The Roles of Cabin1 and Left1 in T Cell Development

by

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ABSTRACT

Cabin1 binds calcineurin and myocyte enhancer factor 2 (MEF2) through the C-terminal region. In cell lines, these interactions inhibit calcineurin activity following TCR signaling and transcriptional activation of Nur77 by MEF2. The role of these interactions in vivo was investigated using a mutant mouse strain that expresses a truncated Cabin1 lacking the C-terminal calcineurin and MEF2 binding domains. Although mutant mice exhibited normal lymphocyte development and thymocyte apoptosis, they had elevated levels of serum IgG1, IgG2b and IgE and produced more IgG1 and IgG2b in response to a T-dependent antigen. The increased antibody production is apparently not due to changes in immunoglobulin class switching as B cells from mutant mice switched to IgG1 at the same frequency as wildtype B cells in vitro in the presence of IL-4. However, in response to anti-CD3 stimulation, mutant T cells expressed significantly higher levels of IL-2, IL-4, IL-9, IL-13, and IFN-γ. Thus, the calcineurin and MEF2 binding domain of Cabin1 is dispensable for thymocyte development and apoptosis, but is required for proper regulation of T cell cytokine expression and Th2 antibody responses.

In our analysis of gene expression in memory CD8+ T cells, we identified a member of the membrane spanning 4A gene family, MS4a4b, which we named Left1. It was also found to be expressed in Th1, but not Th2 cells. To examine the role of Left1 in T cell development, we created transgenic mice which express Left1 in T cells. These mice revealed a role for Left1 at multiple stages of T cell development. Ectopic expression of Left1 in CD4+CD8+ thymocytes promoted CD8+ lineage commitment, although CD4+ thymocyte development was unaffected. Although mature transgenic T cells were impaired in their response to certain stimuli in vitro, there was a dramatic increase in the proportion of memory-phenotype T cells in vivo. Finally, Th2 cytokine and GATA-3 expression was impaired in Left1 transgenic Th2 cells. Transgenic T cells activated under Th2 polarizing conditions maintained some characteristics of Th1 cells, such as the expression of T-bet and IL-12 receptor. Downregulation of Left1 expression is an essential step in commitment to the Th2 lineage.

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Chapter One

Introduction to T Cell Development
Overview of T cell development

The immune system must defend against a variety of foreign agents, such as bacteria, viruses, and parasites, as well as abnormal cells that have become cancerous. Two branches of the immune system have evolved to counter these attacks. Inate immunity is the first line of defense, providing a relatively non-specific response to pathogens that are easily identified as foreign. It can also prime the adaptive immune response to infection, which can mount a more specific response, tailoring itself to the type of pathogen involved.

The main components of the adaptive immune response are T and B lymphocytes. T and B cells bearing unique receptors are able to recognize potentially millions of antigens and undergo clonal expansion to form an antigen-specific response. Unlike the B cell antigen receptor, which can recognize a wide range of soluble antigens (1), the T cell receptor (TCR) recognizes primarily short, processed peptide antigens complexed with major histocompatibility complex (MHC) proteins on the surface of antigen presenting cells (APCs) (2).

The development of an αβ T cell is driven by several processes: rearrangement of functional V, D, and J segments of the TCRα and TCRβ genes in order to generate a diverse repertoire that is able to interact with MHC proteins (3, 4), elimination of autoreactive cells (5), diversification of T cell responses to pathogens into cellular or humoral immunity (6-9), and maintenance of protective immunity in the form of memory T cells (10-12). Figure 1 outlines the major stages of T cell development. T cell precursors from the bone marrow migrate to the thymus, where early T cell development occurs (13). These early progenitor cells can be identified by their surface marker expression, as they are CD4−CD8− double negative (DN), and CD44−CD25− (14). They pass through a CD44+CD25− DN stage, and then to the CD44+CD25+ DN stage, where VDJ recombination at the TCRβ locus occurs (15). Each cell has two alleles, and therefore two chances to rearrange a TCRβ gene that can code for a functional protein. Only the DN thymocytes that successfully rearrange a TCRβ gene and produce a protein are allowed to progress to the next stage of thymocyte development (16, 17), which
FIGURE 1. The basic stages of T cell development in the thymus and peripheral lymphoid tissues. DN, CD4⁺CD8⁻ double negative; DP, CD4⁺CD8⁺ double positive
involves a burst of proliferation and the upregulation of both CD4 and CD8 coreceptors to become CD4\(^+\)CD8\(^+\) double positive (DP) thymocytes (18).

DP thymocytes are programmed to rearrange the TCR\(\alpha\) gene to form TCR\(\alpha\)\(\beta\) heterodimers (19). The clonotypic TCR is then tested for its ability to bind to MHC Class I or Class II complexed to self-peptide and presented on thymic stromal cells (5). DP thymocytes bearing TCRs that are unable to interact with MHC proteins will be useless during an immune response, and those cells are presumed to die from neglect, or the lack of survival signals induced by TCR engagement. However, if the avidity of the TCR for MHC-self-peptide is too high, the cells are potentially autoreactive and are deleted by apoptosis in a process called negative selection (5). Cells with TCRs that are able to bind to MHC-self-peptide, but not too strongly, are positively selected and progress to the CD4\(^+\) or CD8\(^+\) single positive (SP) thymocyte stage (5).

Positively-selected DP thymocytes must also shut off expression of one of the coreceptors and make the decision to become a CD4\(^+\) helper T cell or a CD8\(^+\) cytotoxic T cell (9, 20). CD4\(^+\) helper T cells promote immune responses by secreting effector cytokines that enhance humoral immune responses. CD8\(^+\) cytotoxic T cells are important for cellular immunity, as they are responsible for recognizing and killing virally infected or cancerous cells. The outcome of lineage commitment is determined by the specificity of the TCR for MHC Class I or Class II. T cells expressing TCRs that are MHC Class I-restricted will become CD8\(^+\), and cells with MHC Class II-restricted receptors become CD4\(^+\).

After commitment to the CD4 or CD8 lineage, naive T cells migrate out of the thymus and reside in the peripheral lymphoid tissues. Engagement of TCR by peptide antigen complexed to MHC triggers a signaling cascade that induces proliferation and cytokine production (21), and in the case of CD8\(^+\) T cells, cytotoxic effector ability (22). Costimulatory signals through receptors such as CD28 (23), or adhesion receptors (24) also provide necessary signals for full differentiation to an effector T cell, as TCR activation in the absence of costimulation may lead to anergy, or unresponsiveness (25).

Upon activation, CD4\(^+\) helper T cells choose between two developmental programs, referred to as type 1 (Th1) and type 2 (Th2) (7). These subsets are primarily defined by the cytokines they secrete, and they are important for diversification of the T
helper cell response against different pathogens. Type 1 T helper cells secrete predominantly IFN-\(\gamma\), which promotes cell-mediated immunity and the inflammatory response to intracellular pathogens such as *Leishmania major* (26). Th1 responses have also been implicated in certain types of autoimmune diseases (27). Type 2 T helper cells secrete IL-4, IL-5, IL-10, and IL-13, which promote antibody responses. Th2 responses are essential for successful clearance of extracellular pathogens such as the nematode *Nippostrongylus brasiliensis* (28), and also play a role in allergic diseases (27). Although several factors have been shown to influence the polarization of naive CD4\(^+\) T cells, the cytokine milieu in which T cells are activated is the most important determinant of T helper cell differentiation (8).

After clearance of an antigen, the immune response must be brought to an end, and the majority of effector T cells cleared from the animal. Many effector T cells will die from activation-induced cell death or by the withdrawal of survival signals (29). However, some subset of effector cells manage to survive and differentiate into memory T cells (10-12). Memory T cells provide long-lived, antigen-specific protective immunity, an important feature of the adaptive immune response.

At each stage of T cell development, signals through the TCR are essential for determining the fate of the cell. The cell's response to TCR signals depends on the differentiation state of the cell, but it can also be modified by other signals, such as cytokines or costimulation. A major goal in understanding T cell development is in understanding how TCR signaling in different contexts can lead to such different outcomes.

**T cell signaling during development**

*The TCR signaling complex.* Some of the major signaling pathways activated by TCR engagement are diagrammed in Figure 2. The basic components of the proximal TCR signaling complex are the same throughout development of the T cell. As the \(\alpha\beta\) TCR does not have enzymatic activity of its own, it relies upon the signaling abilities of the associated CD3\(\gamma\), \(\delta\), \(\epsilon\), and \(\zeta\) molecules (21, 30). The CD3 proteins all have immunoreceptor tyrosine-based activation motifs (ITAMs), which are phosphorylated
FIGURE 2. Signaling cascades induced upon T cell receptor engagement. Not all molecules involved in TCR signaling are shown.
by the src family protein tyrosine kinase Lck upon engagement of the TCR (31). The
ZAP-70 protein tyrosine kinase is then recruited to the CD3 ITAMs through its SH2
domain, and is activated by phosphorylation by Lck (32-34). An important substrate of
ZAP-70 is the transmembrane adaptor protein LAT (35). LAT localizes preferentially to
special membrane structures called lipid rafts (36), which are thought to be involved in
the clustering of signaling molecules in order to facilitate signaling (37). LAT may serve
as a scaffold on which downstream signaling molecules can efficiently associate after
TCR engagement, and its localization to lipid rafts is required for its function (36). It is
an essential link between the TCR and downstream signaling cascades, as most TCR-
mediated signaling events after ZAP-70 phosphorylation are blocked in the absence of
LAT (35, 38, 39).

One of the signaling cascades activated downstream of LAT is triggered by is
phospholipase Cγ1 (PLCγ1). PLCγ1 catalyzes the breakdown of phosphatidylinositol
4,5-biphosphate into diacylglycerol (DAG) and inositol phosphates (IP₃) (40). DAG
activates protein kinase C, while IP₃ induces Ca²⁺ release from intracellular stores,
resulting in activation of the Ca²⁺-dependent Ser/Thr phosphatase calcineurin (41-43).
Calcineurin’s importance in T cell receptor signaling was recognized when it was
identified as the target of the immunosuppressive drugs cyclosporine A and FK506 (44-
51). The best-known substrates of calcineurin are the NF-AT family of transcription
factors (52), which are an important link between TCR signaling and changes in gene
expression. In mature T cells, NF-AT transcription factors are essential for transcription
of many cytokine genes, including IL-2, IL-4, and IFN-γ (53).

However, it is not well understood how calcineurin activity is downmodulated
after TCR stimulation. Although the exogenous small molecules CsA and FK506 have
long been known to potently inhibit calcineurin phosphatase activity, only recently have
endogenous protein inhibitors been identified. AKAP79 (54, 55), DSCR1 (54, 56, 57),
and Cabin1/Cain (58-63) all inhibit calcineurin activity in transfection assays in the
presence of calcium signal. The interaction between Cabin1 and calcineurin is dependent
on both calcium signaling and protein kinase C (PKC) activation, which results in Cabin1
hyperphosphorylation (62). As Cabin1 is found primarily in the nucleus in T cells, it may
interact only with activated calcineurin that has translocated into the nucleus. Based on
these results, Cabin1 was hypothesized to function in down-modulating calcineurin activity during T cell activation.

Other important pathways induced by the TCR are the MAP kinase signaling cascades. LAT, through adaptor proteins such as Grb2 (35), associates with Sos, a Ras guanine-nucleotide exchange factor. Recruitment of Sos to LAT results in Ras activation. Ras is coupled to multiple downstream effectors, including the Ser/Thr kinase Raf-1, which can activate the MEK/ERK pathway. In mature T cells, activation of Ras is essential for activation of transcription factors involved in cytokine gene induction (64).

**Positive and negative thymocyte selection.** It is in the thymus that T cells acquire their self-MHC restriction (65, 66), and autoreactive cells are deleted (67, 68). After TCRα rearrangement in DP thymocytes, each TCR is subjected to the processes of positive and negative selection by self-peptide/MHC complexes on thymic stromal cells. The prevailing model for thymocyte selection is the avidity model, in which the outcome of selection is determined by the strength of the TCR-peptide-MHC interaction. However, it is not clear how signals through the same receptor can lead to such different outcomes for the DP thymocyte: apoptosis, in the case of negative selection, or survival and progression to either the CD4+ or CD8+ single positive (SP) thymocyte stage, in the case of positive selection.

Studying the role of TCR-associated signaling molecules in positive and negative selection is difficult to do with gene-targeted mice, as many of the molecules are also required for β-selection and progression from the DN to DP thymocyte stage. However, the use of dominant negative transgenes and pharmacological inhibitors has proved to be informative.

Most mutations in proximal TCR signaling molecules inhibit both positive and negative selection, confirming the requirement for TCR signaling for both events. Expression of a dominant negative lck (69), or deficiency in ZAP-70 in DP thymocytes (70) results in impaired thymocyte selection. Interestingly, mutations in CD3ζ which reduce but do not ablate TCR signaling can shift a transgenic TCR that is normally negatively selected into a positively selected TCR (71, 72), illustrating how the level of TCR signal can influence thymocyte selection.
Signaling pathways downstream of the TCR, however, may be differentially involved in positive versus negative selection. Based on experiments in which dominant negative forms of Ras (73), Raf-1 (74), or MEK-1 (75) were expressed in DP thymocytes, the MAPK pathway was shown to be required for positive, but not negative selection. Expression of an activated Raf-1 resulted in enhanced positive, but not negative selection (74), confirming the importance of the MAPK pathway at this stage of thymocyte development. However, it is difficult to know whether all of the endogenous molecules were outcompeted by the dominant negative transgenes in these experiments, casting some doubt on the conclusion that the MAPK pathway is dispensable for negative selection.

Inhibition of calcineurin in thymocytes by treatment with CsA was shown to block positive selection both in vivo and in vitro (76-80). However, the role of calcineurin in negative selection is less clear. Some studies showed that negative selection could be blocked by CsA also (77-79, 81, 82), but in others there was no effect observed (76, 80, 83). As in the experiments with MAPK inhibitors, it is difficult to know whether the doses of CsA used in the different experiments were sufficient to suppress calcineurin activity completely. In addition, there is evidence for an increased calcium flux in cells undergoing negative selection, compared with positively selected cells (84), which may indicate that a higher dose of CsA is required to inhibit negative selection. However, overexpression of an activated calcineurin transgene in DP thymocytes does enhance positive, but not negative selection (85), supporting the idea that calcineurin signaling is uniquely involved in positive selection.

Little is understood about how negatively selecting signals cause thymocyte apoptosis. Fas/FasL interactions, which are essential for peripheral deletion of autoreactive T cells, do not seem to be required for thymocyte apoptosis (86, 87). However, the Nur77 zinc finger transcription factor is known to be important for thymocyte apoptosis (88). When overexpressed in transgenic mice, Nur77 results in massive thymocyte apoptosis (89). Nur77's target genes are not known, and little is understood about the signals that induce Nur77 expression. However, the MEF2 family of transcription factors has been shown to transactivate the Nur77 promoter (90, 91). MEF2 transactivation is regulated by calcium signaling through CaMKIV (92-94),
Cabin1 (90, 91), and possibly through calcineurin (92, 95, 96), pointing to a potential role for calcium signaling in negative selection.

**Thymocyte lineage commitment.** During lineage commitment, the specificity of TCR for MHC Class I or Class II is matched with the appropriate coreceptor expression, and differentiation into functionally different CD4⁺ and CD8⁺ T cell occurs. Transgenic expression of TCRs with known MHC restriction leads to preferential development of the appropriate lineage, showing that TCR binding to MHC is important in determining lineage commitment (97-103). In addition, MHC Class I⁻ mice have severely reduced numbers of CD8⁺ T cells (104, 105), and MHC Class II⁻ mice have reduced numbers of CD4⁺ T cells (106, 107). As the CD4 and CD8 coreceptors bind selectively to MHC Class II and Class I, respectively, it was suggested that they may be responsible for differential signaling events that determine the outcome of lineage commitment.

A good candidate for mediating differential coreceptor signaling is the src family kinase lck. The cytoplasmic tail of CD4 associates with lck with a higher avidity than the cytoplasmic tail of CD8 (108, 109), so it was suggested that increased recruitment of lck to the TCR signaling complex by CD4 promotes CD4 development. This model was supported by studies showing that altering the activity of lck in DP thymocytes could influence lineage commitment, with increased lck activity promoting CD4 development, and a dominant negative lck promoting CD8 development (110-112).

There is also evidence that TCR signaling through MAPK pathways influences lineage commitment. Inhibitors of MAPK pathways can inhibit CD4⁺ T cell commitment and enhance CD8⁺ T cell commitment (111, 113, 114), while activated MEK-1 or ERK-2 can promote CD4⁺ T cell development (111, 115). Based on these results and the data regarding lck signaling, a strength of signal model was proposed in which lck and TCR signaling together determine the outcome of lineage commitment, with stronger signals promoting CD4⁺ development, and weaker signals allowing CD8 commitment.

However, a modification of the strength of signal model was put forward based on the observation that CD8⁺ commitment requires lineage-specific signals from MHC Class I, while CD4 commitment could occur in response to TCR signaling in the absence of CD4 coreceptor engagement by MHC Class II (116). This led to the assymmetric commitment model, which suggests that unique signals are required to promote CD8⁺
lineage commitment, and TCR signaling in the absence of these signals results in a
default commitment to the CD4+ lineage.

What might the CD8 lineage-specific signal be? One clue came from analysis of
mice deficient in the IRF-1 transcription factor. These mice are deficient in CD8+ SP
thymocyte development, although CD4+ lineage commitment appears unaffected (117).
In addition, transgenic overexpression of an activated Notch-1 protein results in increased
commitment to the CD8+ lineage (118), as does transgenic expression of bcl-2 in
thymocytes (119). However, it is not known how these proteins may be enhancing CD8+
lineage commitment.

**Naive T cell survival.** Positively selected thymocytes emigrate to the peripheral
lymphoid tissues where they reside as naive T cells. Maintenance of the peripheral pool
of naive T cells is subject to homeostatic control, but the nature of the signals involved is
not known. It is clear, however, that naive T cells require TCR engagement with
restricting MHC in the periphery in order to survive (120-125). The signal triggered by
this interaction, and how it differs from antigen activation is not known. Some evidence
suggests that a basal level of CD3ζ ITAM phosphorylation and association of ZAP-70
protein is maintained by the interaction (125), but surprisingly, lck was not necessary for
maintenance of the CD3ζ phosphorylation or survival of naive cells (126).

**T cell activation by antigen** Engagement of TCR by peptide-MHC on APCs such
as dendritic cells results in a cascade of signals (as described in an earlier section) that
trigger proliferation, cytokine production, and differentiation of a naive T cell into an
effector cell. The specificity of the TCR for the antigen determines whether the T cell
maintains contact with the APC, but a host of other membrane receptors, such as CD28,
CD2, and integrins can alter TCR signaling by promoting close contact with the APC,
and by modulating various TCR-triggered signaling events.

Initial contact of a T cell with an APC probably requires contact through CD28
and adhesion receptors. This gives the TCR a chance to “scan” the antigen presented by
the APC. If the TCR recognizes antigen and sends a signal, a reorganization of the actin
cytoskeleton occurs which results in widening of the T cell-APC contact zone, and the
formation of what is termed the “immunological synapse,” a clustering of membrane
receptors and signaling molecules at the point of contact with APC (127, 128). This is presumed to be required for sustained TCR signaling and full activation of the T cell.

TCR engagement without costimulation through a receptor such as CD28 can result in unresponsiveness (129, 130). B7-1 and B7-2, the ligands for CD28, are only expressed by activated APCs (131), which ensures that the T cell is activated in the context of a pathogen that has already been recognized by the innate immune system. Signals from CD28 can amplify TCR signals, enhancing IL-2 and IL-2R expression, for example, but also activate separate signaling pathways which may promote T cell survival (131).

**T helper cell development**  Upon activation with antigen, CD4⁺ T cells can differentiate into either Th1 or Th2 effector cells, which secrete different patterns of effector cytokines that promote distinct types of immune response (7, 8). The polarization of the T helper cell response can have a profound effect on the outcome of an immune response. Although the strength of the signal through the TCR has been proposed to influence this developmental decision (132), the most important factor in determining the pathway the helper T cell takes is the cytokine environment it finds itself in at the time of activation. Figure 3 outlines the major cytokine receptor signaling pathways that influence T helper cell differentiation.

The most important cytokine for induction of Th1 development is IL-12 (133, 134), produced by macrophages and some dendritic cells. Signaling through the IL-12 receptor induces STAT4 activation (135), which is required for Th1 development and for high levels of IFN-γ production in T cells (136, 137). Th2 development is promoted primarily by IL-4 (138, 139). Signaling through the IL-4 receptor induces STAT6 activation (140, 141), which is required for Th2 development and IL-4 production by Th2 cells (142). However, the initial source of IL-4 during an immune response is unclear. As they produce large amounts of IL-4 upon stimulation, NK1⁺ T cells were considered good candidates for the source of early IL-4 (143), but analysis of Th2 responses in mice lacking NK1⁺ T cells showed that they are not essential (144). Mast cells, eosinophils, and basophils are all capable of producing IL-4 (145), but it may be the IL-4 produced by the CD4⁺ T cells themselves that is most important (146).
FIGURE 3. Cytokine receptor signaling pathways involved in T helper cell differentiation.

