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Ce mémoire intitulé:

The effect of Notch1 intracellular domain in T cell development and transformation

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The effect of Notch1 intracellular domain in T cell development and transformation

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The effect of Notch1 intracellular domain in T cell development and transformation

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Résumé

Notch1, l'homologue du gène Notch chez la drosophile, code pour une large protéine transmembranaire. Le domaine extracellulaire contient 36 répétitions du motif EGF et 6 répétitions Lin12/Notch (LNR). Le domaine intracellulaire contient 6 motifs cdc10/ankyrines, une région OPA et une région PEST à l'extrémité C-terminale. Quatres protéines Notch ont été identifiées chez les mammifères : Notch1, Notch2, Notch3, Notch4. Ces quatres protéines, les deux ligands Jagged 1 et Jagged 2, ainsi que Hes1, un gène cible de Notch, ont un rôle dans le développement des thymocytes. Des études ont montré que le domaine intracellulaire de Notch1 est impliqué dans la survie des thymocytes et dans leur différenciation en CD4/CD8 ou TCR$\alpha\beta$/TCR $\gamma\delta$. Les formes tronqués de Notch1, Notch2 et Notch3 sont impliqués dans la lymphomagenèse des cellules T.

C-myc, un membre de la famille des proto-oncogènes *myc*, joue un rôle important dans la prolifération et la différenciation cellulaire. Nos études antérieures ont montré que les souris transgéniques qui expriment *c-myc*, sous le contrôle du promoteur LTR du virus DMBA-LV-MMTV^{D}, développent des thymomes de manière spontanée. En utilisant la mutagenèse par insertion proviral dans ces souris, nous avons trouvé que 52% des tumeurs avaient des mutations dans le gène Notch1. Ces mutations sont produites par des réarrangements casés par l'insertion proviral. Deux régions d'insertion ont été ainsi identifiées. Une de les régions contient la portion génomique codant pour la séquence protéique situé entre la 34ème répétition d'EGF et le segment

transmembranaire. Elle résulte en la formation d'une forme tronquée de Notch1, appelé "type I". En général, les tumeurs avec des insertions de type I produisent un haut niveau d'ARN tronqué de 3-4Kb. Ces ARN commencent au niveau du site d'integration et se terminent à l'éxtrémité 3' de gène. Ils codent pour une protéine tronquée nommé $Notch1^{Intra}$ qui contient une protion du domaine extracellulaire, le domaine transmembranaire et toute la portion intracellulaire. Ces résultats suggèrent que $Notch1^{Intra}$ est impliqué dans la transformation des cellules T et coopère avec *c-myc* danns la tumeurogenèse.

Afin d'évaluer le rôle de $Notch^{intra}$ dans le développement et la transformation des cellules T, nous avons généré des souris transgéniques (Tg) qui expriment le gène $Notch^{intra}$ sous le contrôle du promoteur CD4 humain. Chez les jeunes souris Tg, nous avons constaté une augmentation significative du nombre de cellules double positives $CD4^+$ $CD8^+$ avec une forte expression des gènes CD25 et TCR$\alpha\beta$. L'expression de $Notch^{intra}$ procure une protection contre l'apoptose induite, chez les thymocytes double positives, par la dexaméthasone. Par ailleurs, ces souris Tg développent des thymomes clonales ou oligoclonales de type $CD4^+$ $CD8^+$ après un temps de latence relativement long. Afin d'étudier la coopération entre $Notch^{intra}$ et c-myc dans la transformation cellulaire, des souris Tg CD4C/$Notch^{intra}$ ont été croisées avec des souris Tg MMTVD/c-myc. La progenie de ce croisement développe, très tôt (29-44 jours), des thymomes, en comparaison avec les souris CD4C/$Notch^{intra}$ ou MMTVD/c-myc qui développe ntles mêmes thymomes en 150 jours. Ces résultats indiquent que $Notch^{intra}$ peut

agir comme un oncogène ou coopérer avec d'autres facteurs, comme *c-myc*, pour initier la transformation des thymocytes double positives.

Abstract

Notch1, the mammalian homolog of Drosophila Notch gene, encodes a large transmembrane protein, which is divided into two portion by the cytomembrane. The extracellular portion of the Notch1 protein contains 36 EGF repeats and 6 LNR (Lin-12/Notch/Repeat). The intracellular domain includes RAM domain, six cdc10/ankyrin motifs and OPA, PEST region in its C-terminus. In mammals, the Notch family is consisted of four members, Notch1, Notch2, Notch3, and Notch4. Notch1, Notch2 and Notch3, Notch special ligands (Jagged1 and Jagged2) and Notch target gene (Hes1) have been found to be involved in thymocyte development. A significant role for Notch1 intracellular domain in thymocyte survival as well as in differentiation to a CD4/CD8 or TCR$\alpha\beta$/TCR$\gamma\delta$ phenotype has been described. Moreover, the roles of truncated forms of Notch1, Notch2 and Notch3 have been implicated in the T cell lymphomagenesis.

c-myc is a member of the *myc* proto-oncogene family which plays an important role in growth regulation and differentiation of several cell types. Our previous studies showed that transgenic mice expressing *c-myc* under the control of the DMBA-LV mouse mammary tumor virus (MMTV^{D}) LTR promotor spontaneously developed thymic lymphomas. Using provirus insertional mutagenesis approach in these transgenic mice, it was found that *Notch1* was mutated in a high proportion of tumors (52%). The proviral integration sites focused on two regions and caused the *Notch1* gene rearrangement. One of these regions consisted of genomic regions coding for sequences between the

34th EGF repeat and the transmembrane (TM) of Notch1, resulting in the production of what we termed "type I" truncated *Notch1* alleles. Typically, tumors with type I proviral insertions produced elevated levels of 3-4 kb truncated RNA transcripts, initiating at the integration site and terminating at the 3' end of the gene, and thus encoding truncated Notch1 protein named Notch1^{intra}. The Notch1^{intra} contained a portion of ectodomain, TM and completed cytoplasmic domain. These results suggest that Notch1^{intra} may be involved in T-cell transformation and probably cooperate with *c-myc* in T cell tumor generation.

To determine the role of Notch1^{intra} in T cell development and transformation, we generated transgenic (Tg) mice in which the Notch1^{intra} gene was under the control of the human CD4 (CD4C) promoter. Studies in CD4C/Notch1^{intra} young Tg mice showed an increased number in CD4^{+}CD8^{+} double positive (DP) thymocytes and an increased percentage of CD25^{+}, high level expression of CD2 and intermediant level expression of TCR$\alpha\beta$ in CD4^{+}CD8^{+} cells. Furthermore, the expression of Notch1^{intra} protected DP thymocytes from dexamethasone-induced apoptosis *in vitro* and *in vivo*. Tg mice spontanously developed clonal or oligoclonal CD4^{+}CD8^{+} thymic lymphomas after a relatively long latency. To study the cooperation between Notch1^{intra} and c-myc in tumorigenisis, CD4C/Notch1^{intra} Tg were crossed with MMTV^{D}/c-myc Tg mice to establish a double Tg model. All double Tg mice developed thymic lymphomas at a latency which was much shorter than that in single Notch1^{intra} Tg littermates or single c-myc Tg littermates. These results indicate that Notch1^{intra} can behave as an oncogene to initiate DP thymocyte dysregulation, then transformation. The

acceleration of tumorigenesis in the double Tg mice provided a directed evidence that Notch1^{intra} and c-myc act in cooperation for lymphomagenesis.

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List of Abbreviations

EGFRs	growth factor-like repeats
LNR	Lin/Notch repeats
TM	transmembrane domain
CSL	RBP-Jk/CBF1, Suppressor of Hairless[Su(H)] and Lag-1
ANK	cdc10/ankyrin repeat
Dsh	Dishevelled
NIs	nuclear localization signals
NCR	Notch Cytokine Response
TAD	transcriptional activation domain
DSL	Delta, Serrate and Lag-2
ER	endoplasmic reticulum
TGN	trans-Golgi network
KUZ	metalloprotease-disintegrin Kuzbanian
TACE	TNF-alpha converting enzyme
CR	cysteine-rich region
EBNA2	Epstein-Barr nuclear antigen2
E(spl)	Enhancer of split
BHLH	basic helix-loop-helix
Ac-Sc	Achaete-Scute
JNK	c-jun N-terminal kinase
SOP	sensory organ precursor

Wg	wingless
PTB	phosphotyrosine-binding
T-ALL	T cell acute lymphoblastic leukemia
WAP	whey acidic protein
MMTV	mouse mammary tumor virus
$MMTV^{D}$	DMBA-LV mouse mammary tumor virus
HSCs	hemopoietic stem cells
MHC	major histocompatibity complex
TCR	T cell receptor
SP	single positive
DP	double positive
DN	double negative
Notch1IC	Notch1 intracellular domain
$Notch1^{intra}$ and $Notch1EC^{Mut}$t	Notch1 intracellular domain and extracellular domain specific found in MuLV-infected $MMTV^{D}$/myc Tg mice.
Tg mice	transgenic mice
MuLV	Moloney murine leukemia virus
RT	reverse transcription
FACS	Flow cytometric
FITC	fluorescein isothiocyanate
PE	phycoerythrin
FSC	forward scatter

SSC	side scatter
HE	hematoxylin and eosin
HSA	heat shock antigen
BRK	baby rat kidney
TAD	transcriptional activation domain

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Part I: Introduction

Introduction

Cell-cell interactions play an important role in regulating cell fate decisions during the development of multicellular organisms. One of the evolutionarily conserved pathways in local cell interactions is mediated by the transmembrane receptors encoded by the *Notch* gene of Drosophila, the *lin-12* and *glp-1* genes of *C. elegans* and their vertebrate homologies. Human and mice have four Notch genes. They are *Notch1*, *Notch2*, *Notch3* and *Notch4*. The structures of the proteins encoded by these genes are strikingly similar to Drosophila Notch. Recently, Notch1 intracellular domain has been found to be involved in T cell transformation, differentiation and apoptosis in mouse bone marrow transplantation models, or mouse transgenic models, or T cell lines. This introduction will focus on mammalian Notch function in these fields.

1. Notch Structure

Drosophila Notch is a protein with single-pass transmembrane domain which molecular weight is about 300kDa. The Notch extracellular domain contains 29-36 tandem epidermal growth factor-like repeats (EGFs) and three lin/Notch repeats (LNR), which function in ligand binding and Notch activation. The order of the EGFs has been conserved among the Notch proteins, suggesting it is important for function. Mutations in different EGFs of Drosophila Notch produce different developmental phenotypes (Hartley et al., 1987; Kelley et al., 1987). The LNR is a cysteine-rich region and is located immediately downstream of the EGF-like repeat region. This conserved motif appears to

negatively regulate receptor activation, since deletion of the LNR from Drosophila Notch, and missense mutations in this motif in either Drosophila *Notch* or C. elegans *Lin-12*, produce constitutively active receptor proteins. (Greenwald and Seydoux, 1990; Lieber et al., 1993; Lyman and young, 1993). The conserved cysteines between the LNR and the transmembrane domain (TM) are likely involved in disulfide bonding of the heterodimeric receptor (Greenwald and Seydoux, 1990; Blaumueller et al, 1997).

The Notch intracellular domain contains three identifiable regions that affect function: (1) a subtransmembrane region between the transmembrane domain and the ankyrin repeats (ANK), recently named RAM. RAM is found to interact with CSL {RBP-Jk/CBF1, Suppressor of Hairless[Su (H)] and Lag-1} effectors of Notch signaling pathway (Rheon, et al., 1996; Tamura, et al.,1995; Kato, et al., 1997); (2) six cdc10/ankyrin repeat (ANK), motifs involved in protein-protein interactions. This is the most highly conserved region and is essential for Notch signal transduction (Rebay, et al., 1993; Lieber, et al.,1993; Roehl, et al., 1993; Kopan, et al., 1994). This region also interacts with Notch intracellular effector Deltex. (3) C-terminal region OPA and PEST, has been associated with distinct protein interaction and transactivation. It seems to contain a negative regulatory domain, because removal of the C-terminal sequences to the ANK (including the PEST) in either *Drosophila* Notch or GLP-1 produce dominant gain-of-function phenotypes (Lyman and Young, 1993). The *Dishevelled* (*Dsh*) and Numb proteins, the modifiers of Notch signaling pathway, have been shown to bind to the C-terminal portion of Notch intracellular domain, and genetic

studies suggest that these interactions are inhibitory for Notch signal transduction. (Axelrod et al., 1996; Guo et al., 1996; Zhong et al., 1996). The PEST domain alone is thought to regulate protein stability. Not all the Notch homologies contain OPA, just Drsophila Notch and mammalian Notch1, 2, and 3 contain this motif. OPA is a glutamine-rich domain, but the function for this motif alone is still not clear. Drosophila and mammalian Notch also contain nuclear localization signals (nls). The two nuclear localization sequences have been found to reside on either side of the ANK repeat (Stifani et al., 1992; Lieber et al., 1993; Kopan et al., 1994). Recently, two new functional regions between ANK and OPA had been described in mammalian Notch, named Notch cytokine response (NCR) and transcriptional activation domain (TAD). NCR is associated with distinct effects of Notch1 and Notch2 on myeloid differentiation (Bigas A, et al.,1998). TAD contributes to the transactivation activity of mouse Notch1 mediated by RBP-J (Kurooka H, et al., 1998). The general structure of Notch and its effectors are showed in figure 1 (Milner and Bigas, 1999).

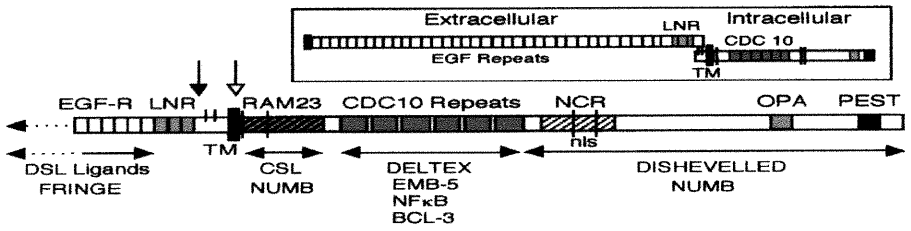

Fig. 1 The general structure of Notch protein

2. Notch signal transduction

During development, multipotent progenitors undergo lineage commitment and maturation. Although many factors contribute to different gene expression, signaling between cells is one of the key components of gene regulation and consequent appropriate cell fate specification. The Notch family members function both as cell surface receptors and direct regulators of gene transcription. Signaling through the Notch pathway allows signal transduction from the cell surface to the nucleus, also leading to direct influence on gene expression of neighboring cells. In general, Notch activation leads to transcriptional suppression of lineage–specific genes, inhibiting differentiation in response to inductive signals.

2.1 Conserved components of the Notch signaling pathway

The gene encoding the Notch receptor was discovered in flies almost 80 years ago by the fact that partial loss of function results in notches at the wing margin. Notch received its notoriety as a result of classic embryonic analyses of lethal loss-of-function mutations. These mutations produced a "neurogenic" phenotype, where cells destined to become epidermis switch fate and gave rise to neural tissue (Artavanis-Tsakonas, et al., 1999). The *Notch* gene first characterized in *Drosophila melanogaster*, and its homologies have been found from *Lin-12* in worms *C. elegans* to *TAN-1* in human being. In addition to Notch receptor, Notch signaling pathway consists of Notch ligands and effectors including extracellular ligands (DSL family, Delta / Serrate / Lag-2), intracellular

effectors (CSL family and Deltex) and target genes. The functional domains of Notch and its ligands are evolutionarily conserved from worms, flies, and mammals. These DSL ligands, and other effectors, targets, as well as modulators in Notch signaling pathway are summarized in table 1 (Milner and Bigas, 1999).

2.2 Activation of Notch and Notch signaling pathway

Several recent studies suggest a model for Notch processing and activation that involves two distinct proteolytic events: the first to generate a functional Notch receptor and the second to activate Notch in response to ligand binding.

Notch is synthesized in the endoplasmic reticulum (ER). Some experiments showed that proteolytic processing of full-length Notch is an essential step in the formation of the biologically active receptor (Blaumueller, et al., 1997). Only the cleaved fragments are present at the cell surface and not the full-length Notch. Full-length Notch just reflects newly synthesized, intracellular and hence, inactive molecules. It has been showed that the post-translation proteolytic events seem to regulate the activities of Notch receptor and its ligands. By using various treatments to block vesicle trafficking, it is demonstrated that the full-length Notch is cleaved in the trans-Golgi network (TGN) to produce two fragments: Notch extracellular domain and Notch intracellular domain. The latter includes a portion of extracellular domain, transmembrane domain and intracellular domain. The molecular weights of these

Table 1. Conserved Components of the Notch Signaling Pathway

	C. elegans	Drosophila	Mammal
Notch receptors	Lin-12 Glp-1	Notch	Notch1 Notch3 Notch2 Notch4
Extracellular ligands	Lag-2	Delta	Delta-1
(DSL proteins)	Apx-1		Delta-like 1 (Dll-1)
			Delta-like 3 (Dll-3)
		Serrate	Jagged1 (Serrate1)
			Jagged2 (Serrate2)
Intracellular effector	Lag-1	Suppressor of Hairless [Su(H)]	CBF-1/RBP-J
		Deltex	Deltex
			NFκB
Target genes		Enhancer of split [E(spl)]	HES (Hairy/Enhancer of split)
		bHLH	bHLH
		Groucho	TLE
Processing molecules	SUP-1	Kuzbanian	Kuzbanian
Modifiers		Fringe	Lunatic Fringe
			Manic Fringe
			Radical Fringe
		Numb	Numb
			Numb-like
		Dishevelled	Dishevelled 1,2,3

two fragments are p200 and p120, respectively. These fragments are tethered together on the plasma membrane via a DTT-sensitive link forming a heterodimeric receptor (Blaumueller, et al., 1997) This cleavage site was confirmed by another group, which worked with mouse Notch1 in cultured mammalian cells (Logeat et al., 1998). This heterodimeric Notch receptor is the basis that Notch interacts with ligands and activates Notch signaling pathway.

Recently, this cleavage site was named site 1 (S1). More experiments gave evidence that the S1 of full-length Notch located at the carboxyl side of the sequence RQRR (amino acids 1651-1654). This site is situated between LNR and TM domain. Two enzymes have been found to be involved in this proteolytic processing. The metalloprotease-disintegrin Kuzbanian (KUZ) has been implicated in the processing of *Drosophila* Notch and mammalian Notch2 (Pan and Rubin, 1997). KUZ belongs to the recently defined ADAM family of transmembrane proteins, members of which contain both disintegrin and metalloprotease domain. This is an emerging gene family that consists of at least 20 members in the mouse genome. The prototypes of the ADAM family, fertilin α and β(also called PH-30 α and β), are sperm surface molecules implicated in sperm-egg binding during fertilization. Another ADAM protein, meltrin, has been implicated in myoblast fusion. More recently, an ADAM protein was identified as a TNF-alpha converting enzyme (TACE) that is responsible for releasing TNF-α from the cell surface by proteolytic cleavage of a transmembrane precursor (Pan and Rubin, 1997). However, the furin-like convertase was found to be responsible for the processing of murine Notch1 (Logeat et al., 1998). These two

results suggested that Kuz is not an invariant part of Notch signaling. Different mechanisms may be involved in processing the different Notch orthologues, or different cell types may use distinct mechanisms, variables that could contribute to specificity of Notch signaling in mammals.

The second proteolytic event to activate Notch receptor and initial Notch signaling pathway depends on the interaction of Notch with its extracellular ligands on the surface of adjacent cells. Recently, two groups independently found the existence of an ectodomain shedding-like cleavage event (S2) induced upon ligand binding. Peptide sequencing showed that S2 cleavage occurs between Ala-1710 and Val-1711 residues, approximately 12 amino acids outside of the TM domain (Mumm et al., 2000; Brou et al., 2000). TACE has been identified as a protease capable of S2 proteolysis *in vitro* (Brou et al., 2000). The S2 cleavage produced a constitutively active, membrane-tethered, deleted Notch extracellular domain protein (NEXT, Notch extracellular domain truncated). This gives rise to a substrate that is readily recognized by the protease responsible for S3 processing. S3 is a ligand binding dependent site, which is located between Gly1743 and Val1744 within the transmembrane domain (Schroeter et al., 1998). S3 cleavage serves to release the Notch intracellular domain (NICD) from the membrane. NICD is then translocated to the nucleus where it functions as a transcriptional activator in concert with CSL family members (Jarriault et al., 1995). It is known that TACE, presenilin1 and a presenilin-1-dependent γ-secretase-like proteases are involved in this cleavage (Brou et al., 2000; Song et al., 1999 Strooper et al., 1999). Kopan's group presented evidence that

production of NEXT and NICD is linked: NEXT is enriched by blocking NICD production via point mutation, γ-secretase inhibitors, and loss of presenilin1, while inhibition of NEXT production eliminates NICD accumulation (Mumm et al., 2000). These data suggest that a ligand-induced proteolytic cascade activates Notch1: ligand binding serving to promote S2 cleavage, which is required for S3 cleavage. The location of these three cleavage site is summarized in fig.2

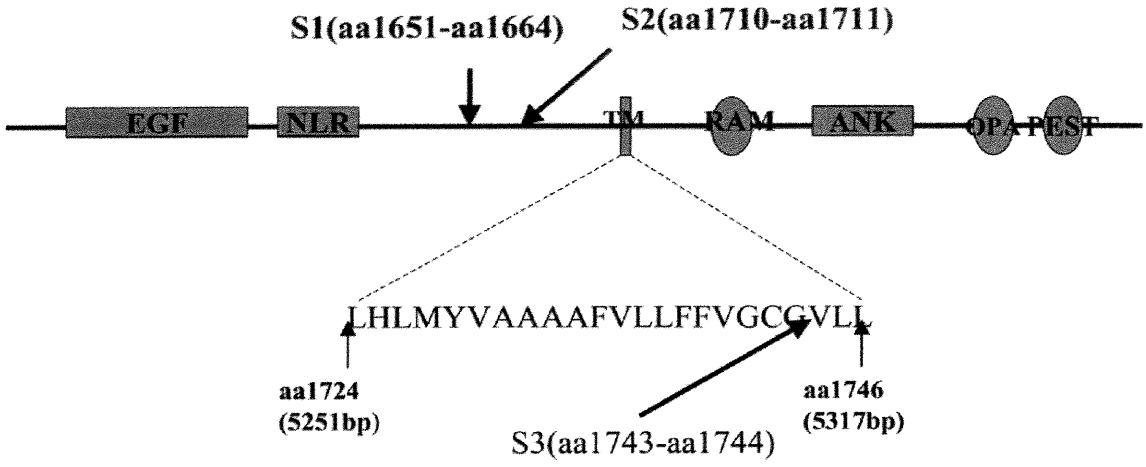

Fig.2 Notch cleavage site

The ligands that bind and activate the Notch/Lin-12/Glp-1 receptors belong to the DSL family, defined by the invertebrate ligands Delta, Serrate, and Lag-2. In Drosophila, the ligands are Delta and Serrate, in vertebrates, Delta and Jagged, and in C. elegans, LAG-2 and APX-1. The DSL family of Notch ligands, like Notch itself, has multiple EGF–like repeats in their extracellular domains. However, unlike Notch, they all possess a characteristic, degenerate EGF N-terminal to the EGFs (known as the DSL domain) which is required for function in invertebrates (Henderson et al., 1994; Lambie and kimble, 1991; Muskavitch, 1994). The ligands have different expression patterns, produce different mutant

phenotypes, and appear to regulate different developmental decisions via activation of a single Notch receptor. Drosophila Delta and Serrate are structurally related, yet differ notably by the presence of additional EGFs repeat and a cysteine-rich region (CR) in Serrate, which is absent in Delta. The CR region may modulate the binding of ligand to Notch. Cell aggregates formed between Delta- and Notch- expressing cells are more stable than aggregates comprising Serrate- and Notch-expressing cells (Fehon et al., 1990; Rebay et al., 1991). Interestingly, Delta-expressing cells form aggregates on their own, while serrate-expressing cells do not exhibit homotypic associations. Perhaps the CR region in Serrate prevents such homotypic interactions. Cell aggregation assays have shown that both Delta and Serrate bind to the extracellular EGF-like repeat region of Notch and that only the extracellular EGF-like repeats 11 and 12 are necessary and sufficient for this interaction (Rebay et al., 1991). Similar binding data have not yet been obtain for Lag-2 and Apx-1, and no such information is available yet for any vertebrate Notch ligands. However, the observation that chimeric Drosophila Notch molecules with the 11/12 EGF-like repeats of vertebrate Notch proteins are capable of interacting with insect Delta and Serrate suggests that there is a high degree of functional conservation in the ligand-binding properties of Notch proteins from different species. A modifier, Fringe, regulates the interaction of extracellular ligands, Delta and Serrate, with Notch receptor. Fringe has been proposed to inhibit Serrate-Notch signaling but to potentiate Delta –Notch signaling (Panin, et, al., 1997). The regulation of Serrate by Fringe occurs at the level of protein and not at Serrate transcription. The

sequences necessary for Fringe to inhibit Serrate-Notch interactions have been mapped to the N-terminal region of Serrate in *Drosophila* (Fleming, et al., 1997). How Fringe affects ligand-Notch signaling is not clear. Recently, Fringe had been found to directly form a complex with Notch receptor through both the Lin-Notch repeats and the epidermal growth factor repeats 22-36 of Notch when they are co-expressed. Results from *in vitro* protein mixing experiments and subcellular colocalization experiments indicate that the Fringe-Notch complex may form before their secretion, probably within the secretory pathway (Ju, et al., 2000). It may be one of the mechanism through which Fringe regulates Notch signaling pathway. Modulation of Notch signaling by Fringe is directly mediated by the complex. Fringe-Notch complex may have preferential affinity or sensitivity to Delta, whereas free Notch may have a higher affinity or sensitivity to Serrate.

