternate expression of cell surface antigens at distinct developmental checkpoints (3, 4). Extensive analysis of these cell surface markers has resulted in a molecular map of T cell development in the thymus. Progenitors that commit to the T cell lineage migrate into the subcapsular region of the outer cortex where they begin to interact with thymic stromal elements (2, 5). The earliest precursors that home to the thymus express Ly-6A.2 (T cell activating protein, Stem Cell Antigen-1), a member of the Ly-6 supergene family and lack the expression of lineage specific markers including CD3, CD4 or CD8 surface antigens and are referred to as the triple negative (TN) subset (6). The most immature thymocytes are $CD44^{+}CD25^{-}$, express high levels of c-kit (7) and stem cell antigen-1 (Ly-6A.2) (8) and low level expression of Thy-1 (9) and heat stable antigen (HSA) (10). Upon migration through the thymic cortex, the next developmental subset is characterized by up-regulated expression of CD25, HSA, Thy-1, and IL-7-receptor α. A majority of $CD44^{+}CD25^{+}$ TN cells express Ly-6A.2 on the surface (10). Down-regulation of CD44, c-kit, and Ly-6A.2 defines the transition of $CD44^{+}CD25^{+}$ to the next developmental stage, $CD44^{-}CD25^{+}$, in which a successfully rearranged TCR_{β} joins the pre-T_{α} on the cell surface to form the pre-TCR. Thymocytes that have passed developmental checkpoints thus far reach the final TN stage marked by down-regulation of CD25. Upon expression of $TCR_{\alpha\beta}$/CD3 on the cell surface, late stage $CD44^{-}CD25^{+}$ TN thymocytes continue developmental processes by inducing expression of both CD4 and CD8 co-receptor molecules on the cell surface, thus transitioning into the DP stage of development (11, 12). These double

positive (DP) cells ultimately cross the cortico-medullary junction to migrate into the medulla where selection and differentiation processes are completed and CD4 or CD8 single positive (SP) thymocytes exit the thymus to enter peripheral circulation.

Stage-specific expression of cell surface signaling proteins is critical for the transitioning of thymocytes into each developmental subset. Mice with targeted disruption of genes encoding components critical for the generation and assembly of the pre-TCR- including recombination activating gene (RAG)-$1^{-/-}$ RAG-$2^{-/-}$, pT_{α}, $CD3_{\varepsilon}$, $CD3_{\gamma}$, $CD3_{\zeta}$ or TCR_{β}-exhibit severe defects in T cell development early within the TN subset (13-18). Double deletion of two key growth/cytokine receptors, c-kit and IL-7 respectively, inhibits maturation of thymocytes past the $CD44^{-}CD25^{+}$ stage, resulting in thymi that are alymphoid, and TCR-β, -γ, and -δ rearrangements are essentially undetectable (19). Transition from the DP to SP subset also requires up-regulation of key cell surface proteins, which enable thymocytes to engage in selection and differentiation processes. Although a functional TCR may be present, altering CD4 or CD8 expression on the cell surface inhibits thymocytes from engaging in meaningful interactions with selecting MHC/peptide complexes, thereby preventing maturation past the DP stage (20, 21). Up-regulation of IL-2 and IL-2R, which are associated with early thymocyte development and survival, have also been implicated in facilitating deletion of self reactive, antigen specific thymocytes in later stages of maturation (22). Finally, differential signaling from Notch1, has been implicated in several aspects of DN and DP thymocyte development, including commitment to the T cell lineage, TCRαβ vs. TCRγδ differentiation, and signaling to influence differentiation of CD8 SP thymocytes (23-26).

The underlying mechanisms for developmental blocks from altering expression of the above molecules can be attributed, in part, to alterations in the signaling cascade emanating from the TCR upon MHC/antigen ligation. Several membrane-proximal signaling molecules have been identified to be critical components of thymocyte maturation. Src family tyrosine kinase, $p56^{lck}$ (Lck) is important in early (TN to DP) and late (DP to SP) transitional stages of thymocyte maturation (27, 28). Additionally, preferential association of Lck with the cytoplasmic domain of CD4 is suggested to support differentiation of CD4 SP thymocytes (29-35). Progression within the TN developmental stage is highly dependent upon the integral membrane protein LAT (36). Mice deficient in LAT are observed to have a profound block at the $CD44^{-}CD25^{+}$ TN stage and neither DP nor SP thymocytes are detected in these animals. Activation of ZAP-70 upon productive TCR engagement has been shown to be crucial for pre-TCR signaling, thymocyte selection and transitioning from the DP to SP developmental stage (37, 38). Downstream effectors, including Itk, PLC-γ1, Grb-2, and Erk, have also been implicated as important regulators of the successive progression of thymocytes (1). Although other cellular processes and cell surface molecules regulating development T cells in the thymus remain largely unknown, examination of differentially expressed molecules may provide valuable clues- as transitioning through stage-specific developmental checkpoints may be dependent on regulated expression and ligation of these surface proteins and subsequent signaling they induce.

We have previously generated mice with the Ly-6A.2 gene under the control of the human CD2 enhancer to drive constitutive expression of Ly-6A.2 on thymocytes expressing Ly-6E on their cell surface (39). Constitutive transgenic expression of Ly-

6A.2 on all developing thymocytes, including ones that normally do not express this protein, results in a marked impairment in thymocyte maturation. Thymocytes are developmentally arrested within the $CD4^-CD8^-$ double negative (DN) stage, which corresponds to the time point subsequent to normal down-regulation of Ly-6A.2. The developmental block in Ly-6A.2 transgenic mice is incomplete, as both $CD4^+CD8^+$ and mature subsets appear in Ly-6A.2 transgenic thymi. However, analysis of the cellularity from this subset reveals their numbers are reduced by >90%. Thus, the loss of Ly-6A.2 expression within the DN and DP cell stages is critical for thymocyte development. The mechanism for this block is not clear, but recently described cell signaling and adhesive properties of this molecule may provide insight.

We report here that unregulated expression of Ly-6A.2 resulted in a decrease in the production and phosphorylation of LAT, which is likely to inhibit the progression from DN to DP T cells in the thymus. Although equivalent amounts of Lck protein were detected in Ly-6A.2 transgenic and non-transgenic thymocytes, diminished localization of this protein to cytoplasmic domains of the CD4 molecule was observed in Ly-6A.2 transgenic thymocytes. Un-regulated expression of the Ly-6A.2 molecule does not inhibit expression of endogenous V_β-chain rearrangement but enhances cell death of thymocytes by apoptosis and alters the T cell repertoire that develops in Ly-6A.2 transgenic mice. Collectively, these data indicate that loss of Ly-6A.2 expression within the DN subset of developing T cells is an important developmental checkpoint during normal thymic maturation, and that continued expression of this protein alters signaling that governs selection and differentiation processes.

MATERIAL AND METHODS

Mice. The Ly-6A.2 transgenic and non-transgenic (BALB/c background; 6-8 weeks) and Ly-6A.2 transgenic and MHC Class I x II-deficient mice (C57BL/6 background; 6-8 weeks) that were used have been previously described (39). Ly-6A.2 transgenic mice were bred to Vß8.2 (34), DO11 TCR (35) and $_{p}56^{lck}$ transgenic mice (40).

Flow Cytometry. One X 10^6 thymocytes were incubated with various antibodies followed by appropriate fluorochrome-conjugated second step reagents. Cells (5-10 X 10^3) were analyzed on a EPICS Elite Analyzer flow cytometer (Beckman-Coulter, Fullerton, CA). Reagents used for this analysis were: Annexin V (PharMingen, San Diego, CA), phycoerythrin-conjugated anti-CD4 (H129.19), FITC-conjugated CD8 (53-6.7), biotin-anti-Ly-6A.2 (D7) (PharMingen, San Diego, CA), biotin-anti-TCR (H57) and biotin-anti-$V_{\beta}3$, 5.1, 5.2, 6, 8.1, 8.2, 9, 10b, 11, and 17a (PharMingen, San Diego, CA) and streptavidin-Red 670 (Life Technologies, Arlington Heights, IL).

Staining of Bone Marrow Cells with CFSE. Prior to adoptive transfer into mice, cells were suspended at 10^7/ml in PBS and labeled with 4μM CFSE dye (5-(and –6)-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, Eugene, OR) at 37 ^{o}C for 10 minutes. CFSE-labeled cells were washed three times in complete RPMI with 10% FBS and re-suspended at 10^7 cells in 0.1 ml of sterile PBS.

Bone Marrow Chimeras. Bone marrow was harvested from femurs of Ly-6A.2Tg- and Ly-6A.2Tg+ mice (BALB/c background). Bone marrow recipient mice were subjected to 900 rads of gamma radiation (135 Cobalt source), and CFSE-labeled or un-labeled donor stem cells ($1x10^7$) were injected intravenously 4 to 8 hours later. Thymi were harvested and analyzed on days 4, 7, 14, 21, and 28 post-injection by immunohistochemistry and flow cytometery.

Tissue Sections and Immunohistochemistry. Reconstituted thymi were removed from bone marrow chimeras and one thymic lobe from each thymus was separated to assay cellularity. The remaining lobe was snap-frozen in liquid nitrogen for 20 s, and stored at -80^{o}C. Frozen tissue sections were embedded in M-1 embedding matrix (Lipshaw, Pittsburg, PA) and 5-8 um-thick sections were cut at -20^{o}C. Sections were then fixed using a graded series of acetone in water (60%, 70%, 80%, 90% acetone) for 3 minutes in each solution. Sections were washed in PBS containing 0.05% Tween 20 for 10 minutes. Endogenous avidin and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) according to vendor's recommendations. Tissue sections were incubated with biotinylated primary antibodies for 1 hour, and washed 3 times in 0.1M PBS containing 0.05% Tween-20. Each wash was carried out for 10 minutes, each wash followed by incubation with avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA). Tissue sections were again washed before visualization with 3',3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) and counter-staining with

hematoxylin (Sigma, St. Louis, MO). Slides were air-dried and mounted permanently using DPX (Aldrich Chemical Company Inc., Milwaukee, WI).

Generation of Lysates and Isolation of Glycolipid-Enriched Microdomains (GEMs). Four x 10^7 cells were used to generate whole cell lysates from Ly-6A.2 transgenic and non-transgenic thymocytes by lysing in buffer containing 1% NP-40, 0.25% deoxycholate, 50 mM Tris, pH 7.5, 12.5 μg/ml leupeptin, 0.5% aprotinin, 1 mM PMSF, 5 mM EDTA and 2.5 mM Na3VO4. Glycolipid-enriched microdomains (GEMs) were isolated as previously described (41). Briefly, thymocytes ($1x10^8$) were washed in PBS and re-suspended in 1ml of ice cold MBS (25mM MES, 150nM NaCl [pH6.5]), 0.5% Triton X-100, 1 mM Na3VO4, 2 mM EDTA, 1 mM PMSF, and 1 μg/ml aprotinin for 30 minutes on ice. Lysates were then homogenized with 10 strokes of a loose-fitting Dounce homogenizer and mixed with 1ml of 85% sucrose (w/v) in MBS. The sample was placed into an SW40 centrifuge tube and was overlaid with 6ml of 35% sucrose and 4ml of 5% sucrose in MBS containing 1mM Na3VO4. Lysates were centrifuged 16 h at 200,000 x g at 4^{o}C. GEMs were harvested by collecting 1ml fractions from the bottom of the gradient tube.

Immunoprecipitations. Thymocytes (40 x 10^6) were solubilized by lysing in buffer containing 1% NP-40, 0.25% deoxycholate, 50 mM Tris, pH 7.5, 12.5 μg/ml leupeptin, 0.5% aprotinin, 1 mM PMSF, 5 mM EDTA and 2.5 mM Na3VO4. Lysates were immunoprecipitated twice with anti-CD4 (PharMingen, San Diego, CA) conjugated with protein-G speharose beads at 4°C. The Ab complexes were generated by incubating protein G-Sepharose (20 μl of swollen gel, 1 mg/ml) with 50 μl of the appropriate Ab containing

supernatants for 45 min on ice before immunoprecipitation. Proteins were resolved by SDS-PAGE and immunoblotted using anti-$p56^{lck}$ antiserum.

Western blots. For protein separation and analysis, total cell lysates or GEM fractions were subjected to SDS-PAGE and transferred onto PVDF membrane. Membranes were blocked in 5% bovine serum albumin, incubated with anti-$_{p}56^{lck}$ (Santa Cruz Biotechnology, Santa Cruz, CA; and a generous gift from Dr. Pauline Johnson), anti-LAT (Santa Cruz Biotechnology, Santa Cruz, CA; and a generous gift from Dr. Larry Samelson), and anti-phosphotyrosine (Sigma, St. Louis, MO) antiserum, and were then followed by washing, detection with NutrAvidin horseradish peroxidase (Pierce, Rockford, Il), and visualized by enhanced chemiluminescence (Pierce, Rockford, Il). Signals were quantitated by densitometric analysis.

RESULTS

The block in thymocyte development in Ly-6A.2 transgenic mice is bone marrow cell-dependent. Continual expression of Ly-6A.2 on all thymic subsets, including cells that normally lack expression, was previously shown to result in developmental arrest of thymocyte maturation within the $CD3^{-}CD4^{-}CD8^{-}$ (TN) subset (39). Since the Ly-6A.2 transgene is under the control of a T cell specific enhancer, the developmental block was expected to be T cell specific. However, in light of the fact that T cell and thymic epithelial cell development are known to be interdependent processes, it was necessary to explore the potential for maturing thymic stromal elements to contribute to the

developmental arrest of thymocytes in Ly-6A.2 transgenic mice (42). To address this issue bone-marrow cells from Ly-6A.2 transgenic mice were transferred into irradiated non-transgenic littermate controls and vice versa. A block in thymocyte development was observed in mice reconstituted with Ly-6A.2 transgenic bone marrow similar to that observed in adult Ly-6A.2 transgenic mice (Figure 1A). In multiple experiments approximately 19%± 1.03 to 40%± 1.22 of DN cells were observed in thymi reconstituted with Ly-6A.2 transgenic donor cells compared to approximately 3.2% in control mice, which is consistent with the percentages present in Ly-6A.2 adult transgenic mice (39). The extent of reduced thymic cellularity and absolute numbers of different thymic subsets defined by the expression of CD4 and CD8 molecules was similar in Ly-6A.2 transgenic ⇒ non-transgenic and Ly-6A.2 transgenic⇒Ly-6A.2 transgenic bone marrow chimeras (Figure 1A and B). In contrast, transfer of bone marrow cells from non-transgenic mice to irradiated Ly-6A.2 transgenic hosts resulted in normal T cell development and cellularity (Figure 1A and B). These experiments demonstrate the block in T cell development resulting from unregulated expression of Ly-6A.2 is dependent on bone marrow-derived cells. Moreover, thymic epithelial cells in the Ly-6A.2 transgenic mice are competent to support proper T cell development and therefore do not contribute to the block in thymic development.

Reduced production of LAT in $CD4^-CD8^-$ thymocytes from Ly-6A.2 transgenic mice. We next tested whether Ly-6A.2 transgenic expression affected the production of critical signaling proteins. Expression of LAT is critical for transition of DN thymocytes into the DP subset. The importance of this molecule is demonstrated in $LAT^{-/-}$ mice, where a block in T cell development within the DN cell stage is observed (36). Analysis of lysates from total

and purified DN subsets from Ly-6A.2 transgenic mice revealed approximately 44% ± 2.12 and 49% ± 1.41 reduction in the amount of LAT produced, respectively, as compared to cells from non-transgenic mice. A significant decrease in phosphorylated LAT was also observed (Figure 2 A, B, C and D). Examination of GEMs revealed a reduced amount of LAT localized to these structures in Ly-6A.2 transgenic thymocytes, which most likely reflected the overall reduced amount of LAT produced (Figure 2 E and F).

Another well-characterized proximal signaling molecule critical for early thymocyte development is the src family tyrosine kinase, $p56^{lck}$ (Lck). Lck is important in early (TN to DP) and late (DP to SP) transitional stages of thymocyte maturation (43,44). Analysis of total and DN thymocytes as well as GEM fractions revealed developing T cells from non-transgenic and Ly-6A.2 transgenic mice expressed similar amounts of Lck, implying that in Ly-6A.2-over-expressing thymocytes, mechanisms other that the amount of Lck present contributed to the block in thymocyte maturation (Figure 2A and B). Collectively, these results demonstrate dysregulated expression of Ly-6A.2 causes reduced production of LAT within the DN subset, which might contribute to the block in development observed in Ly-6A.2 transgenic mice.

Ly-6A.2 transgene expression is necessary but not sufficient to cause decreased LAT production.

In order to determine if the decreased production of LAT observed in Ly-6A.2 transgenic thymocytes was dependent upon TCR-MHC interactions, thymocytes lysates from Ly-6A.2 transgenic and non-transgenic mice lacking expression of MHC I & II were analyzed. Equivalent amounts of LAT were observed in whole cell lysates from transgenic and non-transgenic MHC-deficient mice (Fig 3A and B). These observations suggest that dysregulated expression of Ly-6A.2 in itself is not sufficient to provide

signals resulting in the reduction of LAT. Additional signals emanating from the TCR after interacting with selecting MHC are also necessary for diminished production of LAT.

TCR-$V_ß$ Repertoire in Ly-6A.2 transgenic mice. Suppressed rearrangement of genes encoding TCR-$V_ß$ is known to block development of T cells beyond the DN cell stage (45). Introducing a re-arranged TCR-$V_ß$ or -$V_{αβ}$ transgenes reverses this block in development (45). To test whether dysregulated expression of Ly-6A.2 blocked T cell development at the DN cell stage by suppressing endogenous TCR-$V_ß$ genes, we crossed our Ly-6A.2 transgenic mice with mice expressing the DO-11 TCR. The expression of a transgenic TCR does not reverse the block in T cell development observed in Ly-6A.2 transgenic mice (data not shown). Consistent with these data is the observation that a majority of TCR-$V_ß$s are present in the thymus of Ly-6A.2 transgenic mice (Figure 4), therefore suggesting that the mechanism for the block in T cell development does not involve suppression of endogenous TCR-$V_ß$ expression. Interestingly, we observed some significant alterations in the TCR-$V_ß$ repertoire. Examination of CD4 SP thymocytes revealed there were 8.3% ± 1.67 of TCR- $V_ß$3-bearing cells in Ly-6A.2 transgenic mice compared to 0.8% ± 1.56 in the controls. In addition, only 2.16% ± 3.23 of TCR- $V_ß$8.1,8.2-bearing T cells were observed, which is a striking contrast to the 19% ± 2.03 typically expressed in control mice (Figure 4). Alterations in the other TCR- $V_ß$s analyzed, TCR- $V_ß$5, -$V_ß$6, -$V_ß$9, -$V_ß$10, --$V_ß$11, -$V_ß$17, were either insignificant or not observed. Similar results were obtained upon examination of TCR- $V_ß$s expressed on CD8 SP thymocytes (Figure 4B). Taken together, these observations indicated that continued expression of Ly-6A.2 on the DP subset, which normally do not express this

protein, does not inhibit endogenous TCR-$V_{ß}$ re-arrangement, but rather alters the TCR-$V_{ß}$ repertoire in Ly-6A.2 transgenic mice.