An interesting feature of T helper cell development is that the cytokines produced by each Th subset tend to both stimulate production of that Th subset, and inhibit development of the other Th subset. IFN-γ produced by Th1 cells has the effect of both stimulating Th1 development and inhibiting Th2 development, while Th2-secreted IL-4 has the opposite effect. Fully differentiated Th2 cells become refractory to subsequent Th1-polarization by downregulation of IL-12Rβ2 expression so that they are no longer
able to respond to Th1-inducing signals (147, 148). STAT6-dependent induction of the transcription factor GATA-3 is important for this commitment (149). GATA-3 is selectively expressed in Th2 cells, and its ectopic expression in Th1-committed cells is sufficient to activate Th2-specific cytokines (150, 151) and suppress IFN-γ production (152, 153). Th1 cells normally downregulate GATA-3 in response to IL-12 signaling (153). The Th1-specific transcription factor T-bet, which strongly induces IFN-γ transcription, may also be involved in the suppression of GATA-3 and other Th2 programs in committed Th1 cells (154).

Several other factors have been observed to influence T helper cell development. Contact with different dendritic cell subsets has been found to affect the polarization of naive T cells (155, 156). Costimulation through CD28 and related receptors is supposed to enhance Th2 development (157-159), perhaps by enhancing IL-4 production early in the response (160, 161). In addition, there is evidence to suggest that the strength of antigen stimulation through the TCR can affect polarization, with stronger stimuli promoting Th1 cell development (132). This is consistent with data suggesting that inhibitors of the p38 MAPK pathway inhibit Th1 development, while MAPK activators enhance Th1 development, perhaps through induction of IFN-γ production (162). JNK1- and JNK2-deficient mice also have either defects in Th1 development or an enhancement of Th2 development (163, 164).

Ca²⁺ signaling plays a role in T helper cell development and function as well. There is evidence that Ca²⁺ flux may be different between Th1 and Th2 cells (165, 166), with Th1 cells able to produce strong, transient Ca²⁺ signals after TCR engagement. TCR engagement of Th2 cells, however, induces a reduced Ca²⁺ response. The differential Ca²⁺ signaling in the Th subsets may result in differential activation of Th1 or Th2 cytokine gene transcription. The importance of Ca²⁺ signaling for Th2, but not Th1 development is demonstrated by the effects on IL-4 production in mice with mutations in NF-ATc or NF-ATp transcription factors (167-171). Itk⁺ T cells, which are unable to sustain a Ca²⁺ flux after TCR engagement, are unable to produce IL-4 or develop into Th2 cells, but have no defects in IFN-γ production or Th1 cell development (172).

**Memory T cell development and activation** During an immune response, extensive clonal expansion of antigen-specific effector T cells occurs. After antigen
clearance, however, they must be cleared from the body to make space for other T cells and to prevent them from causing damage to the host. Effector T cells become susceptible to Fas-mediated apoptosis within a few days of activation (29). Most are deleted by Fas-mediated apoptosis, or by the withdrawal of cytokine-induced survival signals. However, some are able to escape this deletion and differentiate into long-lived memory T cells. What signals, if any, are required for this transition are unknown.

However memory T cells arise, their existence is a remarkable aspect of the adaptive immune response. Memory T cells provide long-lived, antigen-specific protective immunity (10-12). Upon rechallenge with an antigen, memory responses are faster and more effective than primary responses. This may in part be due to a higher frequency of antigen-specific precursor cells able to respond. However, memory T cells may also be more sensitive to triggering through the TCR than naive T cells (173-176), and have reduced costimulation requirements (177). In addition, memory T cells are able to produce a more diverse set of cytokines rapidly upon restimulation (10, 178), which may allow them to perform some functions that naive T cells cannot.

In this thesis, I have examined the roles of two proteins, Cabin1 and Left1, in T cell development.

Chapter Two describes the analysis of Cabin1ΔC mice, which produce a truncated Cabin1 protein lacking the C-terminal calcineurin and MEF2 binding domains. The role of the C-terminal domain in regulation of thymocyte selection, T cell activation, and T helper cell development and function were examined.

Chapter Three describes the identification of Left1, a gene found to be preferentially expressed in memory CD8+ T cells, and in type 1, but not type 2 helper T cells. The effect of overexpression of Left1 in T cells in transgenic mice was examined, and Left1 was found to influence T cell development at several stages, including CD4/CD8 thymocyte lineage commitment, T cell activation, memory T cell differentiation, and T helper cell development.
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Chapter Two

Deletion of Calcineurin Binding Domain of Cabin1 Results in
Enhanced Cytokine Gene Expression in T Cells and
Elevated Th2 Antibody Response in Mice

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Introduction

Calcineurin is a calcium and calmodulin-dependent serine/threonine phosphatase (1-3). It is ubiquitously expressed, but found most abundantly in the brain. Its essential role in T cell receptor (TCR)-mediated signal transduction was first recognized when it was identified as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506 (4-11). The primary substrates of activated calcineurin were identified as the NF-AT family of transcription factors (12, 13). Calcineurin directly dephosphorylates NF-AT proteins in the cytosol, resulting in their translocation into the nucleus. Calcineurin remains associated with NF-AT in the nucleus, where its continued activation by elevated calcium levels is required to sustain NF-AT transcriptional activity (14, 15). NF-AT acts in cooperation with other transcription factors such as AP-1 to stimulate the transcription of many genes important for T cell activation, including several cytokine genes such as IL-2, IL-4, and IFN-γ.

As calcineurin is essential for transmitting cytosolic calcium signals into the nucleus in T cells, its activation is tightly controlled. Calcineurin is activated by intracellular calcium and calmodulin (16). In resting T cells, the level of intracellular calcium is low and the C-terminal autoinhibitory domain binds the catalytic domain and blocks calcineurin enzymatic activity. Upon stimulation through TCR, the level of intracellular calcium is elevated and calmodulin becomes activated. Activated calmodulin then binds to calcineurin at a site located between the catalytic and the autoinhibitory domains, thus disrupting their interaction and activating calcineurin’s phosphatase activity. It is not well understood how calcineurin activity is downmodulated under physiological conditions. Although the exogenous small molecules CsA and FK506 have long been known to potently inhibit calcineurin phosphatase activity, only recently have endogenous protein inhibitors been identified. AKAP79 (17, 18), DSCR1 (18-20), and Cabin1/Cain (21-26) all inhibit calcineurin activity in transfection assays in the presence of calcium signal.

Murine Cabin1 (and its rat homolog Cain) was identified in a yeast two-hybrid screen for calcineurin-interacting proteins. Cabin1 is a ubiquitously expressed protein of 2220 amino acids. The interacting domain in Cabin1 was mapped to 23 amino acid
residues (2117-2140) in the C-terminus (23). When the full length of Cabin1 or the C-terminal region was overexpressed in Jurkat T cells, it inhibited the transcriptional activation of calcineurin-responsive elements in the IL-2 promoter and blocked dephosphorylation of NF-AT upon T cell activation. The interaction between Cabin1 and calcineurin is dependent on both calcium signal and protein kinase C (PKC) activation, which results in Cabin1 hyperphosphorylation (23). As Cabin1 is found primarily in the nucleus in T cells, it may interact only with activated calcineurin that has translocated into the nucleus. Based on these results, Cabin1 was hypothesized to function in down modulating calcineurin activity during T cell activation.

In addition to binding to calcineurin, the C-terminal region of Cabin1 was also shown to interact with myocyte enhancer factor 2 (MEF2) and calmodulin in a mutually exclusive manner (27, 28). MEF2 is a transcription factor and is normally bound to its recognition sequence in the promoter of target genes such as the apoptosis-inducing gene Nur77 in thymocytes (27, 29). Binding of Cabin1 to MEF2 suppresses MEF2 transcriptional activity through recruitment of the mSin3 corepressor complex via the N-terminal region of Cabin1 (27). However, in the presence of a calcium signal, calmodulin binds to Cabin1, freeing MEF2 to recruit the coactivator p300 for transcriptional activation of MEF2 target genes. Consistent with this model, overexpression of Cabin1 in a DO11.10 T cell hybridoma prevents induction of Nur77 by MEF2 and protects the cells from TCR-mediated apoptosis (28). As MEF2 is critical for the transcription of Nur77 in thymocytes, Cabin1 may also regulate thymocyte apoptosis during early T cell development.

To examine the role of the calcineurin- and MEF2-binding domains of Cabin1 in T cell development and function under physiological conditions, we generated Cabin1ΔC mice, which produce a truncated Cabin1 lacking the last 123 amino acid residues from the C-terminus. Contrary to expectation, we found that TCR-mediated thymocyte apoptosis was unaffected by the Cabin1ΔC mutation, indicating that the interaction between Cabin1 and MEF2 is not essential for regulating thymocyte apoptosis in vivo. However, cytokine gene expression in activated T cells from Cabin1ΔC mice was enhanced and Cabin1ΔC mice had an enhanced Th2-dependent antibody response. These results demonstrate that Cabin1 normally downmodulates T cell effector functions.
Materials and Methods

Targeting construct and generation of Cabin1ΔC mice  A cDNA fragment encoding the C-terminal calcineurin binding domain of Cabin1 was used to isolate Cabin1 genomic clones from a 129/SvJ library. Sequencing of genomic clones identified the last three exons of Cabin1, 3’ untranslated sequences and polyadenylation site (Fig. 1A). The targeting vector contained 2.5 kb of 5’ homologous sequence, a loxP-flanked, pgk promoter-driven neomycin’ (neo’) cassette, 4.5 kb of 3’ homologous sequence, and the thymidine kinase gene. Homologous recombination in embryonic stem cells should result in the replacement of a 0.7 kb EcoRI-BamHI fragment with the neo’ cassette. The linearized vector was transfected into J1 ES cells by electroporation and transfectants were selected with G418 and gancyclovir. Resistant clones were screened for homologous recombinants by Southern blot with various probes (data not shown). Fifteen homologous recombinant ES clones were obtained and two were injected into blastocysts from C57Bl/6 mice. Chimeras produced from both clones transmitted the mutant allele to their offspring. Chimeras were bred with Cre deleter mice (30) to remove the neo’ cassette by Cre-loxP recombination, and heterozygous offspring were interbred to produce homozygous mutant mice (Cabin1ΔC). Offspring were genotyped by either Southern blot analysis of tail DNA or PCR assays using primers 5’-caatgtgtggacagcctggacccaggc and 5’-gcagctccccaggctgccaggtcc. Mice were housed in specific pathogen-free facilities.

Western blot analysis  The thymus and brain from wildtype and Cabin1ΔC mice were lysed in lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40, and protease inhibitor cocktail (Roche Molecular Biochemical) by Dounce homogenizer. Lysate containing 750μg of proteins was immunoprecipitated with anti-Cabin1 polyclonal antibodies that specifically recognize the C-terminal 77 amino acid residues of Cabin1. Lysate and antibodies were incubated for 2 hrs at 4°C in the presence of protein A/G Sepharose beads and precipitates were washed three times with lysis buffer. Proteins in precipitates were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-Cabin1 polyclonal antibodies.

For detection of Nur77, protein concentration of thymocyte lysates was measured
by BCA protein assay (Pierce), and equal amounts of wildtype and Cabin1ΔC lysate were immunoprecipitated with anti-Nur77 antibody (Santa Cruz Biotechnology). Western blotting was carried out as above except anti-Nur77 antibody was used.

For detection of NF-ATc translocation, nuclear extracts were isolated from T cells as described (31). Protein concentration of the extracts was measured by BCA protein assay, and 50μg of each nuclear extract was resolved by SDS-PAGE, transferred to PVDF membrane, and probed with anti-NF-ATc antibody (7A6, Pharmingen).

**Electrophoretic Mobility Shift Assay** Nuclear extracts were isolated as described (31). Binding reactions were done using 8μg of nuclear protein in the presence of 5 x 10⁴ cpm of the specific ³²P-end-labeled single-stranded oligonucleotide probe. A 100-fold excess of cold competitor oligonucleotide was added to some reactions. Reactions were separated by PAGE on a 4% gel, and quantitation of ³²P was done by exposure to a phosphorimager. The probe was an NF-ATc consensus binding site: 5’-cgcctaaagagggaaattgtttcata-3’ (Santa Cruz Biotechnology).

**Flow Cytometric Analysis** Single-cell suspensions of spleen, thymus, or lymph node were prepared. Erythrocytes were removed from splenocyte suspensions by lysis with 0.14 M NH₄Cl and 17 mM Tris-HCl, pH 7.4. Cells were incubated on ice with FITC-, APC-, or PE-conjugated antibodies (PharMingen) and analyzed on a FACScalibur (Becton Dickenson) with Cell Quest software.

**Thymocyte apoptosis** Thymocytes were plated at 1.5 x 10⁶ cells/ml on plates precoated with 50μg/ml anti-CD3 (2C11) in RPMI medium supplemented with 10% fetal bovine serum (FBS), 10mM HEPES, 50μM 2-mercaptoethanol, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Annexin V-FITC (PharMingen) binding assay was performed according to manufacturer’s instructions.

**Analysis of T cell activation** For T cell proliferation and cytokine secretion assays, 2.5 x 10⁵ lymph node cells were incubated in 0.2 ml RPMI medium plus supplements in a 96-well plate. The cells were stimulated with 10μg/ml plate-bound anti-CD3 antibody (2C11), or 5 ng/ml PMA and 250 ng/ml ionomycin. Cyclosporin A was added at 2μM. Supernatants were collected for cytokine assays 48 hrs after stimulation. Some cultures were pulsed with [³H]-thymidine at 48 hrs and harvested 18 hours later.
**ELISA assays for cytokine and serum antibodies** IL-2 and IFN-γ in culture supernatants were assayed using commercial kits from Biosource and R & D Systems, respectively, according to manufacturer’s instruction. Serum Ig levels of unimmunized mice were measured by coating plates with anti-IgH + IgL antibodies (Southern Biotechnology Associates), and developing with horseradish peroxidase (HRP)-coupled goat antibodies specific for each mouse Ig isotype. ELISA for IgE was performed by coating the plates with anti-IgE antibody (PharMingen) and developing with HRP-conjugated anti-IgE antibody (PharMingen). NP-specific antibody in serum from immunized mice was measured by coating plates with NP-BSA and developing with HRP-coupled goat antibodies specific for each mouse Ig isotype.

**RNase Protection Assay** Total RNA was isolated from freshly isolated T cells or anti-CD3 stimulated T cells (Ultraspec). The levels of cytokine transcripts were measured by RNase protection assay using the Riboquant multiprobe kit (PharMingen) following the manufacturer’s instructions. After overnight hybridization of target RNA with 32P-labeled RNA probe, the protected mRNAs were purified and resolved on a 6% denaturing polyacrylamide gel. Cytokine transcript levels were quantified on a phosphorimager (Molecular Dynamics) using ImageQuant software.

**Immunoglobulin class switching assay** For B cell class switching assays, 5 x 10^5 splenocytes were cultured in 2ml RPMI medium plus supplements in 24-well plate. B cells were stimulated with 10μg/ml lipopolysaccharide (LPS) or with 10μg/ml plate-bound anti-CD40 (PharMingen) in the presence or absence of 50ng/ml IL-4 (R & D Systems). After five days, the cells were stained with antibodies to B220 (PharMingen) and IgG1 (Southern Biotechnology Associates) and analyzed by flow cytometry.

**Immunization** Eight- to twelve-week old mice were immunized with the T-dependent antigen (4-hydroxyl-3-nitrophenyl) acetyl-conjugated keyhole limpet hemocyanin (NP-KLH). 20μg of NP-KLH in 0.2 ml PBS were injected intravenously through the tail vein on day 0 and the mice were bled 14 days later to measure the NP-specific antibody titer in the serum.
Results

**Cabin1ΔC mice** Cabin1 is a large and widely expressed protein that probably has multiple functions in different cell types. We reasoned that deleting the entire protein in mice would be likely to result in severe and pleiotropic effects, making it difficult to assess the importance of Cabin1’s interactions with calcineurin and MEF2 in T cell development and function. Therefore, we targeted only the last two exons of Cabin1, which encode the C-terminal calcineurin, calmodulin, and MEF2 binding domains. As shown in Figure 1A, a 0.7 kb EcoRI-BamHI fragment containing the last two coding exons and part of the 3' untranslated region was replaced by a neo' cassette by homologous recombination in ES cells. Chimeras derived from the targeted ES cell clones were bred with Cre deleter mice (30) to remove the loxP-flanked neo' cassette, and heterozygous mice were interbred to produce Cabin1ΔC mice homozygous for the Cabin1 truncation (Fig. 1A and 1B). In the absence of the neo' cassette, transcription of the truncated allele is expected to go through the downstream intron, into the remaining 3' untranslated region, and terminate at the normal polyadenylation site. Translation of the mutant transcript would continue into the intron until it reaches an in-frame stop codon after 11 nucleotides, producing a truncated Cabin1 protein with 4 additional amino acid residues at the C-terminus (Fig. 1A).

Homozygous Cabin1ΔC mice were born at the expected Mendelian ratio, were healthy, and reproduced normally. Northern blotting using a cDNA probe that hybridized to a region of Cabin1 upstream of the deleted region revealed a Cabin1 transcript at the expected size in brain and thymus of Cabin1ΔC mice, although at a two-fold reduced level (Fig. 1D). Amplification of the mutant transcripts by RT-PCR and cloning and sequencing of the products confirmed that the transcripts have the expected 3' sequence (data not shown). A Western blot analysis using antibodies specific for the C-terminal domain of Cabin1 readily detected Cabin1 in brain and thymus extracts of wildtype but not Cabin1ΔC mice, demonstrating the expected deletion in the mutant mice (Fig. 1C). However, identification of the truncated Cabin1 protein by Western blotting in Cabin1ΔC mice was hampered by the lack of a good antibody specific for the N-terminus of Cabin1 despite our repeated attempts to generate such an antibody.
In targeting the Cabin1 locus, we inserted the neo' cassette in the opposite transcriptional orientation of the Cabin1 gene. As the neo' cassette is known to have a disruptive effect on gene expression (32), we hypothesized that the insertion could cause a more severe disruption of Cabin1 transcription, resulting in a null allele. Indeed, when heterozygous Cabin1-neo mice were interbred, no homozygous pups were obtained in over 200 offspring genotyped. Examination of embryos after timed matings revealed that homozygous Cabin1-neo mice died in utero around embryonic day 12.5. Although we cannot rule out the possibility that the embryonic lethality is caused by interference of the neo' cassette with the expression of a neighboring gene, Cabin1 is ubiquitously expressed and has no known homologs, and a Cabin1 null allele is likely to cause embryonic lethality. The dramatic difference between the phenotype of Cabin1ΔC and Cabin1-neo mice supports our assertion that Cabin1ΔC mice produce a truncated protein, while Cabin1-neo mice have a null allele.

**Normal T and B cell development in Cabin1ΔC mice** To determine the effect of the Cabin1ΔC mutation on lymphocyte development, the cellularity and surface phenotype of cells from various lymphoid tissues were examined. The numbers of thymocytes in Cabin1ΔC mice were similar to those in wildtype littermates at various ages examined (Fig. 2). Distribution of CD4+CD8+ (double negative, DN), CD4+CD8- (double positive, DP), and CD4+, or CD8+ (single positive, SP) thymocytes was also similar between the wildtype and Cabin1ΔC mice (Fig. 2). Within the DN thymocyte population, similar proportions of cells were found to express CD25 and/or CD44 in the wildtype and Cabin1ΔC mice (data not shown). In DP thymocytes, surface expression of CD69 is transiently upregulated when the cells undergo positive selection (33) and the differentiation from DP to SP is associated with upregulation of TCRβ and downregulation of heat stable antigen (HSA). No difference was detected in TCRβ and HSA expression and in percentages of CD69+ cells in DP thymocytes between the wildtype and Cabin1ΔC mice (data not shown).

The total numbers of T cells and the ratio of CD4+ and CD8+ T cells in lymph nodes and spleen did not differ significantly between wildtype and Cabin1ΔC mice (Fig. 2). There was no increase in the proportion of T cells that expressed the activation markers CD69 and CD25 in Cabin1ΔC mice as compared to wildtype mice (data not
shown), indicating that the Cabin1ΔC mutation does not cause spontaneous activation of T cells. We observed no gross defects in B cell development in the bone marrow (data not shown), and there was a normal proportion of B220+ and IgM+ B cells in the spleen of Cabin1ΔC mice (Fig. 2). Together, these results demonstrate that deletion of the C-terminal domain of Cabin1 does not apparently affect T and B cell development.

**Normal thymocyte apoptosis in Cabin1ΔC mice** To test whether the Cabin1ΔC mutation has an effect on TCR-mediated apoptosis of thymocytes, we treated thymocytes with plate-bound anti-CD3 antibody *in vitro*. As measured by propidium iodide (PI) uptake and Annexin V-binding, the number of dead and apoptotic cells in the Cabin1ΔC thymocyte cultures was similar to that of wildtype at 24 or 48 hrs after stimulation (Fig. 3A). Similarly, no significant difference in thymocyte apoptosis was observed between the two types of mice that were injected with anti-CD3 antibodies (data not shown). Analysis of Nur77 induction by immunoprecipitation and Western blotting showed that a similar level of Nur77 protein was induced in wildtype and Cabin1ΔC thymocytes after anti-CD3 treatment while no Nur77 protein was detected in untreated thymocytes (Fig. 3B). Thus, the C-terminal MEF2- and calcineurin-binding domain of Cabin1 is not essential for proper regulation of thymocyte apoptosis.

**Normal proliferation but enhanced cytokine production by Cabin1ΔC T cells**

Next we examined the effect of the Cabin1ΔC mutation on T cell activation. Lymph node T cells from Cabin1ΔC mice and wildtype controls were stimulated *in vitro* with plate-bound anti-CD3 antibody or PMA plus ionomycin. Upregulation of the activation markers CD69 and CD25 on T cells was monitored every day for three days, and no difference in the kinetics or the levels of induction was observed between the wildtype and Cabin1ΔC T cells (data not shown). Proliferation of the stimulated T cells was measured by [3H]-thymidine incorporation. As shown in Fig. 4A, Cabin1ΔC T cells proliferated to the same extent as wildtype T cells in response to either anti-CD3 or PMA plus ionomycin stimulation.