Ligand-receptor interaction is specific in Vertebrates. In contrast to *C.elegans*, where all the Notch-related ligands activate all the receptors, it is found that Jagged1 can activate both Notch1 and Notch2 expressed in myoblasts whereas Delta1 can only activate Notch1 efficiently. Introduction of a CR, unique to Jagged1, into Delta1, generates a chimeric ligand that efficiently activates both Notch1 and Notch2. Moreover, removal of the CR from Jagged1 yields a ligand that can only activate Notch1 as found with Delta1. These data indicate that ligand-Notch interactions can be affected either by the presence or absence of the Jagged1 CR (Weinmaster, 1997).

In many cases, a lateral signaling feedback loop between adjacent precursor cells regulates the Notch receptor family and their ligands. Studies of

the DSL protein X-Delta 1 indicate that the choice of neuronal fate in Xenopus is controlled by a lateral signaling feedback loop involving Notch and Delta that is strikingly similar to the one that operates in nervous system development in *Drosophila* (Chitnis et, al., 1995). Ectopic expression of X-Delta 1, or expression of a constitutively activated form of Notch, leads to a reduction in the number of neurons. Expression of a dominant negative form of X-Delta 1, leads to an increase in the number of neurons, suggesting that Notch activity inhibits the neuronal fate. X-Delta 1 is normally expressed in scattered cells within the neurogenic region in a pattern that prefigures the positioning of neurons. It suggests that X-Delta 1 expression may be upregulated in cells that are choosing the neuronal fate and that these cells might then inhibit their neighbors from becoming neurons by signaling to them through Delta. Moreover, expression of a constitutively active version of Notch in the Xenopus neurogenic region leads to reduced expression of X-Delta 1, suggesting that a self-reinforcing feedback loop of the type described in *Drosophila* (Heitzler et, al., 1991) and *C. elegans* (Wilkinson et, al., 1994) may also operate in this system, such that cells receiving more Notch signal than their neighbors downregulate ligand and upregulate Notch. As the neighbouring cells would be exposed to less ligand, this would then reduce the Notch signal received by these neighbouring cells causing them to upregulate ligand and downregulate Notch, thus reinforcing a difference in Notch signaling between adjacent cells. Indications of autoregulation of Notch signaling have also been observed in mammalian systems (Robey, 1996; De la Poma, et al. 1997).

2.3 Signal transduction through CSL proteins

As mentioned above, Notch is activated through binding with DSL ligands in its extracellular domain. It is proposed that the activated form of Notch allows signal transduction through at least two pathways, one involving CSL proteins and the other independent of CSL proteins. CSL proteins, CBF1/RBP-Jk, Su(H) and Lag-2, bind to RAM region of Notch intracellular domain. The interaction of Notch with CSL proteins results in the cleavage of Notch and the release of NICD. Both CSL protein and NICD translocate to the nucleus and activate downstream effectors. Normally the CSL protein functions as a transcriptional repressor; activation of Notch signaling in cells converts this DNA-binding protein into a transcriptional activator. How Notch signaling activates the CSL proteins is unknown. A clue may come from studies in which either the intracellular domain of Notch or the Epstein-Barr nuclear antigen2 (EBNA2) can activate CBF1 by directly binding to it (Hsieh and Hayward, 1995; Hsieh et al., 1996). A consequence of these protein-protein interactions is derepression of CBF1 and transcriptional activation of genes containing CBF1-binding sites. Interactions between the cytoplasmic domain of Notch and CBF1 have suggested a model for Notch signaling in which ligand activation of full-length Notch induces a proteolytic cleavage allowing the NICD to translocate to the nucleus, either carrying CBF1 with it or interacting with nuclear CBF1 to directly affect gene expression (Jarriault et al., 1995; Kopan et al., 1996). The Su (H)/CBF1 proteins positively regulate the expression of Enhancer of split [E(spl)] in Drosophila, ESR in Xenopus, and HES-1 and HES-5 in mammals. Members of this family of transcriptional repressors are basic helix-loop-helix (bHLH) proteins with a

characteristic proline in their DNA-binding domain (pro-bHLH) that are upregulated in response to Notch signaling and are required for some, but not all, Notch functions (De la Poma, et al., 1997; Wettstein et al., 1997). bHLH products, together with Groucho, can repress the expression of the Achaete-Scute(Ac-Sc), which in turn functions to activate the transcription of the DSL ligand Delta. Since Ac-Sc and the homologous vertebrate genes are required for neural cell fate, it is thought that the encoded proteins also positively regulate downstream neural-specific genes. Consistent with this idea, Notch signaling in cells downregulates the expression of Ac-Sc and its homologs to inhibit neurogenesis in both invertebrates and vertebrates.

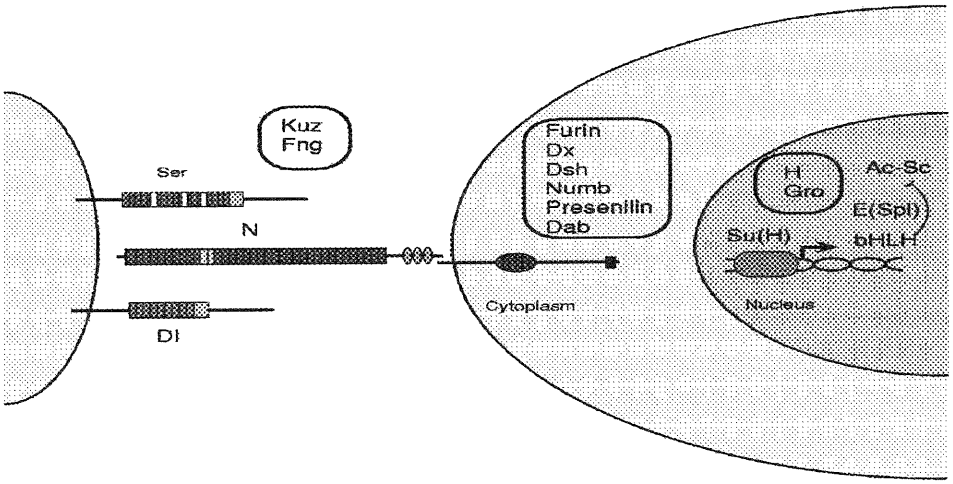

Fig 3. Notch signal pathway

2.4 CSL- independent signaling

Not all the signals from Notch receptors are mediated by CSL proteins. A CSL-independent pathway has been described that involves the intracellular Notch-binding protein Deltex (Ordentlich et al., 1998; Zecchini et al., 1999). The participation of Deltex in Notch signaling was realized as a result of the ability of Deltex mutations to suppress the lethality of certain heteroallelic Notch mutant combinations (Xu and Artavanis-Tsakonas, 1990). Deltex is a conserved, zinc finger cytoplasmic protein with a ubiquitous tissue distribution throughout development (Diederich et al. 1994; Busseau et al 1994). The protein is composed of three domains separated by glutamine-rich stretches. Functional studies in cultured Drosophila S2 cells and in yeast have revealed that all three Deltex domains mediate homotypic interactions between Deltex proteins, and that Deltex domain I binds to the intracellular ANK repeat region of Notch (Diederich et al. 1994). Overexpression of Deltex produces the same phenotype as constitutively active forms of Notch, suggesting that Deltex is a positive regulator of the Notch signaling pathway (Diederich et al. 1994; Busseau et al 1994). Deltex probably acts upstream in this pathway, since Deltex mutant phenotypes can be rescued by overexpression of constitutively active forms of Drosophila Notch. Notch signaling through Deltex is conserved in vertebrates, and this pathway is involved in Notch-mediated inhibition of the bHLH protein E47 through a mechanism involving Ras and c-jun N-terminal kinase (JNK) (Ordentlich et al., 1998). Moreover, it is found that Deltex binding with Notch prevented nuclear translocation of NICD (Matsuno et al., 1998). In addition,

Deltex-Deltex interactions have also been observed, suggesting that dimer formation may be important for function.

2.5 Negative regulators of Notch signaling pathway

Genetic studies with the Hairless gene in Drosophila have suggested that Hairless acts antagonistically to Notch (Posakony, 1994). Hairless is involved in Notch signaling pathway thought regulating Su(H) activity. Hairless encodes a high acidic 109 kDa protein, functions both in the determination of the sensory organ precursor (SOP) cell and in the assignment of proper fates to the progeny of the SOP. Hairless is located in the nucleus. Reductions in the amount of hairless lead to phenotypes of bristle-loss and vein-loss characteristic of Notch gain-of-function in Drosophila. These phenotypes are suppressed by reducing Su(H) and enhanced by increasing Su(H), demonstrating that the activity of the pathway is highly sensitive to the levels of these two proteins (Fortini and Artavanis-Tsakonas, 1994; Schweisguth and posakony, 1992; Bang et al., 1995). It was found that Hairless could inhibit the DNA binding of both Su(H) and RBP-Jk through direct protein-protein interactions. Consistent with this *in vitro* inhibitory effect, transcriptional activation driven by su(H) in transfected Drosophila S2 cells is inhibited by Hairless (Brou et al., 1994). Moreover, overexpression of Hairless can prevent the Notch-induced upregulation of E(spl), presumably by inhibiting Su(H) binding within the E(spl) promoter (Bailey and posakony, 1995). Taken together these results suggest that Hairless functions as a negative regulator of Notch via its negative interaction with Su(H).

In Drosophila, both genetic and molecular studies have suggested that the wingless (Wg) signaling pathway inhibit Notch activity. The Dsh protein may account for some of this antagonism. Genetic analysis has shown that Dsh mutants interact antagonistically with Notch and Delta. The Dsh has been found to bind directly to Notch, both in yeast two-hybrid and in S2 aggregation assays. The N-terminal part of Dsh binds the most C-terminal cytoplasmic region of Notch. Corroborating this Dsh-Notch interaction, expression of the C-terminal of Notch in Drosophila embryos inhibits Dsh-dependent ectopic bristle induction. The direct interaction of Dsh with the Notch C-terminal and the antagonistic genetic interactions suggest that Dsh may block Notch signaling directly, possibly interfering with binding of another cytoplasmic protein interacting with Notch. In addition, overexpression of Dsh blocks Notch signaling during lateral inhibition in *Drosophila*, indicating that Dsh is a negative regulator of the Notch signaling pathway. However, this physical interaction has yet to be confirmed *in vivo* (Axelrod et al., 1996)

Another protein negatively regulating Notch signaling pathway is Numb. Like Notch, Numb is involved in multiple asymmetric divisions during the formation of a sensory organ (Rhyu et al., 1994). The Drosophila Numb protein is a cytoplasmic determinant that is asymmetrically localized between daughter cells during sensory bristle development, and the different cell lineages achieved in this process are also influenced by Notch (Campos-Ortega, 1996). Numb protein has a motif called the phosphotyrosine-binding (PTB) domain (Kavanaugh and Williams, 1994) or phosphotyrosine interaction domain (Bork

and Margolis, 1995). Loss of Numb function transforms the IIb cell into the IIa cell during embryogenesis (Uemura et al., 1989), and overexpression of Numb results in reciprocal cell fate transformation (Rhyu et al., 1994). It is found that Numb is upstream of Notch and negatively regulates Notch. Moreover, the interaction of Numb and Notch occurs through direct protein-protein association. The PTB domain of Numb binds to either the RAM23 region or the Very C-terminal end of Notch (as shown in fig.1) (Guo et al, 1996). These results provided a possible mechanism by which Numb could negatively regulate Notch signaling (Guo et al., 1995; Guo et al, 1996). Numb homologies have been cloned from the mouse and the similar protein-protein interaction has been observed between mouse Numb and mouse Notch (Zhong et al., 1996), suggesting an evolutionarily conserved mechanism. Furthermore, like the *Drosophila* Numb protein, the mouse Numb protein contains a region characteristic of a domain PTB suggesting another plausible connection between protein tyrosine kinase and Notch signaling pathways (Pawson, 1995).

3. Notch function in cell transformation

Several evidences show that alteration of Notch signaling or expression of *Notch* contributes to the generation of neoplasia. Although the Notch1-4 genes are located on different chromosomes, all have been mapped to regions of neoplasia-associated translocation or oncogenic viral insertion, and all of them have been directly associated with malignant transformation. It is firmly established that Notch expression and signaling are altered in spontaneous

human tumors and in tumor models. Two distinct phenomena have been described in the literature. First, increased expression of apparently intact Notch1 and Notch2 has been demonstrated in cervical carcinomas and other epithelial malignancies, as well as in preneoplastic lesions of the cervical epithelium (Zagouras et al., 1995; Daniel et al.,1997). In the cervix, the subcellular distribution of Notch-1 changes dramatically during the progression from a preneoplastic CIN3 lesion to microinvasive carcinoma, with strong nuclear immunoreactivity observed only in carcinoma (Daniel et al., 1997). Notch overexpression was present in 100% of the cervical cancer specimens studied so far. Similarly, increased expression of Notch1 has been described in colon adenocarcinomas and lung squamous carcinomas (Zagouras et al., 1995). Moreover, Notch ligand Jagged1 and Delta1 are also increased in cervical carcinomas concomitantly with Notch1 and Notch2 overexpression (Gray et al., 1999). Abundant expression of apparently intact Notch1 can be detected in transformed cell lines of many different lineages, from cervical cancer to T cell acute lymphoblastic leukemia (T-ALL), acute promyelocytic leukemia, erythroleukemia, neuroblastoma, and medulloblastoma to pleural mesothelioma (Miele and Osborne, 1999). This suggests that increased expression of Notch receptors and ligands is a common molecular consequence of transformation, regardless of cell type.

Second, constitutively active forms of Notch have transforming activity. Such forms of Notch1, resulting from deletions of the extracellular subunit, are associated with approximately 10% of the cases of T-ALL (Ellisen et al., 1991;

Aster et al., 1992) and are oncogenic in mouse T cells *in vivo* (Pear et al., 1996). Similarly, constitutively active forms of Notch3 cause T cell leukemia /lymphoma (Bellavia et al., 2000) as well as constitutively active forms of Notch4 cause breast cancer in mice (Robbins et al., 1992; Jhappan et al., 1992; Smith et al., 1995; Gallahan et al., 1996). Constitutively active Notch1 and Notch2 transform rat kidney cells are associated with adenovirus oncogene E1A *in vitro*. Association of E1A with Notch1 is slightly more potent than with Notch2 (Capobianco et al., 1997). The intracellular domain of Notch1 must accumulate in the nucleus to induce transformation of rat kidney cells (Jeffries and Capobianco, 2000). Active form of Notch1 has also been found to transform mouse epithelial cells (HC11 cells) *in vitro* directly without cooperation with other oncogenes (Dievart, et al., 1999).

The mechanism of Epstein-Barr virus induced oncogenesis is correlated with Notch signaling pathway. Epstein-Barr virus immortalizing protein EBNA-2 is necessary for EBV-induced transformation. EBNA is tethered to responsive promoters through a cellular factor, CBF-1, an intracellular effector of Notch, providing a linkage between EBNA2 function and Notch signaling. Like Notch1 and Notch2 signaling, EBNA2 binds with the transcriptional repression domain of CBF-1. The repression of CBF-1 is masked by EBNA2 binding and converts CBF-1 into a transcriptional activator. It is found that targeting of Notch1 and Notch2 were identical from targeting by EBNA2. Mutation of CBF1 at codon 249 to 251 abolished interaction with both Notch1 and Notch2 proteins but not with EBNA2 (Hsieh and Hayward, 1995; Hsieh et al., 1996; Hsieh et al., 1997).

Recently, it is found that mouse Notch1 intracellular domain transactivates viral promoters known to be regulated by EBNA2. The transactivation may be through Notch-CBF-1 interaction because mutations or deletions of the CBF-1 sites of the promoter diminish or eliminate Notch1IC-mediated transactivation (Hofelmayr et al., 1999). Taken together, all these results indicate that mimicry of Notch signal transduction is involved in Epstein-Barr virus–driven immortalization.

3.1 Notch as a T cell oncogene

Notch1, Notch2, and Notch3 have been implicated in the development of T-lymphoid malignancies (Ellisen et al., 1991; Rohn et al.,1996; Girard et al., 1996; Bellavia et al., 2000). In a subset of T cell acute lymphoblastic leukemias, TAN-1, a homolog of *Notch1* in human, was identified as a locus involved in the t(7;9)(q34;q34.3) chromosomal translocation and was found to be associated with constitutive expression of aberrant Notch1 intracellular domain (Notch1IC) that likely function as constitutively activated forms of Notch1 (Ellisen et al., 1991). A direct association between expression of such truncated Notch1 proteins and the development of T cell malignancies has been confirmed by Pear and coworkers (Pear et al., 1996) using a mouse bone marrow (BM) transplantation model. These investigators found that mice transplanted with BM cells transduced with activated forms of Notch developed T-cell malignancies at a high frequency. Interestingly, equivalent tumorigenesis was observed for Notch constructs containing only the intracellular domain and those including the transmembrane domain, suggesting that either membrane-bound or free

intracellular Notch molecules are oncogenic. This is in contrast to other reports associating malignant transformation primarily with nuclear forms of Notch.

Truncated Notch2 molecules have also been associated with T cell malignancies. Rohn et al (1996) described transduction of Notch2 sequences in thymic lymphomas from cats infected with feline leukemia virus. The transduced region of Notch2 included the conserved extracellular cysteines, the transmembrane domain, and portions of the intracellular domain, including the cdc10 repeat. In contrast to the corresponding Notch1 protein in mice, the truncated Notch2 protein localized to the nucleus, indicating it was not tethered to the membrane. These investigators have proposed that nuclear Notch2 is generated through internal translation initiation at a site immediately downstream of the transmembrane domain and thus would not be membrane-bound. If correct, this observation suggests a nonproteolytic mechanism for generating activated intracellular forms of Notch.

Recently, a constitutively activated form of Notch3 (Notch3 intracellular domain) has been found to induce T cell lymphomas dominated the spleen and lymph nodes in the Tg mice driven by the *lck* proximal promoter (Bellavia et al., 2000). The tumors arising from these Tg mice are characteristics of immature thymocytes, including expression of CD25, pTα and activated NF-κB via IKKα-dependent degradation of IκBα and enhancement of NF-κB-dependent anti-apoptotic and proliferative pathways.

Genetic evidence indicates that Notch1 can cooperate with cellular proto-oncogenes to accelerate tumorigenesis in T cell system (Girard et al.,1996;

Girard et al.,1998). Using provirus insertional mutagenesis to identify putative collaborators of *c-myc,* it was found that *Notch1* mutation was associatied with over 50% T cell lymphomas induced in Moloney MuLV-infected $MMTV^{D}$/*c-myc* Tg mice. The proviral integration sites focused on two regions and caused the *Notch1* gene rearrangement. One of these regions occurred in genomic regions coding for sequences between the 34th EGF repeat and the TM domain, resulting in the production of truncated *Notch1* alleles which encode for *Notch1* extracellular domain and intracellular domain [$N(EC)^{Mut}$ and N(IC)]. named "type I" mutation (Girard et al.,1996; Girard et al.,1998; Hoemann et al, 2000). Almost all of the tumors with type I proviral insertions produced elevated levels of two distinct types of truncated transcripts. The 3- to 4-kb RNAs initiated at the integration site and terminated at the 3' end of the gene and thus encoded the TM and cytoplasmic domains. Another class of transcripts, measuring 6 to 9 kb, appeared to originate at either the *Notch1* promoter and terminate at the integration site or reverse orientation, thus having the capacity to encode only a truncated Notch1 ectodomain [$N(EC)^{Mut}$]. The second type of proviral integration event in *Notch1* is referred to "type II" mutation. This cluster of integrations occurred within an 800-nucleotide span, at the C-terminus-encoding region of *Notch1*. The resulting mutant alleles encoded all of the Notch1 receptor sequence except for the C- terminus, which harbors a PEST domain. These data suggest that these two distinct *Notch1* mutant alleles could be oncogenic and probably cooperate with c-*myc* and accelerate the lymphomagenesis.

3.2 Notch as a mammary tumor oncogene

Both Notch1 and Notch4/Int3 have been found to contribute to the generation of mammary carcinomas in the mouse as well as in directly transforming mouse mammary epithelia *in vitro.* The Int3 gene was originally identified as a common insertion site for infectious MMTV provirus in spontaneous mammary tumors (Gallahan et al., 1987; Robbins et al., 1992; Sarkar et al., 1994). In these tumors, proviral MMTV DNA is inserted into the region of the affected gene, encoding the transmembrane protein domain of the gene product. One consequence of this integration event is the transcription of a intracellular domain of Int-3 RNA initiated in the 3' MMTV LTR element and represents a gain-of-function mutation. This Int3 gene encoded a 200kD protein which shares 60% homology with the mouse homologue of Drosophila Notch, named Notch4. The int3/Notch4 is unique among other members of the Notch family by containing 29 instead of 36 EGF-like repeats in the extracellular domain of the gene product (Gallahan and Callahan, 1997) and by having a significantly shorter intracellular domain (Uyttendaele et al., 1996). In the normal mouse mammary gland, endogenous int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995). Exogenous expression of the Int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Overexpression of the int-3 in mouse mammary epithelial cells (HC11 cells) promotes anchorage-independent growth (Robbins et al., 1992). It is demonstrated that transgenic mice expressing the truncated Int3 gene product from the MMTV LTR exhibited dysregulation of normal developmental controls and hyperpreliferation of glandular epithelia including: the salivary sinus, the

extraorbital lacrimal glands and Harderian glands (Jhappan et al., 1992; Smith et al., 1995). In the MMTV LTR–Int3 transgenic mice, mammary gland development and function were also severely impaired such that both mammary ductal growth and secretory lobule development were curtailed in these mice. All transgenic females developed mammary tumors by 4 months of age. With regard to the effect of truncated Int3 expression on mammary gland development, these results were confirmed and extended in another transgenic mouse line in which Int3 was expressed from the whey acidic protein (WAP) promoter (Gallahan et al., 1996). Furthermore, Notch4/int-3 has been reported to be normally expressed in all adult tissues. It is found that a truncated form of the Notch4/Int3 intracellular domain has high levels of expression in human breast, lung, and colon carcinoma tissue culture cell lines. This truncated Notch4/int-3 protein includes the ANK/CDC10 repeats and C-terminal, but misses the CSL protein binding region RAM (Imatani and Callahan, 2000). The structure of this protein are showed in Fig.4.