Developing thymocytes in Ly-6A.2 transgenic mice exhibit an increased apoptosis. The block in thymocyte development in Ly-6A.2 transgenic mice is characterized by a decrease in cellularity. Enhanced rate of cell death by continued expression of Ly-6A.2 on thymic subsets that normally do not express this protein might contribute to the reduced cellularity. To address this question we performed Annexin V staining on thymocytes from non-transgenic and Ly-6A.2 transgenic mice and cells were examined by flow cytometry. A higher percentage of PI^- Annexin V^+ Ly-6A.2 transgenic thymocytes (54.03% ± 16.6) was undergoing apoptosis than non-transgenic controls (27.84% ± 1.89) (Fig 5A & B). Upon closer examination of thymic subsets it was determined that the ratios of Annexin V^+ DN cells in Ly-6A.2- and non-transgenic mice were similar, 1.13% and 1.45% respectively (Figure 5C & D). Conversely, a strikingly higher number of DP $TCR_{\alpha\beta}^{Low\text{-}Intermediate}$ thymocytes continually expressing Ly-6A.2 were Annexin V^+ as compared to the control mice, 31% ± 1.99 and 7.3 % ± 1.09 respectively (Figure 5 E & F). These data suggests that increased apoptosis is more restricted to the DP thymocytes that express low-intermediate levels of the TCR. Taken together, these results suggest that the increased rate of apoptosis in Ly-6A.2 transgenic mice is not a major mechanism contributing to the thymic block at the $CD4^-CD8^-$ DN cell stage. However it could contribute to the reduced cellularity and altered T cell repertoire observed in later stages of development.

Reduced association of $p56^{lck}$ with CD4 expressed on Ly-6A.2 transgenic thymocytes. Although total amounts of Lck were comparable in lysates harvested from Ly-6A.2 transgenic and non-transgenic thymocytes, immunoprecipitation experiments revealed localization of Lck to the cytoplasmic tails of CD4 in Ly-6A.2 transgenic thymocytes to be reduced by ~35% (Fig 6B and C), even though the surface expression of CD4 was similar (Figure 6A). Lck-associated CD4 is known to enhance signaling through the TCR during thymocyte selection and lineage commitment (46). Interaction of MHC with the CD4 co-receptor brings Lck in proximity to the TCR-CD3 ITAM motifs, resulting in their phosphorylation at tyrosine residues (46). It is speculated that this interaction may favor commitment of thymocytes to the CD4 lineage (29, 33, 34). These observations may provide an explanation as to why reduced numbers of $CD4^+$ T lymphocytes are generated in Ly-6A.2 dysregulated mice. Additionally, these results suggest that reduced association of Lck with CD4 contributes to alterations in thymocyte selection and differentiation observed in the Ly-6A.2 transgenic thymus.

Unaltered trafficking of developing T cells in the thymus of Ly-6A.2 transgenic mice. Developing T cells in the thymus migrate from the outer cortex to the inner medulla. Given the recently described adhesive properties of Ly-6A.2 (47), we hypothesized that extinguished expression of this protein was important for thymocyte trafficking within the thymus. Expressing Ly-6A.2 continually on cells that would normally have down-regulated it might impede the movement of developing T cells in the thymus. To address this issue we injected the CFSE-labeled Ly-6A.2 transgenic bone marrow cells into an adult irradiated non-transgenic host, which allows the placement of the bone-marrow precursor cells in the

inner cortex near the cortico-medullary junction. Movement of the CFSE labeled cells was tracked by staining with anti-CFSE antibodies. Repopulation of the irradiated thymi was evident by day 7-post re-constitution, however thymi lacked defined cortical and medullary regions in both Ly-6A.2- and non-transgenic mice (Figure 7A a-d). Thymic architecture was restored by day 14-post re-constitution, and thymocytes from Ly-6A.2 transgenic and non-transgenic chimeras were first observed to have crossed the cortico-medullary junction into the medulla (Figure 7A e- h). Strong staining was observed in the cortex and medulla in Ly-6A.2 transgenic and non-transgenic chimeras 21 and 28 days post-reconstitution (Figure 7A i-p), indicating that movement of Ly-6A.2 transgenic cells across the cortico-medullary junction into the medulla within the thymi of Ly-6A.2 transgenic mice was unaltered. Medullary T cells from days 14, 21, and 28 chimeras expressed strong levels of TCR-$\alpha\beta^{high}$ staining, which were similar to non-transgenic controls (Figure 7B f-p) as well as those described upon staining Ly-6A.2 adult thymic sections with anti-TCR-αβ antibodies (data not shown). Visualizing with anti-Ly-6A/E antibodies revealed staining to be consistent with that which was observed for CFSE (7C a-p). The movement of bone marrow-derived precursor cells was also tracked by the anti-Ly-6A.2 antibodies. Figure 7C indicates precursor cells and their progeny were present throughout the thymus after day-4 (data not shown) and day 7 of transfer in the thymus of non-transgenic⇒ non-transgenic and Ly-6A.2 transgenic⇒ non-transgenic bone marrow chimeras (Figure 7C a-d). By day 14 of transfer a majority of the staining for Ly-6A.2 was observed in the cortex and the medulla of Ly-6A.2 transgenic thymi (Figure 7C e-h). Expression of the Ly-6A.2 transgene was increased and reached its highest level on day 21 and day 28 in the outer cortex. However, very little Ly-6A.2 expression was observed in the medulla (Figure 7C i-p). Interestingly, almost all

mature T cells in the thymic medulla of Ly-6A.2 transgenic⇒non-transgenic chimeric mice showed down-regulated expression of the Ly-6A.2 transgene (Figure 7C k, l, o, p). Analysis of thymocyte populations from these chimeric mice on days 7, 14, 21 and 28 post transfer showed reduced cellularity and diminished numbers of $CD4^+CD8^+$ DP and mature $CD4^+CD8^-$ and $CD4^-CD8^+$ SP subsets similar to the data displayed in Figure 1B (data not shown). Taken together, these observations suggest trafficking of developing T cells into the medulla is not affected by dysregulated expression of Ly-6A.2, however, cells that are present in the medulla have down-regulated Ly-6A.2 transgene expression.

DISCUSSION

Dysregulation of Ly-6A.2 during thymocyte development results in developmental arrest of maturing thymocytes within the DN stage (39). In order to better understand the underlying mechanism of this block and the biology of Ly-6 proteins, several signaling molecules and processes known to influence DN ⇒ DP stage-specific transition of developing thymocytes were examined. We demonstrate continued expression of Ly-6A.2 inhibits the production of LAT, an adapter protein critical for development past the DN cell stage. In contrast, production of src-family kinase $p56^{lck}$ remained unaltered. Unregulated expression of Ly-6A.2 did not suppress rearrangement of endogenous TCR_β genes, nor did it alter the entry and movement of developing T cells from the outer cortex into the inner medulla. Finally, we provide evidence that dysregulated Ly-6A.2 expression on DP thymocytes increased the rate of apoptosis within the DP subset and modified the selection

processes known to shape the TCR-V_β-repertoire. Collectively, these findings suggest that downregulation of Ly-6A.2 is important for continued growth, maturation and selection of T cells in the thymus.

Thymocytes and thymic epithelial cell (TEC) development are interdependent processes. Establishment of normal thymic architecture and properly differentiated TECs is dependent upon interactions between developing T cells and TECs (3). Studies with human CD3 transgenic mice show a block in T cell development at the $CD44^+CD25^-$ DN stage (stage I). These mice have abnormal thymic architecture and differentiation of TEC (15, 18). In contrast, $RAG^{-/-}$ and SCID mice that show block in T cell development at the $CD44^-CD25^+$ DN stage (stage III) have unaffected thymic architecture and normal distribution of TEC (48, 49). The block in T cell development in Ly-6A.2 dyregulated mice occurs in the transition of $CD44^+CD25^+$ DN (stage II) to the $CD44^-CD25^+$ DN (stage III) cells whereas development of $CD44^+CD25^+$ DN (stage II) cells is unaffected (39). In light of the observations that normal cortical organization accompanies thymocyte development, we sought to determine whether the block in development in the Ly-6A.2 dysregulated mice at the $CD44^+CD25^+$ $CD4^-CD8^-CD3^-$ stage (stage II) affected the development of thymic epithelial cells. Our results demonstrate the block in T cell development in Ly-6A.2 dysregulated mice is bone marrow cell-dependent (Figure 1), and that the presence of $CD44^+CD25^+$ DN cells (stage II) in the thymus is sufficient to support the generation of organized thymic architecture and competent epithelial cells in the thymus.

Additionally, our data suggest that the continued expression of Ly-6A.2 on thymic subsets that normally fail to express this protein does not impede trafficking of T cells from the outer cortex to the inner medulla during the time course tested, and therefore could not

contribute to the block in T cell development that is observed in Ly-6A.2 dysregulated mice. However, it was intriguing that cells present in the medulla showed marked down-regulated expression of Ly-6A.2, which was first observed from Ly-6A.2 Tg^+ BM $\Rightarrow$ Tg- host chimeras examined on day 21 post-transfer (Figure 7C). $TCR_{\alpha\beta}$-bearing cells were clearly observed in the medulla at this time point (Figure 7B). It is unclear why the Ly-6A.2 transgene is down-regulated on medullary T cells in the Ly-6A.2 dysregulated mice, however several possible explanations exist. First, interaction of TCRs on developing cells with MHC/self-peptide and Ly-6 proteins with candidate intrathymic ligand(s) may trigger the loss of cell surface expression. Support for this comes from a previous study suggesting that TCR/MHC interactions down-regulate expression of Ly-6d (50). It is possible that Ly-6A.2 binding to its putative ligand, which is expressed both in the thymus and spleen, may also result in loss of expression on the cell surface. Future investigation of recently identified ligands for mouse Ly-6 proteins may provide insight into the function and effects of ligand binding. Second, expression of the Ly-6A.2 transgene in T cells is under the control of human CD2 enhancer and Ly-6A.2 promoter. Therefore, it is possible that differences in the expression of the transgene reflect the differences in the nature and/or quantity of trans-acting regulatory factors at different developmental stages. Alternatively, it is also possible that Ly-6A.2 is stochastically down-regulated, and only those cells with down-regulated Ly-6A.2 traverse the cortico-medullary junction and migrate into the medulla. Further experiments are needed to resolve this issue.

Analyses of proximal cell signaling molecules revealed that continual expression of Ly-6A.2 on DN thymocytes resulted in decreased production of LAT, an important adapter protein shown to conjoin TCR-induced proximal to distal signaling (36). A previous study

has demonstrated that mice deficient in LAT exhibit a block in thymocyte development and the T cells do not progress past the $CD44^{-}CD25^{+}$ DN cell stage (38). Therefore LAT deficiency in the DN cells might contribute to the block in thymocyte development in Ly-6A.2 dysregulated mice. It is intriguing that the reduction of LAT in total thymocytes appears to require the presence of selecting MHC/self peptide ligands, indicating diminished LAT production in Ly-6A.2-dysregulated thymocytes is dependent upon signaling emanating from a functional pre-TCR or TCR-$\alpha\beta$. Thus, continued expression of Ly-6A.2 alters signaling initiated from TCR-MHC/self-peptide interactions.

Analyses of whole thymocyte lysates, which contain DP and SP cell populations also revealed an overall lower amount of total and phosphorylated LAT. Additionally, LAT localization to the GEMs, which is required for its participation in signal transduction, was significantly decreased in Ly-6A.2 transgenic thymocytes compared to controls. These results suggest that Ly-6A.2 transgene-dependent reduction in LAT production also occurs in thymocytes that have progressed beyond the DN cell stage. Although the regulatory effects of LAT signaling have not been clearly defined in the thymus, studies examining peripheral T cell activation have shown that participation of LAT in TCR-induced signal transduction is required for full activation and clonal expansion of these lymphocytes. Thus, it is conceivable that alterations in production and activation of LAT could inhibit proliferation of DP thymocytes undergoing selection and differentiation. Future experiments will determine if the LAT deficiencies are due to alterations in transcription/translation or post-translational modifications of this protein. However, these results collectively demonstrate that continued expression of Ly-6A.2 results in alterations to LAT, which are likely to contribute to the block in thymocyte maturation.

The thymic block in $Lck^{-/-}$ and Ly-6A.2 dysregulated mice appear to be similar (44), which therefore provided an impetus to examine whether $p56^{lck}$ was altered in Ly-6A.2 dysregulated mice. Signal transduction emanating from the pre-TCRα is facilitated by Lck, the outcome of which is the activation of genes necessary for TCR-$V_{\alpha\beta}$ rearrangement and transition from the DN $\Rightarrow$ DP stage of development (43). Deficiency of this molecule inhibits pre-TCR signaling, which subsequently results in a major, but incomplete block in thymocyte development (44). The importance of this protein for initiating signaling necessary for facilitating up-regulation of intracellular and cell surface molecules necessary for continual development past the DN stage is well established. Lysates from total and purified DN subsets revealed equivalent proportions of Lck were generated in Ly-6A.2 transgenic and non-transgenic thymocytes. Consistent with these observations is the finding that the thymic block in development remained unaffected by introducing the $p56^{lck}$ transgene in Ly-6A.2-dysregulated mice (data not shown). This finding suggested that mechanisms separate from the amount of Lck present in Ly-6A.2 over-expressing thymocytes contributed to the block in thymocyte development.

Preferential localization of Lck to the cytoplasmic tails of CD4 has been associated with bringing this src-family kinase into the TCR/CD3 complex, where it would have access to the activating immunoreceptor tyrosine-based activation motifs (ITAMS) (40, 46). Although production of Lck in total and DN thymocytes was unaltered in developing thymocytes from Ly-6A.2 transgenic mice, immunoprecipitation experiments revealed that the observed localization of Lck to the cytoplasmic tails of CD4 in non-transgenic thymocytes was significantly reduced in thymocytes over-expressing the Ly-6A.2 protein. Expression of an activated form of transgenic Lck in thymocytes over-expressing Ly-6A.2 did not reverse the thymic block observed in Ly-

6A.2 transgenic mice, which suggested the block in T cell development was independent of Lck. It is possible that reduced association of Lck with the CD4 co-receptor expressed on Ly-6A.2 transgenic thymocytes contributes to the reduced development of SP cells in these mice.

Mice that lack the machinery necessary to re-arrange the V_β gene locus exhibit a block at the DN cell stage, which is reversed by the expression of a TCR- V_β or TCR-α β transgene (17). To test this possibility, the DO-11 TCR was transgenically expressed in Ly-6A.2 transgenic mice. However, bypassing the requirement for Lck-activation of RAG-1/-2 genes through incorporation of a pre-rearranged TCR did not reverse the developmental arrest, indicating suppression of endogenous TCR-V_β re-arrangement was not a contributing factor to the block in Ly-6A.2 transgenic thymocyte development. These results were further supported by examination of repertoire of the TCR-V_βs present in Ly-6A.2- and non-transgenic controls, in which Ly-6A.2 transgenic thymocytes showed a diverse selection of TCR- V_β chains expressed on the surface.

Mice of the H-2^d background encode for mouse mammary tumor viruses (mtv) –6, -8, and –9, which mediate deletion of thymocytes bearing TCR-V_β-3, -5, and –11. It is intriguing that unregulated expression of Ly-6A.2 rescued thymocytes expressing mtv-6 SAg-reactive -V_β3 regions. Escape from SAg-mediated deletion has been described in several systems, which suggests that modulation of an array of surface molecules mediate this process. A number of molecules influence the V_β TCR repertoire: down-regulation of CD4 during thymocyte selection results in escape from elimination of mtv-8 and-9-reactive thymocytes bearing TCR-V_β-4, -7, -11, and –12. The level of CD2 on the surface of peripheral T cells also influences the propensity for these cells to undergo apoptosis as down-modulation of this cell signaling/adhesion molecules is known to delay the deletion of SEB

SAg-response TCR-V_β8 T cells. The mechanisms by which these proteins regulate escape from SAg deletion are not clear, however there is evidence to support a role for Lck (51). Although CD4 cell surface expression levels in Ly-6A.2 dysregulated thymocytes was comparable to non-transgenic thymocytes, it is plausible that the diminished association of Lck to the cytoplasmic tails of CD4 in thymocytes over-expressing Ly-6A.2 caused decreased signaling through TCR/CD3 and therefore contributed to rescue of TCR-V_β3-bearing cells in a similar manner. Alternatively, it is possible that rescue of -V_β3 in the Ly-6A.2 transgenic thymus may reflect alterations in the kinetics of SAg-mediated deletion of reactive thymocytes. Although thymocytes expressing TCR- V_β3 are present in the thymus of Ly-6A.2 dysregulated mice, it is possible that these cells will be deleted in the periphery. Recent examination of the kinetics of SAg deletion has revealed this process is not restricted to the thymus and can occur in the periphery (52). Elimination of Mtv-17-reactive T cells occurs after thymocytes exit the medulla and begin trafficking in the peripheral circulation. Only partial deletion of Mtv-8- reactive TCR-V_β12 is observed in the thymus, but other T cells subsets eliminated by this SAg appear to be deleted only in the periphery. Further analysis of the TCR-V_β repertoire from peripheral T cells should resolve this issue.

The almost complete absence of TCR-V_β8-bearing thymocytes (Figure 4), which normally constitute approximately 19% of the selected population, was also surprising. It is not clear why thymocytes expressing -V_β8 regions in Ly-6A.2 transgenic mice are absent as potentially autoreactive cells. The precise mechanisms underlying this regulation remain unknown. This could suggest deletion of this population of cells, or down-regulation of the TCR receptor such that it is not detected by the anti-TCR-V_β8 antibody. Further studies are needed to address this issue. The contributions of co-stimulatory proteins and cytokines in

the disappearance of specific V_β-expressing cells have been reported in $CD5^{-/-}$ and $CD40^{-/-}$ mice. It is possible that cell signaling through ectopically expressed Ly-6A.2 on $CD4^+CD8^+$ DP cells specifically induces deletion of $V_\beta 8$-bearing cells by an unknown mechanism.

Accelerated cell death in the Ly-6A.2 dysregulated mice could also contribute to the reduced cellularity observed in the Ly-6A.2 transgenic mice. The increased rate of apoptosis was not observed in the DN cell population. Instead other immature thymic subsets expressing low-intermediate levels of $TCR_{\alpha\beta}$ showed enhanced cell death by apoptosis. The mechanism by which ectopic expression of Ly-6A.2 may accelerate the cell death process is unclear, but these observations appear to parallel previously published reports that antibodies directed to the Ly-6 proteins induce cell death by apoptosis (53-55).

This study reports the importance of regulated expression of Ly-6A.2 during normal T cell development in the thymus. Termination of Ly-6A.2 expression within the DN subset may be necessary to prevent Ly-6A.2-derived signaling from interfering with those mediating differentiation of thymocytes transitioning from the DN to the DP cell stage and selection of DP thymocytes.

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Figure 3.1. Analysis of bone marrow chimeras. Bone marrow chimeras were generated by injecting bone marrow cells from non-transgenic or Ly-6A.2 transgenic irradiated syngeneic host (Non Tg BM > Non Tg and Non Tg BM > Ly-6A.2 Tg) or vice versa. Thymocytes were stained with anti-CD4-PE and anti-CD8-FITC and analyzed by flow cytometer for thymic subsets (A) and cellularity (B) on day 28 post-transfer as described in materials and methods. Data shown are representative of three experiments. Error bars in panel B indicate standard deviation.