Culture supernatants of T cells were analyzed for the levels of IL-2 and IFN-γ by ELISA. After anti-CD3 stimulation, approximately 3 fold higher levels of both IL-2 and IFN-γ were detected in cultures of Cabin1ΔC T cells than that of wildtype T cells (Fig. 4B). However, after stimulation with more potent stimuli, such as anti-CD28 plus anti-
CD3 or PMA plus ionomycin, the levels of IL-2 and IFN-γ produced by Cabin1ΔC T cells were similar to those of wildtype T cells (Fig. 4B and data not shown). In the presence of CsA, no IL-2 or IFN-γ were detected in the supernatants of either Cabin1ΔC or wildtype cultures, indicating that the increased levels of cytokines observed depend on phosphatase activity of calcineurin.

To determine whether the increased levels of IL-2 and IFN-γ in the supernatants were a result of increased transcription of these genes in Cabin1ΔC T cells, we quantified cytokine transcripts in anti-CD3 stimulated T cells by RNase protection assay (Fig. 4C). The levels of cytokine transcripts in each sample were normalized to the level of transcript of ribosomal protein L32. Transcripts for IFN-γ, IL-2, IL-9, IL-13, and IL-4 were consistently found to be 2-3 fold more abundant in Cabin1ΔC T cells than in wildtype T cells at 48 hrs. The increase for some cytokine transcripts was already detectable at 24 hrs. These results clearly demonstrate that deletion of the C-terminal domain of Cabin1 results in an enhanced cytokine gene expression in T cells following TCR-mediated activation.

NF-AT translocation in Cabin1ΔC T cells Of the three NF-AT family members selectively expressed in T cells, NF-ATc (NF-ATc1, NF-AT2) has been shown to be responsible primarily for positive regulation of calcineurin-dependent cytokine transcription in mature T cells (34, 35). To investigate whether the increased cytokine expression in Cabin1ΔC T cells is mediated through increased activation of NF-AT by calcineurin, we examined NF-ATc translocation to the nucleus after stimulation of T cells with anti-CD3. No NF-ATc was detected by Western blotting in the nucleus of unstimulated T cells (Fig. 5). Following stimulation, however, several isoforms of NF-ATc were detected but no significant difference in the level was detected between wildtype and Cabin1ΔC T cells. By gel mobility shift assay for total nuclear NF-AT activity, no significant difference was detected between mutant and wildtype T cells at 4, 24 or 48 hrs (Fig. 6). Thus, the enhanced cytokine gene expression in Cabin1ΔC T cells does not appear to correlate with an increased nuclear NF-AT activity.

Enhanced IgG1, IgE, and IgG2b production in Cabin1ΔC mice The enhanced cytokine gene expression by activated T cells could result in an enhanced T-dependent antibody response in Cabin1ΔC mice. We assayed the levels of the various
immunoglobulin isotypes in the serum of 3-month old Cabin1ΔC and wildtype mice. As shown in Figure 7, the level of IgG1, IgG2b, and IgE was 3, 2, and 6 fold higher, respectively, in Cabin1ΔC mice than in wildtype mice. No significant difference was observed in the levels of IgM, IgG3 or IgG2a. We also measured the antibody response to a T-dependent antigen NP-KLH in Cabin1ΔC mice. Fourteen days after immunization, NP-specific IgG1 and IgG2b, but not IgM, IgG2a or IgG3, were significantly higher in the serum of Cabin1ΔC than wildtype mice (Fig. 8). Together with the data from unimmunized mice, these results show that T-dependent IgG1 and IgG2b antibody responses are selectively enhanced in Cabin1ΔC mice.

**Normal IgG1 class switching in Cabin1ΔC B cells** The enhanced IgG1 and IgG2b response in Cabin1ΔC mice could result from the increased cytokine production by T cells, or an alteration in B cells that favors class switching to IgG1, IgG2b and IgE. It is known that CD40 signaling in B cells is CsA-sensitive (36, 37) and that stimulation through CD40 strongly enhances IL-4 dependent class switching to IgG1 (38). Stimulation of B cells with LPS plus IL-4 also induces IgG1 class switching, but LPS signaling is not sensitive to CsA (37). To examine whether B cells from Cabin1ΔC mice are intrinsically more efficient in class switching, we compared IgG1 class switching between wildtype and mutant B cells in vitro under the same stimulation conditions. In the absence of IL-4, fewer than 1% of B cells from either wildtype or Cabin1ΔC mice expressed cell surface IgG1 after 5 days of stimulation with LPS or anti-CD40 (Fig. 9). In the presence of IL-4, approximately 15% and 35% of B cells expressed IgG1 after LPS and anti-CD40 stimulation, respectively. However, no difference was detected in the frequency of class switching between wildtype and mutant B cells, indicating that the elevated levels of IgG1 in the serum of Cabin1ΔC mice is not due to an alteration of B cell signaling that results in more efficient class switching.
Discussion

Studies in T cell hybridomas have shown that Cabin1 interacts with calcineurin and MEF2 and regulates their activity in various T cell processes. Cabin1/Cain has also been implicated in regulation of neurotransmitter endocytosis in neuronal cells (25), and muscle cell development (21, 22, 24). We have now examined the role of the C-terminus of Cabin1, encompassing the calcineurin- and MEF2-binding domain, in lymphocyte development and function under physiological conditions. Although the gross phenotype of the Cabin1ΔC mice gave no indication of a major defect in the development and function of muscle cells or the nervous system, we have yet to examine the effect of the mutation on these processes.

Based on previous studies in T cell lines, DP thymocytes from Cabin1ΔC mice were expected to be more susceptible to TCR-mediated apoptosis. Presumably, the truncated Cabin1 cannot bind MEF2 and recruit the mSin3 corepressor complex to inhibit MEF2 from activating the transcription of the pro-apoptotic gene Nur77. However, compared to wildtype thymocytes, Cabin1ΔC thymocytes were not more susceptible to apoptosis and were not induced to express a higher level of Nur77 after TCR stimulation. Thus, while overexpression of Cabin1 or its C-terminus in a T cell hybridoma can suppress TCR-mediated apoptosis, Cabin1's interaction with MEF2 is not uniquely required for the negative regulation of MEF2 in developing thymocytes. The lack of effect of Cabin1ΔC on thymocyte apoptosis is not surprising, however, in light of the recent reports of redundant MEF2 transcriptional corepressors including MEF-2 interacting transcription repressor (MTRR), histone deacetylases 4 and 5 (39-43). The inhibition of MEF2 by these corepressors is also calcium-dependent but does not require Cabin1 as an intermediary (40, 43). It is likely that the redundant MEF2 transcriptional corepressors can compensate for the Cabin1 C-terminal deletion.

In contrast to our observations in Cabin1ΔC thymocytes, anti-CD3 stimulation of mature T cells from Cabin1ΔC mice resulted in an enhanced transcription of several cytokine genes, including IL-2, IL-4, IL-9, IL-13 and IFN-γ. All of these cytokine genes are target genes of NF-AT transcription factors. The increased cytokine gene expression was inhibited by the presence of CsA, indicating the requirement for calcineurin activity.
Considering that overexpression of the C-terminal region of Cabin1 inhibits calcineurin activity in T cell lines, the enhanced cytokine gene transcription in Cabin1ΔC T cells seems likely to be mediated by increased calcineurin activity and associated NF-AT activities. These results are complementary to those observed in T cells deficient in both NF-ATc (NF-ATc1, NF-AT2) and NF-ATp (NF-ATc2, NF-AT1), in which production of many cytokines is reduced after stimulation through TCR (44). Although we did not detect any significant increase in nuclear NF-AT activity in mutant T cells as compared to wildtype T cells, the assays may not be sensitive enough to detect a relatively small difference, which may be sufficient to account for the observed 2-3 fold increase in cytokine gene expression. Consistently, the effect of the Cabin1ΔC mutation on cytokine gene expression was only detected under suboptimal stimulation conditions such as anti-CD3 treatment in the absence of costimulation through CD28. It is also possible that other transcription factors may be primarily responsible for the increased cytokine gene transcription either through calcineurin signaling or an unknown Cabin1-dependent pathway. Nevertheless, our observation that cytokine gene transcription is enhanced in Cabin1ΔC T cells after anti-CD3 stimulation demonstrates that Cabin1 normally downmodulates T cell effector function.

The Cabin1ΔC mutant mice have elevated levels of serum IgG1, IgG2b and IgE and produce higher levels of IgG1 and IgG2b in response to a T-dependent antigen NP-KLH. The enhanced antibody response is apparently not due to an intrinsic alteration of B cells in the mutant mice. Following anti-IgM or LPS stimulation in vitro, B cells from Cabin1ΔC and wildtype mice expressed similar levels of activation markers CD25 and proliferated to the same extent (data not shown). Furthermore, the proportion of mutant and wildtype B cells that switched to IgG1 in response to anti-CD40 and IL-4 stimulation was the same although stimulation of IgG1 class switch through CD40 in B cells is known to involve calcineurin (36, 37). The observed elevation in serum Ig levels in Cabin1ΔC mice is likely to be promoted by the increased levels of cytokine secreted by Cabin1ΔC T cells. It is also possible that cytokine production by non-T cells could be affected by the Cabin1ΔC mutation and contributed to the elevated serum Ig isotypes. However, because the increased IgG1 production was observed even when Cabin1ΔC
mice were immunized with NP-KLH in the absence of adjuvant, cytokines produced by non-T cells were probably not a major contributor.

During a T-dependent antibody response, class switching to IgG1 and IgE isotypes is normally induced by IL-4 secreted by Th2 cells, and class switching to IgG2a isotype is promoted by IFN-γ produced by Th1 cells. Although production of both Th2- and Th1-type cytokines were increased in Cabin1ΔC T cells after anti-CD3 stimulation in vitro, no significant increase in IgG2a was observed in the serum of nonimmunized mice or in response to NP-KLH immunization. The difference in cytokine expression in vitro and T helper cell development in vivo probably reflects differences in activation conditions during an immune response in mice and T cell activation in culture. However, the enhanced Th2 cell development in Cabin1ΔC mice may be due to an increased calcineurin activity in the mutant mice. Some evidence suggests that calcineurin positively regulates Th2 development by enhancing expression of signaling molecules in the IL-4 receptor pathway (45). In addition, targeted deletion of NF-ATc, which is normally activated by calcineurin, results in impaired Th2 but not Th1 development (34, 35). Thus, Cabin1 may function as a negative regulator of calcineurin during Th2 cell differentiation under physiological conditions.
FIGURE 1. Generation of Cabin1ΔC mice. (A) Schematic diagrams of the 3' region of the murine Cabin1 genomic locus and the Cabin1-neo and Cabin1ΔC alleles. Filled box (■), coding sequence; open box (□), 3' untranslated region; triangle (△), loxP site; and filled circle (●), poly A site. DNA sequences at the exon-intron boundary on the Cabin1ΔC allele are shown. Capital letters refer to nucleotides from the exon and lowercase refers to nucleotides from the intron. Reading frames and the in frame stop codon (bold) 11 nucleotides into the intronic sequence are indicated. (B) Southern blot analysis for Cabin1ΔC deletion. Tail DNA was digested with BamHI and the filter was hybridized with a 0.8kb BamHI-EcoRI probe as diagrammed in (A). DNA fragments generated from the wildtype (wt) and Cabin1ΔC (ΔC) allele are indicated. (C) Western blot analysis for Cabin1 protein. Equal amount of lysates from brain and
thymus of Cabin1ΔC and wildtype mice were immunoprecipitated with polyclonal antibodies specific for the C-terminal 77 amino acid residues of Cabin1. Precipitates were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-Cabin1 polyclonal antibodies. Anti-actin monoclonal antibody was used for the detection of actin as a control. (D) Northern blot analysis for Cabin1 transcripts. 5 μg of poly(A)+ RNA from various tissues of wildtype and Cabin1ΔC mice were fractionated by formaldehyde-agarose gel and the filters was hybridized with a 1.2kb Cabin1 cDNA fragment located 5' to the deleted Cabin1 sequence. The same filter was stripped and rehybridized with a GAPDH probe.
FIGURE 2. B and T cell development proceeds normally in Cabin1ΔC mice. Flow cytometric analysis of lymphoid tissues from Cabin1ΔC mice (ΔC) and wildtype controls (+/+). The numbers above the plots are the average number of thymocytes in Cabin1ΔC mice (n=5) and wildtype littermates (n=4). Thymocytes, lymph node cells, or splenocytes were stained with the indicated antibodies, and the numbers indicate the percentages of cells within the quadrants or gated area.
FIGURE 3. Comparison of TCR-mediated apoptosis and induction of Nur77 in thymocytes of Cabin1ΔC and wildtype mice. (A) Thymocytes from six-week-old Cabin1ΔC mice (n=5, grey bars) and wildtype littersmates (n=4, black bars) were stimulated in vitro with 50μg/ml plate-bound anti-CD3 for the indicated times. Annexin V binding of thymocytes was measured by flow cytometry. The percentages shown are after subtraction of background (Annexin V+ thymocytes in unstimulated cultures). (B) Pooled thymocytes from six-week-old Cabin1ΔC mice and wildtype littersmates were stimulated as above for three hours. Cell lysates containing equal amounts of protein were immunoprecipitated with anti-Nur77. Precipitates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the same anti-Nur77 antibody.
**FIGURE 4.** Enhanced cytokine production by Cabin1ΔC T cells. (A) Proliferation of Cabin1ΔC T cells in response to various stimuli. After stimulation with 10μg/ml plate-bound anti-CD3 or PMA and ionomycin (P + I) for 48 hrs, cultures containing Cabin1ΔC lymph node cells (black bars) or wildtype cells (grey bars) were pulsed with [³H]-thymidine for 18 hrs and [³H]-incorporation into DNA was measured. CsA, cyclosporine A. The assay was performed in triplicate in each experiment and was repeated five times with similar results. Data from one representative experiment is shown. (B) Production of IL-2 and IFN-γ by Cabin1ΔC T cells. The levels of IL-2 and IFN-γ were measured in the culture supernatants of Cabin1ΔC T cells (black bars) and wildtype T cells (grey bars) after 48 hrs of stimulation. The assay was performed in triplicate in each experiment and was repeated three times with similar results. Data from one representative experiment is shown. (C) RNase protection assay for cytokine transcripts. Lymph node cells from Cabin1ΔC (ΔC) and wildtype controls (+/+ ) were stimulated for 24 and 48 hrs with 10μg/ml
anti-CD3, and total RNA was isolated. The levels of cytokine transcripts were assayed by RNase protection. Transcript levels were quantified by phosphorimager and normalized to the levels of transcript for the ribosomal protein L32 in each lane. The numbers indicate the fold-increase of specific cytokine transcript in Cabin1ΔC T cells over that in the wildtype T cells at 48 hrs. The entire assay was repeated three times with similar results.
FIGURE 5. Nuclear translocation of NF-ATc. Purified T cells from Cabin1ΔC mice (ΔC) and wildtype controls (+/+), were either not stimulated or stimulated with plate-bound anti-CD3 for 4, 24, and 48 hrs. Nuclear extracts containing equal amounts of protein were separated by SDS-PAGE, transferred to membrane, and probed with an antibody specific for NF-ATc.
FIGURE 6. NF-AT DNA binding. Purified T cells from Cabin1ΔC mice (ΔC) and wildtype controls (+/+) were either not stimulated or stimulated with plate-bound anti-CD3 for 4, 24, and 48 hrs. Nuclear extracts were prepared and analyzed by EMSA using an oligonucleotide containing an NF-AT consensus binding site. In some reactions, cold competitor oligonucleotide was added in excess.
**FIGURE 7.** Serum immunoglobulin levels are elevated in Cabin1ΔC mice. The levels of Ig isotypes in the serum of three-month-old Cabin1ΔC (grey circles) and wildtype mice (black circles) were measured by ELISA. Each symbol represents one mouse. An unpaired Student t test was used to determine the p values.
FIGURE 8. The T-dependent antibody response to NP-KLH is enhanced in Cabin1ΔC mice. Mice immunized i.v. with NP-KLH in PBS were bled 14 days later and the levels of NP-specific Ig isotypes were measured by ELISA with NP-BSA coated plates. Shown is the average OD_{450nm} at different serum dilutions. Cabin1ΔC mice (n=6, grey circles) produce more NP-specific IgG1 than wildtype mice (n=6, black circles).
FIGURE 9. Class switching to IgG1 is unaffected in Cabin1ΔC B cells. Splenocytes from wildtype (+/+ ) and Cabin1ΔC (ΔC) mice were stimulated in vitro for five days with LPS or anti-CD40 in the presence or absence of IL-4. Cells were stained with antibodies specific for IgG1 and B220, and analyzed by flow cytometry. Splenocytes from three wildtype and three Cabin1ΔC mice were assayed, and a representative plot for each stimulation is shown. The numbers indicate percentages of B220+ cells that are IgG1+.
References

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Chapter Three

The Role of Left1 in T Cell Development and T Helper Cell Effector Function

Katrin Dürr and Chunjeih Ryu also contributed to the work described in this chapter.
Introduction

Upon activation with antigen, CD4$^+$ helper T cells differentiate into either Th1 or Th2 effector cells (1, 2). The two subsets are defined primarily by the pattern of cytokines they secrete, and whether an antigen elicits a Th1 or Th2 response has a profound effect on the ability of the immune system to clear the infection. Th1 cells make IFN-γ and TNF-α, which promote inflammatory responses and cellular immunity, while Th2 cells make IL-4, IL-5, IL-10, and IL-13, which promote humoral immunity and are involved in allergic diseases. The polarization of T helper cells to Th1 or Th2 is determined primarily by the cytokine environment in which the T cell finds itself at the time of activation. Signaling through the IL-12 receptor and activation of Stat4 promotes Th1 development by inducing IFN-γ transcription and suppressing IL-4 production (3). IL-4 receptor signaling through Stat6 promotes Th2 development by inducing IL-4 transcription and preventing IFN-γ production (3).

Two transcription factors are known to be essential for determining the differentiation pathway a naive helper T cell takes. GATA-3 is a Th2-specific transcription factor whose expression is induced by STAT6 (4), and can activate transcription of Th2 cytokines such as IL-4, IL-5, and IL-13 (5, 6). It is also important for suppression of Th1-specific gene expression in Th2 cells. Its expression in Th1 cells is sufficient to suppress IFN-γ transcription and induce IL-4 (7, 8). The T-bet transcription factor plays an analogous role in Th1 cells. It is a potent inducer of IFN-γ transcription (9), and when expressed in Th2 cells is sufficient to suppress IL-4 transcription and induce IFN-γ (9). As the expression of these transcription factors appears to play a determining role in the decision of a naive CD4$^+$ T cell to differentiate into a Th1 or Th2 effector cell, understanding the regulation of GATA-3 and T-bet expression should provide insight into the molecular basis for Th1/Th2 differentiation.

During an immune response, antigen-specific T cells undergo extensive clonal expansion to create an army of effector T cells. After clearance of the antigen, however, the majority of effector T cells must be removed from the animal, most of them by activation-induced cell death (AICD) (10). A few, however, survive and become long-lived memory cells. These cells are able to respond more rapidly and effectively upon
reencounter with the same antigen (11-13). What signals, if any, are required for effector T cells to escape AICD, survive, and become memory cells after the clearance of antigen is obscure. We were interested in characterizing the changes in gene expression that take place as an effector T cell differentiates into a memory T cell, in order to better understand this transition. The 2C TCR transgenic system was used to generate large numbers of antigen-specific effector and memory CD8+ T cells, which were used to perform a cDNA subtractive hybridization to identify genes upregulated in memory T cells compared to effector cells.

We chose one novel gene identified in this screen for further study. This gene is expressed specifically in lymphocytes, and the predicted protein sequence is that of a transmembrane protein with four transmembrane domains. We have named this gene Left1, for Lymphocyte Expressed Four Transmembrane protein. We also discovered that Left1 expression in CD4+ T cells was restricted to Th1 effector cell. Another group also reported that Left1 was expressed in Th1 cells, but downregulated in Th2 cells in response to IL-4 signaling through STAT6 (14). This suggested that Left1 may play a role in regulation of Th1/Th2 helper cell polarization. In order to examine the role of Left1 in T helper cell responses and memory T cell development, we constructed transgenic mice in which Left1 is overexpressed in T cells. Analysis of Left1 transgenic mice revealed a role for Left1 in regulation of several stages of T cell development. First, ectopic expression of Left1 in CD4+CD8+ double positive (DP) thymocytes promoted CD8+ single positive (SP) thymocyte lineage commitment, although CD4+ SP thymocyte differentiation was apparently unaffected. Second, mature Left1 transgenic T cells are impaired in their ability to respond to some stimuli in vitro. In spite of this, there is an increase in the proportion of memory T cells in the transgenic mice. Finally, we found that Th2 effector function was indeed impaired in T cells from Left1 transgenic mice. In addition, the transgenic T helper cells activated under Th2 polarizing conditions maintained some characteristics of Th1 cells, such as expression of the Th1-specific transcription factor T-bet, and the ability to respond to IL-12. These data show that downregulation of Left1 expression is an important step in commitment of T cells to the Th2 lineage.
Materials and Methods

Purification of effector and memory CD8+ T cells In order to generate large numbers of CD8+ memory T cells, 2C TCR transgenic mice on the RAG1−/− background (2C/RAG) were used (15). The 2C TCR recognizes the SIYRYGL peptide in association with Kβ, and can be specifically identified with the clonotypic antibody, 1B2.