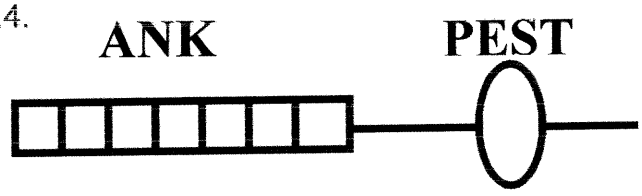

Fig. 4 The structure of truncated Notch4/int-3 protein

Similar to Notch4/Int-3, Notch1 is found to contribute to mammary tumor development. Some mammary tumors contain viral induced rearrangement of Notch1 in c-erbB2 transgenic mice infected with MMTV (Jolicoeur et al., 1998). As describe above, the activated form of Notch1 also transformed mouse HC11

mammary epithelial cells *in vitro* (Dievart et al., 1999). Deletion analysis revealed that the ankyrin-repeats and the domain I are required, while a signal peptide, the two conserved cysteins and the OPA and PEST sequence are dispensable for transformation.

4. Notch function in T cell development, differentiation and apoptosis

All lymphoid cells derive, ultimately, from multipotent hemopoietic stem cells (HSCs) found in the liver during fetal life and then in the bone marrow of the adult. The T lymphocytes develop from pluripotent stem cells and mature in the thymus. The earliest progenitors slowly divide in the subcapsular region, before showing signs of maturation. At this stage, these cells can give rise to intrathymic dendritic cells as well as α:β and δ:γ thymocytes. Later, they express the first T cell specific surface molecules: CD2 and Thy-1 (in mice). Then, they express CD44 and, at a later stage CD25. These early T cells commit to either the T cell receptor (TCR) $\alpha\beta$ or $\gamma\delta$ lineage in $CD44^{low}CD25^{+}$ stage. Successful commitment is in large part determined by whether they complete the appropriate rearrangement at the TCR β, γ or δ loci. T cells that make an in-frame rearrangement of γ and δ TCR genes develop as $\gamma\delta$ T cells, whereas cells that make an in-frame β TCR gene rearrangement develop along the $\alpha\beta$ T cell pathway. Cells that adopt the TCR$\alpha\beta$ T cell fate progress from the $CD4^{-}CD8^{-}$ double negative (DN) to the $CD4^{+}CD8^{+}$ double positive (DP) stage after they receive a signal from the pre-TCR. The pre-TCR heterodimeric receptor is

comprised of the TCRβ and pre-Tα molecules assembled with the CD3 signal-transducing chains. Signals from the pre-TCR are crucial for the efficient formation of DP thymocytes. Rearrangement of V and J segments at the TCRα locus allows DP thymocytes to express a αβ TCR on their surface (Bevan et, al 1997, A). After the expression of αβ TCR, they can test their newly formed αβ TCRs for ability to bind to the major histocompatibity complex (MHC) protein. During this stage, DP thymocytes are faced with three choices in the thymus: life by positive selection, death by negative selection, and death by neglect. Recognition of either class I or class II MHC proteins presented by thymic cortical epithelial cells directs the CD8 versus CD4 lineage choice, respectively. Thymocytes whose antigen receptors can recognize class I MHC proteins develop as CD8 lineage cells, whereas thymocytes whose antigen receptors can recognize class II MHC develop as CD4 lineage cells. This process is called positive selection. The positive selection occurs in deeper layers of the thymic cortex. In contrast to the positive selection which leads to cells survival, the negative selection or death by neglect leads to cells died. Several different cell types mediate negative selection in the thymic medulla. The most important cells are the bone marrow derived dendritic cells and macrophages, the professional antigen–presenting cells. Negative selection removes those T cells whose receptors recognize the complex of self-MHC molecules with self peptides to give a self-tolerant population of thymocytes (Bevan et, al 1997, B; Jameson and Bevan 1998). Recent experiments indicated that Notch signaling plays a critical role during the commitment of T cell differentiation including T cell versus B cell

lineage, αβ versus γδ T cell lineage, and $CD4^+$ versus $CD8^+$ T cell lineage and T cell apoptosis.

4.1 Notch function in T cell development.

It is known that mammalian Notch1 is expressed at all stages of thymus development. Immunohistochemistry study in thymus of embryos (13.5 to 15.5 days postcoitum) showed that most early thymocytes were immunoreactive for Notch1, with a subset of cells showing particularly intense cytoplasmic staining (Hasserjian et al., 1996). As thymic maturation progresses in the embryo, the strongest staining was observed in the thymic cortex, although weak staining persisted within the developing medulla. In neonatal, 4 weeks and 7 weeks thymuses, strong staining was confined to a population of relatively large cells in the superficial cortex, with the deep cortex being negative and the medulla showing weak equivocal staining. Analysis in each subpopulation of thymus showed that expression of Notch1 is the highest in immature $CD4^-CD8^-$ thymocytes and lowest in less immature $CD4^+CD8^+$ thymocytes, whereas intermediate levels of expression were observed in $CD4^+CD8^-$ and $CD4^-CD8^+$ cells. These data indicate a dynamic pattern of Notch1 expression during T cell differentiation and suggest that downregulation of Notch1 may be required for maturation of cortical thymocytes (Hasserjian et al., 1996). Notch signaling is important for the thymocyte development. Inducible deletion of Notch1 in newborn mice or in bone marrow stem cells resulted in a severe block in thymocyte development (Radtke et al., 1999). At 4 weeks of age, these mice had

a markedly smaller thymus with a 5-fold reduction in thymocyte numbers and an abnormal architecture, where medullar and cortical regions could no longer be distinguished. Cytofluorometric analysis of cell markers on thymocytes showed a moderate decrease in the percentages of mature single positive (SP) $CD4^+CD8^-$ and $CD4^-CD8^+$cells, and a considerable decrease in $CD4^-CD8^-$ double negative (DN) T cells. In absolute numbers, $CD4^+$ SP thymocytes were reduced 5-fold, $CD8^+$ SP 4.4-fold and $CD4^+CD8^+$ double positive (DP) thymocytes 9-fold, whereas the $CD4^-CD8^-$ DN population remained largely unaffected (Radtke et al., 1999). These results indicated that Notch1 is expressed in thymocytes and is important for thymocyte development and differentiation. However, in a new study by MacDonald and colleagues (Wolfer et al., 2001), which used a conditional knockout approach, it has been shown that all the events specific to TCR$\alpha\beta$ T cell development — from TCRβ selection through positive deletion to survival in the periphery — proceed quite normally in the complete absence of Notch1. They found that once thymocytes have reached the $CD25^+CD44^-CD4^-$ $CD8^-$ pro-T stage, deletion of Notch1 has no further effect on their ability to produce different subsets of T cell progeny. Lack of Notch1 does not alter the proliferation states or viabilities of these subsets or, to any significant extent, the rates at which these subsets are generated. In addition, loss of Notch1 at this stage does not give cells any competitive disadvantage, compared to wild-type cells, in long-term mixed chimeras. Together these different results from the same system indicate that a stage of T cell development, before TCRβ selection, is dependent of Notch1. When the cells reach to $CD25^+CD44^-CD4^-CD8^-$ pro-T

stage and finish the rearrangment of their TCRβ gene, they become independent of Notch (Rothenberg 2001).

The effect of Notch signaling in regulating normal T cell development is further supported by the expression of Jagged2, a ligand for Notch1, in thymus (Luo et al., 1997). Expression of Jagged2 was found in fetal and 2-week-old thymus. Furthermore, immunohistochemistry revealed co-expression of Jagged2 and Notch1 within thymus, consistent with interaction of the two proteins *in vivo* (Luo et al., 1997). As we know, the DSL domain of Jagged2 is required for interaction with Notch receptors. In Jagged2$^{\Delta DSL}$ transgenic mice (Jagged2 mutant deleted of exons encoding the DSL domain of the protein), homozygotes for this deletion died at birth because of defects in craniofacial morphogenesis and exhibited impaired differentiation of γδ lineage T cells. In fetal thymus from Jaged2$^{\Delta DSL}$ homozygotes, the percentage of total γδ T cells, as well as those among the $CD4^-CD8^-$ DN subset, was approximately one-half that observed in wild-type and heterozygous mutation control littermates. Histological analyses also revealed alteration in thymic morphology in the Jaged2$^{\Delta DSL}$ homozygous embryos. The amount and number of the lighter-staining medullar regions appeared reduced compared to the wildtype and heterozygous. (Jiang et al., 1998).

However, kinetic studies done by Robey and coworkers did not found any evidences for alteration in thymocyte proliferation or life-span in transgenic mice expressed activated Notch1 under the control of LCK proximal promoter (Robey et al., 1996). Bromodeoxyuridine (BrdU) is incorporated in the DNA of dividing

cells and can be detected using a monoclonal antibody. Tg mice and non-Tg littermates when exposed to BrdU in their drinking water and then analyzed thymocyes for the presence of BrdU and for the expression of CD4, CD8, and TCR. In normal mice, the $CD4^+CD8^+$ precursors present a short-lived population reflected by the rapid appearance of BrdU, and almost all are $BrdU^+$ after 3-4 days. Expression of activated Notch1 in thymocytes does not alter the labeling kinetics of the $CD4^+CD8^+$ precursor population, neither in mature single positive $CD4^+$ or $CD8^+$. Hence, activated Notch1 does not lead to the inappropriate proliferation of mature thymocytes in this transgenic mouse model (Robey et al., 1996).

4.2 Notch function in T cell differentiation

4.2.1 Notch1 signaling and B cell versus T cell lineage commitment

Notch1 signaling is important to the early stage of T cell versus B cell lineage commitment. Inducible deletion of Notch1 in newborn mice or in bone marrow stem cells resulted in a severe block in thymocyte development and the B cell proliferation. Although the number of $CD25^-CD44^+$ $CD4^-CD8^-$ cells in the thymus was normal, the majority of these cells did not express thymocyte precursor markers such as CD117 and CD90, but instead expressed typical B cell markers such as B220, CD19, IgM and MHC class II. Normally, thymic B cells represent only a small subset (0.2%) of thymic cells, and these thymic B cells that are found in the thymic medulla and have a specific phenotype ($B220^+$ IgM^{low} $classII^{int}CD5^+CD43^+$) which distinguishes them from peripheral B cells. These thymic B cells may play a role in T cell negative selection. The B cell

population found in the thymus of inducible Notch1 knockout mice gave a phenotype that was different from this phenotype and resembled B cells normally found in the bone marrow. These results indicate that Notch1 signaling is necessary for lymphoid progenitor cells to commit to the T cell lineage. Moreover they suggest that, in the absence of Notch signals, lymphoid progenitor cells that enter the thymus differentiate into the B cell lineage (Radtke et al., 1999; Deftos and Bevan 2000). Conversely, the experiments from another group suggested that expression of constitutively active Notch1 in bone marrow stem cells induce T cell lineage commitment (Pui et al. 1999). When bone marrow stem cells expressing constitutively active Notch1 were transferred into irradiated hosts, they gave rise to a thymus-independent population of cells in the bone marrow that expressed markers of T cell lineage commitment, including CD4, CD8 and Thy-1. In some cases, a proportion of these cells also expressed CD3 and TCRβ. Expression of constitutively active Notch1 also completely inhibited the differentiation of stem cells into the B cell lineage but had no effect on differentiation into the myeloid lineage. These results suggest that Notch1 signaling blocks the differentiation of lymphoid progenitor cells into the B cell lineage and may be sufficient to induce differentiation into the T cell lineage (Pui, et al., 1999; Deftos and Bevan, 2000). Taken together, these loss- and gain-of-function experiments suggest that Notch1 signaling plays a critical role in determining whether lymphoid progenitor cells differentiate into the T cell or B cell lineage.

4.2.2 Notch signaling and $\alpha\beta$ versus $\delta\gamma$ T cell lineage commitment

There are two checkpoints of T cell development in the thymus. The first occurs as T cell receptor β, γ and δ genes rearrangement. If a cell makes productive γ and δ rearrangements, it can express the heterodimeric γδ receptor. If it makes a productive β rearrangement, it can make the pre-T cell receptor that consists of the β chain and the pre-T cell receptor α protein, which is encoded by a non-rearranging gene. Cells that can produce neither the γδ receptor nor the pre-T cell receptor will die at this stage of development. Cells that express the γδ or the pre-T cell receptor can enter the γδ or αβ T cell lineage. The choice between αβ or γδ lineage is controlled by Notch as well as by the types of antigen receptor expressed by the processor cells. Mutation of the β TCR gene produces a block in αβ development when Notch1 is normally regulated. A constitutively active form of Notch1 (containing a portion of Notch1 intracellular domain), however, overrides the normal requirement for the TCR β chain and allows normal numbers of αβ thymocytes to develop in β TCR mutant mice (Washburn T et al., 1997). Using mice with a chimaeric haemopoietic system populated by a mixture of equal portions of fetal liver or bone marrow cells from $Notch1^{+/-}$ and $Notch1^{+/+}$ donor mice, the $Notch1^{+/-}$ stem cells produced three times fewer αβ T cells than did $Notch1^{+/+}$ donor stem cells. Conversely, the Tg mice overexpressing activated *Notch1* under the control of *Lck* proximal promoter had significant increases in the number of αβ lineage $CD4^{+}CD8^{+}$ cells in the thymus, even in the absence of TCR β locus (Washburn T et al., 1997). However, this change caused by activated Notch1 was TCR gene rearrangement dependent, since Notch1 Tg / Rag-/- (TCR rearrangement deficiency) mice did not show any

accumulation of αβ lineage cells. Together, the activated Notch1 under the regulation of the *Lck* proximal promoter. can overcome the block of αβ T cell development in TCR β mutant mice, but can not drive αβ T cell development in the absence of TCR gene rearrangement (Washburn T et al., 1997; van Boehmer et al., 1999).

4.2.3 Notch signaling and $CD4^+$ versus $CD8^+$ T cell lineage commitment

In thymocyte development, the second checkpoint occurs when the CD4 or CD8 co-receptor binds to thymic MHC molecules. The cell fate in dividing into single positive $CD4^+$ or $CD8^+$ T cells is determined at this point. In αβ lineage cells, the pre-T-cell receptor rescues cells from death, induces a strong wave of proliferation, followed eventually by rearrangements at the α chain locus and expression of CD4 and CD8 co-receptors. These $CD4^+CD8^+$ DP cells express the T cell receptor, cease dividing and are programmed to die after three to four days unless their TCR and CD4 or CD8 co-receptor binds to thymic MHC molecules. While the TCR binds to peptides presented by either MHC class I or class II molecules, CD4 binds only to MHC class II and CD8 only to MHC class I molecules. Appropriate binding of the TCR and co-receptor to the same MHC molecule results in rescuing of the cells from apoptotic cell death, and the rescued cells differentiate into either $CD4^+CD8^-$ cells or $CD4^-CD8^+$ cells, depending on whether their TCR is specific for class II or class I MHC presented peptides. The Notch1 signaling has also been implicated at this stage of T cell development. This was first suggested by an analysis of $CD4^+$ and $CD8^+$ cell

development in Tg mice expressing activated Notch1 in the thymus as we described above (Robey et al., 1996). These mice had approximately 10-fold increase in the number of $CD8^+$ SP thymocytes and slight decrease in the number of $CD4^+$ SP thymocytes. BrdU labeling experiments showed a 3-fold increase in the rate of production of $CD8^+$ SP thymocytes and a 5-fold decrease in the rate of production of $CD4^+$ SP thymocytes. However, the lymph nodes in these Tg mice had a normal $CD4^+$ versus $CD8^+$ ratio and a 4-fold reduction in total T cell numbers, suggesting that the excess $CD8^+$ SP thymocytes may not be fully mature and that the Notch1 transgene may affect the survival of mature T cells. Furthermore, the excess $CD8^+$ SP thymocytes developing in these Notch1 Tg mice deficient for either MHC class I or MHC class II expression but not in Tg mice deficient for both MHC classes, suggesting that their development is dependent upon TCR stimulation (Robey et al., 1996). On the basis of these results, the Notch1 signaling was proposed to regulate the $CD4^+$ versus $CD8^+$ cell fate choice during normal T cell development. DP thymocytes that are selected on MHC class I receive a Notch1 signaling that directs them to the $CD8^+$ lineage, whereas DP thymocytes that interact with MHC class II do not receive a Notch1 signaling and develop along the $CD4^+$ lineage. In the presence of constitutive activated Notch1, DP thymocytes selected on MHC class II are diverted from the $CD4^+$ to the $CD8^+$ lineage. Recently, HES-1, a target gene in Notch signaling pathway, has been found to silence CD4 expression through specific binding to the CD4 silencer of the CD4 promoter (Kim and Siu, 1998). These data provide evidences that Notch1 signaling can result in the silencing of CD4 expression

through interaction with the silencer and further suggest one possible mechanism that explains the increase of $CD8^+$ SP thymocytes and the decrease of $CD4^+$ SP thymocytes in Notch1 Tg mice produced by Robey's group.

However, controversial results were reported by Bevan's group, which showed that the expression of an actived form of Notch1 promoted the maturation of both $CD4^+$ and $CD8^+$ SP thymocytes. (Deftos et al., 2000). This conclusion came from Tg mice whose Notch1 intracellular domain was deleted in the PEST region and under the control of LCK proximal promoter. The results showed a large decrease in the percentage of DP thymocytes and a corresponding increase in the percentage of $CD8^+$ SP thymocytes. The magnitude of this effect was variable, with the percentage of DP thymocytes ranging from 75% to 24% (compared to 80% ± 5% for control mice) and the percentage of CD8+ SP thymocytes ranging from 8% to 44% (compared to 3% ± 1% for control mice). In mice that had a "weak" phenotype (a small change in the percentage of DP and $CD8^+$ SP thymocytes), a small reduction in the percentage of $CD4^+$ SP thymocytes had been found. However, in mice that had a strong phenotype (a larger change in the percentage of DP and $CD8^+$ SP thymocytes), there was an increase in the percentage of $CD4^+$ SP thymocytes. To determine whether the development of $CD4^+$ and $CD8^+$ SP thymocytes promoted by active Notch1 is dependent upon TCR-MHC interactions, the bone marrow cells from Tg mice and their control littermates were transferred into irradiated MHC-deficient hosts and T cell development was analyzed. A significant but highly variable number of $CD4^+$ and $CD8^+$ SP thymocytes developed in MHC-deficient

hosts reconstituted with bone marrow from Tg mice. In contrast, MHC-deficient hosts reconstituted with bone marrow from control mice did not develop a significant number of mature $CD4^+$ or $CD8^+$ SP thymocytes. These results indicate that Notch1 signaling promotes $CD4^+$ and $CD8^+$ SP thymocyte maturation in the absence of MHC expression on thymic epithelial cell in this mouse model (Deftos et al., 2000).

Comparing the results from these two groups, they both observed that the expression of active Notch1 leaded to the generation of excess in $CD8^+$ SP thymocytes. However, in contrast to Robey's group findings that active Notch1 inhibited the development of mature $CD4^+$ SP thymocytes and that the effect of active Notch1 in $CD4^+$ versus $CD8^+$ lineage commitment was dependent on the expression of either MHC class I or MHC class II on thymic epithelial cells, the Bevan's group found that the active Notch1 promoted, rather than inhibited, the development of mature $CD4^+$ SP thymocytes. Moreover, in their Tg line, mature $CD4^+$SP and $CD8^+$ SP thymocytes developed in the absence of MHC expression on thymic epithelial cells. The reason of the different conclusions in these two groups is unknown but may be due to the differences in the region of Notch1 expressed as a transgene. The transgene used by Robey and colleagues contains only the ANK repeat region and the nuclear localization sequences (aa1750 - aa2293), whereas the transgene used by Bevan and colleagues contains the RAM, ANK repeat, C-terminal transcriptional activation domain (TAD) (aa2155 – aa2374) and OPA(aa2370 - aa2393), but lacks the C-terminal PEST domain (aa 2481 – aa2503).

Recently, a report from MacDonald and colleagues showed that tissue-specific inactivation of the gene encoding Notch1 in immature ($CD25^+CD44^-$) T cell precursors does not affect subsequent thymocyte development (Wolfer et al., 2001). Neither steady-state numbers nor the rate of production of $CD4^+$ and $CD8^+$ mature thymocytes is perturbed in the absence of Notch1. Together the results which are from overexpression a constitutively active Notch1 indicate that cells can respond to a " gain-of–function " mutation, like a constitutively active transgene, without necessarily depending on the normally expressed products of that gene.

Taken together, the effect of Notch1 in T cell development and differentiation can be summarized in Fig. 4. The upper part of the figure shows implied effects when constitutively active forms of Notch1 and Notch3 are overexpressed. The lower part of the figure indicates stages in which conditional

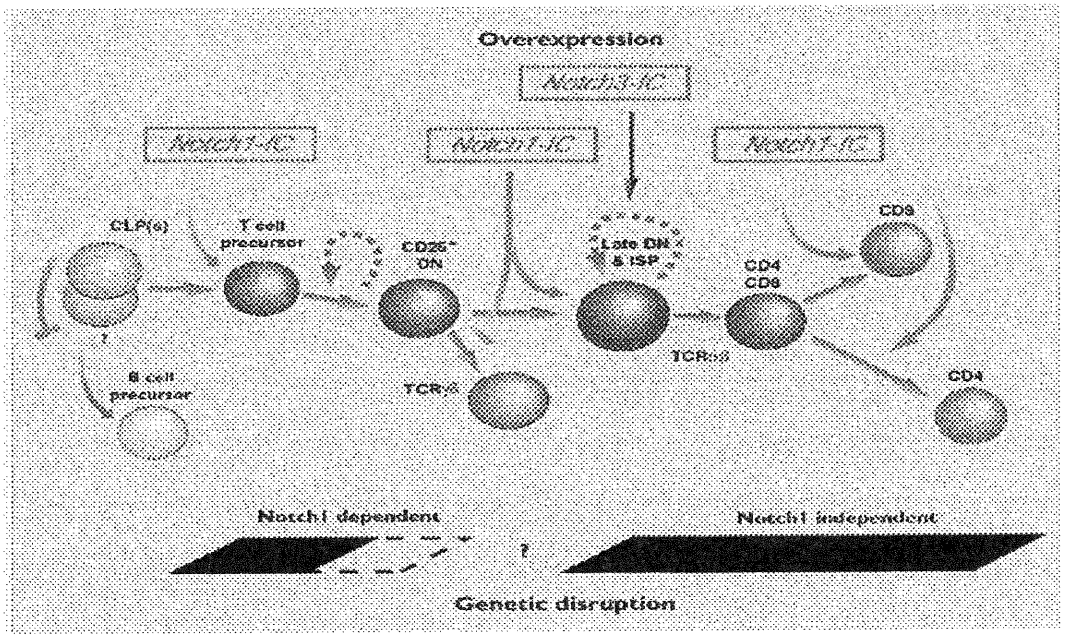

Fig. 5 Notch effects defined by overexpression and by genetic disruption

deletion shows Notch1 to be essential or dispensable (Rothenberg 2001). Thus, Notch1 is completely indispensable for the initial specification of T-lineage precursors at an early stage, before TCRβ selection. When cells pass this stage, they "graduate" to being independent of Notch1.

4.3 Notch signaling and T cell apoptosis

Recently, *Notch1* has been reported to regulate cell death. These observations suggest that, at least in some systems, Notch signaling regulates cell survival besides differentiation. Two groups have independently discovered that Notch1 intracellular domain (Notch1IC) has anti-apoptotic properties in T cell systems. One group reported that retrovirally transduced Notch1IC inhibited dexamethasone-induced cell cycle arrest and apoptosis in a thymic lymphoma line (AKR1010) and a T cell hybridoma (2B4.11) *in vitro* (Deftos et al., 1998). Glucocorticoid receptor levels or known downstream effector genes were not affected. The RAM region of Notch1 was shown to be necessary for this effect, suggesting that it is CBF-1 dependent. However, Deltex expression was upregulated by Notch1IC expression, suggesting that Deltex mediated events may also participate in this effect. Interestingly, TCR and Bcl-2 expression was upregulated by Notch1. These data indicate that Notch1 signaling has complex effects on molecules involved in T cell development and that activation of Notch1 may protect thymocytes from "death by neglect" mediated by glucocorticoids. Another group showed that Notch1IC protected TCR-induced apoptosis through inhibiting Nur-77-dependent apoptosis in a T cell hybridoma (DO11.10) (Jehn et

al., 1999). Nur-77 is a zinc finger transcription factor of the NGFI-B family, which includes Nur-77/NGFI-B, Nurr1, and NOR-1. Nur-77 and Nor1 are upregulated during apoptosis induced by TCR engagement and are required for TCR-mediated apoptosis during negative selection. These transcription factors have structural features similar to steroid receptors. Since specific ligands for these molecules have not been identified, they are often called orphan nuclear receptors. Notch1IC expression reduced Nur77 expression triggered by PMA and Ca_2^+ ionophore or TCR ligation. Using yeast two hybrid experiments, they identified Notch1IC as a candidate for Nur-77-binding protein. Since Nur77 is known to positively regulate its own expression through interaction with a Nur77 site in its own promoter, these data suggest a possible mechanism for the anti-apoptotic activity of Notch1: Notch1 may be directly interacting with Nur77/NGFI-B family members. This interaction may reduce nur77 expression by interfering with the autoregulatory loop through which Nur77 induces its own expression. Additionally, Notch1 expression was found to result in the repression of Nur77 induced transcription (Jehn et al., 1999). Taken together, those data suggest that Notch signaling may regulate apoptosis during thymocyte maturation by preventing death by neglect and negative selection in cells destined to die.