Figure 3.1

Figure 1

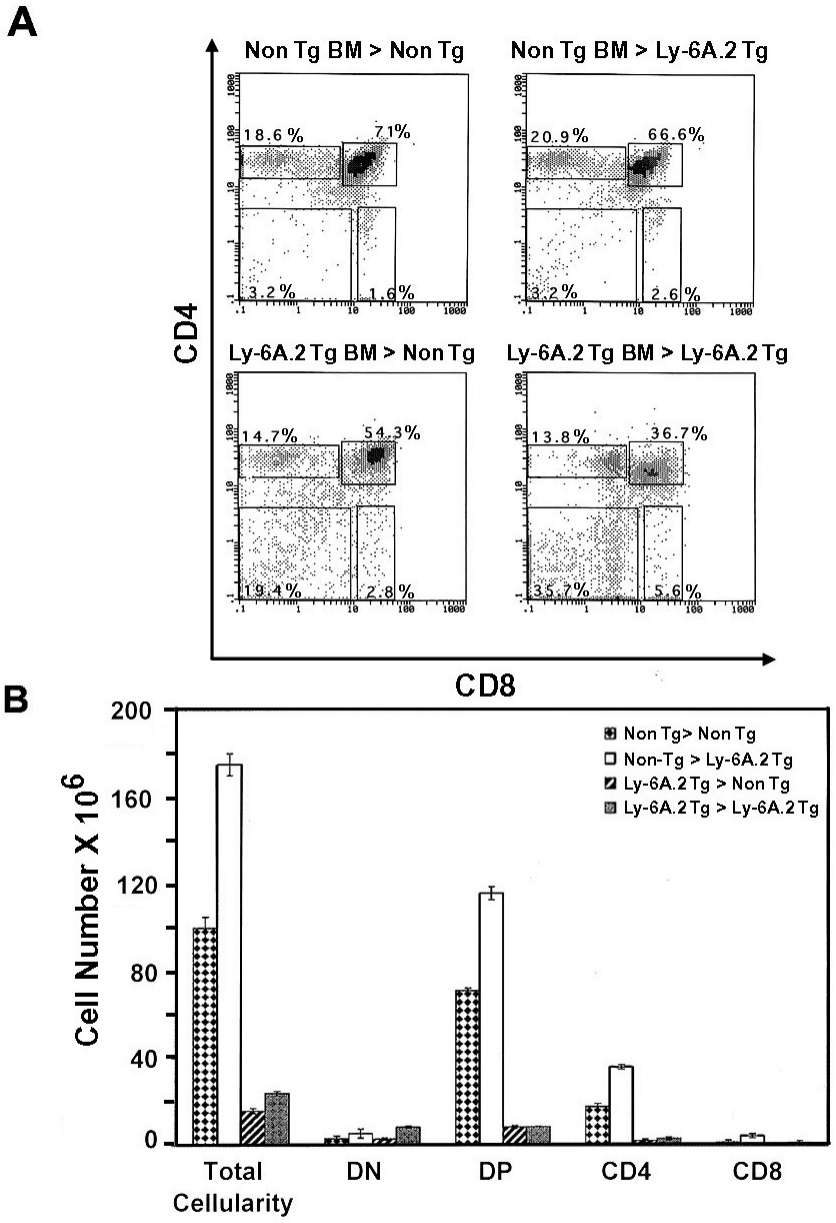

Figure 3.2. Expression of LAT and $p56^{lck}$ in Ly-6A.2 transgenic thymocytes. Total and purified $CD4^-CD8^-$ DN thymocytes from non-transgenic and Ly-6A.2 transgenic mice were solubilized with lysis buffer containing 1% NP-40 and 0.25% deoxycholate. The lysates, equivalent to 2 x 10^6 cells, were run on SDS-PAGE and immunoblotted with antibodies against LAT, $p56^{lck}$ and phosphotyrosine (A and C). Triton X 100 insoluble GEM fractions were purified from either non-transgenic or Ly-6A.2 transgenic thymocytes. Purified GEM fractions from 4 x 10^7 cells were run on SDS-PAGE after concentration and subjected to western blotting with antibodies to Ly-6A/E, LAT and $p56^{lck}$ as described in Materials and Methods (E). Densitometric analyses of the data is shown in panels to the right (B, D and F) and are represented as percent signal of the control. Each mouse analyzed is represented by a filled circle. Cumulative data from 7 (A-D) and 4 (E, F) experiments are shown.

Figure 3.2

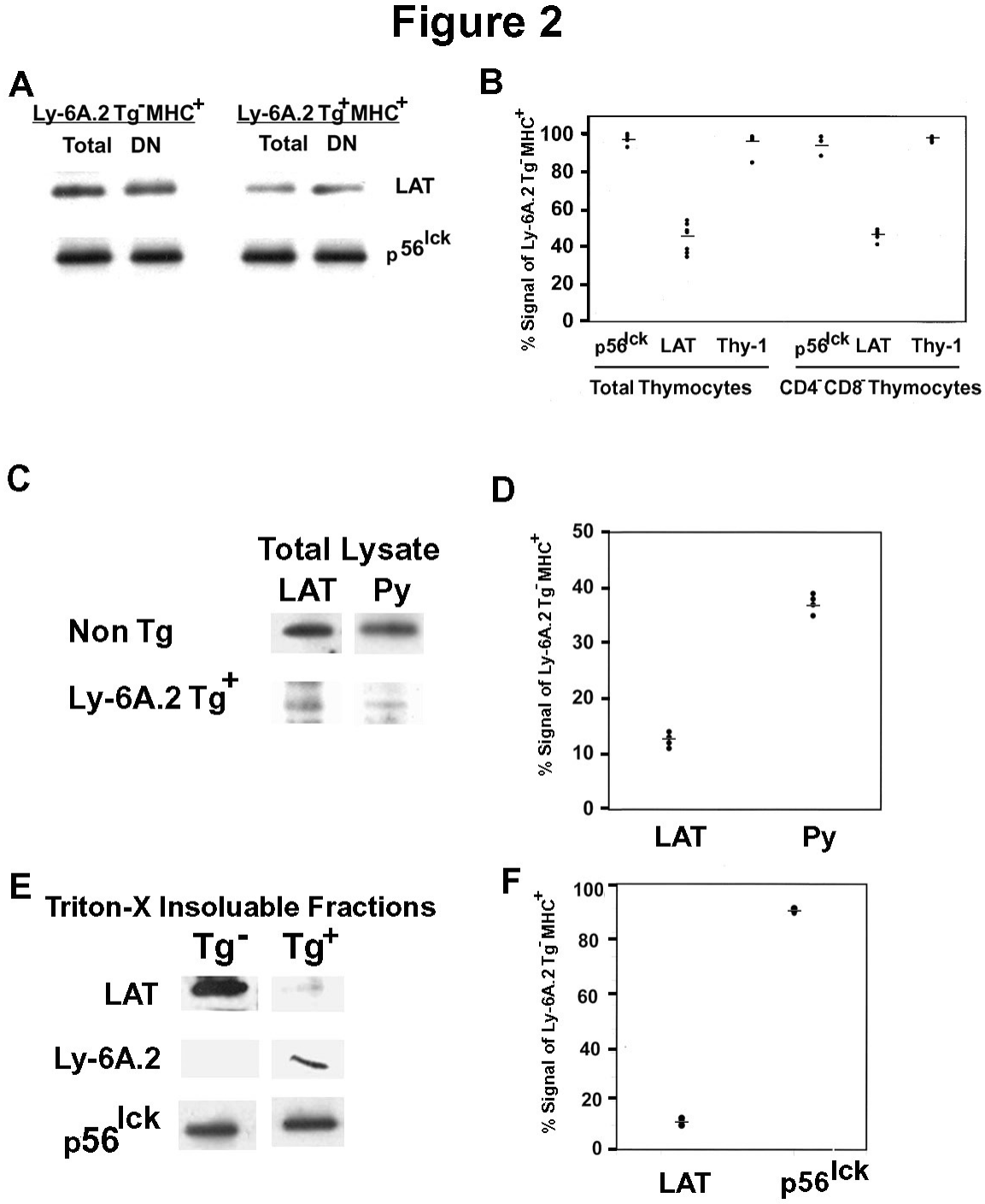
Figure 2
A
Ly-6A.2 Tg-MHC+
Total DN
Ly-6A.2 Tg+MHC+
Total DN
LAT
p56lck
B
% Signal of Ly-6A.2 Tg-MHC+
100
80
60
40
20
0
p56lck LAT Thy-1
p56lck LAT Thy-1
Total Thymocytes
CD4-CD8- Thymocytes
C
Total Lysate
LAT Py
Non Tg
Ly-6A.2 Tg+
D
% Signal of Ly-6A.2 Tg-MHC+
50
40
30
20
10
0
LAT
Py
E
Triton-X Insoluable Fractions
Tg- Tg+
LAT
Ly-6A.2
p56lck
F
% Signal of Ly-6A.2 Tg-MHC+
100
80
60
40
20
0
LAT
p56lck

Figure 3.2. Decreased LAT production in Ly-6A.2 transgenic thymocytes requires presence of selecting MHC I and II. Ly-6A.2 Transgenic mice were bred with MHC I and II-double deficient mice (in which the $A\beta^b$ gene and β_2-microglobulin were disrupted by homologous recombination), and their transgenic progeny were backcrossed with MHC I and II-deficient mice for 8-9 generations. Thymocytes from Ly-6A.2 Tg^-MHC^- and Ly-6A.2Tg^+MHC^- were solubilized as described in Materials and Methods, the lysates were run on SDS-PAGE and immunoblotted with antibodies against LAT, $p56^{lck}$ (A), and Thy-1 (shown in B) as percent signal of the control. Each filled circle represents one mouse. Densitometric analysis of the data is shown in panel B. Data from four independent experiments are represented. (Note: For each analysis, four data points are represented. These data points overlap for the $p56^{lck}$ and Thy-1 analyses)**.**

Figure 3.2

Figure 3

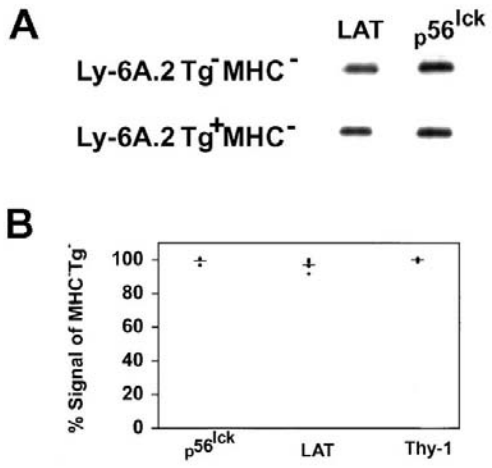

Figure 3.4. The TCR-V_β repertoire in Ly-6A.2 Transgenic mice. Non-transgenic (■) and Ly-6A.2 transgenic (□) thymocytes were stained with anti-CD4-PE, anti-CD8-FITC and anti-TCR-V_β (V_β3, -5, -6, -8.1 /-8.2, -9, -10, -11, and -17)-biotin followed by streptavidin-Red 613 in a three color immunofluorescence. Percent V_β expression on gated $CD4^+CD8^-$ (A) and $CD4^-CD8^+$ (B) SP cells is shown. Error bars indicate standard deviation (n=4). Significance of these data was determined by student T-test. * indicates a p-value of ≤ 0.01 ** indicates a p-value of ≤0.002 or less.

Figure 3.4

Figure 4

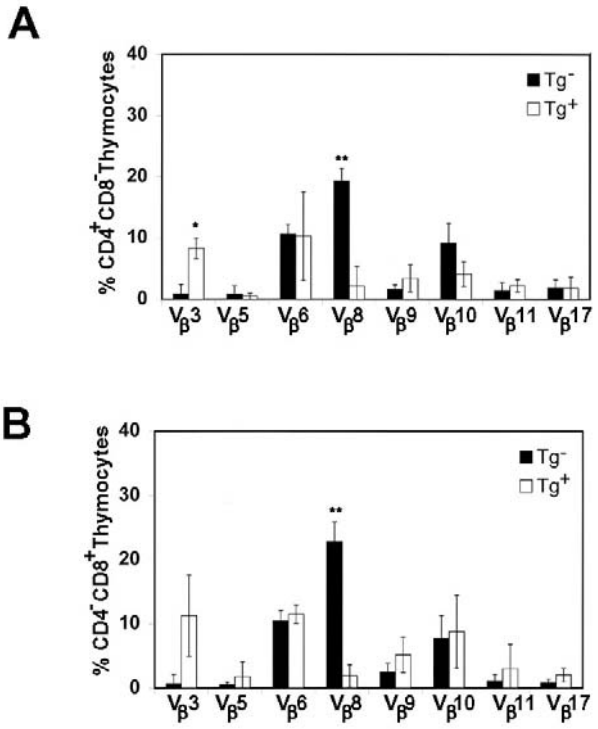

Figure 3.5. Developing thymocytes in Ly-6A.2 transgenic mice undergo enhanced apoptosis. Non-transgenic (□) and Ly-6A.2 transgenic (■) thymocytes were stained with Annexin V-FITC in the presence of propidium iodide (A), or a combination of anti-$TCR_{\alpha\beta}$ streptavidin-CD4-PE or anti-$TCR_{\alpha\beta}$ alone (F) and analyzed by flow cytometry. Densitometric analysis was performed (shown in panel B) as described in Materials and Methods. A representation of four experiments is shown. Appropriate subsets were gated to show total (B) or DN (D) or $TCR^{-/low}$ (F) undergoing apoptosis. Error bars indicate standard deviation (n=4).

FIGURE 3.5

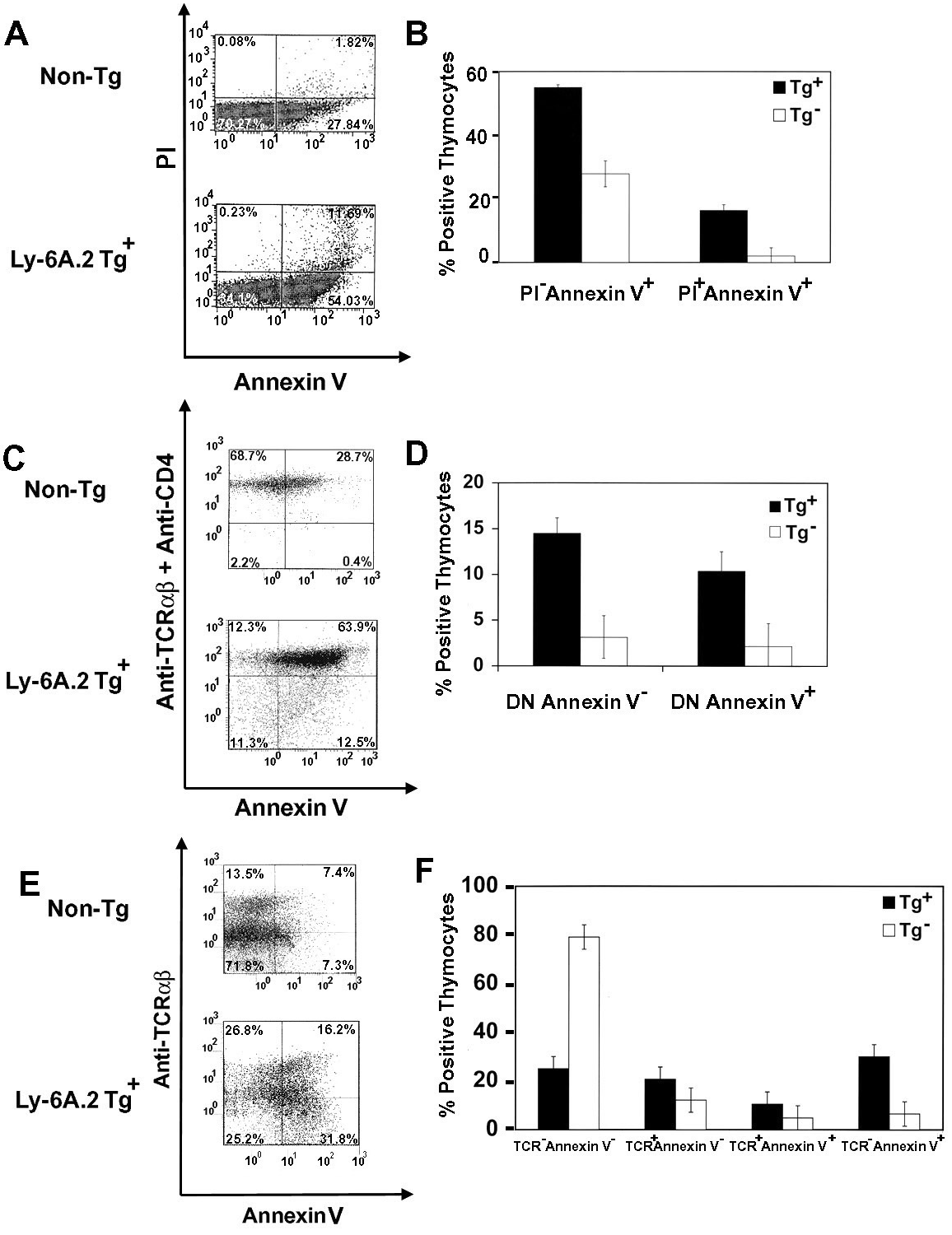
A
Non-Tg
Ly-6A.2 Tg+
PI
0.08%
1.82%
27.84%
0.23%
11.69%
54.03%
Annexin V
B
% Positive Thymocytes
Tg+
Tg-
PI-Annexin V+
PI+Annexin V+
C
Non-Tg
Ly-6A.2 Tg+
Anti-TCRαβ + Anti-CD4
68.7%
28.7%
2.2%
0.4%
12.3%
63.9%
11.3%
12.5%
Annexin V
D
% Positive Thymocytes
Tg+
Tg-
DN Annexin V-
DN Annexin V+
E
Non-Tg
Ly-6A.2 Tg+
Anti-TCRαβ
13.5%
7.4%
71.8%
7.3%
26.8%
16.2%
25.2%
31.8%
Annexin V
F
% Positive Thymocytes
Tg+
Tg-
TCR-Annexin V-
TCR+Annexin V-
TCR+Annexin V+
TCR-Annexin V+

Figure 3.6. Association of $p56^{lck}$ with CD4 expressed on Ly-6A.2 transgenic thymocytes. Non-transgenic (thick line peak) and Ly-6A.2 (grey-filled peak) transgenic thymocytes were stained with anti-CD4-PE or control antibodies (thin line and dashed line, respectively) and analyzed by flow cytometry to visualize surface expression of CD4. (A). CD4 proteins were immunoprecipitated from either non-transgenic or Ly-6A.2 transgenic thymocyte lysates with anti-CD4 (GK1.5)-Protein G-sepharose beads. Two sequential immunoprecipitates (marked 1 and 2) were run on SDS-PAGE and immunoblotted (B) with anti-$p56^{lck}$ antiserum. Densitometic analysis of immunoblots is shown in panel C. Error bars indicate standard deviation (n=3)

