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Transcription mechanisms and functions of NFATc1 in T lymphocytes

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Jiming Liu

8th May 2007

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SUMMARY

TRANSCRIPTION MECHANISMS AND FUNCTIONS OF NFATC1 IN T LYMPHOCYTES

By Jiming Liu

The Nuclear Factors of Activated T cells (NFATs) are critical transcription factors that direct gene expression in immune and non-immune cells. Interaction of T cells with Ag-presenting cells results in the clustering of T-cell antigen receptor (TCR), co-receptors and integrins. Subsequent signal transduction resulting in NFAT activation leads to cytokine gene expression. Among the NFATs expressed in T cells, NFATc1 shows a unique induction property, which is essential for T cell differentiation and activation. It was revealed before that 3 major isoforms of NFATc1 are generated in activated T cells – the inducible short NFATc1/A, and the longer isoforms NFATc1/B and C. However, due to alternative splicing events and the existence of two different promoters and two alternative polyadenylation, we show here that 6 isoforms are synthesized in T cells which differ in their N-terminal and C-terminal peptides. In these experiments, we have identified these 6 isoforms by semi-quantitative long distance RT-PCR in several T cells subsets, and the inducible properties of 6 isoforms were investigated in those cells. The short NFATc1/αA which is under control of the P1 promoter and the proximal pA1 polyadenylation site was the most prominent and inducible isoform in T effector cells. The transcription of the longer NFATc1/βB and βC isoforms is constitutive and even reduced in activated T lymphocytes. In addition to NFATc1 autoregulation, we tried to understand the NFATc1 gene regulation under the control of PKC pathways by microarray analysis. Compared to treatment of T cells with ionomycin alone (which enhances Ca^{++} flux), treatment of cells with the phorbolester TPA (leading to PKC activation) enhanced the induction of NFATc1. Microarray analysis revealed that PKC activation increased the transcription of NF-κB1, Fos and JunB, which are important transcription factors binding to the regulatory regions of the NFATc1 gene. Besides the promoting effect of these transcription factors, we provided evidence that p53 and its targeting gene,

Gadd45α, exerted a negative effect on NFATc1 gene transcription. Summarizing all these results, we drew novel conclusions on NFATc1 expression, which provide a more detailed view on the regulatory mechanisms of NFATc1 transcription. Considering the high transcription and strong expression of NFATc1 in various human lymphomas, we propose that similar to NF-κB, NFATc1/αA plays a pivotal role in lymphomagenesis.

ZUSAMMENFASSUNG

TRANSKRIPTIONSMECHANISMEN UND FUNKTIONEN VON NFATC1 IN T LYMPHOZYTEN

Jiming Liu

NFATs (Nuclear Factors of Activated T cells) sind entscheidende Transkriptionsfaktoren für die Genexpression in Immun- und Nichtimmunzellen. Die Interaktion von T-Zellen mit Antigen-präsentierenden Zellen hat eine Klusterbildung von T-Zellrezeptoren, Korezeptoren und Integrinen zur Folge. Die nachfolgende Signaltransduktion führt zur NFAT-Aktivierung und dies wiederum zur spezifischen Zytokin-Genexpression. Von den in T-Zellen exprimierten NFATs weist NFATc1 eine besondere Regulation auf. Diese ist wichtig für die T-Zell-Aktivierung und -Differenzierung. Frühere Daten zeigten, dass drei Isoformen von NFATc1 in aktivierten T-Zellen gebildet werden: die induzierbare kurze Isoform NFATc1/A und die längeren Isoformen NFATc1/B und C. In dieser Arbeit wird nachgewiesen, dass in aktivierten T-Zellen sechs Isoformen exprimiert werden. Diese Isoformen unterscheiden sich N- und C-terminal infolge der Aktivität von zwei verschiedenen Promotoren, dem Vorhandensein von zwei unterschiedlichen Polyadenylierungsstellen sowie alternativer Spleißereignisse. Die Identifikation der sechs Isoformen erfolgte mittels semi-quantitativer “long distance” RT-PCR. Ferner konnte mit dieser Methode die induzierbaren Eigenschaften der Isoformen untersucht werden. Die prominenteste und am stärksten induzierbare Isoform in T-Effektorzellen ist NFATc1/αA. NFATc1/αA wird unter Kontrolle des P1-Promoters und der proximalen pA1-Polyadenylierungsstelle gebildet. Die Transkription von NFATc1/βB und βC erfolgt hingegen konstitutiv, wobei eine Reduktion ihrer Synthese in aktivierten T-Lymphozyten zu beobachten ist. Neben der NFATc1-Autoregulation interessierte uns die NFATc1-Genregulation unter der Kontrolle des PKC-Weges. Durchgeführte Microarray-Analysen zeigten, dass T-Zellen, die mit dem Phorbolester TPA stimuliert wurden, eine stärkere Induktion von NFATc1 aufweisen als Zellen, die nur mit Ionomycin stimuliert wurde. Es ist bekannt, dass

TPA die Aktivierung von PKC stimuliert, und wir beobachteten eine verstärkte Transkription von NF-κB1, Fos und JunB, die an die regulatorischen Region von NFATc1 binden können. Für p53 und seinem Zielgen Gadd45α zeigen wir unterstützende Beweise für einen negativen Einfluß auf die NFATc1-Transkription. Zusammenfassend erlauben die Resultate neue Schlussfolgerungen für die NFATc1-Genexpression und ermöglichen damit eine detailiertere Sicht auf den Regulationsmechanismus der NFATc1-Transkription. Bezug nehmend auf die hohe Expressionsrate von NFATc1 in verschiedenen humanen Lymphomen vermuten wir, dass - ähnlich NF-κB - NFATc1/αA eine entscheidende Rolle bei der Lymphomagenese spielt.

DEDICATED TO MY PARENTS AND MY TEACHER, MY…

献给我的父母和老师，
以及. . .

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LIST OF SYMBOLS / NOMENCLATURE

A	Adenosine
αA	NFATc1/αA isoform
βA	NFATc1/βA isoform
aa	Amino acid
Ab	Antibody
AEBSF	4-(2-Aminoethyl)-benzensulfonylfluoride
Ag	Antigen
AHR	Airway hyperresponsiveness
AP	Alkaline Phosphatase
APC	Ag-presenting cell
AP-1	Activator protein 1
APS	Ammoniumperoxidisulfate
Aqua dest.	distilled water
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
αB	NFATc1/αB isoform
βB	NFATc1/βB isoform
BAF	Brahma-related gene (BRG)/Brahma (BRM)-associated factor
BCR	B cell receptor
BCS	Bathocuproine disulfonate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	complementary DNA
CpG	cytosine and guanine separated by a phosphate
C	Cytosine
°C	Degree Celsius
αC	NFATc1/αC isoform
βC	NFATc1/βC isoform
CMV	Cytomegalovirus
CNS	Central nervous system
ConA	Concanavaline A
cpm	Counts per minute
CREB	cAMP response element binding
CTL	Cytotoxic T lymphocytes
CTLA	Cytotoxic T lymphocyte-associated
Da	Dalton
dd	double distilled
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DNP	Dinitrophenyl
dNTP	2´-deoxynucleoside 5´-triphosphate
ds	double-stranded

DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylendiamintetraaceticacid (Disodiumsalt)
e.g.	for example (*L. exampli gratia*)
EGTA	Glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic moility shift assay
ER	Endoplasmic reticulum
ES	Embryonic stem cells
et al.	and others
F	Forskolin
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcR	Fc Receptor
Fig.	Figure
FITC	Fluorescine isothiocyanate
FL	Fluorescence
FSC	Forward Scatter Light
G	Guanosine
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
H-89	N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinoline-sulfonamide hydrochloride
HBSS	Hanks´ balanced Salt solution
HCL	Hierarchical clustering
HEK	Human Embryonic Kidney Cell line
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethansulfonic Acid
HRP	Horse radish peroxidase
IFN	Interferon
IgG	Immunglobulin G
IκB	Inhibitory NF-κB
IL	Interleukin
i.p.	intraperitoneal
IPTG	Isopropyl-β-D-thiogalactoside
I	Ionomycin
i.v.	intravenous
Kb	Kilobase
Kbp	Kilobase pair
Kd	Dissociation constant
Kda	Kilodalton
KMC	K-means clustering
LB	Luria-Bertani
LFA-1	Leukocyte function associated Ag-1
LIF	Leukemia inhibition factor
LPS	Lipopolysaccharide
Luciferin	4, 5-dihydro-2-[6-hydroxy-2-benzothiazolyl]-4-thiazol-carboxylacid; C11H8N2O3S2

mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MEM	Minimal essential medium
2-ME	2-mercaptoethanol
MEFs	Mouse Embryo Fibroblast
MES	Morpholinoethansulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MHC	Major histocompatibility complex
ml	milliliter
µg	microgram
mg	milligram
µl	microliter
µM	micromolar
mM	millimolar
Mr	Relative molecular mass
mRNA	messenger RNA
miRNA	microRNAs
mw	Molecular weight
N	Nuclear
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
ncRNAs	noncoding RNA transcripts
n.d.	not determined
ng	nanogram
nM	nanomoles
NK cells	Natural killer cells
nt	Nucleotide
OCT	Octamer-binding factor
OD	Optical density
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrine
PEG	Polyethylene glycol
per se	intrinsically or by itself (*L.per se*)
PFA	Paraformaldehyde
PI	Propidium iodide
PIPES	Piperazine-N,N´-bis(2-ethanesulfonic acid)
Polybrene	1,5-dimethyl-1,5-diazaundecamethylene polymethobromide
PMA	Phorbol myristate acetate
pmol	picomoles
PMSF	Phenylmethylsulfonyl fluoride
p-ONPG	O-nitrophenyl-β-D-galactopyranoside
r	Recombinant
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RNase	Ribonuclease

rpm	revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SAM	Significance analysis microarray
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SOM	Self-organizing maps
SOTA	Self organizing tree algorithm
SSC	Sideways scatter light
STAT	Signal transducer and activator of transcription
T	Thiamidine
Tab.	Table
TAE	Tris-acetate EDTA buffer
TBS	Tris buffered saline
TBS-T	TBS with Tween 20
T_{CM}	Central memory T cells (Non-polarized T cells)
TCR	T cell receptor for Ag
T_{EM}	Effector memory T cells
TEMED	N,N,N´,N´-tetramethylethylenediamine
TESS	Transcription Element Search System
TGF	Transforming growth factor
α-TG	α-thioglycerol
TNF	Tumor necrosis factor
Th	T helper cells
T/I	TPA and ionomycin
Tnp	Non-polarized T cells (central memory T cells)
TPA	12-O-tetradecanoyl-phorbol-13-acetate
Tris	2-Amino-2-(hydroxylmethyl)-1,3-propandiol
Triton X-100	Alkylphenylpolyethyleneglycol
tRNA	Transfer RNA
U	Unit
U/min	Revolutions per minute
U/ml	Unit per millilitre
UV	Ultraviolet
vs.	versus
WT	Wild type
w/v	Weight per Volume
YFP	Yellow fluorescence protein

CHAPTER I - INTRODUCTION

1.1 Overview

Two general systems of immunity to infectious agents have been selected during evolution: innate (Aderem and Ulevitch 2000; Beutler 2004) or natural immunity, and acquired (adaptive) or specific immunity (Pancer and Cooper 2006). The former is phylogenetically older, with some forms presumably present in all multicellular organisms, whereas the latter evolved about 400 million years ago and is found only in cartilaginous and bony fish, amphibians, reptiles, birds, and mammals (Fearon and Locksley 1996; Magnadottir 2006). The essential difference between the two systems is the means by which they recognize microorganisms. Innate immunity has been considered only to provide rapid, incomplete antimicrobial host defense until the slower, more definitive acquired immune response develops. However, innate immunity may have an additional role in determining which Ag the acquired immune system responds to and the nature of that response. Adaptive (acquired) immunity refers to antigen-specific defense mechanisms that take some days to become protective. There are two types of the adaptive immune responses: humoral immunity and cellular immunity, which is mediated by lymphocytes. In response to antigen, humoral immunity involves the production of antibody molecules, and cellular immunity involves the production of cytokines.

T- and B-cell are two major types of lymphocytes. Each T cell and B cell is specific for a corresponding antigen. This means that each is able to bind a particular molecular structure. Lymphocyte receptors are the specific binding proteins for antigen, and T and B cell specific receptors are referred to TCR and BCR, respectively. The specific recognition between receptor and Ag corresponds to lymphocyte mediating specific immunity.

B cells are produced in the bone marrow, spleen and lymph nodes. The most important function of B cells is antibody production and humoral immunity mediation. Unlike B cells, the majorities of T cells develop in the thymus, and mediate cellular

immunity. They orchestrate the specific immune response to infected or malignant cells.

A critical event in the initiation of a specific immune response is the encounter of Ag-specific T cells with the DCs (dendritic cells) that present the corresponding Ag. Confronted with the low frequency of the two protagonists, the immune system has evolved efficient strategies for the two cell types to meet within organized lymphoid organs. On one hand, lymphocytes continuously patrol the entire body, achieving a complete tour of the lymphatic system. On the other hand, DCs that pick up the Ag at the site of infection are stimulated by pathogen-derived signals to migrate through the afferent lymph toward the draining LNs (Banchereau and Steinman 1998; Banchereau and Palucka 2005). DCs present the processed peptides to T-cell receptors by MHC. Formation of a MHC-Ag-TCR complex can elicit T lymphocyte activation.

Typically, T lymphocytes are classified into two subsets, and these subsets are defined by exclusive surface expression of either the MHC-II-binding protein CD4 (helper or regulatory $CD4^+$ T cells) or the MHC-I-binding protein CD8 (cytotoxic $CD8^+$ T cells) (Germain 2002). There are two types of TCR; each T cell subsets are further divided into two lineages according its TCR expression: alpha/beta (αβ) T cells and gamma/delta (γδ) T cells. This essay deals with αβ $CD4^+$ T cell differentiation, activation, and transcriptional control by NFAT transcription factors.

1.2 $CD4^+$ T cells

The immune system has evolved to combat infectious organisms (Marrack and Kappler 1994). During the evolution of the immune systems, T cells with Ag-specific receptors have emerged. An acquired immune system was established to support the innate immune system (Janeway and Medzhitov 2002). As a subtype of T lymphocytes, $CD4^+$ T cells (also called helper T cells) are essential for both the cellular and humoral response of the immune system. The activated $CD4^+$ T cells can release cytokines and chemokines that attract other cells to the area. The result is inflammation: the accumulation of cells and molecules that attempt to clear and destroy the Ags. In humoral immunity, $CD4^+$ T cells bind to antigen presented by B

cells. The result is the clonally expansion of plasma cells secreting antibodies directed against Ags.

In respect to $CD4^+$ T cells, the critical events are T cell development, activation and homeostasis/apoptosis.

1.2.1 $CD4^+$ T cell development

The thymus is the central organ for T cell development (Fig.1). Lymphoid progenitors which have developed from hematopoietic stem cells in the bone marrow migrate to the thymus to complete their Ag-independent maturation into functional T cells (Kondo, Weissman et al. 1997; Igarashi, Gregory et al. 2002). Recent studies have challenged whether progenitors in the bone marrow are mainstream progenitors for post-natal thymocytes (Allman, Sambandam et al. 2003). In the thymus, T cells develop their specific T cell markers, including TCR, CD2, CD3, and CD4 or CD8. The thymic microenvironment directs differentiation, and T cells also undergo thymic education through positive and negative selection. The thymus is a multi-lobe organ composed of cortical and medullary areas surrounded by a capsule. T cell precursors enter the subcapsular cortical areas, where they encounter networks of cortical epithelial cells (the thymic stoma) and undergo a period of proliferation. As they differentiate, they move from the cortex towards the medulla of the thymus; different microenvironments within the thymus direct T cell development. Most cells that enter the thymus die by apoptosis without successfully completing the steps required for becoming a mature naïve T cell (Petrie 2003). How is this cortical migration controlled? The CXC-chemokine receptor 4 (CXCR4) provides essential signals that guide thymocytes on their developmental journey through the thymus (Plotkin, Prockop et al. 2003).

T cells that strongly recognize self-peptide MHC complexes undergo apoptosis, whereas cells that receive an "appropriate" weak signal differentiate further in the thymus, which prevents self-reactivity of mature T cells – negative selection (Hogquist 2001; Ohashi 2003). Furthermore, the immature T cells undergoing

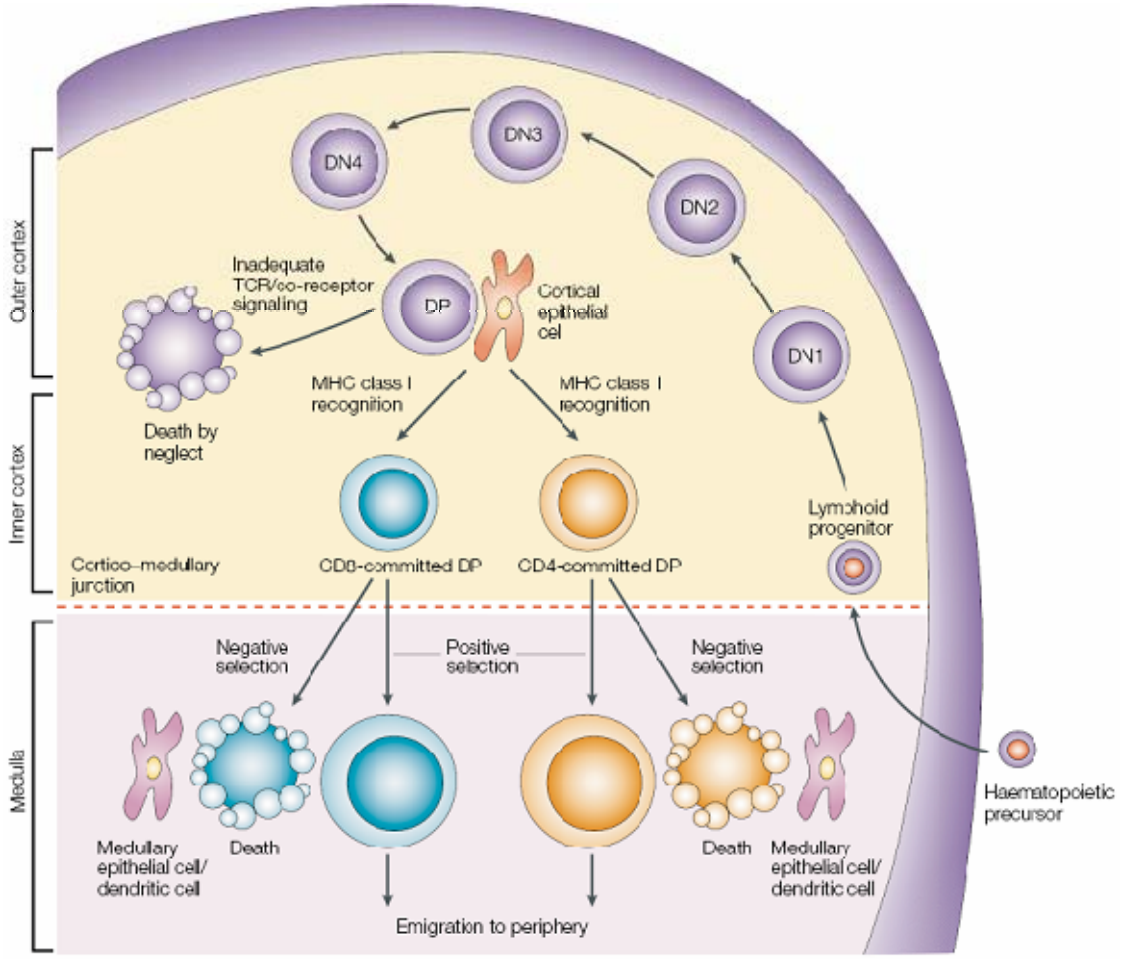


Fig.1 Overall scheme of T-cell development in the thymus

Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$) (Godfrey, Kennedy et al. 1993). As cells progress through the DN1 to DN4 stages, they all express the pre-TCR, which is composed of the pre-Tα chain and a rearranged TCR β-chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain, which yields a complete αβTCR. The αβ-$TCR^{+}CD4^{+}CD8^{+}$ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signaling that is mediated by interaction of the TCR with these self-peptide–MHC ligands. Too weak signaling results in delayed apoptosis (death by neglect). Low/missing signaling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands on hematopoietic cells, particularly dendritic cells. The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind peptide–MHC-class-I complexes become $CD8^{+}$ T cells, whereas those that express TCRs that bind peptide–MHC-class-II ligands become $CD4^{+}$ T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites. SP, single positive cells (Germain 2002; Kaech, Wherry et al. 2002).

Positive selection commit to the CD4 vs. CD8 lineages based on the duration/strength of intracellular signals arising from the interaction between the TCR and self-MHC molecules (Yasutomo, Doyle et al. 2000; Germain 2002; Bosselut 2004). If so, T cells undergo a series of well-documented differentiation steps, which are typically defined based on the cell-surface expression of CD4 and CD8, with thymocytes first starting as $CD4^-CD8^-$ double negative (DN), then becoming $CD4^+CD8^+$ double positive (DP), and lastly maturing to single-positive (SP) $CD4^+$ or $CD8^+$ T cells. The DN stage can be further subdivided based on CD25 and CD44 (or CD117) cell-surface expression (Fig.1). After completion of T cell maturation in the thymus, naïve $CD4^+$ or $CD8^+$ T cells migrate into peripheral lymphoid organs and further differentiate into a variety of effector T cells to eliminate pathogens by recognizing pathogen-derived Ags presented by their own MHC (Abbas, Murphy et al. 1996; Murphy 1998; Murphy and Reiner 2002). $CD4^+$ T cells can regulate the 'quality' of the $CD8^+$ T cells generated through lymphokine production and direct of contact (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003).

1.2.2 $CD4^+$ T cell differentiation and activation

Full activation of Th cells requires two signal pathways: First, binding of the TCR to the peptide-MHC complex on APCs causes stabilization of lipid rafts, and signaling through CD3. This involves the activation of protein tyrosine kinases Lck, Zap70 and Itk, of PLCγ, and PKCθ, and Ras. Second, the co-stimulatory molecules CD80 and CD86 on the APC bind CD28 on the T cell and trigger the activation of PI3K. This triggers changes in the cytoskeleton which activate Akt. It can also bring about NF-κB activation through activation of the MAGUK (membrane-associated guanylate kinase) protein Carma-1 by the protein kinase-θ. In response to both signals, activated T cells express CD40 ligand (CD154), and activation of CD40 on the APC allows for continued APC survival. Another response to activation by the T cell is the production of IL-2 and expression of receptor α chain (IL-2Rα), which now enables physiologic signaling through the IL-2 receptor and induces T cell proliferation (Abbas and Sen 2003; Jun, Wilson et al. 2003; C. van Oers and Chen 2005; Lee, D'Acquisto et al. 2005; Matsumoto, Wang et al. 2005). The Ca^{2+}-

calcineurin-NFAT pathways are discussed in detail in the following text (chapter 1.3.5).

The most-studied differences between peripheral T-cell subsets are the distinct panels of cytokines expressed by $CD4^+$ T helper 1 (Th1; type 1) and Th2 (type 2) cells upon activation. There is extensive plasticity in the T-cell response to Ag (Woodland and Dutton 2003). After Ag priming, naïve $CD4^+$ T cells are skewed into at least two types of helper T cells - Th1 and Th2 cells characterized by the production of IFN-γ and IL-4 (Fig.2), respectively (Murphy and Reiner 2002). The polarization of T cell cytokine profiles toward Th1 or Th2 responses is one of the mechanisms by which the immune system responds to microbial challenge. Protective immunity against pathogens requires the differentiation of naïve Th cells into Th1 and Th2 like cells.

Cellular differentiation is a process of gradual acquisition of cell type specific properties governed by extracellular factors and cell–cell interactions. In principal, differentiation is tightly interconnected with the cell cycle machinery. T cell differentiation is a multistep process. The generation of effector Th1 cells requires repeated TCR and cytokine signaling. In vivo, effector T cell differentiation is completed within lymphoid organs (Bajenoff and Guerder 2003). When naïve $CD4^+$ T cells recognize peptides on MHC, they proliferate and differentiate into functional subsets called Th1 and Th2 cells. In Th1 cells, INF-γ promotes the effective eradication of pathogens through the induction of antimicrobial activity in macrophages. So Th1 cells are essential for protection against a variety of intracellular infections including Leishmania major, Toxoplasma gondii, and Mycobacterium tuberculosis, perhaps also against certain tumors. In contrast, Th2 responses are protective against certain extracellular infections. Th2 cells secrete IL-4 and enhance humoral immunity that takes part in protection against extracellular pathogens. The balance of $CD4^+$ T cell differentiation toward Th1 and Th2 cells is a critical determinant in most infections or autoimmune diseases (Liew 2002; Murphy and Reiner 2002; Wynn 2004; Kalinski and Moser 2005).

The differentiation of naïve $CD4^+$ T cells into cells with IFN-γ- or IL-4–producing capacity is a complex process involving the interaction of specific cytokines with cell signaling proteins and transcription factors, with subsequent chromatin remodeling. For Th1 differentiation, a key player in governing the

development of Th1 responses is IL-12, a cytokine largely produced by Ag-presenting cells (APC), such as specialist Ag-presenting dendritic cells (DCs) (O'Garra 1998; Kapsenberg, Hilkens et al. 1999) in response to Toll-like receptor stimulation. IL-12 acts through the activation of signal transducer and activator of transcription 4 (STAT4) (Thierfelder, van Deursen et al. 1996) and T-bet (Szabo, Kim et al. 2000). Similarly, IL-4, STAT6 and the transcription factor GATA-3 (Ting, Olson et al. 1996; Zheng and Flavell 1997) are essential in controlling the generation of Th2 cells. In short, Th1/Th2 polarization is based on the balance between Th1- and Th2-specific transcription factors activated by the extracellular cytokines IL-12 and IL-4, respectively (Rengarajan, Szabo et al. 2000). These extensive regulatory mechanisms put considerable constraints on the relative efficiency of generating large numbers of IFN-γ- and IL-4-producing $CD4^+$ T cells.

In the past few years, several lines of evidence have indicated that the chromatin structure has an integral role in Th-cell differentiation (Lee, Takemoto et al. 2000; Miyatake, Arai et al. 2000; Wilson, Makar et al. 2002). Thereafter, T cell development and differentiation are controlled at the epigenetic level (Wilson, Makar et al. 2002). Ag and cytokine stimulation of naïve T cells activates diverse intracellular signaling pathways, and undergo programmed alterations in their patterns of gene expression, which result in chromatin remodeling and demethylation of cytokine genes. T cell differentiation results in long-range changes in the chromatin structure of effector cytokine genes, which persist in resting Th1 and Th2 cells in the absence of further stimulation. Differentiation of naïve T helper cells into mature Th2 cells is associated with chromatin remodeling of the IL-4 and IL-13 genes, whereas differentiation into Th1 cells evokes remodeling of the IFN-γ gene. Remodeling of the IL-4 locus is accompanied by DNA demethylation and requires both Ag stimulation and STAT6 activation (Agarwal and Rao 1998). There are two main categories of enzymes that modulate chromatin structure (Fry and Peterson 2001; Narlikar, Fan et al. 2002). Modification enzymes can covalently modify histones. The modifications function, in part, by creating docking sites for other regulatory proteins, ultimately leading to alterations in the nucleosome structure. Various modifications have been documented, including acetylation, methylation, phosphorylation, ubiquitylation, glycosylation and ADP ribosylation, and distinct modifications have different biological consequences. Chromatin-remodeling complexes (CRCs) belong to the second class of chromatin-regulatory enzymes.

CRCs are powerful molecular motors that use energy derived from ATP hydrolysis to disrupt histone–DNA contacts, thereby exposing nucleosomal DNA. With increasing cell divisions, the cytokine expression patterns of Th1 cells and Th2 cells become epigenetically fixed and no longer regulated by the environment. Chromatin-based regulatory mechanisms may explain several features of cytokine gene expression in effector versus naïve T cells, including their monoallelic expression, coordinate regulation, and stable maintenance in memory T cells (Agarwal, Viola et al. 1999). The changes in chromatin structure of the genes associated with lineage commitment and terminal differentiation are often stably maintained and propagated to daughter cells. This unique property of chromatin is the molecular basis of transcriptional memory (Muller and Leutz 2001; Fisher 2002). It enables cells to remember transient signaling events that occurred in the past, such as an interaction with neighboring cells during development or an encounter with a pathogen during primary immune responses (Agarwal, Viola et al. 1999; Richter, Lohning et al. 1999; Lohning, Richter et al. 2002). For such memory, unique for each cell type, also provides an explanation for why ubiquitous signaling pathways elicit distinct transcriptional responses in different cell types (Muller and Leutz 2001; Fisher 2002).