5. Aim of the project

As we described above, our previous study showed that over 50% of thymic lymphomas which were induced in Moloney MuLV-infected $MMTV^D$/*c-myc* Tg mice presented a targeted *Notch1* by provirus insertion. The proviral

integration sites focused on two regions and caused the *Notch1* gene rearrangement. One of these regions occurred in genomic regions coding for sequences between the 34th EGF repeat and the transmembrane (TM) resulting in the production of two distinct types of truncated Notch1 proteins, Notch1EC^{Mut} and Notch1^{intra}. The later contained a portion of ectodomain, TM and cytoplasmic domain. These results indicated that the truncated forms of Notch1 alleles might be involved in T-cell transformation and probably cooperate with *c-myc* in T cell tumor generation. The purpose of this project is to develop biological systems to study the effect of the gain-of-function truncated mutant of Notch1 (Notch1^{intra}) in T cell development, T cell transformation and collaboration with *c-myc* in T cell transformation.

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Zaher S Hanna		July 19, 2001
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The student (first author) worked independently in most of the experiments including in this paper: RNA and protein expression of the Tg mice, FASC analysis, tumorigenicity, apoptosis *in vivo* and *in vitro,* establishment of Notch1^{intra}/c-myc double Tg mice. She also participated in DNA mapping of the Tg mice and histological study. She has a major contribution in this paper.

Involvement of Notch1 intracellular domain in T cell development and transformation

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Résumé

Dans nos ètudes sûr les lymphomes thymiques des souris MMTV^{D}/*c-myc* transgéniques (Tg) infectes par Moloney MuLV, nous avons montré que le géne *Notch1* été muté dans 52% des tumeurs. Nous avons trouvé le provirus inserté dans deux régions different du géne *Notch1*. Une de les régions contient la portion génomique codant pour la séquence protéique situé entre la 34ème répétition d'EGF et le segment transmembranaire. Elle résulte en la formation d'une forme tronquée de Notch1, appelé "type I". En général, les tumeurs avec des insertions de type I produisent un haut niveau d'ARN tronqué de 3-4Kb. Ces ARN commencent au niveau du site d'integration et se terminent à l'éxtrémité 3' de gène. Ils codent pour une protéine tronquée nommé Notch1^{Intra} qui contient une protion du domaine extracellulaire, le domaine transmembranaire et toute la portion intracellulaire. Ces résultats suggèrent que Notch1^{Intra} est impliqué dans la transformation des cellules T et coopère avec *c-myc* danns la tumeurogenèse. Pour étudier le rôle de *Notch1^{intra}* pendant le dévéloppement et la transformation des cellules T, et aussi la synergie avec *c-myc* dans la génération de lymphomes, nous avons généré des souris Tg avec *Notch1^{intra}* sous le contrôle du promoteur humain CD4 (CD4C). Le croisement de ces souris avec les souris MMTV^{D}/*c-myc* Tg nous procure un modèle d'étude double Tg. L'analyse des jeunes souris CD4C/ *Notch1^{intra}* révèle un accroissement du nombre de thymocytes CD4^{+}CD8^{+}, une augmentation du porcentage de CD25^{+}, une haute expression de CD2 et une expression moyenne de TCR$\alpha\beta$, dans la population totale de thymocytes et dans les tymocytes CD4^{+}CD8^{+}. Nous avons aussi

montré, dans les jeunes souris Tg, une protection des thymocytes $CD4^+CD8^+$ contre l'apoptose induite *in vitro* et *in vivo* par la dexamethasone. Les souris Tg développe de manière spontanée des lymphomes thymmiques clonales ou oligoclonales, avec une latence relativement longue. Toutes les souris double Tg développent des lymphomes avec une latence beaucoup plus courte par rapport aux souris transgéniques simples de la même potée, avec les transgènes $Notch1^{intra}$ ou *c-myc*. Ces résultats suggèrent que $Notch1^{intra}$ se comporte comme un oncogène qui inite la dérégulation des thymocytes DP et leur transformation. L'accélération dans la formation des tumeurs, dans les souris double Tg, démontre la cooperation entre $Notch1^{intra}$ et *c-myc* dans la production de lymphomes.

Summary

In our previous studies on thymic lymphomas arising in Moloney MuLV-infected MMTV^{D}/*c-myc* Tg mice, we reported that *Notch1* was mutated in a high proportion of tumors (52%). The inserted provirus were found in two regions of the *Notch1* gene, inducing the *Notch1* gene deletion. One of these regions consisted of genomic regions coding for sequences between the 34th EGF repeat and the transmembrane (TM) of Notch1, resulting in the production of what we termed "type I" truncated *Notch1* alleles. Typically, tumors with type I proviral insertions produced elevated levels of 3-4 kb truncated RNA transcripts, initiating at the integration site and terminating at the 3' end of the gene, and thus encoding truncated Notch1 protein named Notch1^{intra}. These Notch1^{intra} mutants contain a small portion of extracellular domain, the transmembrane domain (TM) and the complete cytoplasmic domain. These results strongly suggested that this truncated Notch1^{intra} might be involved in T cell transformation and may cooperate with *c-myc* in T cell oncogenesis. To determine the role of Notch1^{intra} during T cell development, T cell transformation as well as synergy with *c-myc* in lymphomagenesis, we have generated Notch1^{intra} transgenic (Tg) mice under the control of the human CD4 (CD4C) promoter and crossed them with MMTV^{D}/*c-myc* Tg mice to establish a double Tg model. Studies in these CD4C/Notch1^{intra} Tg young mice showed an increase number of CD4^{+}CD8^{+} thymocytes, an increase in the percentage of CD25^{+}, high level expression of CD2, and intermediate level expression of TCR$\alpha\beta$ in total thymocytes and CD4^{+}CD8^{+} thymocytes. It was also showed a protection of CD4^{+}CD8^{+} thymocytes from

dexamethasone-induced apoptosis *in vitro* and *in vivo* in Tg young mice. Tg mice spontaneously developed clonal or oligoclonal $CD4^+CD8^+$ thymic lymphomas after a relatively long latency. All double transgenic mice bearing $Notch1^{intra}$/*c-myc* transgenes developed thymic lymphomas at a latency which was much shorter than that in single $Notch1^{intra}$ or *c-myc* Tg littermates. These results indicate that $Notch1^{intra}$ behaves as an oncogene to initiate DP thymocyte dysregulation, then transformation. The acceleration of tumorigenesis in the double Tg mice provided a directed evidence that $Notch1^{intra}$ and *c-myc* act in cooperation for lymphomagenesis.

Introduction

The Notch transmembrane receptors have been well conserved throughout evolution and the best characterised are those of *Drosophila* encoded by *Notch,* those of *C. elegans* encoded by *Lin-12* and *Glp-1*, that of *Xenopus* encoded by *Xnotch* and those of mammals encoded by *Notch1, Notch2, Notch3* and *Notch4.* The structure of these proteins are strikingly similar. The extracellular domain contains 29-36 tandem epidermal growth factor (EGF) -like repeats which function in ligand binding and three Lin/Notch repeats (LNR) of unknown function. The intracellular (intra) domain includes the RAM domain (RAM), the six cdc10/SW16/ankyrin motifs, a transcriptional activated domain, and OPA, PEST sequence at its C-terminus, which is responsible for the signal transduction to the nucleus. A truncated form of *Notch1* representing this intracellular domain ($Notch^{intra}$) has been found to function as a gain-of-function mutant in several species. The Notch receptors are broadly expressed throughout embryonic development and control cell fate decisions in many different tissues [1,2].

The function of the mammalian Notch receptors has been explored in T cell development. Notch1-3 have been found to be involved in thymocyte development [3 4 5 6 7 8 9]. Notch1 is expressed at all stages of thymus development. In adult mouse thymus, the expression of *Notch1* is the highest in immature $CD4^-$ $CD8^-$ double negative (DN) thymocytes and lowest in less immature $CD4^+CD8^+$ double positive (DP) thymocytes, whereas intermediate levels of expression were observed in more mature single positive (SP) $CD4^+CD8^-$ and $CD4^-CD8^+$

thymocytes [3]. Inducible deletion of *Notch1* in newborn mice or in bone marrow stem cells resulted in a markedly smaller thymus exhibiting an abnormal architecture and 5-fold reduction in cell numbers : a considerable decrease in $CD4^-CD8^-$ DN T cells and a moderate decrease in the percentages of mature SP $CD4^+CD8^-$ and $CD4^-CD8^+$ cells were observed in mice of 4 week-old [4]. Overexpression of a constitutively activated form of Notch1 (containing a portion of Notch1 intracellular domain) under the control of *lck* proximal promoter favors the cell fate of TCRαβ cells in the choice of TCRαβ and TCRδγ lineage [5] , and promote the maturation of CD8 SP thymocytes [6] or both CD4 and CD8 SP thymocytes [7]. Overexpression of a constitutively activated form of Notch1 or Notch3 was also found to induce high expression of CD25 in DP thymocytes [7 8]. Furthermore, overexpression of a constitutively activated form of Notch1 in a thymic lymphoma line (AKR1010) and in a T cell hybridoma (2B4.11) *in vitro* [10] has been reported to inhibited dexamethasone-induced apoptosis and to protect against TCR-induced apoptosis in a T cell hybridoma (DO11.10) by inhibiting Nur-77-dependent apoptosis[11]. However, in a new study by MacDonald and colleagues, [12] involving a conditional knockout approach, it was found that once thymocytes have reached the $CD25^+CD44^-CD4^-CD8^-$ pro-T stage and have finished to rearrange the TCRβ gene, deletion of *Notch1* has no further effect on altering the proliferation states or viabilities of these subsets or, to any significant extent, the rates at which these subsets are generated. Together, these results indicate that T cells can respond to a "gain-of–function" *Notch1* mutation without necessarily depend on the normally expressed Notch1.

In addition to their effect on T cell development, gain-of–function mutants of Notch1-3 have been implicated in the development of T-lymphoid malignancies [8 13 14 15]. TAN-1, the homologue of *Notch1* in human, was identified as a locus involved in the t(7;9)(q34;q34.3) chromosomal translocation present in a subset of T-cell acute lymphoblastic leukemias [13 16]. In these human leukemia cells, an aberrant *Notch1* intracellular domain was constitutively expressed and most likely functioned as constitutively activated forms of Notch1. Similarly, using a mouse born marrow transplantation assay, it was found that expression of truncated Notch1 proteins in donor cells led to the development of T cell malignancies [16]. In this assay, the ANK repeat and C-terminal transactivation domains are required for T cell leukemogenesis [17]. The truncated Notch2 molecules have also been associated with thymic lymphomas arising in cats infected with feline leukemia virus [14]. In this system, the Notch2 sequences which were transduced on the retrovirus genome included the conserved extracellular cysteines, the transmembrane domain, and portions of the intracellular domain, including the ANK repeats. Finally, a third member of the Notch family, Notch3, has recently been found to induce T cell lymphomas when expressed as a gain-of-function mutant ($Notch3^{intra}$) in Tg mice under the regulatory sequence of the *lck* proximal promoter [8]. Interestingly, the leukemic process mainly involved the spleens and lymph nodes.

In our previous studies on thymic lymphomas arising in Moloney MuLV-infected $MMTV^{D}$/*c-myc* Tg mice, we reported that *Notch1* was mutated in a high proportion of tumors (52%). The inserted provirus were found in two regions of

the *Notch1* gene, inducing the *Notch1* gene deletion. One of these regions consisted of genomic regions coding for sequences between the 34th EGF repeat and the transmembrane (TM) of Notch1, resulting in the production of what we termed "type I" truncated *Notch1* alleles. Typically, tumors with type I proviral insertions produced elevated levels of 3-4 kb truncated RNA transcripts, initiating at the integration site and terminating at the 3' end of the gene, and thus encoding truncated Notch1 protein named Notch1^{intra}. These Notch1^{intra} mutants contain a small portion of extracellular domain, the transmembrane domain (TM) and the complete cytoplasmic domain [15 18,19]. These results strongly suggested that this truncated Notch1^{intra} might be involved in T cell transformation and may cooperate with *c-myc* in T cell oncogenesis. To assess the effect of Notch^{intra} on T cell and myeloid cell development and its oncogenic potential for T cells and myeloid cells, Tg mice expressing this gene under the regulatory sequences of the human CD4 gene (CD4C) were generated. Studies in the thymus of CD4C/Notch1^{intra} young Tg mice showed an increased number of CD4^{+}CD8^{+} double positive thymocytes (DP thymocytes); as well as an increased percentage of CD25^{+}, high level expression of CD2, and intermediate level expression of TCR$\alpha\beta$ in total thymocytes and DP thymocytes. It was also showed a protection of DP thymocytes from dexamethasone-induced apoptosis both *in vitro* and *in vivo*. Subsequently, Tg mice spontaneously developed clonal or oligoclonal CD4^{+}CD8^{+} thymic lymphomas after a relatively long latency. All double transgenic mice bearing Notch1^{intra} and c-myc transgenes developed thymic lymphomas at a short latency, which was much shorter than that of single

Notch1^{intra} Tg littermates or single c-myc Tg littermates. These results indicate that Notch1^{intra} can behave as an oncogene or collaborate with other factors to initiate DP thymocyte dysregulation, then transformation. The acceleration of tumorigenesis in the double Tg mice provides a strong evidence that Notch1^{intra} and c-myc act in cooperation for lymphomagenesis.

Materials and Methods

Construction of Notch1^{intra} transgenic mice

The 14.9 kb CD4C regulatory sequences of the human CD4 gene have been described previously [20]. The 3.0 kbp truncated *Notch1* fragment was cleaved from mouse *Notch1* cDNA (kindly provided by Dr Weintraub) [21] at the NcoI site (nt 5051) and at the EcoRI site (nt 8064) at the end of C-terminus and inserted downstream of the CD4C sequences (1.9kbp enhancer and 13kbp CD4 promoter from plasmid pBR322/CD4C-HIV) [22]. The simian virus 40 polyadenylation sequences (0.88kbp from plasmid NL4/SV40) [23] were ligated at the 3' end and cloned in the pBR322 vector. The 18.8 kbp transgene CD4C/Notch1^{intra} DNA used for microinjection was cleaved with EcoRI, then isolated by preparative agarose gel electrophoresis and further purified on CsCl gradients, as described previously [24 25].

One-cell (C57BL/6 X C3H) F2 embryos were collected, microinjected, and transferred into pseudopregnant CD1 females essentially as described previously [24 25]. The presence of the transgene was confirmed by Southern hybridization with tail DNA, using *Notch1* cDNA as a probe. A 2.7 kbp fragment was detected in Tg mice by HindIII digestion. Six CD4C/Notch1^{intra} Tg founders (F30856, F35748, F35735, F35753 and other two untapped founders) were produced from the pups born. The Tg founders were bred on a C3H and on a CD1 backgrounds. All founders transmitted the transgene in a Mendelian fashion and appeared phenotypicaly normal. The Tg mice were observed for spontaneous thymic lymphoma development and the thymic lymphomas were collected for analysis.

Establishment of Notch1^{intra}/c-myc double Tg mice

The generation of MMTV^{D}/c-myc Tg mice has been described previously [26]. To obtain Notch1^{intra} */c-myc* double Tg mice, CD4C/Notch1^{intra} Tg mice were crossed with MMTV^{D}/*c-myc* Tg mice. Both of these two Tg lines were on a CD1 background. The Notch1^{intra} */c-myc* double Tg mice and their littermates (Notch1^{intra}, or *c-myc* single Tg mice, and non-Tg mice) were observed for spontaneous thymic lymphoma development and the thymic lymphomas were collected for analysis.

Analysis of the transgene RNA expression

Total RNA was extracted from thymus or thymic lymphoma samples with 1ml Trizol reagent (Gibco BRL product), according to the manufacturer's protocol. To study the expression of Notch1^{intra} transgene, RNA was separated on 1% formaldehyde-agarose gels, transferred to Hybond-N membranes (Amersham Co) and hybridized with *Notch1* probe K, as previously described [15]. The *Notch1* probe K is a 2.7 kbp BamHI-EcoRI cDNA fragment covering the entire *Notch1* intracellular domain. This probe was prepared as previously described [19].

For analysis of *c-myc* Tg RNA expression, RT-PCR was performed on the RNA of thymic lymphomas arising in Notch1^{intra}/*c-myc* double Tg mice. Total RNA (5μg) was used for cDNA synthesis with reverse transcriptase (RT) (200 U, MMLV, BRL, 200U/μl). The cDNA obtained was used as template for PCR

reaction employing primers specific for the $MMTV^D$ LTR (sense: 5'-GCAACAGTCCTAACATTCACCT-3') and the *c-myc* exon 3 (antisense: 3'-CGGAATGGAGATGAGCCCGAC-5'). PCR reactions were performed in 2.5 mM $MgCl_2$, 1mM dNTP, 1U Taq DNA polymerase and 0.5 pM of each primer. DNA was amplified for 35 cycles at an annealing temperature of 60°C. PCR products were separated on 1 % agarose gels and stained with ethidium bromide.

Protein extraction and Western blotting

Total protein extractions were done in RIPA buffer(12 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP40, 1% sodium desoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, 10.5 mM EGTA) [27] containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml Nα-p-Tosyl-L-Lysine chloro-methylketone(TLCK) and 100 μg/ml Phenylmethylsulfonylfluoride (PMSF). Extracts were cleared by centrifugation at 150,000 × *g* for 30 minutes. Protein extracts were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 20 %Glycerol, 2 % SDS, 5 % β-mercaptoethanol), boiled for 5 min, subjected to 6% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nylon membranes. Protein molecular mass standards myosin (205 kDa) was purchased from Bio-Rad. Filters were blocked with 5% milk powder in TBST [10 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Tween 20] overnight at 4°C and then probed with primary antiserum in 0.5% milk powder in TBST. Immunodetection was performed essentially as described previously, using rabbit anti-Notch1-intra1 antibodies, and secondary horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma A0545), followed by

chemiluminescent detection (NEN Inc). The anti-Notch1-intra1 antibodies are specific for the Notch1 intracellular domain and their generation and characterization have been described previously [19]. The resulting autoradiograms were scanned with HP Deskscan II and reproduced for publication using PowerPoint (Microsoft) software.

Thymocyte apoptosis induced by dexamethasone and other treatments

The CD4C/Notch1^{intra} Tg mice and their littermates used in this experiment were 6-12 week-old. For *in vitro* experiments, thymus were removed and single cell suspensions were prepared. The cells were treated without or with dexamethasone in 96 well plates with final concentrations of 500 nM, 250 nM, 100 nM and 50 nM. After incubation for 18 hrs at 37°C, cells were labeled with 7-Amino Actinomycin D (7-AAD) and analyzed by flow cytometry (FACS). Viable cells incubated in medium alone were considered to be 100% viability. The relative viability in each dosage was calculated by percentage of cells were determined as being 7-AAD negative. The percentage of viable viable cells in cultures containing dexamethasone as compared with the percentage of viable cells in cultures containing medium alone.

The thymocytes were also treated by γ- radiation (500 rads, 250 rads, 125 rads and 50 rads), PMA (5ng/ml, 2.5ng/ml and 1ng/ml) and ionomycin (300ng/ml, 200ng/ml, 100ng/ml and 50ng/ml) *in vitro*, and processed the same way as mentioned above.

For *in vivo* study, 0.3mg dexamethasone was injected intraperitoneally (i.p.) to Tg and nTg littermates, while the same volume vehicle (RPMI medium + 6% ethanol) was injected to the control group. The mice were sacrificed after 48 hrs. Cells were labeled with 7-AAD and mAb anti-CD4 (PE) and anti-CD8 (FITC) and analyzed by FACS. The number of viable cells in control mice determined the 100% viability. The relative viability in Tg or non-Tg mice was calculated by number of viable cells in treated mice compared with the number of viable cells in control mice.

Transplantation of tumor cells into to nude mice

Thymic lymphomas from CD4C/Notch1^{intra} or Notch1^{intra}/c-myc double Tg mice were Inoculated into *nude* mice. The tumors were cut into small pieces and passed through 18G needle (D=0.3mm) in 1 x PBS buffer. This cell suspension (~ 2 x 10^7 cells in 0.2ml) was injected subcutaneously to 40-50 day-old CD1 *nude* mice. The tumor cells were injected on the right site and PBS buffer on the left site as a control.

Flow cytometic (FACS) analysis

Thymus, spleen and lymph nodes (LN) from young Tg mice and their littermates, or mice bearing thymic lymphomas and control mice were disaggregated by pressing through a 70 μm nylon mesh filter to obtain single cell suspensions. To remove the red blood cells from spleen cells, cells were submitted to lysis buffer (NH4Cl, KHCO3, EDTA, pH: 7.2) for 5 minutes on ice.

Antibodies conjugated to either fluorescent isothiocyanate (FITC), phycoerythrin (PE) or biotin were obtained from Pharmingen or Cedarlane. Biotinylated antibodies were revealed using streptavidin-TRICOLOR (Caltag) or FITC. Thymic lymphoma cells, splenocytes, and LN cells at $1X10^6$ cells per sample were stained for surface expression of Thy-1.2, CD2, CD44, CD25, TCR$\alpha\beta$, TCR$\gamma\delta$, CD4, CD8, HAS, B220, Mac-1, CD11c, F4/80, CD69, anti-NK. Cells were stained in FACS buffer containing antibodies and analyzed on a FACS Calibur apparatus (Becton Dickinson). Live cells were gated according to their FSC and SSC profiles after staining with propidium iodide (PI). Data were analyzed using CellQuest software.

Gene rearrangement analysis

DNA was extracted from thymic lymphomas. The DNA from the kidney of the same mouse was used as control. For studying the TCR β gene and immunoglobulin gene rearrangement, DNAs were digested with EcoRI and HindIII, respectively, and Southern blot analysis was performed with ^{32}P-labelled probes, as described previously [26]. The probe used for analyzing TCR β gene was a 700 bp RBL-5 DNA fragment containing most of the murine C region and 3' untranslated sequences of $C_{\beta 1}$. The J_H region of immunoglobulin gene was analyzed with a 6.2 kbp EcoRI germ line J_H DNA probe.

Histological study

Tissues used for histology were fixed in 3.7% formaldehyde, sectioned and stained with hematoxylin and eosin (HE) as described previously [22].

Results

Construction of CD4C/Notch1^{intra} Tg mice

To assess the effect of mutated truncated Notch^{intra} on T cell and myeloid cell development and its oncogenic potential for T cells and myeloid cells, Tg mice expressing this gene under the regulatory sequences of the human CD4 gene (CD4C) were generated (Fig. 1A). The CD4C regulatory sequences have previously been shown to allow expression of surrogate genes in immature $CD4^+CD8^+$ and mature $CD4^+CD8^-$ T cells as well as in cells of the macrophage/myeloid lineage, notably in macrophages and in dendritic cells .[20] The Notch1^{intra} gene contained the complete intracellular domain of Notch1 and was not deleted of C-terminal sequences. Six Tg founders harboring the CD4C/Notch1^{intra} transgene were produced. Mice from two founders were sacrificed and not studied further because they did not exhibit transgene RNA expression. Two other founders died with thymic lymphoma prior to breeding. Founder lines F30856 and F35748 were established by mating Tg founder mice with inbred C3H and outbred CD1 mice. All mice were heterozygous for the transgene. In each of the two independent founder lines, the transgene was transmitted to progeny in a Mendelian ratio. Southern blot analysis showed that the structure of the transgene was grossly intact (data not shown).