To generate effector CD8+ T cells, splenocytes from 2C/RAG mice on a C57Bl/6 background were activated with 10−8 M SIYRYGL peptide for 3 days. After removal of dead cells on a Ficoll column, 98% of the remaining cells stained positive for the 2C TCR with the 1B2 antibody. >98% of the cells expressed the activation markers CD25 and CD69.

To generate memory CD8+ T cells, lymph node cells from 2C/RAG mice were transferred intravenously into RAG1−/−C57Bl/6 congenic mice. Three days later, the mice were immunized with 50 μg SIYRYGL in Freund’s adjuvant at the base of the tail and the scruff of the neck. After one month, memory 2C CD8+ T cells were purified magnetically from lymph nodes and spleen with anti-CD8α magnetic microbeads (Miltenyi Biotec, see below). Approximately 60% of the cells were CD8+CD44hi, or memory phenotype T cells.

cDNA subtraction A PCR-based subtractive hybridization protocol was performed using the PCR-Select cDNA subtraction kit (Clontech), according to the instructions of the manufacturer. Total RNA was isolated from the purified effector and memory 2C CD8+ T cells with Trizol (Life Technologies), then mRNA was isolated using a MicroPoly(A) Pure kit (Ambion). The mRNA was then used to create cDNA following the instructions of the PCR-Select kit. Effector T cell cDNA (the driver population) was subtracted from memory T cell cDNA (the tester population) in order to enrich for genes upregulated in memory CD8+ T cells. At the end of the protocol, the memory T cell-expressed, PCR-amplified cDNA fragments were cloned into the pT-Adv T/A plasmid (Clontech). Positive clones were used as probes on a Northern slot blot to compare the expression of the gene in effector and memory CD8+ T cells. Genes which showed increased expression in memory T cells on the Northern slot blot were then sequenced and the gene identified by searching the BLAST databases.
Rapid amplification of cDNA ends (RACE) Marathon cDNA Amplification kit (Clontech) was used to create a cDNA library from polyA+ RNA purified from Balb/c lymph nodes. The 5' and 3' regions of Left1, Left2, and Left3 cDNAs were amplified using primers in the most variable regions of the highly similar genes.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) RT-PCR was performed using the Titan One-Tube RT-PCR kit (Roche Molecular Biochemical). Typically, 100ng total RNA were used for each reaction, except for β-actin, in which 10ng total RNA were used. The primers used were as follows:
Left1: 5'-acaaccttattttctgaactacccacttcagtgta-3' and 5'-ccttgtacagattctggaacattgg-3' 
Left2: 5'-gcaaatctatctttcagacttcctttgtgag-3'and 5'-gcgtcacagggggtccatcacagtcacag-3' 
Left3: 5'-atatattaatagattctgaagctatgta-3' and 5'-tagcgtctgggtggctgtttctcattaattggtg-3' 
β-actin: 5'-ctctaaggccacaccgtgaagaag-3' and 5'-ctctcatggctgacagcc-3' 
T-bet: 5'-gatccacagcctattgtgcttg-3' and 5'-ccttccaatcagccgattttc-3'

Construction of transgenic mice Nucleotides encoding the hemagglutinin (HA) peptide epitope tag was introduced into the Left1 gene at either the 5' or 3' end by PCR, and the resulting construct was cloned into the human CD2 promoter/enhancer construct. VA-hCD2 (16). Bacterial plasmid sequences were removed by digesting with XhoI and NotI, and the resulting 13 kb construct was cotransfected into J1 embryonic stem (ES) cells with a pgk-puro' cassette by electroporation. The transfected ES cells were selected with puromycin, and resistant clones were screened by Southern blotting. HindIII-digested genomic DNA was probed with a 5kb HindIII fragment from the human CD2 promoter. Several ES cell clones were chosen to inject into RAG2Δ blastocysts, and the resulting chimeras were screened for expression of the transgene by Western blotting of lysates from lymphoid tissues with an anti-HA epitope antibody (Roche Molecular Biochemical). Chimeras from expressing lines were bred with C57Bl/6 mice for germline transmission. Genotyping of transgenic mice was performed by PCR with the following primers: Left1: 5'-gaaccccagcagctaatgagttcagtcacc-3' and hCD2: 5'-gagggccacccctttatagcagcc-3'.

Antibodies, intracellular staining, and flow cytometry For flow cytometry, single-cell suspensions of spleen, thymus, or lymph node were prepared. Erythrocytes were removed from splenocyte suspensions by lysis with 0.14 M NH₄Cl and 17 mM Tris-
HCl, pH 7.4. Cells were incubated on ice with FITC-, APC-, or PE-conjugated antibodies (PharMingen) in PBS with 4% fetal bovine serum (FBS) and analyzed on a FACScanLibur (Becton Dickenson) with Cell Quest software.

For flow cytometric analysis of intracellular cytokines or HA-tagged Left1, cells were first stained for surface antigens, washed, and then fixed with 4% paraformaldehyde in PBS for 20 min. at RT. After washing with PBS, the cells were incubated with the appropriate antibody in PBS with 0.1% saponin for 30 min. at RT. Cells were then washed once and analyzed by flow cytometry.

To assay for intracellular cytokine production, the T cells were first stimulated for four hours with 25ng/ml PMA and 500ng/ml ionomycin, and 10μg/ml brefeldinA was added for the last two hours. Antibodies used were anti-IFN-γ (XMG1.2, Pharmingen), anti-IL-4 (11B11, Pharmingen), or high affinity anti-HA (3F10, Roche Molecular Biochemical).

BrdU incorporation in vivo Mice were injected daily for three days with 1mg BrdU, sacrificed on the fourth day, and thymocytes and lymph node cells were isolated. After surface staining, the cells were washed with PBS and resuspended in 0.5ml ice cold 150mM NaCl. To fix the cells, 1.2ml ice cold 95% ethanol was added dropwise while vortexing the cells. After incubation on ice for 30 min., 2ml PBS was added and the cells were pelleted in a tabletop centrifuge for 5 min. The pellet was resuspended in PBS with 1% paraformaldehyde and 0.01% Tween-20, and incubated at RT for 30 min. The cells were then pelleted and resuspended in 1ml DNase solution (150mM NaCl, 4.2mM MgCl₂, 10nM HCl) containing 50 Kunitz units DNase I (Sigma), freshly prepared, for 10 min. at RT. After washing once with PBS, anti-BrdU antibody (BDPharmingen) was added directly to the pellet and incubated for 30 min. at RT. After two washes with PBS, the cells were analyzed by flow cytometry.

Purification of T cells For in vitro activation assays, spleen and lymph node cells were pretreated with anti-FcR antibody (Pharmingen), and then incubated with anti-CD4 or anti-CD8α magnetic microbeads (Miltenyi Biotec). Cells were purified on either a SuperMACS cell sorter or AutoMACS machine (Miltenyi Biotec). Cell purity was typically 90-95%. To purify naive CD4⁺CD44hi T cells for in vitro T helper cell differentiation assays, CD4⁺ cells from spleen and lymph node cells were first purified by
magnetic separation, and then stained with antibodies for CD4 and CD44 (Pharmingen) and sorted on a MoFlo cell sorter (Cytomation).

**Analysis of T cell activation** For T cell proliferation and cytokine secretion assays, 10^5 purified CD4^+ or CD8^+ T cells were incubated in 0.2 ml RPMI medium supplemented with 10% FBS, 10mM HEPES, 50μM 2-mercaptoethanol, 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 96-well plate. Some wells were pre-coated with anti-CD3 (2C11) and anti-CD28 (Pharmingen) antibody in PBS overnight at 4°C. 5μg/ml ConA, or 10ng/ml PMA (Sigma) + 250ng/ml ionomycin (Calbiochem) were used for some stimulations. For wells in which cytokines were added, 20u/ml IL-2, 25ng/ml IL-4 (R&D Systems), or 5ng/ml IL-12 (R&D Systems) were added to the cultures. Some cultures were pulsed with 1μCi [³H]-thymidine (NEN) at 48 hrs and harvested 18 hours later.

Alternatively, purified T cells were labeled with 15μM carboxyfluorescein diacetate-succinimidyld ester (CFSE, Molecular Probes) at 37°C for 10 min., washed, and activated under the same conditions as described above. Proliferation was monitored by flow cytometry for loss of the CFSE dye.

**ELISA assays for cytokine and serum antibodies** Serum Ig levels of unimmunized mice were measured by coating plates with anti-IgH + IgL antibodies (Southern Biotechnology Associates), and developing with horseradish peroxidase (HRP)-coupled goat antibodies specific for each mouse Ig isotype. ELISA for IgE was performed by coating the plates with anti-IgE antibody (PharMingen) and developing with HRP-conjugated anti-IgE antibody (PharMingen). NP-specific antibody in serum from immunized mice was measured by coating plates with NP-BSA and developing with HRP-coupled goat antibodies specific for each mouse Ig isotype.

**Immunization** Five-week old mice were immunized with the T-dependent antigen (4-hydroxy-3-nitrophenyl) acetyl-conjugated keyhole limpet hemocyanin (NP-KLH). 50μg of NP-KLH in alum were injected intraperitoneally on day 0 and the mice were bled 14 days later to measure the NP-specific antibody titer in the serum.

**T helper cell differentiation** Sorted wildtype and Left1-N27 transgenic CD4^+CD44^lo T cells were activated for three days in 24-well plates precoated with 5μg/ml anti-CD3 and 5μg/ml anti-CD28 antibody. 10^6 purified T cells were plated per
well. Wildtype splenocytes which had been depleted of T cells and γ-irradiated (1000 rad) were added at 10⁶/well. Th1-polarized cultures had 5ng/ml IL-12 (R&D Systems) added. Th2-polarized cultures had 25ng/ml IL-4 (R&D Systems) and 10µg/ml anti-IFN-γ antibody (XMG1.2, Pharmingen) added. After 24 hours, 40u/ml IL-2 was added to all cultures. After three days, the cells were split 1:3, removed from the anti-CD3 and anti-CD28 stimulation, and maintained another three days in IL-2 plus polarizing cytokines. At six days, the cells were washed extensively with PBS and restimulated with 10µg/ml platebound anti-CD3 for six hours and RNA was isolated for RNase protection analysis of cytokine transcripts. Alternatively, the cells were restimulated with PMA + ionomycin and cytokine production was analyzed by intracellular staining and flow cytometry (see above).

Alternatively, splenocytes from DO11.10 TCR transgenic Balb/c mice were cultured in RPMI media plus supplements and 1µM OVA<sub>323-339</sub> peptide. 2 x 10⁶ cells were cultured per well in a 24-well plate. Th1- and Th2- polarizing conditions were as described above. For neutral conditions, cells were cultured without any polarizing cytokines.

**RNase Protection Assay** Total RNA was isolated from Th1- or Th2-polarized CD4<sup>+</sup> T cells with Ultraspec reagent (Biotecx). The levels of cytokine transcripts were measured by RNase protection assay using the Riboquant multiprobe kit (PharMingen) following the manufacturer’s instructions. After overnight hybridization of target RNA with ³²P-labeled RNA probe, the protected mRNAs were purified and resolved on a 6% denaturing polyacrylamide gel. Cytokine transcript levels were quantified on a phosphorimager (Molecular Dynamics) using ImageQuant software, and the transcript levels were normalized to levels of L32 or GAPDH transcripts.

**Western Blotting** Polarized Th1 or Th2 cells were lysed in STAT lysis buffer (20mM Tris pH 8.0, 150mM NaCl, 1% TritonX-100, 0.5% NP-40, 1mM EDTA, 1mM EGTA, 1mM sodium vanadate, 1mM sodium fluoride, and protease inhibitor cocktail (Roche Molecular Biochemical)) after no treatment or after treatment with 5ng/ml IL-4 for 15 min., or 5ng/ml IL-12 for 30 min. Protein concentration was determined by BCA protein assay (Pierce). For STAT6 Western blotting, lysate containing 100µg protein was separated by SDS-PAGE, transferred to PVDF membrane, and blotted with antibody
to Tyr617-phosphorylated STAT6 (Upstate Biotechnology) or total STAT6 (Pharmingen). For STAT4 analysis, lysate containing 200μg protein was immunoprecipitated with anti-STAT4 antibody (Santa Cruz Biotechnology), separated by SDS-PAGE, transferred to PVDF membrane, and blotted with either STAT4 antibody or anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). Western blots were developed using an ECL reagent (Amersham).

**Retroviral Infection** Left1 cDNA or N-terminally HA-tagged Left1 (Left1N) cDNA were cloned into pMIG-W retrovirus (17). Lipofectamine reagent (Qiagen) was used to cotransfect the retroviral construct and pCL-Eco plasmid into 293T fibroblasts. Supernatants of transfected fibroblasts were used to spin-infect DO11.10 splenocytes at days one and two after initial activation with peptide.
Results

Screen for genes upregulated in CD8* memory T cells as they differentiate from effector T cells  In order to identify genes upregulated when memory T cells differentiate from effector cells, we used the 2C transgenic TCR system to generate large numbers of memory T cells ((18) and see Materials and Methods). Naive 2C/RAG CD8* T cells were transferred into congenic C57Bl/6 RAG-1+ mice, and three days later were immunized with 50 µg SIYRYYGL peptide in Complete Freund's adjuvant. Two months later, the memory cells were harvested from the lymph nodes and spleen, purified with CD8 magnetic beads, and RNA was isolated. The effector cells were generated by stimulating 2C/RAG splenocytes with SIYRYYGL peptide for 3 days. Clontech's PCR-Select system was used to perform the PCR-based cDNA subtraction. The memory cDNA was used as tester, and the effector cDNA was the driver population. The resulting cDNA fragments were subcloned and used as probes on a Northern slot blot to verify their increased expression in memory T cells, and fragments which showed an increased level of transcription in memory compared to effector 2C CD8* T cells were sequenced. Table 1 lists 81 genes, including 22 novel genes, identified in the screen, grouped according to their known or possible functions. Three additional novel genes were identified which had no corresponding mouse ESTs.

Cloning of Left1 We selected one novel gene identified in the screen to examine more closely. The predicted protein sequence of this gene has homology to both FceRIβ, a transmembrane protein important in IgE receptor-mediated signaling in mast cells (19), and CD20, a B-cell specific transmembrane protein which may be involved in B cell activation (20). The predicted structure of this protein, like FceRIβ and CD20, consists of four transmembrane domains, with both the C- and N-termini found in the cytoplasm. We named the gene Lymphocyte-Expressed, Four-Transmembrane protein 1 (Left1), although another group independently identified Left1 in a screen for Th1-specific genes and called it Chandra (14). Full-length Left1 cDNA clones were isolated by RACE (see Materials and Methods) and also by screening a lambda phage mouse spleen cDNA library. This revealed the presence of at least two highly-related genes, which we named
Left2 and Left3. Figure 1 shows an alignment of the deduced amino acid sequences of the three Left proteins.

*Expression pattern of Left1 and related genes* As the three genes are so similar at the DNA level (>90%), it was impossible to do a Northern blot for each gene individually to analyze their mRNA expression patterns. Primers were designed at regions of the cDNA sequence that were most variable among the genes, and the mRNA expression pattern of each gene was characterized by RT-PCR (Fig. 2). To verify that the correct gene was being amplified by each primer set, the amplified fragments were subcloned, and five clones from each primer pair were sequenced. All sequences were found to correspond to the expected Left gene (data not shown). Of the tissues examined in Figure 2A, Left1 expression is confined to the thymus, spleen, and lymph nodes. Left2 expression is also restricted to the lymphoid compartment, but it is found primarily in the spleen and lymph node and is barely detectable in the thymus. Left3 is more widely expressed, as it is detectable in heart, lung, and testis, as well as spleen and lymph node.

In Figure 2B, a more careful assessment of expression within lymphoid tissues shows that Left1 is modestly expressed in mature B cells. During thymic development, it is not induced until the CD4⁺ or CD8⁺ single positive (SP) thymocyte stage, and the expression is maintained in naive peripheral T cells. Although we expected the level of Left1 mRNA to drop in activated T cells and effector 2C CD8⁺ T cells, there was either no decrease or a modest decrease in Left1 mRNA levels in three different populations of activated T cells: anti-CD3 treated T cells (αCD3-T), ConA treated lymph node cells (ConA-LN), or peptide-MHC activated 2C TCR transgenic CD8⁺ T cells (2C effector). Memory 2C CD8⁺ T cells do express Left1 mRNA, but at only slightly higher levels than effector 2C CD8⁺ T cells. Neither the EL4 mouse thymoma cell line or the 2C T cell clone 2C88 expressed Left1.

In contrast to Left1, Left2 was expressed predominantly in B cells. Left2 mRNA was not found in thymocytes, but it was detected in mature peripheral T cells. It was downregulated in response to ConA or peptide-MHC activation, but anti-CD3 treatment alone is apparently not sufficient to induce the downregulation. Left2 was not re-expressed in 2C CD8⁺ memory T cells.
As Left1 was also isolated in a screen for genes expressed in CD4\(^+\) Th1, but not Th2 cells (14), we also tested for the expression of Left1 and Left2 in polarized Th1 and Th2 cells (Fig. 2C). We confirmed that Left1 is downregulated in Th2 cells after 3 days, but the expression is maintained in Th1 cells. As Left2 is downregulated after T cell activation, it was not found in either Th1 or Th2 effector cells.

**Construction of Left1 transgenic mice** In order to examine the effect of Left1 on T cell development and function, transgenic mice were created in which an epitope-tagged Left1 gene was under the control of the human CD2 promoter/enhancer (16) (Fig. 3A). This promoter should restrict expression of the transgene to T cells, beginning at the CD4\(^-\)CD8\(^-\) double negative (DN) stage of thymocyte development. We were able to analyze the level of epitope-tagged Left1 expression in individual cells by flow cytometry after fixing and permeabilizing the cells, and staining with an anti-HA antibody. Although it was reported that the N- and C-termini of Left1 are located extracellularly (14), we were unable to detect either N- or C-terminally tagged Left1 protein by flow cytometry without first fixing the cells. Several lines were screened, but one line, N27, gave the highest level of expression at the earliest stage of thymocyte differentiation (Fig. 3B). Two other lines expressed the transgene, although at lower levels, even when bred to homozygosity (Fig. 3C). All the studies reported here were performed with the N27 transgenic line. Transgene expression in the N27 line is detectable in DN thymocytes, and peaks in CD4\(^-\)CD8\(^+\) double positive (DP) thymocytes (Fig. 3B). Expression is maintained in both CD4\(^+\) and CD8\(^+\) SP thymocytes, and in the periphery, with the expression in CD4\(^+\) T cells higher than in CD8\(^+\) T cells (Fig. 3B). No Left1 transgene expression was detected in B cells or other non-lymphoid splenocytes in the N27 line (data not shown).

**Enhanced CD8\(^+\) lineage commitment in Left1 transgenic mice** Analysis of the distribution of DN, DP, and SP thymocytes in Left1-N27 transgenic mice by flow cytometry revealed an increase in the proportion of CD8\(^+\) SP thymocytes compared to wildtype littermates (Fig. 4A). In transgenic mice younger than two months, there was consistently a two- to three-fold increase in the proportion of CD8\(^+\) SP thymocytes compared to wildtype littermates, with a corresponding decrease in the percentage of DP thymocytes. The number of thymocytes in the transgenic mice compared to wildtype
littermates was not significantly different in younger mice, at least up until 7 weeks of age. In older transgenic mice (5 months or older), however, the thymus was often much smaller than wildtype littermates, although the size varied (Table 2). In these older mice, the percentage of DP thymocytes compared to that found in wildtype littermates was also reduced (Fig. 4A), and an accumulation of both SP thymocytes (Fig. 4A) and of CD44⁺CD25Thy-1⁺ DN thymocytes was observed (Fig. 4B).

**Reduced numbers of peripheral CD4⁺ T cells in Left1-N27 transgenic mice**
Analysis of the distribution of CD4⁺ and CD8⁺ T cells in peripheral lymphoid tissues in young transgenic mice revealed a modest reduction in the total number of CD4⁺ T cells compared to wildtype littermates. The cellularity of the spleen and lymph nodes were similar to wildtype, but the ratio of CD4⁺ to CD8⁺ T cells in the lymph nodes was reduced in the transgenic mice compared to wildtype mice (Fig. 5). In wildtype mice, the ratio of CD4⁺:CD8⁺ T cells in the lymph node is almost 2:1, but in transgenic mice the ratio was 2:3. The ratio of CD4⁺ to CD8⁺ T cells in the spleen of transgenic mice was normal (Fig. 5).

**Increased proportion of functional memory T cells in Left1 transgenic mice**
An examination of surface marker expression by peripheral CD4⁺ and CD8⁺ T cells in the transgenic mice by flow cytometry revealed a markedly increased proportion of cells expressing memory T cell surface markers. By 4 weeks of age, the transgenic mice had a significant increase in the proportion of CD8⁺ T cells that were CD44⁺hi, IL-2Rβ⁺, and Ly-6C⁺hi, compared to wildtype littermates (Fig. 6A). Depending on the marker, there was an increase of between two- and four-fold in the proportion of cells that were positive for each memory T cell marker compared to wildtype, and the proportion of memory-phenotype cells increased as the mice aged (Fig. 6A). CD8⁺ T cells from transgenic mice did not show an increased proportion of cells expressing the activation markers CD69 or CD25 when analyzed by flow cytometry (data not shown and Figure 7). Compared to wildtype, the transgenic mice also had a larger proportion of CD4⁺ T cells that were CD44⁺hi and CD62L⁺lo, markers characteristic of memory CD4⁺ T cells, and as many as 80% of the transgenic CD4⁺ T cells had a memory phenotype by the age of 6 months (Figure 6B). The transgenic CD4⁺ T cells also had a 2-fold increase in the proportion of
cells expressing the activation marker CD69, but there was no increase in the proportion of cells expressing the activation marker CD25 (Fig. 7).