Ag properties also influence T cell differentiation. In vivo, dendritic cells (DCs) control polarized (Th) and non-polarized T cell (Tnp) differentiation through strength of Ag binding (Langenkamp, Messi et al. 2000). In addition to Th1, Th2 and Tnp differentiation, naïve $CD4^+$ T cells can differentiate into Th0 cells and regulatory T cells (Treg) by Ag recognition (Krauss, Chen et al. 2003; Park, Paik et al. 2004). Under polarized conditions, Tnp cells are able to further differentiate into polarized Th1 and Th2 cells. As Ag-priming T cells, the property of Tnp re-differentiation provides a fast way in generating polarized T helper cell, act as a pool of polarized T cells. Treg cells have a suppressing role in preventing autoimmunity and inflammation by excessive immune responses.

What is different between $CD4^+$ and $CD8^+$ T cell differentiation and activation? It is well known that $CD4^+$ and $CD8^+$ T cells recognize different MHC molecules (De Boer, Homann et al. 2003). In contrast to $CD8^+$ T cells, naïve $CD4^+$ T cells develop into effector cells more slowly after short-term primary stimulation (Seder and Ahmed 2003). A model useful for studying both $CD4^+$ Th1 and CD8+ T cell effector cell differentiation is the transfer of naïve T cell receptor−transgenic $CD4^+$ and $CD8^+$ T cells specific for ovalbumin into normal mice subsequently infected with the

bacterium Listeria monocytogenes expressing ovalbumin (Foulds, Zenewicz et al. 2002). At day 8 after infection, >85% of ovalbumin-specific $CD8^+$ T cells but <7% of ovalbumin-specific $CD4^+$ T cells produced IFN-γ. In addition, $CD8^+$ T cells underwent extensive proliferation, whereas $CD4^+$ T cells divided a limited number of times. Another point regarding differences in the generation of effector cytokine production by $CD4^+$ and $CD8^+$ T cells is illustrated by an examination of how the factors involved in polarizing $CD4^+$ T cell responses regulate the differentiation of $CD8^+$ T cells. Similar to their effect on $CD4^+$ T cells, IL-12 and IL-4 generate in vitro $CD8^+$ effector T cells called Tc1 or Tc2 cells based on their production of IFN-γ or IL-4, respectively. However, in contrast to results with $CD4^+$ T cells, there is less evidence demonstrating that these cytokines are essential in polarizing $CD8^+$ T cell responses in vivo. Also, it is notable that T-bet, the main transcription factor controlling Th1 differentiation has little effect on the generation of $CD8^+$ IFN-γ producing T cells (Szabo, Sullivan et al. 2002). These observations reflect substantial differences in the efficiency and factors regulating the induction of $CD4^+$ and $CD8^+$ cytokine-producing cells.

Finally, costimulatory requirements also seem to be different for the activation of naïve $CD4^+$ and $CD8^+$ T cells. This has been demonstrated best in studies examining antiviral responses using mice deficient in various costimulatory pathways (Homann, Teyton et al. 2001). The frequency of virus-specific $CD8^+$ IFN-γ producing cells is substantially higher than that for $CD4^+$ IFN-γ producing cells both at the peak and throughout the memory response in mice infected with LCMV (Homann, Teyton et al. 2001), Sendai (Cauley, Cookenham et al. 2002) or vaccinia viruses (Harrington, Most et al. 2002). These notable differences in magnitude of $CD4^+$ and $CD8^+$ T cell responses to such infections could be due to many factors, such as due to their intrinsic proliferating capacity (Iezzi, Karjalainen et al. 1998; Homann, Teyton et al. 2001), efficiency of Ag presentation and susceptibility to apoptosis (Chambers, Sullivan et al. 1997; Seder and Ahmed 2003). Although primary $CD8^+$ T cell responses to acute infections are independent of $CD4^+$ cell help, it is still controversial if $CD4^+$ T cell help is required to generate functional $CD8^+$ T cell memory. In early reports, it needs $CD4^+$ T cell help to generate functional $CD8^+$ T cell memory (Carbone, Kurts et al. 1998; Shedlock and Shen 2003). Recently, there are also published reports contradictory to the conclusion that $CD4^+$ T cell help is

required for in generating functional $CD8^+$ T cell memory (Marzo, Vezys et al. 2004; Jones, Carvalho-Gaspar et al. 2006).

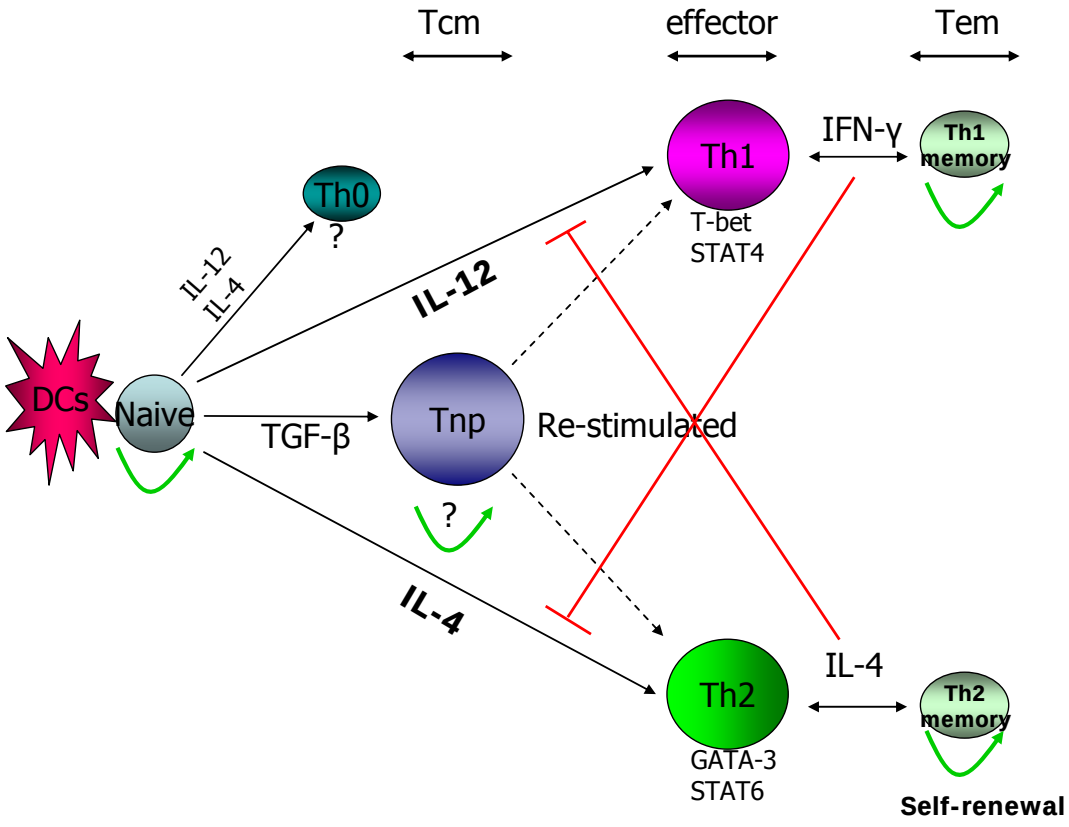


Fig.2 Scheme of $CD4^+$ T cell differentiation and homeostasis
After activation by Ag presenting DCs, naïve $CD4^+$ T cells differentiate into Th1, Th2, Tnp, Treg and Th0 cells. Under normal differentiation conditions, the main populations of differentiated T cells are Tnp, Th1 and Th2 cells, and small amounts of Th0 and Treg cells. Naïve $CD4^+$ T cells stimulated under Th1-polarizing (IFN-γ) or Th2-polarizing (IL-4) conditions undergo progressive differentiation towards IFN-γ- or IL-4- producing cells. Th1 and Th2 cells development is controlled by IL-12-dependent STAT4 and IL-4-dependent STAT6 activation, respectively. Thus, naïve T cells can be differentiated either into the most common T cell effector subsets, i.e. Th1 cells or Th2 cells using IL-12 and IL-4, respectively. TGF-β induces Ag priming T cell of differentiation into Tnp cells through unknown transcription factors, and Tnp cells also know as central memory T cells (T_{CM}) (Sallusto, Geginat et al. 2004). Small parts of polarized T cells can be further differentiated into memory T cells after re-stimulation, which is so called effector memory T cells (T_{EM}). Effector Th cells with a short live span and limited proliferation, but there is self-renewal capacity in naïve, Tnp and memory T cells.

Proliferation and differentiation of $CD4^+$ T cells are differently regulated by TCR signaling (Murphy and Reiner 2002). Transient exposure to APC induces a program of proliferation that can proceed in the absence of Ag both in vitro and in

vivo. A 20-h stimulation with plate-bound MHC complexes is sufficient to commit naïve T cells to proliferation (Iezzi, Karjalainen et al. 1998). The programmed proliferation induced by transient exposure to Ag can proceed in an Ag- and cytokine- independent fashion (Bajenoff, Wurtz et al. 2002). Indeed, activated T cells continue to divide even when reincubated in medium alone. This suggests that the continuous presence of IL-2, IL-4, IL-7 and IL-15, i.e. of four γ-chain-dependent cytokines involved in the proliferation and survival of naïve or memory T cells (Rebollo, Gomez et al. 1996; Zhang, Sun et al. 1998; Ku, Murakami et al. 2000; Schluns, Kieper et al. 2000), is not necessary to sustain the proliferation of these activated $CD4^+$ T cells. This observation is in agreement with the γ-chain independence of the clonal expansion of naïve T cells in vivo (Lantz, Grandjean et al. 2000). However, the continuous presence of IL-2 in vitro is essential for survival of activated $CD4^+$ naïve T cells (Iezzi, Karjalainen et al. 1998). This may suggest that in vivo, other not yet defined receptor(s) may deliver the necessary survival signals to activated $CD4^+$ T cells. The programmed proliferation induced by initial Ag stimulation is, however, not endless and will cease after a total of six to seven divisions when proliferation can be reinduced by re-exposure to Ag. This early programmed proliferation may ensure a significant clonal expansion (64- to 128-fold increase) of rare Ag-specific T cells even when Ag is limiting, as during the early phase of an infection. In sharp contrast, the differentiation of naïve $CD4^+$ T cells into effector Th1 cells requires repeated contacts with Ag-loaded APCs. Indeed, they found both in vitro and in vivo that when Ag and cytokines are removed 48 h after primary stimulation, activated T cells did not further differentiate and produced mainly IL-2 but limited IFN-γ. Clearly, both cytokine- and TCR-derived signals were essential at this stage to induce optimal differentiation. Nonetheless, differentiation, although less efficient, was also induced by TCR re-engagement or cytokine signaling alone. In lymphoid organs, dendritic cells (DCs) are the most likely source of IL-12. Mature DCs do not constitutively produce IL-12 but are induced to do so by innate and T cell-derived signals including MHC class II and CD40 ligation (Cella, Scheidegger et al. 1996; Langenkamp, Messi et al. 2000; Schulz, Edwards et al. 2000). Under these conditions, $CD4^+$ T cells would be exposed to IL-12 mainly upon direct interaction with DCs. Differentiation of $CD4^+$ T cells in vivo may be therefore mainly driven by Ag availability. In agreement, T cells activated for 48 h did not

differentiate into IFN-γ producing Th1 cells when transferred into hosts preinjected with CFA, that due to microbial constituents ought to induce IL-12 production (Fearon and Locksley 1996).

1.2.3 Non-polarized T cells and T cell memory

Specificity and memory are two important features in immune response. Ag-specific $CD4^+$ T cells exist at very low frequencies in naïve hosts. After infection or immunization, naïve T cells undergo clonal expansion, culminating in a higher frequency of Ag-specific cells with more rapid effector function. These quantitative and qualitative changes in T cell numbers and function constitute the immunological memory. Memory T cells mediate faster and stronger responses that follows re-exposure to the same Ag.

Memory T cells express a different pattern of cell surface markers, and they respond in several ways that are functionally different from those of naïve cells. Murine memory T cells are CD44 high, and low in the expression of other activation markers, such as CD25 (IL-2R), whereas human memory cells are $CD45RA^-$, $CD45RO^+$. In contrast to naïve T cells, memory T cells secrete a full range of T cell cytokines and can be polarized to secrete particular cytokines for both $CD4^+$ and $CD8^+$ T cells. The requirements for the activation of memory T cells for proliferation and cytokine production are not as strict as those of naïve T cells, but costimulation in the broad sense is required for optimum responses and for responses to suboptimum Ag concentrations. Memory T cells can persist in the absence of antigenic stimulation as non-dividing cells. Re-encounter with the same Ag can expand the population to a new, stable, higher level and generate a separate population of CD44 high effectors that may be required for protection, while competition by other Ags can drive it down to a lower stable level.

Recent evidence indicated that $CD4^+$ memory T cells are heterogeneous consisting of at least two subsets (Fig.3) with different migratory capacity and effector function (Sallusto, Lenig et al. 1999; Langenkamp, Messi et al. 2000; Rivino, Messi et al. 2004). Central-memory T cells (TCM), which express the lymph node homing receptors CCR7 and CD62L, are largely devoid of effector functions but acquire characteristics of effector-memory T cells (TEM) (i.e., $CCR7^-$ T helper cells).

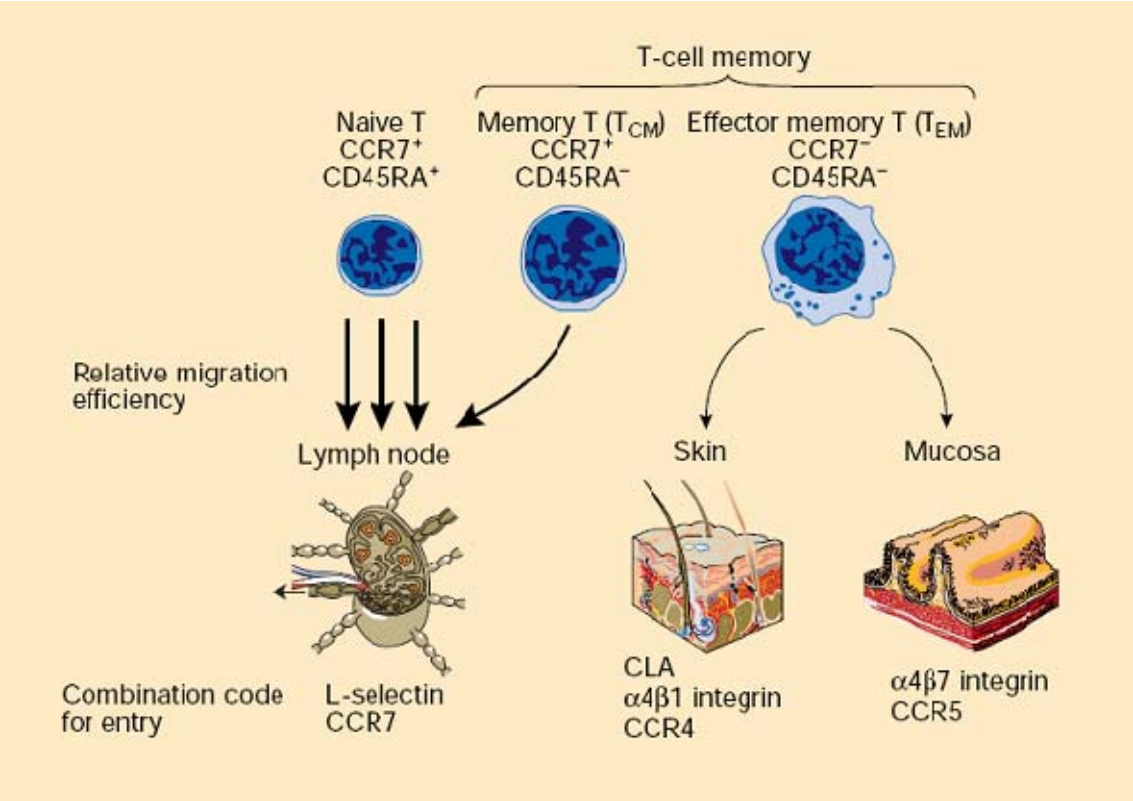


Fig.3 Two types of memory T cells with different migration preferences
Expression of CCR7 and the CD45RA isoform distinguishes three subsets of T cells: naïve T cells ($CCR7^+$& $CD45RA^+$), central memory T cells (T_{CM}: $CCR7^+$& $CD45RA^-$), and effector memory T cells (TEM: $CCR7^-$ &$CD45RA^-$). Naïve T cells migrate very efficiently through lymph nodes using a 'combination code' of L-selectin (an adhesion molecule) and CCR7 (a CC chemokine receptor). Central memory T cells probably migrate through lymph nodes, whereas effector memory T cells migrate through peripheral tissues, such as the skin and mucosa. It is likely that there are many combinations which code for homing of T cells to the skin and mucosal tissues. CLA - cutaneous lymphocyte Ag (Mackay 1999; Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004).

It is unclear how or where memory cells arise (Fig.4), but once generated they have different pathways of recirculation and homing (Dutton, Bradley et al. 1998). Memory T cells have been traditionally viewed as survivors of effector cells that revert to a quiescent state (Zinkernagel, Bachmann et al. 1996). The question is whether memory T cells come from naïve T cells, or linear differentiation from naïve, effector cells, and then finally differentiate into memory T cells. For $CD8^+$ T cells, linear differentiation was observed (Opferman, Ober et al. 1999). $CD4^+$ cells differ from $CD8^+$ effector T cells in their transition to resting memory cells. $CD4^+$ IFN-γ producing cells do not efficiently develop into resting memory cells. Although activated $CD4^+$ T cells stimulated under Th1-polarizing conditions that have not yet

become IFN-γ producing cells (Th1-lineage cells or uncommitted) are able to develop into long-term memory cells (Seder and Ahmed 2003). For Th2 cells, a heterogeneous population of activated $CD4^+$ T cells cultured under Th2 conditions develops into resting memory cells (Hu, Huston et al. 2001).

Which factors could promote T cell memory generation and what is required to sustain $CD4^+$ and $CD8^+$ memory T cells? Activated cells must escape cell death if they are determined to develop to memory cells. Thus, factors that promote survival of otherwise death-susceptible T cells are candidates for memory factors. For $CD8^+$ memory T cells, IL-7 and IL-15 are important in regulating T cell survival and turnover, respectively. In sustaining the survival of memory $CD4^+$ T cells, IL-7 is important (Seddon, Tomlinson et al. 2003). IL-2 participates in T cell survival, but it also keeps T cells in an activated state in which they are susceptible to death. The cytokine TGF-β1 both promotes CD4 and CD8 effector survival and also tends to push activated effector cells to a more resting phenotype. In vitro, non-polarized T cells (Tnp or TCM) can be generated by adding TGF-β in T cell differentiation (Sallusto, Lenig et al. 1999; Geginat, Sallusto et al. 2001; Sallusto, Geginat et al. 2004). However, $CD4^+$ memory T cells do not require expression of the common cytokine receptor beta chain for survival, providing indirect evidence that cytokines, such as IL-2, IL-4, IL-7, IL-9 and IL-15 may not be required for the maintenance of $CD4^+$ memory cells. With regard to costimulatory molecules, there is evidence that OX-40 and OX-40 ligand may be involved in sustaining $CD4^+$ T cell survival. For $CD4^+$ and $CD8^+$ memory cells, Ag and MHC class I or II are not essential for survival.

In Th cell differentiation, there are always large amounts of non-polarized T cells (Tnp), even under strict polarized condition. Tnp cells show neither INF-γ and IL-4 secretion after re-stimulation, nor effector T cell function. Since Tnp cells do not produce effector cytokines, they were considered as non-functional T cells. Tnp can be generated and expanded by active suppression of polarization in the presence of TGF-β, limiting amounts of Ag and the absence of polarizing cytokine co-stimulation (rather than active suppression, such as TGF-β alone) (Sallusto, Lenig et al. 1999; Unsoeld, Krautwald et al. 2002; Rivino, Messi et al. 2004).

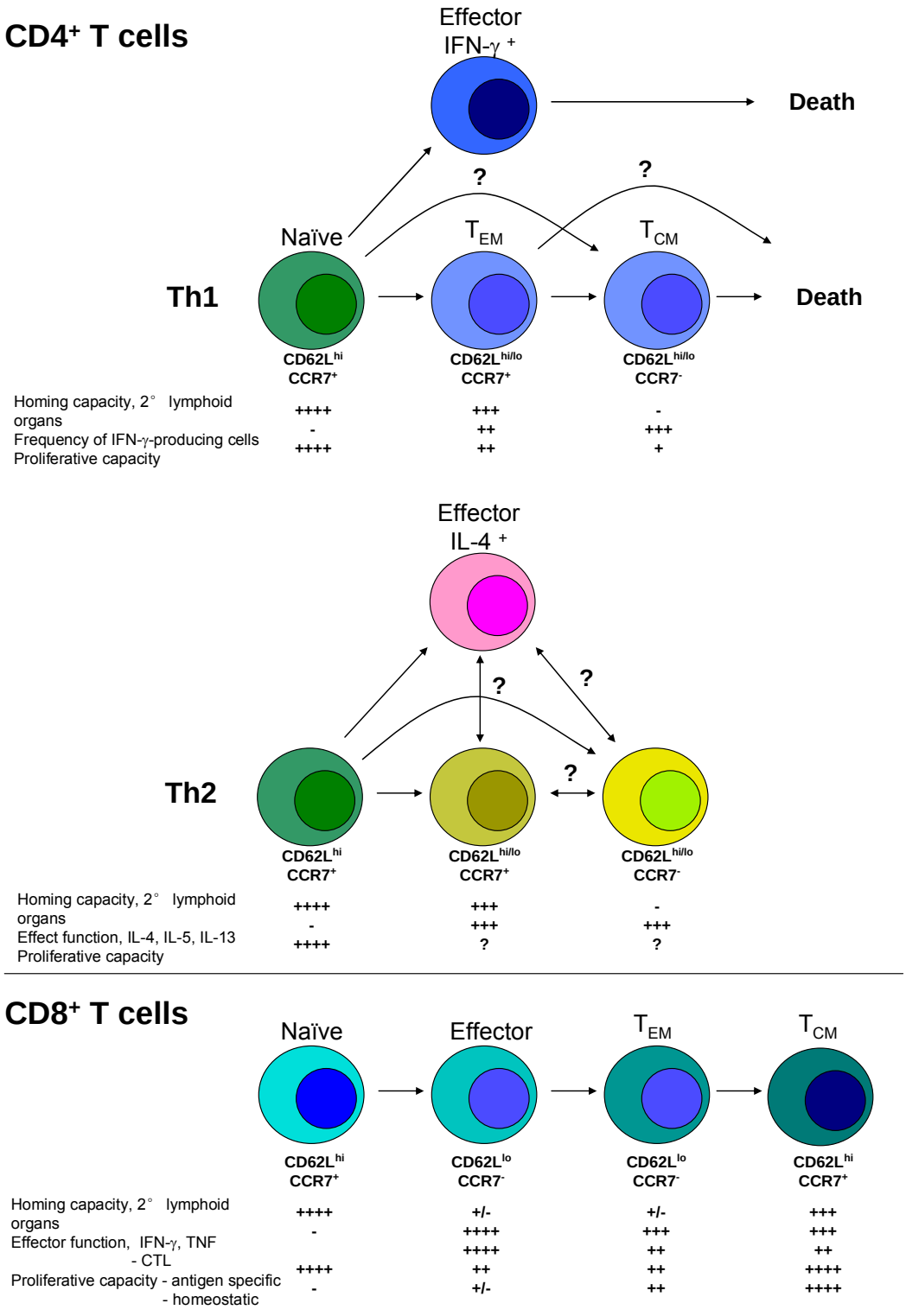


Fig.4 Lineage differentiation of memory T cells

Naïve $CD4^+$ T cells have multiple potential pathways for differentiating from naïve to effector and then to memory cells. For Th1 cells, differentiation is likely to follow a progressive and linear pathway, because $CD4^+$ IFN-γ-producing effector cells are relatively short-lived in vivo and do not efficiently develop into memory cells. $CD4^+$ IL- 4-producing T

cells may have multiple potential pathways for memory cell differentiation. $CD8^+$ T cells readily develop into effector cells after activation. After the contraction phase of the response, $CD8^+$ T cells seem to proceed to effector T_{EM} and then T_{CM} cells. T_{EM} and T_{CM} cells have comparable cytolytic and cytokine producing capacity, but the T_{CM} population has enhanced proliferative capacity (Sallusto, Lenig et al. 1999; Seder and Ahmed 2003; Wherry, Teichgraber et al. 2003; Zaph, Uzonna et al. 2004; Zhang, Joe et al. 2005).

Tnp cells could easily be re-polarized to Th1 or Th2 cells under polarized condition (Fig.2). In addition, Tnp cells have the capacity of long-term survival as the memory T cells (Sallusto, Geginat et al. 2004). As Ag-priming T cells, Tnp cells could re-polarize to Th1 or Th2 cells, and differentiate into effector memory T cells (T_{EM}). As a dominant T cells subtype population in vivo, they show Ag-priming, self-renewal and re-differentiated properties, which give the potential roles of Tnp cells in T cell memory and the central roles of Tnp cells in T cell homeostasis. In general, Tnp cells are considered as the “pool” of effector T cells, so called “central memory T cells” – T_{CM}. Unlike the patrol effector T cells, Tnp cells are mainly located in secondary lymph nodes (Fig.3).

Despite of functional differences between naïve and memory T cells, a common property of naïve and memory T cells is long-life survival and self-renewal. Studies on the similarities and differences between naïve and memory T cells may provide the clues to find out the factor in supporting long-living survival. In addition, the mechanisms involved in memory cell generation are still mostly unknown, and in particular the process that determines which of the Ag-specific T cells will survive and become memory cells and which will die has yet to be elucidated. This pursuit has been hindered mainly because of a lack of definitive markers that distinguish between these two cellular fates (death or survival). Understanding the multiple facets of immunological memory and its relationship to protection remains a central issue in immunology.

One critical event of immunity is T cell homeostasis. T cells are selected in the thymus and released as mature naïve T cells into the periphery. However, the thymus shrinks with age, and, as a consequence, there is a reduced output of new T cells. As such, the maintenance of the existing mature T cell pool becomes an important issue. This problem is resolved in part by the generation of long-lived naïve T cells, which ensures the opportunity to form immune responses to new pathogens throughout life. Secondary, this problem is resolved by maintaining long-lived memory T cells, which ensures the fast immune responses to old pathogens.

Recently evidence indicates two factors. MHC and IL-4 contribute to the establishment of long-lived naïve $CD4^+$ T cells (Boursalian and Bottomly 1999). Another report describes a novel mechanism by which naïve Th cells expand but accumulate in an arrested, uncommitted state maintained by CTLA-4 until relieved by the simultaneous delivery of both TCR/CD28 and a STAT signal from either of the polarizing cytokines, IL-4 or IL-12 (Mohrs, Lacy et al. 2003).

1.2.4 $CD4^+$ T cell phenotype and function

Naïve, effector and memory T cells represent different stages in a developmental pathway and can be clearly distinguished by functional assays and also by cell-surface markers. Chemokine receptor expression on T lymphocyte is related to function and defining T cell subtypes (Baggiolini 1998; Baggiolini and Loetscher 2000). Studies on chemokine receptors (CKRs) suggested that Th1 and Th2 cells have homing potentials quite different from each other. The existence of characteristic Th1 versus Th2 homing properties would be attractive, because it might provide a mechanism for amplification of Th1- or Th2-associated inflammation at tissue sites of appropriate chemokine expression. There are several classes of Th1- or Th2-associated CKRs. The first is expressed by almost all Th1 cells or all Th2 cells (CXCR3 for Th1 and CCR4 for Th2). In addition, CCR5 is characteristic for Th1 cells and CCR3 is characteristic for Th2 cells (Sallusto, Mackay et al. 1997; Bonecchi, Bianchi et al. 1998; Loetscher, Uguccioni et al. 1998). Immunohistochemical staining of inflamed tissues indicated that these receptors can indeed determine the development of Th1- and Th2-mediated pathologies. The role of other chemokine receptors that were shown to be expressed differentially on Th subsets (i.e. CCR8) is less clear.

It has also been reported that the expression of CCR7 distinguishes two distinct subsets of memory T cells (Fig.2): $CCR7^+$ memory T cells, which are called central memory T cells, are lymph-node-homing, non-effector cells whereas $CCR7^-$ memory T cells are tissue-homing effector cells (Sallusto, Lenig et al. 1999). CCR7+ T cells are defective in immediate effector function and home exclusively to secondary lymphoid tissues, while cells producing effector cytokines are CCR7– and home only in nonlymphoid tissues. Expression of CCR7 may be necessary for targeting $CCR7^+$ T cells into lymphoid tissues, but is not sufficient to prevent the

homing of polarized $CCR7^+$ T cells into nonlymphoid tissues. Indeed, $CCR7^+$ memory T cells express other CKRs such as CXCR3 and CCR4 that can target cells to nonlymphoid tissues (Kim, Rott et al. 2001). Thus, many polarized $CCR7^+$ Th1 or Th2 cells may be able to home to lymphoid organs as well as extralymphoid tissues. In line with this, Randolph et al. have reported that Th1 cells generated in vitro express CCR7 and migrated well into T cell areas of the spleen (Randolph, Huang et al. 1999). Homing of polarized T cells into lymphoid tissues may lead to amplification of Th1 or Th2 cell generation from naïve T cells. It has also been reported that CCR7 does not discriminate IFN-γ producers from non–IFN-γ producers among $CD8^+$ T cells (Champagne, Ogg et al. 2001).