Expression of the transgene was first assessed by Northern blot analysis with a Notch-1-specific probe. This analysis revealed a relatively intense signal of the expected size (3.5 kb) in the thymus (Fig. 1B) and a lower expression in the

spleen (data not shown) from mice of the two remaining founder lines. Similarly, Western analysis with anti-Notch1-intra1 antibodies showed that the 110 kDa Notch1^{intra} protein was at higher levels in the thymus of Tg mice than in their wildtype littermates (Fig. 1C). It has been shown previously that the mutated truncated Notch1 protein co-migrates with the 110 kDa processed wild-type intracellular Notch1 protein in this gel system [19].

Thymic phenotype of CD4C/Notch1^{intra} Tg mice

To determine whether the expression of this gain-of-function Notch1 mutant affects T cell development, the lymphoid organs [thymus, spleen and peripheral lymph nodes (LN)] of young (6 - 12 week old) C3H Tg mice and their control non-Tg littermates were studied. A reproducible increase in the total thymocyte number was observed in both Tg lines (Table 1). Although the absolute numbers of $CD4^-CD8^-$ double negative (DN) and $CD4^+$, $CD8^+$ single positive (SP) subsets tend to be higher in Tg than in non-Tg mice, these differences were significant only for the $CD4^+CD8^+$ DP subset ($P<0.05$, Table 1).

To further study the phenotype of these cells, a FACS analysis was performed with various cell surface markers (Fig. 2, Table 2) in C3H and CD1 background mice. In the thymus, there was no significant impairment in the FACS profile with mAb anti-CD4 versus anti-CD8 staining. There were no significant differences ($P>0.05$) in the percentage of Thy-1^+, $CD5^+$, $CD44^+$, HSA^+, Mac-1^+CD11c^+, $B220^+$ cells between the Tg mice and their littermates (table 2). However, there was an increase in the percentage of $CD2^{high}$ and a decrease in

the percentage of $CD2^{low}$ and $CD2^{medium}$cells (Fig. 2, table 2) in Tg mice. Three-color analysis of anti-CD4/anti-CD8/anti-CD2 revealed these changes in DP subpopulation (Fig.2, table 2). There was also an increase of percentage of $TcR\alpha\beta^{medium}$ cells and a decrease of $TcR\alpha\beta^{low}$ cells, but no decrease of $TcR\delta\gamma$ thymocytes in Tg mice (table 2). Interestingly, the percentage of $CD25^+$ was increased in total thymocytes from Tg mice. Typically about 5% of normal thymocytes express CD25 while it was elevated up to 18% in CD4C/$Notch1^{intra}$ Tg mice (Fig. 2, table 2). Three-color analysis of anti-CD4 / anti-CD8 / anti-CD25 revealed that the percentage of $CD25^+$ was increased in $CD4^+CD8^+$ subpopulation. In the spleen and peripheral LN of CD4C/$Notch1^{intra}$ Tg young mice, no significant difference as compared to normal non-Tg control was observed in the numbers and proportions of T cells and B cells (data not shown). These results are similar in both background mice. Together, this analysis showed a significant increase of the $TCR\alpha\beta^{medium}$ and a decrease of $TCR\alpha\beta^{low}$ thymic population, but no change in the $CD8^+$ or the $\gamma\delta$ T cell population previously observed with a gain-of-function Notch1 mutant [5-7]

$Notch1^{intra}$ protects thymocytes from dexamethasone-induced apoptosis in CD4C/$Notch1^{intra}$ Tg mice *in vitro* and *in vivo*

Since Notch1 insertional mutation was initially observed by our group in tumors of Tg mice overexpressing *c-myc* in the thymus, we speculated that these gain-of-function insertional Notch1 mutants could be involved in apoptosis. To test this hypothesis, thymocytes from C3H CD4C/$Notch1^{intra}$ Tg young mice and

from their nTg littermates were cultured in the presence of different stimuli (dexamethasone, γ-radiation, PMA and ionomycin) for 18 hrs at 37°C *in vitro* and analyzed by FACS to determine their viability. The viability of thymocytes was no different between Tg and nTg mice when treated with different dosages of γ-radiation, PMA and ionomycin (data not shown). However, the viability of these Tg thymocytes following treatment was higher than that of the nTg littermates at all concentrations of dexamethasone tested, reaching statistical significance ($P < 0.05$) with the 100nM treatment (Fig 3A). Furthermore, *in vivo*, Tg thymocytes were protected after an intraperitoneal injection of 0.3mg dexamethasone exhibiting a 3-fold highter number of their total thymocytes and a 5-fold higher number of $CD4^+CD8^+$ thymocytes than those in non-Tg littermates, 48 hours after the treatment (table 3). FACS analysis of thymocytes of these mice revealed a higher proportion ($P<0.05$) of remaining $CD4^+CD8^+$ thymocytes in Tg (65.98% ± 2.47%, n=4) than that in the control non-Tg (33.48% ± 22.46%) littermates (Fig 3B). Therefore, $Notch1^{intra}$ protected thymocytes, especially the $CD4^+CD8^+$ subpopulation, from dexamethasone-induced apoptosis both *in vitro* and *in vivo*.

Increased incidence of thymic lymphomas in CD4C/$Notch1^{intra}$ Tg mice on CD1 background

Groups of C3H CD4C/$Notch1^{intra}$ Tg mice and their littermates were observed for 12 months for the development of tumors. On this C3H background, about 5% of CD4C/$Notch1^{intra}$ Tg mice developed thymic lymphomas spontaneously in both Tg lines assessed (5/88 and 6/93, respectively in line

F30856 and F35748), most after a long latency period (149.75 ± 85.11 days) This long latency and low incidence of thymic lymphoma development indicated that Notch1^{intra} transgene is not very active in oncogenesis or that some resistance factors may exist in the C3H mouse strain.

To determine whether the genetic background was one of the reasons affecting the incidence of thymic lymphoma in CD4C/ Notch1^{intra} Tg mice, both founder lines were transferred to the outbred CD1 mouse background for at least five generations and then observed for up to 9 months for the incidence of thymic lymphomas. A high incidence of thymic lymphoma was observed : 42% (5/12, line F30856) and 87.5% (7/8, line F35748) of Tg mice, died of or were sacrificed with thymic lymphoma at age of 58 to151 days and 74 to 137 days of age, respectively. In contrast, the non-Tg littermates from both founder lines survived beyond 9 months without tumor development (Fig. 4A). The thymic lymphoma filled the thoracic cage, leading to dyspnea and eventually death. No other signs of illness were observed at the time of death. Histopathological assessment confirmed the presence of thymic lymphomas and showed destruction of thymus architecture and the accumulation of atypical lymphoblasts, accompanied by either scattered single died cells (Fig. 4B, panel B) or areas of marked cell death. Northern blot analysis revealed high expression of Tg Notch1^{intra} in all these thymic lymphomas analyzed (n=10) (data not shown).

The secondary lymphoid organs of most CD1 Tg animals bearing thymic lymphomas were normal. In one of these Tg mice (1 out 12), the spleen and LN showed gross enlargement. Histologic examination showed the relative

preservation of the normal architecture of the spleen, liver and LN, but infiltration of lymphoblasts was found in the spleen and liver. In the spleen, single or clusters of lymphoblasts infiltrated the red pulp region, especially near the capsule (Fig. 4B, panel D). In the liver, lymphoblasts were characteristically found concentrated in perivascular area (Fig. 4B, panel F). FACS immunophenotyping of these lymphoblasts infiltrating the spleen showed that they were $CD4^+CD8^+$ DP expressing CD4 and CD8 at different levels. This phenotype is consistent with that of a disseminated thymic lymphoma (Fig. 4C). In Tg LN, the architecture appeared normal. No lymphoblast infiltration was found by histology or FACS analysis.

Thymic lymphomas belong to the T cell lineage.

To determine the nature of cells being transformed, T cell receptor (TCR) and immunoglobulin (Ig) gene rearrangement were studied on DNA from thymic lymphomas arising in CD4C/Notch1^{intra} Tg mice bred on both C3H and CD1 backgrounds. In DNA tested from all of five thymic lymphomas from Tg mice on a C3H background, the TCR β-chain gene was found to be rearranged or deleted in both alleles (data not shown). Two alleles of the immunoglobulin heavy-chain gene were found to be rearranged in one of five thymic lymphomas tested, but not in the other four tumors (data not shown). In Tg mice from the CD1 background, 9 thymic lymphomas were tested. The TCR β-chain gene was found to be rearranged or deleted in both alleles in all these samples (Fig. 5A), while the Ig heavy-chain gene were found to be rearranged in two alleles in 1/9 of the

samples and one allele in 2/9 of the samples (Fig. 5B). These results indicate that these thymic lymphomas are clonal or monoclonal in origin and that they belong to the T cell lineage.

This result was further confirmed by the characterization of the transformed thymic cell population FASC analysis. Cells isolated from thymic lymphomas were analyzed for expression of various cell surface markers of T cells [Thy-1, CD2, CD5, CD44, CD25, CD4, CD8, TCR$\alpha\beta$, TCR$\delta\gamma$, and heat shock antigen (HSA, CD24)], B cells (B220), macrophages (F4/80, Mac-1), dendrictic cells (Mac-1/CD11c), NK cells (DX-5). The lymphoma cells were distinguished from normal thymocytes by their larger size. Normal thymocytes consistently revealed a large proportion of small cells (85%-90%, mean channel=65) and a small proportion of large cells (10%-15%, mean channel=130) which include precursors and proliferating thymocytes. Forward light scatter showed that the lymphoma cell population consisted of 80%-90% of large cells (mean channel=142) (Fig. 6A).

All thymic lymphomas tested (n=15) were of the $CD4^+CD8^+$ DP phenotype and were Thy-1^+, $CD2^+$, and $CD5^-$, However, the phenotype varied based on the TCR$\alpha\beta$ and HSA expression: type A (TCR$\alpha\beta^{low}HSA^-$) and type B (TCR$\alpha\beta^{hi}HSA^+$) (Fig. 6B). In thymic lymphomas arising in Tg mice of a C3H background, all five thymic lymphomas showed a type A phenotype ($CD4^+CD8^+$ TCR$\alpha\beta^{low}HSA^-$), indicating a less mature double positive stage. In thymic lymphoma arising in Tg mice of a CD1 background, 5/10 thymic lymphomas showed type A phenotype, while 5/10 showed type B phenotype ($CD4^+CD8^+$TCR$\alpha\beta^{hi}HSA^+$), indicating a

more mature double positive stage. The CD25 and CD44 cell surface molecules are normally expressed in $CD4^-CD8^-$ DN thymocytes or by activated T cells. In thymic lymphomas, DP lymphoma cells expressed high levels of $CD25^+$ and/or $CD44^+$ (Fig.6C), but did not express another T cell activation marker CD69 (data not shown). These thymic lymphomas also showed absence of B220, F4/80, Mac-1, DX-5 and CD11c staining (data not shown), ruling out an origin from B cells, macrophages, NK cells, or dendritic cells.

Tumorigenesis is accelerated in Tg mice bearing $Notch1^{intra}$ and c-myc double transgenes

Our initial screen of provirus insertional mutants in Tg mice ($MMTV^D$/*c-myc*) overexpressing *c-myc* in DP thymic T cells revealed a truncation of the *Notch1* gene by provirus insertion leading to overexpression of $Notch1^{intra}$ in large proportion of thymic lymphomas (52%).[15] This result not only suggested that this $Notch1^{intra}$ truncated form might be involved in T cell tumor generation, but also that this truncated molecule may be cooperating with *c-myc* for oncogenesis. To test directly whether $Notch1^{intra}$ is one of the c-myc cooperators in lymphomagenesis, CD4C/$Notch1^{intra}$ Tg mice (F30856) were crossed with $MMTV^D$/*c-myc* Tg mice to generate double Tg mice. Both Tg lines were on a CD1 background. All (100%) the double Tg mice analyzed (n=17) developed thymic lymphomas with a latency of 29-44 days (mean=35 days) (Fig.7). This latent period for tumor development is much shorter than that in single $Notch1^{intra}$ or single *c-myc* Tg littermates [110 ± 35 days (56.25%) and 135 ± 41 days

(88.88%), respectively], within 210 days for observation. (Fig.7). No significant difference was found in the dissemination and/or infiltration of spleen and liver between the double Tg mice and Notch1^{intra} or *c-myc* single Tg mice bearing thymic lymphomas. Northern blot analysis or RT-PCR showed that Tg Notch1^{intra} and *c-myc* were expressed in these thymic lymphomas (data no showed). Interesting, a novel phenotype of weight loss and wasting, not present in single Notch1^{intra} or *c-myc* Tg mice, was observed in a significant proportion (4/17) of Notch1^{intra}/myc double Tg mice bearing thymic lymphomas. Together, these results indicated a clear collaboration of Notch1^{intra} and *c-myc* in accelerating the oncogenic process in thymic T cells.

Thymic lymphomas arising in double Notch1^{intra} and *c-myc* Tg mice are DP T cells

Molecular and phenotypic (FACS) analysis of all thymic lymphomas (n=17) from double Notch1^{intra} /*c-myc* Tg mice showed that they belong to the T cell lineage, having all a rearrangement or a deletion of the T cell receptor β (TCR β) gene (data not shown), and expressing T cell specific markers. These tumors (n=9) were CD4$^+$CD8$^+$. However, In contrast to CD4C/Notch1^{intra} or MMTV^D/c-myc single Tg thymic lymphomas, Notch1^{intra}/c-myc double Tg thymic lymphomas could not be clearly divided into two phenotypes. Some (2/9) were TCRαβ$^{int\text{-}high}$ with a mixed expression of HSA$^+$ and HSA$^-$ cells; one (1/9) was TCRαβ$^{low\text{-}int}$ HSA$^+$; some (3/9) were TCRαβ^{Int} HSA$^+$; and others (3/9) were TCRαβ$^{low\text{-}int}$ HSA$^-$. Similar to the lymphomas arising from CD4C/Notch1^{intra} single

Tg mice, these lymphomas also expressed the T cell activation markers CD25 and CD44, but not CD69 (data not shown).

Malignant potential of thymic lymphomas arising in both Notch1^{intra} single and Notch1^{intra} /*c-myc* double Tg mice

The malignant potential of thymic lymphomas arising from Notch1^{intra} Tg mice or Notch1^{intra} /*c-myc* double Tg mice was tested by inoculation of the tumor cells into 40-50 days old CD1 *nude* mice. Of three tested thymic lymphomas arised in Notch1^{intra} Tg mice (CD1 background), one grew in nude mice, reaching a tumor diameter of 2.2cm within 40 days postinoculation. All of three thymic lymphomas arising from Notch1^{intra}/*c-myc* double Tg mice tested grew in nude mice, reaching a diameter of 3cm-3.5cm within 40 days postinoculation. The tumors which grew from *nude* mice also belonged to T cell lineage according to the TCRβ gene rearrangement or deletion (data not shown). The phenotype of cell surface markers analyzed by FACS was similar to that of the primary thymic lymphomas (data not shown). These data indicate that the thymic lymphomas arising in either Notch1^{intra} single Tg mice or Notch1^{intra}/*c-myc* double Tg mice are malignant.

Discussion

In this study, we have demonstrated that the Notch1^{intra}, a truncated form of Notch1 which was found in thymic lymphomas developed in Moloney murine leukemia virus(MuLV) infected MMTV^{D}/myc transgenic mice, [15] plays a role in alteration of T cell development in young CD4C/ Notch1^{intra} transgenic mice and eventually leads to lymphomagenesis in those old Tg mice. The specific altered thymocyte phenotype includes an increase in total number of thymocytes, especially in the CD4^{+}CD8^{+} DP subpopulation; an increased percentage of intermediate level TCR$\alpha\beta^{+}$ and CD25^{+} in total thymocytes and DP subpopulation; resistance of DP thymocytes to dexamethasone-induced apoptosis *in vitro* and *in vivo*. Finally, the Tg mice developed CD4^{+}CD8^{+} DP thymic lymphomas. All these results indicated that the DP thymocytes are the initial target of Notch1^{intra}-induced dysregulation. Such dysregulation may be the basis of the lymphomagenesis in these transgenic mice.

The phenotype of CD4C/ Notch1^{intra} Tg mice is different from the other two reported Notch1 transgenic mouse lines which expressed a portion of Notch1 intracellular domain and were under the control of the LCK proximal promoter [5-7]. In the Tg mice produced by Robey's group, active Notch1 induced an increase in CD8^{+} SP thymocytes and a decrease in CD4^{+} thymocytes [6]. Using these Tg mice as well as a chimeric hemopoietic system populated by an equal proportion mixture of Notch1$^{+/-}$ and Notch1$^{+/+}$, it was found that overexpression of active Notch1 favored the $\alpha\beta$ T cell fate [5]. In another Tg line produced by Bevan's group, [7] an increase in both CD4^{+} and CD8^{+} SP thymocytes as well as the

expression of CD25 and CD44 on DP thymocytes was observed. Furthermore, activated Notch1 is found to inhibit dexamethesone-induced apoptosis in a thymic lymphoma line (AKR1010), in T cell hybridomas and in Tg mice [10]. Consistent with these studies, we observed that expression of Notch1^{intra} lead to an increase percentage of TCR$\alpha\beta^+$ cells and CD25$^+$ cells in total thymocytes and DP thymocytes. Moreover, we also provided evidences that Notch1^{intra} protected thymocytes from dexamethasone-induced apoptosis *in vitro* and *in vivo.* However, we found no impairment of CD4 and CD8 distribution in neither thymocytes nor T cells in periphery lymph organs in CD4C/Notch1^{intra} Tg mice. We did observe an increased number of total thymocytes and CD4$^+$CD8$^+$ DP thymocytes in the Tg mice. The reason for the differences between CD4C/ Notch1^{intra} transgenic mice and other two Notch1 transgenic mice is not clear, but may be due to the different promoter which control the transgene expression, or the different region of Notch1 expressed as a transgene. For example, in adult mouse thymus, the expression of lck proximal promoter is higher in immature CD4$^-$CD8$^-$, and CD4$^+$CD8$^+$ thymocytes and lower in more mature single positive thymocytes, particularly low in CD4$^+$CD8$^-$ SP thymocytes and peripheral T cell indicating its dominant expression in immature T cells [28,29]. In constrast to lck proximal promoter, CD4C promoter is expressed in CD4$^+$CD8$^+$, CD4$^+$CD8$^-$ thymocytes, and CD4$^+$ T cells, as well as in dendritic cells and macrophages, but not in CD4$^-$CD8$^-$ thymocytes, indicating its expression in both immature and mature T cells as well as other cell types [20]. The different expression pattern in SP thymocytes may affect the differentiation from DP thymocytes to SP

thymocytes. Since the increase of $CD8^+$ and/or $CD4^+$ SP thymocytes is independent on the interactions of TCR and CD4 or CD8 co-receptors with MHC molecules when activated Notch1 is overexpressed [7]. Although the differentiation from DP thymocytes to SP thymocytes is activated Notch1 specific, it is likely to be controlled by the expression level of activated Notch1 in the DP and SP thymocytes.

Another reason for the differences between CD4C/Notch1^{intra} Tg mice and the other two Notch1 transgenic lines may be due to the different region of Notch1 expressed as a transgene. Our construct mimics the mutation spontaneously arising by provirus insertion containing a portion of extracellular domain, the transmembrane domain and intact intracellular domain including RAM, ankyrin-repeat, OPA and PEST regions (aa1659-aa2533). The transgene used by Robey's group contains only the ankyrin repeat region and the nuclear localization sequences (aa1750 - aa2293) [6], whereas the transgene used by Bevan and colleagues contains the RAM, ankyrin repeat, and C-terminal transcriptional activation domain (TAD) and OPA, but lacks the C-terminal PEST domain (aa1751-aa2444) [7]. The truncated Notch1 protein containing a transmembrane region may influence the primary localization of the protein and further influence the transduction of Notch1 signaling and then, the regulation if downstream effectors. The completely C-terminal including OPA and PEST region, has been associated with Numb and Disheveled proteins, the modifiers of Notch signaling pathway [30 31 32]. Genetic studies suggest that these interactions are inhibitory to Notch signaling transduction. PEST domain alone is thought to

regulate protein stability. Deletion of PEST domain seems to stabilize the protein and increase the phenotype. However, the further function of PEST in Notch1 is unknown.

The increased number of total thymocyte and DP thymocyte in CD4C/ Notch1^{intra} Tg mice is novel. This is the significant difference in the phenotype between CD4C/ Notch1^{intra} Tg mice and the other two Notch1 Tg lines. The mechanism of this thymocyte dysregulation is not clear. There are two possibilities: Notch1^{intra} promotes thymocyte proliferation and/or inhibits thymocyte apoptosis. In CD4C/ Notch1^{intra} Tg mice, the increase number of thymocytes is only in DP subpopulation, which are the target cells of the CD4C promoter. It is reasonable to propose that the proliferation of thymocytes is transgenic Notch1^{intra} specific. However, this hypothesis needs to be supported by more experiments of kinetics analysis. Another possible mechanism is the inhibition of thymocyte apoptosis by Notch1^{intra} transgene. The majority of $CD4^+CD8^+$ DP thymocytes, probably up to 95%, cannot recognize self-MHC and do not undergo positive selection, hence are never signaled to continue development and die by neglect. Death by neglect is thought to be mediated, at least in part, by glucocorticoids which are endogenously produced in the thymus. Previously, the role of Notch1 in protection of DP thymocyte from dexamethasone-induced apoptosis has only been studied in cell lines or thymocytes from transgenic mice *in vitro* [10]. Our study is the first to examine it *in vivo*. Comparing the phenotype between Tg mice reported here and Tg mice produced by Robey's group, we noted that activated Notch1 can prevent

thymocyte from dexamethasone-induced apoptosis *in vitro* [10], but do not promote thymocyte proliferation [6]. Therefore, the number of thymocyte was not increased and thymic lymphomas only developed occasionally in Tg mice produced by Robey's group [6]. Hence, it is possible that Notch1^{intra} contributed to the number of thymocytes increased and the development of thymic lymphomas in CD4C/ Notch1^{intra} Tg mice is based on promoting proliferation, rather than inhibiting of "death by neglect" in thymocytes.

CD4C/Notch1^{intra} Tg mice spontaneously developed thymic lymphomas suggesting that this truncated form of Notch1 can behave as an oncogene. All the thymic lymphomas tested belong to the T cell lineage because they express T cell surface markers and have TCR β-chain gene rearrangement or deletion in both alleles. All the tumors analyzed were composed of $CD4^+CD8^+$ T cells. No other cell phenotype was found in those lymphomas. These results indicated that Notch1^{intra} was efficient in transforming some ($CD4^+CD8^+$ thymocyte) but not all ($CD4^+$ thymocyte, $CD4^+$ T cell, macrophage) of the target cells in which the CD4C promoter was expressed. The observation that the thymic lymphoma cells expressed a single or double TCR-β chain indicated that these tumors were monoclonal or clonal rather than polyclonal. The monoclonal of the origin as well as a long latency in the thymic lymphoma development suggested that Notch1^{intra} itself was not sufficient to fully transform T cell. Additional co-factors (second hit) are required for the tumorigenesis. Our previous studies suggested that Notch1^{intra} is a cooperator of c-myc for T cell transformation [15]. Other genes which have been identified as collaborators of Notch1 in different tissue types are

neu, and *E1A* [33,34]. Here we reported a double Tg system produced by crossing CD4C/Notch1^{intra} Tg mice with MMTV^{D}/c-myc Tg mice. The double Tg mice bearing both Notch1^{intra} and c-myc transgenes 100% developed thymic lymphomas with a latency of average 35 days. This latency of double Tg mice is much shorter than that in single Notch1^{intra} transgenic littermates or single c-myc transgenic littermates. The high incidence and short latency of the thymic lymphomas developing in these double Tg mice provided a direct evidence that c-myc works as a second hit in Notch1^{intra} induced thymic lymphomas, and c-myc and Notch1^{intra} are cooperators in lymphomagenesis, although the mechanism of synergy between c-myc and Notch1^{intra} needs to be elucidated.

Taken together, our results suggest that overexpression of Notch1^{intra} can induce the dysregulation in thymocyte development and eventually cause thymocyte transformation and thymic lymphomas development in CD4C/ Notch1^{intra} Tg mice. The acceleration of tumorigenesis in the Notch1^{intra}/c-myc double Tg mice provided a strong evidence for the cooperation of Notch1^{intra} and c-myc in lymphomagenesis.