Compared to naive T cells, memory T cells are able to produce a broader range of cytokines after only a short stimulation (11, 21). To test whether the memory phenotype CD4⁺ and CD8⁺ T cells in the transgenic mice were functionally memory cells, lymph node cells from wildtype and transgenic mice were isolated and activated with PMA and ionomycin for four hours, with brefeldin A added to the culture for the last two hours. The cells were then fixed and stained with antibodies against IFN-γ, IL-2 or IL-4 and analyzed by flow cytometry. Figure 8A shows the production of IFN-γ by naive and memory CD8⁺ T cells. Only 7% of wildtype naive CD8⁺ T cells made IFN-γ upon restimulation, but 30% of transgenic naive CD8⁺ T cells were IFN-γ⁺. Of the memory phenotype CD8⁺ T cells, 30% of wildtype and 67% of transgenic cells were able to produce IFN-γ upon restimulation, indicating that transgenic memory-phenotype cells are functionally memory T cells. Figure 8B shows the production of IL-2, IL-4, or IFN-γ by naive and memory CD4⁺ T cells. The percentage of naive and memory CD4⁺ T cells that produced IL-2 upon restimulation was similar for wildtype and transgenic mice. Very few naive CD4⁺ wildtype or transgenic cells made IFN-γ upon resimulation. However, while 6% of wildtype memory CD4⁺ T cells produced IFN-γ, 22% of transgenic memory CD4⁺ T cells were IFN-γ⁺. While less than 1% of wildtype naive CD4⁺ T cells made IL-4, 8% of transgenic naive CD4⁺ T cells were IL-4⁺. 3% of wildtype memory CD4⁺ T cells made IL-4, while 7% of transgenic memory CD4⁺ T cells were IL-4⁺. These data show that transgenic memory-phenotype CD4⁺ T cells are also functionally memory cells.

*Left1 transgenic T cells are defective in proliferation in vitro* We suspected that the increased number of memory T cells was due to a lowered threshold for activation in transgenic T cells, resulting in spontaneous activation by environmental or autoantigens. To further examine the proliferative responses of the transgenic T cells, purified CD4⁺ T cells were activated *in vitro* with various stimuli, and their proliferative responses monitored by [³H]-thymidine incorporation (Fig. 9A). In addition, some cells were labeled with CFSE, and their proliferation was monitored by analyzing the loss of CFSE by flow cytometry (Fig. 9B). The CD4⁺ T cell proliferative responses to PMA + ionomycin and ConA are normal in the transgenic T cells compared to wildtype.
Transgenic CD4\(^+\) T cell responses to different doses of anti-CD3 antibody are also comparable to wildtype. However, while the addition of anti-CD28 antibody to the anti-CD3 stimulated wildtype cultures enhances the proliferative response, there is no enhancement observed in the transgenic CD4\(^+\) T cell cultures. Addition of IL-2 or IL-4 to the anti-CD3 stimulated wildtype cultures also enhances the proliferative response. While the proliferation of transgenic CD4\(^+\) T cells under these conditions is slightly enhanced, it is not enhanced to the same degree as the wildtype CD4\(^+\) T cell cultures. Figure 9C shows that the defects in transgenic CD4\(^+\) T cell proliferation correspond to an inability to enhance CD25 expression in response to anti-CD28 or cytokine treatment. The transgenic CD4\(^+\) T cells could upregulate CD25 in response to anti-CD3 treatment as well as wildtype T cells. Curiously, the kinetics of CD25 upregulation by transgenic CD4\(^+\) T cells in response to PMA + ionomycin was faster than wildtype cells, peaking after 2 days and disappearing by day 3. In contrast to our expectation that the transgenic T cells would be hyperresponsive to stimulation \textit{in vitro}, these data show that transgenic CD4\(^+\) T cells have selective defects in their ability to enhance expression of the high affinity IL-2 receptor and proliferate in response to anti-CD28 and cytokines.

In comparison to the transgenic CD4\(^+\) T cells, CD8\(^+\) T cells from transgenic mice have a more severe proliferative defect \textit{in vitro}. Figure 10A shows proliferation of purified CD8\(^+\) T cells from wildtype and transgenic Left1-N27 mice after three days of \textit{in vitro} stimulation, measured by pulsing the cultures with \(^{3}H\)-thymidine. Proliferation of the CD8\(^+\) T cells measured by loss of CFSE after three days is shown in Figure 10B. Although their proliferative responses to PMA + ionomycin and ConA were largely normal compared to wildtype CD8\(^+\) T cells, the proliferative responses of transgenic CD8\(^+\) T cells to anti-CD3 stimulation were markedly impaired. Addition of anti-CD28 or IL-2 to the culture was not able to overcome the defect or enhance proliferation at all. Like the CD4\(^+\) transgenic T cells, the defects in CD8\(^+\) transgenic T cell proliferation corresponded to an inability to upregulate the CD25 activation marker (Fig. 10C). Left1 transgenic CD8\(^+\) T cells have severe defects in expression of the high affinity IL-2 receptor and proliferation in response to TCR stimulation.

\textbf{Impaired response to T-dependent antigen in Left1 transgenic mice} In order to measure the ability of Left1 transgenic T cells to promote B cell antibody responses \textit{in
vivo, five-week-old transgenic and wildtype littermates were immunized with NP-KLH in alum. Fourteen days after immunization, the level of NP-specific IgGs were measured by ELISA. NP-specific IgG1, IgG2b, and IgG3 levels were significantly lower in the serum of transgenic mice compared to wildtype mice (Fig. 11). The average level of NP-specific IgG2a in the transgenic mice was not significantly lower than wildtype mice, however. These data show that the in vivo CD4+ helper T cell responses in the transgenic mice are impaired.

**Increased proportion of in vivo proliferating T cells in Left1 transgenic mice**

We were puzzled that although the transgenic T cell responses in vitro and in vivo were impaired, there was a dramatic increase in the number of memory T cells. In order to determine whether the acquisition of memory phenotype was associated with proliferation in vivo, wildtype and transgenic Left1-N27 mice were injected with bromodeoxyuridine (BrdU) daily for three days. On the fourth day lymph node cells isolated from the mice were fixed and stained with anti-BrdU antibodies, and the level of BrdU incorporation was analyzed by flow cytometry. As shown in Figure 12A, although the proportion of Left1-N27 transgenic thymocytes that incorporated BrdU was not significantly different from thymocytes of wildtype littermates, the proportion of peripheral CD4+ and CD8+ cells that incorporated BrdU was clearly higher in the transgenic compared to wildtype. However, when the absolute numbers of lymph node T cells in the transgenic are taken into account, the number of proliferating CD4+ and CD8+ lymph node cells in the transgenic was only slightly increased compared to wildtype T cells (Fig. 12B). These data show that the transgenic T cells are able to proliferate in vivo.

**High serum immunoglobulin levels in 6 month old Left1 transgenic mice**

We assayed the levels of the various immunoglobulin isotypes in the serum of 6-month old Left1-N27 transgenic mice and wildtype littermates. At this age, the majority of the CD4+ T cells in the transgenic mice are memory-phenotype. As shown in Figure 13, the levels of IgM and IgG3 in the serum of transgenic mice were comparable to wildtype, although the modest increase in IgM in the transgenic was statistically significant. However the average level of IgG2b in the transgenic mice was almost six times higher than the average level in wildtype mice. The levels of IgG1 and IgG2a were elevated in
some transgenic mice, but not all. Of the five mice tested, two had elevated IgG1 but not IgG2a, two had elevated IgG2a but not IgG1, and one mouse had high levels of both IgG1 and IgG2a. Of the transgenic mice which had elevated serum IgG1 levels, the average level was nine times higher than wildtype mice. Of the transgenic mice which had elevated serum IgG2a levels, the average level was four times higher than wildtype mice. Two of the five transgenic mice had levels of serum IgE that were on average 30-fold higher than wildtype levels, although the high IgE level did not correlate with high IgG1 levels, as only one of the two mice with high IgE levels also had high levels of IgG1. Class switching in B cells to IgG1 and IgE are both promoted by IL-4, and mice with elevated serum IgE usually have elevated IgG1 as well. However, that was not the case in the Left1 transgenic mice.

Expression of Left1 in Th2 cells inhibits Th2 cell effector function  As Left1 is normally expressed in Th1, but not Th2 effector cells (14), it may play a role in regulation of Th1/Th2 differentiation or effector function. We tested the effect of ectopic expression of Left1 in Th2 cells on their development and function in the Left1-N27 transgenic mice. Purified naive CD44<sup>hi</sup>CD4<sup>+</sup> T cells from wildtype and transgenic mice were activated with platebound anti-CD3 and anti-CD28 antibodies in the presence of irradiated APCs and polarizing cytokines for three days. The cells were then removed from the anti-CD3 and anti-CD28 stimulation and rested for another three days in the presence of IL-2 and polarizing cytokines. Figure 14A shows that expression of the transgene is maintained in polarized transgenic Th2 cells, as measured by flow cytometry. Analysis of T helper cell effector function was performed by restimulating the cells on day six with platebound anti-CD3 for six hours, isolating total RNA, and measuring the transcript levels of a panel of cytokines by an RNase protection assay. Figure 14B shows the results of such an experiment. Wildtype Th1-polarized cells produce primarily IFN-γ, and some IL-2 upon restimulation. Transgenic Th1-polarized cells also produce IFN-γ, although normalization to levels of L32 transcripts shows a modest decrease (25%) in IFN-γ transcript levels compared to wildtype. Both wildtype and transgenic Th2 cells produce several Th2-specific cytokines, including IL-4, IL-5, IL-10, and IL-13. Transgenic Th2 cells, however, have reduced levels of transcripts of all of these cytokines compared to wildtype Th2 cells. Figure 14C shows the average
decrease in Th2 cytokine production taken from three such experiments. In addition, there was a small amount of IFN-γ transcript detectable in the Th2-polarized cells, indicating that Left1 transgene expression can not only inhibit Th2 cytokine production, but may induce Th1 cytokine production in cells that have been under Th2-polarizing conditions for six days.

Similar results were obtained by intracellular staining for cytokine production after restimulation of the polarized wildtype and transgenic T helper cells on day six. Instead of restimulation with anti-CD3, the polarized cells were stimulated with PMA + ionomycin for four hours with brefeldinA, fixed and stained for intracellular IFN-γ and IL-4. Figure 14D shows that of the transgenic Th1 cells, 26% were positive for IFN-γ after stimulation, compared to 46% of wildtype Th1 cells. Of the wildtype Th2 cells, 38% were IL-4+ after restimulation. However, only 7% of the transgenic Th2-polarized cells produced IL-4 upon restimulation. Again, this shows that ectopic expression of Left1 in Th2 cells can inhibit Th2 cytokine production in these cells. It can also cause a reduction in IFN-γ production by transgenic Th1 cells.

**Left1 transgenic Th2 cells have decreased GATA-3 expression but normal activation of STAT6 in response to IL-4** As GATA-3 expression is essential for Th2 cytokine transcription, we next examined whether altered expression of GATA-3 was responsible for impaired induction of Th2-specific cytokines in Left1 transgenic Th2 cells. RNA from restimulated wildtype and transgenic Th2 cells was probed on a Northern blot for the expression of GATA-3. As Figure 15 shows, although transgenic Th2 cells express GATA-3, the levels are three-fold lower than wildtype Th2 cells after normalization to levels of the L32 gene. Neither wildtype or transgenic Th1 cells expressed GATA-3 upon restimulation. This experiment shows that Left1 expression in Th2 effector cells inhibits GATA-3 expression.

As GATA-3 induction in Th2 cells is dependent on STAT6 activation through the IL-4 receptor (4), it was possible that Left1 expression was interfering with IL-4 receptor signaling. In order to measure the responsiveness of the Left1 transgenic Th2 cells to IL-4, we assayed STAT6 activation in response to IL-4 treatment by Western blotting. Figure 16 shows that both wildtype and transgenic Th2 cells are able to phosphorylate STAT6 in response to IL-4 treatment. When normalized to the total level of STAT6 on
the blot, the amount of activated STAT6 in transgenic lysates is comparable to wildtype, indicating that the transgenic Th2 cells are able to activate STAT6 in response to IL-4 normally.

**Left1 transgenic Th2 cells maintain the ability to respond to IL-12 and inappropriately express T-bet** We next examined whether ectopic expression of Left1 in transgenic Th2 cells results in the inappropriate expression of the Th1-specific transcription factor T-bet. We assayed for the expression of T-bet mRNA in wildtype and transgenic Th2 cells by RT-PCR. Although T-bet expression in wildtype Th2 cells was barely detectable at the highest amount of RNA used for the reaction, T-bet mRNA was readily amplified from nine-fold less RNA from transgenic Th2 cells, indicating that transgenic Th2 cells have considerably higher levels of T-bet expression (Fig. 17).

Th2 cells are normally unresponsive to IL-12, as they have downregulated expression of the IL-12Rβ2 gene (22, 23). To measure the responsiveness of Left1 transgenic Th2 cells to IL-12, we assayed STAT4 activation in response to IL-12 by immunoprecipitation and Western blotting. Figure 18 shows that after IL-12 treatment, STAT4 in lysates from both wildtype and transgenic Th1 cells undergoes a mobility shift, indicating activation by phosphorylation. In wildtype Th2 cells, there was less STAT4 protein detectable, and what could be seen did not undergo a mobility shift in response to IL-12 treatment. However, in the transgenic Th2 cells there was a substantial amount of STAT4 protein detected, and it underwent a mobility shift in response to IL-12, indicating that transgenic Th2 cells probably maintain the expression of the IL-12 receptor.

**Expression of Left1 in Th2 cells by retroviral-mediated gene transfer** We attempted to independently confirm our results in Left1-N27 transgenic Th2 cells by performing similar experiments using retroviral gene transfer of Left1. Either the Left1 coding sequence or an epitope-tagged Left1 gene (Left1N) were cloned into the pMIG-W retrovirus (17), which contains a bicistronically linked GFP gene to allow the detection of retrovirally infected cells. αβTCR transgenic CD4+ DO11.10 T cells (24) were infected with either pMIG-W retrovirus, or pMIG retrovirus containing Left1 or Left1N on days 1 and 2 after stimulation with APC plus OVA323-339 peptide in the presence of Th1- or Th2-polarizing cytokines, or under neutral conditions. After 7 days, approximately 20-40% of
the T cells were GFP⁺ (Fig. 19A). The cells were restimulated on day 7 with PMA + ionomycin and assayed for the production of IL-4 or IFN-γ by flow cytometry (Fig. 19A). Table 3 summarizes the results of this experiment, showing the percentage of GFP⁺CD4⁺ cells that were IL-4⁺ or IFN-γ⁺, and comparing that to the percentage of GFP⁺CD4⁺ or total CD4⁺ T cells that stained positive for either cytokine. As the table shows, there was no significant decrease in the percentage of IL-4 producing cells in GFP⁺ cells cultured under Th1, Th2, or neutral conditions, compared to GFP⁺ cells in the same cultures. There was no increase in the percentage of IFN-γ producing cells in GFP⁺ cells cultured under any of the three conditions, compared to GFP⁺ cells in the same cultures. The retrovirally transduced Left1 gene did not inhibit Th2 development or promote Th1 development in DO11.10 transgenic T cell cultures. Analysis of the expression level of the epitope-tagged Left1N protein in the transduced cells after seven days revealed that Left1 expression in GFP⁺ cells was very low compared to the Left1-N27 transgenic mice (Fig. 19B and Fig. 3), which may account for our inability to replicate the results we observed in the transgenic mice.
Discussion

Screen for genes upregulated in memory T cells  We have reported here the results of a subtractive hybridization screen for genes upregulated in memory CD8⁺ T cells as they differentiate from effector cells. Many genes already known to be involved in T cell signaling and costimulation were identified, such as the membrane proteins CD2 and LAT, and the intracellular signaling molecules Fyn, Lyn, and SKAP55. Increased expression of these signaling molecules may in part explain how memory T cells are able to respond more effectively upon reactivation with antigen. Many other signaling molecules that have not been previously examined for a role in memory T cell development and function were identified, and may provide additional clues to how memory T cells differ from effector cells. The other categories of genes identified in the screen can also provide some clues to the changes a T cell undergoes when it becomes a memory cell. Several genes that code for cytoskeletal components were isolated, which may be related to the different migration abilities and circulation patterns of memory T cells compared to effector T cells. The number of genes identified that are involved in RNA processing and chromatin remodeling also suggests that memory T cells undergo global changes in gene expression as they differentiate from effector T cells. In addition, many novel genes of unknown function were identified which may prove fruitful in the future for understanding memory T cell differentiation, survival, and function.

Characterization of Left1  We chose one novel gene, Left1, for further study. Left1 shares homology with the FceRIβ subunit, part of the high affinity IgE receptor expressed on mast cells, and with the B cell expressed membrane protein CD20. Like these proteins, its predicted structure is of a membrane protein with four-transmembrane domains and both N- and C-termini located in the cytoplasm. Although its membrane topology is similar to proteins of the tetraspanin superfamily (25, 26), Left1 lacks the conserved amino acid sequences that define the tetraspanins. However, like the tetraspanins, both FceRIβ and CD20 operate as components of larger complexes of membrane proteins, in which their roles may be to modify signaling through their associated proteins rather than to directly bind to their own extracellular ligands. Indeed, the predicted membrane structure of Left1 leaves only two extracellular loops of not
more than 11 and 16 amino acids. While Left1 may have its own extracellular ligand, it is likely to be more important as part of a larger signaling complex. We have attempted to identify Left1-associated proteins by surface biotinylation of cells expressing an HA-tagged Left1 protein, followed by co-immunoprecipitation with an anti-HA antibody (data not shown), but so far have had no success.

**Enhanced CD8+ lineage commitment in Left1 transgenic mice** Although we initially identified Left1 as a gene prominently expressed in memory CD8+ T cells, analysis of transgenic mice overexpressing Left1 in T cells revealed a role for Left1 in regulation of several stages of T cell development. RT-PCR analysis of Left1 expression indicates that Left1 normally isn’t transcribed until the SP thymocyte stage in T cell development. However, when Left1 is ectopically expressed in DP thymocytes, we observed a slight enhancement in CD8+ SP thymocyte development, showing that expression at the DP thymocyte stage can influence signaling pathways that affect lineage commitment. CD4+ SP thymocyte development was apparently unaffected.

According to the “strength of signal” model for thymocyte lineage commitment, the relative amounts of TCR signaling and Lck activation by the CD4 and CD8 coreceptors determine the commitment of DP thymocytes to one or the other lineage (27, 28). This is a modification of the instructive model, which assumes that CD4 and CD8 coreceptor engagement instructs the T cell to the appropriate lineage in order that it maintains the correct MHC specificity. Differential activation of Lck by the coreceptors is thought to be important for regulation of the fate decision. CD4 binds Lck with a higher avidity than CD8 (29, 30), so CD4 binding to MHC Class II on selecting thymic stromal cells induces a stronger Lck signal. In addition, many of the CD8 molecules in DP thymocytes are alternatively spliced so that they do not bind Lck at all (31), suggesting that a reduction or absence of Lck signaling may be necessary for CD8 development. In addition, there is evidence that the duration and strength of TCR signaling can influence CD4/CD8 lineage commitment (32), with stronger, or prolonged TCR signaling preferentially inducing CD4+ T cell differentiation. Weaker TCR engagement promotes CD8+ lineage commitment, but DP thymocytes which are unable to make any MHC contacts of sufficient strength will not be positively selected and will die by neglect.

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Although we cannot say precisely how Left1 may be enhancing CD8\(^+\) lineage commitment, we can speculate about how our data may fit into these models. Left1 may be amplifying signals, either through the TCR, or through a costimulatory pathway, which rescue thymocytes that would normally die by neglect, and redirecting the thymocyte to the CD8\(^+\) pathway. Alternatively, Left1 could be providing an inhibitory signal that redirects some cells that would normally become CD4\(^+\) T cells to the CD8\(^+\) T cell lineage. This would be consistent with the data showing that mature Left1 transgenic T cells have some defects in their ability to respond to some stimulations \textit{in vitro}. We might expect a corresponding decrease in the proportion of CD4\(^+\) T cells in the transgenic thymus, unless cells which would normally be negatively selected were then redirected to become CD4\(^+\) T cells, resulting in similar numbers of CD4\(^+\) SP thymocytes. Breeding the Left1 transgene to MHC Class I-restricted and MHC Class II-restricted TCR transgenic mice should allow us to address this question.

There is also evidence for an "asymmetric" model of lineage commitment, in which separate, lineage-specific signals are required for CD8\(^+\) T cell differentiation, and the absence of these signals leads to default CD4\(^+\) T cell development (27, 28, 33). This is supported by the observation that overexpression of bcl-2 (34) or activated Notch (35) in DP thymocytes seems to exclusively enhance CD8\(^+\) T cell differentiation. Mice deficient for the IRF-1 transcription factor also have selective defects in CD8\(^+\), but not CD4\(^+\) T cell development (36), suggesting that the requirements for CD8\(^+\) T cell development may be distinct. Similar to these proteins, Left1 could be promoting a signal that specifically enhances CD8\(^+\) T cell differentiation. Regardless of which model forr lineage commitment is correct, identification of the signaling pathways induced or inhibited by Left1 expression in DP thymocytes could provide valuable new information about the regulation of thymocyte lineage commitment.