Figure 3.6

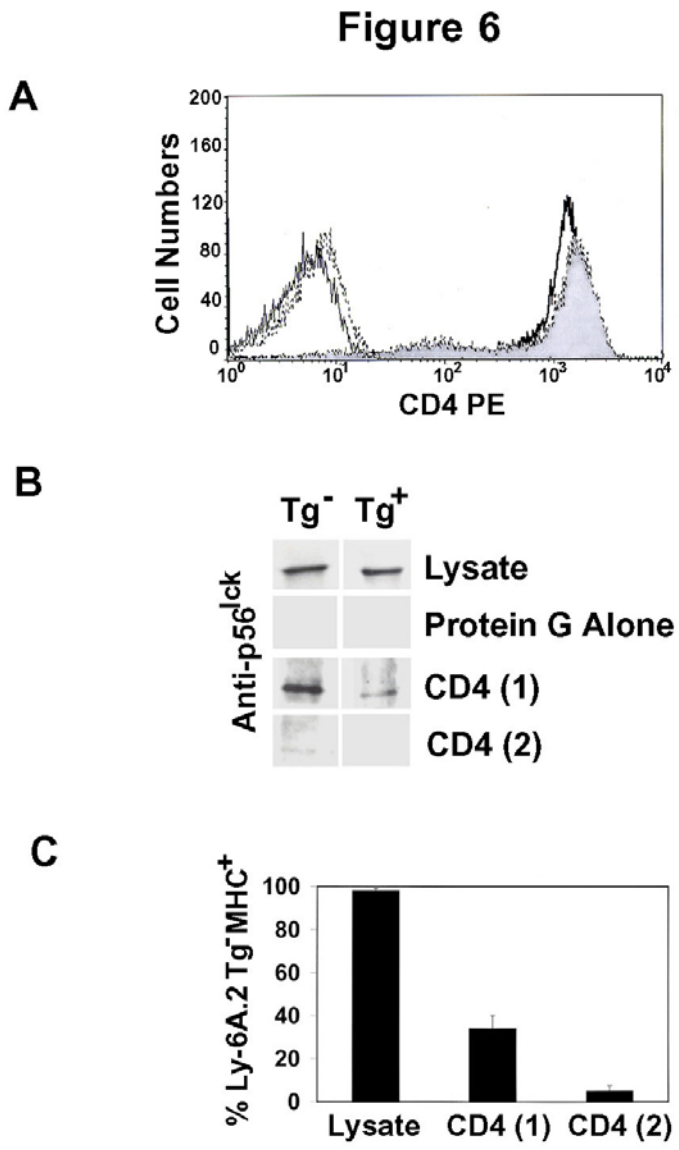
Figure 6
A
Cell Numbers
200
160
120
80
40
0
10^0
10^1
10^2
10^3
10^4
CD4 PE
B
Tg^-
Tg^+
Anti-$p56^{lck}$
Lysate
Protein G Alone
CD4 (1)
CD4 (2)
C
% Ly-6A.2 Tg^-MHC^+
100
80
60
40
20
0
Lysate
CD4 (1)
CD4 (2)

Figure 3.7. Analysis of trafficking of developing T cells in bone marrow chimeras by immunohistochemistry. 5-8 μM sections from thymi of chimeric mice were stained with biotinylated antibodies directed against CFSE (A), anti-$TCR_{\alpha\beta}$ (B), and anti-Ly-6A.2 (C) followed by neutravidin-HRP. Control staining in absence of primary antibody is shown in panel D. The staining was visualized with hydrogen peroxide as substrate and di-amino-benzidine as chromogen as described in Materials and Methods. The larger micrographs within each panel depict the cortex and medulla, C and M respectively (magnification, *X*100. Medullary staining, indicated by arrows, is shown at higher magnification (magnification, *X*200). Data shown is representative of three experiments.

Figure 3.7A

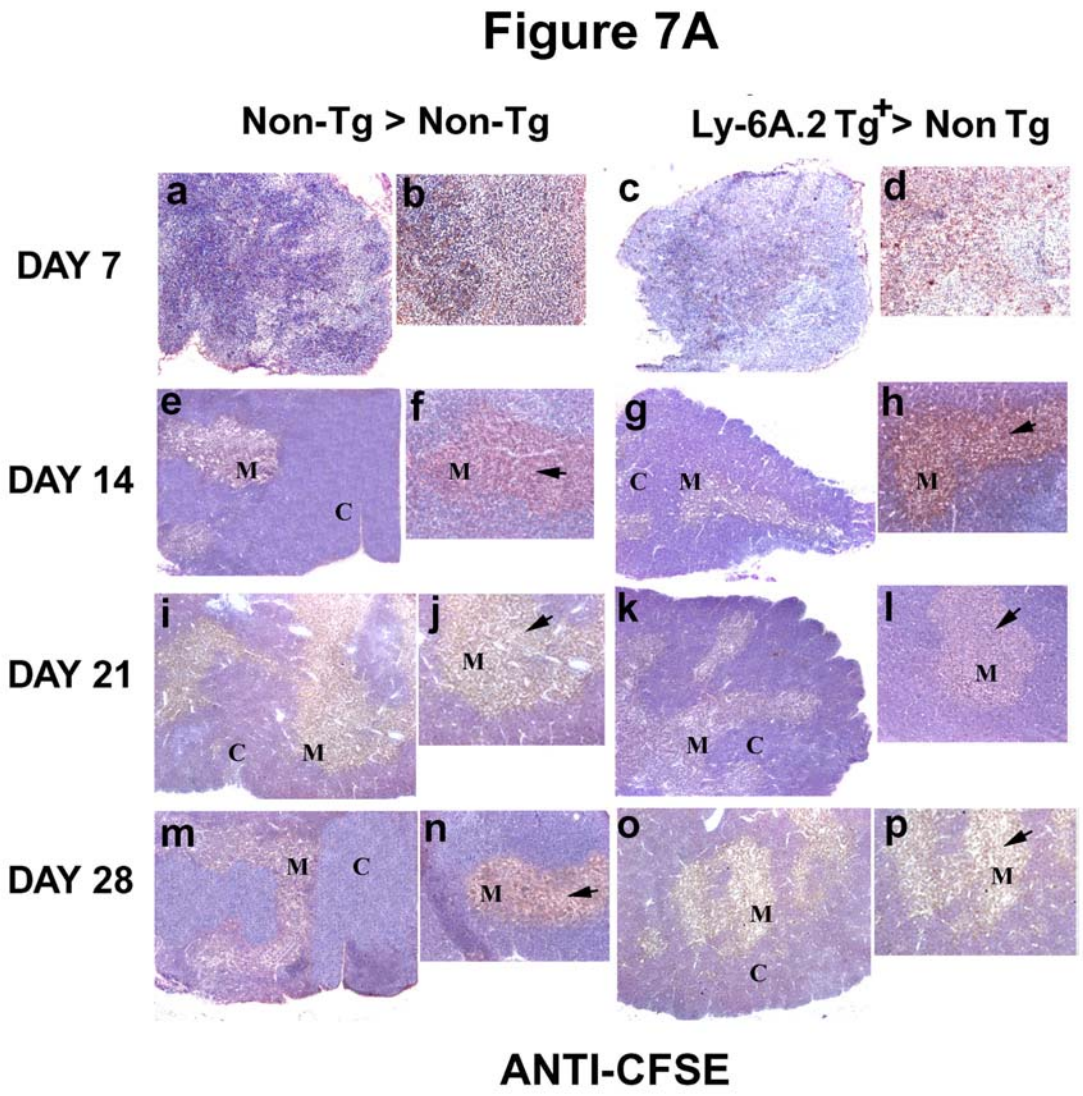
Figure 7A
Non-Tg > Non-Tg
Ly-6A.2 Tg+> Non Tg
DAY 7
DAY 14
DAY 21
DAY 28
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
M
C
ANTI-CFSE

Figure 3.7B

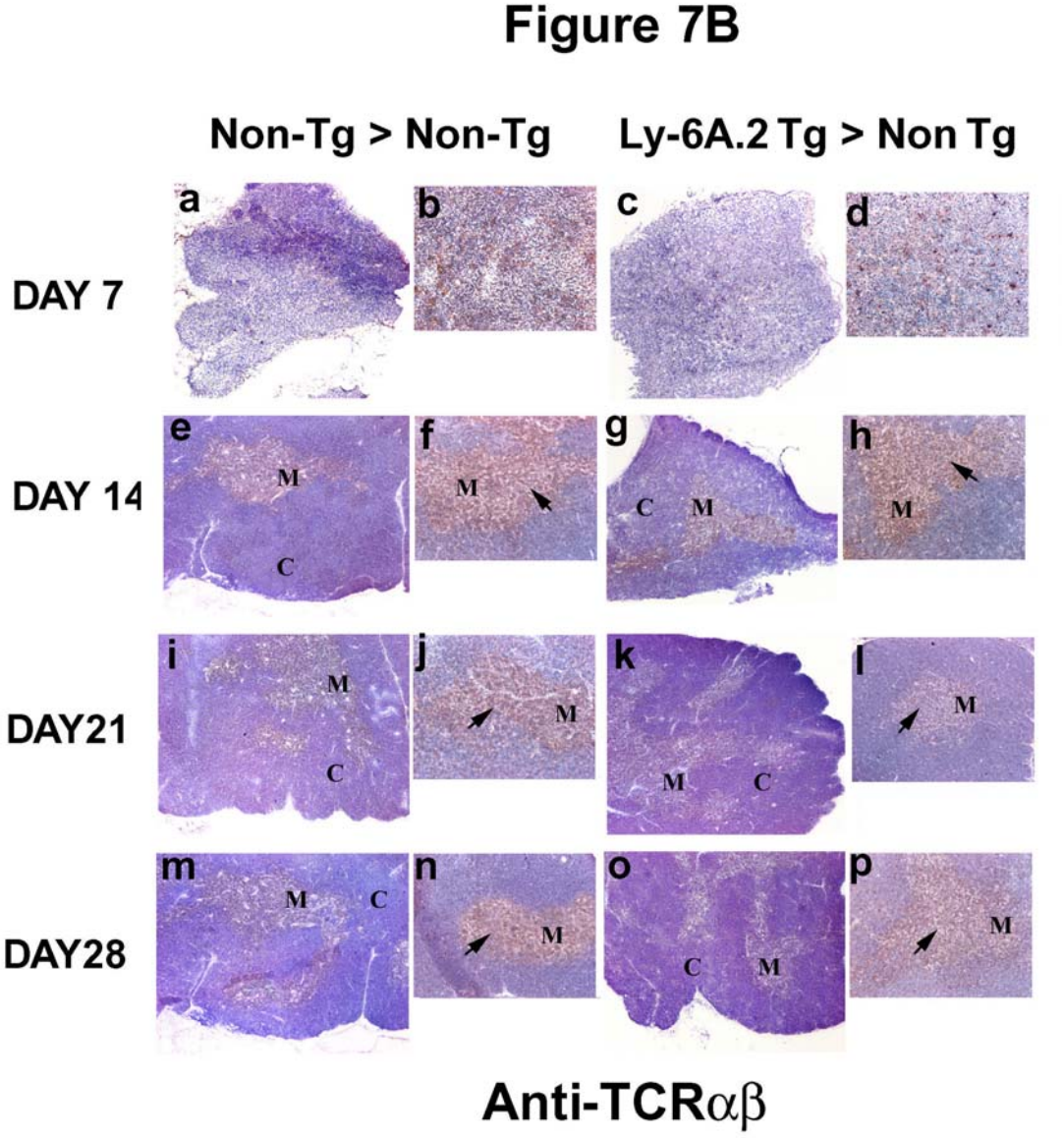
Figure 7B
Non-Tg > Non-Tg
Ly-6A.2 Tg > Non Tg
DAY 7
DAY 14
DAY21
DAY28
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
M
C
Anti-TCRαβ

Figure 3.7C

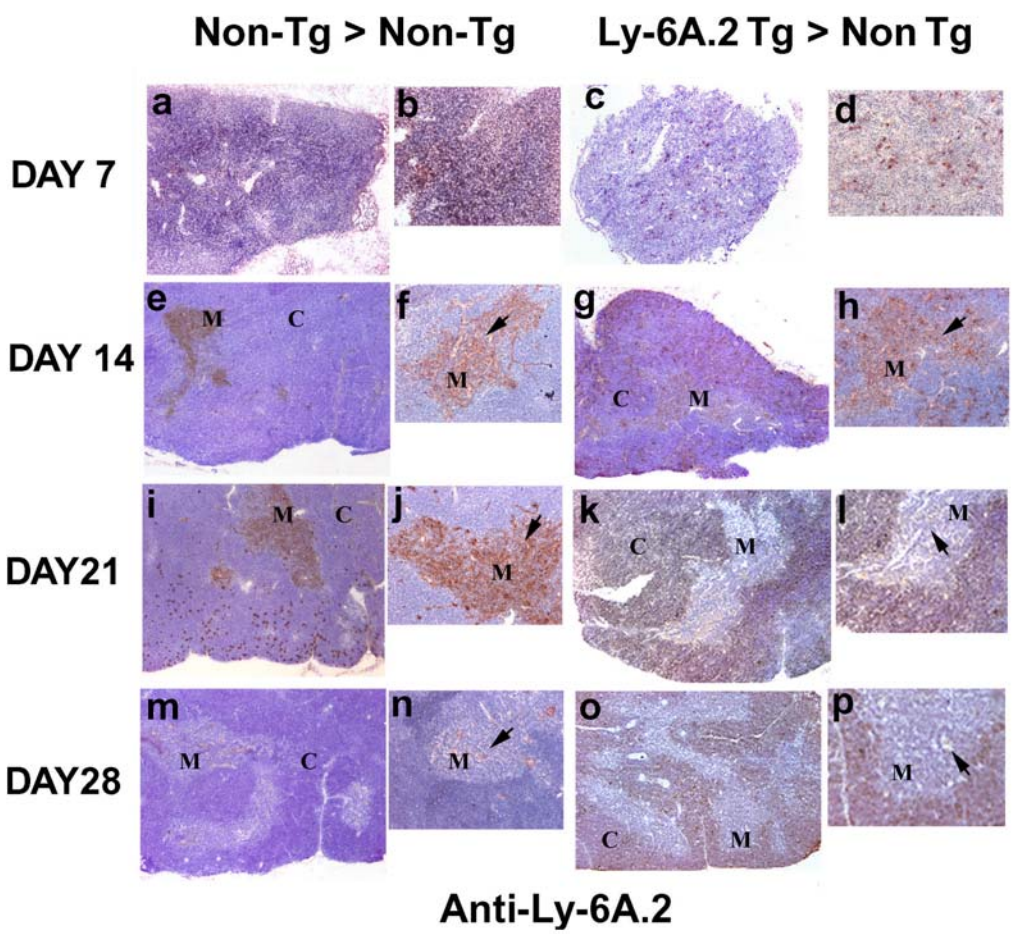
Figure 7C
Non-Tg > Non-Tg
Ly-6A.2 Tg > Non Tg
DAY 7
DAY 14
DAY21
DAY28
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
M
C
Anti-Ly-6A.2

Figure 3.7D

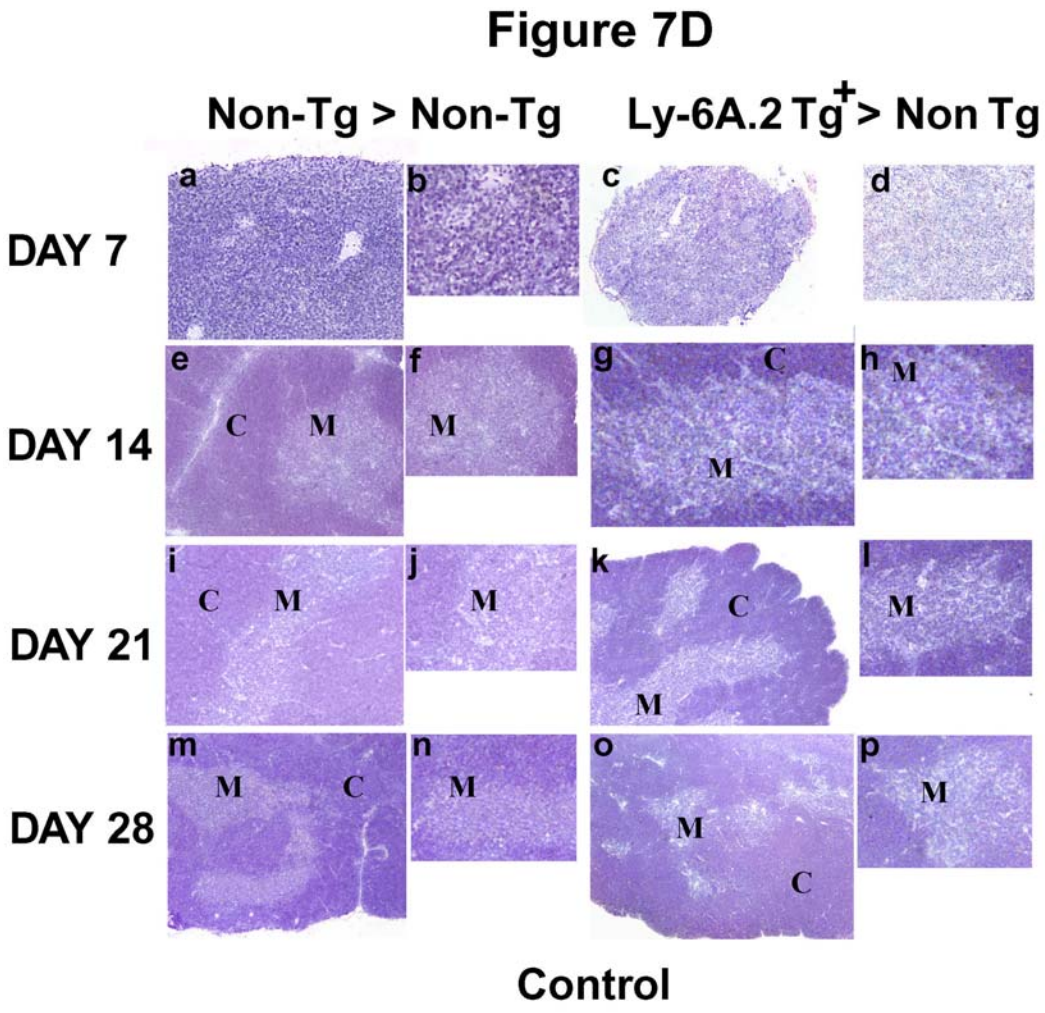
Figure 7D
Non-Tg > Non-Tg
Ly-6A.2 Tg+ > Non Tg
DAY 7
DAY 14
DAY 21
DAY 28
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
C
M
Control

CHAPTER 4

LY-6A.2 EXPRESSION REGULATES ANTIGEN-SPECIFIC $CD4^+$ T CELL PROLIFERATION AND CYTOKINE PRODUCTION.