Figure 1

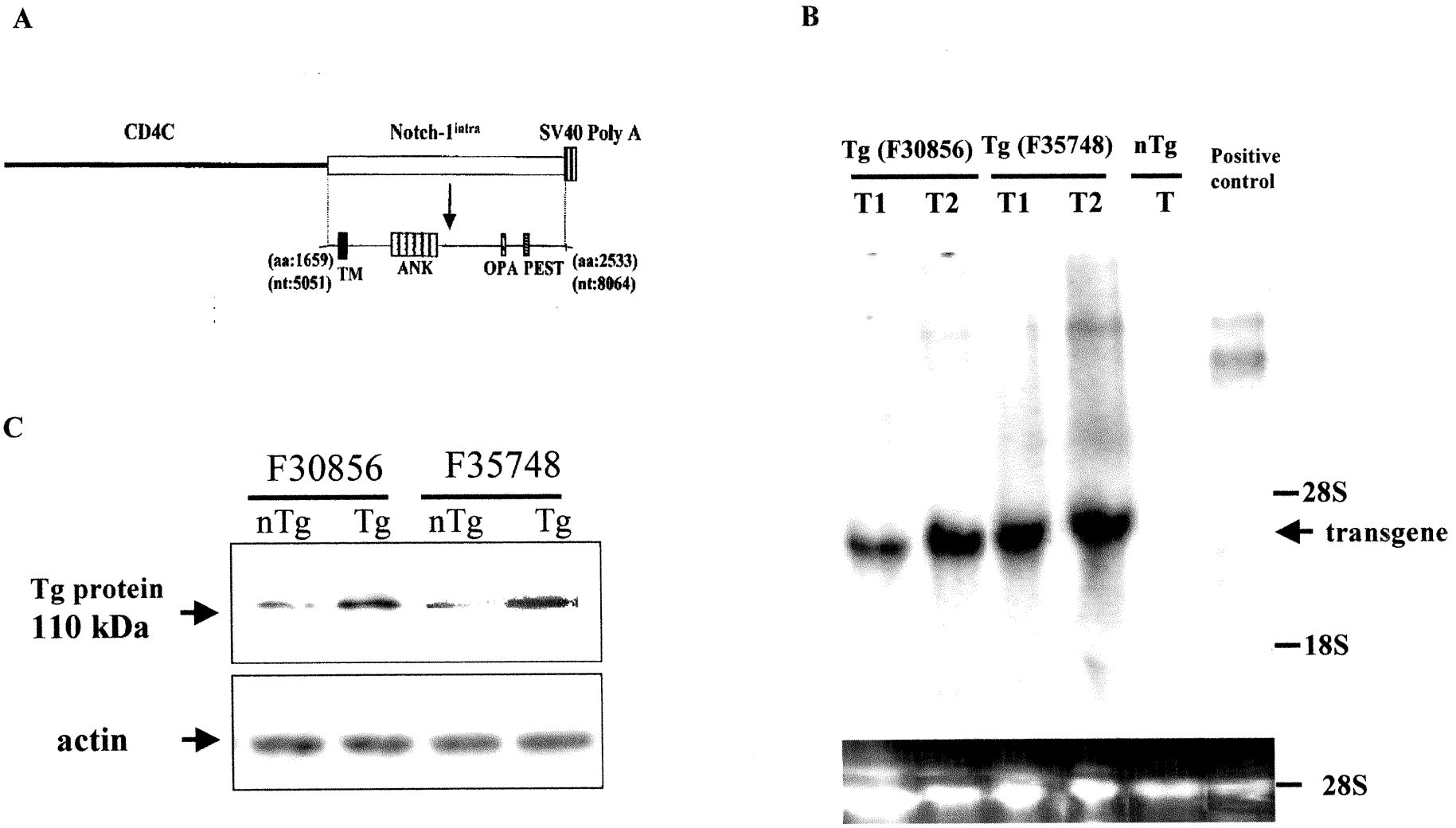

A
CD4C
Notch-1intra
SV40 Poly A
(aa:1659)
(nt:5051)
TM
ANK
OPA PEST
(aa:2533)
(nt:8064)
B
Tg (F30856)
Tg (F35748)
nTg
Positive control
T1
T2
T1
T2
T
28S
transgene
18S
28S
C
F30856
F35748
nTg
Tg
nTg
Tg
Tg protein
110 kDa
actin

Table 1: The number of thymocyte in CD4C/Notch1^{intra} Tg mice (x 10^7)

Thymocytes subsets	nTg	Tg
Total	**11.6 ± 2.4**	**15.4 ± 3.8 ***
$CD4^-CD8^-$	0.9 ± 0.6	1.4 ± 1.4
$CD4^+CD8^+$	**9.3 ± 2.0**	**12.2 ± 2.1 ***
$CD4^+CD8^-$	1.0 ± 0.4	1.2 ± 0.7
$CD4^-CD8^+$	0.3 ± 0.2	0.5 ± 0.4

Eleven pairs of CD4C/Notch1^{intra} Tg mice and their littermates (F30856, C3H background) were studied at 6 to 12 weeks of age for their number of thymocytes and thymocyte subsets based on the CD4 and CD8 cell surface expression. The mean ± SD (x10^7) is shown Significant ($P < 0.05$) differences were noted in total and in the DP thymocytes subset.

Table 2: Cell surface markers in thymocyte of CD4C/Notch1^{intra} Tg mice (%)

Markers	nTg	Tg	
Total thymocyte (n=5)			
Thy-1$^+$	93.3 ± 2.4	94.7 ± 1.4	
CD2 ^{low}	**7.1 ± 5.4**	**3.0 ± 3.6**	*
CD2 ^{medium}	**63.2 ± 17.0**	**49.9 ± 21.0**	*
CD2^{high}	**29.5 ± 12.3**	**47.2 ± 17.8**	*
CD5$^+$	83.8 ± 14.6	94.9 ± 2.8	
CD44$^+$	4.3 ± 0.4	4.1 ± 0.7	
CD25$^+$	**4.6 ± 1.4**	**18.1 ± 4.4**	*
CD44$^+$CD25$^+$	1.2 ± 0.7	1.7 ± 0.6	
TCR$\alpha\beta^{low}$	**46.2 ± 5.2**	**26.6 ± 6.0**	*
TCR$\alpha\beta^{medium}$	**39.4 ± 6.1**	**61.8 ± 6.0**	*
TCR$\alpha\beta^{high}$	13.3 ± 1.5	12.0 ± 2.0	
TCR$\delta\gamma^+$	0.6 ± 0.2	0.6 ± 0.2	
CD4$^-$CD8$^-$	3.2 ± 1.3	2.9 ± 1.5	
CD4$^+$CD8$^+$	79.5 ± 4.1	83.0 ± 4.5	
CD4$^+$CD8$^-$	12.6 ± 3.6	9.3 ± 3.0	
CD4$^-$CD8$^+$	4.7 ± 2.8	4.5 ± 4.0	
HSA$^+$	84.0 ± 19.8	88.0 ± 10.3	
Mac-1$^+$CD11c$^+$	0.7 ± 0.2	0.5 ± 0.2	
B220$^+$	1.0 ± 0.6	1.3 ± 0.7	
DP thymocyte (n=4)			
CD2^{low}	**3.3 ± 1.9**	**0.3 ± 0.2**	*
CD2^{medium}	**83.9 ± 1.5**	**66.1 ± 11.5**	*
CD2^{high}	**13.0 ± 3.2**	**34.1 ± 11.7**	*
CD25$^+$	**2.1 ± 1.2**	**13.0 ± 5.8**	*
TCR$\alpha\beta^{low}$	**56.5 ± 4.2**	**30.5 ± 6.5**	*
TCR$\alpha\beta^{medium}$	**41.1 ± 5.7**	**66.8 ± 7.6**	*

Five pairs of CD4C/Notch1^{intra} Tg mice and their littermates (F35748, CD1 background) were studied at 5 to 6 weeks of age for cell surface markers, as described in Materials and Methods. The data are expressed in percentage as the mean ± SD. *P ≤ 0.05.

Figure 2

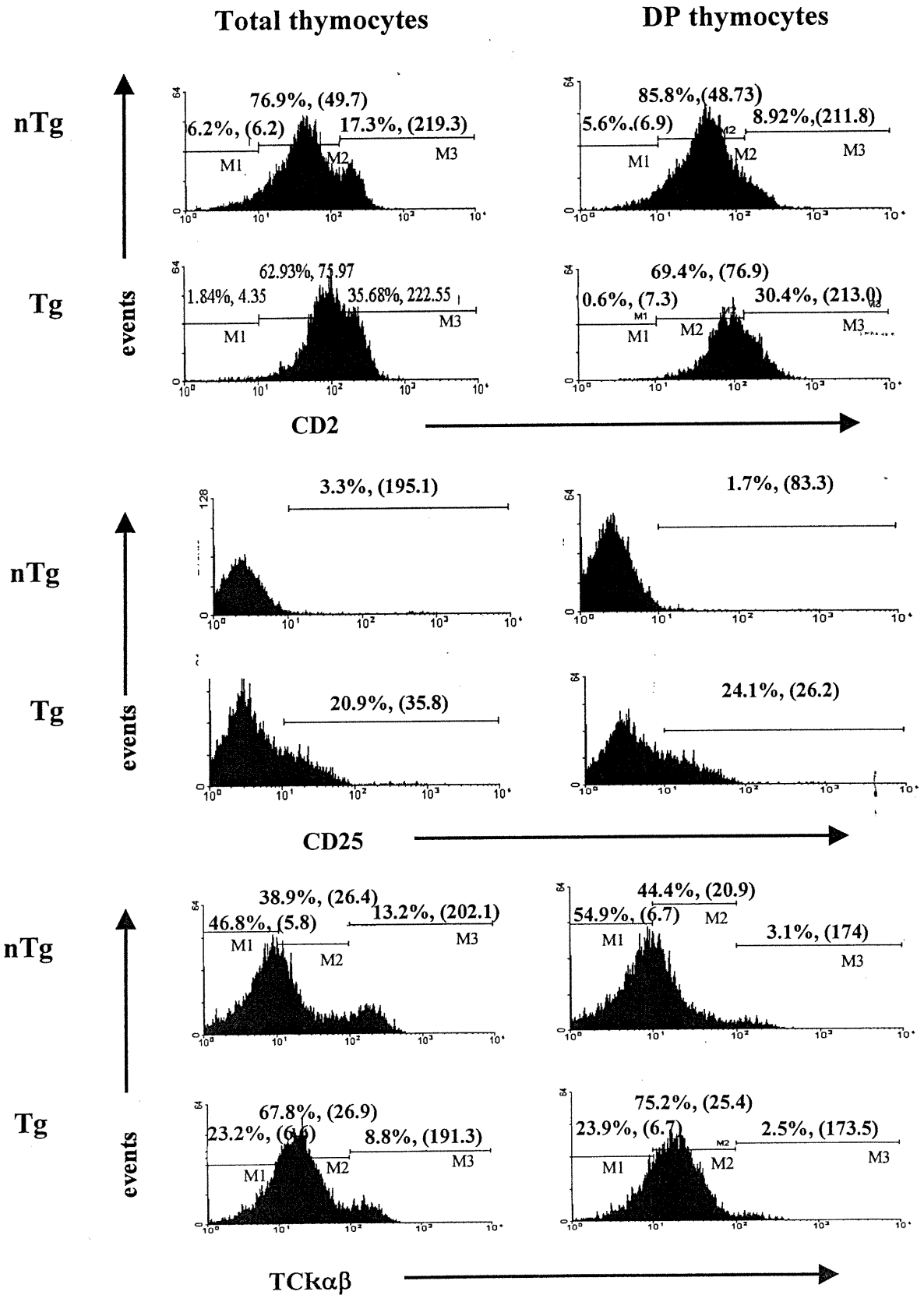

Total thymocytes
DP thymocytes
nTg
Tg
events
76.9%, (49.7)
6.2%, (6.2)
17.3%, (219.3)
M1
M2
M3
85.8%,(48.73)
5.6%,(6.9)
8.92%,(211.8)
62.93%, 75.97
1.84%, 4.35
35.68%, 222.55
69.4%, (76.9)
0.6%, (7.3)
30.4%, (213.0)
CD2
3.3%, (195.1)
1.7%, (83.3)
20.9%, (35.8)
24.1%, (26.2)
CD25
38.9%, (26.4)
46.8%, (5.8)
13.2%, (202.1)
44.4%, (20.9)
54.9%, (6.7)
3.1%, (174)
67.8%, (26.9)
23.2%, (6.6)
8.8%, (191.3)
75.2%, (25.4)
23.9%, (6.7)
2.5%, (173.5)
TCKαβ

Table 3: Number of thymocytes in CD4C/Notch1^{intra} Tg mice treated with dexamethasone *in vivo*

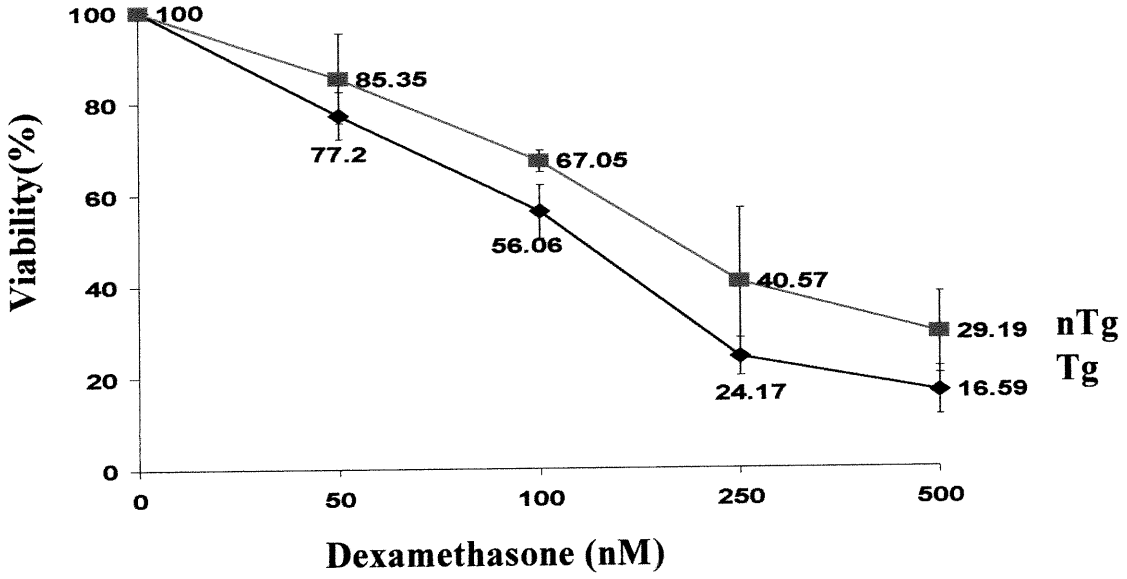
Mice	Dexamethasone [a]				Relative Viability [b] (treatment/control, %)	
	-		+			
	Total	DP	Total	DP	Total	DP
nTg	13.5±4.5	10.8±3.4	1.5±1.2	0.5±0.8	9.2± 7.1	3.2± 2.9
Tg	14.8±7.7	12.1±6.4	4.4±3.5	2.8±2.2	26.4±17.1*	19.1±12.7*

a. The number (10^7) of total and DP viable (7-AAD negative) thymocytes from Tg and nTg mice (F30856, C3H background) injected i.p. with dexamethasone (0.3mg) (+) or with vehicle (-) (RPMI medium with 6% ethanol). The mice were sacrificed after 48hrs. Thymocytes were labeled with 7-AAD and anti-CD4 (PE), anti-CD8 (FITC) mAb. The mean±SD (X 10^7) is shown.

b. The ratio represents the number of thymocytes in treated mice compared to the number of thymocytes in control mice. The data from four independent experiments are shown. * $P \leq 0.05$

Figure 3

A


100
80
60
40
20
0
Viability(%)
100
85.35
77.2
67.05
56.06
40.57
24.17
29.19
16.59
nTg
Tg
0
50
100
250
500
Dexamethasone (nM)

B

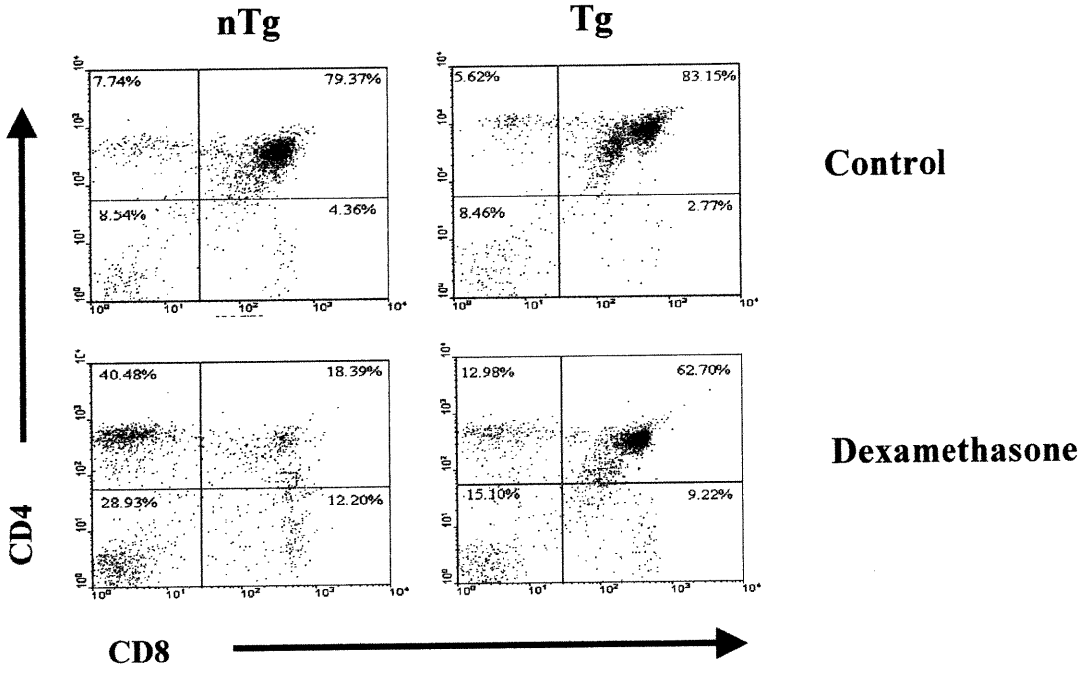
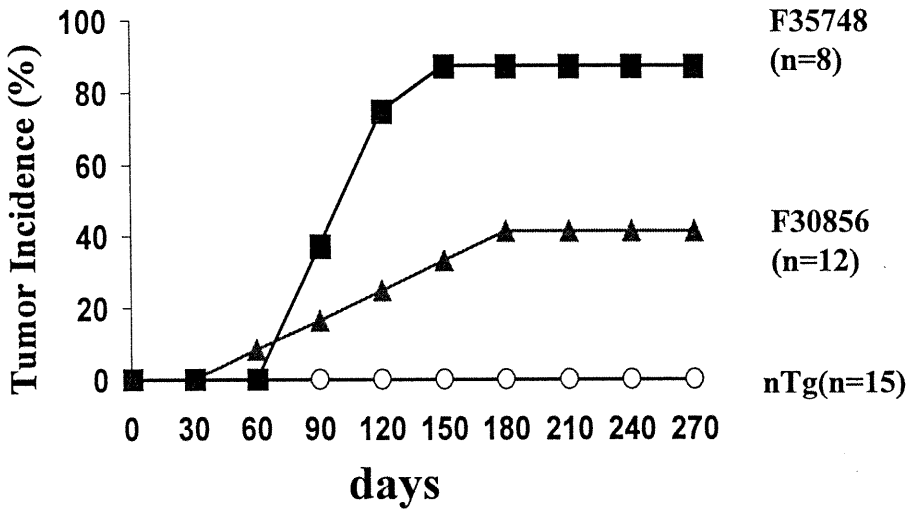
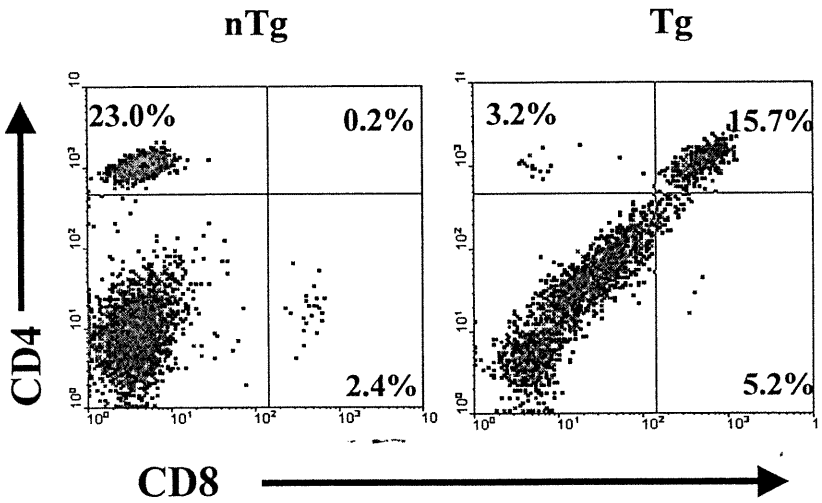

nTg
Tg
7.74%
79.37%
8.54%
4.36%
5.62%
83.15%
8.46%
2.77%
Control
40.48%
18.39%
28.93%
12.20%
12.98%
62.70%
15.10%
9.22%
Dexamethasone
CD4
CD8

Figure 4

A


100
80
60
40
20
0
Tumor Incidence (%)
0 30 60 90 120 150 180 210 240 270
days
F35748
(n=8)
F30856
(n=12)
nTg(n=15)