\textbf{Increased number of memory T cells in Left1 transgenic mice} A model in which Left1 overexpression is providing some costimulatory signals might be consistent with the observed increase in the proportion of memory phenotype T cells in the periphery of Left1 transgenic mice. A likely explanation for the increase in memory T cells is that expression of Left1 lowers the threshold for TCR stimulation, resulting in hyperreactivity of the T cells to environmental or self antigens. The observed increase in
CD69$^{+}$CD4$^{+}$ T cells suggests that the T cells are being activated, although there was no corresponding increase in CD69$^{+}$CD8$^{+}$ T cells. Alternatively, development of Left1 transgenic T cells in the thymus may have been altered in such a way as to reset the threshold of TCR stimulation in mature T cells.

**Proliferation of Left1 transgenic T cells** It is puzzling that although the increase in memory T cells suggests the cells are hyperresponsive, the Left1 transgenic T cells have some defects in proliferation and activation *in vitro*. The CD4$^{+}$ T cells are able to proliferate in response to anti-CD3 treatment normally, but costimulation through CD28 doesn’t enhance the response at all, and their ability to express the high affinity IL-2 receptor and proliferate in response to cytokines is also reduced. The CD8$^{+}$ T cells have even more severe proliferative defects, as they are unable to respond even to anti-CD3 treatment. Both CD4$^{+}$ and CD8$^{+}$ transgenic T cells were able to proliferate and express CD25 in response to phorbol ester and calcium ionophore treatment, however, indicating that the defect in the transgenic cells is likely to be membrane proximal. The defects cannot be compensated by addition of APCs to the T cell cultures *in vitro*, however, because similar results were obtained when unpurified lymph node cells were activated *in vitro*. In addition, the antibody response to a T-dependent antigen was reduced, suggesting that *in vivo* T cell responses in the transgenic mice are also impaired. These data indicate that the Left1 transgene is suppressing T cell activation.

Why is there a difference in the proliferative responses between transgenic CD4$^{+}$ and CD8$^{+}$ T cells? One possibility is that the different levels of Left1 transgene expression lead to different magnitudes of effect on the cells. Fig. 3B shows that the expression level of transgenic Left1 in CD4$^{+}$ cells is at least two-fold higher than CD8$^{+}$ T cells. This argues against a linear relationship between increased Left1 expression and the severity of the impairment in *in vitro* T cell responses. However, the percentage of CD4$^{+}$ memory-phenotype cells peaks in the transgenic mice at an earlier age compared to CD8$^{+}$ memory cells, and the CD4$^{+}$ cells have an increased number of CD69$^{+}$ cells that CD8$^{+}$ T cells do not, which may suggest that the *in vivo* activated and memory phenotype does correspond to expression of the transgene. Another possible explanation for the difference between CD8$^{+}$ and CD4$^{+}$ T cell responses is that Left1 associates with different sets of proteins and affects different signaling pathways in the two cell types.
Finally, it is possible that the development of the transgenic T cells in the thymus was abnormal in some way which has resulted in the resetting of TCR signaling thresholds in the periphery. The CD8⁺ and CD4⁺ T cells may have been differentially affected by the pathways they have taken through thymic development.

*Inhibition of Th2 effector cell function in Left1 transgenic mice* We also evaluated the effect of Left1 overexpression on T helper cell differentiation, and found that Th2 cytokine production was severely impaired. Normally Left1 would be downregulated in response to IL-4 signaling, but in the transgenic mice Left1 expression is ectopically maintained in Th2 cells. As IL-4 signaling is responsible for both inducing Th2-specific gene expression and for repression of Th1-promoting pathways, it is perhaps not surprising that downregulation of Left1 by IL-4 is an important step in regulation of Th2 effector cell polarization.

How does Left1 expression in Th2 cells result in reduced Th2 cytokine production? According to the Western blot for STAT6 activation, Left1 expression apparently does not interfere with IL-4R-mediated STAT6 activation, although in *in vitro* proliferation assays transgenic CD4⁺ T cells were impaired in their ability to proliferate and upregulate CD25 in response to IL-4, suggesting that IL-4 signaling is not completely intact. However, STAT6 activation is not required for the mitogenic response to IL-4 (37, 38), indicating that other IL-4 receptor induced pathways must be affected. In any case, expression of the Th2-expressed transcription factor GATA-3 was reduced in the transgenic Th2 cells. It’s not clear how Left1 transgene expression results in reduced GATA-3 transcripts, but the reduced GATA-3 levels are certainly at least partially responsible for the observed decrease in Th2 cytokine production in the transgenic Th2 cells. GATA-3 is known to be important for IL-4, IL-5, and IL-13 transcription (5), although it’s not clear what role it plays in IL-10 transcription, which was the most reduced of the cytokines assayed.

Along with the decreased expression of Th2-specific genes in the transgenic T cells is an inappropriate induction of Th1-specific gene expression. The Th1-specific transcription factor T-bet was found at levels that, while less than wildtype Th1 cells, were still at least nine times higher than transgenic Th2 cells. As expression of GATA-3 and T-bet transcription factors seem to be mutually antagonistic (39), it is not clear
whether Left1 suppression of GATA-3 leads to some T-bet expression, or whether Left1 induction of T-bet leads to repression of GATA-3 in the transgenic Th2 cells. Maintenance of the ability to respond to IL-12 in the transgenic Th2 cells, measured by STAT4 activation, could also be a consequence of GATA-3 downregulation. However, maintenance of IL-12R expression, and even activation of the pathway in the presence of IL-4 signaling, is not enough to suppress Th2 development (40, 41). This was the conclusion of two groups that ectopically expressed the IL-12Rβ2 gene in Th2 cells either in transgenic mice or by retroviral infection. Both groups found that although the Th2 cells were responsive to IL-12 and able to activate STAT4, culturing the cells with IL-4 and IL-12 together still resulted in Th2 development and very little IFN-γ transcription in response to IL-12. GATA-3 was not downregulated in these cells, either, suggesting that inhibition of Th2 developmental programs in the presence of IL-4 signaling involves other signaling pathways besides IL-12. This suggests that Left1 is acting to suppress GATA-3 transcription and Th2 cytokine production not simply through maintenance of IL-12R signaling in Th2 cells, but through another pathway. The maintenance of IL-12R expression in the transgenic Th2 cells may simply be a consequence of the downregulation of GATA-3.

There is also some suppression of IFN-γ expression in transgenic Th1 cells, which may call into question whether the decreased Th2 cytokine production by the transgenic T cells is really an impairment in Th2 development, or simply reflects the general suppression of T helper cell responses we observed in vitro and in vivo. Although the impaired proliferative responses of the CD4+ T cells certainly contribute to the cytokine suppression in Th2 cells, the fact that in the transgenic Th2 cells we observe Th1-specific gene expression, including IFN-γ production, argues that the transgenic Th2 cells have specific defects in Th2 effector cell polarization. In the in vivo T-dependent antibody response, although the levels of NP-specific IgG production were much lower in the transgenic mice, the exception was the IgG2a isotype, which was only marginally lower. Class switching to the IgG2a isotype in B cells is strongly promoted by IFN-γ, and therefore Th1 type responses, indicating some preference in the transgenic mice for the development of Th1 responses in vivo.
**High serum immunoglobulin in older Left1 transgenic mice**  An observation that is difficult to explain is the high levels of serum immunoglobulin in the 6-month-old transgenic mice. Although the response to a T-dependent antigen in the younger mice was slightly impaired, by the time the transgenic mice are six months old they all have six-fold higher levels of IgG2b, and high levels of either IgG1 or IgG2a. The presence of high levels of IgG1 in some older mice is also a surprise, as IgG1 class switching in B cells is IL-4-dependent and promoted by Th2 cells. As the development of IL-4-producing Th2 cells seems to be impaired in these mice, it is not clear how so much IgG1 antibody production is induced. Several of the older transgenic mice examined exhibited varying degrees of splenomegaly, and we have so far been unable to determine the origin of the infiltrating cells. They don't express surface markers characteristic of lymphocytes, NK cells, macrophages, granulocytes, or dendritic cells. It is possible that these cells may be producing cytokines or factors that are in part responsible for the high serum immunoglobulin levels found in these mice.

**Expression levels of Left1 in transgenic mice**  We attempted to reproduce these experiments in a second transgenic line, N63, but were unable to do so. Experiments in which a Left1 gene was introduced into Th2 effector cells by retrovirus-mediated gene transfer were also unsuccessful in inhibiting Th2 cytokine production. However, in both cases we could show that significantly lower levels of Left1 were expressed in the Th2 cells, compared to the N27 transgenic line. We are currently creating more transgenic lines in order to find another with similar expression levels to the N27 line. If Left1 requires such high levels of expression in order to have an effect on T cell development, what does that suggest about how the transgene may be causing these effects? It is difficult to know without being sure what proteins it associates with or what its ligand might be. As mentioned before, we predict that it will be involved in a complex with other membrane receptors, based on the homology with FcεRIβ and CD20. As only a small piece of Left1 is exposed on the membrane, it may not have a ligand independently of its associated receptors. Left1 does not have an immunoreceptor tyrosine-based activation motif (ITAM), as FcεRIβ does. CD20 doesn't have an ITAM, but it has been shown to co-immunoprecipitate with several src family protein tyrosine kinases through an unknown associated protein (42-44). As the cytoplasmic tail of CD20 was not
required for the interaction (43, 44), it was assumed to be associated through lateral interactions with other membrane proteins. The high levels of expression observed in the Left1-N27 transgenic mice may result in spontaneous aggregation of Left1 and its associated proteins, causing constitutive signaling. Or, it may act as a dominant negative, segregating an associated receptor so that it is unable to function. Experiments to find both intracellular and transmembrane Left1-associated proteins are in progress, but so far have had no success. Identification of such proteins will be essential for understanding the role of Left1 in T helper cell development and memory T cell function.
Membrane proteins and receptors
CD2
CD8β
CD82
CLCh, Chloride intracellular channel protein
E-cadherin
EGFR-154
IL-2Rβ
LAT, linker for activation of T cells
LAPTm5
Left1/Chandra
Ly6A/Sca-1
MHC Class I
ribophorin II
TSA-1/Sca-2

Cytokines and Chemokines
calgizarin
RANTES

RNA Metabolism
dyskerin
hnRNP F
hnRNP U/SAF-A
NonO/p54nrp
RNase P
SRp20

Lipid Metabolism
Gpi1
SCP-2
S1P, site-1 protease

Chromatin Remodeling
BAF60b
NAP-2, nucleosome assembly protein 2

Other
Brrp, brain-expressed proline-rich protein
C3
Copz1
hsp84
Nsg2/p19
PA28α
homolog of Aspergillus sudD
Tbr2
topoisoerase I
ubiquitin
vacuolar proton-ATPase subunit
VDUP1

Signal Transduction
ALG-2
ARF3
Fyn
GTP cyclohydrolase I
Igf-204, IFN-activatable protein 204
Lyn
PP2A
Rap1B
SKAP55
Tak1 binding protein 2
TGTP
TIC
VCP

Cytoskeleton
β-actin
coronin 1
L-plastin
LSP1/pp52
moesin
Septin 6
SH3P7/Drebrin-like

Novel Genes
Genbank Accession Number of most homologous mouse EST:
AA667037
AA615175
AI607226
AK003694
AK004612
AK011759
AK014391
AK017908
AW456459
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BE692425
BF099988
BF102382
BF178332
BF583401
BF714912
BG095349
BG100988
BG298884
W61477

**TABLE 1.** Genes upregulated in memory CD8+ T cells as they differentiate from effector cells, identified by subtractive hybridization.
FIGURE 1. Comparison of the Left family deduced amino acid sequences. Left1, Left2, and Left3 amino acid sequences are aligned to show the high similarity among the three genes. Amino acids in the Left2 and Left3 sequences which differ from Left1 are highlighted in pink. Boxes are drawn around the predicted transmembrane domains.
FIGURE 2. Analysis of Left mRNA expression. (A) Left mRNA expression in mouse tissues measured by RT-PCR. LN, lymph node. The levels of β-actin expression were measured as a control. (B) Left1 and Left2 gene levels in lymphocyte subsets measured by RT-PCR. DN thym, CD4+CD8− thymocytes. DP thym, CD4+CD8+ thymocytes. SP thym, CD4+ and CD8− thymocytes. αCD3-T, T cells activated with platebound anti-CD3 for 48 hours. ConA-LN, lymph node cells activated with ConA for 48 hours. The levels of β-actin expression were measured as a control. (C). Left1 and Left2 expression in Th1 and Th2 effector cells measured by RT-PCR. The levels of β-actin expression were measured as a control.
FIGURE 3. Creation of Left1 transgenic mice. (A) HA-tagged Left1 transgene construct. The coding sequence for N- or C-terminally hemagglutinin epitope tagged Left1 was cloned into the VA-hCD2 vector. (B) Expression of the HA-tagged Left1 transgene in the N27 transgenic mouse line. After staining for surface CD4 and CD8, fixed and permeabilized thymocytes and lymph node cells from Left1-N27
transgenic mice and wildtype littersmates were stained with anti-HA peptide antibody and analyzed by flow cytometry. Cells in each histogram were gated as indicated above the plot. Shaded histograms are cells from wildtype littersmates. The bold line represents the cells from the N27 transgenic mouse. (C) Expression of the HA-tagged Left1 transgene in the N63 and C19 lines. Flow cytometry was performed as described in (B). Shaded histograms are from wildtype littersmates. The bold line represents the cells from homozygous transgenic mice.
FIGURE 4. Altered lineage commitment in Left1-N27 transgenic mice. (A) Flow cytometric analysis of thymocytes from young (5 weeks old, left plots) and old (5 months old, right plots) Left1-N27 transgenic mice and wildtype littersmates, stained with antibodies to CD4 and CD8. The numbers indicate the percentage of cells that fall into the indicated quadrants. (B) Flow cytometric analysis of thymocytes from young (5 weeks old, left plots) and old (5 months old, right plots) Left1-N27 transgenic mice and wildtype littersmates, stained with antibodies to CD44 and CD25 and gated on CD4<sup>−</sup>CD8<sup>−</sup> double negative thymocytes. The numbers indicate the percentage of cells that fall into the indicated gated areas.
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**TABLE 2.** Summary of thymocyte numbers and surface phenotype in Left1-N27 transgenic mice and wildtype littermates. Each table represents data from littermates at a different age. Expression of CD4 and CD8 was measured by flow cytometry. DN, CD4^−/CD8^− double negative. DP, CD4^+^CD8^+^ double positive.
FIGURE 5. Distribution of CD4$^+$ T cells in Left1-N27 transgenic mice. Flow cytometric analysis of lymph node cells and splenocytes from 5 week old Left1-N27 transgenic mouse and wildtype littermate, stained for CD4 and CD8. The numbers indicate the percentage of cells that fall into the indicated gated areas.
FIGURE 6. Increased proportion of memory-phenotype T cells in Left1-N27 transgenic mice. (A) Lymph node cells from young (5 weeks old, left plots) or old (6 months old, right plots) Left1-N27 transgenic mice and wildtype littermates were analyzed by flow cytometry for various CD8+ T cell memory surface markers. In each histogram, the shaded plot represents wildtype CD8+ T cells, the bold line represents N27 transgenic CD8+ T cells. The numbers indicate the percentage of the gated cells that fall
into the indicated region. The first number (regular typeface) represents wildtype, the second number (bold typeface) represents transgenic. (B) Lymph node cells from young (5 weeks old, left plots) or old (6 months old, right plots) Left1-N27 transgenic mice and wildtype littermates were analyzed by flow cytometry for various CD4+ T cell memory surface markers. Labeling of the histograms is as described in (A).
FIGURE 7. Expression of the activation markers CD25 and CD69 by Left1-N27 T cells. Splenocytes from a 5-month old Left1-N27 transgenic mouse and a wildtype littermate were analyzed by flow cytometry for expression of the activation marker CD25 on CD4+ T cells, and the early activation marker CD69 on both CD4 and CD8 T cells. The numbers indicate the percentage of CD4+ or CD8+ T cells that fall into the indicated gated areas.
FIGURE 8. Memory-phenotype cells from Left1-N27 transgenic mice rapidly secrete cytokines upon stimulation. Lymph node cells from 5-month old wildtype and Left1-N27 transgenic mice were stimulated for 4 hours with PMA + ionomycin. BrefeldinA was added for the last 2 hours, then the cells were fixed, permeabilized and stained with the indicated antibodies and analyzed by flow cytometry. (A) IFN-
\( \gamma \) production by gated naive or memory phenotype CD8\(^+\) T cells. The shaded histograms are wildtype cells, the bold line represents Left1-N27 transgenic cells. The numbers indicate the percentage of the gated cells that fall into the indicated region. The first number (regular typeface) represents wildtype, the second number (bold typeface) represents transgenic. (B) IL-2, IFN-\( \gamma \), or IL-4 production by gated naive or memory phenotype CD4\(^+\) T cells. Labeling of the histograms is as described in (A).
A

$[^3\text{H}]-\text{thymidine incorporation}$

\(\text{(cpm} \times 10^3)\)

- none
- 1$\mu$g/ml αCD3
- 5$\mu$g/ml αCD3
- αCD3 + αCD28
- αCD3 + IL-4
- αCD3 + IL-2
- PMA + ionomycin
- ConA

- wildtype
- transgenic

B

- none
- 1$\mu$g/ml αCD3
- 5$\mu$g/ml αCD3
- αCD3 + αCD28
- αCD3 + IL-2
- αCD3 + IL-4
- PMA + ionomycin
- ConA