Cytokines have crucial functions in the development, differentiation and regulation of immune cells. Defining the cytokine secretion patterns of $CD4^+$ T cell subsets (Table 1.1) provides a framework for understanding T cell subset functions, the heterogeneity of normal and pathological immune responses. Peripheral T lymphocytes die every day. Cytokine-regulated lymphocyte homeostasis and differentiation maintains a constant lymphocyte population size in vivo. A lymphocyte acquires survival signals through receptors for cytokines, and the intracellular signaling pathways need both cytokine secretion and its receptor expression (Khaled and Durum 2002). Dysregulation of cytokine production or action is thought to have a central role in the development of autoimmunity and autoimmune disease. Some cytokines, such as IL-2, TNF and IFN-γ, are well known for the promotion of immune and inflammatory responses (Leonard 2001; O'Shea, Ma et al. 2002).

Tab.1 Patterns of Cytokine secretion by T helper subsets

cytokine	*Naïve*	*Treg*	*Tnp*	*Th0*	*Th1*	*Th2*	*Th3*
IL-2	(+)	-/+	+	+	+		
IL-3				+	+		
IL-4				+		++	
IL-5		+		+		+	
IL-6				+		+	
IL-10		+		+		+	
IL-13				+		+	
IL-17				+			+
INF-γ				+	++		
TNF-α				+	+		
TNF-β				+	+	+	
TGF-β		+		+			
GM-CSF				+	+	+	

Th cells producing interleukin-17 (IL-17) are distinct from the traditional Th1 and Th2 cell subsets. Here, we designed these cells as Th3 cells (Hunter 2005; Dong 2006). The data in this table are compiled from the following references (Feldmann, Londei et al. 1989; Firestein, Roeder et al. 1989; Kaczmarski and Mufti 1991; Abbas, Murphy et al. 1996; Wood and Seow 1996; Carter, Zhang et al. 1998; Viola and Rao 1999; Kim, Campbell et al. 2001; McKee and Pearce 2004; Sallusto, Geginat et al. 2004)

1.3 The Nuclear Factors of Activated T cells (NFATs)

The Nuclear Factors of Activated T cells (NFATs) are critical transcription factors that direct gene expression in immune and nonimmune cells. Essentially, all the biological functions of DNA depend on the activity of transcription factors binding to their targets (Halford and Marko 2004).

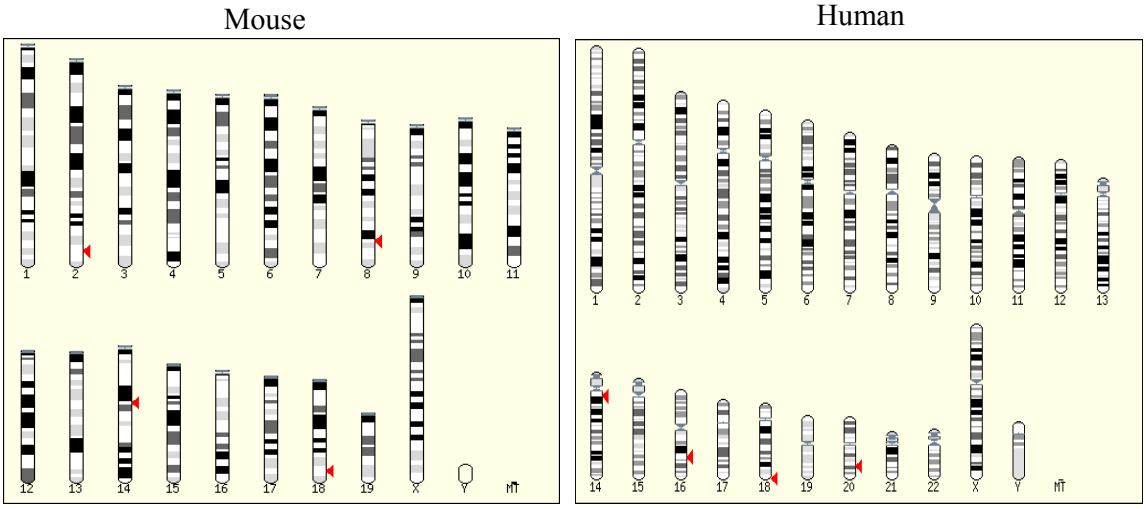
T cell activation depends on signals transduced through the T-cell receptor (TCR) and costimulatory receptors, such as CD28, which leads to production of IL-2 and further lymphokines. Studies on T cells have produced a consensus pathway in which signals transduced by each receptor are necessary to activate the transcription factors that bind to distinct sites in the IL-2 gene promoter/enhancer and are essential for maximal transcription of the IL-2 gene. NFATs were first characterized as transcription factors that bind to the interleukin-2 (IL-2) promoter (Durand, Shaw et al. 1988; Shaw, Utz et al. 1988; Serfling, Barthelmas et al. 1989; Serfling, Avots et al. 1995) in T cells, but also in other classes of lymphoid cells. NFAT proteins are activated by stimulation of receptors coupled to calcium mobilization and calcineurin. Calcineurin (also called protein phosphatase 2B), a major calmodulin-binding protein, is conserved in all eukaryotes and is unique among phosphatases for its ability to sense Ca^{2+} through its activation by calmodulin. Calcineurin was identified and characterized by the pioneering work in the Claude Klee´s and Philip Cohen´s laboratories in the late 1970s (Klee, Crouch et al. 1979). Calcineurin catapulted to the centre stage when the research groups of Stuart L. Schreiber and Irving Weissman discovered that it is the target of the immunosuppressants cyclosporin A and FK506 (Liu, Farmer et al. 1991). In 1989/1990, it has been shown that NFATs are the CsA-target by Crabtree's and Serfling's lab (Shaw, Utz et al. 1988; Emmel, Verweij et al. 1989; Randak, Brabletz et al. 1990). In 1992/1993, it was shown that these proteins are calcineurin's target and dephosphorylated by calcineurin (Clipstone and Crabtree 1992; O'Keefe,

Tamura et al. 1992; Jain, McCafffrey et al. 1993). These findings revealed a central pathway that coupled calcineurin to transcriptional regulation. Since then, calcineurin and NFAT proteins have been shown to participate in signaling cascades that govern the development and function of the immune, nervous, cardiovascular and musculoskeletal systems (Serfling, Berberich-Siebelt et al. 2000; Aramburu, Heitman et al. 2004).

Unlike other phosphatases, calcineurin has a relatively narrow substrate specificity (Klee, Ren et al. 1998). As substrates for calcineurin, NFAT proteins respond to TCR-CD28 signals and are major targets of the immunosuppressive drugs cyclosporin A (CsA) and FK506.

1.3.1 Structure NFATc proteins

The NFAT transcription complex consists of two components: a cytoplasmic Rel domain protein (NFATc family member - NFATc) and a nuclear component consisting of activating protein 1 (AP-1) transcription factors (Fos- and Jun-related proteins), or other transcription factors, such as GATA-3, c-maf, ICER, Foxp3 and others. The NFAT family of transcription factors encompasses five proteins evolutionarily related to the Rel/NF-κB family (Serfling, Berberich-Siebelt et al. 2000; Graef, Gastier et al. 2001; Hogan, Chen et al. 2003; Macian 2005). There are four closely related NFAT proteins (NFATc1-NFATc4, also known as NFAT1-4), which are designated collectively as NFATc. The nomenclature for the calcineurin-dependent CsA-sensitive cytoplasmic proteins of NFAT transcriptional complexes (NFATc proteins) used here is that used by HUGO and GenBank: NFATc1 (c1) = NFATc = NFAT2; NFATc2 (c2) = NFATp = NFAT1; NFATc3 (c3) = NFATx = NFAT4; NFATc4 (c4) = NFAT3 (see HGNC Gene Family Nomenclature online at: http://www.gene.ucl.ac.uk/nomenclature/genefamily/NFAT/NFAT.%20shtml). The primordial family member is NFAT5, the only NFAT-related protein represented in the Drosophila genome. NFAT5 is identical to TonEBP (tonicity element binding protein), a transcription factor crucial for cellular responses to hypertonic stress (López-Rodríguez et al. 1999; Miyakawa et al. 1999). The Chromosomal localization of the different NFAT family genes is given in Fig.5.



	mouse		*human*	
Gene Name	**Gene ID**	**Genome Location**	**Gene ID**	**Genome Location**
Nfatc1	ENSMUSG00000033016	Chromosome 18: 80.73m	ENSG00000131196	Chromosome 18: 75.26m
Nfatc2	ENSMUSG00000027544	Chromosome 2: 167.99m	ENSG00000101096	Chromosome 20: 49.44m
Nfatc3	ENSMUSG00000031902	Chromosome 8: 105.36m	ENSG00000072736	Chromosome 16: 66.68m
Nfatc4	ENSMUSG00000023411	Chromosome 14: 50.34m	ENSG00000100968	Chromosome 14: 23.91m

Fig.5 NFATc nomenclature and chromosome distribution
Information retrieved from EMBL

The proteins of NFATc family are weakly related to the Rel/NF-κB family of transcription factors over a 300-amino acid region, called the Rel homology domain (RHD). This region governs DNA binding and association with AP-1 transcription factors. Within the RHD, sequence conservation among the NFAT proteins is very high. Upstream of the RHD, NFAT proteins are less closely related, but they do share several serine- and proline-rich segments. Downstream of the RHD, NFAT proteins are variable in length and in sequence (Serfling, Berberich-Siebelt et al. 2000; Graef, Gastier et al. 2001). The regions of NFATc proteins located outside the DNA-binding and regulatory domains show relatively little sequence conservation. The ~100 amino acid region at the very N-terminus of each protein, which behaves as a strong transactivation domain (TAD) in NFATc2 (Rao, Luo et al. 1997; Wolfe, Zhou et al. 1997; Serfling, Berberich-Siebelt et al. 2000), is not conserved in sequence (Fig.6).

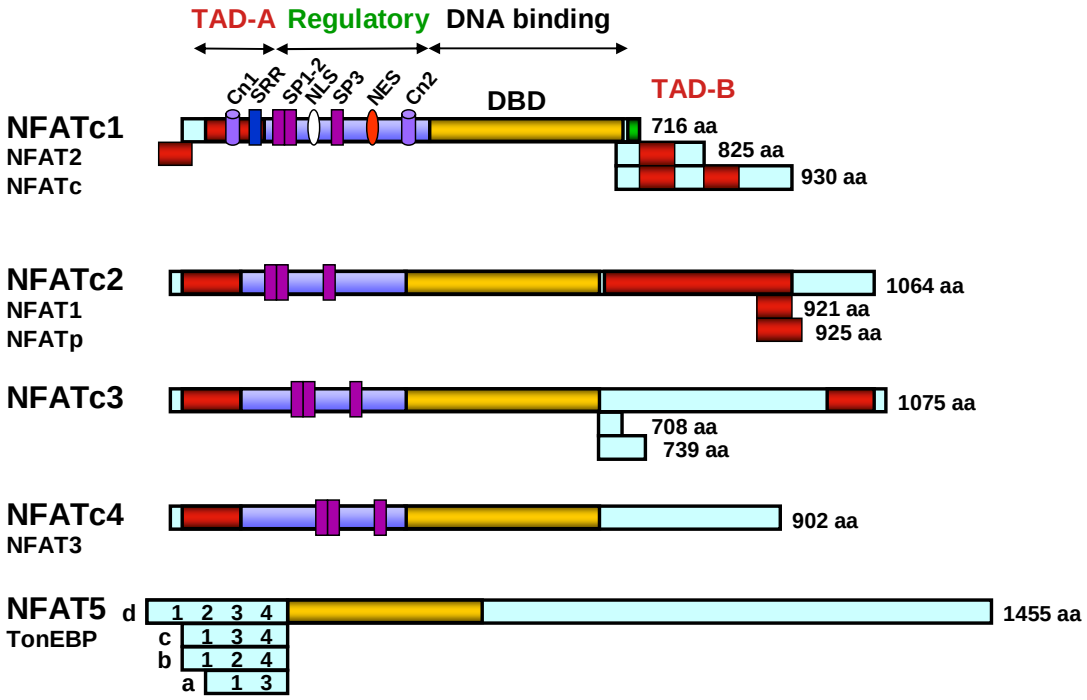


Fig.6 Schematic alignment of NFATc proteins predicted from their cDNAs
The region of highest homology between NFAT proteins is the DNA-binding domain (DBD), which includes the RHD (Rel homology domain) and a newly defined IPD (Immunoglobulin-like fold, Plexins, Transcription factors like domain). In addition, the following motifs are indicated: the calcineurin-docking site (Cn), the nuclear localization signal (NLS), the nuclear export signal (NES), the serine-rich regions (SRRs) and the SPXX-repeat motifs (SPs), where X denotes any amino acid. A second region of homology is the regulatory region, which binds to calcineurin (CN) and is found only in the NFATc1-4 family proteins. The N- and C-terminal transactivation domains (TADs) are indicated. For protein isoforms, identical shading patterns represent similar sequences. The boundary of each region is labeled above the sequences with numbering referring to position in the human protein. The lengths indicated are also for the human proteins. The GenBank accession numbers for these sequences are U02079 (murine NFAT1a), U43341 (NFAT1b), U43342 (NFAT1c), U08015 (NFATc), U59736 (NFATc), L41066 (NFAT3), U14510 (NFATx), L41067 (NFAT4c), U28807 (NFATc3, the murine homolog of NFATx) (Serfling, Berberich-Siebelt et al. 2000).

The core structural motif of NFATc-DBD consists of a ten antiparallel β-barrels. NFAT family members share extensive sequence homology (-70%) over a -300-amino-acid region comprising the entire DNA-binding domain, the N-terminal half of which corresponds to NFATc-DBD. A potential sequence relationship between the DNA-binding domain of NFAT and members of the NF-κB Rel protein family has been noted. However, the extent of sequence similarity (<20%) falls below that considered to be statistically significant. Comparison of the solution structure of NFATc-DBD with the X-ray structure of NF-κB p50 DNA-binding domain (Fig.7)

reveals that the two molecules share an essentially identical overall fold (Fig.7a or b) and strand topology (Fig.8c or d). Many of the residues that are invariant between the NFATc family members and p50 (Fig.7c, bold) form the hydrophobic core of the β-barrel (Wolfe, Zhou et al. 1997).

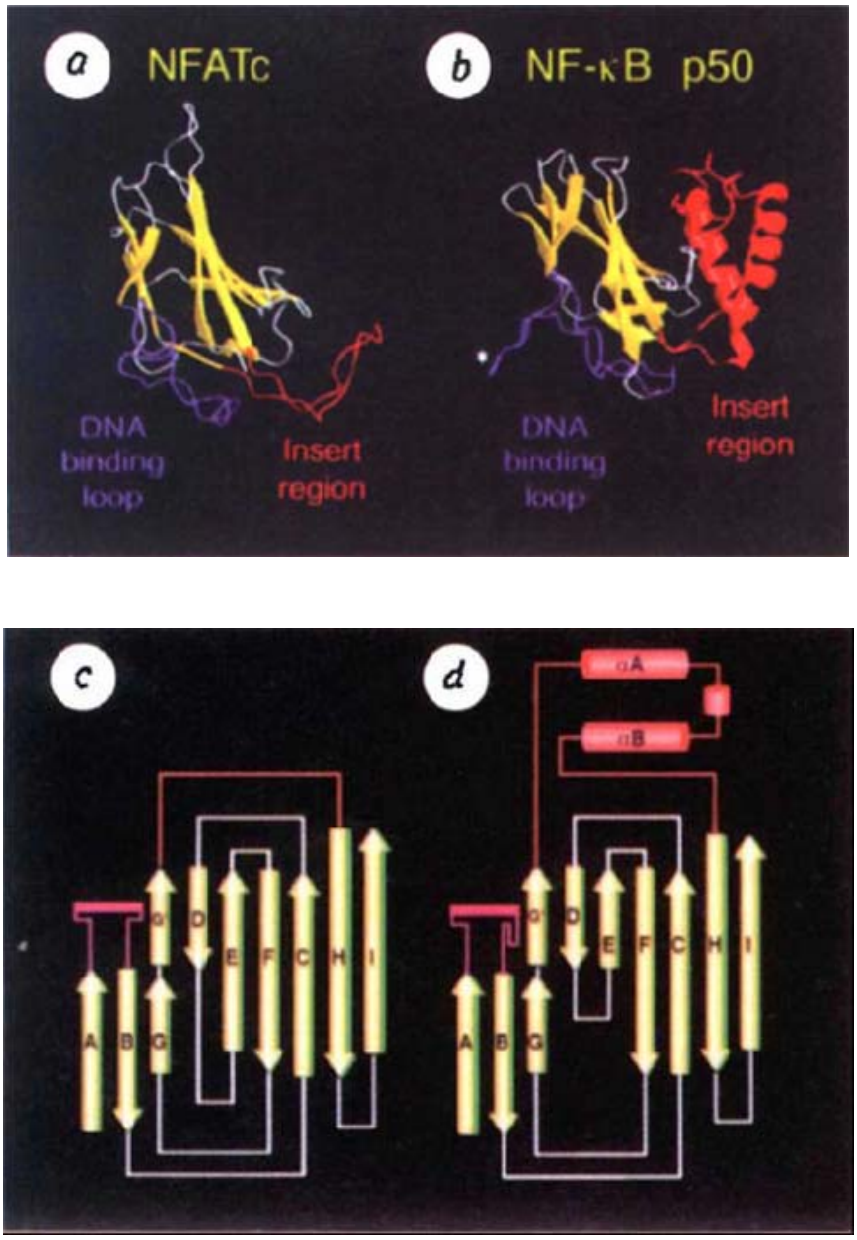


Fig.7 Comparison of solution structure between NFATc2 and NF-κB
(a) NFATc2-DBD X-ray crystal structure. (b) The DNA-binding domain of NF-κB p50. Yellow, β-Strands; magenta, DNA-binding loop (DBL); red, insert region. The asterisk denotes a basic pentapeptide in the p50 DBL with no counterpart in NFATc-DBD. (c, d) Topological maps of NFATc2-DBD (c) and DNA-binding domain of NF-κB p50 (d). β-

Strands are shown as arrows, α-helices as cylinders. Color scheme in c,d corresponds to that in a, b (Wolfe, Zhou et al. 1997).

A remarkable feature of the binary NFAT/DNA complex is the conformational flexibility exhibited by NFATc in the four independent copies of the NFAT/DNA complex in the crystal structure, which may reflect a mechanism by which NFATc interacts with a variety of protein partners as it mediates disparate biological responses (Stroud and Chen 2003). A representative model is the cooperative complex formed between NFATc2 and AP-1 on the interleukin-2 promoter/enhancer (Fig.8).

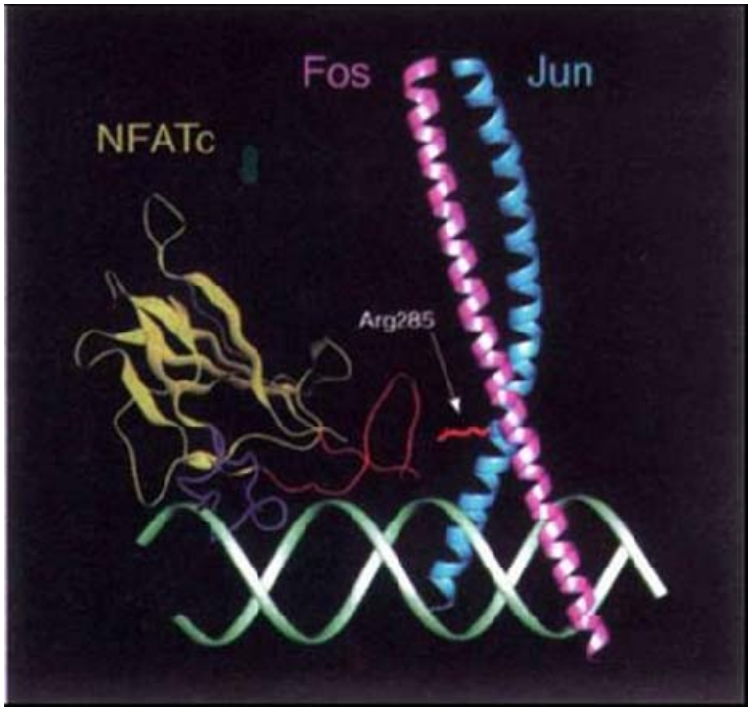


Fig.8 Model of the core NFATc2-AP-1 DNA structure
The solution structure of NFATc2-DBD was positioned with respect to the DNA backbone (green) by least-square superposition of the β-barrel (yellow), and with respect to the IL-2 sequence and AP-1 (Wolfe, Zhou et al. 1997).

The tonicity-responsive enhancer binding protein (TonEBP), also known as NFAT5, is a unique member of the NFAT family of transcription factors that regulates gene expression induced by osmotic stress in mammalian cells. Unlike monomeric members of the NFAT family, TonEBP exists as a homodimer and binds asymmetric TonE binding DNA (TonEBP) sites. Furthermore, the affinity of TonEBP for DNA is much lower than that of other NFATc proteins. TonEBP adopts a NF-κB-like

structure upon binding to DNA, providing a direct structural link between the NFAT and NF-κB families of transcription factors. TonEBP completely encircles its DNA target and presents biochemical evidence that the DNA encirclement may lead to increased kinetic stability of the TonEBP-DNA complex (Stroud, Lopez-Rodriguez et al. 2002).

In addition to the similarity in DNA binding (Rel homology) domain, the genomic (Ho, Thomas et al. 1995; Graef, Gastier et al. 2001) and tree views (Fig.9) show that NFATc1 and NFATc2, and NFATc3 and NFATc4 are the evolutionary closest relatives of the NFATc proteins. The close position between NFATc1 and NFATc2 may explain the functional redundancy between NFATc1 and NFATc2.

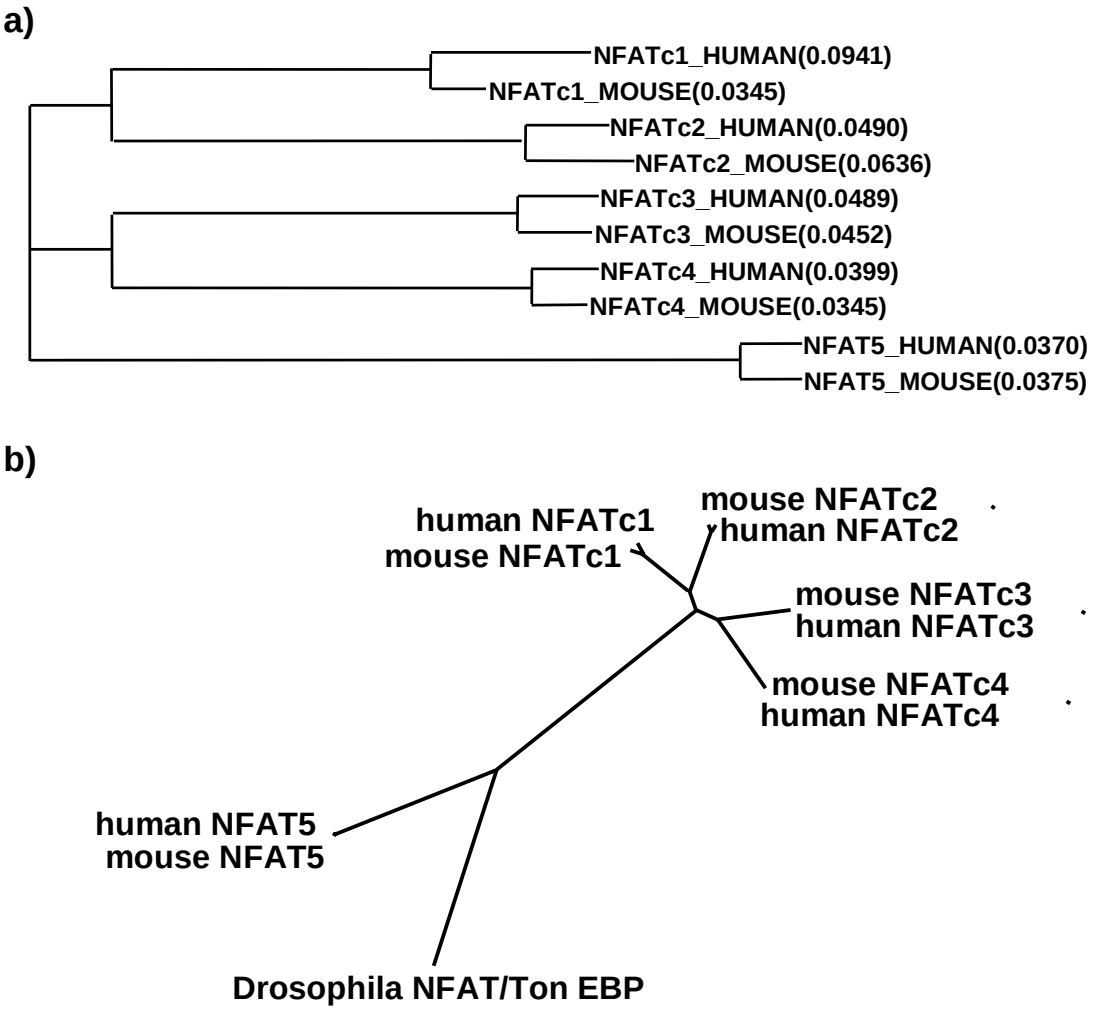


Fig.9 Tree analysis of NFATc proteins
a) Alignment tree view of NFAT family proteins; b) Phylogenetic tree analysis for the DNA binding (Rel homology) region of NFAT family proteins (Asagiri, Sato et al. 2005).

1.3.2 NFATc expression and development

Although the calcineurin–NFATc signaling pathway was first defined in lymphocytes (Serfling, Berberich-Siebelt et al. 2000), it is now clear that it has far more extensive roles.

NFATc1 and NFATc2 are predominantly expressed in lymphoid tissues (thymocytes, T cells, B cells, mast cells and NK cells), but NFATc1 is also expressed in muscle cells. NFATc3 is expressed mainly in the thymus (Oukka, Ho et al. 1998) and NFATc4 is expressed primarily in nonlymphoid tissues. The major NFAT proteins expressed in peripheral T cells, which producing IL-2, correspond to the isoforms NFATc1 and NFATc2. In the thymus, NFATc3 is expressed at highest levels in DP thymocytes, while NFATc1 is preferentially expressed in DN thymocytes and NFATc2 in SP thymocytes.

There are significant differences in the expression patterns of NFATc2 mRNA reported by different laboratories: Although NFATc2 mRNA has been variably detected in brain, heart and skeletal muscle, NFATc2 protein expression has not been detected in bulk extracts of these tissues (Ruff and Leach 1995; Wang, McCaffrey et al. 1995). NFATc1 mRNA is upregulated in activated T cells and NK cells via a CsA-sensitive mechanism that may involve an NFAT-regulated positive feedback loop (Chuvpilo, Jankevics et al. 2002; Zhou, Cron et al. 2002).

In addition to T cell activation and differentiation (Serfling, Berberich-Siebelt et al. 2000), NFATs are involved in organ development, such as in dorsoventral axis formation (Saneyoshi, Kume et al. 2002), cardiac valve formation (de la Pompa, Timmerman et al. 1998; Ranger, Grusby et al. 1998), muscle differentiation (Abbott, Friday et al. 1998), skeletal myocyte and cardiac hypertrophy (Musaro, McCullagh et al. 1999; Xia, McMillin et al. 2000), angiogenesis (Hernandez et al, 2001) and bone and teeth formation (Asagiri, Sato et al. 2005). They all utilize calcium influx and calcineurin activation pathways leading to the nuclear localization of the cytoplasmic components of the NFAT transcription complexes (Crabtree 1999; Serfling, Berberich-Siebelt et al. 2000; Serfling, Chuvpilo et al. 2006).

1.3.3 NFATc function in lymphocytes

The majority of NFAT proteins in peripheral lymphocytes consist of NFATc1 and NFATc2 (Rao, Luo et al. 1997). In mice, NFATc3 mRNA was expressed in all T lymphocyte subsets and was highest in CD4+CD8+ double positive (DP) thymocytes. Conversely, NFATc2 mRNA was preferentially expressed in mature CD4+ single positive (SP) cells. NFATc1 mRNA was presented at low levels in all subsets, but strongly induced upon treatment with phorbol ester and calcium ionophore in effector T cells (Amasaki, Masuda et al. 1998). In human, NFATc2 (135 kDa) is constitutively expressed in human T cells, while synthesis of short NFATc1 isoform (86 kDa) is induced by phorbol ester and ionomycin treatment. NFATc3 was very weakly expressed in unstimulated cells, and its level did not increase upon treatment with activating agents. NFATc4 protein was not observed under any conditions. Higher-molecular-weight isoforms of NFATc1 (of 110 and 140 kDa) were also detected (Lyakh, Ghosh et al. 1997).