C


nTg
Tg
23.0%
0.2%
2.4%
3.2%
15.7%
5.2%
CD4
CD8

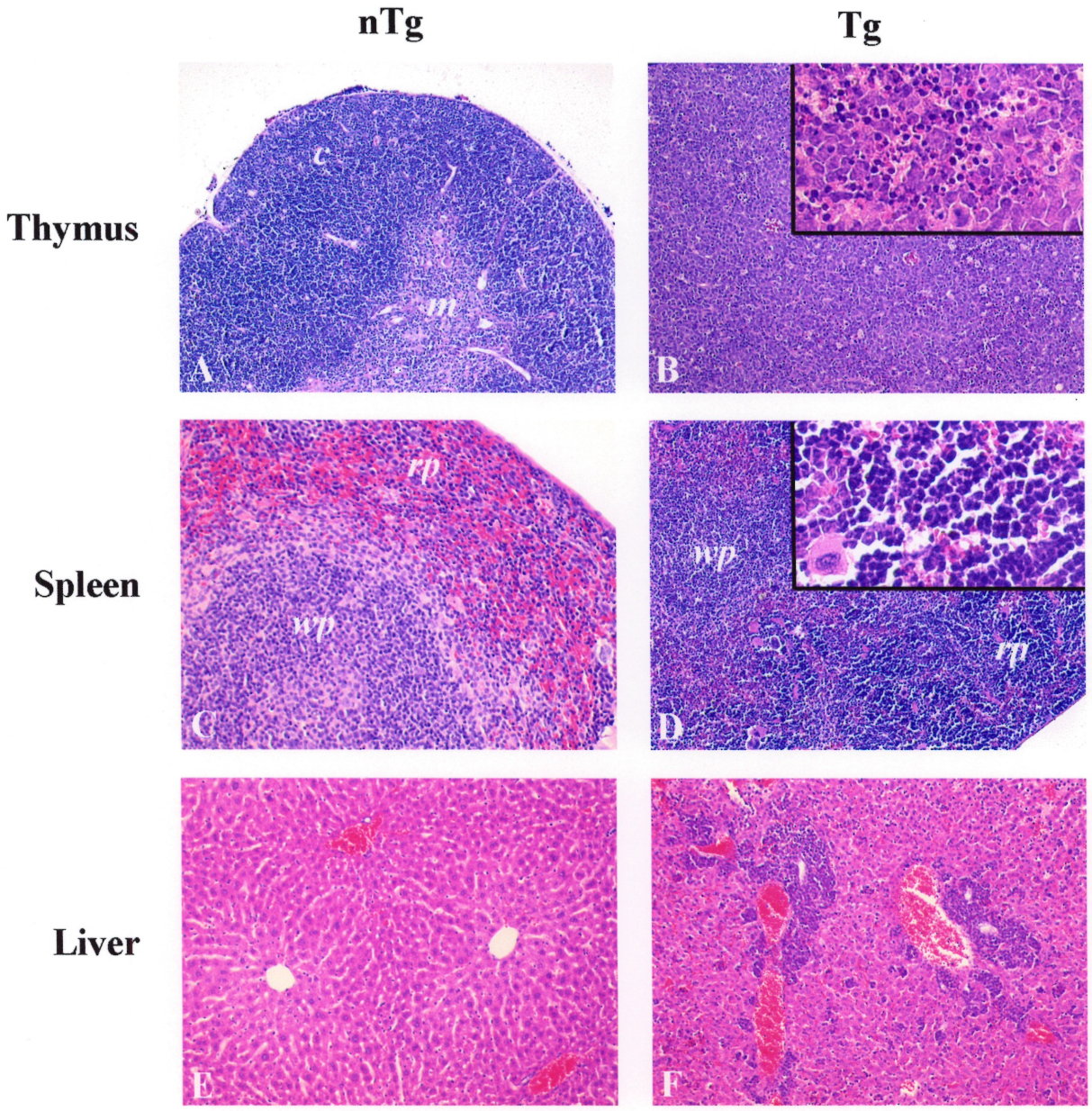
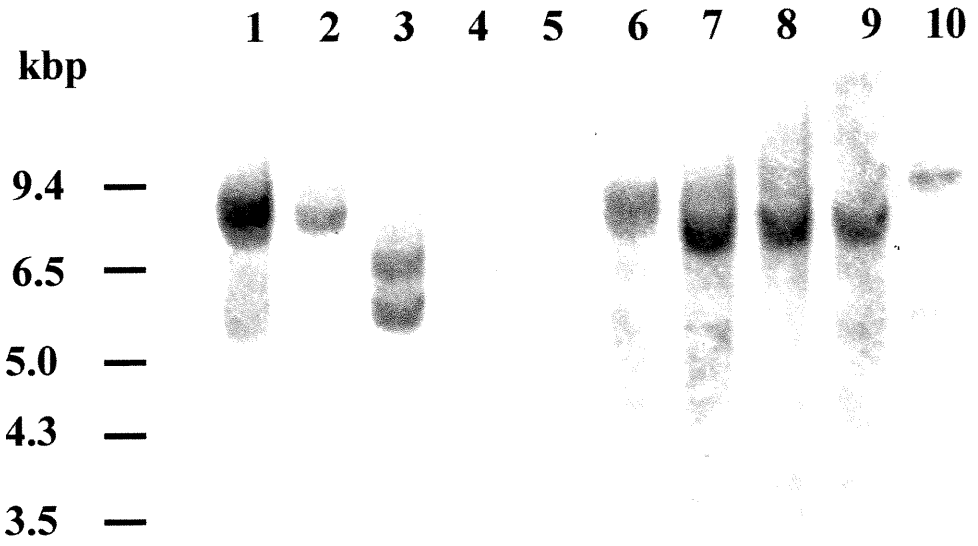


Figure 4B

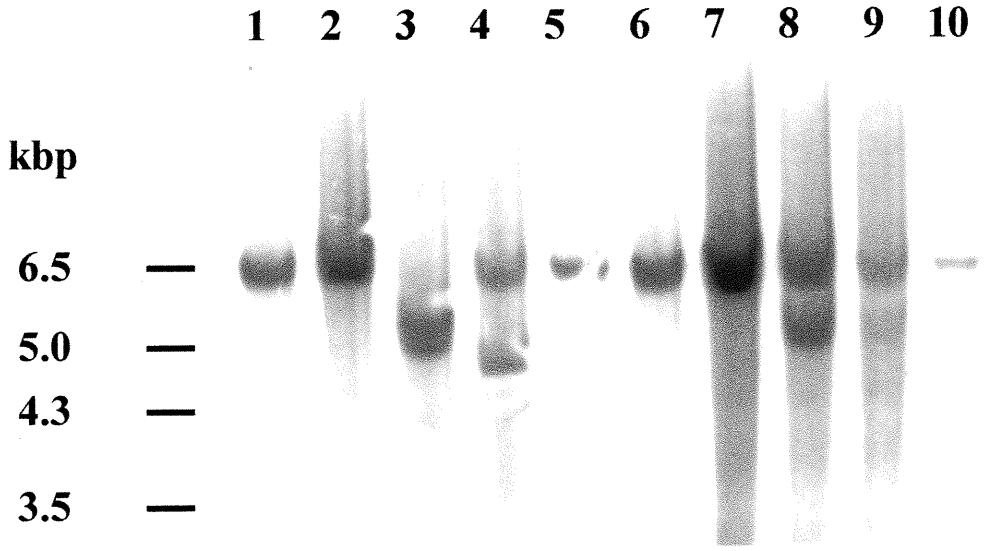
Figure 5

A



TCR gene

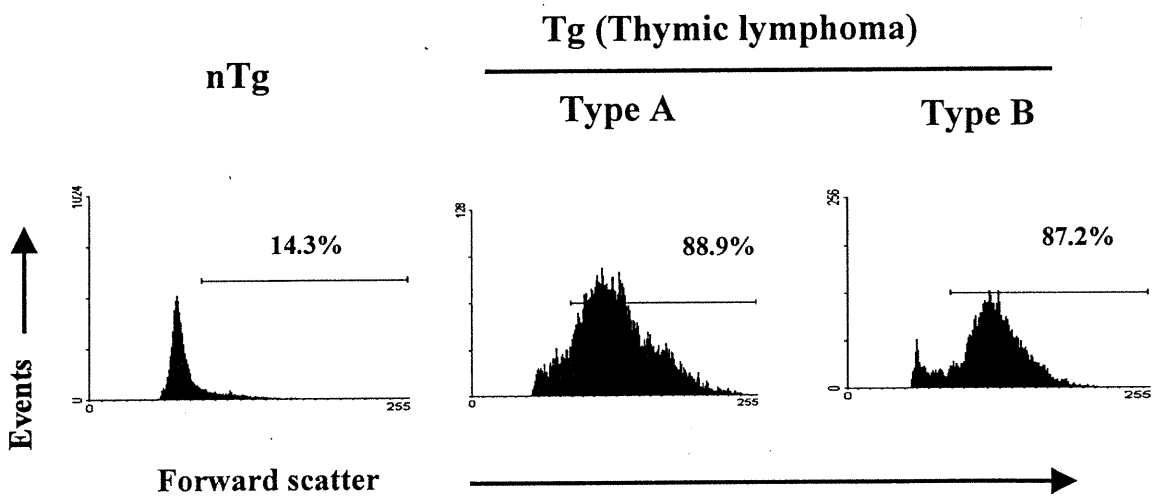
B



Ig gene

Figure 6

A


nTg
Tg (Thymic lymphoma)
Type A
Type B
14.3%
88.9%
87.2%
Events
Forward scatter

C

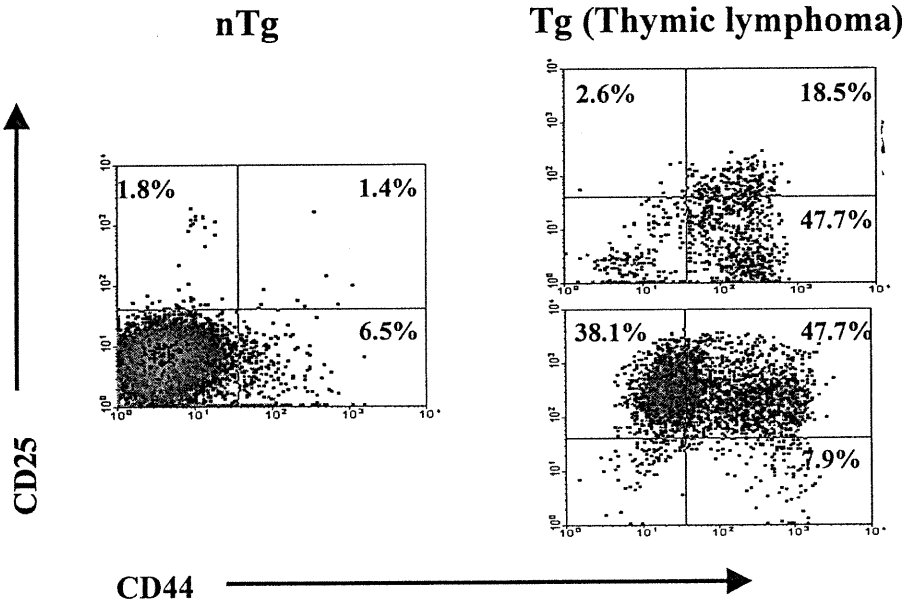

nTg
Tg (Thymic lymphoma)
1.8%
1.4%
6.5%
2.6%
18.5%
47.7%
38.1%
47.7%
7.9%
CD25
CD44

Figure 6

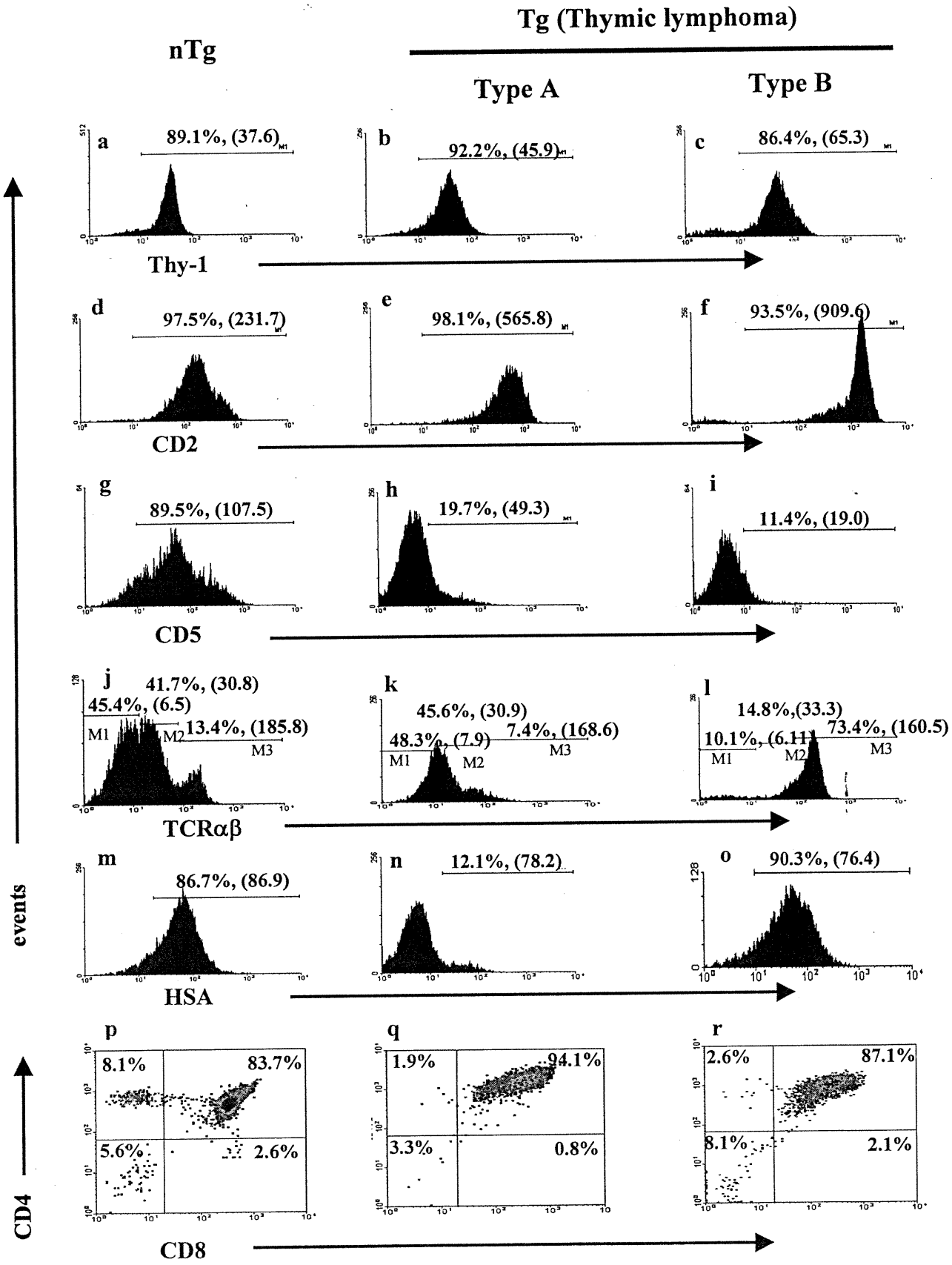

B
nTg
Tg (Thymic lymphoma)
Type A
Type B
a
89.1%, (37.6)
b
92.2%, (45.9)
c
86.4%, (65.3)
Thy-1
d
97.5%, (231.7)
e
98.1%, (565.8)
f
93.5%, (909.6)
CD2
g
89.5%, (107.5)
h
19.7%, (49.3)
i
11.4%, (19.0)
CD5
j
41.7%, (30.8)
45.4%, (6.5)
M1
M2
13.4%, (185.8)
M3
k
45.6%, (30.9)
48.3%, (7.9)
7.4%, (168.6)
l
14.8%,(33.3)
10.1%, (6.11)
73.4%, (160.5)
TCRαβ
m
86.7%, (86.9)
n
12.1%, (78.2)
o
90.3%, (76.4)
HSA
events
p
8.1%
83.7%
5.6%
2.6%
q
1.9%
94.1%
3.3%
0.8%
r
2.6%
87.1%
8.1%
2.1%
CD4
CD8

Figure 7

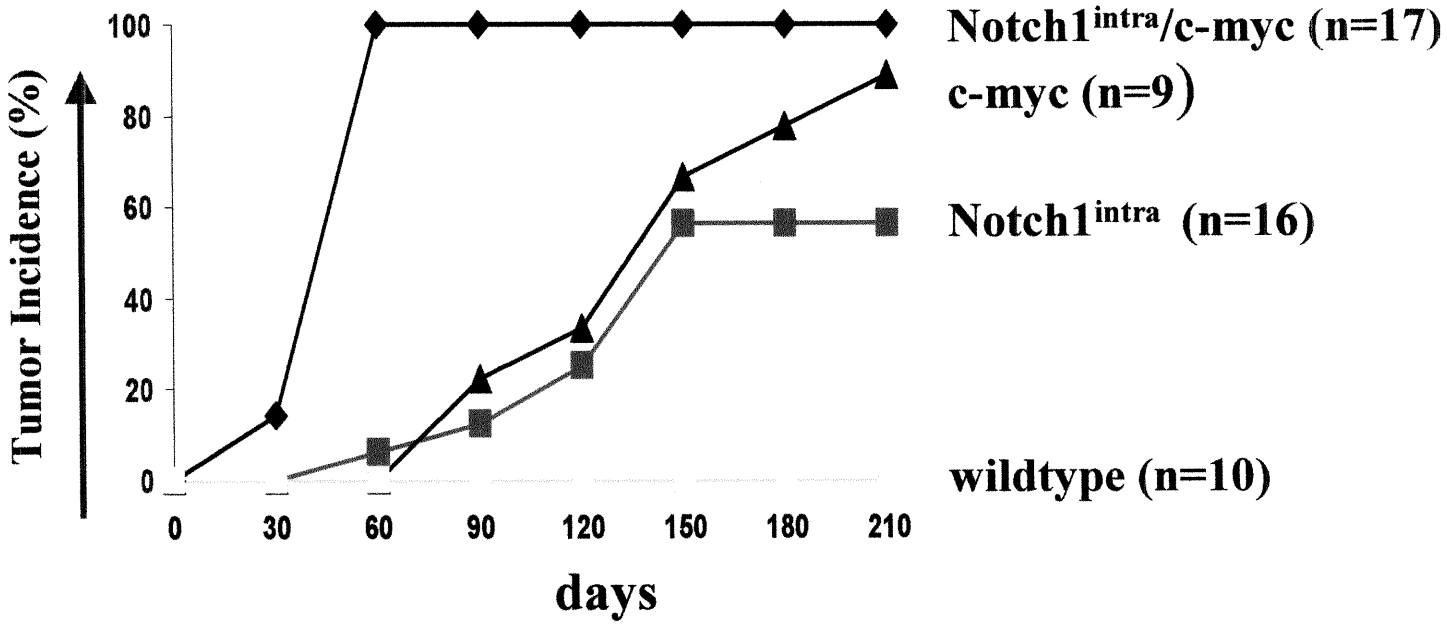

Tumor Incidence (%)
100
80
60
40
20
0
0
30
60
90
120
150
180
210
days
Notch1intra/c-myc (n=17)
c-myc (n=9)
Notch1intra (n=16)
wildtype (n=10)

Fig.1: Transgene expression in CD4C/Notch1^{intra} Tg mice

A. Construct of the CD4C/Notch1^{intra} transgene. Symbols: close bar, human CD4C promoter; open bar, Notch1 intracellular domain (amino acid 1659 to 2533), including the transmembrane (TM) domain, ankyrin (ANK) repeats, OPA and PEST domains; hatched bars, SV40 polyA signal.

B. Northern blot analysis of Notch1^{intra} transgene RNA expression in thymocytes. Thymus (T) RNA from two animals of each Tg founder line (F30856 and F35748) and from wildtype nTg littermates were hybridized with Notch1 probe K (2.7 kbp fragment of Notch1 intracellular domain). Note the detection of the 3.5 kbp Notch1^{intra} RNA in Tg thymuses. The 28S rRNA is to monitor loading.

C. Western blot analysis of transgene protein expression in thymocytes. Whole-thymocyte lysates from Tg mice of two founder lines (F30856 and F35748) or from control nTg littermates were reacted with anti-Notch1-intra1 antibodies, as described in materials and methods. The membrane was then stripped and then reacted with anti-actin antibodies.

Fig. 2: Cell surface marker analysis of thymocytes from CD4C/Notch1^{intra} Tg Mice

FACS analysis was carried out on total thymocytes by simple staining with anti-CD2 (PE), anti-CD25 (PE) and anti-TCRαβ (FITC) antibodies. The DP thymocytes were analyzed by three-color staining with anti-CD4(CY. 5) / anti-CD8 (FITC or PE) plus anti-CD2 (PE), anti-CD25 (PE) or anti-TCRαβ (FITC), and

gating on the $CD4^+CD8^+$ DP population. The numbers over the markers indicate the percentage and the mean fluorescence intensity.

Figure 3 : Notch1^{intra} inhibits dexamethasone-induced apoptosis of CD4C/Notch1^{intra} Tg thymocytes *in vitro* and *in vivo*

A. Thymocytes from CD4C/Notch1^{intra} Tg mice and their nTg littermates (F30856, C3H background) were incubated *in vitro* in medium alone or in medium containing dexamethasone at the indicated concentration and analyzed for viability after 18 hrs with 7-AAD staining and FACS. The numbers show the average relative viability (7-AAD negative) from three independent experiments. The percentage of viable thymocytes in culture containing medium alone was considered by 100%. The differences between groups reached significance ($P<0.05$) at the 100nM concentration.

B. FACS analysis of CD4 and CD8 expression in thymocytes from Tg and nTg mice (F30856, C3H background) treated *in vivo* with dexamethasone (0.3mg) or with vehicle (RPMI medium with 6% ethanol). Animals were inoculated i.p. and sacrificed 48 hrs later. Thymocytes were stained with anti-CD4 (PE) and anti-CD8 (FITC) mAb as well as with 7-AAD. The numbers in the panels showed the percentage of viable cells (first gated as 7-AAD negative cells) in each subset. Similar results were obtained in four independent experiments.

Fig 4. Development of thymic lymphoma in CD4C/Notch1^{intra} Tg mice

A. Cumulative incidence of thymic lymphoma in CD4C/Notch1^{intra} Tg mice (CD1 background). Data are shown for Tg mice of the F30856 (closed triangles), and the F35748 (closed squares) lines as well as for their nTg littermates (open circle). Results are indicated as the percentage of mice which were found dead and had thymic lymphoma or were sacrificed because of dyspnea caused by thymic lymphomas.

B. Histological analysis of tissues from a thymic lymphoma bearing mouse. Thymic lymphoma (B), spleen (D), and liver (F) are compared with nTg organs (A, C, and E). Note the disruption of the thymic architecture (B) and the accumulation of atypical lymphoblasts and dead and dying cells (inset). The splenic red pulp (shown at high magnification in the inset to D) and perivascular areas of liver are infiltrated with lymphoblasts. *wp*, white pulp; *rp*, red pulp; *c*, cortex; *m*, medulla. Counterstain: hematoxylin and eosin. Magnification: panels A-F 100X, inserts 400X,

C. FASC analysis of spleenic cells isolated from a nTg mouse and from a Tg mouse with thymic lymphoma. Cells were stained for surface expression of CD4 and CD8.

Fig 5. TCR and Ig gene rearrangement in thymic lymphomas of CD4C/Notch1^{intra} Tg mice

DNAs from thymic lymphomas were digested with HindIII (A) or EcoRI (B) and analyzed with ^{32}P-labeled TCR β chain (RBL5) (A) or Ig H chain (J_H-6.2) (B)

probe. Lanes 1 to 9 represent independent thymic lymphomas from different Tg mice. Lane 10, control DNA from a kidney of Tg mouse bearing thymic lymphoma. Molecular weight markers (kbp) are shown at the left.

Fig 6. FASC analysis of thymic lymphomas of CD4C/Notch1^{intra} Tg mice.

A. Forward scatter (FSC) analysis. Notice that thymic lymphoma cells are larger than most normal thymocytes. No difference is observed between type A and type B tumors. The numbers over the markers show the percentage of larger cells.

B. Cell surface markers on thymic lymphoma cells. Cell surface markers included Thy-1(FITC) (a-c), CD2 (PE) (d-f), CD5 (PE) (g-i), TCR$\alpha\beta$(FITC) (j-l), HAS (FITC) (m-o), and two-color staining of CD4(PE) versus CD8 (FITC) (p-r). The numbers in the panels or over the markers show the percentage of positive cells and the mean fluorescence intensity. Type A (TCR$\alpha\beta^{LOW}$HSA^{LOW}) and B (TCR$\alpha\beta^{high}$HSA^{intermedium}) define two patterns of staining observed for the thymic lymphoma.

C. Two-color staining of anti-CD25(PE) versus anti-CD44(FITC) in thymic lymphoma cells. Notice that the thymic lymphoma cells are either CD44^{+}CD25^{+}, CD44^{+}CD25^{-} or CD44^{-}CD25^{+}. No difference between the type A and B in this phenotype.

Fig 7 : Enhanced development of thymic lymphoma in Notch1^{intra}/ c-myc double Tg mice

CD4C/Notch1^{intra} (F30856) and MMTV^{D}/c-myc Tg mice (CD1 background were crossed together to obtain double Notch1^{intra}/c-myc (closed diamonds) Tg mice and the control littermates single CD4C/Notch1^{intra} (closed squares) and MMTV^{D}/myc (closed triangles) Tg mice, as well as non-Tg mice (open squares). Results are indicated as the percentage of mice which were found dead and had thymic lymphoma or were sacrificed because of dyspnea caused by thymic lymphomas. Cumulative incidence of tumor is shown.

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Part III: General Discussion

General Discussion

Involvement of Notch1^{intra} in T cell development In this study, we demonstrate that the truncated form of Notch1^{intra}, which was previously found in thymic lymphomas of Moloney murine leukemia virus (MuLV) infected MMTV^{D}/c-myc transgenic mice (Girard et al., 1996), plays a role in promoting T cell development in young CD4C/Notch1^{intra} Tg mice. First, we show that expression of Notch1^{intra} leads to an increase in total number of thymocytes, especially in the $CD4^+CD8^+$ DP subpopulation. Second, we find an increase percentage in $CD2^{high}$, $TCR\alpha\beta^{medium}$ and $CD25^+$, a decrease percentage in $CD2^{low}$, $CD2^{medium}$ and $TCR\alpha\beta^{low}$ in total thymocytes as well as DP thymocytes. Third, Notch1^{intra} protects DP thymocytes from dexamethasone-induced apoptosis *in vitro* and *in vivo*. Together, these results indicate that activation of Notch1^{intra} influences thymocyte development and survival.