[1]Henderson, S. C., M. M. Kamdar, and A. Bamezai. 2002. Journal of Immunology. 168:118 Reprinted here with permission of publisher

ABSTRACT

Ly-6A.2 belongs to a multi-gene family, which encodes for several proteins that are expressed differentially on cells of hematopoietic origin. Investigations into the role of Ly-6A.2 during T cell stimulation have offered conflicting data, which supports both activation and inhibition of T cell signaling. In order to determine the role of Ly-6A.2 during an antigen-induced stimulatory response, Ly-6A.2 transgenic mice were bred to animals transgenically expressing a c-ovalbumen (cOVA)-specific DO-11 T cell receptor (TCR). In this study we report reduced proliferation of $CD4^+$ T cells over-expressing Ly-6A.2 in response to a peptide antigen. Moreover, the Ly-6A.2 over-expressing $CD4^+$ cells generated elevated levels of Interleukin-4, a key factor that propels the differentiation of naïve $CD4^+$ T cells into Th2 subset. The hypo-responsiveness of Ly-6A.2 transgenic $CD4^+$ T cells is dependent on the interaction of Ly-6A.2 T cells with the antigen presenting cells (APC) and can be reversed by blocking the interaction between Ly-6A.2 and a recently reported candidate ligand. Over-expression of Ly-6A.2 in $CD4^+$ T cells reduced their Ca^{2+} responses to T cell receptor stimulation, therefore suggesting effects of Ly-6A.2 signaling on membrane proximal activation events. In contrast to the observed antigen-specific hypo-responsiveness, the Ly-6A.2 transgenic $CD4^+$ T cells produced IL-4 independent of the interactions between Ly-6A.2 with the candidate Ly-6A.2-ligand. Our results suggest that interaction of Ly-6A.2 with a candidate ligand expressed by APC regulates clonal expansion of $CD4^+$ helper T cells in response to an antigen, which provides further functional evidence for Ly-6A.2-ligand interaction. Additionally, Ly-6A.2 expression on $CD4^+$ T cells promotes production of IL-4, therefore suggesting this molecule participates in effector differentiation of CD4 T-helper cells.

INTRODUCTION

Growth and differentiation of T cells following an encounter with an antigen is critical for proper immune function. The specificity of the clonal expansion and differentiation of lymphocytes is determined by the interaction of T cell receptor with its ligand, a peptide-MHC complex expressed on the antigen presenting cells. Clonal expansion of $CD4^+$ T cells is tightly regulated as unchecked growth can cause toxic effector functions and unbalanced immune responses to foreign antigens. Although the mechanism of regulation of T cell growth remains largely unknown previous studies suggest that surface expression of CTLA-4, Fas/APO-1, and IL-2R proteins may regulate $CD4^+$ T cell growth (1-3). Mice lacking expression of CTLA-4, Fas or IL-2R show hyper-lymphoproliferation and abnormal accumulation of activated T cells, therefore suggesting their importance in regulating homoeostasis of either naïve and/or cycling T cells. (4-7). Moreover, a single gene mutation of Cbl, SLP, HPK1, or Csk in mice is known to cause hyper-proliferation of T cells, suggesting a role for these kinases in inhibiting T cell stimulation (8-11). The cell surface proteins that regulate these intracellular kinases have not been identified. Many of these regulatory molecules or growth inhibitory pathways may function in different T cell subsets or at different stages of clonal expansion or even work synergistically. The mechanisms underlying these intricate regulation of clonal expansion following an encounter with foreign antigen remains unclear.

Following activation the naive $CD4^+$ T cells undergo differentiation that is influenced by the type and relative concentrations of cytokines present. The presence of IL-4 at the initiation of immune response enhances the development of a Th2 response accompanied by production of IL-4, IL-5 and IL-13 (reviewed in 12, 13). These cytokines activate mast cells and eosinophils

and promotes the production of IgE (reviewed in 14, 15). Th2 responses are crucial in eradicating extracellular parasites and also mediate allergic reactions (reviewed in 16). The Th2 differentiation promoting factors inhibit the differentiation of naïve T cells to Th1-like cells that are critical for the eradication of intracellular pathogens and regulation of self-tolerance (12, 13). In a progressing immune response against a foreign antigen the development of Th1 and Th2 responses are often mutually exclusive (17). The role of accessory molecules expressed on naïve or activated T cells in development of Th1 and Th2 remains unresolved.

The mouse Ly-6 locus encodes a family of GPI-anchored, developmentally regulated cell surface proteins (reviewed in references 18, 19). Members of the Ly-6 gene family are excellent markers of different lineages of hematopoietic origin, including lymphocytes (20-25), monocytes (26, 27), bone marrow cells (20, 27, 28), and granulocytes (29). There are shared motifs among the mouse Ly-6 proteins including 8-10 conserved cysteine residues which are also found in human CD59, epidermal growth factor (EGF), urokinase plasminogen activator receptor (uPAR), squid Sgp-2, SP-10 (sperm antigen), (reviewed in reference 30), snake neurotoxins/cytotoxins (29) and *C. elegans* odr-2 (31). All these proteins from different species have been grouped together into the Ly-6 supergene family (Ly-6SF) based on their limited amino acid similarity and the presence of conserved cysteine residues. Published reports have suggested a role for Ly-6 protein in T cell signaling (32, 33) and cell adhesion (34-36). Surface expression of Ly-6A.2 is important for immunoresponsiveness of both T-T hybridomas (37) and normal T cells (38). Interestingly, surface expression of TCR/CD3 expression on T-T hybridomas is important for stimulation through the Ly-6 protein (39, 40). Moreover, ectopic expression of Ly-6A.2 transgene on $CD4^+CD8^+$ thymocytes promotes maturation of $CD4^+$ (not $CD8^+$) T cells in the thymus in the absence of TCR-MHC interaction (41). These data suggest that Ly-6A.2

expression influences cell growth and differentiation that is dependent or independent of signaling through the antigen receptor. Contrary to some published reports, the $CD4^+$ T cells from Ly-6A mutant mice show modest increased proliferation in response to anti-CD3 antibody in comparison to controls (42). Moreover, antibodies against Ly-6A.2 inhibit anti-CD3 induced IL-2 production by T-T hybridoma (43). The role of Ly-6A.2 expression in the antigen-specific response of primary $CD4^+$ T cells remains untested and the mechanism by which Ly-6A.2 expression may augment or inhibit TCR-induced activation is unknown.

Ly-6A.2 and other members of the Ly-6 gene family, including E48 protein and Ly-6C, participate in cell-cell adhesion (35, 36). We recently reported biochemical characterization of a candidate ligand that binds Ly-6A.2 (44). The Ly-6A.2 candidate ligand is expressed on the majority of B cells and macrophages (34, 44, Henderson et. al., unpublished data). Moreover a ligand for Ly-6d (Ly-6dL), another member of the Ly-6 gene family was recently identified and shown to be expressed in almost all mouse tissues analyzed (45). Ly-6dL shows similarity to epidermal growth factor domain of mouse notch (motch-1) protein, known for its role in directing cell fate decisions in variety of cell types during development (reviewed in 46). These observations raise the possibility that Ly-6 proteins may mediate cell function by binding to a ligand, but the consequences of these Ly-6 – ligand interactions are unknown.

To examine the role of Ly-6A.2 expression on the function of $CD4^+$ T cells, we bred the Ly-6A.2 transgenic mice with DO11 TCR transgenic mice. Here we report the overexpression of Ly-6A.2 on $CD4^+$ T cells inhibits responses initiated by the TCR in the presence of peptide antigen presented on APC's. Surprisingly, the same transgenic $CD4^+$ T cells are hyper-responsive to a combination of anti-TCR/CD3 and CD28 antibodies, in the absence of APC. Our antibody against a candidate Ly-6A.2 ligand reversed the antigen-specific hypo-responsiveness.

These observations suggest that Ly-6A.2 expression exerts both inhibitory and activating role depending on how T cells are stimulated. Interaction of Ly-6A.2 with a candidate Ly-6A.2-ligand negatively regulates T cell proliferation. Moreover, Ly-6A.2 transgenic DO11 $CD4^+$ T cell primary cultures produce large amounts of IL-4 in response to the $OVA^{323-339}$ peptide, therefore suggesting that Ly-6A.2 expression may participate in differentiation of $CD4^+$ T cells into Th2 subset.

MATERIALS AND METHODS

Mice. The Ly-6A.2 gene under the control of the human CD2 promoter was used to generate transgenic mice as previously described (47). The Ly-6A.2 transgenic mice were bred to mice homozygous for the DO11 TCR transgene (48) and MRL-lpr/lpr (Jackson Laboratory. Bar-Harbor, ME, USA) for testing the role of Ly-6A.2 expression in antigen-specific responses. Mice ages 4-6 weeks were used in this study. The following PCR primers were used to identify the lpr transgene: 5' GTA AAT AAT TGT GCT TCG TCA G 3' and 5' TAG AAA GCT GCA CGG GTG TG 3', yielding PCR fragments of 212 bp (lpr) and 184 bp (wildtype).

Cell Preparation. $CD4^+$ T cells from Ly-6A.2 transgenic or non-transgenic mice were prepared from the lymph nodes. Lymph node cells were incubated with 100 µl of anti-CD8 (3.155) and anti-MHC Class II (M5/114) antibodies for 30 minutes at 4^oC. Samples were washed three times with the phosphate buffered solution supplemented with 0.1% bovine serum albumin (PBS-BSA 0.1%). Following the washing step, cells were incubated with Dynal beads M-450 coupled with sheep anti-mouse IgG Ab, as per manufactures instructions (Dynal A. S., Oslo, Norway) for 45 minutes at 4^oC. Depletion of contaminating cells was achieved by magnetic separation, and purity of $CD4^+$ cells ranged from 85 to 95%.

Flow cytometry. One X 10^6 lymph node or purified $CD4^+$ cells were incubated with anti-CD4-PE, anti-CD8-FITC, anti-Ly-6A/E (D7) (PharMinigen, San Diego, CA), and anti-DO-11 T cell receptor (KJ1-26) (49) antibodies followed by appropriate fluorochrome-conjugated second step reagents. Cells were analyzed on an EPICS Elite Analyzer flow cytometer (Beckman-Coulter, Fullerton, CA).

ELISA for detection of anti-DNA antibodies and cytokines. For detection of anti-DNA antibodies, the microtiter wells were coated with poly-L-lysine (25 μg/ml) for 24 hrs at 4^oC. Excess of poly-L-lysine was removed by washing with Tris-buffer saline 0.1M containing 0.1% Tween-20 (TTBS) prior to coating of dsDNA at 5 μg/ml for 2 hrs at room temperature (RT). Sera from mice were analyzed at 1:10, 100, 1:1000 dilution by incubating for 60 min at RT. The presence of anti-dsDNA antibodies was detected by incubation with Protein-G-alkaline phosphatase at 1:4000 dilution for 1 hr at RT and the assay was developed in the presence of the substrate, p-nitrophenyl phosphate.

For cytokine ELISA, microtitre wells (Costar) were coated with appropriate capture antibody in 0.1 M Na2HPO4 binding buffer (pH 9.0) overnight at 4°C. Plates were washed 5 times with PBS-0.05% TWEEN-20 and blocked with 100 ml of 1% BSA in PBS for 30 minutes at room temperature. After 5 washes, cytokine standards and samples diluted in bocking buffer/ TWEEN-20 were added to wells for overnight incubation at 4°C. Plates were washed 6 times before adding appropriate capture antibody for 1 hour incubation at room temperature. Incubation was followed by 6 washes after which 100 μl of streptavidin-HRP conjugate (Vector, Burlingame, CA) was added at a 1:2000 concentration. Plates were incubated for 30 minutes at room temperature, washed 8 times prior to addition of the substrate 2,2,-Azino-bis-(3-

ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemicals, MO, USA). The assays were read at 405 nm by an ELISA reader.

Antibody Immobilization. Microculture wells (Costar, Cambridge MA) were coated either with purified anti-CD3 (145-2C11), anti-CD28 (37N) (50) or anti-DO11 TCR (KJ1-26) for 2 hours at 37^{o}C in carbonate-bicarbonate buffer, pH 9.6. Unbound antibodies were removed by washing with PBS 0.1M, pH 7.4, five times prior to the addition of the purified CD4^{+} T cells.

Antigen Stimulation and Cell Culture. 1 X 10^5 purified CD4^{+} T cells were co-cultured with 5 X 10^5 irradiated antigen presenting cells (APC) in RPMI 1640 based culture media (Irvine Scientific, Santa Ana CA). The cultures were carried out in the presence of either stimulating c-Ovalbumin$^{323-339}$ (SQAVHAAHAEINEAGRE) or non-stimulating cOVA$^{324-334}$ (QAVHAAHAEIN) peptides (synthesized at Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA). The precise culture condition is listed in the legend to the appropriate figures. Cultures were pulsed after 72 hours with 1 μCi ^{3}H-thymidine for the last 20 hours of culture.

Calcium responses. CD4^{+} T cells purified from the lymph nodes of BALB/c mice were loaded with Indo-1 AM (Molecular Probes, Eugene, OR) at 2 μg/mL final concentration. The cell loading was carried out at cell concentration of 2 x 10^6 cell/ml in loading medium consisting of Hank's balanced salt solution (HBSS) with 1% BSA for 30 minutes at 37^{o}C. Following incubation, cells were centrifuged for 8 minutes at 1000 rpm, and pellet was resuspended in cell loading medium to a final concentration of 2x10^6 cells/ml. Cells were then stored at 22^{o}C and protected from light until analysis. Indo-1 AM-loaded cells were warmed to 37^{o}C for 5-10 minutes prior to analysis. Agonists were added at varying time points and the cells were analyzed on an Epics 753 Flow Cytometer (Coulter, Hialeah, FL) at 37^{o}C in cell loading

medium. Anti-CD3 (145-2C11) and control antibody, anti-H-$2K^k$ (10.2.16) were used at a concentration of 10 μg/ml. Rabbit anti-mouse IgG and ionomycin were used at final concentrations of 25 μg/ml and 2 μg/ml respectively.

RESULTS

Ly-6A.2-overexpressing $CD4^+$ T cells are hypo-responsive to c-$OVA^{323-339}$ peptide presented by splenic APC. Previous studies have suggested both activating and inhibitory effects of Ly-6A.2 expression on $CD4^+$ T cell responses. Moreover, the role of Ly-6A.2 expression on antigen specific T cell activation of normal T cells is unclear. To clarify these issues and to fully understand the role of Ly-6A.2 expression in antigen-specific responses we bred the Ly-6A.2^{high} transgenic mice to DO11 TCR transgenic mice. $CD4^+$ T cells were isolated from the lymph nodes of transgenic mice and tested for their responsiveness to c-$OVA^{323-339}$ peptide in the presence of irradiated syngeneic APC. Ly-6A.$2Tg^{high}$ DO11 TCR^+ $CD4^+$ T cells proliferated 7-8 fold lower than $CD4^+$ T cells from Ly-6A.2 Tg^- DO11 TCR^+ control littermates (Figure 1A). Hypo-responsiveness of Ly-6A.2 Tg^{high} $CD4^+$ T cells was observed at 1 μM (Figure 1A) and lower (0.5 μM and 0.25 μM) c-$OVA^{323-339}$ peptide concentration (data not shown). DO11 TCR transgenic $CD4^+$ T cells did not respond to c-$OVA^{324-334}$ control peptide, as expected (Figure 1A). Ly-6A.$2Tg^+$ $CD4^+$ T cells also showed reduced responses to anti-CD3 mAb (Figure 1B). Similar levels of hypo-responsiveness of Ly-6A.2 Tg^{high} $CD4^+$ T cells was observed when mitomycin treated splenic cells were used to present the cOVA peptide (data not shown).

To examine whether the reduced proliferation in Ly-6A.$2Tg^+$ DO11Tg^+ $CD4^+$ T cells was not due to a general unresponsive (refractory) status of these cells, we tested the ability of the Ly-6A.2 Tg^{high} DO11 transgenic $CD4^+$ T cells to proliferate in the presence of PMA and calcium

ionophore, that induce receptor independent signaling in T cells. $CD4^+$ T cells from Ly-6A.2 Tg^{high} mice were fully responsive to activation by PMA and calcium ionophore (Figure 1B). Moreover, cross-linking of Ly-6A.2 with an anti-Ly-6A.2 antibody induced greater proliferation in the $CD4^+$ Ly-6A.2 transgenic T cells than the $CD4^+$ T cells from non-transgenic mice (Figure 1C). These results indicate that the hypo-responsiveness observed in $CD4^+$ Ly-$6Tg^+$ T cells is $OVA^{323-339}$ peptide-APC specific.

High expression of the Ly-6A.2 transgene ameliorates the lymphoproliferative disorder in lpr/lpr mice. Lymphoadenopathy and autoimmunity in the MRL-lpr/lpr mice is a consequence of spontaneous mutation in the Fas gene (5). T lymphocytes lacking the expression of Fas protein undergo activation-mediated cell death after recognizing an unidentified autoantigen bound to appropriate MHC class II molecule (51). Interaction of the TCR with the self-MHC proteins is critical for initiation of this autoimmune phenotype (52, 53). We sought to examine whether the overexpression of the Ly-6A.2 on T cells from the lpr mice might inhibit autoreactivity through the TCR and reverse lymphoproliferative disorder in these mice. For these experiments the Ly-6A.2 Tg^{high} mice were backcrossed to the MRL-lpr/lpr mice for 7-8 generations, Ly-6A.2 Tg^{high} lpr/lpr and Ly-6A.2Tg^- lpr/lpr mice were analyzed. The Tg^- MRL-lpr/lpr mice develop large lymph nodes at around 16-18 wk of age as expected. Interestingly, the expression of high levels of Ly-6A.2 in the MRL-lpr/lpr mice results in lymph nodes of normal cellularity (Figure 2A). Consistent with the previous observation (54), the MRL-lpr/lpr mice have an abnormal subset of $CD4^-CD8^-$Thy-1^+ T cells in the lymph node (Figure 2B). The abnormal subset is not observed in the Ly-6A.2Tg^{high} MRL-lpr/lpr and normal mice (Figure 2B). Moreover, the presence of anti-DNA antibodies that is a signature of the autoimmune phenotype was significantly reduced in the lpr/lpr mice over-expressing Ly-6A.2 (Figure 2C). These results

suggest that the over-expression of Ly-6A.2 in the MRL-lpr/lpr mice suppresses the proliferation of T cells in the lymph node and reverse the autoimmune phenotype. Consistent with the above observation is the finding that $CD4^+$ T cells from Ly-6A.2Tg^{high} MRL-lpr/lpr mice do not proliferate in the presence of syngeneic APC (Figure 2D). These results indicate that the over-expression of Ly-6A.2 suppress lymphoproliferation and autoimmune phenotype in MRL-lpr/lpr mice.

Reduced proliferation of Ly-6A.2 transgenic $CD4^+$ T cells in response to peptide antigen requires interaction with antigen presenting cells. The finding that Ly-6A.2 over-expression decreased peptide-stimulated but not the PMA + calcium ionophore responses might suggest that the Ly-6A.2 affects signaling through the TCR or that a Ly-6A.2 ligand on the APC mediates this inhibition. To distinguish between these two possibilities purified $CD4^+$ T cells were cultured with anti-TCR + anti-CD28 antibodies bound to the microtitre wells, in the absence of antigen presenting cells. Previous experiments have demonstrated that crosslinking of TCR and CD28 with the antibodies directed against them was necessary and sufficient to activate naive T cells (50). Figure 3B shows that $CD4^+$ T cells from Ly-6A.2 transgenic mice proliferate in response to the stimulation through the TCR and co-stimulatory molecule. Indeed much higher proliferation was observed with Ly-6A.2 transgenic T cells in comparison to the responses of $CD4^+$ T cells from non-transgenic mice. Similar results were obtained with a combination of plate-bound anti-DO11 TCR (KJ1-26) and anti-CD28 (Figure 3B) or anti-TCR (H57) and anti-CD28 (data not shown). Taken together these data suggest that Ly-6A.2 transgenic $CD4^+$ T cells are capable of proliferating well (even more than the non-transgenic $CD4^+$ T cells) in response to anti-TCR stimulation in the absence of APC. The antigen-specific hypo-responsiveness of Ly-

6A.2 transgenic $CD4^+$ T cells occurs by a non-cell autonomous mechanism that requires interactions with the APC.