What is the NFAT function in T cells? To answer this question, different mice deficient for one or more NFAT family members were established by several groups. NFATc1 is not required for the generation and basal activation status of T and B lineage cells (Ranger, Hodge et al. 1998). NFATc1-deficient lymphocytes demonstrated an impaired proliferative and Th2-like responses, and a decrease in IL-4 production and failure to produce IgG1 and IgE (Ranger, Hodge et al. 1998; Yoshida, Nishina et al. 1998), while NFATc2-deficient animals displayed modestly enhanced immune responses with Th2-like characteristics (Hodge, Ranger et al. 1996; Xanthoudakis, Viola et al. 1996; Kiani, Viola et al. 1997; Schuh, Kneitz et al. 1997). NFATc3 deficiency caused in increased apoptosis of thymocytes and hyperactivation of peripheral T cells (Oukka, Ho et al. 1998; Ranger, Oukka et al. 1998). These findings suggest that NFATc1 might provide principally a proliferative function, while NFATc2 and NFATc3 provide repressive functions in the immune system. Accordingly, NFATc1 is rapidly induced, while NFATc2 and NFATc3 are rapidly repressed, by anti-CD3-mediated stimulation of T cell clones (Ranger, Oukka et al. 1998). Animals deficient in both NFATc2 and NFATc3 showed significant defects in lymphocyte apoptosis and developed a profound lymphadenopathy and Th2-predominant syndrome including allergic blepharitis and pneumonitis (Ranger, Oukka et al. 1998). Mice double deficient for NFATc1 and NFATc2 displayed a

significantly impaired production of T cell–dependent effector functions yet developed significant B cell activation. Double deficient T cells could not generate final mature T effector cells, and were characterized by a loss of IFN-γ and IL-4 production. Such findings emphasize the importance of the NFATc transcription factors in the balanced regulation of immune responses (Peng, Gerth et al. 2001).

How can it be explained that NFATs are important in IL-2, IFN-γ and IL-4 production, but double NFATc2 and c3 deficiency results in Th2 hyper-responses? There must be a downregulation of some factors, such as cytokines or transcription factors, which control the balance of Th1 and Th2 cells. The functions of NFATc1 and NFATc2 are largely redundant but not identical, as evidenced by the following fact: NFATc2-deficient animals displayed dysregulated expression of IL-4 but diminished expression of IFN-γ, while NFATc1-deficient T cells produced less IL-4 and IL-6 but largely normal amounts of TNF-α and IFN-γ. But both of them control the T cell effector functions, including IL-2 (Chow, Rincon et al. 1999; Peng, Gerth et al. 2001).

1.3.4 NFATc regulation and TCR signaling

T-cell activation and development require signaling through very similar receptors, the T-cell antigen receptor (TCR) and the pre-TCR. T-cell development requires the pre-TCR as well as the mature TCR, whereas T-cell activation uses the TCR exclusively (Chu, Morita et al. 1998). TCR triggering results in the activation of both calcium/calcineurin-NFATc, NF-κB and Ras mediated signaling pathways, which ultimately result in differentiation, proliferation, activation and developing of mature T cells. Unlike NF-κB activation, NFATc activation is predominantly activated by TCR engagement. There is no evidence that NFATs play a role in cytokine induced gene expression, but NFATs control TCR-induced gene expression.

Signaling through the Ag receptors of T lymphocytes is initiated upon binding of the Ag receptor to cognate Ags in the context of MHC complex (Fig.10). For more than a decade, numerous reports of biochemical studies, spontaneous human mutations, and the use of specific inhibitors, gene targeting and transgenic approaches in mice have shown that there are adaptor proteins of TCR signaling pathways. Propagation of signals from TCR involves a number of adaptor molecules. Ligation of TCR complex activates protein tyrosine kinases (PTKs) including the Src

family PTKs Fyn (Cooke, Abraham et al. 1991) and Lck (Veillette, Bookman et al. 1988; Glaichenhaus, Shastri et al. 1991) to phosphorylate immunoreceptor tyrosine-based motifs (ITAMs) (Johnson, Pleiman et al. 1995) located in cytosolic domains of the TCR and associated CD3 chains. This leads to the recruitment and activation of the Syk family kinase ZAP-70 and possibly other proteins such as Shc to phosphorylated CD3. These activated PTKs in turn phosphorylate a variety of proteins, and thereby couple TCR ligation to a number of intracellular signaling cascades culminating in T cell activation.

There are two direct consequences of protein tyrosine phosphorylation in TCR engagement. First, tyrosine phosphorylation can activate enzymes. Second, tyrosine phosphorylation creates sites for binding by proteins with SH2 domains (Pawson 1995). Principal downstream substrates/effectors for TCR-activated PTKs include phospholipase C-γ1 (Park, Rho et al. 1991), the 85-kD subunit of the phosphatidylinositol 3-kinase (Fukazawa, Reedquist et al. 1995), the Src homology (SH)2 domain-containing leukocyte protein of 76 kDa (SLP-76) (Motto, Ross et al. 1994; Jackman, Motto et al. 1995), the proto-oncogene product Cbl (Fukazawa, Reedquist et al. 1995; Chiang, Kole et al. 2000), the Vav guanine nucleotide exchange factor (GEF) of the Rho/Rac/CDC42 family of GTP-binding proteins (Margolis, Hu et al. 1992; Fischer, Zmuldzinas et al. 1995; Wu, Zhao et al. 1997), and the linker for activation of T cells (LAT) (Zhang, Sloan-Lancaster et al. 1998; Delgado, Fernandez et al. 2000; Werlen, Hausmann et al. 2000). GrpL (Grb2-related protein) and SLP-76 interacts with the guanine nucleotide exchange factor Vav to activate NFATc, and its expression is required for normal T cell development (Law, Ewings et al. 1999). Functional interactions between these phosphorylated effector molecules are believed to be necessary for the operation of major pathways required for T cell maturation including (a) intracellular free Ca^{2+} release, (b) activation of NFAT, and (c) mitogen-activated protein kinase (MAPK) activation. These events ultimately converge to induce gene expression, proliferation and differentiation of T cells (Law, Ewings et al. 1999).

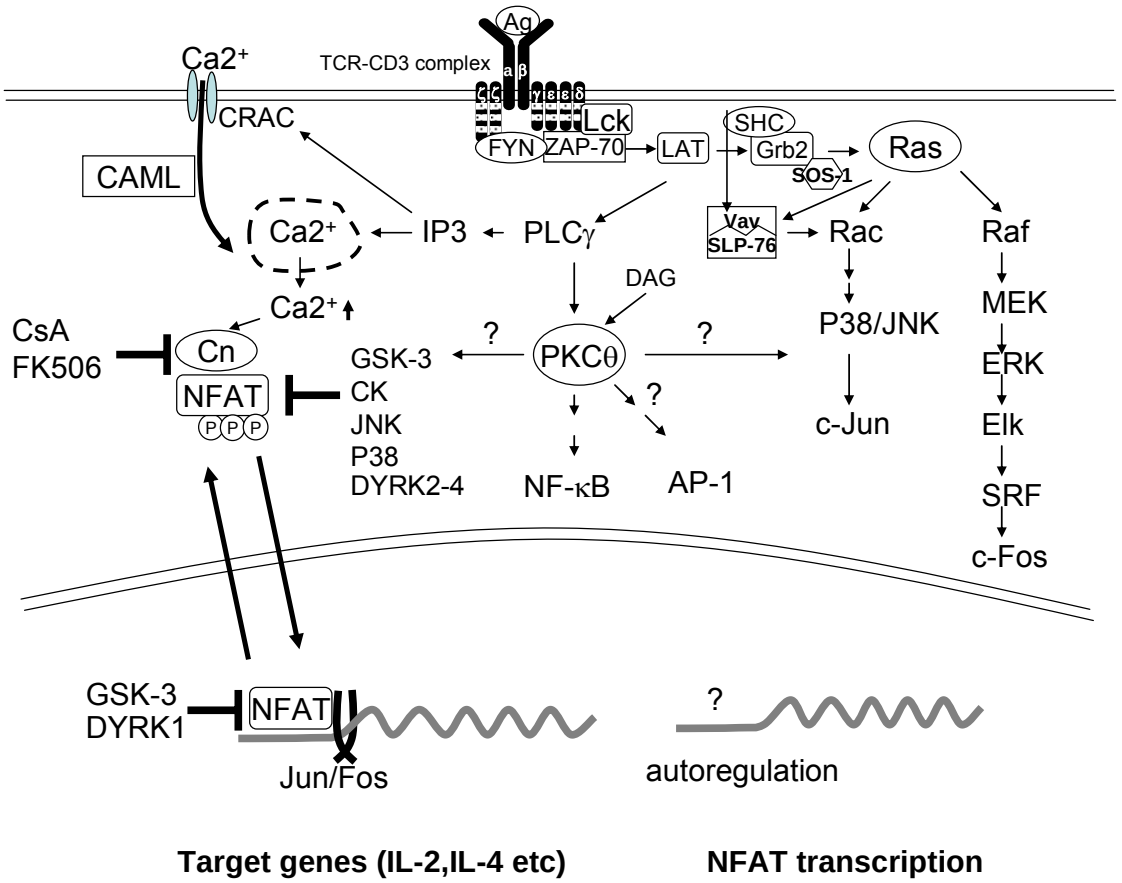


Fig.10 A simplified view of signaling pathways that control NFAT
Molecules discussed in the text are boxed. Unidentified signaling molecules and pathways are indicated by question marks. Key: CaMK, multifunctional Ca^{2+}/calmodulindependent protein kinases; CAML, calcium-signal modulating cyclophilin ligand; Cn, calcineurin; JNK, c-Jun N-terminal kinase; (Rao, Luo et al. 1997; Acuto and Cantrell 2000; Serfling, Berberich-Siebelt et al. 2000; Bromley, Burack et al. 2001; Isakov and Altman 2002; Gwack, Sharma et al. 2006).

In these pathways, Ca^{2+}/calcineurin signaling is integrated with and depended on other signaling pathways, such as Ras, Rac, and PKC signaling (Fig.9). Prolonged Ca^{2+} signaling in lymphocytes requires the function of the Ca^{2+} release–activated Ca^{2+} (CRAC) channels, which open in response to depletion of intracellular stores through the IP3 receptor. NFATc activity requires sustained Ca^{2+} stimuli provided by CRAC channels. Brief Ca^{2+} signals are discriminated from sustained signals by the rapid export of NFATc family members from the nucleus (Timmerman, Clipstone et al. 1996; Rao, Luo et al. 1997). Cells need to distinguish between transient Ca^{2+} signals that induce molecular events, such as muscle contraction, secretion, adhesion and synaptic transmission, and sustained Ca^{2+} signals that are

involved in cell proliferation and differentiation. A rise in intracellular Ca^{2+} triggered by ligand binding to a cell membrane receptor leads to the activation of calcineurin's phosphatase activity, which dephosphorylates cytoplasmic NFATc family members. Dephosphorylated NFATc family members enter the nucleus and cooperatively bind to DNA with AP-1, c-MAF, GATA3/4, and other co-factors. NFAT transcription complexes are assembled in the nucleus from cytoplasmic (NFATc) and nuclear (co-factors) components allowing them to integrate numerous signaling pathways.

It should be noted that JNK1 and JNK2 play different roles during $CD8^+$ T cell activation, and these roles differ from those in $CD4^+$ T cells. Unlike in $CD4^+$ T cells, the absence of JNK2 causes an increase IL-2 production and proliferation of $CD8^+$ T cells. In contrast, JNK1-deficient $CD8^+$ T cells are unable to undergo Ag-stimulated expansion in vitro, even in the presence of exogenous IL-2. The hypoproliferation of these cells is associated with impaired IL-2 receptor α chain (CD25) gene and cell surface expression. The reduced level of nuclear AP-1 complexes in activated JNK1-deficient $CD8^+$ T cells can account for the impaired IL-2 receptor α chain gene expression (Conze, Krahl et al. 2002).

Activation of $CD4^+$ T cells can lead to several functional outcomes: activation and cytokine secretion, death, or functional non-responsiveness (anergy). Activation of NFATc2 in the absence of AP-1 appears to be required and sufficient for the induction of functional anergy in T cells (Macian, Garcia-Cozar et al. 2002). In Th1 cells, NFATc family members may induce various genetic profiles in the absence or presence of AP-1. The function of NFATc family members as repressors of transcription in both lymphocytes and other cell types has been highlighted by recent microarray analyses, and is an emerging observation from several laboratories. Thus, NFATc2 directly represses cyclin-dependent kinase 4 (cdk-4) transcription by binding to the cdk-4 promoter and functioning independently of AP-1 (Bakhshi, Goel et al. 2001).

In general, there are four different ways in control of NFATc protein: phosphorylation/dephosphorylation, nuclear translocation, increase in affinity for DNA and NFATc transcriptional level. The consensus from these studies is that, in resting cells, NFATc proteins are phosphorylated, reside in the cytoplasm, and show a low affinity for DNA binding. Stimuli that elicit calcium mobilization result in the rapid dephosphorylation of NFATc proteins and their translocation into the nucleus;

the dephosphorylated proteins show increased affinity for DNA. It should be noted that cytokines and LPS receptors, which are not linked to Ca^{2+} mobilization, are not involved in activation of calcineurin and NFATc.

The activation of NFATc proteins is determined by their phosphorylation state, which reflects the intensity of Ca^{2+}/calcineurin signaling and the activities of several kinases. Phosphorylation of these serines in NFATc are involving one or more protein kinases: GSK3 (Beals, Sheridan et al. 1997), DYRK (Gwack, Sharma et al. 2006), JNK (Ortega-Perez, Cano et al. 2005), p38 (Yang, Xiong et al. 2002) and the combination of MEKK1 and Casein kinase 1α (Zhu, Shibasaki et al. 1998). Hence, current observations are consistent with two possible mechanisms for the nuclear export of NFATc family members.

The NFAT transcription complex is composed of NFATc family members and "co-factors" (Rao, Luo et al. 1997; Crabtree 1999; Serfling, Berberich-Siebelt et al. 2000). Transcription factors, like AP-1, c-Maf, and GATA3/4 as well as other unidentified components can function as "co-factor" and cooperatively bind to DNA with members of the NFATc family (Wolfe, Zhou et al. 1997; Zhou, Sun et al. 1998; Crabtree 1999). So, the transcriptional activity of NFATc can be regulated through its interactions with partner proteins on DNA (Hogan, Chen et al. 2003). Activators of PKC, such as phorbol 12-myristate 13-acetate (PMA), induce the synthesis of numerous co-factors (e.g., of Fos and Jun family proteins).

The immunosuppressive drugs CsA and FK506 inhibit calcineurin (Liu, Farmer et al. 1991). In addition, CsA and FK506 exert their immunosuppressive effects through targeting calcineurin-independent activation pathways for JNK and p38 (Matsuda, Shibasaki et al. 2000; Matsuda and Koyasu 2003).

A variety of biological and pharmacological agents, including protein kinase A (PKA) activators, glucocorticoids, transforming growth factor-β (TGF-β), retinoic acid, and vitamin D3 are known to inhibit IL-2 production by activated T cells. They often affect NFATc activation (Rao, Luo et al. 1997).

At the transcriptional level, autoregulation is one of the important mechanisms in NFATc1 transcription (Chuvpilo, Zimmer et al. 1999; Chuvpilo, Jankevics et al. 2002). A stimulatory effect of p38 MAPK on at least four other stages of NFATc activation was found. First, the p38 MAPK cascade activated the NFATc promoter and induced the transcription of NFATc mRNA. Second, p38 MAPK mildly increased

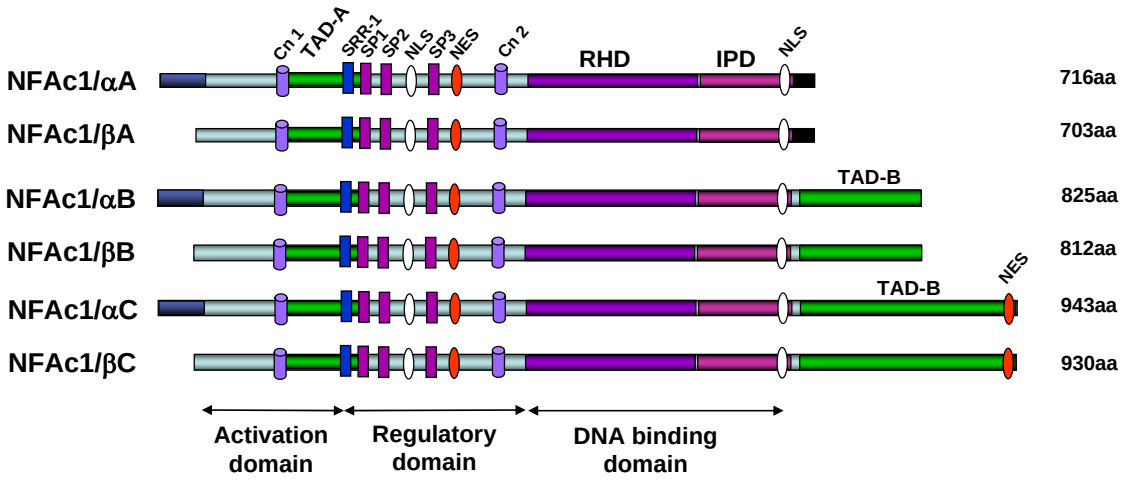
the mRNA stability of NFATc. Third, p38 MAPK enhanced the translation of NFATc mRNA. Fourth, p38 MAPK promoted the interaction of NFATc with the coactivator p300/CBP. In contrast, p38 MAPK moderately enhanced the expulsion of NFATc from the nucleus in T cells. Therefore, p38 MAPK has opposite effects on different stages of NFATc activation. All together, the overall effect of p38 MAPK on NFATc in T cells is activation (Wu, Hsu et al. 2003).

There are many potential target genes for NFATc proteins. NFATc involvement is well established for the IL-2, IL-4, IL-5, GM-CSF, and TNF-α cytokine genes in T cells. There is good but less extensive evidence for NFAT regulation of the IL-3, IL-8, interferon-γ (IFN-γ) and CD40L genes in T cells, the TNF-α gene in B cells, and the IL-4 and IL-5 genes in mast cells (Rao, Luo et al. 1997).

1.3.5 NFATc1 isoforms and T cells

NFATc1 is synthesized in 6 isoforms (Fig.11). These differ in the length of their N and C terminal peptides, and mode of synthesis. Due to a switch from the use of a 3' polyA site to a more proximal polyA site, NFATc1 expression switches from the synthesis of the two longer isoforms B and C in naïve T cells to that of short isoform A in T effector cells. The relative low binding affinity of cleavage stimulation factor CstF-64 to the proximal polyA site seems to contribute to its neglect in naïve T cells. These alternative polyadenylation events ensure the rapid accumulation of high concentrations of NFATc1 necessary to exceed critical threshold levels of NFATc1 for gene induction in effector T cells.

Threshold levels of individual NFATc factors appear to be critical for apoptosis induction in effector T cells. In these cells, the short isoform A of NFATc1 is induced to high levels due to the autoregulation of the NFATc1 promoter P1 by NFATs. P1 is located within a CpG island in front of exon 1, represents a DNase I hypersensitive chromatin site, and harbors several sites for binding of inducible transcription factors, including a tandemly arranged NFAT site. A second promoter, P2, before exon 2, is not controlled by NFATs and directs predominantly the synthesis of the longer NFATc1/B+C isoforms. Contrary to other NFATs, NFATc1/A is unable to promote apoptosis, suggesting that NFATc1/A enhances effector functions without promoting apoptosis of effector T cells (Chuvpilo, Avots et al. 1999; Chuvpilo, Zimmer et al. 1999; Chuvpilo, Jankevics et al. 2002).



Calcineurin-binding region 1 (Cn1): SPRIEIT (PxIxIT)
Calcineurin-binding region 2 (Cn2): DQYLAVPQHPYQWAKPKPLSP (LxVP)

NFATc1	*A*	*B*	*C*
alpha	mouse, human	mouse, human	human
beta	mouse	human	human

Motif	*Amino-acid sequence*	*Maintenance kinase*	*Export kinase*
SRR1	spasslssrscnse	CK1	CK1
SP2	sprhspstsprasvtee	ND	GSK3
SP3	sptpsphgsprvsvtdd	DYRK2-4	DYRK1

Fig.11 A scheme of NFATc1 isoform structure
The 6 NFATc1 isoforms that are expressed in T cells from mouse and human (table list), which are generated by alternative splicing and the use of alternative promoters and polyA sites. A schematic representation of the location of the transactivation, regulatory, DNA-binding and carboxyl (C)-terminal domains of NFATc1 is shown. In addition, the following motifs are indicated: Rel homology domain (RHD); Immunoglobulin-like fold, plexin, transcription factors like domain (IPD); the calcineurin-docking site (Cn), the nuclear localization signal (NLS), the nuclear export signal (NES), the serine-rich regions (SRRs) and the SPXX-repeat motifs (SPs), where X denotes any amino acid. An inducible phosphorylation site has also been described in the N-terminal transactivation domain (TAD-A) of NFATc2. In addition to the DNA-binding, the RHD and IPD domains contain points of contact with AP-1, which allows the formation of the synergistic NFAT–AP-1–DNA quaternary complex. The phosphoserines that are targeted by calcineurin dephosphorylation (underlined) are located in the SP2, SP3 and SRR1 motifs. These serines are phosphorylated in the cytosol by maintenance kinases and in the nucleus by export kinases. CK1, casein kinase 1; GSK3, glycogen-synthase kinase 3; DYRKs, dual-specificity tyrosine-phosphorylation regulated kinases; ND, not determined (Chuvpilo, Zimmer et al. 1999; Serfling, Berberich-Siebelt et al. 2000; Chuvpilo, Jankevics et al. 2002; Macian 2005; Gwack, Sharma et al. 2006).

1.3.6 NFATc proteins and human diseases

Given the fact that NFATc are transcription factors controlling cytokine gene expression, lymphoid cell differentiation and activation, and non-lymphoid tissue and organ development, it is not difficult to deduce that NFATc are involved in many diseases. Until now, many reports showed that NFATc proteins are related to chronic graft rejection (Murphy and Hughes 2002), HIV replication (Managlia, Landay et al. 2006), superantigen-induced lethal shock (Tsytsykova and Goldfeld 2000), down's syndrome (Arron, Winslow et al. 2006), bone and tooth loss (Asagiri, Sato et al. 2005), severe combined immunodeficiency disease (Castigli, Geha et al. 1993), infection and Th1/Th2-releated diseases (Kiani, Viola et al. 1997; Viola, Kiani et al. 1998; Erb, Twardzik et al. 2003), etc.

In tumorigenesis, there is experimental evidence indicating that NFATc may involved in the development of several type of lymphomas (Zhu, Hollmen et al. 2002; Cannon, Philpott et al. 2003; Ding, Kim et al. 2003; Kondo, Harashima et al. 2003; Marafioti, Pozzobon et al. 2005) and leukemias (Yoshimura and Diem 1995; Kneitz, Goller et al. 2002; Glud, Sorensen et al. 2005; Niinuma, Higuchi et al. 2005). But it is still controversial whether and which NFATc proteins are tumor suppressors or oncoproteins (Serfling, Berberich-Siebelt et al. 2004; Marafioti, Pozzobon et al. 2005).

1.4 Goals of the experimental work

Given the central role played by NFATc1 in cytokine production, lymphoid cell differentiation and activation, it is important to understand its expression in lymphoid cells in detail. Many reports have shown that NFAT activation could be controlled by phosphorylation/dephosphorylation, nuclear translocation, and NFATc transcription complex formation. But few experimental data indicated that NFAT regulation was also controlled at the transcriptional level. To study NFATc1 gene transcription in lymphoid cells, we investigated the synthesis of different NFATc1 mRNAs in primary T cell subsets and lymphoma cell lines by using high sensitive RT-PCR assays. In other ways, we studied NFATc1 gene regulation by analyzing transcription factor binding sites and CpG islands in its promoter regions. In addition, we started to investigate NFATc1 isoform function in vivo by establishing conditional knockouts for the NFATc1 promoters 1 and 2, as well as for its intronic enhancer.

p53-deficient mice show an increase in tumor incidence. The most common type of tumors in those mice are T-cell lymphomas (Donehower, Harvey et al. 1992; Harvey, McArthur et al. 1993; Philipp-Staheli, Kim et al. 2004). Moreover, in *p53* and *decorin* double knockout mice, 95% of double homozygotes develop thymic lymphomas (Iozzo, Chakrani et al. 1999). In regard to the pleiotropic NFATc function in lymphocytes and their possible role in lymphoma genesis, we have studied the interplay between p53 and NFATc proteins by establishing *p53*-deficent T cell lymphoma cell lines.

PKC activation is one critical event in TCR-mediated NFATc activation. PKC-θ is a predominant PKC isoform in T lymphocyte activation (Hermann-Kleiter, Thuille et al. 2006). It has been shown that PKC-θ activation leads to activation of NF-κB and AP1 factors (Baier-Bitterlich, Uberall et al. 1996; Sun, Arendt et al. 2000), which are prominent transcription factors controling transcription of lymphokine genes. In addition, PKC activation also activates NFAT (Pfeifhofer, Kofler et al. 2003). Our previous results have revealed that autoregulation is one of the important mechanisms of NFATc1 gene transcription. These data suggest that PKC actvity may control NFATc1 gene transcription by influencing these transcription factors.

Unlike calcineurin having a relative specific target – NFAT, PKC signaling pathways in T cell activation is such complex affair (Baier 2003). The prominent effect in activating NFAT, NF-κB and AP-1 reveal that PKC activation has an incomparable position in T cell activation. Further experiments indicate that PKC-θ functions in a unique pathway that links the TCR signaling complex to the activation of NF-κB in mature T lymphocytes (Sun, Arendt et al. 2000). But the precise mechanism is not clear. The broad effects of PKC imply that a fine turns are needed to carry on within the PKC and the other transcription factor. To have a global view of PKC activation, we analysis the PKC-mediated gene transcriptions of lymphocyte by microarray, which including 4,551 well-characterized genes. In such way, we tried to find out the candidate genes in regulated NFATc1 gene transcription, make a comprehensive review on the interaction between PKC and NFAT in T lymphocyte.