Two other Notch1 Tg mouse lines have been previously reported by Robey's and Bevan's groups. In these Tg lines a portion of Notch1 intracellular domain was expressed under the control of the LCK proximal promoter. Using these Tg mice and other cell lines, Notch1 intracellular mutants have been found to generate an increase in $CD8^+$ SP thymocytes and a decrease in $CD4^+$ thymocytes (Robey, et al, 1996,) or an increase in both $CD4^+$, $CD8^+$ SP thymocytes (Deftos et al., 2000); to favor the $\alpha\beta$ T cell fate in a chimaeric haemopoietic system populated by a equal portions mixture of fetal liver or bone marrow cells from Notch1$^{+/-}$ and Notch1$^{+/+}$ donor mice (Washburn, et al. 1997); to induce the expression of CD25 and CD44 on DP thymocytes (Deftos et al., 2000); as well as to inhibit dexamethesone-

induced apoptosis in a thymic lymphoma line (AKR1010), T cell hybridoma and transgenic mice (Deftos et al., 1998). Consistent with the work of these two groups, we observe that expression of Notch1^{intra} when under the control of CD4C promoter leads to an increased percentage of CD2 high level expression cells, TCR$\alpha\beta$ intermediate level expression cells and CD25^{+} cells in thymocytes. Moreover, we also provide evidences that Notch1^{intra} inhibits dexamethasone-induced apoptosis of thymocytes, not only *in vitro,* but also *in vivo* experiments. However, in contrast to their results, we could not find impairment of CD4 and CD8 distribution in either thymocytes or T cells in peripheral organs of CD4C/Notch1^{intra} Tg mice. Interestingly, the number of both total thymocytes and CD4^{+}CD8^{+} DP thymocytes reproducibly increased in CD4C/ Notch1^{intra} Tg mice. This phenomenon was not observed in other two Tg lines produced by other groups. The similarities and differences of Notch1 Tg lines from Robey's group, Bevan's groups and our group are summarized in table 1 (Robey, et al, 1996; Washburn, et al. 1997; Deftos et al., 1998; Deftos et al., 2000).

The reason for the differences between CD4C/Notch1^{intra} Tg mice and others is not clear, but may be due to differences in the promoter which controls the transgene expression, or to differences in the region of Notch1 expressed as a transgene. For example, in mouse adult thymus, LCK proximal promoter expression is higher in immature CD4^{-}CD8^{-} and CD4^{+}CD8^{+} thymocytes and lower in more mature single positive thymocytes, particularly low in CD4^{+}CD8^{-} SP thymocytes and peripheral T cells (Reynolds, et al., 1990; Sentman et al., 1991). The CD4C promoter is expressed in CD4^{+}CD8^{+} and CD4^{+}CD8^{-} thymocytes, CD4^{+} T cells in the

Table 1. The similarities and differences of three Notch1 Tg lines

	Lck/Notch1IC (Robey's group)	Lck/Notch1IC (Bevan's group)	CD4C/Notch1^{intra}
Transgene	ANK and nls	RAM,ANK,TAD and OPA	a portion of EC,TM,RAM, ANK,TAD, OPA and PEST
	(aa1750 - aa2293)	(aa1751-aa2444)	(aa1659-aa2533)
mouse strain	C57	B6	C3H and CD1
thymic phenotype			
No.of thymocytes	No change	no change	increase in total thymocyte and DP thymocytes
CD4/CD8	increase in CD8 decrease in CD4	increase in both CD4 and CD8	no impairnent in CD4, CD8 distribution
TCR αβ	increased	no data	Percentage of TCRαβ^{medium} cells increased and TCRαβ^{low} cells decreased.
γδ	normal number of γδ TCR$^+$ cells	no data	no change
other markers	no data	CD25$^+$, CD44$^+$ increased in DP thymocytes	CD2^{high}, CD25$^+$ cells increased in DP thymocytes
resistance of DMX -induced apoptosis	yes (*in vitro*)	no data	yes (*in vitro* and *in vivo*)
periphery	no data	a reduction in the No. of both CD4$^+$ and CD8$^+$T cells but no change in the ratio	no change in the No. of T cells, B cells , and CD4/CD8 ratio.
thymic lymphomas			
Incidence	occation	some (4/8 founder lines loss by thymomas)	C3H strain : 5% CD1 strain : 42% and 87% in two founder lines
phenotype	TCR^{low}CD4^{low}CD8$^+$	CD3^{medium}HSA^{high}CD25^{high} CD69^{low} with varible levels of CD4 and CD8.	Thy-1$^+$CD2$^+$CD5$^-$ CD4$^+$CD8$^+$ Type A : TCRαβ^{low}HSA$^-$ Type B : TCRαβ^{high}HSA^{medium}

periphery organs as well as macrophages and dendritic cells. No expression, however, was found in $CD4^-CD8^-$ thymocytes (Hanna et al., 1994; unpublished data) (see detail in table 2). The different expression levels in mature SP thymocytes may affect the differentiation from DP thymocytes to SP thymocytes, since overexpression of activated Notch1 can override the interactions of TCR and co-receptors with MHC molecular to induce an increase of $CD8^+$ and/or $CD4^+$ SP. Although differentiation from DP thymocytes to SP thymocytes in Tg mice is transgenic Notch1 specific, it is likely to be controlled by the expression level of transgenic Notch1 in the DP and SP thymocytes.

Table 2. Difference of the cell type expression pattern between Lck proximal promoter and CD4C promoter

	Thymus				periphery
	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$	
LCK proximal promoter	+	+	-	±	-
CD4C promoter	-	+	+	±	CD4+ T cells Macrophage, DC

Another reason causing the differences between CD4C/$Notch1^{intra}$ Tg mice and other Notch1 Tg mice may be due to the differences in the region of Notch1 expressed as a transgene (see table 1). Our construct mimics the mutation spontaneously arising by provirus insertion containing a portion of extracellular domain, the transmembrane domain and intact intracellular domain including RAM, ANK-repeat, OPA and PEST regions. The truncated Notch1 protein containing a transmembrane region may influence the primary localization of the protein and further influence the transduction of Notch1 signaling and then, the regulation of

downstream effectors. The complete C-terminus including OPA and PEST region, has been found to associate with Numb and Dishevelled proteins, the modifiers of Notch signaling pathway. Genetic studies suggest that these interactions are inhibitory to Notch signaling transduction (Axelrod et al., 1996; Guo et al., 1995, Guo et al., 1996). PEST domain alone is thought to regulate protein stability. Deletion of PEST domain seems to stabilize the protein and to increase the phenotype. However, the further function of PEST in Notch1 is unknown.

The increased number of total thymocytes and DP thymocytes in CD4C/ Notch1^{intra} Tg mice is novel. This is the significant difference in the phenotype between CD4C/ Notch1^{intra} Tg mice and the other two Notch1 Tg lines in thymocyte development. The mechanisms of this thymocyte dysregulation need to be studied. There are two possibilities: Notch1^{intra} promotes thymocyte proliferation and/or inhibits thymocyte apoptosis. A link between proliferation events and the member of Notch family has been seen in several instances. In *C. elegans*, hermaphrodites and males homozygous for a constitutively active form of GLP-1 have germ cells that never exit the mitotic cycle (Berry et al., 1997). Additionally, the expression of an activated form of the Notch receptor along the dorsal-ventral or the anteriorposterior boundary of the *Drosophila* Wing induces mitotic activity (Go et al., 1998). Moreover, Notch3, a member of Notch family in mammal, is found to be involved in proliferation of thymocytes in Tg mice models. The activated form of Notch3 can increase the absolute number of each subset of thymocytes in Tg mice under the control by LCK proximal promoter (Bellavia et al, 2000). In CD4C/ Notch1^{intra} Tg mice, the increase of thymocytes is only in DP subpopulation, which

is one of the target cell type of the CD4C promoter. It is reasonable to propose that transgenic Notch1^{intra} induces this phenomenon. However, the hypothesis needs to be supported by further experiments of kinetics study.

Another possible mechanism is the inhibition of thymocyte apoptosis by Notch1^{intra} transgene. During the CD4^{+}CD8^{+} double positive stage, thymocytes express CD4 and CD8 co-receptor as well as TCR. These cells are competent to bind to MHC and undergo positive selection and negative selection. The CD8 co-receptor binds to MHC class I while the CD4 co-receptor binds to MHC class II. Cells that bind to MHC class I become CD8 single positive T cells while those that bound to MHC class II become CD4 single positive T cells. This process is known as positive selection. On the other hand, some thymocytes can bind self-MHC with high affinity and then cause negative selection or the induction of apoptosis. Thus, CD4^{+}CD8^{+} DP thymocytes can either be positively selected for further development and export to the peripheral immune system or undergo negative selection and death within the thymus. However, the majority, probably up to 95% DP thymocytes, cannot recognize self-MHC and hence are never signaled to continue development. These cells die by neglect. "Death by neglect" is thought to be mediated, at least in part, by glucocorticoids endogenously produced in the thymus. Previously, the role of Notch1 in protecting DP thymocyte from dexamethasone-induced apoptosis has been described, but only been studied in cell lines or Tg thymocytes *in vitro* (Deftos et al., 1998). Our study is the first report to examine the anti-apoptosis function of Notch1 *in vivo*. Comparing the phenotype between CD4C/Notch1^{intra} Tg mice reported here and Tg mice produced by Robey's group,

we noted that thymocytes from Tg mice produced by Robey's group presented a phenotype of resistance to dexamethasone-induced apoptosis *in vitro* (Deftos et al., 1998), but less proliferation (Robey et al., 1996). Therefore, no increased number of thymocytes is found in those Tg mice. Hence, it is possible that the Notch1^{intra} by promoting proliferation, rather than inhibiting "death by neglect" contribute to the increased number of thymocyte in CD4C/ Notch1^{intra} transgenic mice.

T lymphocytes begin life in the bone marrow as pluripotent stem cells. These cells migrate to the thymus and the process of T cell receptor rearrangement begins, committing the cells to the T cell lineage. T cells possess two different types of TCR known as the TCR$\alpha\beta$ and TCR$\gamma\delta$. These earliest thymic pre-T cells are thought to commit to either the $\alpha\beta$ or the $\gamma\delta$ lineage by the rearrangement of the appropriate TCR genes. This checkpoint which T cells choose the cell fate in TCR$\alpha\beta$ cells or TCR$\gamma\delta$ cells occurs at the CD4$^-$CD8$^-$ DN stage. Notch1 has been found to be involved in this checkpoint by Robey's group. Overexpression of the activated form of Notch1 favors the cell fate in TCR$\alpha\beta$ T cell (Washburn, et al. 1997). We also found an increased percentage of intermediate level of TCR$\alpha\beta^+$ cells, but it was not accompanied by a decrease of TCR$\gamma\delta$ cells in CD4C/Notch1^{intra} Tg mice. This increase of intermediate level of TCR$\alpha\beta$ expression cells is thought to be through a different mechanism, because Tg Notch1^{intra} under the control of CD4C promoter is not expressed in CD4$^-$CD8$^-$ thymocytes. We believe that the increase of the intermediate level of TCR$\alpha\beta$ expression cells is due to the dysregulation of TCRα and TCRβ gene expression by Notch1^{intra} in DP stage.

Taken together, the increased number of DP thymocyte, the persistence of $CD25^+$ cells in DP subset, the increased percentage of $CD2^{high}$ and TCR$\alpha\beta$ intermediate level expression cells in total thymocytes and DP thymocytes, as well as the inhibition of DP thymocytes from dexamethasone-induced apoptosis suggest that the DP thymocytes are the initial target of $Notch1^{intra}$-induced dysregulation. Such dysregulation may be the basis of the lymphomagenesis in these Tg mice.

Contribution of $Notch1^{intra}$ in lymphomagenesis Our results show that CD4C/$Notch1^{intra}$ Tg mice spontaneously develop thymic lymphomas, suggesting that this truncated form of Notch1 can behave as an oncogene. All the thymic lymphomas tested belong to the $CD4^+CD8^+$ cell lineage. No other cell phenotype was found in those lymphomas. These results indicated that $Notch1^{intra}$ was efficient in transforming some ($CD4^+CD8^+$ thymocyte) but not all ($CD4^+$ thymocyte, $CD4^+$ T cell, macrophage, dendritic cell) of the target cells in which CD4C promoter was expressed. The observation that the thymic lymphoma cells expressed a single or double TCR-β chain indicated that these tumors were monoclonal or clonal rather than polyclonal. The clonal or monoclonal origin as well as the long latency in the thymic lymphoma development suggested that $Notch1^{intra}$ itself was not sufficient to fully transform T cell. This is not unusual. A single genetic event rarely directly transforms primary cells and additional events (second hit) are required for the tumorigenesis.

These thymic lymphomas belong to the T cell lineages. This conclusion is supported by the presence of T cell surface markers and TCR β-chain gene rearrangement or deletion in both alleles in the tumor cells. However,

Immunoglobulin heavy-chain (IgH) gene rearrangement is found in some of these thymic lymphomas. This phenomenon is not unique in the thymic lymphomas arising in CD4C/ Notch1^{intra} Tg mice. It was also observed in thymic lymphomas arising in MMTV^{D}/c-myc Tg mice and other lymphoid tumors (Pelicci et al. 1985; Zuniga et al., 1982). Furthermore, IgH gene segments are rearranged not only in B lymphocytes but also frequently in T lymphocytes in mice and human (Forster et al., 1980; Kurosawa et al. 1981; Zuniga et al., 1982). The IgH rearrangements occur in the thymus after T cell receptor gene and T cell specific γ- gene rearrangements but before thymocyte maturation is completed (Born et al., 1988). In CD4C/Notch1^{intra} Tg mice, the thymocytes were transformed at the $CD4^{+}CD8^{+}$ DP stage. This stage matched that which IgH rearrangement occurred in thymocyte development. Therefore, it is not surprising that IgH rearrangement occurred in the tumors tested. However, IgH rearrangement occurred in some, but not all thymic lymphomas tested in both cell surface marker phenotypes as we described in the paper (type A and type B). It seems that such rearrangements occur at random, rather than at a distinct T cell stage in thymic lymphomas reported here.

In our Tg model, Notch1^{intra} expresses higher in founder line F35748 in both RNA and protein level than those in founder line F30856, and the incidence of thymic lymphomas is correspondingly higher in founder line F35748 on CD1 background. These results suggest the expression level of the Tg is responsible for the incidence of thymic lymphomas. However, even in the higher expression Tg founder line, there is not 100% Tg mice spontaneously development thymic lymphoma. Since CD1 mice are outbred mice, they are not completely uniform in

genetic background among the lettermates, it is possible those different genetic events contribute to the development of thymic lymphoma.

Although several N-terminally truncated Notch1 mutants have been reported to be involved in transformation in several cell types, how these Notch1 mutants induced cell transformation is not completely understood. Those Notch1 mutants as well as Notch1^{intra} used here lack most or all of Notch1 extracellular domain which is though to be involved in DSL ligand binding, therefore activating Notch signaling pathway. Hence, cell transformation by these Notch1 mutants are thought to occur through a DSL ligand binding-independent mechanism. Interestingly, these Notch1 mutants were found to locate in the nucleus, but whether this nuclear localization is necessary for cell transformation is still controversial (Jeffries and Capobianco, 2000; Pear et al., 1996). Previously, we have reported that deletion analysis of Notch1^{intra} revealed RAM and ANK domains were required for transformation in HC11 mouse mammary epithelial cells (Dievart et al., 1999). However, studies in E1A immortalized baby rat kidney (BRK) cells by another group showed that ANK domain, but not RAM domain was necessary for transformation in that cell type (Jeffries and Capobianco, 2000). These results indicated transformation is through CBF1-dependent pathway in HC11 and CBF1-independent pathway in BRK cells. Nevertheless, using a robust murine bone marrow reconstitution assay, the ANK and TAD were found to be required for T cell leukemogenesis, whereas the RAM domain was nonessential, but all of the transforming forms of Notch1 including the "RAM-less" form can activate Su(H)/CFB-1 – sensitive promoter elements. It is likely that Su(H)/CBF-1 – dependent signaling is required for transformation in T cell

progenitor (Aster et al. 2000). Taken together, the mechanism of transformation by Notch1 mutants is complicated. It seems lineage specific. Different effectors interact with Notch1 mutants in different cell types.

Collaboration of Notch1^{intra} and c-myc in lymphomagenesis Our previous studies suggested that Notch1^{intra} is a cooperator of c-myc for T cell transformation (Girard et al. 1996). Other genes which have been identified as collaborators of activated Notch1 in differerent tissue types are *neu, E1A, ras*, (Dievart et al.,1999; Capobianco et al., 1997; Fitzgerald et al., 2000). We observed here that all of the double Tg mice bearing Notch1^{intra} and c-myc developed thymic lymphomas with a latency average of 35 days. This latency is much shorter than that in single Notch1^{intra} transgenic littermates or single c-myc transgenic littermates. The high incidence and short latency of development of thymic lymphomas in double Tg mice provide a direct evidence that Notch1^{intra} and c-myc are cooperators in lymphomagenesis.

Although the mechanism of synergy between c-myc and Notch1^{intra} is not clear at this point, the following two possibilities could be discussed. First, c-myc and Notch1^{intra} expression is directly upregulated by their interaction. This hypothesis can be proved by studying c-myc and Notch1^{intra} expression in thymocytes without transformation and thymic lymphoma cells from double Tg mice. Second, c-myc and Notch1^{intra} interact through an indirect mechanism, for example, through increasing the expression of Bcl-2. Dysregulated expression of c-myc is known to induce proliferation and apoptosis. It is capable to drive quiescent cells into S phase (Eilers et al., 1989) and results in ungoverned progression

through the cell cycle. On the other hand, overexpression of c-myc can accelerate the rate of cell apoptosis in serum or glucose deprivation conditions, but this effect can be repressed by Bcl-2 (a pro-oncogene which function as an apoptosis resistant gene) co-expression (Fanidi et al., 1992; Wagner et al., 1993). Furthermore, Bcl-2 can cooperate with c-myc in cell immortalization (Vaux et al., 1988) and tumor progression (Strasser et al., 1990). The expression of Bcl-2 abrogates c-myc-induced apoptosis without affecting the c-myc mitogenic function (Fanidi et al., 1992). The mechanism of synergy between Bcl-2 and c-myc provided a clue for our future study. In our study, Notch1^{intra} protects thymocytes from dexamethasone-induced apoptosis in CD4C/Notch1^{intra} Tg mice, and Bcl-2 is found to be upregulated in the cell line that overexpressed activated Notch1. This cell line is resistant to dexamethasone-induced apoptosis *in vitro*. Hence, it is possible that the cooperation of Notch1^{intra} and c-myc in tumorigenesis is through Notch1^{intra} block of c-myc-induced apotosis by upregulating Bcl-2 expression. Therefore, these double Tg mice provide a model for studying the mechanism of synergy between Notch1^{intra} and c-myc in T cell transformation.

Different phenotype between C3H and CD1 background Tg mice

Different phenotypes of thymic lymphoma development are found in CD4C/Notch1^{intra} transgenic mice whether they are on C3H or CD1 background. Firstly, incidence of thymic lymphoma is lower in Tg mice from C3H background (about 5%) than in Tg mice from CD1 background (42% and 87.6%, respectively, in two founder lines). Secondly, the thymocyte population transformed is different. In Tg mice of C3H background, only one phenotype was seen in all the thymic

lymphomas tested which showed $CD4^+CD8^+TCR\alpha\beta^{low}HSA^-$ (type A) indicating the DP thymocytes were transformed in less mature DP stage. However, in thymic lymphoma arising in Tg mice of CD1 background, lymphoma cells presented two phenotypes, $CD4^+CD8^+TCR\alpha\beta^{low}HSA^-$ (type A) and $CD4^+CD8^+TCR\alpha\beta^{hi}HSA^+$ (type B), indicating DP thymocytes could have been transformed in both less mature and more mature stages. Thirdly, 100% double Tg mice bearing $Notch1^{intra}$ and c-myc transgenes on a CD1 background spontaneously developed thymic lymphomas with a very short latency. No double Tg mice on a C3H background developed this tumor. These dramatic differences indicated that the genetic events, which are presented here by our findings with the background of the mice, contribute to the oncogenesis. For example, CD1 mice could express specific effectors required for Notch1 and c-myc transformation, or C3H mice could express specific effectors that suppress Notch1 and c-myc transformation.

In summary, we present evidence here that an N-terminal truncated form of Notch1 ($Notch1^{intra}$) arising in Moloney MULV provirus insertion in $MMTV^D$/c-myc transgenic mice behaves as an oncogene. Under the control of the CD4C promoter, $Notch1^{intra}$ can induce dysregulation in thymocyte development, eventially causing thymocyte transformation and thymic lymphoma development in Tg mice. $Notch1^{intra}$ works as a cooperator of c-myc in lymphomagenesis. We postulate that

this activated form of Notch1 would bypass signaling by its nomal ligands and alter the pattern of gene expression in T cell development and T cell transformation.

Part IV: Conclusion

Conclusion

The *Notch1*^{intra} can behave as an oncogene to

1. dysregulate thymocyte development by increasing the number of total thymocytes and DP thymocytes, by increasing the percentage of $CD2^{high}$, $CD25^{+}$ and $TCR\alpha\beta^{int}$ cells and decreasing the percentage of $CD2^{low}$, $CD2^{medium}$ and $TCR\alpha\beta^{low}$ cells in DP thymocytes; by inhibiting dexamethasone-induced apoptosis of DP thymocytes.
2. induce transformation of double positive cells. There are two types of tumors. Type A tumor cells expressed $Thy\text{-}1^{+}CD2^{+}CD5^{-}CD4^{+}CD8^{+}TCR\alpha\beta^{low}HSA^{-}$, indicating the thymocytes were transformed in less mature DP stage. Type B tumor cells expressed $Thy\text{-}1^{+}CD2^{+}CD5^{-}CD4^{+}CD8^{+}TCR\alpha\beta^{high}HSA^{medium}$, indicating the thymocytyes were transformed in more mature DP stage.
3. cooperate with *c-myc* for lymphomagenesis.

Part V: Future Work

Future work

1. The proliferation of thymocytes

Since the number of thymocytes is increased in CD4C/Notch1^{intra} Tg mice, it is possible that an increased rate of proliferation of thymocytes contributes to this phenotype. To test this hypothesis, thymocyte proliferation following stimulation by anti-CD3 will be studied.

2. The expression of Bcl-2 in thymocytes

We found that Notch1^{intra} protects thymocytes from dexamethasone-induced apoptosis both *in vitro* and *in vivo.* Additionally Notch1^{intra} induces thymocyte transformation and cooperates with c-myc in lymphomagenesis. The mechanism of these effects of Notch1^{intra} may be through increased Bcl-2 expression. If this hypothesis is confirmed by Northern blot or Western blot analysis in RNA or protein level used thymocytes of Tg mice as well as thymic lymphomas arising in CD4C/Notch1^{intra} Tg mice and *Notch1^{intra}/c-myc* double Tg mice. The CD4C/ Notch1^{intra} Tg mice would be crossed with Bcl-2 knockout mice. The thymocytes from mice bearing Notch1^{intra} Tg/ Bcl-2 knockout will be studied in apoptosis.

3. The expression of Notch1^{intra} and c-myc in thymocytes of double Tg mice

We found that *Notch1^{intra}* and *c-myc* cooperated in lymphomagenesis. To study the mechanism of synergy between these two oncogenes, the expression of *Notch1^{intra}* and *c-myc* (RNA and Protein) will be studied in thymocytes and

thymic lymphomas from double Tg mice and compared to those from *Notch1*^{intra} or *c-myc* single Tg mice.

4. The cooperation of *Notch1(EC)*^{mut}, *Notch1*^{intra} and *c-myc*

Our previous study (Girard, et al., 1996) showed *Notch1* is associated with over 50% of thymic lymphomas which are induced in Moloney MuLV - infected MMTV^{D}/c-myc Tg mice, indicating that truncated forms of Notch1: Notch1EC^{mut} and Notch1^{intra} may be cooperating with c-myc in lymphomagenesis. We propose to test if another mutant, *Notch1EC*^{mut}, is a cooperator of c-myc and/or Notch1^{intra} in lymphomagenesis. CD4C/ Notch1EC^{mut} Tg mice will be crossed with CD4C/Notch1^{intra} or MMTV^{D}/c-myc Tg mice to generate double Tg mouse models. The incidence and immunophenotype of thymic lymphomas will be observed in these mouse models.

Part VI: References

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