Reduced Ca^{2+} responses in $CD4^+$ T cells over-expressing Ly-6A.2. To characterize the mechanism underlying hypo-responsiveness we first sought to test the effects of Ly-6A.2 over-expression on the Ca^{2+} response of these cells after loading with a calcium sensitive dye, Indo-1 (55) followed by crosslinking of the TCR/CD3 complex. As calcium binds to Indo-1, the peak emission wavelength shifts from 500 nm to 400 nm. This event is quantified in flow fluorocytometer and data is displayed as a ratio of emission fluorescence at 515 nm and 400 nm as function of time. Lower Ca^{2+} responses were observed in Ly-6A.2 transgenic $CD4^+$ T cells than non-transgenic $CD4^+$ T cell controls (Figure 4B). Both the mean intensity of Ca^{2+} flux (Figure 4B) as well as the total number of Ly-6A.2Tg^+ $CD4^+$ T cells responding (data not shown) to TCR/CD3 stimulation was significantly reduced. The lack of any Ca^{2+} response with the control antibody indicates the specificity of the responses observed (Figure 4A). These results suggest that Ly-6A.2 expression affect early signaling events.

Cytokine production by Ly-6A.2 transgenic $CD4^+$ T cells. Impaired anti-TCR mediated Ca^{2+} responses and proliferation of Ly-6A.2 transgenic $CD4^+$ T cells suggests a decrease in the production of cytokines that are key regulators of T cell growth. We therefore sought to quantitate the production of IL-2, IL-4 and IFN-gamma. Figure 5 demonstrates that Ly-6A.2 transgenic and non-transgenic T cells produce IL-2, a key growth factor. Comparable amounts of IL-2 was produced by $CD4^+$ T cells form non-Ly-6A.2 transgenic and Ly-6A.2 Tg^{high} mice on day–1 and 2 post-activation, but significantly reduced IL-2 was detected in day-3 cultures with Ly-6A.2 transgenic cells. In contrast, Ly-6A.2 transgenic $CD4^+$ T cells produced comparable or even more IFN-gamma than their non-transgenic controls. To our surprise the $CD4^+$ T cells

from Ly-6A.2 transgenic mice generated more IL-4 in response to the $OVA^{323-339}$ peptide than their non-transgenic controls. High amounts of IL-4 were detected in day-2 & 3 cultures, therefore suggesting that Ly-6A.2 transgene expression promotes generation of IL-4 that is undetectable in the primary cultures in which $CD4^+$ T cells were stimulated through the antigen receptor (our detection sensitivity; 62.5 pg/ml). Taken together these results suggest that Ly-6A.2 expression influences $CD4^+$ T cell growth by inhibiting full production of IL-2. Ly-6A.2 expression may also influence the differentiation of naïve T cells by producing elevated levels of IL-4.

The elevated production of IL-4 by Ly-6A.2 transgenic $CD4^+$ T cells in response to stimulation through the antigen receptor is independent of APC. To test whether the elevated production of IL-4 was dependent on interaction of the $CD4^+$ T cell with antigen presenting cells or reflected intrinsic ability of these cells to produce IL-4 in response to TCR stimulation we stimulated $CD4^+$ cells with anti-CD3 and anti-CD28 in the absence of APC. Figure 6 shows that plate-bound antibodies induce large amounts of IL-4 in cultures with Ly-6A.2 transgenic $CD4^+$ T cells and not the non-transgenic controls. These observations strongly suggest that ability of IL-4 production by Ly-6A.2 transgenic T cells is induced by a cell-autonomous mechanism that does not require interaction with APC.

Role of Ly-6A.2 – ligand interactions in antigen-specific inhibition of proliferation and IL-4 production by Ly-6A.2 transgenic $CD4^+$ T cells. We have generated a monoclonal antibody against the Ly-6A.2 ligand. This antibody recognizes a 66 kDa protein expressed in majority of professional antigen presenting cells in the spleen and blocks the binding of a candidate ligand expressing cells to Ly-6A.2 over-expressing CHO cells (44). We sought to test whether the anti-Ly-6A.2 ligand antibody reversed the peptide-specific hypo-responsiveness that was observed

with Ly-6A.2 transgenic $CD4^+$ T cells. Figure 7A shows that an antibody against the candidate Ly-6A.2 ligand (9AB2) but not the hamster control antibody (9E3) reverses this inhibition. These results strongly suggest that inhibition of $CD4^+$ T cells to peptide antigen may be mediated through the interaction of the over-expressed Ly-6A.2 with a candidate Ly-6A.2-ligand expressed on the APC. In contrast, the presence of anti-Ly-6A.2 ligand antibody did not alter the production of IL-4 in response to $OVA^{323-339}$ peptide (Figure 7B). These later results are consistent with our observation that IL-4 production is independent of APC (Figure 6). Taken together these data suggest that hypo-responsiveness and elevated production of IL-4 show differential dependence on ligand interaction.

DISCUSSION

Previous reports suggest that mouse Ly-6 proteins have both cell adhesion (34) and cell signaling (32, 33) function. Although the ligands that might bind Ly-6 proteins have been proposed (44, 45), the functional consequence of their interaction with Ly-6 proteins remains unclear. We demonstrate that Ly-6A.2 expression negatively regulates antigen-specific $CD4^+$ T cell responses by interacting with a candidate ligand. Ly-6A.2 transgenic $CD4^+$ T cells show lower Ca^{2+} fluxes and IL-2 production than $CD4^+$ T cells from non-transgenic controls in response to TCR stimulation. To our surprise the primary cultures of Ly-6A.2 transgenic $CD4^+$ T cells generated IL-4 in response to peptide stimulation that was independent of interaction with the candidate Ly-6A.2 ligand. These data suggest that Ly-6A.2 expression regulates T cell proliferation and possibly differentiation of $CD4^+$ T cells after encountering an antigen. This data also provides functional evidence of a candidate ligand expressed on APC that is recognized by our recently reported hamster anti-ligand antibody.

Our previous reports suggest the presence of Ly-6A.2 ligand on majority of B cells and macrophages (34, 44). APC-dependent inhibition of $CD4^+$ transgenic T cell responses to peptide antigen is consistent with these published data, since splenic APC is comprised of B cells and macrophages. Suppression of lymphoproliferative disorder and autoimmune symptoms in the Ly-6A.2 Tg^+ lpr/lpr mice that are normally observed in MRL-lpr/lpr mice in the absence of Ly-6A.2 transgene support these in vitro observations. The inhibitory responses are proportional to the level of Ly-6A.2 transgene expression, in as much as 10 fold lower transgene expression on peripheral $CD4^+$ T cells (from Ly-6A.2Tg^{low} mice) showed reduced proliferation in response to $OVA^{323-339}$ peptide (ranging from 0-40% inhibition compared to the wild type controls) (Henderson et. al., unpublished observations; data not shown). Consistent with this finding is the observation that Ly-6A.2^{low} lpr/lpr mice did not show abrogation of lymphoproliferative disorder (Henderson et. al., unpublished observation; data not shown). These results corroborate with a previously published report that shows a modest hyper-responsiveness by T cells lacking expression of Ly-6A.2 to anti-CD3 stimulation (35). Taken together these observations demonstrate that Ly-6A.2 over-expression inhibits antigen specific responses of $CD4^+$ T cells that is primarily mediated by interaction of Ly-6A.2 with its candidate ligand. These experiments with transgenic mice may be physiologically relevant since similar high levels of Ly-6A.2 expression on normal $CD4^+$ T cells is achieved during T cell activation or treatment with type I and II interferons (56, 57; Henderson et. al., unpublished observations).

Analyses of cell lines and normal T cells suggest either activating (38, 39, 58) or inhibitory (42, 43) role of the Ly-6A protein. It is unclear why the expression of Ly-6A.2 has these opposing affects. Our data may provide some insights into these apparently contradictory observations. Our results suggest that one candidate Ly-6A.2 ligand expressed on splenic APC

can inhibit TCR mediated responses in $CD4^+$ T cells by interacting with Ly-6A.2 (Figure 1 & 2), whereas the same Ly-6A.2 transgenic $CD4^+$ T cell shows hyper-responses to TCR signaling, (an opposite outcome) in the absence of Ly-6A.2 – ligand interactions (Figure 3). These data do not rule out the possibility that hyper-responsiveness may also be induced by interaction with another Ly-6 ligand. Interaction of Ly-6A.2 with a recently reported ligand for Ly-6d (45), another member of Ly-6 gene family has not been examined. Taken together our results suggest that hypo- or hyper-proliferation of Ly-6A.2 in Ly-6A.2 transgenic $CD4^+$ T cells may depend on how these cells are stimulated.

The antigen-stimulated T cells double every 4.5 hrs and therefore have a potential to generate $1x10^{12}$ cells in a week's time. This profound proliferation compounded with a limited space available in the lymphoid compartment may potentiate the toxic effects and autoimmune consequences, therefore these processes are under tight regulation. Up-regulation of CTLA-4 and Fas on activated $CD4^+$ T cells is known to negatively regulate T cell proliferation (1, 2). Even though IL-2 has been recognized as a T cell growth factor, recent observations suggest its importance in propriocidal regulation of T cell growth by inducing apoptosis in cycling T cells (reviewed in 3). These results strongly suggest that the expression of CTLA-4, Fas and IL-2 receptor on T cells exert their role in regulation of homoeostasis of naïve or antigen-stimulated T cells. A number of published studies have suggested growth inhibitory role of GPI-anchored proteins (including Ly-6) (reviewed in 59). Our results suggest that the interaction of Ly-6A.2 with the candidate ligand inhibits T cell proliferation and therefore regulate clonal expansion of T cells following their encounter with a foreign antigen. These later observations are consistent with the expression pattern of Ly-6A.2. Naive $CD4^+$ T cells express low levels of Ly-6A.2 protein that is profoundly increased (100-200 fold) upon T cell activation and by treatment with

type I and type II interferon's (46, 47; Henderson, unpublished observations). We propose that Ly-6A.2 – ligand interactions do not affect the initiation of T cell proliferation but instead down-regulate their proliferation when high level expression of endogenous Ly-6A.2 is achieved following T cell activation. These results may suggest that the regulation of T cell proliferation may occur once the proliferating T cells have performed their effector function. Further studies are needed to determine the precise stage at which Ly-6A.2 expression contributes to T cell proliferation.

To begin to understand the mechanism of antigen-specific inhibitory responses of $CD4^+$ T cells over-expressing Ly-6A.2 protein we focused on early signaling events. We report that Ca^{2+} responses are significantly affected. Further experiments are needed to precisely determine the mechanism of reduced Ca^{2+} fluxes in Ly-6A.2 transgenic cells. We suspected that reduced initial signaling in T cells would affect IL-2 production. To our surprise, the production of IL-2 on day-1 and day-2 of T cell response were unaltered. Significant effects were observed in the production of this growth factor on day-3 of the culture. It is possible that production of IL-2 is reduced on day-1 and –2 of the culture but our assays were unable to detect these differences. These results suggest that overall IL-2 production is significantly diminished, that in turn is likely to reduce the clonal expansion of T cells. Reduced production of IL-2 was observed on day-3 but not the day-1 & 2 of the culture suggesting that regulation of T cell proliferation by Ly-6A.2 may occur at a later stage where normal expression is highly up-regulated (typically 48 hrs after the initial culture with antigen). Alternatively the Ly-6A.2 – ligand interactions alter signaling through the IL-2 receptor and therefore inhibit growth of T cells regardless of IL-2 production, as has been previously observed with antibodies against GPI-anchored proteins

(reviewed in 59). Further experimentation is needed to completely and precisely address this question.

How cell proteins expressed on naïve or activated T cells participate in T cell differentiation is not entirely clear. A number of factors influence development of Th1 and Th2 effector T cells including relative concentration of cytokines present. IL-4 is a key regulator of differentiation of naïve $CD4^+$ T cells into the Th2 subset while IL-12 and IFN-gamma promote differentiation into Th1 effector cells. Factors known to influence differentiation of Th1 and Th2 subsets are derived from varied sources, including macrophages and dendritic cells (IL-12), NK and T cells (IFN-gamma). T cells are known to generate initial bursts of IL-4, perhaps not in large enough amounts to be detectable in the primary cultures (60). In addition, the nature, dose and route of administration of the antigen as well as nature of interacting APC influence the process of T cell differentiation (reviewed in 61). Some recent reports suggest that interaction of CD28 with B7-2 (62-65), CD4 with MHC class-II (66, 67) and OX-40 with OX-40 ligand (68, 69) promote differentiation to Th2 but not the Th1 cells. In contrast, interaction of LFA-1 with ICAM-1 or –2 (70, 71), CD28 with B7-1 (62, 72), CD40 with CD40L (73) promote Th1 differentiation. Moreover, naïve T cells lacking expression of CTLA-4 differentiate into Th2 subset, therefore suggesting role of CTLA-4 in Th1 differentiation pathway (74). To our surprise the Ly-6A.2 transgenic $CD4^+$ T cells generated large amounts of IL-4 in response to appropriate peptide ligand in the primary cultures. IL-4 was not detected on day-1 of the culture, however > 3.5 ng/ml of IL-4 was detected on day-2 and day-3 of culture. These observations raise the possibility that elevated cell surface expression of Ly-6A.2 may regulate differentiation of naïve T cell differentiation into the Th2 type by increasing IL-4 concentrations during primary stimulation. More studies need to be carried out to completely establish the Th2 phenotype of

the antigen-stimulated Ly-6A.2 transgenic $CD4^+$ T cells and the mechanism underlying IL-4 production. It is interesting that other cell surface proteins appear to influence differentiation of naïve T cells by interacting with their ligand, our study suggests that surface expression of Ly-6A.2 promotes IL-4 production that is independent of its interaction with its candidate ligand by some unknown cell-autonomous mechanism. Further studies are required to define differentiation status of the IL-4 producing Ly-6A.2 transgenic $CD4^+$ T cells in these primary cultures as well as the cell-autonomous mechanisms that may be involved in these processes.

Several members of Ly-6 supergene family appear to participate in regulating important functions in other tissues by yet unidentified mechanisms. A human Ly-6 protein inhibits osteoclast formation and bone resorption (75), mutation in odr-2 gene that encodes a Ly-6 related protein causes defect in the ability to chemotax to odorants that are recognized by the two AWC olfactory neurons in C. elegans (76) and murine lynx1 that is expressed on hippocampus, cortex and cerebellum modulates nichotinic acetylcholine receptors (nAChRs) in mammalian brain (77). Moreover, mutation in SLURP-1, another member of human Ly-6 superfamily causes a rare autosomal recessive skin disorder, Mal de Meleda, characterized by transgressive palmoplanter keratoderma (PPK), keratotic skin lesions and perioral erythema (78). Our results suggest that one way Ly-6 proteins modulate signaling and mediate their function is by interacting with their ligand(s).

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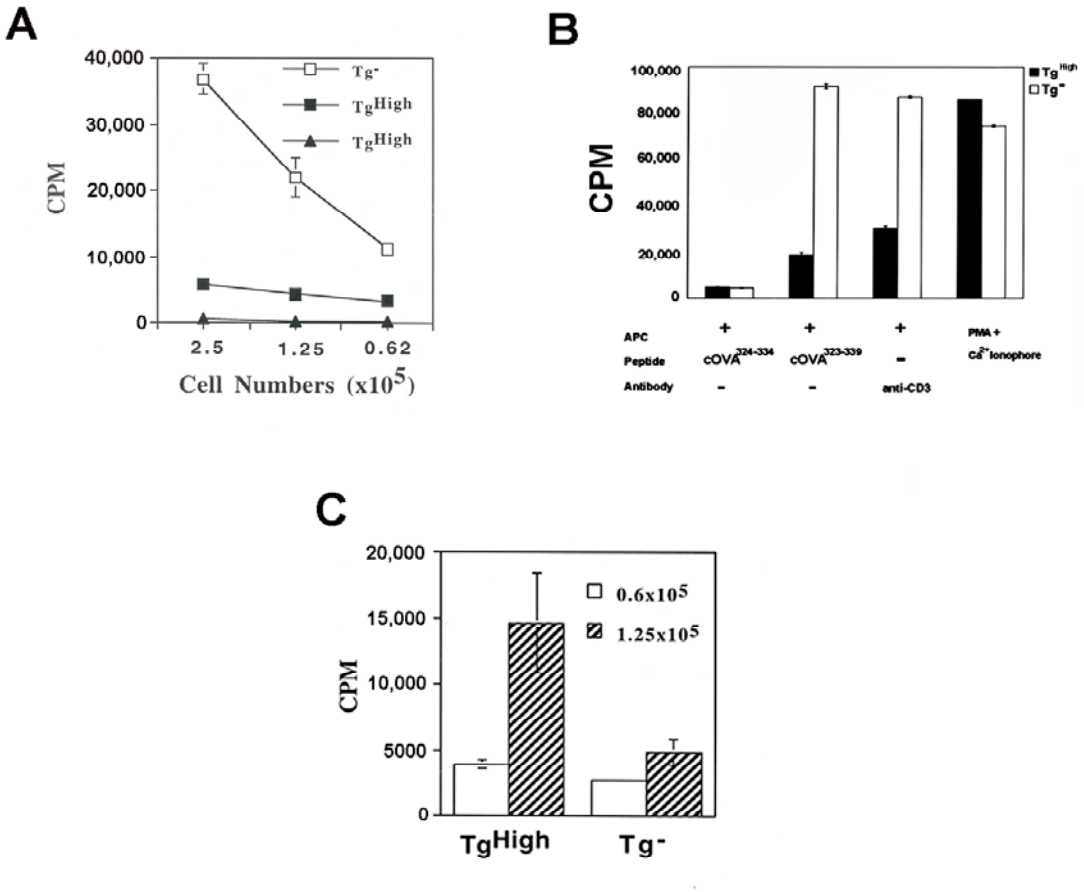
Figure 4.1. Antigen specific responsiveness of the purified Ly-6A.2 transgenic $CD4^+$ T cells. Variable numbers of purified $CD4^+$ T cells from Ly-6A.2 Tg^- (□)or Ly-6A.2Tg^{high} (■and▲)mice were co-cultured with 5 x 10^5 irradiated syngeneic spleen cells. A. The cultures were carried out in the presence of either $cOVA^{324-334}$ (control, ▲) or $cOVA^{323-339}$ (specific peptide, □ and ■) at 1 μM concentration. B. Cultures were also set up with anti-CD3 (145-2C11; 1:100 dilution of culture supernatant) $cOVA_{323-339}$, or $cOVA_{324-334}$ (1 μM), or a combination of PMA (20 ng/ml) and ionomycin (0.125ug/ml0. C. Additional cultures with a combination of anti-Ly-6A/E + 20 ng/ml of PMA were also conducted. Cells were harvested after 72 hr of culture. The data is expressed as arithmetic mean counts per minute of incorporated [^{3}H] thymidine incorporated. Error bars indicate standard deviation. T cells from either Tg^- or Tg^{high} mice did not proliferate when cultured with $OVA^{323-329}$ in the absence of the irradiated syngeneic APC (cpm<350). A representative experiment of at least five independent experiments is shown.