CHAPTER II - MATERIALS AND METHODS

2.1 Materials

2.1.1 General materials

Cell strainer (70 µM)	BD Falcon
2 ml cryotubes	Greiner bio-one
Disposable needles, cuvettes & syringes	Hartenstein
FACS tubes	Hartenstein
Glasswares	Schott
Nitrocellulose membrane	Schleicher & Schuell
Nytran® SuPerCharge	Schleicher & Schuell
Polypropylene tubes	Greiner bio-one, Nunc
Parafilm	Hartenstein
Pipette tips	Eppendorf
Pipettes	Sarstedt
X-ray film (13x18 cm, BioMax)	Kodak
Sterile filters (0.2 µM/ 0.45 µM)	Schleicher & Schuell
Tissue culture plates	Greiner bio-one, Falcon
Tissue culture flasks (50, 250, 500 ml))	Greiner bio-one
Tissue culture dishs (60 mm, 90 mm)	Falcon, Greiner bio-one
Tubes (1.5 & 2 ml)	Sarstedt, Eppendorf
Whatman paper 3MM	Schleicher & Schuell

2.1.2 Chemical materials

Acetic acid [$C_2H_4O_2$]	Carl Roth
Acrylamide solution	Carl Roth
Agar-Agar	Carl Roth
Agarose	Sigma-Aldrich
Ampicillin	Hoechst
APS	Merck Eurolab
ATP-disodiumsalt [$C_{10}H_{14}N_5O_{13}P_3Na_2$]	Sigma-Aldrich
ß-glycerophosphate [$C_3H_7O_6PNa_2$]	Carl Roth
ß-mercaptoethanol	Carl Roth
BioRad protein assay (5x Bradford reagent)	BioRad
Boric acid	Merck Eurolab
Bromophenol blue	Merck Eurolab
BSA fraction V	Carl Roth
Butanol [$C_4H_{10}O$]	Carl Roth
Calcium chloride [$CaCl_2$]	Carl Roth
CD62L MACS beads	Miltenyi Biotech
Chloroform [$CHCl_3$]	Carl Roth
Citric acid [$C_6H_8O_7 \cdot H_2O$]	Carl Roth
Columns for isolating murine $CD4^+$ cells	Miltenyi Biotech
Coomassie brilliant blue R-250	Roche Applied Science
Cyclosporin A [CsA]	Novartis Pharma
DEPC	Carl Roth
Diethanolamine	Roth
Disodiumhydrogenphosphate [$Na_2HPO_4 \cdot 7H_2O$]	Merck Eurolab
D-luciferin [$C_{11}H_8N_2O_3S_2$]	AppliChem
DMEM	Gibco BRL
DMSO	Carl Roth
dNTPs	MBI-Fermentas
DTT	Carl Roth
ECL Chemiluminiscence Kit	Amersham, Roche
	Carl Roth

EDTA [Na_2 EDTA $\cdot 2H_2O$]	Sigma-Aldrich
EGTA	
ELISA Reagents	
• Phosphatase substrate (Disodium 4-nitrophenylphosphate hexahydrate)	Sigma DAKO
• StreptABComplex/AP	Carl Roth
Ethanol [C_2H_5OH]	Sigma-Aldrich
Ethidium bromide [EtBr]	Carl Roth
FCS	Amersham Pharmacia
Ficoll	Carl Roth
Formaldehyde [CH_2O]	Calbiochem
Forskolin	Genomed
Gel extraction kit (Jetsorb)	Sigma-Aldrich
Glutathione sepharose	Carl Roth
Glycerin (87%)	Merck Eurolab
Glycine [$C_2H_5NO_2$]	Sigma
H-89	Gibco BRL
HBSS	Gibco BRL
HEPES	Merck Eurolab
Hydrochloric acid [HCl]	Carl Roth
Hydrogen peroxide [H_2O_2]	Sigma-Aldrich
4-Hydroxytamoxifen [$C_{26}H_{29}NO_2$, Z-isomer]	Tocris
ICI-118 551	Sigma-Aldrich
Ionomycin	Boehringer-Ingelheim
IPTG	Carl Roth
Isoamylalcohol	Carl Roth
Isopropanol [2-Propanol, C_3H_8O]	Sigma
Isoproternol	Roche Applied Science
Leupeptin hydrochloride	Gibco BRL
L-glutamine	Serva
L-glutathione [M_r: 307,3]	Sigma-Aldrich
Lithium chloride [LiCl]	Saliter
Milk powder	Sigma-Aldrich

Magnesium acetate [$Mg(C_2H_3O_2)_2 \cdot 4H_2O$]	Carl Roth
Magnesium chloride [$MgCl_2$]	Carl Roth
Magnesium sulfate [$MgSO_4 \cdot 7H_2O$]	Fluka
Manganese chloride [$MnCl_2$]	Gibco BRL
MEM [100x]	Sigma-Aldrich
MES [$C_6H_{13}NO_4S$]	Carl Roth
Methanol [CH_4O]	Serva
Metrizamide [$C_{18}H_{22}I_3N_3O_8$]	Sigma
Mitomycin C	Carl Roth
MOPS	Merck Eurolab
Paraformaldehyde	Qiagen
PCR purification kit	NEB
PEG 4000	Hoechst
Penicillin (10,000 IU/ml)	Carl Roth
Phenol [C_6H_6O, TE equilibrated]	Serva
Plasmid-DNA Isolation kit (Maxi)	Genomed
Plasmid DNA Isolation kit (Mini)	Serva
PMSF	Boehringer Ingelheim
Poly dI/dC	Sigma Aldrich
Polybrene	Sigma Aldrich
Ponceau Red	Sigma Aldrich
p-ONPG	Carl Roth
Potassium acetate [$C_2H_3KO_2$]	Sigma-Aldrich
Potassium chloride [KCl]	Sigma-Aldrich
Potassium dihydrogen phosphate [KH_2PO_4]	Sigma-Aldrich
Potassium hydrogen phosphate [$KHPO_4$]	Carl Roth
Potassium hydroxide [KOH]	Sigma Aldrich
Propidiumiodide (PI 1 mg/ ml ddH_2O)	Roche Applied Science
Protease inhibitor tablet (complete mini)	Santa Cruz
Protein-A/G sepharose	
Radioactive nucleotides [γ^{32}P-ATP, α^{32}P-dCTP, α^{32}P-UTP]	Amersham Pharmacia
RPMI 1640 (DUTCH modified)	Gibco BRL
	Gibco BRL

RPMI 1640	Sigma
Saponin	Sigma
Sodium acetate [$CH_3COONa{\cdot}3H_2O$]	Merck Eurolab
Sodium azide [NaN_3]	Merck Eurolab
Sodium carbonate [Na_2CO_3]	Carl Roth
Sodium chloride [NaCl]	Carl Roth
Sodium fluoride [NaF]	Sigma-Aldrich
Sodium hydrogen phosphate [$NaH_2PO4{\cdot}H_2O$]	Merck Eurolab
Sodium hydroxide [NaOH]	Carl Roth Fluka
Sodium orthovanadate [Na_3VO_4]	Gibco BRL (100 mM)
Sodium pyruvate [$C_3O_3H_3Na$]	Carl Roth
Sodium citrate [$C_6\ H_5Na_3O_7{\cdot}2\ H_2O$]	Carl Roth
SDS	Amersham Pharmacia
Sephadex G50	Hoechst
Streptomycin sulfate (10 mg/ml)	Sigma Aldrich
Sulfosalicylic acid	Sigma
TaqDyePrimer sequencing Kit	Carl Roth
TEMED	Sigma Aldrich
TPA	Qiagen
Transfection reagents (SuperFect™, PolyFect™)	Sigma Aldrich
Trichloroacetic acid [$C_2HCl_3O_2$]	Carl Roth
Tris	Sigma Aldrich
Triton X-100	Gibco BRL
TRIZOL Reagent	Gibco BRL
Trypan blue 0.1% Trypsin EDTA	Gibco BRL
Tween 20	Carl Roth
Western blotting substrate (Lumi-light)	Roche
X-VIVO 15	BioWhittaker
Xylene cyanol FF	Serva
2-YT Broth	Gibco BRL
Zeocin (100 mg/ml)	Invitrogen

2.1.3 Instruments

Autoclave	Stiefenhofer
Balance machine	Sartorius, Hartenstein
Cold centrifuge	Heraeus
DNA sequencer 373A	Perkin Elmer
ELISA plate reader	BioRad
FACScan™	Becton Dickinson
Fluorescence microscope (Dialux 20)	Leica
Gel documentation system	Herolab
Gel camera	Stratagene, Hoefer
Gel dryer	H.Hölzel
Hemocytometer	Brand
Heating blocks	Hartenstein
Humidified tissue culture incubators	Nuaire US, Heraeus
Hybridization oven	Bachofer
Ice machines	Genheimer
Intensifying screen	DuPont
Laminar hoods	Heraeus, Gelaire
Light microscope	Olympus, Leica
Liquid nitrogen tank	Tec-lab
Luminometer	Berthold
Microliter pipettes	Eppendorf, Brand
Microcentrifuge	Eppendorf
Multichannel pipette	Eppendorf
Multi dispenser pipette	Eppendorf
PCR machine	Perkin Elmer, MWG
pH meter	Ingold, Hartenstein
Phosphorimager	Molecular Dynamics
Quartz cuvettes	Hellma
Refrigerators (-20°C; -70°C)	Privileg, Bosch, Heraeus
Rotors (JA-10, JA-14)	Beckman
Scintillation counter	Canberra Packard
Shaking incubator	Hartenstein

Power supplier	Amersham Pharmacia
SDS-PAGE apparatus	BioRad
Spectrophotometer	Amersham Pharmacia
Ultracentrifuge	Beckman
UV lamp (UVT-20M)	Herolab
Vortexer	Hartenstein
Waterbath	Hartenstein
Water filtration unit (MilliQ Plus)	Millipore
Western blot apparatus	Hoefer

2.1.4 Mice

In generating T helper cell subtypes, DO11.10 OVA-TCR transgenic mice were used. These mice carry the MHC class II restricted rearranged T cell receptor transgene specific for the OVA323–339 peptide Ag.

DO11.10 OVA-TCR transgenic mice, C57BL/6 and BALB/c wild-type mice were maintained under specific pathogen-free conditions in the mice facility of the Institute of Pathology. Animals used in these experiments were between 5 and 7 weeks of age.

2.1.5 Cell lines

All mammalian cell lines were procured from either the German collection of microorganisms and cell cultures (DSMZ), or the European collection of cell cultures (ECACC) or the American Type Culture Collection (ATCC).

Cell lines	***Source***	***Notes***
293	DSMZ	human embryonic kidney cells
EL-4	DSMZ	murine thymoma cell line
Jurkat	DSMZ	human T cell leukemia
DOHH2	DSMZ	follicular B cell lymphoma
L428	DSMZ	Hodgkin's disease cell lines
KH-H2	DSMZ	Hodgkin's disease cell lines
STO	ATCC	Mouse SIM embryo fibroblast
SNL 76/7	ATCC	Mouse feeder cell line

E14	ATCC	Mouse stem cell
WS4	ATCC	Mouse stem cell

p53T cell line belongs to *p53*$^{-/-}$ T cells, which was established from fresh lymphomas of *p53*-deficient mice in our laboratory. JSRG4 and JSRG7 are stably transfected pSRG-1 and pSRG-2 into Jurkat cells respectively, and pSRG-1 and pSRG-2 is the knock-down vector of human Gadd45α.

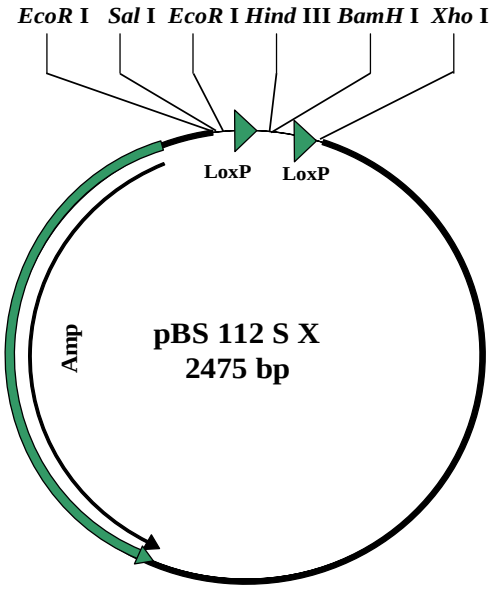
2.1.6 Bacteria

All bacterial strains were *E. coli*, procured from Stratagene. XL-blue and DH5α strains were used in the usual transformation of plasmid DNAs for gene cloning purposes. For the amplification of retroviral expression vectors and accessory vectors, the XL-10 Gold strain was used.

2.1.7 Plasmids

2.1.7.1 pBS112 SX

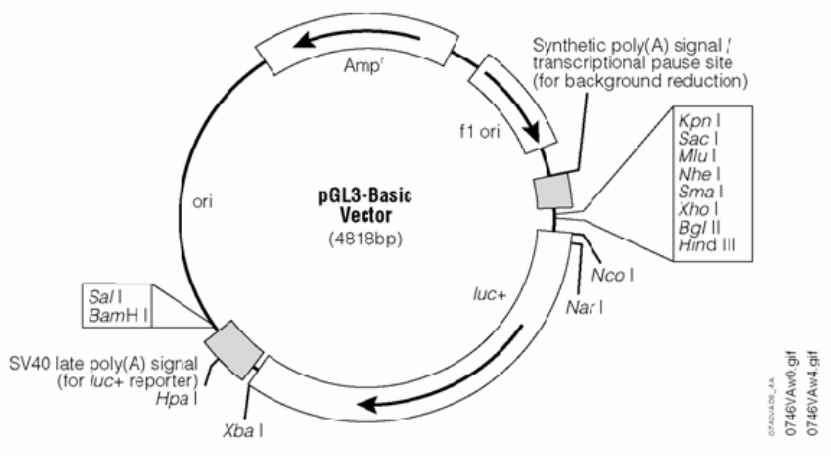
The loxP segment was isolated from vector pBS112SX (a gift of B. Sauer and modified by J. Pelkonen). Correct orientation of the loxP segments was confirmed by sequencing.



LoxP: **ATAACTTCGTATAATGTATGCTATACGAAGTTAT**

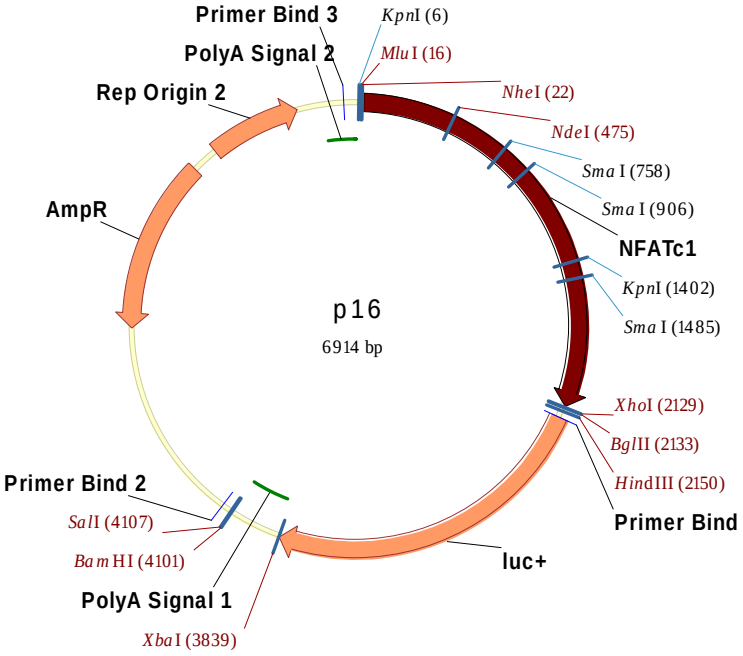
2.1.7.2 pGL3-Basic

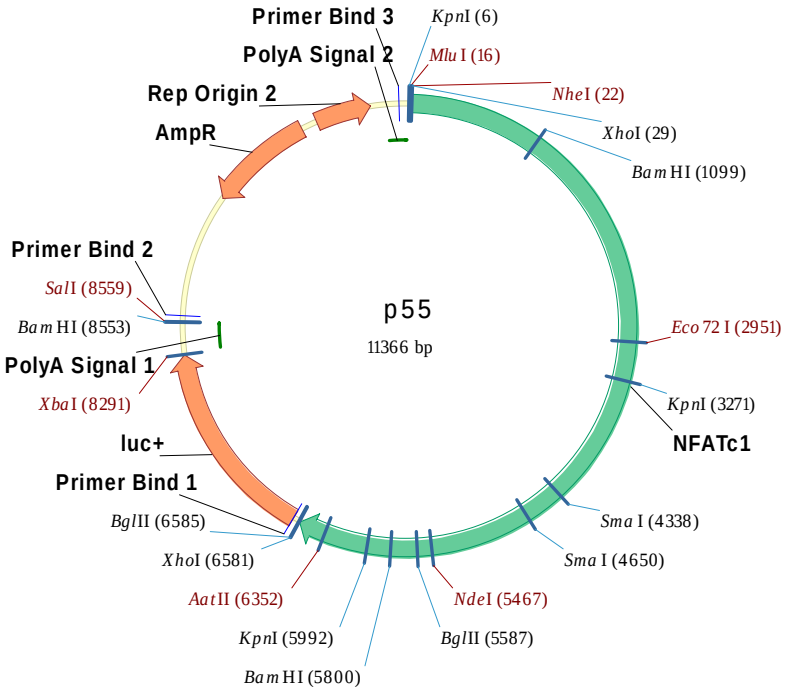
The pGL3-Basic vector is from Promega Corp. A multiple cloning site (MCS) region is positioned upstream of the reporter gene. In the pGL3 vectors, the *Sma I* site is positioned near the center of the MCS. This feature allows DNA fragments inserted by blunt-ended ligation, which usually destroys the site, to be subsequently subcloned or analyzed using the flanking restriction sites. We used the plasmid for subcloning of different NFATc1 fragments.



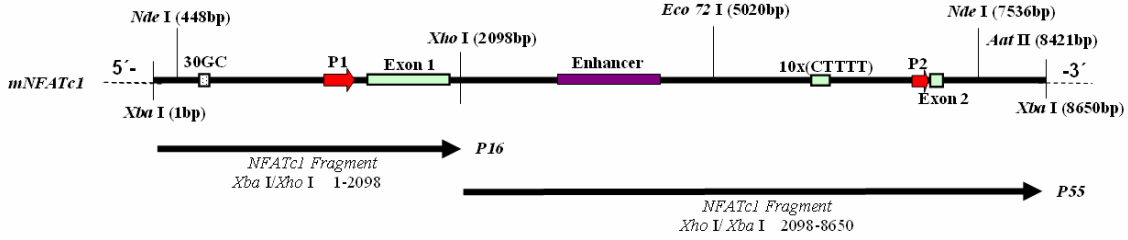
2.1.7.3 p16 and p55 vectors

These two vectors were used to subclone the mouse NFATc1 promoter regions and loxP insertions. p16 contains the NFATc1 *Xba I* - *Xho I* (2098bp) fragment, inserted into the pGL3 basic Sma I site by blunt end cloning. P55 contains the NFATc1 *Xho I* to *Xba I* (6552bp) fragment which was inserted into the *Sma I* site of pGL3-basic by blunt end cloning.



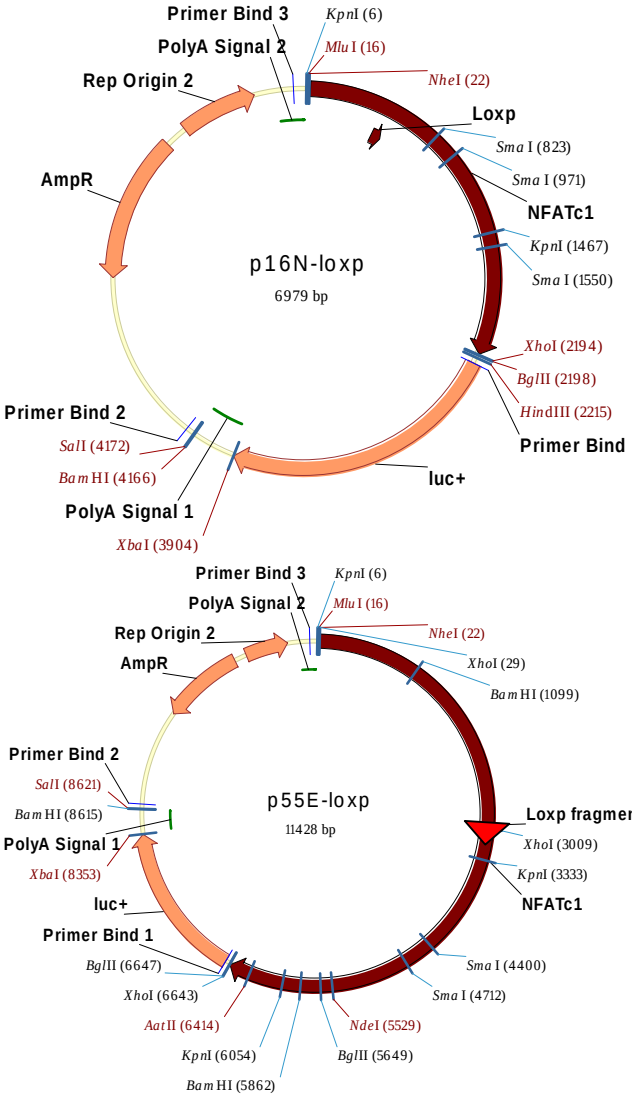


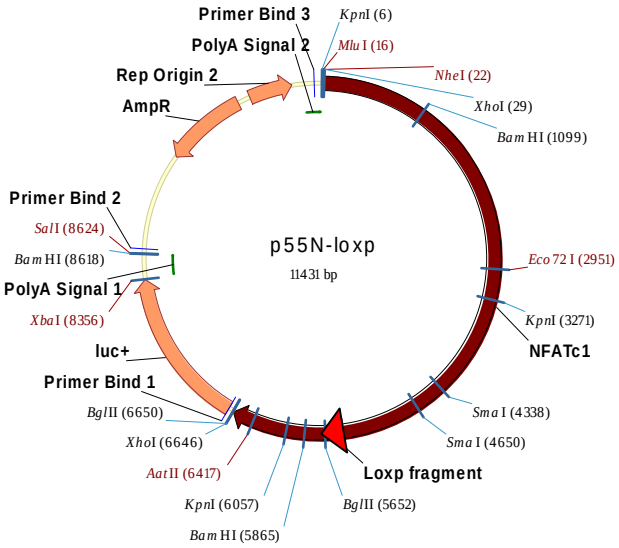
Murine genomic NFATc1 DNA



2.1.7.4 p16E-loxp, p55E-loxp and p55N-loxp vectors

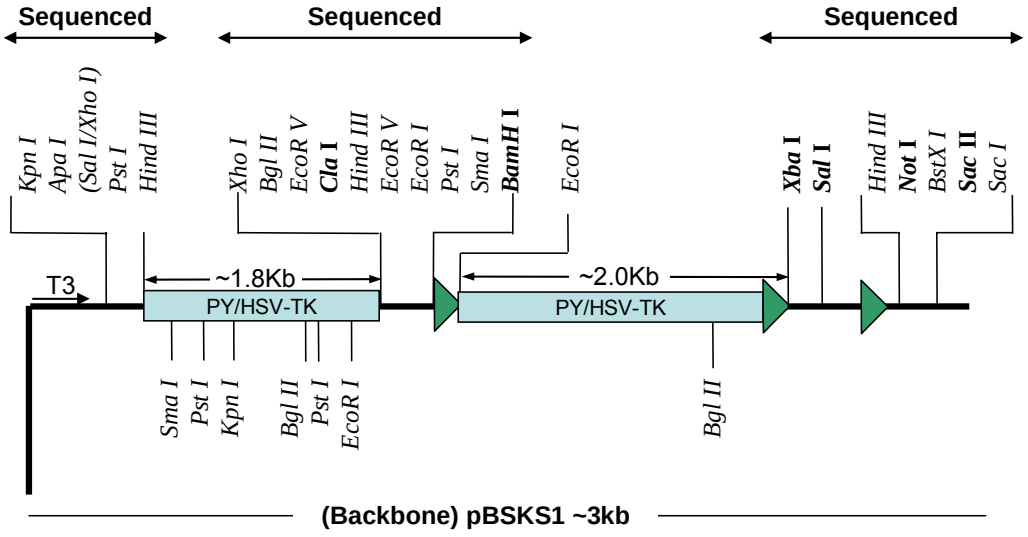
These three vectors are modified versions of p16 and p55. A loxP oligo nucleotide was inserted into 3 different sites. To construct the p16N-loxp vector, pBS112SX was digested by *Hind III* and *Xho I*, and the loxP fragment was inserted into the p16 *Nde I* site by blunt end cloning. To construct the p55E-loxp and p55N-loxp vectors, pBS112SX was digested by *Hind III* and *Xho I*, and blunt end fragments were cloned into the p55 *Eco72 I* and *Nde I* sites, respectively.





2.1.7.5 pKSTKneoloxp vector

This plasmid is the basal vector for the construction of the NFATc1 targeting vectors. To construct the different NFATc1 targeting vectors, left arms were cloned into a *Cla I* or *BamH I* site, right arms were cloned into the *Xba I* site. There are two selection cassettes in pKSTKneoloxp, one is the herpes simplex virus thymidine kinase gene, and another is the neomycin resistance gene flanked by Cre recombinase recognition (loxP) sequences. The unique restriction enzymes are indicated in bold: ***Cla I, BamH I, Xba I, Sal I, Not I, Sac II***. With respect to *Kpn I, Hind III, Bgl II, EcoR I, Sma I*, all cutting sites are indicated

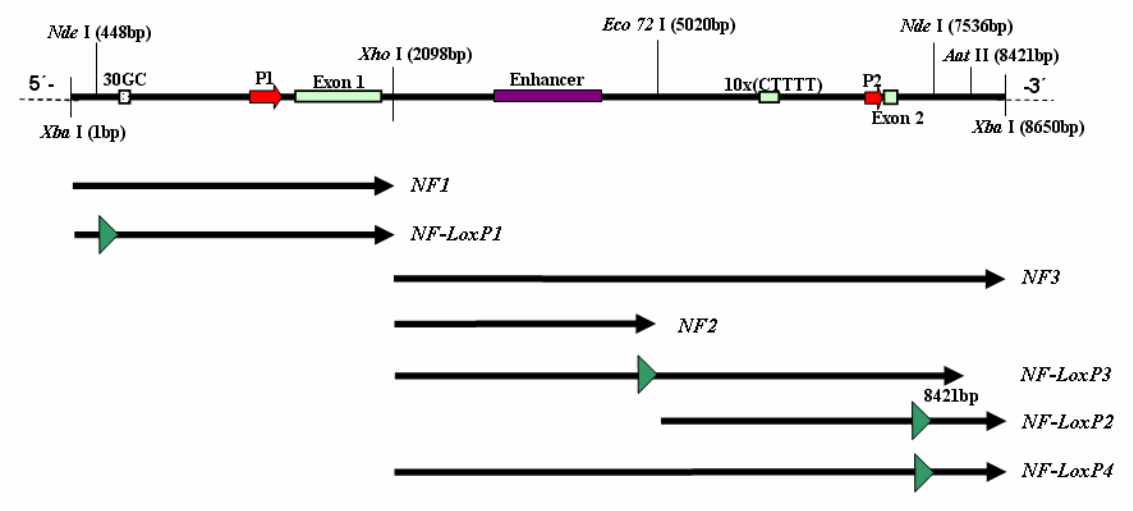


LoxP: **5'-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3'**

2.1.7.6 Fragments for vector construction

To construct the targeting vectors, the following fragments were prepared from the corresponding vectors: NF1 - from p16 (digested by *Mlu I*, *Xho I*), NF2 - from P55 (digested by *Mlu I*, *Eco72 I*) ; NF3 - from p55 (digested by *Mlu I*, *Xho I*) ; NF-LoxP - from p16E-loxp (digested by *Mlu I*, *Xho I*) ; NF-Loxp2 - from p55N-loxp (digested by *Eco72 I*, *Xho I*) ; NF-LoxP3 - from p55E-loxp (digested by *Mlu I*, *Aat II*, *Sal I*), NF-LoxP4 - from p55N-loxp (digested by *Xho I*, *Mlu I* and *Sal I*) ; Loxp fragment - from pBS112sx (digested by *BamH I*, *Xho I*). Before insertion, all fragments were blunted by T4 DNA Polymerase.

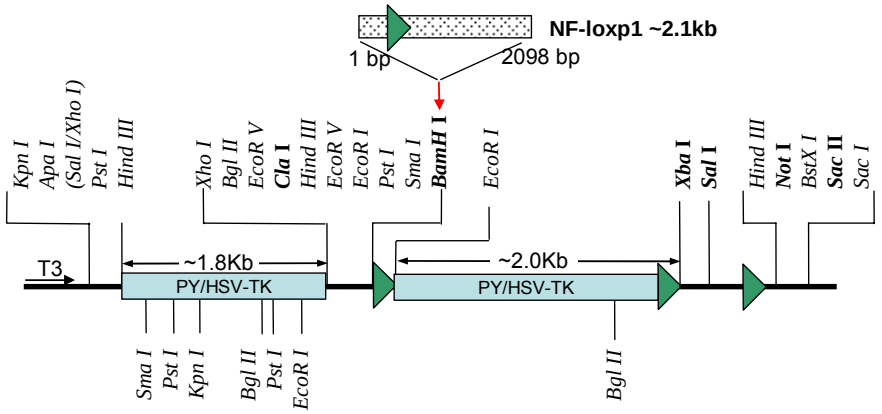
Murine genomic NFATc1 DNA and modified fragments



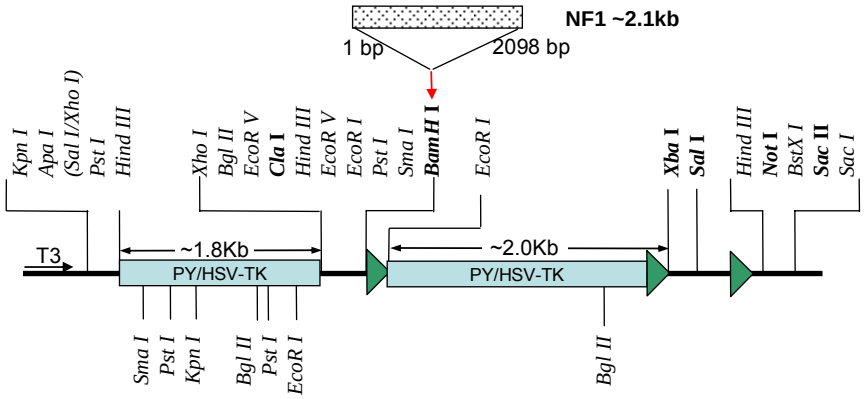
2.1.7.7 pKN1, pKN2, pKN3 and pKN4 vectors

Mentioned vectors are the pre-vectors for constructing the mNFATc1 final targeting vectors. For that purpose, different fragments from the murine NFATc1 gene locus were inserted into the pGKTKneoloxp vector as indicated.