CFSE

103
FIGURE 9. Specific defects in in vitro proliferation of CD4+ Left1-N27 transgenic T cells. (A) After stimulation with the indicated treatment for 48 hrs, cultures containing purified Left1-N27 transgenic (dark bars) or wildtype (light grey bars) CD4+ T cells were pulsed with [3H]-thymidine for 18 hrs and [3H] incorporaton into DNA was measured. The assay was performed in triplicate in each experiment. One representative experiment of three is shown. (B) Purified transgenic and wildtype CD4+ T cells were labelled with CFSE, then stimulated in vitro with the indicated treatment for three days. The cells were then analyzed for loss of CFSE by flow cytometry. The shaded histograms are wildtype cells, the bold line represents Left1-N27 transgenic cells. (C) Purified transgenic and wildtype CD4+ T cells were stimulated in vitro with the indicated treatment for three days. The cells were then stained with antibody against CD25 and analyzed by flow cytometry. Plotted is the geometric mean of CD25 fluorescence for wildtype (light grey bars) and transgenic (dark bars) T cells.
FIGURE 10. Specific defects in in vitro proliferation of CD8⁺ Left1-N27 transgenic T cells. (A) After stimulation with the indicated treatment for 48 hrs, cultures containing purified Left1-N27 transgenic (dark bars) or wildtype (light grey bars) CD8⁺ T cells were pulsed with [³H]-thymidine for 18 hrs and [³H]-incorporaton into DNA was measured. The assay was performed in triplicate in each experiment. One representative experiment of three is shown. (B) Purified transgenic and wildtype CD8⁺ T cells were labelled with CFSE, then stimulated in vitro with the indicated treatment for three days. The cells were then analyzed for loss of CFSE by flow cytometry. The shaded histograms are wildtype cells, the bold line represents Left1-N27 transgenic cells. (C) Purified transgenic and wildtype CD8⁺ T cells were stimulated in vitro with the indicated treatment for three days. The cells were then stained with antibody against CD25 and analyzed by flow cytometry. Plotted is the geometric mean of CD25 florescence for wildtype (light grey bars) and transgenic (dark bars) T cells.
FIGURE 11. Impaired T-dependent antibody responses in Left1-N27 transgenic mice. 5-week-old mice immunized i.p. with NP-KLH in alum were bled 14 days later and the levels of NP-specific immunoglobulin isotypes in the serum of Left1-N27 (●) and wildtype mice (◆) were measured by ELISA with NP-BSA coated plates. Each symbol represents one mouse, and the lines represent the average of either the transgenic or wildtype mice for each Ig isotype. An unpaired Student t test was used to determine the p values.
FIGURE 12. Increased frequency of BrdU incorporation in Left1-N27 transgenic T cells in vivo. (A) Left1-N27 transgenic mice (n=4) and wildtype littermates (n=3) were injected i.p. with 1mg BrdU daily for three days. On the fourth day, incorporation of BrdU into thymocytes and lymph node T cells was assayed by flow cytometry. Shown is the average percentage of cells within the indicated cell population that stained positive for BrdU incorporation. (B) The number of BrdU+ CD4+ and CD8+ T cells found in the lymph nodes of wildtype and Left1-N27 transgenic mice.
FIGURE 13. Enhanced serum immunoglobulin levels in six-month-old Left1-N27 transgenic mice. The levels of Ig isotypes in the serum of six-month-old Left1-N27 (●) and wildtype mice (◆) were measured by ELISA. Each symbol represents one mouse.
FIGURE 14. Ectopic expression of Left1 in Th2 cells inhibits Th2 effector function. (A) Expression of Left1 in N27 transgenic Th1 and Th2 cells. Purified naive CD4⁺CD44⁺ transgenic and wildtype T cells were stimulated with platebound anti-CD3 and anti-CD28 under Th1- or Th2-polarizing conditions for
three days, then rested for another three days in IL-2 under polarizing conditions. Fixed and permeabilized cells were then stained with an anti-HA antibody and analyzed by flow cytometry. The shaded histograms are wildtype cells, the bold line represents Left1-N27 transgenic cells. (B) RNase protection assay for cytokine production. After six days of stimulation under Th1- or Th2-promoting conditions as described in (A), the polarized wildtype and transgenic cells were restimulated with anti-CD3 for six hours and RNA was isolated from the cells. An RNase protection assay for a panel of cytokines was performed. Transcript levels were quantitated and normalized to the amount of L32 transcript in each lane. (C) Average decrease in Th2 cytokine production in Left1-N27 transgenic Th2 cells. Shown is the average level of various Th2 cytokines produced by Left1-N27 transgenic Th2 cells as a percentage of wildtype Th2 cytokine production. The data are averaged from three experiments. (D) Analysis of IL-4 and IFN-γ production by Left1-N27 Th1 and Th2 cells by intracellular staining and flow cytometry. Naive CD4+ T cells from wildtype and transgenic mice were stimulated under Th1- or Th2-promoting conditions as described in (A). At day six, the cells were restimulated with PMA + ionomycin for four hours in the presence of brefeldin A, fixed, stained for intracellular IL-4 or IFN-γ, and analyzed by flow cytometry. The numbers indicate the percentage of cells that fall into the indicated gates.
FIGURE 15. Northern blot analysis for GATA-3 expression in Left1-N27 transgenic Th1 and Th2 cells. Naive CD4+ T cells from wildtype and Left1-N27 transgenic mice were stimulated with anti-CD3, anti-CD28, and irradiated APCs for three days in the presence of Th1- or Th2-polarizing cytokines, rested for another three days in IL-2 plus polarizing cytokines, and then restimulated for six hours with 5μg/ml platebound anti-CD3. RNA was isolated from each cell sample, and 10μg total RNA was used for Northern blotting. The blot was probed sequentially with cDNA probes to GATA-3 and L32.
FIGURE 16. Normal STAT6 activation in Left1-N27 transgenic Th2 cells. Naive CD4+ T cells from wildtype and Left1-N27 transgenic mice were stimulated with anti-CD3, anti-CD28, and irradiated APCs for three days under Th2-polarizing conditions, then rested for another three days in IL-2 plus Th2-polarizing cytokines. After extensive washing with PBS, the cells were then cultured in medium containing low serum for five hours. The cells were divided in two and half were treated for 15 min. with IL-4. The cells were lysed and equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membrane, and blotted sequentially with antibodies to STAT6 or phosphorylated STAT6.
FIGURE 17. Inappropriate T-bet expression in Left1-N27 transgenic Th2 cells. Naive CD4+ T cells from wildtype and Left1-N27 transgenic mice were stimulated with anti-CD3, anti-CD28, and irradiated APCs for three days in the presence of Th1- or Th2-polarizing cytokines, rested for another three days in IL-2 plus polarizing cytokines, and then restimulated for six hours with 5μg/ml platebound anti-CD3. RNA was isolated from each cell sample, quantitated, and various dilutions were used in an RT-PCR reaction with primers specific for T-bet cDNA. Three-fold dilutions of total RNA, beginning with 200ng were used for each sample. RT-PCR was performed on 10ng total RNA of each sample with primers specific for β-actin as a control.
FIGURE 18. Left1-N27 transgenic Th2 cells maintain the ability to respond to IL-12. Naive CD4+ T cells from wildtype and Left1-N27 transgenic mice were stimulated with anti-CD3, anti-CD28, and irradiated APCs for three days in the presence of Th1- or Th2-polarizing cytokines, then rested for another three days in IL-2 plus polarizing cytokines. After extensive washing with PBS, the cells were then cultured in medium containing low serum for five hours. The cells were divided in two and half were treated for 30 min. with IL-12. The cells were lysed and equal amounts of protein immunoprecipitated with anti-STAT4 antibody. The resulting precipitates were separated by SDS-PAGE, transferred to PVDF membrane, and blotted with antibody to STAT4.
FIGURE 19. Expression of Left1 in primary T cells by retrovirus-mediated gene transfer. (A) Splenocytes from DO11.10 αβ TCR transgenic mice were activated with OVA<sub>323-339</sub> peptide under neutral, Th1-promoting, or Th2 promoting conditions. At one and two days after activation, the splenocytes were infected with empty pMIG-W retrovirus, or pMIG retrovirus containing Left1 or Left1N genes. At day seven after initial activation, the cells were restimulated with PMA + ionomycin for four hours in the presence of brefeldin A, fixed, stained for intracellular IL-4 or IFN-γ, and analyzed by flow cytometry. (B) DO.11.10 αβ TCR transgenic splenocytes infected with the indicated retroviral vectors were fixed and stained with anti-HA antibody at day seven after initial activation with OVA<sub>323-339</sub> peptide.
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**TABLE 3.** Summary of cytokine production by retrovirally transduced DO11.10 transgenic CD4⁺ T cells. The table summarizes the data from the experiment described in Figure 18A. The percentage of IL-4⁺ or IFN-γ⁺ cells within the indicated CD4⁺ T cell population seven days after initial activation is shown. The polarizing conditions (neutral, Th1, or Th2) are indicated in the upper left of each table.
References


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Chapter Four

Discussion
Calcineurin regulation by Cabin1  Calcineurin is a calcium and calmodulin-dependent serine/threonine phosphatase (1-3). It is ubiquitously expressed, but found most abundantly in the brain. Its essential role in T cell receptor (TCR)-mediated signal transduction was first recognized when it was identified as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506 (4-11). The primary substrates of activated calcineurin were identified as the NF-AT family of transcription factors (12, 13). Calcineurin directly dephosphorylates NF-AT proteins in the cytosol, resulting in their translocation into the nucleus. Calcineurin remains associated with NF-AT in the nucleus, where its continued activation by elevated calcium levels is required to sustain NF-AT transcriptional activity (14, 15). NF-AT acts in cooperation with other transcription factors such as AP-1 to stimulate the transcription of many genes important for T cell activation, including several cytokine genes such as IL-2, IL-4, and IFN-γ.

As calcineurin is essential for transmitting cytosolic calcium signals into the nucleus in T cells, its activation is tightly controlled. Calcineurin is activated by intracellular calcium and calmodulin (2). In resting T cells, the level of intracellular calcium is low and the C-terminal autoinhibitory domain binds the catalytic domain and blocks calcineurin enzymatic activity. Upon stimulation through TCR, the level of intracellular calcium is elevated and calmodulin becomes activated. Activated calmodulin then binds to calcineurin at a site located between the catalytic and the autoinhibitory domains, thus disrupting their interaction and activating calcineurin’s phosphatase activity. However, it is not well understood how calcineurin activity may be downmodulated in the presence of a calcium signal. Although the exogenous small molecules CsA and FK506 have long been known to potently inhibit calcineurin phosphatase activity, only recently have endogenous protein inhibitors been identified. AKAP79 (16, 17), DSCR1 (18, 19), and Cabin1/Cain (20-25) all inhibit calcineurin activity in transfection assays in the presence of calcium signal.

Murine Cabin1 (and its rat homolog Cain) was identified in a yeast two-hybrid screen for calcineurin-interacting proteins. Cabin1 is a ubiquitously expressed protein of 2220 amino acids. The calcineurin interacting domain in Cabin1 was mapped to 23
amino acid residues (2117-2140) in the C-terminus (24). When the full length Cabin1 or the C-terminal fragment was overexpressed in Jurkat T cells, it inhibited the transcriptional activation of calcineurin-responsive elements in the IL-2 promoter and blocked dephosphorylation of NF-AT upon T cell activation. The interaction between Cabin1 and calcineurin is dependent on both calcium signal and protein kinase C (PKC) activation, which results in Cabin1 hyperphosphorylation (24). As Cabin1 is found primarily in the nucleus in T cells, it may interact only with activated calcineurin that has translocated into the nucleus. It was also noted that Cabin1's calcineurin binding domain contains a peptide sequence motif (PEITVT) similar to the conserved motif found in the calcineurin binding domains of NF-AT family members (PxIxIT), suggesting that Cabin1 binding may prevent calcineurin from interacting with NF-AT (26). Based on these results, Cabin1 was hypothesized to function in down modulating calcineurin activity after T cell activation. However, all the assays examining the effect of Cabin1 on calcineurin activity have been done by overexpression of Cabin1 in cell lines. Often, only the C-terminal fragment of Cabin1 was transfected. From these experiments, it was not clear how important the Cabin1-calcineurin interaction is for proper regulation of calcineurin activity in primary T cells after activation.

**MEF2 regulation by Cabin1** In addition to binding to calcineurin, the C-terminal region of Cabin1 was shown to interact with myocyte enhancer factor 2 (MEF2) and calmodulin in a mutually exclusive manner (27, 28). MEF2 is a transcription factor that is normally bound to its recognition sequence in the promoter of target genes such as the apoptosis-inducing gene Nur77 in thymocytes (27, 29). Binding of Cabin1 to MEF2 suppresses MEF2 transcriptional activity through recruitment of the mSin3 corepressor complex via the N-terminal region of Cabin1 (27). However, in the presence of a calcium signal, calmodulin binds to Cabin1, freeing MEF2 to recruit the coactivator p300 for transcriptional activation of MEF2 target genes. Consistent with this model, overexpression of Cabin1 in a DO11.10 T cell hybridoma prevents induction of Nur77 by MEF2 and protects the cells from TCR-mediated apoptosis (28). As MEF2 is critical for the transcription of Nur77 in thymocytes, Cabin1 may also regulate thymocyte apoptosis during early T cell development. However, there are also several reports of additional MEF2 corepressors that directly bind MEF2, including MEF2 interacting transcription
repressor (MTR), and histone deacetylases 4 and 5 (30-34). The inhibition of MEF2 by these corepressors is also calcium-dependent but does not require Cabin1 as an intermediary (31, 34).

Calcium-dependent regulation of MEF2 transcription factors is well-established, and it is known to be at least in part dependent on calmodulin-dependent kinases such as CaMKIV (35-37). It has also been suggested that calcineurin plays a role in enhancing MEF2 transactivation. Calcineurin may act on MEF2 directly (38), but it is more likely to be mediated through NF-AT (35, 39). NF-AT may cooperate with MEF2 to coactivate target genes, or it may act indirectly. Either way, it suggests that Cabin1 can affect MEF2 both directly and indirectly, through its interactions with calcineurin. The relative importance of the different pathways in which Cabin1 is involved in vivo is difficult to know, however, as again all studies have been done in cell lines by overexpressing Cabin1.

**Creation of Cabin1ΔC mice** To examine the role of the calcineurin- and MEF2-binding domains of Cabin1 in T cell development and function under physiological conditions, we generated Cabin1ΔC mice, which produce a truncated Cabin1 lacking the last 123 amino acid residues from the C-terminus. We reasoned that deleting the entire protein in mice would be likely to result in severe and pleiotropic effects, making it difficult to assess the importance of Cabin1’s interactions with calcineurin and MEF2 in T cell development and function. Therefore, we targeted only the last two exons of Cabin1, which encode the C-terminal calcineurin, calmodulin, and MEF2 binding domains.

Although Cabin1ΔC mice have the pgk-neo' cassette removed from the locus, we also examined mice with the neo' cassette remaining at the locus. We inserted the neo' cassette in the opposite transcriptional orientation of the Cabin1 gene, between the coding sequence and the polyadenylation site. We suspected that this would result in a disruption of the Cabin1 transcript, either by interfering with polyadenylation or through an antisense RNA mechanism. In fact, mice homozygous for the Cabin1-neo allele died in utero by day E12.5 of undetermined causes. Unfortunately, the lack of an antibody specific for the N-terminal region of Cabin1 made it impossible for us to verify that it is truly a Cabin1 null allele. We also cannot rule out the possibility that a neighboring gene
is disrupted by the neo' cassette, accounting for the embryonic lethality of the allele. However, in light of Cabin1's ubiquitous expression, the lack of any known homologs, and its probable involvement in brain and muscle development, it is not surprising that a Cabin1 null mutation would result in embryonic lethality. However, an unambiguous Cabin1 null mutant mouse must be generated to be certain.

**Thymocyte selection in Cabin1ΔC mice**  MEF2 is thought to be important for expression of the apoptosis-inducing gene Nur77, and so we had predicted that deletion of Cabin1’s MEF2 binding domain would result in enhanced negative selection of Cabin1ΔC thymocytes. However, we did not observe any difference in induction of Nur77 or apoptosis in thymocytes from Cabin1ΔC mice after TCR stimulation, showing that the Cabin1 C-terminal domain is not uniquely required for proper regulation of thymocyte apoptosis. It is likely that the redundant MEF2 transcriptional corepressors can compensate for the deletion of the Cabin1 MEF2-binding domain.

It is not clear how Cabin1 regulation of calcineurin would be expected to influence thymocyte selection. Most evidence suggests that calcineurin signaling is important for positive, but not negative selection (40-42). If this is the case, we would predict that the Cabin1ΔC mutation would result in enhanced positive selection. However, some evidence implicates calcineurin in signaling negative selection as well (43-47), in which case we might expect both positive and negative selection to be enhanced.

It is possible that breeding the Cabin1ΔC mutation onto a TCR transgenic system such as the H-Y TCR may reveal an alteration in positive or negative selection. However, it is not clear what we expect to see. The MEF2 data suggest we should see enhanced negative selection, but calcineurin data suggest enhanced positive, and possibly negative selection. Either way, we would be assaying for an enhancement of positive or negative selection, which is more difficult to measure than an impairment of selection. Given the subtlety of the phenotype observed in mature T cells, the change in selection efficiency is likely to be slight and difficult to detect.

**Cytokine production by Cabin1ΔC T cells**  As predicted, after T cell activation we saw an increase in transcription of many cytokine genes, suggesting that Cabin1’s interaction with calcineurin is important for regulation of effector function. However, we
were not able to conclusively prove that the increase in cytokine transcription was actually mediated through calcineurin and NF-AT. Analysis of nuclear translocation of NF-ATc in Cabin1ΔC T cells after activation did not reveal any detectable difference. However, the difference in cytokine transcription in the Cabin1ΔC T cells is so slight, it may be that a slight increase in NF-ATc is responsible, but the assay is not sensitive enough to detect it. We also assayed for NF-ATp nuclear translocation (data not shown) and were again unable to detect an increase in CabinΔC T cells. It is possible that the increase in cytokines is caused by other Cabin1-interacting proteins. In particular, little is known regarding the role of MEF2 in mature T cells, and we have recent data showing that MEF2 nuclear localization and phosphorylation status is altered in Cabin1ΔC T cells after activation, showing that changes in MEF2 are correlated with increased cytokine transcription. It is not clear whether these changes are mediated through calcineurin, or are a direct result of deletion of Cabin1’s interaction with MEF2. There is still a possibility that other calcineurin target proteins, or other unidentified Cabin1-interacting proteins are responsible for the increased cytokine production by Cabin1ΔC T cells.

**Antibody response and Th2 development in Cabin1ΔC mice** Consistent with the cytokine data, we also observed an increased antibody response to T dependent antigen in Cabin1ΔC mice. We first noticed high serum immunoglobulin levels in unimmunized Cabin1ΔC mice, in particular of the IgG1 and IgE isotypes induced by IL-4 during a Th2 antibody response. The NP-specific IgG1 antibody response to the T-dependent antigen NP-KLH was also most prominently increased. However, we measured an increase in production of both Th1-promoting (IFN-γ) and Th2-promoting (IL-4) cytokines by Cabin1ΔC T cells *in vitro*. This suggests that Cabin1 may have a special role in the negative regulation of Th2 development or effector function *in vivo*.

One explanation for the selective enhancement of Th2 responses in Cabin1ΔC mice may be the relative importance of T cell-produced cytokines for Th1 and Th2 development. Th1 development is induced most potently by IL-12, which is produced by monocytes and dendritic cells – not by T cells themselves. Although IFN-γ can encourage Th1 development by inducing antigen presenting cells to produce IL-12 (48) and by maintaining expression of the IL-12 receptor (49), it is not sufficient to induce Th1 polarization. In contrast, although the original source of the IL-4 that drives Th2
development is not entirely clear, there is evidence that the IL-4 produced by T cells themselves is most important (50). It may be that although many cytokines are overproduced by Cabin1ΔC T cells, IL-4 plays a dominant role in influencing T helper cell development in vivo.

The selective enhancement of Th2 development in Cabin1ΔC mice is not surprising given the evidence that calcium signaling and NF-AT play a preferential role in regulation of Th2, but not Th1 responses. For example, T cells deficient in the Tec family kinase itk are defective in their ability to sustain a calcium flux and have corresponding defects in NF-ATc nuclear localization and IL-4 production (51). IFN-γ production by itk-deficient T cells is unaffected. As a consequence, itk−/− mice have defective Th2 responses, although Th1 responses are normal, confirming the importance of sustained calcium signaling for Th2, but not Th1 development.

Although NF-ATc and NF-ATp together are essential for induction of many cytokines, individually they have special roles in the regulation of IL-4 transcription and Th2 development. While NF-ATc−/− mice have severe defects in IL-4 production and Th2 development, NF-ATp−/− mice actually have increased IL-4 production and Th2 development. This surprising result has led to the theory that the ratio of the two proteins determines the level of IL-4 transcription. In the NF-ATp−/− T cells, the IL-4 promoter is occupied by NF-ATc with no opposition from NF-ATp, resulting in enhanced IL-4 transcription. If NF-ATp dephosphorylation by calcineurin is normally more sensitive to inhibition by Cabin1, it may be that deletion of Cabin1’s calcineurin binding domain has altered the balance of NF-AT transcription factors, allowing sustained IL-4 transcription in the Cabin1ΔC T cells.

It has also been noted that Th1 and Th2 cells produce different patterns of calcium signaling upon stimulation (52). Th1 cells produce a strong but transient calcium signal, while Th2 cells produce a lower but sustained calcium signal. This has been suggested to reflect the differential inducibility of several signaling pathways in Th1 versus Th2 cells. A strong, transient calcium signal may preferentially induce the MAPK pathway, which is known to be important for IFN-γ expression. Mutations in MAPK pathways affect Th1, but not Th2 development, probably through regulation of IFN-γ (53-55). However, the sustained calcium signal found in Th2 cells is necessary to maintain NF-AT in the
nucleus, which is important for IL-4 transcription. Consistent with this, one report found that although similar levles of NF-AT protein were present in Th1 and Th2 cells, NF-AT transcriptional activity was higher in Th2 cells than Th1 cells (56). This indicates that NF-AT plays a more important role in Th2 cytokine transcription than Th1. If this is the case, it would suggest that the enhanced Th2 responses we see in Cabin1ΔC mice are not due to an enhancement of Th2 development, but rather that after differentiation the Cabin1ΔC Th2 cells have enhanced IL-4 production.

A potential caveat is that we did not show directly that Th2 development is enhanced in Cabin1ΔC mice in vivo. We inferred from the pattern of Ig isotype switching that Th2 development or effector function is enhanced, but it is also possible that B cell signaling is altered, resulting in the observed pattern of isotype switching. Immunization with the T-independent antigen NP-Ficoll showed no difference in Cabin1ΔC mice compared to wildtype (data not shown), suggesting that B cell responses are normal. However, both BCR and CD40 signaling are CsA-sensitive. Although we did not observe any significant increase in Ig production by Cabin1ΔC B cells in vitro in response to anti-IgM or anti-CD40 treatment, there may be other signaling pathways responsible for the increased IgG1, IgG2b, and IgE observed in vivo. We did show that isotype switching to IgG1 and IgE in response to anti-CD40 + IL-4 was apparently not enhanced in Cabin1ΔC B cells in vitro, but it is not clear that the stimulation conditions used in vitro mimic the in vivo situation closely enough for us to draw conclusions about the in vivo B cell responses.

There is also a strong possibility that other cell types are affected by the Cabin1ΔC mutation, and could be responsible for promoting isotype switching to IgG1 and IgE, independent of the T helper cell response. In particular, both mast cells and eosinophils produce Th2-promoting cytokines, including IL-4 and IL-5, and that production is CsA-sensitive (57). More work needs to be done to examine the effect of the Cabin1ΔC mutation on signaling in non-lymphoid cell types.

Other considerations Several issues further complicate our interpretation of these results. First, although we could show the absence of the C-terminal domain of Cabin1 by Western blotting, the unavailability of a specific antibody against the N-terminus of Cabin1 made it impossible to show that a truncated protein was produced by the
Cabin1ΔC mice. Although Cabin1ΔC transcripts were amplified by RT-PCR and sequenced, showing that the structure of the transcript is as we predicted, we have no way to show conclusively that a truncated Cabin1 protein is being produced. Further attempts to make an antibody specific for the N-terminus of Cabin1 must be made.

Second, Northern blotting for Cabin1 transcripts showed a two-fold reduction in several tissues from Cabin1ΔC mice. We cannot rule out the possibility that the phenotype we observe is due more to the reduction in total Cabin1 protein than to the deletion of the C-terminal domain. This question is difficult to address, however. It might be resolved by examining the phenotype of mice heterozygous for a Cabin1 null mutation.

Finally, given the subtle phenotype we observe in the Cabin1ΔC mice, the background of the mice used in the experiments may be important. The mice used in these experiments were on a mixed C57Bl/6-129 background, and often the wildtype and Cabin1ΔC mice were not littermates. Although the data shown were reproducible with mice from several sets of breeding pairs, many of these experiments should be repeated with backcrossed mice. In fact, crossing the Cabin1ΔC mutation onto various mouse strains may reveal more about Cabin1’s C-terminal domain in specific signaling pathways as its function becomes more or less important in different mouse strains.

**Future directions** Our data show that the C-terminal domain of Cabin1 normally plays a role in negative regulation of T cell effector function and antibody response. However, Cabin1 certainly plays a role in many other tissues, including brain and muscle, where calcineurin and MEF2 are known to be important. Expression of Cabin1 in the brain coincides with calcineurin expression (22), and a study in which the calcineurin binding domain of Cabin1 was conditionally expressed in the brain of transgenic mice showed that Cabin1 can enhance LTP in vivo (58). Transgenic expression of the calcineurin binding domain in the heart was able to reduce cardiac hypertrophy (59, 60). In addition, nothing is known about Cabin1 outside of the C-terminal calcineurin and MEF2-binding domain. It is a large and probably multifunctional protein which will certainly provide many surprises in the future.
Memory T cell development  During an immune response, extensive clonal expansion of antigen-specific effector T cells occurs. After antigen clearance, however, they must be cleared from the body to make space for other T cells and to prevent them from causing damage to the host. Effector T cells become susceptible to Fas-mediated apoptosis within a few days of activation (61). Most are deleted by Fas-mediated apoptosis, or by the withdrawal of cytokine-induced survival signals. However, some are able to escape this deletion and differentiate into long-lived memory T cells. What signals, if any, are required for this transition are unknown.