FIGURE 4.1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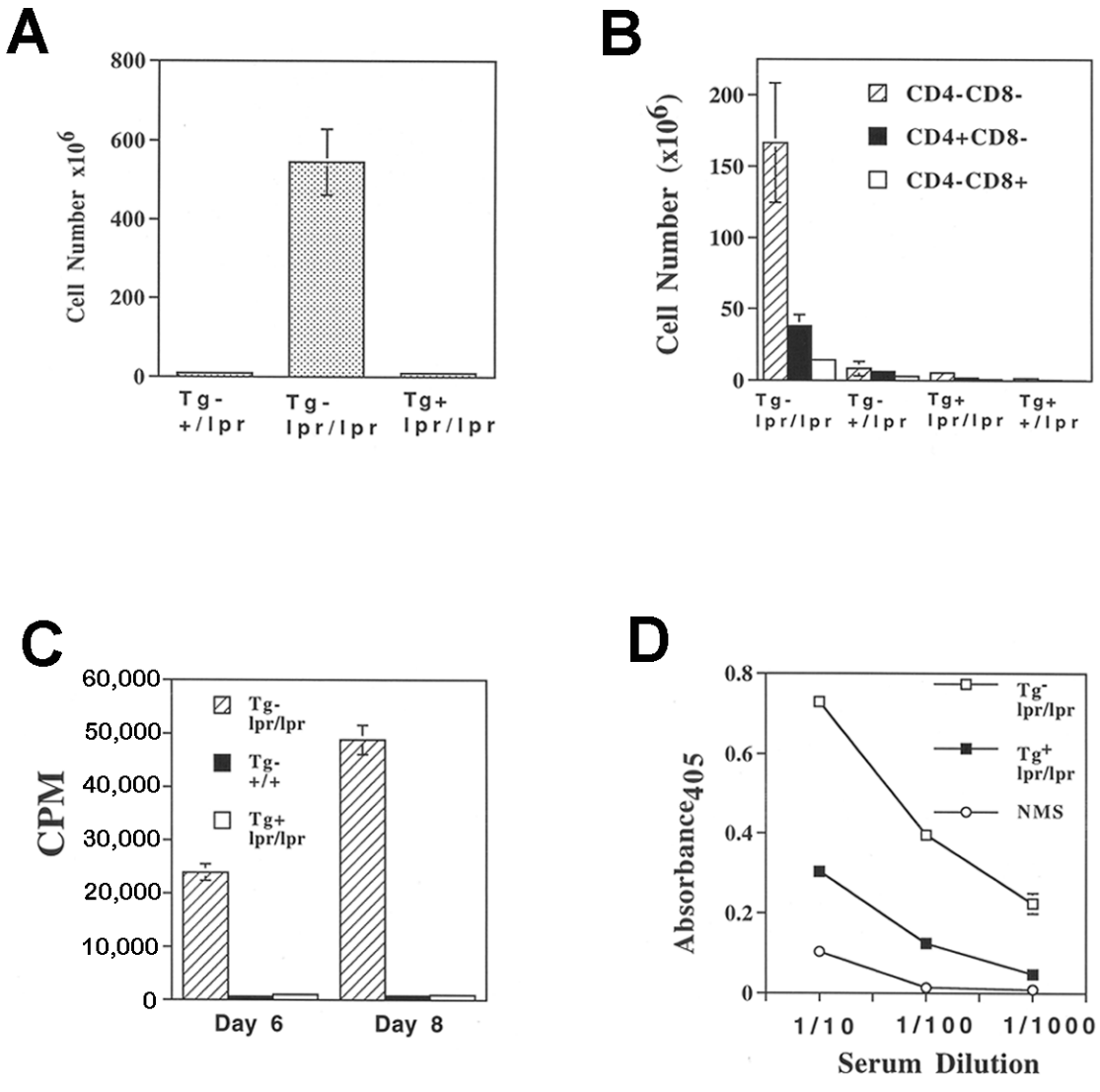
30,000
20,000
10,000
0
CPM
Tg-
TgHigh
TgHigh
2.5
1.25
0.62
Cell Numbers (x10^5)
B
100,000
80,000
60,000
40,000
20,000
0
CPM
TgHigh
Tg-
APC
Peptide
Antibody
cOVA 324-334
cOVA 323-339
anti-CD3
PMA + Ca2+ Ionophore
C
20,000
15,000
10,000
5000
0
CPM
0.6x10^5
1.25x10^5
TgHigh
Tg-

Figure 4.2. Ly-6A.2 transgene expression ameliorates lymphoadenopathy in MRL-*lpr/lpr* mice. A. Cellularity of lymph nodes from normal, Ly-6A.2 $Tg^{-/+}$*lpr*, Ly-6A.2 Tg^{-}*lpr/lpr*, or Ly-6A.2 Tg^{high}*lpr/lpr* mice was analyzed. The total number of cells obtained from four lymph nodes per mouse is shown and at least five mice from each group were analyzed. B. Lymph node cells were stained with anti-CD4-PE and anti-CD8 FITC. $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$ and $CD4^{-}CD8^{-}$ subsets were gated and the number of cells present in the gates (n=3 experiments) are shown. C. Lymph node cells (5 $x10^{5}$) were cultured with 5 $x10^{5}$ irradiated syngeneic splenic APC for 6 or 8 days followed by pulsing with [^{3}H] thymidine and harvesting for the last 6-8 h of the culture. The data is expressed as the arithmetic mean counts per minute of [^{3}H] thymidine incorporated +/- SD (n=3 experiments). D. Anti-dsDNA Ab titers (1/10 to $1/10^{3}$) were analyzed from the sera of Ly-6A.2 Tg^{-}*lpr/lpr* (□) or Ly-6A.2 Tg^{+}*lpr/lpr* (■) mice by ELISA as described in Materials and Methods. The pooled normal mouse serum was used as a control (O).

FIGURE 4.2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200
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Tg+ lpr/lpr
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150
100
50
0
Cell Number (x10^6)
CD4-CD8-
CD4+CD8-
CD4-CD8+
Tg- lpr/lpr
Tg- +/lpr
Tg+ lpr/lpr
Tg+ +/lpr
C
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50,000
40,000
30,000
20,000
10,000
0
CPM
Tg- lpr/lpr
Tg- +/+
Tg+ lpr/lpr
Day 6
Day 8
D
0.8
0.6
0.4
0.2
0
Absorbance405
Tg- lpr/lpr
Tg+ lpr/lpr
NMS
1/10
1/100
1/1000
Serum Dilution

Figure 4.3. 1 x10^5 Purified $CD4^+$ T cells from the Ly-6A.2 Tg^- or the Ly-6A.$2Tg^{high}$ mice were cultured in wells coated with different concentrations of (A) anti-CD3 (145-2C11) or (B) anti-TCR (KJ1-26) and 10 μg/ml of anti-CD28 (37N). Cells were pulsed and harvested after 48 hr of culture. The data is expressed as arithmetic mean counts per minute of [^{3}H] thymidine incorporated +/- SD. A representative experiment of five experiments is shown.

Figure 4.3

Figure 3

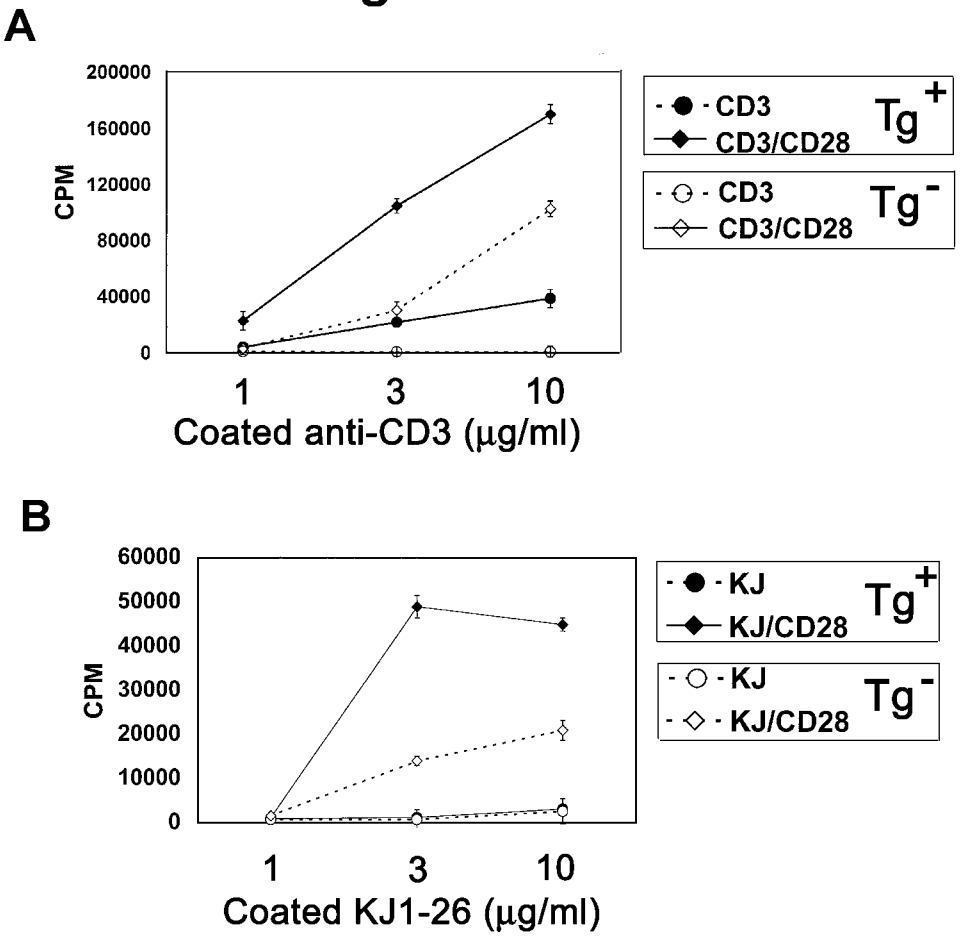


Figure 4.4. Over-expression of Ly-6A.2 on $CD4^+$ T cells inhibits calcium responses mediated through the T cell receptor complex. Purified $CD4^+$ T cells from either $Ly\text{-}6Tg^-$ (dotted line) or $Ly\text{-}6Tg^+$ (solid line) were loaded with Indo-1 and exposed to (A) anti-CD3 or (B) isotype control (anti-$H\text{-}2K^k$) at 10 µg/ml, followed by rabbit anti-mouse IgG (cross reactive with hamster IgG). Mean calcium response (ratio of 515/400 nm) by the stimulated cells is shown and total calcium present in the cells is evaluated by addition of Ionomycin at 2 µg/ml concentration.

Figure 4.4

Figure 4

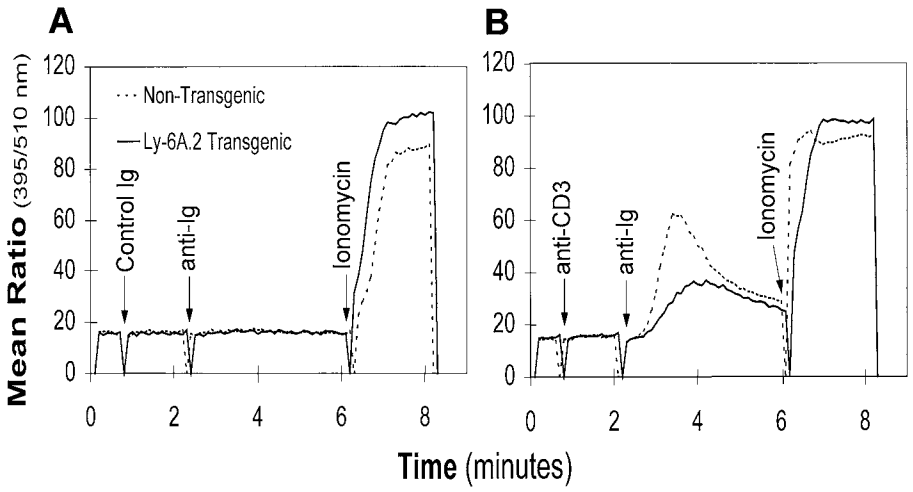


Figure 4.5. Ly-6A.2 transgenic $CD4^+$ T cells produce elevated levels of IL-4 in response to $Ova^{323-339}$ presented by splenic APC. IL-2 (A), IFN-gamma (B), and IL-4 (C) was quatitated in supernatants from day 1-3 cultures of either Ly-$6Tg^-$ (open diamond) or Ly-6A.2 transgenic (closed diamond) mice stimulated with $OVA^{323-339}$ peptide in the presence of splenic APC by ELISA as described in material and methods. A representative of total three experiments is shown.

Figure 4.5

Figure 5

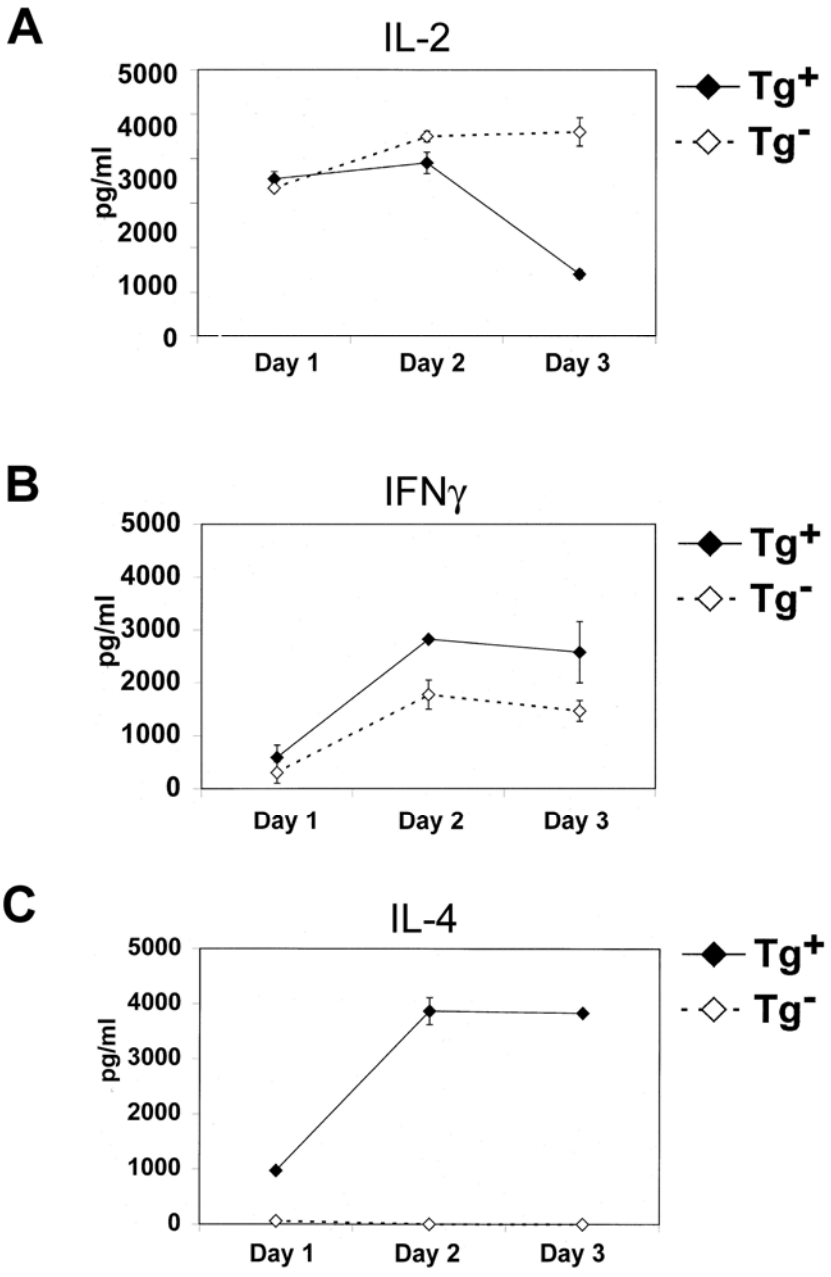


Figure 4.6. Ly-6A.2 transgenic $CD4^+$ T cells produce elevated levels of IL-4 by cell-autonomous mechanism in the absence of APC. IL-4 was quantitated in supernatants from day 3 cultures of either $Ly\text{-}6Tg^-$ (open) or Ly-6A.2 transgenic (closed) mice stimulated with either anti-CD3 alone or combination of anti-CD3 and anti-CD28 in the absence of APC by ELISA as described in material and methods. A representative of total three experiments is shown.