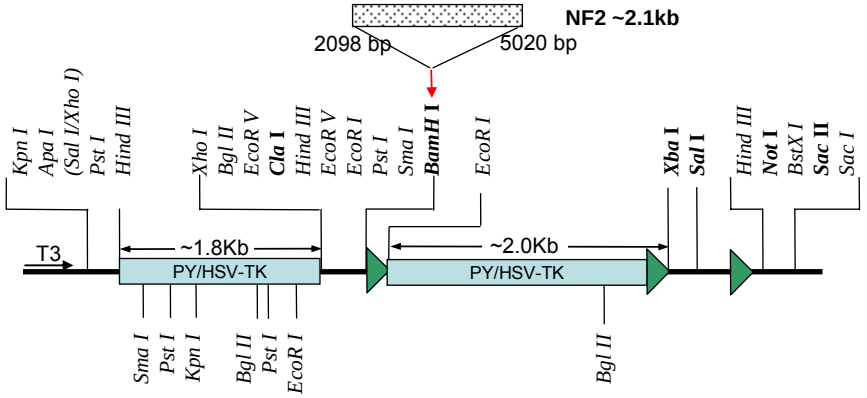
pKN1



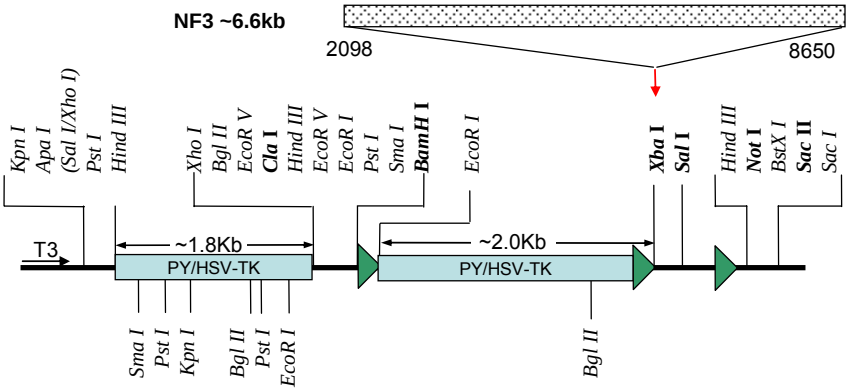
pKN2



pKN3



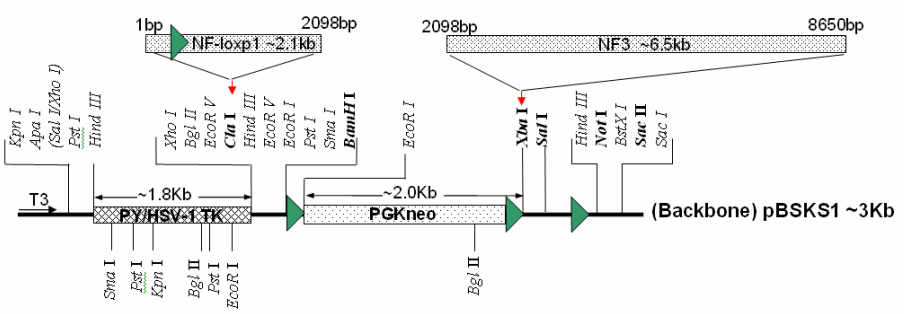
pKN4



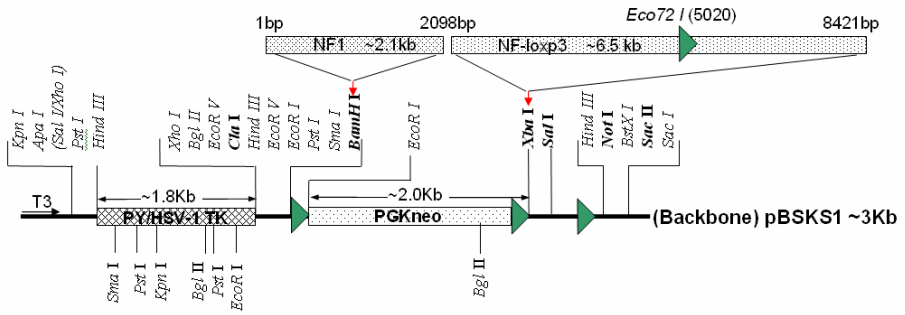
2.1.7.8 pKNF1, pKNF3 and pKNF4 vectors

These are the targeting vectors for the conditional deletion of murine NFATc1 promoter 1 (pKNF1), enhancer (pKNF4) or promoter 2 DNA (pKNF3). The left arms were cloned into *Cla I* or *BamH I* sites, the right arms were cloned into the *Xba I* site. The insertion, position and direction of DNA fragments were checked by restriction enzyme digestion and DNA sequencing.

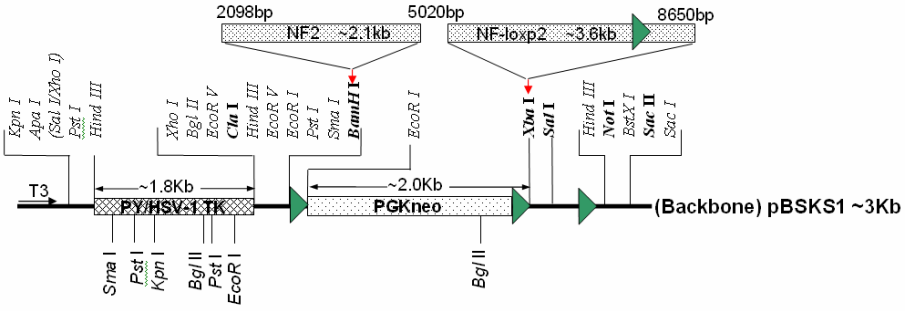
pKNF1



pKNF3



pKNF4



2.1.7.9 pMC-Cre and pTriEx-HTNC vectors

The pMC-Cre vector was constructed in Prof. Klaus Rajewsky's laboratory (Gu, Zou et al. 1993). The pTriEx-HTNC vector is a gift from Prof. Frank Edenhofer (Peitz, Pfannkuche et al. 2002). Both vectors are designed for the expression of the Cre enzyme and the deletion of DNA segments flanked by loxP sites.

2.1.7.10 CMV-p53wt vector

The CMV-p53wt is an expression vector for human wild type p53 (Baker, Markowitz et al. 1990; Gu, Zou et al. 1993).

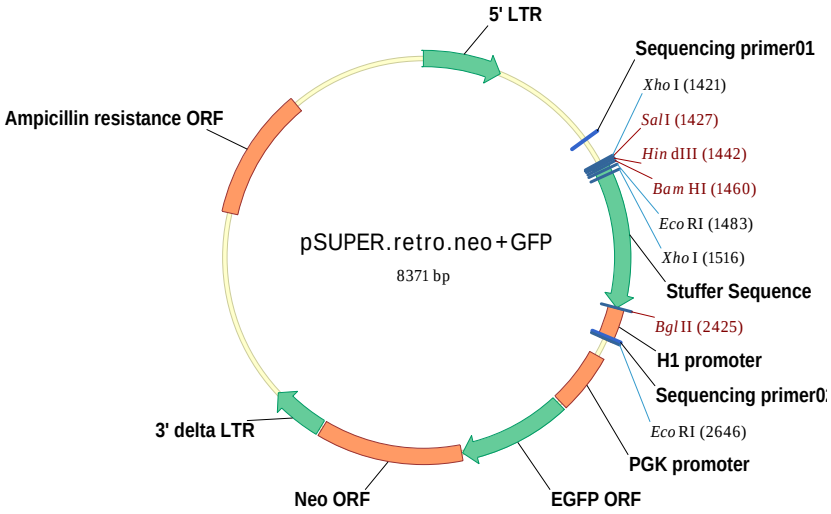
2.1.7.11 The luciferase vectors

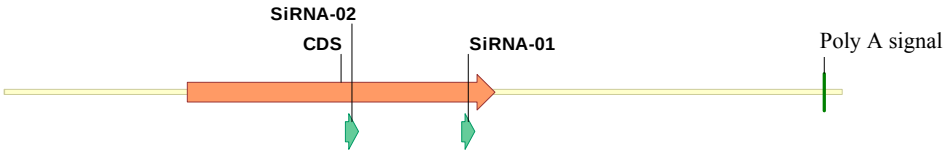
7xNF-κB and 4xPu-box, both vectors are designed for luciferase activity driven by multiple binding sites NF-κB and NFATc transcription factors, respectively.

Two NFATc1 P1-luciferase constructs – P1-0.8 and P1-5.5, whcih contain murine NFATc1 core promoter P1 segments of 0.8 and 5.5 kb, respectively (Chuvpilo, Jankevics et al. 2002).

2.1.7.12 pSRG-1 and pSRG-2 vector

These vectors are the knock-down vectors for human *Gadd45α*. To construct the pSRG vector, the region (*Hind III* and *Bgl II*) of pSUPER·retro·neo^{+}GFP vector (Brummelkamp, Bernards et al. 2002; Brummelkamp, Bernards et al. 2002) was replaced by the synthetic oligonucleotides siRNA-01 and siRNA-02, the vectors are designated as pSRG-1 and pSRG-2, respectively. In both synthetic oligonucleotides siRNA-01 and siRNA-02, there is only one nucleotide difference between human and murine sequence. Because one nucleotide mutation in knockdown oligo usually increases knockdown efficiency, for the same purpose these vectors also could be used in knock-down *Gadd45α* in murine.





hGadd45α (NM_001924)
1866bp

Human siRNA-01 fragment: 66 bp.

Bgl II *Hind III*

5'-GATCCCCGTTGATCCATGTAGCGACTTTCTTCAAGAGAGAAAGTCGCTACATGGATCAATTTTTTA-3'

3'-GGGCAACTAGGTACATCGCTGAAAGAAGTTCTCTCTTTCAGCGATGTACCTAGTTAAAAAATTCGA-5'

^| Antisense | Loop | Sense |Termination Signal

Human siRNA-02 fragment: 71 bp.

Bgl II *Hind III*

5'-GATCCCCGTGACGCGCAGGATGTTGATGTTTGATATCCGACATCAACATCCTGCGCGTCATTTTTTTCCAAA-3'

3'-GGGCACTGCGCGTCCTACAACTACAAACTATAGGCTGTAGTTGTAGGACGCGCAGTAAAAAAGGTTTTCGA-5'

^| Antisense | Loop | Sense |Termination Signal

2.1.8 Oligonucleotides

All oligonucleotides and primers were purchased from MWG Biotech. The lyophilized oligos were dissolved in 10 mM TE buffer, pH 7.5, to the final concentration of 100 pmol/µl and stored at –20°C.

For sequencing:

ID	*Orientation*	*Sequence (5´- 3´)*	*notes*
2012	sense	GGCCGAGGAAGGTAAGC	Sequencing region behind 2098 bp
4923	sense	CTATTATTAGAAACGAAATCAGGC	Sequencing region behind 5020 bp

8320	sense	GGAGGGCACAAAAGGAAG	Sequencing region behind 8421 bp
7433	sense	TATTCCACTGACGCTCACTACTA	Sequencing region behind 7534 bp
351	sense	AGCACCTACTGGCTCACTACA	Sequencing region behind 2098 bp
8553	sense	CGCTGAACTCCCTATTGCT	Sequencing region behind 8650 bp
Primer4(p55-loxp)	sense	ATGACACTTGTAGAGCATCTCCGA	sequencing P55-loxp (Nde I: 7534 bp)
Primer5/NFATc	sense	GAACTATAGTTTATCACTGGAGAAA	sequencing P55-loxp(Eco72I: 5020 bp)
nfat114	anti-sense	CCATCACGCGGACTCTGTA	sequencing region outside Xba (1 bp)
pKSneo-right arm	sense	GATGATACCAGAGTGCTCGCTGAAC	Sequencing region behind 8516 bp
L-R primer (L)	sense	CATGAAGGCGGCCGAGGAAGGTAAG	Sequencing region behind 2003 bp
nfat28	anti-sense	TGGACGCACTCACCGAGGTA	sequencing upstream of mNFATc1
nfat116	anti-sense	CCATCACGCGGACTCTGTACA	sequencing upstream of mNFATc1
nfat132	anti-sense	TACAACCCGTGACGGACACC	sequencing upstream of mNFATc1
nfat92	anti-sense	GAGGTACACTCATCGTCCGGT	sequencing upstream of mNFATc1

Probes for colony lifts:

LoxP probe: 5´- TATAATGTATGCTATACGAAGT -3´

LoxP zone: 5´- ATAACTTCGTATAATGTATGCTATAC -3´

RT-PCR primers for detecting mNFATc1 isoforms:

alpha_forward: 5´-ATGCCAAGCACCAGCTTTCCAGTCCCTTCC-3´

beta_ forward: 5´-ATGACGGGGCTGGAGGACCAGGAGTTCGAC-3´

A_reverse: 5´-TTAGAAAAAGCACCCCACGCGCTCATGTTC-3´

C(orB)_reverse: 5´-CAGGTTGGAGCAGGCTCATAATCATCAGTG -3´

Primers for probe preparation used in southern blotting:

Probe 1_forward (5´): 5´- TGGGGTGAAAAGTTATTTATTG - 3´

Probe 1_reverse (5´): 5´- TCAGTGAGTCCTAGTGTCGC - 3´

Probe 2_forward (3´): 5´- GCTTATTTGCGGACCATT - 3´

Probe 2_reverse (3´): 5´- CAGCCAACTTTCACCTTTCA - 3´

Probe 3_forward (neo): 5´- TCGCACACATTCCACATCCACC - 3´

Probe 3_reverse (neo): 5´- CGTGCAATCCATCTTGTTCAA - 3´

Probe 4_forward (5´): 5´- GGGTGGATAACCAAATGTGCC - 3´

Probe 4_reverse (5´): 5´- AGCCCTTTCCTGTCTGTCTCA - 3´

Oligos for mice genotyping:

Name	*Orientation*	*Sequence (5´- 3´)*	*notes*
NFAT17a	sense	AGCACCTACTGGCTCACTACAC	cover 448, check for loxP insert
NFAT17b	anti-sense	AAATCTATTGGCCTACACCTCA	cover 448, check for loxP insert
NFAT18a	sense	ATTCCACTGACGCTCACTACTATT	cover 7536, check for loxP insert
NFAT18b	anti-sense	ACCTATCCGAAAGGGTCTTATG	cover 7536, check for loxP insert
NFAT19a	sense	GGAGCGGAGAAACTTTGCG	cover 2098, check for neo insert,
NFAT19b	anti-sense	AGACTCACTTGGGAGGATGC	cover 2098, check for neo insert
NFAT20a	sense	ACGCTCCAACTCGCATCTC	cover 5020, check for loxP (neo) insert
NFAT20b	anti-sense	TCTGCCACCAGATGCAAGTG	cover 5020, check for loxP (neo)insert

2.1.9 Antibodies

For flow cytometry

Antigen	*Type*	*Format*	*Clone*	*Source*
CD4	rat Anti-mouse CD4	APC	RM4-5	BD Pharmingen
CD8	rat Anti-mouse CD8	PE	53-6.7	BD Pharmingen
CD3	rat Anti-mouse CD3	APC	G4.18	BD Pharmingen
TCRβ	rat Anti-mouse TCRβ	PE	H57-597	BD Pharmingen
IFN-γ	rat Anti-mouse IFN-γ	APC	XMG1.2.9	BD Pharmingen
IL-4	rat Anti-mouse IL-4	PE	BVD4-1D11	BD Pharmingen
Isotype control	rat Anti-mouse IgG1	FITC		BD Pharmingen
Isotype control	rat Anti-mouse IgG1	APC		BD Pharmingen
Isotype control	rat Anti-mouse IgG1	PE		BD Pharmingen

For Th differentiation

Antigen	*species*	*Source*
IFN-γ (XMG1.2.9)	Mouse	Prof. E. Schmitt (Mainz)
IL-12 (C17.8)	Mouse	
IL-4 (11 B11)	Mouse	Prof. E. Schmitt (Mainz)

For western blotting

Antigen	*species*	*Source*
NFATc1 (7A6)	Mouse	Alexis Biochemicals
NFATc2 (4G6-G5)	Mouse	Santa Cruz (sc-7296)
β-Actin (AC-15)	Mouse	Sigma-Aldrich

2.1.10 Cytokines

Cytokine	*Species and type*	*Source*
IL-12	murine, recombinant, E.coli	Prof. E. Schmitt, Mainz
IL-2	murine, recombinant, E.coli	Prof. A. Schimpl, Wuerzburg
IL-4	murine, recombinant, E.coli	Prof. E. Schmitt, Mainz
TGF-β1	human, recombinant, *E.coli*	PeproTech EC LTD
LIF	Recombinant , CHO	ESGRO^{TM}, CHEMICON

2.1.11 Enzymes

All restriction endonucleases and DNA/RNA modifying enzymes are purchased from MBI Fermentas. Proteinase K and RNase (Ribonuclease) Type I-A is bought from Sigma-Aldrich.

2.1.12 Markers

1kb DNA marker (Fermentas) with 14 fragments of the following sizes (in bp): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250.

Protein marker: PageRuler™ prestained protein ladder (Fermentas) with 10 bands of the following sizes (in kDa): 170, 130, 100, 70, 55, 40, 35, 25, 15, and 10.

2.1.13 Solutions and buffers

Except of statements, the regular solutions and buffers are prepared in following the Molecular Cloning (3rd edition).

For colony lifts

1. **Lysis solution:**
   0.5 M NaOH

2. **Neutralizing solution I:**
   0.2 M Tris-HCl
   1 M NaCl
   pH 7.5

3. **Neutralizing solution II:**
   0.5 M NaCl
   0.05 M EDTA
   25 mM $NaH_2PO_4 \cdot H_2O$
   Adjust the pH to 8.0 with 10 N NaOH

For Nucleic Acid Transfers

The following solutions are noted in the transfer protocols. All solutions should be prepared with sterile, double-distilled deionized water with high-grade reagents. Filter stock solutions through a cellulose acetate filter with a glass fiber prefilter (e.g., bottle-top filter).

ELECTROPHORESIS BUFFERS

Tris-acetate (TAE) buffer-1x
- 40 mM Tris-acetate
- 1 mM EDTA, pH 8.0
- Adjust pH with glacial acetic acid

Tris-borate (TBE) buffer-1x
- 89 mM Tris-base
- 89 mM boric acid
- 2 mM EDTA, pH 8.0

TRANSFER/WASH BUFFERS

SSPE (1x)
- 0.18 M NaCl
- 10 mM sodium phosphate, pH 7.7
- 1 mM EDTA

(20x)
- 3.6 M NaCl
- 0.2 M sodium phosphate, pH 7.7
- 20 mM EDTA

(Na_2HPO_4 is added to NaH_2PO_4 to bring sodium phosphate to pH 7.7.)

SSC (1x)
- 0.15 M NaCl
- 15 mM sodium citrate pH 7.0

(20x)
- 3.0 M NaCl
- 0.3 M sodium citrate pH 7.0

TBS (1x)
- 50 mM Tris
- 150 mM NaCl (pH 10x stock to 7.5)

TurboBlotter Alkaline Transfer Buffer
- 3 M NaCl
- 8 mM NaOH

BLOCKING AGENTS

50 x Denhardt's Solutions:
- 5g of Ficoll
- 5g of Polyvinylpyrrolidone
- 5g of BSA
- Adding water to 500 ml

SAMPLE BUFFERS

1x SET buffer
- 0.1 M NaCl
- 10 mM Tris-HCl, pH 8.0
- 1 mM EDTA

TE buffer
- 10 mM Tris-HCl, pH 8.0
- 1 mM EDTA

ALKALINE TRANSFER BUFFERS

To make one liter:

Denaturing buffer:	**Transfer buffer:**	**Neutralizing buffer 5x:**
3 M NaCl, 0.4 M NaOH	3 M NaCl, 8 mM NaOH	1 M phosphate buffer, pH 6.8
175.5 g NaCl	175.5 g NaCl	79.25 g Na_2HPO_4
16.0 g NaOH	0.32 g NaOH	60.25 g $NaH_2PO_4 \cdot H_2O$
873.0 ml ddH_2O	879.0 ml ddH_2O	Bring to 1 liter with ddH_2O

For isolation of genomic DNA from ES cells in 96 well plates

Required Reagents and Materials:

1. Lysis Buffer for 100 ml:

10 mM Tris-HCl pH 7.5	0.5 ml of 2 M stock
10 mM EDTA	2 ml of 0.5 M stock
10 mM NaCl	0.2 ml of 5 M stock
0.5% Sarcosyl* or SDS w/v	0.5 g / 100 ml
1 mg/ml proteinase K	5ml of 20mg/ml proteinase K (add fresh each time, stored in –80° freezer)

2. Precipitation Buffer for 100 ml (fresh):

150 mM NaCl	3 ml of 5 M stock
100% EtOH	97 ml 100% ETOH
	(This solution remains slurry)

3. Wash Buffer for 200 ml:

70% EtOH	140 ml 100% EtOH
	60 ml ddH_2O

4. Resuspension Buffer: for 25 ml:

10 mM Tris-HCl pH 8.0:	0.125 ml of 2 M stock
10 mM EDTA:	0.5 ml of 0.5 M stock

5. PBS without Ca^{2+} and Mg^{2+}

6. 4 x Restriction enzyme digestion cocktail (100 μl):

4 x mM restriction enzyme reaction buffer:	40 μl of 10 x restriction enzyme buffer
4 mM Spermidine:	4 μl of 0.1 M Spermidine (stored in -80°C)
100 μg/ml RNase A:	4 μl of 10 mg/ml RNase A
2 U/μl restriction enzyme (20/per well)	20 μl 10 /μl enzyme
	32μl of dH_2O

*Sarcosyl (Sarkosyl): N-Lauroylsarcosine sodium salt

2.1.14 Bacterial culture medium

LB medium (Luria-Bertani medium)***:***

deionized H_2O	to 950 ml
tryptone	10 g
yeast extract	5 g
NaCl	10 g

To prepare LB medium shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (approx. 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

Preparation of agar plates:

Prepare LB medium. Just before autoclaving, add agar 15 g/liter. Sterilize LB by autoclaving for 20 min at 15 psi (1.05 kg/cm^2). When the medium is removed from the autoclave, swirl it gently to distribute the melted agar evenly throughout the solution. Be careful! The fluid may be superheated and may boil over when swirled. Allow the medium to cool to 50-60°C before adding thermolabile substances (e.g., antibiotics). Store them at 4°C until needed.

2.1.15 Mammalian cell culture medium

For suspension cell lines (Jurkat, EL-4, and other lymphoid cells):

Reagents	***Volume(ml)***
RPMI1640	459
FCS	25
ß-Mercaptoethanol (50 mM)	1
100 x L-Glutamine (200 mM)	5
100 x Penicillin/Streptomycin (Gibco BRL)	5
Na Pyruvate	5
Total	500

For hybridoma cells:

a) Regular medium (to maintain hybridoma)

Reagents	***Volume(ml)***
*DMEM (with 4500 mg/L glucose, L-glutamine, without Na Pyruvate)	434
10% FCS	50
ß-Mercaptoethanol (50 mM)	1
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Na Pyruvate	5
Total	500

*could be replaced by RPMI1640, it depends on each hybridoma

b) Serum free medium (to produce mAb)

Reagents	***volume(ml)***
CD hybridoma medium(GIBCO. Cat.no. 11279-023)	479
L-Glutamine	5
ß-Mercaptoethanol (50 mM)	1
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Na Pyruvate	5
Total	500

For primary CD4$^+$T cells:

Reagents	***volume(ml)***
x-vivo-15	459
5% FCS	25

ß-Mercaptoethanol (50 mM)	1
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Na Pyruvate	5
Total	500

For ES cells:

a) MEF media (500ml):

Reagents	***Volume(ml)***
DMEM (with 4500 mg/L glucose, L-GlutaMAX™, without Na Pyruvate)	440
10% FCS	50
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Total	500

b) ES cell media (500ml):

Reagents	***Volume(ml)***
DMEM (with 4500 mg/L glucose, L-GlutaMAX™, without Na Pyruvate)	408
15% FCS	75
ß-Mercaptoethanol (50 mM)	1
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
10^7 u/ml LIF (ESGRO™, CHEMICON International Inc)	1
Nucleoside stock (100x)	5
Total	500

c) ES cell media with G418 (500ml):

Reagents	***Volume(ml)***
DMEM (with 4500 mg/L glucose, L-GlutaMAX™, without Na Pyruvate)	431.95
10% FCS	50
ß-Mercaptoethanol (50 mM)	0.5
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Nucleoside stock (100x)	5
10^7 u/ml LIF (ESGRO™, CHEMICON International Inc)	0.05
G418 (50mg/ml)	2.5
Total	500

d) Selection Media (500ml):

Reagents	***Volume(ml)***
DMEM (with 4500 mg/L glucose, L-GlutaMAX™, without Na Pyruvate)	431.45
10% FCS	50
ß-Mercaptoethanol (50 mM)	0.5
100 x non-essential amino acids	5
100 x Penicillin/Streptomycin	5
Nucleosides stock (100x)	5
10^7 u/ml LIF (ESGRO™, CHEMICON International Inc)	0.05
G418(50mg/ml)	2.5
2mM ganciclovir (1000 x)	0.5
Total	500

e) 100 x Nucleoside Stock(100ml)

Adenosine	80 mg	3mM	Sigma A 4036
Guanosine	85 mg	3mM	Sigma G 6264
Cytidine	73 mg	3mM	Sigma C 4654
Uridine	73 mg	3mM	Sigma U 3003
Thymidine	24 mg	1mM	Sigma T 1895

Add to 100 ml ddH_2O and dissolve by warming to 37°C. Filter to sterilize and aliquot while warm. Store at 4°C.

For maintaining cells after electroporation:

Electroporation medium (E-medium): standard medium (RPMI 1640, 10% FCS, 20 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin) supplemented with 50mM Trehalose, 1.25% DMSO, 1 mM sodium pyruvate, 50 μM α-thioglycerol (α-TG) and 20 nM bathocuproine disulfonate (BCS).

2.1.16 Radioactive materials

[γ-^{33}P]-dATP for 5'-termini primer labeling, [α-^{33}P]-dCTP for random labeling (HexaLabel™ DNA Labeling Kit) has been purchased from Amersham.

2.2 Methodology

2.2.1 DNA cloning

Bacterial Culture and Stock preparation:

For minipreps of plasmid DNA, E. coli colonies or 5-10 µl of previous cultures were inoculated in 3 ml liquid medium (LB or 2YT) supplemented with selective antibiotics. They were incubated overnight (8-16 h) at 37°C, 220 U/min in a shaking incubator. Bacterial cells were generally stored at -70°C. In an Eppendorf tube, 500 µl of overnight culture was mixed with 500 µl of 70% glycerol and immediately stored at -70°C.

DNA ligation:

All cloning was performed using blunt ends. Using the manufacture's manual, the right incubation buffer and temperature for the appropriate enzyme were chosen.

The cleavage vector was dephosphorylated by alkaline phosphatase (prevents self-ligation), and DNA fragments were polished by T4 DNA polymerase in order to obtain blunt ends. Prepare the following reaction mixture:

5X reaction buffer	4µl
digested DNA	1µg
2mM dNTP Mix	1µl (0.1mM - final concentration)
T4 DNA Polymerase	1u
Water, nuclease-free	to 20µl

The mixture was incubated at 11°C for 20 min or at room temperature for 5 min. The reaction was stopped by heating at 70°C for 10 min.

To ligate vector and a DNA fragment, the following reaction mix was prepared in an Eppendorf tube:

Vector DNA	50-400ng
Foreign DNA	3:1 with vector
10X ligation buffer	2µl
50% PEG 4000 solution (for blunt ends only)	2µl
Water, nuclease-free	to 20µl
T4 DNA Ligase	5u (for blunt ends)

The tube was vortexed and spun down in a microcentrifuge for 3-5 seconds. The mixture was incubated for 1 hour at 22°C, and the T4 DNA Ligase was inactivated by heating the reaction mixture at 65°C for 10 min. The mixture was used for transformation.

Preparation of competent cells and bacterial transformation:

In routine subcloning experiments, the Calcium Chloride Method was adopted. To increase transformation efficiency, fresh competent cells were prepared each time for reasonable transformation efficiencies (10^6 colonies per μg of DNA). One colony was inoculated into 50 ml 2xYT with shaking at 37°C, and then 5 ml overnight culture was inoculated into 200 ml of pre-warmed 2xYT in a 1 liter flask. Shake at 37°C for 3-4 h. The growing bacteria were spun in 50 ml Falcon tube for 5 min at 5000 rpm at 4°C. The cell pellet was resuspended in 50 ml ice cold 100 mM $MgCl_2$, and Incubated for 10 min on ice. Then cells were spun for 5 min at 5000 rpm at 4°C, and the cell pellet was resuspended in 50 ml ice cold 100 mM $CaCl_2$. Suspension was incubated for 30 min on ice. Cell suspension was spun down again, and

resuspended in 10 ml ice cold 100 mM $CaCl_2$. The suspension was split into 200 µl/aliquots on ice. It was ready for transformation.

Transformation:

- Added plasmid DNA into competent cells (mix by flicking with a finger).
- Incubated for 30 min on ice.
- Heat shock: Placed tubes at a 42°C water bath for 90 seconds
- Immediately transferred tubes into ice for 5 min.
- Added 0.8 ml 2xYT (with no antibiotics) and mixed briefly.
- Incubated samples in a 37°C shaker for 1h.
- Plated 100 µl onto an ampicillin containing 100-mm LB plate.
- Incubated plate overnight at 37°C.