We were interested in identifying changes in gene expression as a T cell differentiates from an effector to a memory T cell, in the hope of shedding light on this transition. The approach we used was a PCR-based cDNA subtractive hybridization to identify genes upregulated in memory CD8\\(^{+}\) T cells as they differentiate from effector CD8\\(^{+}\) T cells. The identification of known memory-specific genes such as IL-2R\\(\beta\\) showed that our approach worked, and many interesting genes were identified. For example, it was striking that many genes coding for cytoskeletal proteins were identified. In addition to the known cytoskeletal proteins listed in Table 1, Chapter 3, many of the novel genes identified had predicted protein sequences with significant homology to cytoskeletal proteins. This suggests that changes in the composition of the cytoskeleton may be important in the transition from effector to memory T cell.

However, it is important to remember that the transition from effector to memory T cell is also a transition from a dividing to a resting cell. Our screen probably identified some genes whose differential expression is not related specifically to memory T cell function, but simply to the transition to a resting state. It may not be surprising then that so many cytoskeletal proteins were identified in our screen, as one would expect an effector T cell to undergo dramatic changes in its cytoskeleton as it exits the cell cycle. Also, after examining the expression of many of the genes identified in the screen at different stages of T cell development, some of them were found to be expressed in both memory and naive T cells, which are both resting T cell populations. It may be fruitful in the future to do a screen in which cDNA from both naive and effector T cells is
subtracted from memory T cell cDNA to avoid the identification of genes related to
to changes in the cell cycle, and limit the screen to genes specifically related to memory T
cell differentiation.

**Identification of Left1** We selected one novel gene identified in the screen to examine more closely. The predicted protein sequence of this gene has homology to both FcεRIβ, a transmembrane protein important in IgE receptor-mediated signaling in mast cells (62), and CD20, a B-cell specific transmembrane protein which may be involved in B cell activation (63). The predicted structure of this protein, like FcεRIβ and CD20, consists of four transmembrane domains, with both the C- and N-termini found in the cytoplasm. We named the gene Lymphocyte-Expressed, Four-Transmembrane protein 1 (Left1), although another group independently identified Left1 as a gene expressed in Th1, but not Th2 cells, and called it Chandra (64). It has since been identified as a member of the MS4A protein superfamily, which includes CD20 and FcεRIβ, and assigned the name MS4a4B under a new standardized system of nomenclature (65).

Although the membrane topology of MS4A proteins is similar to proteins of the tetraspanin superfamily (66, 67), they lack the conserved amino acid sequences that define the tetraspansins. However, like the tetraspansins, both FcεRIβ and CD20 operate as components of larger complexes of membrane proteins, in which their roles may be to modify signaling through their associated proteins rather than to directly bind to their own extracellular ligands. Indeed, the predicted membrane structure of Left1 leaves only two extracellular loops of not more than 11 and 16 amino acids. While Left1 may have its own extracellular ligand, it is likely to be more important as part of a larger signaling complex.

In order to examine the role of Left1 in T helper cell responses and memory T cell development, we constructed transgenic mice in which Left1 is overexpressed in T cells. Analysis of Left1 transgenic mice revealed a role for Left1 in regulation of several stages of T cell development.

**Lineage commitment in Left1-N27 transgenic mice** In young Left1-N27 transgenic mice, there was a 2-3 fold increase in the proportion of CD8+ thymocytes, although the percentage of CD4+ thymocytes was unaffected. As the total number of thymocytes in the young mice was comparable to wildtype littermates, the absolute
number of CD8* thymocytes is increased in young Left1-N27 transgenic mice. In this line, transgenic expression is high in double positive (DP) thymocytes, although endogenous Left1 is not normally expressed until the single positive (SP) thymocyte stage of development. Apparently, ectopic expression of Left1 in DP thymocytes causes enhanced CD8* lineage commitment.

Only a few mutations are known to affect CD8* lineage commitment. Transgenic expression of activated Notch in the thymus promotes CD8* lineage commitment (68), and it is possible that Left1 expression at the DP thymocyte stage can induce or enhance Notch signaling, enhancing CD8* thymocyte differentiation. This possibility can be tested by monitoring Notch signaling pathways in Left1 transgenic DP thymocytes.

Intriguingly, mice deficient for the transcription factor IRF-1 have selective defects in CD8* lineage commitment (69). IRF-1 is a transcription factor which binds at the promoter of IFN genes and several IFN-inducible genes (70), and therefore plays an important role in promoting cell-mediated immunity. Transgenic overexpression of IRF-1 in mice has not yet been done to determine whether it can enhance CD8* lineage commitment. As the expression pattern of endogenous Left1 suggests that it plays a role in promoting cell-mediated immunity in CD8* T cells and CD4* Th1 cells, it is possible that Left1 signaling involves IRF-1 in DP thymocytes. It will be interesting to examine IRF-1 activation in Left1 transgenic DP thymocytes.

However, without knowing what signaling pathways Left1 is involved in, it is difficult to guess how it may be influencing CD8* lineage commitment. Breeding the Left1 transgene with several TCR transgene models could solve the puzzle of where the extra CD8* thymocytes come from. Are they cells which should have died from neglect which are now rescued by Left1 expression? Analysis of selection in a Left1 transgenic expressing a TCR in a non-selecting background would answer this question. Are they cells which would have been CD4* thymocytes, but are now redirected to become CD8* due to either an enhancement of CD8*-promoting signals or a repression of CD4+-promoting signals? Analysis of a TCR transgenic line which normally produces CD4* T cells would address this question. Are they cells which have been rescued from negative selection, and are therefore potentially autoreactive? Examination of a TCR transgenic line on a negatively selecting background would address that question.
Although the increase in CD8+ lineage commitment in younger Left1-N27 transgenic mice was clear, as the mice aged the defects in thymocyte development became pleiotropic. Thymic atrophy was accelerated compared to wildtype, and an accumulation of CD44+CD25- double negative (DN) thymocytes was evident. A corresponding decrease in DP thymocytes was observed. It seems that over time, Left1 accumulation in DN thymocytes blocked their expansion at a stage before TCRβ rearrangement, resulting in a decrease in thymic output. The block occurred at a different pace for each mouse. Understanding why this block occurs may shed light on the normal role of Left1 in mature T cells.

**Memory T cell development in Left1-N27 transgenic mice** A striking phenotype of the Left1-N27 transgenic mice was the dramatic increase in both CD4+ and CD8+ memory phenotype cells. The proportion of memory T cells increased dramatically as the mice aged in Left1-N27 mice compared to wildtype. However, the phenotype was more pronounced in CD4+ T cells, with greater than 60% of the CD4+ T cells exhibiting a CD62L+CD44hi memory phenotype by 4 weeks of age. This may be related to the higher levels of Left1 transgene expression in CD4+ T cells compared to CD8+ T cells in the N27 line.

How are the Left1-N27 T cells becoming memory cells? It was possible that the Left1-N27 transgenic memory T cells were arising from cells that had undergone spontaneous activation in vivo, in the absence of TCR engagement. To test this possibility, we bred the Left1-N27 transgenic with the 2C TCR transgenic line, which produces primarily CD8+ T cells. We found that the 2C TCR could suppress development of the memory phenotype in CD8+ Left1 transgenic 2C T cells (data not shown). As the 2C TCR has minimal cross reaction with environmental and auto antigens, this shows that the development of memory T cells in the Left1 transgenic is dependent on TCR signaling.

These data suggest that after TCR stimulation through cross reaction with environmental or auto antigens, more memory T cells are generated in the transgenic mice. One explanation may be that memory T cell differentiation after TCR stimulation is more efficient in the Left1 transgenic, leading to an accumulation of memory T cells which eventually displace naïve T cells in the periphery. Left1 may also be acting to
selectively promote survival of memory T cells, perhaps by making them more responsive to memory-specific growth factors such as IL-15, in the case of CD8\(^+\) T cells.

Another alternative is that more T cells are cross reacting with endogenous antigens in the Left1 transgenic due to a lowering of the threshold for TCR signaling, resulting in an increase in memory T cells. In support of this theory, we observed an increase in the proportion of CD4\(^+\) T cells expressing the early activation marker CD69 in Left1-N27 transgenic mice. We did not find an increase in CD69\(^+\)CD8\(^+\) T cells, which may reflect the milder phenotype they exhibit. However, in vitro we did not observe a difference in the ability of Left1 transgenic CD4\(^+\) T cells to proliferate or upregulate surface activation markers in response to TCR stimulation. This appears to argue against the hypothesis that Left1 is acting to lower TCR activation thresholds. However, if Left1, or a Left1-associated receptor, requires ligand engagement in order to influence TCR signaling, in vitro stimulation may not be optimal for this interaction, whereas the ligand may be present on dendritic cells or other antigen presenting cells which the transgenic T cells come into contact with.

One concern we had about the Left1-N27 T cells was that the increase in memory T cell numbers was a result of resetting TCR thresholds due to abnormal thymocyte development. If there is a defect in negative selection in the thymus, autoreactive cells may be escaping to the periphery, responding to self antigen, and producing memory T cells, regardless of the expression of Left1 in the mature T cells. In fact, we have anecdotal evidence that some older transgenic mice exhibit inflammation and thickening of their skin, possibly caused by autoimmune disease, which needs further investigation. Creation of a transgenic mouse in which high levels of Left1 are expressed only in mature T cells would be the ideal way to address this question.

We also noted that there were fewer CD4\(^+\) T cells in the lymph nodes of Left1-N27 transgenic mice compared to wildtype. CD4\(^+\) T cell numbers in the spleen and peripheral blood were normal, however. Most of the CD4\(^+\) T cells were memory phenotype, or CD62L\(^-\). As CD62L, or L-selectin, confers the ability to migrate into the lymph nodes (71), this may account for the reduced CD4\(^+\) T cell numbers in lymph nodes of transgenic mice. However, based on the reduced ability of the CD4\(^+\) T cells to respond to survival-promoting costimulatory and cytokine signals in vitro, we suspected that
survival of transgenic CD4\(^+\) T cells may also be impaired \textit{in vivo}. We did perform thymectomies on Left1-N27 transgenic mice and wildtype littermates and followed the numbers of CD4\(^+\) and CD8\(^+\) T cells in the peripheral blood for 6 months (data not shown). We found no difference in the numbers of T cells in the peripheral blood of transgenic mice, however, suggesting that survival of Left1-transgenic T cells is not seriously impaired \textit{in vivo}.

**Cytokine receptor signaling and T helper cell development** Upon activation with antigen, CD4\(^+\) T cells can differentiate into either Th1 or Th2 effector cells, which secrete different patterns of effector cytokines that promote distinct types of immune response (72, 73). The polarization of the T helper cell response can have a profound effect on the outcome of an immune response. Type 1 T helper cells secrete predominantly IFN-\(\gamma\), which promotes cell-mediated immunity and the inflammatory response to intracellular pathogens such as \textit{Leishmania major} (74). Th1 responses have also been implicated in certain types of autoimmune diseases (75). Type 2 T helper cells secrete IL-4, IL-5, IL-10, and IL-13, which promote antibody responses. Th2 responses are essential for successful clearance of extracellular pathogens such as the nematode \textit{Nippostrongylus brasiliensis} (76), and also play a role in allergic diseases (75). Although several factors have been shown to influence the polarization of naïve CD4\(^+\) T cells, the cytokine milieu in which T cells are activated is the most important determinant of T helper cell differentiation (73), and most work in this area has focused on the role of cytokines in controlling Th1/Th2 differentiation.

The most important cytokine for induction of Th1 development is IL-12 (77, 78), produced by macrophages and some dendritic cells. Signaling through the IL-12 receptor induces STAT4 activation (79), which is required for Th1 development and for high levels of IFN-\(\gamma\) production in T cells (80, 81). IFN-\(\gamma\) promotes Th1 development by stimulating IL-12 production by monocytes and dendritic cells (48) and by upregulating expression of the \(\beta2\) subunit of the IL-12 receptor (49). IL-12 receptor signaling through STAT4 is also responsible for suppressing the expression of Th2-specific genes such as the GATA-3 transcription factor and IL-4 (82). The Th1-specific transcription factor T-bet is initially induced by TCR signaling (83), but it may require IL-12 signals to sustain
expression in Th1 cells, because it is rapidly downregulated in Th2 polarized effector cells.

Th2 development is promoted primarily by IL-4 (84, 85). Signaling through the IL-4 receptor induces STAT6 activation (86, 87), which is required for Th2 development and IL-4 production by Th2 cells (88). STAT6 promotes IL-4 transcription through its induction of the Th2-restricted GATA-3 transcription factor, which can induce several Th2 cytokines (89). Fully differentiated Th2 cells become refractory to subsequent Th1-polarization by downregulation of IL-12Rβ2 expression so that they are no longer able to respond to Th1-inducing signals (49, 90). STAT6-dependent induction of the transcription factor GATA-3 is important for this commitment (91).

The expression of T-bet and GATA-3 transcription factors in Th1 and Th2 cells, respectively, is mutually exclusive, and appears to play a determining role in the decision of a naive CD4+ T cell to differentiate into a Th1 or Th2 effector cell. For this reason, considerable effort has been made to understand the regulation of their expression in the expectation that it will provide insight into the molecular basis for Th1/Th2 differentiation.

**Left1 and T helper cell development** Although we initially identified Left1 in a screen for genes expressed in memory CD8+ T cells, it was also identified by another group in a screen for genes expressed in Th1, but not Th2 cells (64). This group also showed that downregulation of Left1 in Th2 cells required IL-4 signaling through STAT6 (64). As IL-4 signaling through STAT6 is known to be important for suppression in Th2 cells of several Th1-promoting genes, we suspected that this downregulation may be required for Th2 effector cell development. This was confirmed by our analysis of the Left1-N27 transgenic mice which maintain expression of Left1 in T cells under Th2-polarizing conditions. The Left1 transgenic Th2 cells had severely reduced levels of transcription of the Th2 effector cytokines IL-4, IL-5, IL-10, and IL-13. There were modestly reduced levels of GATA-3 transcripts, which may in part be responsible for the reduced cytokine production. However, STAT6 activation in response to IL-4 receptor signaling was not disrupted by Left1 expression. We also found that the transgenic Th2 cells maintained some characteristics of Th1 cells. Expression of the Th1-specific T-bet transcription factor was observed, and the transgenic Th2 cells maintained the ability to
activate STAT4 in response to IL-12 treatment, indicating that they had not downregulated IL-12Rβ2 expression.

Based on these data, Left1 may regulate T helper cell development through various mechanisms. Left1 may normally promote Th1 responses, with ectopic expression in Th2 cells causing inappropriate Th1-specific gene expression. Left1 cannot promote Th1 development simply through maintenance of IL-12Rβ2 expression, however, because maintenance of IL-12R expression or activation of the pathway in the presence of IL-4 signaling, does not suppress Th2 development and cytokine production (92, 93). It was recently suggested that IL-12Rβ2 upregulation and STAT4 activation are secondary to T-bet expression in commitment of Th1 cells (94). Left1 may normally act to promote Th1 effector function through upregulation of T-bet, resulting in IFN-γ and IL-12Rβ2 transcription and suppression of Th2 cytokines. This model is consistent with our observations, although we might have expected enhanced levels of IFN-γ production in the transgenic Th1 cells, which we did not observe. However, there were also general defects in activation of Left1 transgenic T cells which may account for this result.

The other possible explanation for Left1 action is that it triggers signals which are normally responsible for inhibiting Th2 effector function. These signals would have to act downstream of STAT6 activation, since STAT6 phosphorylation in response to IL-4 was normal in the transgenic Th2 cells. Left1 expression does result in inhibition of GATA-3 expression, and possibly other Th2-specific genes such as c-maf, which we have not yet examined. The diminished Th2 cytokine production would follow as a consequence of GATA-3 downregulation by Left1. The downregulation of GATA-3 by Left1 may also account for the maintenance of T-bet and IL-12Rβ2 expression in the transgenic Th2 cells.

These possibilities may be tested by examining the phenotype of Left1-deficient Th1 effector cells. If Left1 normally acts to promote Th1 development or effector function, and its action is nonredundant, we would expect Left1 knockout mice to have impaired Th1 responses and perhaps produce reduced levels of IFN-γ. We would not expect to see any enhancement of Th2 responses. However, if it is primarily involved in suppression of Th2 gene expression, we may observe an incomplete polarization of Th1 cells, inappropriate Th2 gene expression in Left1 knockout Th1 cells, and perhaps
enhanced Th2 responses in the knockout mice. Of course, as the promotion of Th1 development and suppression of Th2 development are so intertwined, it may be difficult to separate the two, as we have found in the Left1 transgenic Th2 cells.

**Other considerations** We were unable to reproduce these experiments in a second transgenic line, and experiments in which a Left1 gene was introduced into Th2 effector cells by retrovirus-mediated gene transfer were also unsuccessful in inhibiting Th2 cytokine production. However, in both cases we showed that significantly lower levels of Left1 were expressed in the Th2 cells, compared to the N27 transgenic line. Left1 apparently requires a threshold level of expression in order to inhibit Th2 effector function. What does that tell us about how it may act to inhibit Th2 effector function? We predict that Left1 is a component of a receptor complex, based on the homology with FcεRIβ and CD20. As only a small piece of Left1 is exposed on the membrane, it may not have a ligand independently of its associated receptors. Left1 does not have an immunoreceptor tyrosine-based activation motif (ITAM), as FcεRIβ does. Although CD20 doesn’t have an ITAM, it has been shown to co-immunoprecipitate with several src family protein tyrosine kinases through an unknown associated protein (95-97). As the cytoplasmic tail of CD20 was not required for the interaction (95, 97), it was assumed to be associated through lateral interactions with other membrane proteins. Left1 may also act to amplify signaling through associated receptors, promoting Th1 development.

We also cannot rule out the possibility that the observed phenotype in the N27 transgenic line are due to transgene integration that has altered expression of a nearby gene. It would be surprising, however, if disruption of one copy of a neighboring gene by transgene integration, through haploinsufficiency, would cause such a dramatic phenotype only in T cells, and affect T helper cell development in such a specific way. Nonetheless, this possibility cannot be formally excluded until a second transgenic line is produced with the same phenotype, and efforts are being made to do this.

Another potential problem in interpreting our results is the presence of the HA epitope tag at the N-terminus of Left1. As we don’t yet know anything about how Left1 associates with other proteins, we cannot predict how the epitope tag will affect its function. Further biochemical analysis of Left1 is required to resolve this potential problem.
Future directions Based on the prominent expression of Left1 in CD8\(^+\) T cells
and CD4\(^+\) Th1 cells, it is likely that Left1 normally plays a role in promoting cell-
mediated immunity or inflammatory responses. This may be mediated by upregulation of
T-bet and promotion of IFN-\(\gamma\) expression, as overexpression of Left1 in CD8\(^+\) T cells
results in increased IFN-\(\gamma\) production after stimulation, and in CD4\(^+\) T cells results in
impaired Th2 development and function. The known role of IRF-1 in CD8\(^+\) lineage
commitment suggests that IFN signaling pathways may also be involved in promoting
CD8\(^+\) T cell development, which might explain how ectopic expression of Left1 in DP
thymocytes results in enhanced CD8\(^+\) lineage commitment.

It is not surprising then that we would identify Left1 in a screen for genes
expressed in memory CD8\(^+\) T cells. Left1 expression may be in part responsible for the
enhanced effector function of CD8\(^+\) and CD4\(^+\) Th1 memory cells, as its expression
apparently makes cells more easily triggered to produce IFN-\(\gamma\) after TCR stimulation.

In order to learn more about the role of Left1 in regulation of cell-mediated
immunity, it will be informative to identify Left1-associated proteins and the signaling
pathways that Left1 can regulate. We have tried co-immunoprecipitation experiments
after surface biotinylation of EL4 mouse thymoma cells transfected with Left1, but our
attempts under several different detergent conditions were unsuccessful. It may be
fruitful to try the experiment after metabolic labeling of the cells instead of surface
biotinylation. It is also a concern that EL4 cells may not express some of the proteins
that Left1 normally associates with, so it may help to try other cell lines or primary T
cells. Production of a stimulating monoclonal Left1 antibody would also allow us to
induce signaling through Left1 in transfected cell lines and identify the signaling
pathways it can modulate.

As mentioned earlier, making a Left1 knockout mouse would clarify the role of
Left1 in T helper cell development. It will also be useful for understanding the role of
Left1 in CD8\(^+\) T cell function. Will IFN-\(\gamma\) expression by CD8\(^+\) T cells be affected in the
knockout mice? We will want to examine whether CD8\(^+\) memory T cell development or
function is impaired in the knockout as well.

Although we do not yet know the signaling pathways through which Left1 is
inhibiting Th2 development and effector function, it is clear that Left1 downregulation is
essential for inhibition of the Th1-specific transcription factor T-bet and commitment to the Th2 lineage. Future investigations into the mechanism of Left1 function should provide new insights into the molecular basis of T helper cell differentiation.
References

5. Friedman J, Weissman I. 1991. Two cytoplasmic candidates for immunophilin action are revealed by affinity for a new cyclophilin: one in the presence and one in the absence of CsA. Cell 66: 799-806