Figure 4.6

Figure 6

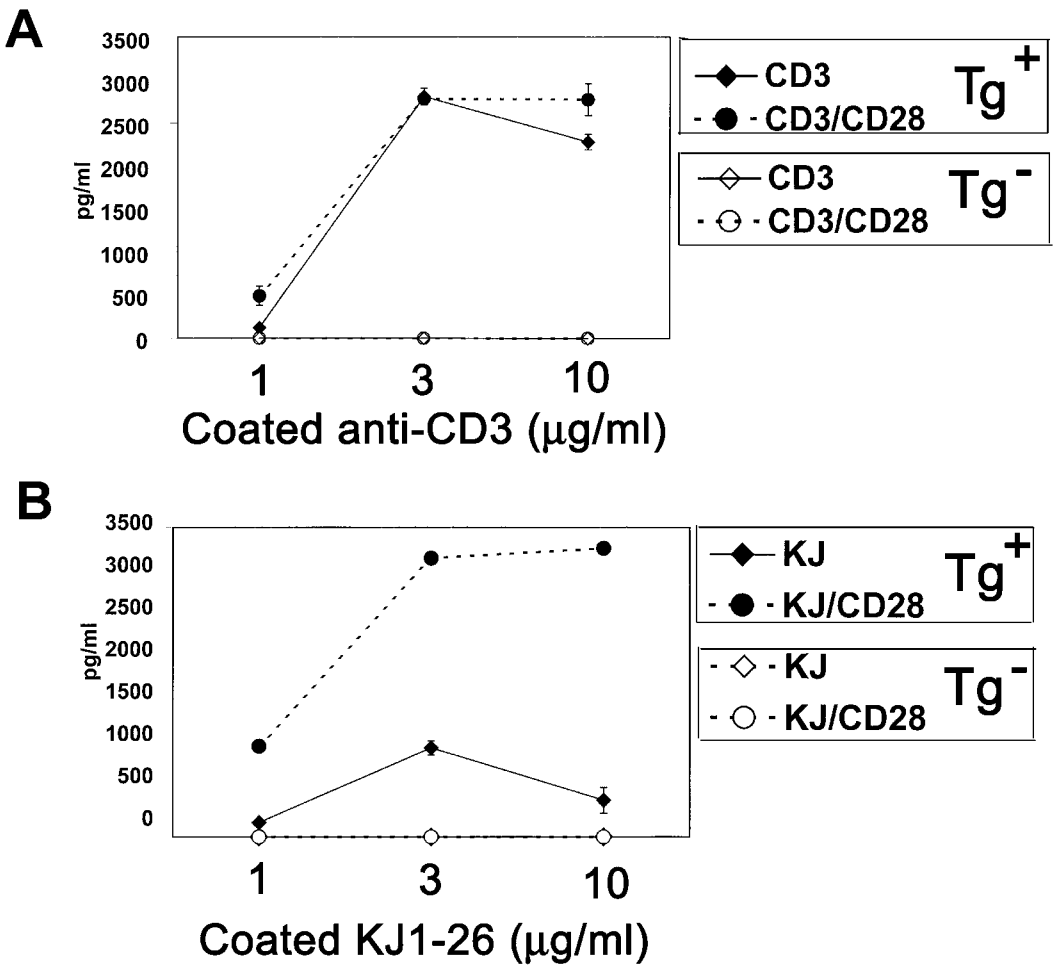
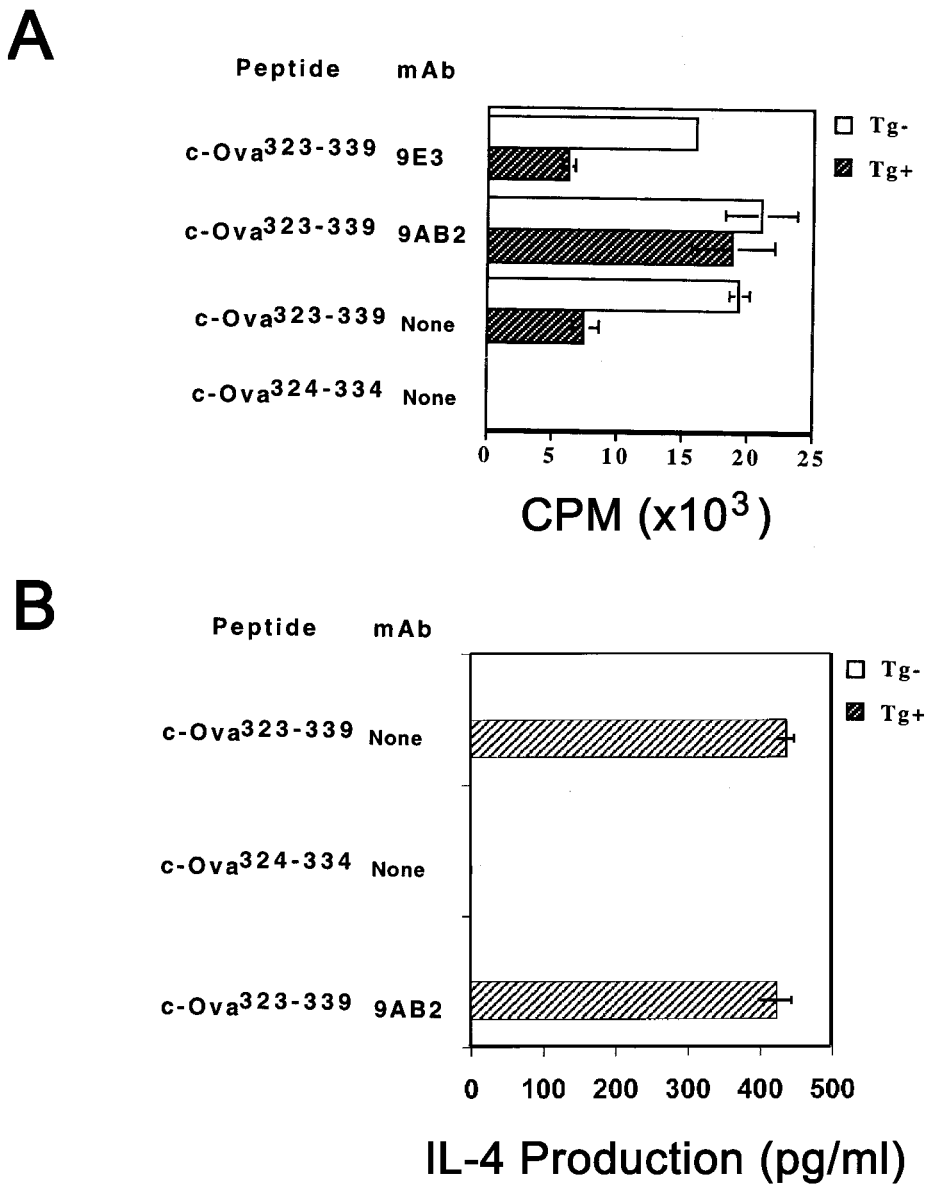


Figure 4.7. Role of the candidate Ly-6A.2 ligand in $OVA^{323-339}$ peptide specific $CD4^+$ T cell proliferation and IL-4 production. (A) Purified $CD4^+$ T cells from the Ly-6A.2 Tg^- or Ly-6A.2Tg^{high} mice were co-cultured with 5 x 10^5 irradiated syngeneic spleen cells. These cultures were carried out in the presence of either $cOVA^{324-334}$ (control) or $cOVA^{323-339}$ (specific-peptide) at 1 μM concentration either in the absence or the presence of anti-ligand (9AB2, 1:4 dilution of supernatant) or control hamster (9E3 at 1:4 dilution of supernatant) antibody. Cells were harvested after 72 hr of culture. The data is expressed as the arithmetic mean counts per minute of [^{3}H] thymidine incorporated. A representative experiment of four independent experiment of is shown. (B) IL-4 production was quatitated in the supernatants of these day-3 cultures with the $^{OVA323-339}$ peptide as descibed in materials and methods. A representative of total three experiments is shown.

Figure 4.7

Figure 7



CHAPTER 5

CONCLUSIONS

Summary

T lymphocytes have a central role in the cell-mediated immune response against infectious agents. The processes that regulate the immune response are not completely understood. Moreover, gaining insight into the mechanisms that govern growth and differentiation of T lymphocyte development in the thymus and periphery will provide ways to enhance beneficial immunity against pathogens and inhibit unwanted immune responses to self-tissues. We demonstrate that Ly-6A.2 expression on T cells inhibits their growth, both in the thymus and periphery, which is mediated by interaction with a candidate ligand. The growth inhibitory effects observed in the thymus occur when Ly-6A.2 is expressed ectopically on developing cells that normally do not express this protein. Ly-6A.2 exerts antigen specific growth and inhibitory effects on peripheral $CD4^+$ T cells when over-expressed precociously on the cell surface. A major significance of these observations is that Ly-6A.2 might regulate clonal expansion of $CD4^+$ T cells induced by a foreign antigen. Surprisingly, Ly-6A.2 expression also appears to induce production of a Th2 differentiation promoting factor, IL-4, by naïve $CD4^+$ T cells.

Mechanisms of Thymic Block in Ly-6A.2 Dysregulated Mice

Proper development of T cells in the thymus requires orderly induction or down-regulation of signaling and adhesion proteins at specific stages of development. Regulated expression of several proteins is required during transitioning within the $CD4^-CD8^-$ (DN) subset. The IL-7R, expressed on c-$kit^+CD44^+CD25^+$ pro-thymocytes has been implicated in both cell proliferation and cell survival (1, 2). Additionally, several reports have implicated IL-7 in initiation of TCR re-arrangements (3). The importance of c-kit, the tyrosine kinase receptor for stem cell factor (SCF), has also been demonstrated, as these interactions have been shown to drive expansion of immature thymocytes *in vivo* (4). Surface expression of the pre-Tα is critical for transduction of signals to initiate TCR-V_β re-arrangement (5). Notch plays a critical role during the commitment of progenitor cells to the T cell lineage, as well as differentiation into $\alpha\beta$ versus $\gamma\delta$ T cells (6, 7). As thymocytes begin to transition from the DN to the $CD4^+CD8^+$ cell stage, expression of a complete pre-TCR_α on the cell surface is required (8). Without this completed receptor complex, thymocytes could not undergo the selection and differentiation processes critical for generating the TCR repertoire. Signal transduction emanating from the pre-TCR requires participation of several membrane-proximal signaling molecules, many of which have been identified as critical components of thymocyte maturation within this early subset. Best characterized is the src family tyrosine kinase, $p56^{lck}$, which has been shown to mediate cellular expansion and allelic exclusion at the TCR_β chain gene locus during the transition from the DN to the DP subset (9, 10). Analysis of molecules downstream from $p56^{lck}$ have revealed Linker for Activation of T cells (LAT) to be a component critical for the continual progression of thymocytes within the DN subset (11). More recently, a member of the hedgehog (Hh) family of secreted proteins, Sonic hedgehog (Shh), has been shown to play a major part in regulating the differentiation

of the DN subset into the DP cell stage (12). Additionally, beta-catenin ($_{\beta}$-catenin), a central effector of the Wnt signaling pathway, is capable of substituting for pre-TCR signaling and may be a key mediator regulating the transitioning of DN and DP subsets (13).

Stage-specific differential expression of Ly-6A.2 is also critical for thymocyte development. One of the earliest lineage markers of thymic progenitors, Ly-6A.2 can be detected on pluripotent hematopoietic stem cells that colonize the thymus and continues to be expressed until thymocytes begin transitioning to the $CD4^{+}CD8^{+}$ DP cell stage (14, 15). Regulation ensures that DN thymocytes turn off expression of Ly-6A.2 before progressing to the DP subset. Following selection and differentiation processes, Ly-6A.2 is re-expressed on mature, $CD4^{+}$ or $CD8^{+}$ single positive (SP) thymocytes as they begin to move out of the thymus. Previous studies have shown that dysregulation of Ly-6A.2 during thymocyte development results in developmental arrest of maturing thymocytes at the DN stage and reduced thymic cellularity (15). The goal of this study was to gain insight into the mechanisms underlying this block in development observed in Ly-6A.2 transgenic mice.

Our studies demonstrate that the block in T cell development resulting from unregulated expression of Ly-6A.2 is primarily dependent on bone marrow cells. Thymic epithelial cells in these transgenic mice are competent to support proper T cell development and therefore do not contribute to the block in thymocyte development. The trafficking of developing T cells from the cortex into the medulla in Ly-6A.2 transgenic mice appeared to be normal. Noteworthy is the observation that thymocytes that did cross into the medulla showed down-regulated expression of the Ly-6A.2 transgene, however the mechanism for down-regulation of transgenically expressed molecules remains unclear.

Analyses of whole thymocyte lysates, which contain DP and SP cell populations, and purified DN thymocyte lysates revealed an overall lower amount of total and phosphorylated LAT. Additionally, localization of LAT to the GEMS, which is required for its participation in signal transduction, was significantly decreased in Ly-6A.2 transgenic thymocytes compared to controls. Although the regulatory effects of LAT signaling have not been clearly defined in the thymus, studies examining peripheral T cell activation have shown participation of LAT in TCR-induced signal transduction is required for full activation and clonal expansion of these lymphocytes. Thus, it is conceivable that alterations in production and activation of LAT could inhibit proliferation of DP thymocytes undergoing selection and differentiation. Future experiments will determine if the LAT deficiencies are due to alterations in transcription/translation or post-translational modifications of this protein. However, these results collectively demonstrate that continued expression of Ly-6A.2 results in alterations to LAT, which are likely to contribute to the block in thymocyte maturation.

Dysregulated expression of Ly-6A.2 did not inhibit endogenous TCR-V_β gene rearrangement, which occurs late within the DN cell stage. Examination of the TCR- V_β repertoire revealed a presence of diverse V_β chains on the surface of T cells. However, we were surprised to find TCR-V_β3 bearing T cells in Ly-6A.2 transgenic BALB/c mice, which along with TCR-V_β5, -11, and –17 are typically deleted by Mtv-6, -8, and –9 SAg products. We were equally surprised to observe the near complete absence of TCR-V_β8 bearing T cells in the thymus of Ly-6A.2 transgenic BALB/c mice, which normally constitute approximately 20% of all T cells in non-transgenic BALB/c mice. These results indicated that unregulated expression of Ly-6A.2 did not alter expression of a re-arranged TCR-V_β, and therefore was

not the prime mechanism for the block in thymocyte development. Nonetheless, the dysregulated expression of Ly-6A.2 resulted in altered T cell selection.

We observed some striking effects of continued expression of this molecule within the DP subset. Analyses of cells from total and purified DN cells showed that equivalent amounts of $p56^{lck}$ were produced in Ly-6A.2 transgenic and non-transgenic thymocytes, indicating $p56^{lck}$ did not contribute to the block in development. However, immunoprecipitation experiments of lysates containing DP and SP populations showed preferential localization of Lck to cytoplasmic tails of CD4 in non-transgenic thymocytes, which suggested that this src family kinase may contribute to altered selection and differentiation of DP thymocytes that continue to express Ly-6A.2.

Accelerated cell death in the Ly-6A.2 dysregulated mice could also contribute to the reduced cellularity observed in the Ly-6A.2 transgenic mice. The increased rate of apoptosis was not observed in the DN cell population. Instead other immature thymic subsets expressing low-intermediate levels of $TCR_{\alpha\beta}$ showed enhanced cell death by apoptosis. It is plausible that the increase in apoptosis observed within the DP subset may reflect, in part, the elimination of a majority of thymocytes expressing TCR-$V_{\beta}8$. However, the mechanism by which Ly-6A.2 contributes to apoptosis remains to be defined.

Based upon the findings presented here, we propose the following model. Under normal developmental conditions, thymocyte progenitors entering the thymus express high levels of Ly-6A.2. As developing thymocytes begin to express the necessary receptors for receiving differentiating and selective signals, expression of Ly-6A.2 is down-regulated. The inferred consequences of this decreased expression are two-fold: down-modulation may be necessary to prevent Ly-6A.2-derived signaling from interfering with those mediating the

transition of thymocytes from the DN to DP subset. Decreased expression of Ly-6A.2 on DP thymocytes would also ensure signaling from this protein would not interfere with TCR-MHC/peptide-governed selection. Together these findings suggest a model in which termination of Ly-6A.2 expression and its signaling is required for the DN to DP transition and complete development of DP and SP subsets in the thymus.

Regulated Ly-6A.2 Expression of Peripheral T cells

Regulation of T cell activation and clonal expansion is critical for preventing abnormal immunity and autoimmune consequences. The principal mechanisms of regulation involve signaling through co-stimulatory molecules that are up-regulated upon T cell activation. Although these mechanisms are not completely understood, characterization of several regulatory proteins has provided insight to these processes. Ligation the TNF super-family member Fas ligand transduces signals leading to apoptosis of T cells by a pathway called “activation induced cell death” (16). Interaction of CTLA-4 with B7-1/-2 expressed by antigen presenting cells (APCs) transmits signals that inhibit T cell activation, thereby down-modulating the immune response (17). Even though IL-2 has been recognized as a T cell growth factor, recent observations suggest its importance in propriocidal regulation of T cell growth by inducing apoptosis in cycling T cells (18). Mechanisms such as these are considered to be important for the down-regulation of immune responses and for the maintenance of self-tolerance.

In order to determine the function of Ly-6A.2 on antigen-stimulated T cells, we bred Ly-6A.2 transgenic mice to animals expressing the c-ovalbumen (cOVA)-specific TCR. *In*

vitro cultures generated to test the responsiveness of T cells over-expressing Ly-6A.2 to c-OVA peptide being presented by irradiated syngeneic APC demonstrated that CD4 cells constitutively expressing high levels of transgenically expressed Ly-6A.2 proliferated 7-8 fold lower than CD4 cells from non-transgenic mice. Providing the appropriate and necessary signals to activate T cells through Ab-mediated cross-linking resulted in a higher proliferative response from CD4 cells expressing dysregulated Ly-6A.2, demonstrating the antigen-specific hypo-responsiveness of Ly-6A.2 transgenic CD4 cells was a non-cell autonomous mechanism requiring interactions with APC. Moreover, the ability of Ly-6A.2 to proliferate in response to anti-TCR stimulation in the absence of APC suggested that ligand binding of Ly-6A.2 facilitated this response.

Further supporting an inhibitory function of Ly-6A.2 on T cell proliferation is the observation that transgenic expression of Ly-6A.2 in MRL-lpr/lpr mice ameliorates the lymphoproliferative disorder in these animals. Thus, the suppression of lymphoproliferation and autoimmune phenotype in this mouse model supports *in vitro* observations, which suggest Ly-6A.2 functions to negatively regulate T cell activation.

In order to characterize the mechanism underlying the hypo-responsiveness, we began to investigate the potential for over-expression of Ly-6A.2 to alter T cell signaling following antigenic stimulation. Both the mean intensity of Ca^{2+} flux as well as the mean number of Ly-6A.2 transgenic T cells responding to TCR/CD3 cross-linking was significantly reduced, suggesting transgenic expression of Ly-6A.2 affected early signaling events, which could subsequently impair downstream cascades and gene transcription.

There is a growing appreciation for the concept that lymphocytes are regulated by inhibitory as well as activating signals, which may reflect expression of regulatory cytokines.

Given the data that supports a role for Ly-6A.2 on inhibiting proliferation, we suspected reduced initial signaling would affect production of cytokines that are key regulators of T cell growth. We therefore sought to quantitate the production IL-2, IL-4 and IFNγ. Comparable amounts of IL-2 from Ly-6A.2 transgenic and non-transgenic cultures were observed during early time points, however significantly reduced IL-2 production from Ly-6A.2 transgenic cells was detected at later time points. In contrast, Ly-6A.2 transgenic CD4 cells produced comparable or even higher amounts of IFNγ than non-transgenic controls. To our surprise CD4 cells from Ly-6A.2 transgenic mice generated more IL-4 in response to cOVA than the non-transgenic controls, suggesting over-expression of Ly-6A.2 promoted the generation of IL-4. Antibody-stimulation of cultures further demonstrated this elevated production of IL-4 by Ly-6A.2 transgenic CD4 T cells was a cell-autonomous effect that did not require the presence or interaction with an APC. Consistent with our observation that IL-4 production is an APC-independent process, is the observation that although the presence of the candidate anti-Ly-6A.2 ligand in antigen-stimulated cultures reduced proliferation, it did not alter production of IL-4. Taken together, these results suggest Ly-6A.2 expression influences CD4 T cell growth by inhibiting membrane proximal signaling events and altering production of growth and differentiation cytokines IL-2 and IL-4.

Our data perhaps also provide explanations to the apparently contradictory data found in the literature regarding the function of Ly-6A.2 signaling during T cell stimulation (14, 19-26). Ligand binding to Ly-6A.2 mediates inhibitory signals, whereas the absence of ligand binding may result in the opposite outcome. Construction of a model for Ly-6A.2 regulation, based upon our observations, has been and continues to be an on-going process. The model we propose is that under normal conditions of antigenic

stimulation naïve $CD4^+$T cells initially express low levels of Ly-6A.2. As T cell stimulation continues, Ly-6A.2 is upregulated to maximal levels, which may be enhanced by IFNγ secretion. Upon binding with its ligand, Ly-6A.2 transmits regulatory signals to inhibit antigen-dependent clonal expansion, due to diminished Ca^{2+} flux and IL-2 secretion, thereby preventing autoimmune consequences. Moreover, we also propose that proliferating $CD4^+$ T cells with high Ly-6A.2 expression generates IL-4, a cytokine known to promote differentiation of naïve CD4 T cells to a Th2 phenotype.

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