Colony lifting

The transformed E. coli culture was plated onto a 100-mm LB plate and grown up for one night at 37°C. 3 pieces of Whatman 3MM paper were prepared to an appropriate size and shape, and saturated each of paper with one of the following solutions: Lysis solution, neutralizing solution I and neutralizing solution II

Bacterial colonies growing on agar plates were transferred to nitrocellulose filters. A dry nitrocellulose filter (marked by a pen according to the number) was placed onto each plate to lift and tap down enough so that the filter wets all over (no air bubbles between the paper and filter), left for 5 min. The filter positions on plate were stabbed by a needle to mark the position. After that, you could quickly lift the filters out and place colony-side up onto the lysing paper. The filters were incubated for 10 min at room temperature, and then the filters were transferred to neutralizing solution I for 10 min and neutralizing solution II for 15 min sequentially. The filters were placed onto the dry 3MM paper for 15 min, and then baked in a heating oven for 2 h at 80°C. After that, the air dry filters were rinsed in an excess of 1x TBS for 5 min at room temperature, preceding the hybridization. The success of colony hybridization depends on the high specificity of the synthetic oligonucleotide probe used. Meanwhile, the radiolabeled probe was prepared by labeling of oligonucleotides by polynucleotide kinase (PNK). The following reaction mixture was prepared:

dephosphorylated DNA	1-20 pmol of 5'-termini
10X reaction buffer A	2 µl
[gamma-^{32}P or gama-^{33}P]-ATP	20 pmol
Water, nuclease-free	to 19 µl
T4 Polynucleotide Kinase (10 u)	1 µl

The mixture was incubated at 37°C for 30 min. To stop the reaction, 1µl 0.5 M EDTA (pH 8.0) was added, and the mixture was extracted with an equal volume of chloroform. The labeled DNA from unincorporated label was separated by gel filtration on Sephadex G-50. Prehybridization was done at the T_H of the hybrid in 6x SSPE/0.5% SDS/5x Denhardt's solution with salmon sperm DNA (Sigma) for approximately 30 min - 1h. The prehybridization/hybridization temperature (T_H) was calculated using formulas: T_H = [2° C (A:T bp) + 4° C(G:C bp)] – 5° C. If the melting temperature (T_M) of probe is known, then T_H can be calculated by: $T_H = T_M – 5°C$. To start hybridization, the rehybridization buffer was removed and fresh hybridization buffer was added. Hybridizing for 12–20 h with 10^6–10^7 cpm/ml end-labeled probe at T_H was performed. After that, one could wash the blots in 6x SSPE/1% SDS three times for approximately 5 - 10 min each (the washing period may be decreased for lower stringency). At the end, a final wash should be carried out in 1x SSPE/1% SDS for 1–3 min at the T_H of the hybrid. The filters were exposed to Kodak MS film at –70° C with an intensifying screen.

2.2.2 Plasmid DNA Isolation

To analyze plasmid DNA, the positive clones from the colony lift were inoculated in 3 ml LB ampicillin medium and cultured overnight at 37°C. 2.5 ml of bacterial culture was harvested and plasmid DNA was isolated using the JETSORB kit.

After confirmation by restriction enzyme digestion and sequencing, the positive clones were inoculated in 3 ml LB ampicillin medium and cultured overnight at 37°C. Then, an entire overnight culture was inoculated into 1 liter of pre-warmed LB ampicillin medium in a 2 liter flask overnight at 37°C. Plasmid DNA was isolated by using the Genomed isolation kit, and dissolved in 10 mM TE and stored at -80°C.

DNA fragments were isolated by using JETSORB isolation kit, according to manufacture’s protocol.

2.2.3 DNA sequencing

For DNA sequencing, the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) was used. Before sequencing, the amount of purified DNA was quantitated by measuring the absorbance at 260 nm. Mixing the following reagents in a PCR tube:

Sample	*concentration*	*Vol (μl)*
DNA	0.01~0.1 μg/μl	12.0
Primer	10 pmol	4.0
Mixture		4.0
H2O		up to 20.0 μl

Transferring the tube to PCR machine, running the temperature cycling:

Step	*Temp(°C)*	*Time(min)*	*cycling*
Initial denaturation step	95	5:00	1 X
Denaturation step	95	0:30	50 X
Primer annealing Step	50	0:15	
Extending step	60	4:00	
Final Extending step	60	7:00	1 X
End	4	∞	1 X

Cycling products were purified through a Sephadex G-50 spin-column (3000 rpm x 5 min). The passed DNA was directly used for sequencing on an ABI PRISM® 3700 DNA analyzer (without precipitation). DNA sequences were analysed by using the Vector NTI and DNAsis software.

2.2.4 RT-PCR assays

From 1 x 10^6 cells, mRNA was isolated and cDNA synthesis was performed in one step by the use of μMACS™ one-step cDNA Kit (Miltenyi Biotec). To normalize and quantify the difference between all samples, we included small amounts of α^{32}P-dCTP in the cDNA synthesis reaction. The labeled cDNA samples were dotted on a nitrocellulose paper and exposed to a Fujitsu phosphorimager for 2 h. Samples were quantificated by using the software AIDA.

cDNA synthesis was performed with four combinations of NFATc1 isoform specific primers (αA,βA,αCB,βand CB) and a Long PCR enzyme mix.

Temperature cycling:

Step	*Temp(°C)*	*Time(min)*	*cycling*
Initial denaturation step	94	5:00	1 X
Denaturation step	94	0:20	30 X
Annealing and extending step	66	3:00 +0:05	
Final extending step	68	10:00	1 X
End	4	∞	1 X

2.2.5 PCR assays

The PCR reactions were setup on ice, all solutions were gently vortex and briefly centrifuge after thawing. To minimize the possibility of pipetting errors and to save time, a master mix was prepared containing water, buffer, dNTPs, primers and template DNA (or MgCl2) in a single tube. The mix can then be aliquoted into individual tubes. The PCR enzyme mix should be added last. The cocktail for PCR contained the following components:

DNA-template (plasmid ~ 9 Kb)	100ng - 1μg
Forward primer (10 pmol/μl)	1μl
Reverse primer (10 pmol/μl)	1μl
25mM $MgCl_2$	1-4μl(end conc. 2 - 8mM)
10 x buffer include $(NH_4)_2SO_4$	5μl
dNTP-mix (10 mM)	1μl
Tag DNA polymerase(5u/μl)	0.4μl
ddH2O	Up 50 μl

The PCR cocktail was immediately incubated in a PCR machine for amplification by using the following cycles:

Temperature cycling

Step	*Temp(°C)*	*Time(min)*	*cycling*
Initial denaturation step	94	5:00	1 X
Denaturation step	94	0:30	30-35 X
Primer annealing step	54-60	0:20	
Extending step	72	0:45	
Final extending step	72	7:00	1 X
End	4	∞	1 X

2.2.6 Maintenance of cell lines

All cell lines were handled under sterile conditions in a laminar hood and cultivated in appropriate media supplemented with antibiotics. Cells were incubated at 37°C, 5 % CO_2 in a humidified incubator. Before experiments or further passages, cells were frozen with 15 % FCS and 10 % DMSO in liquid nitrogen. The 293 HEK cell line, being an adherent cell line, was split by trypsin-EDTA treatment every 3-4 d at 80% confluency. Suspension cell lines, like EL-4 and Jurkat T cells, were split every 3-4 d just by refreshing with complete RPMI medium.

2.2.7 Induction and inhibition of cells

T lymphocytes were induced either by plate bound αCD3 Ab plus soluble αCD28 Ab or the pharmacological agents TPA (or PMA) and ionomycin that activate MAPK and calcium-dependent pathways, respectively. These mimic signals through the TCR and CD28 co-receptors.

αCD3 Ab : 5 µg/ml for primary stimulation of naïve T cells, 10 µg/ml for restimulation of primary effector T cells. Coating of 24 well plates (1000 µl/well) or 48 well plates (500 µl/well) for O/N at 4°C. Afterwards, coated wells were washed with BSS/BSA 2x immediate and 1x for 5 min.

αCD28 Ab : 1:100 dilution of clone 37.51 hybridoma culture supernatant obtained from Prof. A. Schimpl, or 5 µg/ml soluble Ab (BD Biosciences) for costimulation.

TPA: dissolved in DMSO. 20 ng/ml for stimulation of stable cell lines, 10 ng/ml for primary cells and EL-4 cells

PMA: dissolved in DMSO. Phorbol myristate acetate (PMA; Sigma) was used at 5 to 10 ng/ml for primary cells and EL-4 cells, and 20 ng/ml for Jurkat or stable cell lines.

Ionomycin: dissolved in DMSO. Ionomycin at 0.25 to 1 µg/ml for primary cells and EL-4 cells, and 2 µg/ml for Jurkat cells etc.

CsA: CsA is dissolved in DMSO. Cyclosporin A (100 ng/ml) was added 30 min before addition of the other stimuli (Ghosh, Sica et al. 1996).

2.2.8 Preparation of primary $CD4^+$ T lymphocytes

Preparing lymphocytes from lymph nodes

CD4 naïve T lymphocytes were isolated from LNs of 5-7 weeks old healthy mice. Combined lymph node (axillary, inguinal, brachial, popliteal, and mesenteric) cells from at least 6 DO11.10 TCR-transgenic mice were used for each experiment. The lymph nodes were gently disrupted and passed through cell strainer (70 µm) to

remove aggregates. Lymph node lymphocytes were collected in cold PBS buffer. Lysis of red blood cells was performed before further treatment.

Lysis of red blood cells

For the Preparation of red blood cell (RBC) lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$ and 0.1 mM EDTA), dissolve the following reagents in 800 ml distilled H_2O: 8.3g NH_4Cl 1.0g $KHCO_3$, 200µl 0.5M EDTA. To sterilize, filter through 0.2 µm membrane filter and add distilled H_2O to 1000ml.

- Diluted one volume of cell suspension (freshly drawn blood, buffy coat, leukapharesis material, murine or rat spleen cells, etc.) with 5–10 volumes of lysis buffer and incubated for 5 min at room temperature.
- Centrifuged at 300×g for 10 min at 20 °C.
- Washed cells twice by adding buffer. Centrifuge at 200×g for 10 min at 20°C and carefully removed supernatant.
- Resuspended the cell pellet in an appropriate buffer. Counted the cells and proceeded to magnetic separation.

Enrichment of naïve $CD4^+$ T lymphocytes

To isolate naïve $CD4^+$ T cells, total lymphocytes from LNs were first passed though the $CD4^+$ lymphocyte negative selection MACS kit (Miltenyi Biotec). Untouched $CD4^+$ T lymphocytes were further enriched by the use of the CD62L positive selection MACS kit (Miltenyi Biotec). The cells isolated in this way belonged to the $CD62L^+$ $CD4^+$ naïve T cell population.

Preparing APCs

To prepare 1×10^6 purified B or dendritic cells as Ag-presenting cells (APC), the cells were pulsed with 5 µg/ml OVA323–339 peptide in 100 µl X-VIVO 15 medium for 10 min, then incubated with 50 µg/ml mitomycin C for 1 h and washed 5 times with PBS. To stimulate naïve T cells, 5×10^5 APCs were cultured with 1×10^6 $CD4^+$ T naïve cells at 37°C in a 5% CO_2 atmosphere

$CD4^+$ T cell activation and differentiation:

$CD4^+$ naïve T lymphocytes were induced to proliferate and differentiate in vitro by Ag stimulation in the presence of the respective cytokines and neutralizing antibodies. The establishment of stable phenotype was achieved after 7-10 d of

culture under skewing conditions. The culture of $CD4^+CD62L^+$ T cell was set up at a density of $1x10^6$ cells per ml with a maximum volume of 3 ml per well in a 12-well plate. The following cocktail was used to activate $CD4^+$ T lymphocytes isolated from ova-tg mice:

	Th1	*Th2*	*Tnp*	*Th0*
$CD4^+CD62L^{hight}$	1×10^6	1×10^6	1×10^6	1×10^6
OVA priming APC	5×10^5	5×10^5	5×10^5	5×10^5
Cytokine	IL-2 100u/ml IL-12 10ng/ml	IL-2 100u/ml IL-4 1000u/ml	IL-2 100u/ml TGF-β 10ng/ml	IL-2 100u/ml
antibody	αIL-4 10μg/ml	αIFN-γ 10μg/ml αIL-12 10μg/ml	αIFN-γ 10μg/ml αIL-4 10μg/ml	αIFN-γ 10μg/ml αIL-12 10μg/ml
Start medium	2ml	2ml	2ml	2ml
Skewing time	7 d	7d	7 d	7 d
Final medium	3ml	3ml	3ml	3ml

Format: 12-well plate, check the cell condition, add fresh medium with IL-2 over these days.

2.2.9 Intracellular FACS staining

Cells were resuspended at 1 x 10^6/ml in 24-well plates and stimulated with TPA/ionomycin or plate bound CD3/CD28 Abs. After 4 h stimulation, brefeldin A (Sigma) was added at 10 μg/ml for another 6 h using a stock of 1 mg/ml in ethanol. Cells were harvested, washed, and resuspended in PBS with brefeldin A before adding an equal volume of 4% formaldehyde fixative (final concentration = 2%). After fixing for 20 min at room temperature, they were stained immediately for cytokines. Staining cells were performed with αIL-4-PE and αIFN-γ-APC, or isotype antibody as control. After 20 min, cells were washed twice with PBS/BSA/saponin and then with PBS/BSA without saponin to allow membrane closure. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

Stain buffer = 2% FCS, 0.2% NaN_3, in PBS (pH 7.2 – 7.4)

Perm buffer = Stain Buffer + 0.5% saponin

2.2.10 Luciferase reporter gene assay

The cells were centrifuged at 1200 rpm for 3 min and resuspended in 1 ml of PBS. After that, the cells were again centrifuged at 6,000 rpm for 2 min. To lysis the cells, 100 µl of harvesting buffer was added to the pellet, and mixed thoroughly by vortexing and centrifuged at 14,000 rpm for 4 min. In the next, 50 µl of this supernatant was mixed with 50 µl of assay buffer in the luciferase plate, which was placed in a luminometer, injected with 100 µl of luciferin solution and luciferase activity was measured.

2.2.11 Cell cycle and apoptosis analysis

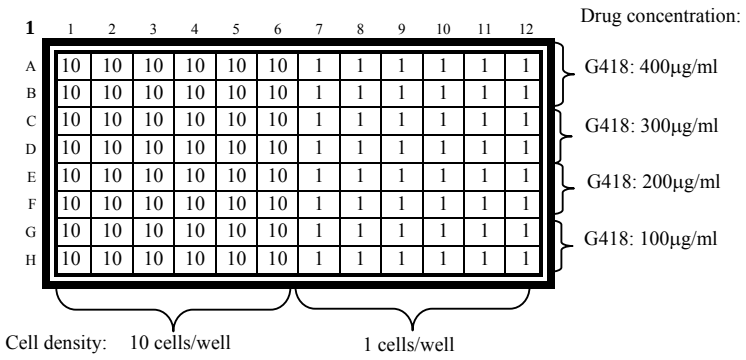
To have the best condition for cell cycle analysis, cells were grown in 24-well plates at a density of 2 x 10^5/2ml/well one day before. The second day, these cells are ready for different experiments. After experiments, cells were harvested at FACS tubes (1500 rpm x 5 min), and then 0.5 ml of cold 70% ETOH was immediately added drop by drop (keep at –20°C). After carefully mixing, cells were kept at 4°C for 1 h. The fixed cells were washed with 3ml of PBS at 1800 rpm x 5min. The pelleted cells were resuspended in a 250 µl propidium iodide solution with 5 µl of 10 mg/ml RNase A (MBI Fermentas, DNase free) (keep at 4°C). After keeping at 37°C for 4 h or 4°C overnight, the cells are ready for FACS analysis. To measure cell cycle progression and apoptosis, the results were analysed by using ModFit LT™ software.

2.2.12 Stable transfection by electroporation

Transient transfection was performed by electroporation using a Bio-Rad Gene Pulser II (Bio Rad, Hercules, CA) at 200 mV and a capacity of 960 µFarads in a 0.4 cm electroporation cuvette. 1x10^7 cells were suspended in 800 µl E-medium in the presence of 50 µg plasmid DNA. After electroporation, cell were kept waiting 15 min at room temp in order to allow resealing before pipetting the cells, it increased cell viability. After that, 200 µl cells were transferred to a 12-well plate with 1ml E-medium as following:

- Day 0: cells were recovered in a 12-well plate overnight.

- Day 1: Counting of surviving of cell and plating at 10 and 1 cell/well in 96-well plates in 100µl standard medium containing PTB (1 mM sodium pyruvate, 50 µM a-thioglycerol and 20 nM bathocuproine disulfonate)



- Days 2-4: Microscopical evaluation of cell recovery. The selective drug was added as 10 x stock solution in fresh medium when the plated cells have undergone two to three cell divisions.
- Days 7-14: Microscopical evaluation of clonal outgrowth. Addition of 50 µl fresh medium containing PTB.
- Weeks 3-5: Evaluation of clonal outgrowth. Addition of 50 µl fresh medium containing PTB if medium turned yellow. To obtain single clones, cells were expanded from the plates with the lowest number of clones.

Stably transfected cells were identified by GFP expression. In a similar way, transfected cells were enriched with a GFP marker by using cell sorting. To gain more purity of stably transfected cells, the cells were subcloned and enriched several times before use.

2.2.13 ES cell culture and manipulation

Stem cell culture

Two stem cell lines (E14 and WS4) were adopted in these experiments, both are derived from the 129X1/SvJ mouse strain. These ES cell lines have been proven to generate germline chimeras with high efficiency. To maintain ES cells integrity, ES cells were carefully expanded and subsequently frozen in liquid nitrogen before screening. All ES cells were grown on MEF feeder layers plus LIF cytokine in high quality medium. This medium prolonged the embryonic phenotype of the cell line. ES cells grew rapidly, dividing every 18 to 24 h. The cells were kept at relatively high

densities to ensure that a high rate of cell division was maintained as this seems to minimize the level of spontaneous differentiation. The cultures were re-fed daily, and subcultured every 2 d. When passaging, it was important to trypsinize the cells well to ensure that a single cell suspension was generated.

Preparation of MEFs

Fresh MEFs were isolated from mouse embryos. MEFs were cultured until passage 3, then frozen in liquid nitrogen as stock. Since MEFs have a limited life span in culture after 3 passages, they can be used to support ES growth without mitomycin treatment.

Preparation of targeting vector DNA

A large scale preparation of gene targeting vector DNA was prepared and purified by the Maxprep kit (QIAGEN). 100 µg of targeting construct DNA was digested with an enzyme to linearize. 0.5 µl of digested DNA were fractionated on an agarose gel to control that the vector DNA was completely digested. The DNA was extracted 2X with phenol/chloroform, afterwards 0.15 volumes of 2M NaOAc and 2 volumes EtOH were added for precipitation. The DNA was pelleted, and then washed with 70% EtOH. The DNA was resuspended in 100 µl sterile H_20 or TE in a laminar flow hood. Checked 0.5 µl DNA on an agarose gel, and determined DNA concentration.

Preparation of ES cells for transformation and electroporation

Approximately 6 x 10^7 ES cells (~3 confluent 10 cm plates) were needed for one electroporation. Splitted one plate of subconfluent ES cells into four dishes such that the next day the cells are ready to be split again. Fed the cells in the morning, as the cells were plated more confluent than usual. The cells were ready for electroporation after a few hours. Trypsinized the cells: removed the medium and rinsed 2 times briefly with 10 ml of calcium-free PBS (37°C), added 3 ml of fresh trypsin-EDTA and incubated for 5 min at 37°C. Digested cells were checked under the microscope to see if the colonies looked like grapes (not single cell). Then the cells were pipetted gently in trypsin-EDTA to break up the clumps, after that added 7 ml medium and pipetted gently up and down again. The cells were pelleted for 5 min at 270g. Aspirated the supernatant and resuspended the cells in 50ml of PBS.

Count cells in hemocytometer. The cells were centrifuged and resuspended to a final concentration of 2.0~2.5 x 10^7 cells/1ml PBS. Transferred 0.8 ml of cell suspension into a 4 mm electroporation cuvette (gap width 0.4 cm) with 25~40 μg of DNA (1 μg/μl of linearized and purified DNA). Waited for 5 min and proceeded at room temp as below: Bio-Rad electroporator (Gene Pulser II System, Bio-Rad, Inc., CA) -- 250V, 500μF. Disconnected the resistance and used the capacity extender to achieve these settings. Monitored time constants, which was ~7 seconds. Let cuvettes sat on ice for 20 min and plated onto three 10-cm feeder dishes in regular ES media (without G418).

Drug selection and cell culture

The day after electroporation there should be some floating dead cells but most cells should sit down on the feeder cells. Start selection 24 h after the electroporation with ES cell media containing 250 μg/ml G418 and 0.5 μM FIAU (or 2 μM Ganciclovir) for 3-4 d, then changed the ES selection medium (G418 only) every day. After about 6-8 d of selection, individual drug-resistant colonies should have appeared and be large enough to pick and subcloned for screening.

Picking of ES cells and 96-well plate culture

Washed plate with PBS. Picked the ES colonies when they were easily seen microscopically but have not yet begun to show any signs of differentiation. This was usually after ~7-9 d under selection, but some variability was not unusual. Picked colonies into a 96 well plate (U shaped wells) with 35 μl of trypsin / well. Picked 24 colonies and incubated at 37 ºC for 10 min. Used a multichannel pipette to break up colonies. Transferred directly into a 96-well plate with feeders and 150 μl of ES medium (without selection drug). Fed every day with ES medium containing selection drug (250 μg /ml G418). Repeated this step on second and third picking days, used the original plates of electroporated cells. If the colonies have not grown to ~80% confluence in two to three days, trypsinized the cells using the following procedure: Aspirated the medium and washed the cells with PBS 2 times. Added 35 μl of trypsin/EDTA and incubated for 5 min at 37°C, or until the cells lift off the plate. Added 150 μl ES cell medium and broke colonies up by gently pipetting up and down. Cultured another 3 -5 d.

Freezing in 96-well plates

Working one row at a time using a multichannel pipette. Changed the medium 2-3 h prior freezing. After 2 days cells, was ready (~80% confluence) to be frozen. Washed with PBS, added 35 µl of trypsin, incubated at 37 ºC for 10 min, added 65 µl of ES medium. Used multichannel pipette to disperse cells. Transferred 65 µl into replica plate (U shaped wells) containing 65 µl of cold 2 x ES freezing medium. Wrapped with parafilm, placed in a styrofoam box with dry ice for 20 min, and transferred to -80 ºC. After removing cells for freezing, added 150 µl of ES medium to remaining 35 µl cells. Grew cells without feeder for 3 - 5 d, the medium became yellow. Splitted into 2 - 4 x 96 well plates for southern blotting and PCR assays.

96-well plate DNA extraction

When cells were confluent, aspirated the medium from each well on the plate and washed twice with PBS. Added 50 µl of lysis buffer to each well. Dampened some paper towels and placed in the bottom of a tupperware container. Placed the plates on paper towels and sealed the lid of the tupperware container. Incubated the plates (with lids on) in this sealed humid container in a 56°C incubator overnight. Added 120 µl of fresh precipitation buffer to each well; leaved at RT for 4 h to allow the DNA to precipitate, avoiding vibration. DNA precipitates were visible against a black background or web-like DNA fibers were seen under a low power microscope. Removed lid, took five layers of paper towel and pre-wetted one side by 70% EtOH, then put the wetted-side on plate over open wells. Inverted plate together with paper towels slowly and kept it on bench for 30 seconds. Lift the plate, blotted away liquid. The precipitated DNA remained attached to the bottom of wells. Washed the precipitated DNA by carefully adding 200 µl of wash buffer 3 times, kept at room temp for 10 min for each washing. Each time removed alcohol by inversion of plate on paper towels. At this point, DNA could be stored in 70% ethanol in a parafilm sealed plate at –20 °C (for four to five weeks) until proceeding with diagnostic restriction enzyme digestion and PCR assays (or southern blotting). Added 60 µl dH_2O, left at RT overnight. Mixed up a 10 µl restriction enzyme cocktail with 30 µl DNA sample for a final volume of 40 µl. Digested overnight at 37°C (seal carefully). Checked on a 0.8 % agarose mini-gel, and if not completely digested, added 10 u/per well restriction enzyme mix again. Added 4-5 µl of loading buffer to each well,

electrophoresed the digested DNA on an agarose gel at 20 V, overnight. For PCR, use 1~2 μl of DNA sample. To run a PCR reaction (nested-PCR), external PCR: 25~30 cycle, internal PCR: 15~25 cycle. The expected yield of DNA from each plate well was about 10 μg.

Make up digest cocktail (100μl):

4 x restriction enzyme reaction buffer:	40 μl of 10 x restriction enzyme reaction buffer
4 mM spermidine:	4 μl of 0.1 M spermidine (stored in -80°C)
100 μg/ml RNase A:	4 μl of 10 mg/ml RNase A
2 U/μl restriction enzyme (20/per well)	20 μl 10 u/μl enzyme
	32μl of dH_2O

2.2.14 Southern blotting

Separated DNA fragments on a 0.8% agarose gel in TAE buffer overnight. Immersed gels in 0.25 N HCl (500 ml for a 200 ml gel) for 15 min at room temperature, agitated gently. Denatured gel by soaking in denaturation solution (3 M NaCl, 0.4 M NaOH) two times for 30 min each (Shaken the dish containing the gel slowly), then soaked in transfer buffer (8 mM NaOH, 3 M NaCl) for 15 min. Floated nitrocellulose filter membrane in distilled water for 15 min. (If dry spots remain, heat membrane in water briefly until boiling.) Soaked membrane in 3 M NaCl, 8 mM NaOH transfer buffer. The membrane should be thoroughly wetted with transfer buffer before using. Set up downward capillary transfers, continued the transfer for 4 h by alkaline buffer transfer. Then, the membrane was ready for hybridization. Prepared probe by random-primed labeling.

High Temperature Hybridization Solution (72°C)

Pre- or hybridization Solution	***Stock***	***Volume(10ml)***
2 x Denhardt's solution	50 x	0.4ml
6 x SSC (or SSPE)	20 x	3.0ml
0.5% SDS	10%	0.5ml
1mM CTAB	0.1M	100μl
100 μg/ml denatured DNA (freshly denatured)*	10mg/ml	100μl
dH_2O to 10 ml		5.9ml

*Salmon testis DNA (Sigma D-9156), 10.4 mg/ml, boiled 5 min before use, and added freshly each time.

Place the membrane in hybridization bottle with 10 ml (minimal 100 μl/cm^2 filter) of prehybridization buffer (use nylon mesh as a spacer). Incubated for 1–2 h at 72° C. Denatured probe at 95°C for 5 min, then added probe in hybridization bottle, hybridized at 72° C overnight. Washed membrane three times for at least 15 min

each with at least 125 ml/100 cm2 of 7x SSPE/0.1–0.5% SDS at room temperature. Then washed membrane twice for at least 15 min each in 1x SSPE/0.5-1.0% SDS at 37° C. Washed finally for 15 min~1 h in 0.1x SSPE/1% SDS at 65° C. Measured the counts coming off the membrane with a Geiger monitor. Stopped washing when the counts dropped to 200 to 700 cpm. If the counts were above this, continued washing as outlined below. Be sure to check the counts between each change of wash buffer. After washing, blotted filters with filter paper (Whatman 3MM) to remove most of the excess moisture. Wrapped moist blots in plastic wrap prior to autoradiography. Exposed filters to Kodak MS film at –70° C with an intensifying screen for 1 day.

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2.2.15 Northern blotting

To avoid RNase contamination, always wore gloves and used DEPC-treated H_2O in the whole procedure. Sample RNAs were fractionated in the following step:

- Prepared gel: The 1.2% agarose gel and running buffer were made with 1X TBE containing 1μg/ml of ethidium bromide (10mg/ml stock) and 20 mM guanidine thiocyanate.
- Prepared sample: 15 μl RNA samples (at least 10 μg) were mixed with 2.5 μl of sterile 6X loading buffer [0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% w/v glycerol, 1.2% SDS, 60mM sodium phosphate (pH 6.8)]. Samples were denatured at 75°C for 5 min and immediately loaded onto a 1.2 % agarose gel.
- Run gel: The gel was electrophoresed at 7-10 V/cm (approximately 90-100 V) for 1 h and 15 min. Gel electrophoresis was stopped and the gel was photographed under U.V. light

Then rinsed the gel with DEPC-treated H_2O, soaked in 50 mM NaOH transfer buffer for 10 min. Set up downward capillary transfers, and continued the transfer no more than 2.5 h. After that, the membrane was neutralized with 0.2 M sodium phosphate, pH 6.8, for 5 min. Baked nitrocellulose membranes in a vacuum oven at 65-80° C for 20 min-2 h. this membrane was ready for hybridization.

Placed membrane in hybridization bottle with 20 ml of prehybridization buffer for 2 h at 42° C with rolling. Denatured DNA probe (prepare by RT-PCR) at 95°C for 5 min, then added probe in hybridization bottle, hybridized at 42° C overnight. For washing and detection, followed the step of southern blotting.

Prepared hybridization buffer:

Pre- or hybridization Solution	***Stock***	***Volume (50ml)***
50% formamide	100%	25.0ml
5 x Denhardt's solution	50 x	5.0ml
5 x SSPE	20 x	12.5ml
1% SDS	10%	5.0ml
0.5% Sodium pyrophosphate	10%	2.5ml
100 µg/ml denatured DNA (freshly denatured)	10mg/ml	0.5ml
dH_2O added to 10 ml		

2.2.16 Western blotting

Samples were fractionated on a SDS-PAGE gel, then electrotransferred onto a nitrocellulose membrane at 100 mA for 1 h at 4°C. The air dried membrane was incubated in blocking solution (5% fat free milk in 1X TBS-T) for 30 min to 1 h at RT. The membrane was directly incubated in primary antibody solution (e.g., 1:2000 in blocking solution) for 2 to 3 h at RT. After incubation, membrane was washed in 1X TBS-T for 1 x 20 min and 3 x 5 min each. Now, the membrane was incubated in secondary antibody conjugate solution (1:10,000 in blocking solution) for 1 to 2 h at room temperature and washed in 1X TBS-T for 1 x 20 min and 3 x 5 min each. The image was developed with ECL Pierce chemiluminescent method according to the instructions of the manufacturer (Pierce).

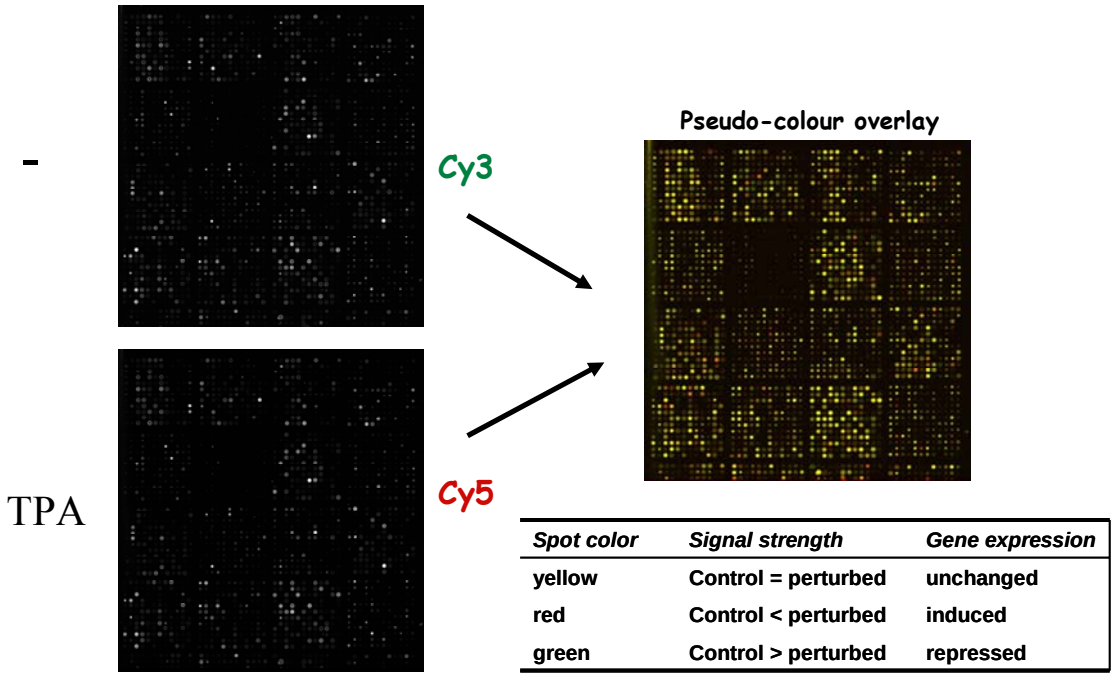
2.2.17 cDNA microarrays

mRNA preparation

RNA isolation was done in a clean, ribonuclease-free environment. All glasswares were baked overnight at 180°C. Double distilled water and all solutions except Tris-containing solutions were treated with 0.1% DEPC solution, followed by autoclaving. Disposable plastic ware was used. Chloroform resistant plastic wares were rinsed in chloroform. The cuvettes were washed every time with 0.1 M NaOH and 1 mM EDTA, followed by washing with RNase-free water. Gloves were worn all times during the experiment. Approximately 10^7 cells of each T cell subtype were used as sources for the preparation of mRNA. TRIzol reagent (Gibco BRL) was used to extract total RNA according to the manufacturer's instructions.

Generation of cDNA and microarray hybridization

Microarray hybridization was performed in the core facility of IZKF, University of Würzburg. General array design: mostly unidentified genes (basically a combination from one known onco-chips) ca. 11,410 Genes. Quality control steps included: dye swaps and replicates. The hybridization started with 7.5 μg of purified total RNA. RNA was labeled according to the Amersham First Strand Labeling – Kit. The protocol and conditions used during hybridization, blocking and washing were the following: Blocking: array 45 min, 42°C, in 25% formamide, 1 % BSA, 5 x SSC, 0.1 % SDS immersed in water and immersed in isopropanol to dry slides. Slides: GAPS CMT by Corning Hybbuffer: 25% Formamide, 5X SSC, 0.1 % SDS. a hybridization station (Amersham, Lucidea Slide Pro) was used. Hybridization was performed with the following conditions: Hybridization: at 42°C for 15 h. Washes in 3 steps: first, 2x SSC, 0.1 % SDS at 42°C, second, 1x SSC, then: 0.5 x SSC and rinsed with water, followed by rinsing with EtOH. The following types of scanning hardware and software were used: scanner: ScanArray 4000, Perkin Elmer, Scan Software: QuantArray (belongs to the Scanner). Image analysis software used: ScanAlyze, Mike Eisen, Stanford, USA. High qulity pictures were used as follow:



Spot color	*Signal strength*	*Gene expression*
yellow	Control = perturbed	unchanged
red	Control < perturbed	induced
green	Control > perturbed	repressed

Data and statistical analysis

Dye bias can be most obviously seen in an experiment where two identical mRNA samples are labeled with different dyes and subsequently hybridized to the same slide, especially in low level expression genes. To balance the fluorescence intensities of the two dyes (green Cy3 and red Cy5 dye) as well as to allow the comparison of expression levels across experiments (slides), normalizations were needed. Analyses were performed using BRB ArrayTools developed by Dr. Richard Simon and Amy Peng Lam. All data were normalized by Lowess smoother algorithm using BRB Array Tools software (Fig.S2). Cut off value: IA and IB>100, Flag=1, 16%>1.5, percent missing>7.5%. The significance modulating gene identifying in different cell lines and phenotype rely on Significance Analysis Microarray (SAM) (Tusher, Tibshirani et al. 2001). Hierarchical Clustering (HCL) (Eisen, Spellman et al. 1998) with average linkage was used to construct the dendrograms for the comparison of the differentially expressed genes in all samples. Genes that were co-regulated (i.e. genes whose expression values follow a similar pattern) with the top-ranked genes in different lymphoma cell lines were identified using Self-Organizing Maps (SOM) (Toronen, Kolehmainen et al. 1999) or Self Organizing Tree Algorithm (SOTA) (Herrero, Valencia et al. 2001) combining with visual inspection in TIGR Multiple Array Views (Quackenbush 2002; Dudoit, Gentleman et al. 2003; Saeed, Sharov et al. 2003). The statistical analysis tools were written in R through the Bioconductor project (http://www.bioconductor.org). The Java-based TM4 software system is available from The Institute for Genomic Research (http://www.tigr.org/software). An overview of data analysis was shown in the figure (supplements Fig.S1)

CHAPTER III - RESULTS

3.1 NFATc1 isoform expression in primary T cells

The transcription of the NFATc1 gene is under control of two different promoters and alternative polyadenylation sites, which results in the generation of 6 different transcripts (Fig.11). While activity of promoter P1 results in the generation of NFATc1/α transcripts encoding the N-terminal α-peptide of 42 aa, promoter P2 directs the synthesis of NFATc1/ß transcripts which code for the N-terminal ß-peptide of 29 aa. In addition, alternative splicing events and the use of two poly A sites, pA1 and pA2, lead to the synthesis of individual NFATc1 RNAs and proteins which differ in the 3` ends/C-termini. Whereas use of the weak proximal poly A site pA1 results in the formation of NFTc1/A RNAs in effector T cells, alternative splicing and use of the distal polyA site pA2 results in the predominant formation of NFATc1/B and C RNAs in naïve and resting T cells. In order to determine the distribution of NFAT isoforms in primary T cells, we generated isoform-specific primers and amplified the NFATc1 isoforms by semi-quantitative long distance RT-PCR. In freshly isolated primary $CD4^+$ T cells, only the long NFATc1/βB or βC isoforms were detected on mRNA level (Fig. 12a), while the NFATc1/αA, βA and αB+C isoforms were detected after T cells differentiated for 2 days (Fig. 12b). All isoform expression reached maximal level after 4 days of differentiation (Fig. 12c). On day 7, when the primary T cells were turned into resting, the α isoforms which controlled by P1 promoter were not detected any more, but the β isoforms still existed (Fig. 12d). When resting primary T cells were stimulated by immobilized CD3 antibody alone for 4 h, there was minor increase in α isoforms (Fig. 12e), and further incubation led to a loss of βB isoform (Fig. 12i). There were no significant differences between immobilized CD3 alone and plus soluble CD28 (Fig. 12e and 12f). Ionomycin alone or in combination with TPA exerted a strong induction at 4h, but only the αA isoform was strongly induced by TPA/ionomycin (Fig. 12g and 12h). In primed T cells, the induction of different isoforms was faster compared to un-primed T cells. In general, the inducible NFATc1 isoforms in primed T cells reached

maximal transcription at 4 h induction, no further increase was observed with the prolong time. In resting T cells, the β isoforms were predominantly expressed. These implies that the long constitute β isoforms may have an important role in maintaining T cells homeostasis, whereas the inducible αA are correspond to Th differentiation and effector function.

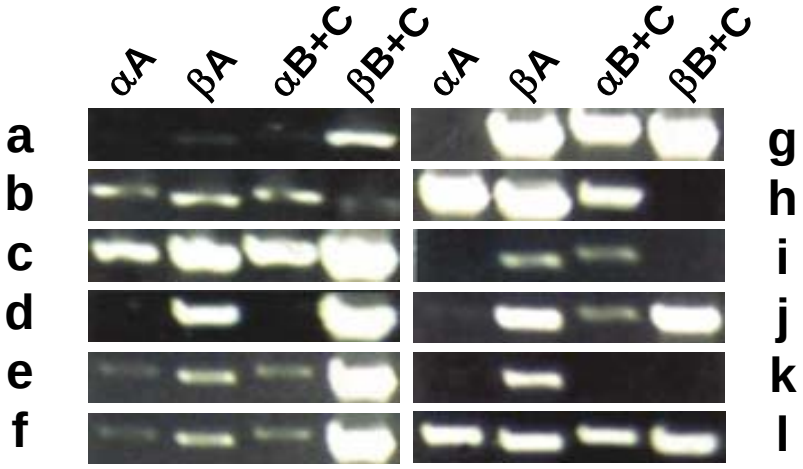


Fig.12 Induction properties of different NFATc1 isoforms in T cell differentiation
Primary $CD4^+$ T lymphocytes were activated by ova-DC on different day in the presence of exogenous IL-2 (50 U/ml). After priming by ova for 7 d, primed $CD4^+$ T cells were re-stimulated by immobilized CD3/CD28 antibodies or TPA/Ionomycin in the absence of exogenous IL-2. RT-PCR was used to identify the transcriptional level of different NFATc1 isoforms, which are NFATc1/αA (αA), NFATc1/βA (βA), NFATc1/αB and αC (αB+C) and NFATc1/βB and βC (βB+C). Samples were processed in the flowing condition: (a). Fresh $CD4^+$ T cells without any stimulation. (b). $CD4^+$ T cells are stimulated with ova-APC for 2 d. (c). $CD4^+$ T cells are stimulated with ova-APC for 4 d. (d). $CD4^+$ T cells are stimulated with ova-APC for 7 d. (e). After 7^{th} d differentiation, $CD4^+$ T cells were re-stimulated with CD3 for 4 h. (f). Re-stimulated with CD3 and CD28 for 4 h. (g). Re-stimulated with Ionomycin for 4 h. (h). Re-stimulated with TPA plus Ionomycin for 4 h. (i). Re-stimulated with CD3 for 8 h. (j). Re-stimulated with CD3 + CD28 for 8 h. (k). Re-stimulated with Ionomycin for 8 h. (l). Re-stimulated with TPA + Ionomycin for 8 h.

To gain insight into the induction properties of different NFATc1 isoforms in resting primary T cells, murine T cells were primed with Ova-Ag for 7 d followed by a rest for another 7 d. Again, the NFATc1/ß isoforms were found to be predominantly expressed in these resting cells (Fig.12). When the resting T cells were re-stimulated by CD3 Ab, or a combination of CD3 and CD28 Abs., the α isoforms, especially NFATc1/αA, showed a strong induction. This is much more pronounced upon TPA + ionomycin treatment which, as also shown in the former experiment, leads to a

strong induction of P1 promoter and the synthesis of α isoforms. In contrast, ionomycin alone is less active in P1 induction but appears to stimulate the synthesis of ß-isoforms and, therefore, P2 activity (Fig.12). Similar with the last experiment, there were almost no any different between immobilized CD3 alone and plus soluble CD28. However, the transcriptions of NFATc1 isoforms decreased in stimulated T cells after a prolong incubation (8 h).

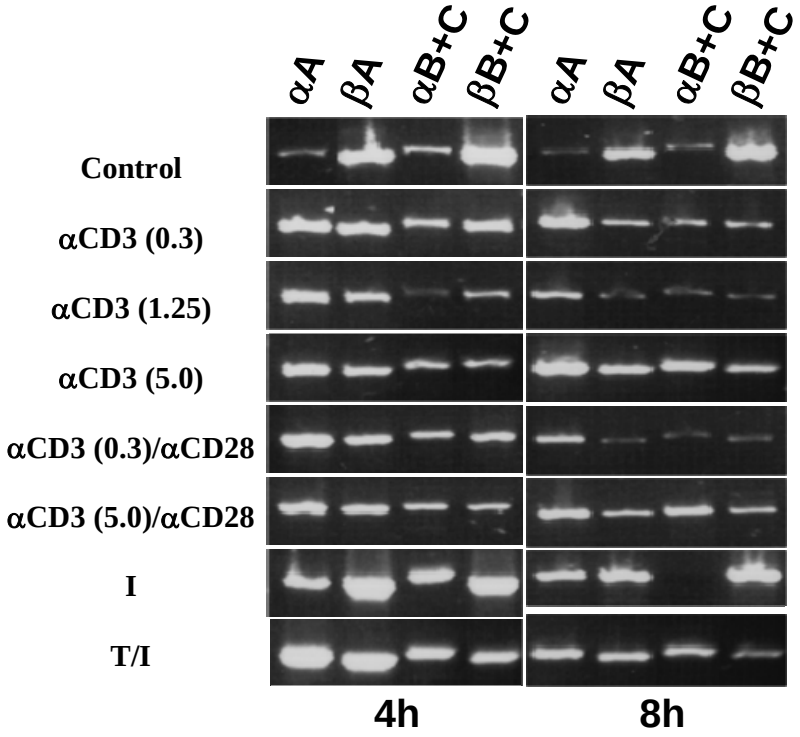


Fig.13 Induction properties of NFATc1 isoforms in Ag-primed CD4$^+$ T cells
Primary CD4$^+$ T lymphocytes were activated by ova for 7 d, and then rested for another 7 d in the presence of exogenous IL-2 (50U/ml). The resting CD4$^+$ T cells were used for different re-stimulations as shown for 4 h or 8 h, RT-PCR assays were used to identify different NFATc1 isoforms at the transcriptional level. Lanes were represented by NFATc1/αA (αA), NFATc1/βA (βA), NFATc1/αB and αC (αB+C) and NFATc1/βB and βC (βB+C). The resting CD4$^+$ T cells were used as control.

3.2 NFATc1 isoform expression in T cell subsets

In freshly isolated primary CD4$^+$ T cells from lymph nodes, there are several T cell subsets, such as naïve, Th1, Th2 and non-polarized T cells, etc. Without separation, it is difficult to deduce in which cells the synthesis of different NFATc isoforms is most active. To answer the question of the potential roles of different

NFATc1 isoforms in T cell subtypes, we generated different $CD4^+$ T help subtypes under polarized and non-polarized conditions (Fig.14).

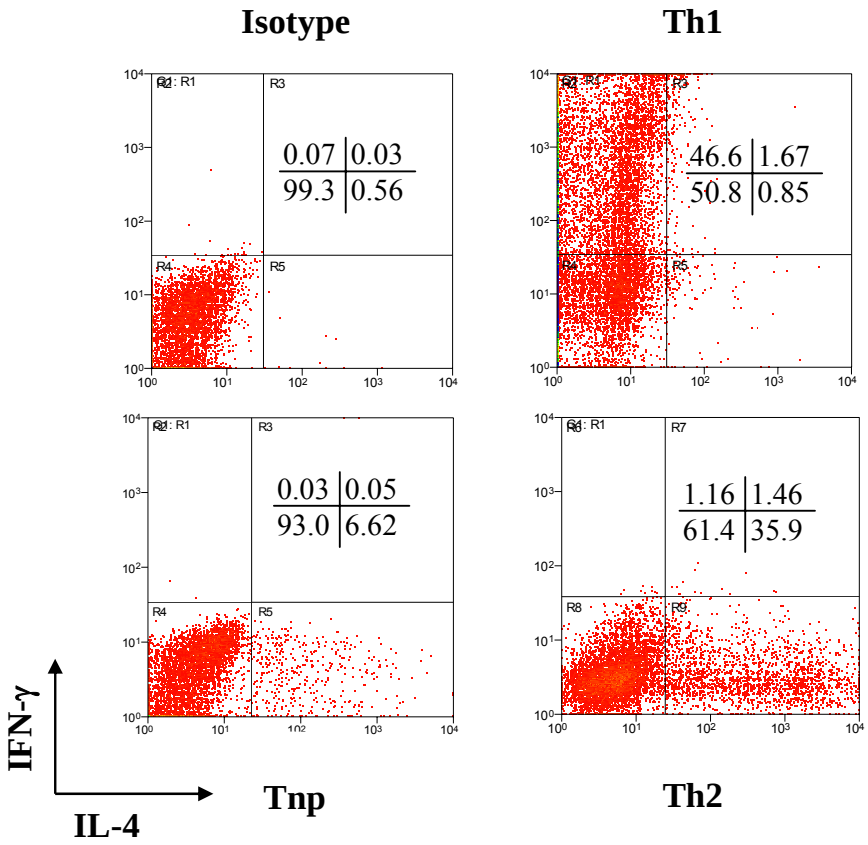
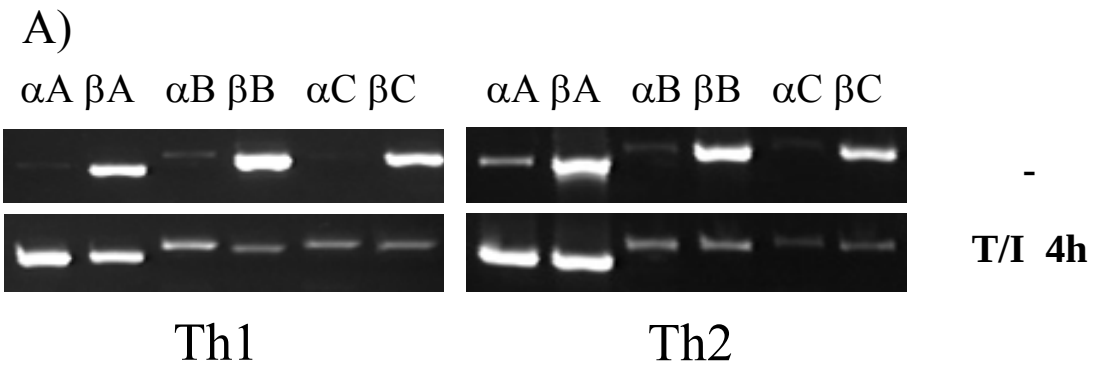


Fig.14 Preparation of T cell subsets
Intracellular staining for IL-4 and IFN-γ from $CD4^+$ T cell subtypes. $CD4^+CD62L^+$ naïve T cells were isolated from LNs and spleen, and primed with ova peptide under different coordinations of cytokines and antibodies for 7 d. The generated T cell subtypes were used for intracellular FACS staining for IL-4 and IFN-γ.



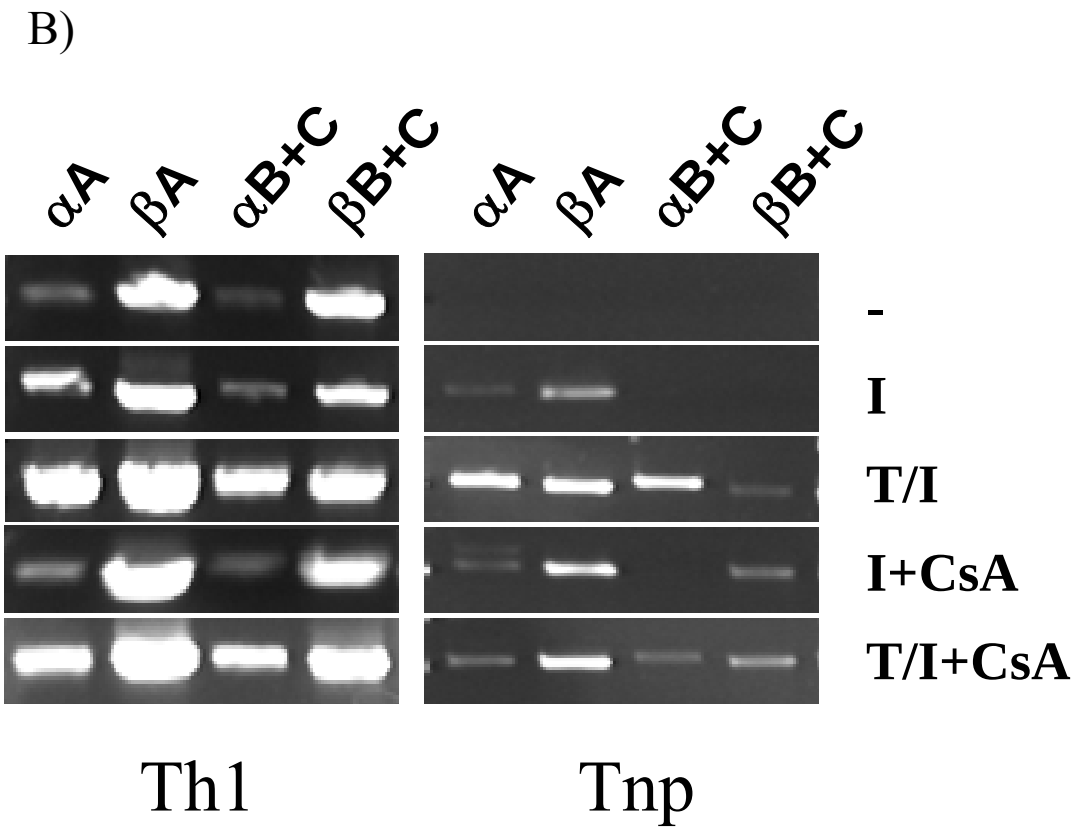


Fig.15 Induction properties of different NFATc1 isoforms in distinct T subsets
These results were coming form "culture" where, according the FACS data, were 47% of Th1, 36% of Th2 and 93% of Tnp cells. A) Transcriptional levels of different NFATc1 isoform were checked by RT-PCR. Resting T cells were stimulated with I or T/I for 4 h as shown, un-stimulated T cell subtypes as control. Lanes were indicated by NFATc1/αA (αA), NFATc1/βA (βA), NFATc1/αB (αB), NFATc1/βB (βB), NFATc1/αC (αC) and NFATc1/βC (βC). B) To see the effect of CsA on each T cell subtype, cells were pretreated with CsA overnight before stimulation. Lanes are represented by the following NFATc1 isoforms: NFATc1/αA (αA), NFATc1/βA (βA), NFATc1/αB and αC (αB+C) and NFATc1/βB and βC (βB+C).

Th1 and Th2 all were found to have a similar pattern in NFATc1 isoform transcription. When stimulated by T/I for 4 h, NFATc1/αA shows a strong induction in both Th1 and Th2 cells. In addition, weaker induction of NFATc1/αB and αC was observed. In reverse, T helper cells show a decrease in expression of long isoforms (βB and βC) after T/I stimulation. There was almost no change in βA expression (Fig.15a). To see how NFATc1 is expressed in T memory cells, we generated non-polarized T cells (Tnp), which are characterized by a loss in IL-4 and INF-γ production (Fig.14). Recently it has been shown that Tnp cells are a type of memory

T cells (Sallusto, Lenig et al. 1999; Langenkamp, Messi et al. 2000; Kim, Campbell et al. 2001; Sallusto, Geginat et al. 2004), so called central memory T cells (T_{CM}). T_{CM} cells are a different population compared to effector memory T cells (T_{EM}). A large different NFATc1 isoform transcription was observed between Th1 and Tnp cells. There was not any NFATc1 isoform expression in Tnp without stimulation, and no long isoform transcription was detected which constitutes most NFATc1 RNA in Th1/Th2 cells. Compared to Th1 cells, Tnp cells show a weak αA isoform induction after stimulation, and Ionomycin alone was unable to induce the αA isoform transcription (Fig.15). Another interesting observation is that the induction of α isoforms was partially blocked by pretreatment of CsA, a calcineurin inhibitor, whereas that of β isoforms remained unaffected. This indicates that synthesis of α isoform controlled by promoter 1 is under NFAT autoregulation, whereas the synthesis of β isoforms controlled by promoter 2 is not controlled in NFAT autoregulation in T cells.

3.3 NFATc1 isoforms in EL4 T cells

The former experiments showed that the synthesis of NFATc1 αA isoform was highly inducible in primary T cells, whereas that of the long isoforms βB/C was constitutively transcribed in effector T cells. The question arises whether there is a similar transcription pattern in T lymphoma cells. The mouse EL4 lymphoma cells were used in these experiments, since they have been intensely used in investigations of T cell function. The results indicated that there were similar induction properties between primary T cells and T-cell lymphomas. Thus, there was a basal transcription of NFATc1 β isoform, whereas transcription of α isoforms, especially of αA isoform, is highly inducible. The marked difference between primary T cells and EL-4 lymphoma cells was that the inducible isoforms have a low level of constitute transcription in EL-4 lymphomas (Fig.16) but not in primary T cells (Fig.12a).

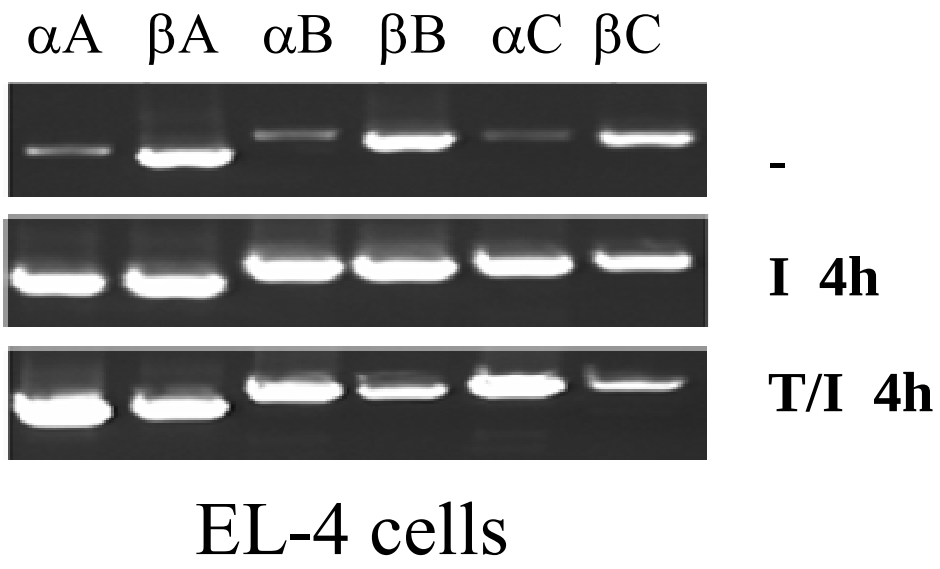


Fig.16 Transcripional levels of different NFATc1 isoforms in EL4 cells
EL4 T lymphoma cells were treated with Ionomycin and TPA for 4 h. The transcription levels of the NFATc1 isoforms were detected by RT-PCR. Lanes were indicated by NFATc1/αA (αA), NFATc1/βA (βA), NFATc1/αB (αB), NFATc1/βB (βB), NFATc1/αC (αC) and NFATc1/βC (βC). RNA from EL-4 cells stimulated by the phorbol ester TPA and ionomycin was isolated followed by cDNA synthesis and long distance PCR assays using the μMACS mRNA isolation and cDNA synthesis kits from Miltenyi.

According to these experiments and our former reports (Chuvpilo, Zimmer et al. 1999; Chuvpilo, Jankevics et al. 2002), we gained more insight into NFATc1 isoform transcription control in effector T cells (Fig.17). The transcription of NFATc1 gene is controlled by two promoters, P1 and P2 and two poly A sites, pA1 and pA2. P1 are inducible promoter controlling alpha isoform synthesis, and P2 are basically transcription promoter controlling longer B and C isoform synthesis. In all T cell subtypes, the transcription of NFATc1/αA isoform are highly inducible and always controlled by the inducible promoter P1 and the poly A site pA1. The B and C isoforms arise from alternative splicing and poly A addition at the distal site pA2. Whereas the short isoform A results from polyadenylation at the proximal poly A site pA1. Since α isoform transcription decreases when treated by CsA, it implies that the activity of inducible P1 promoter is autoregulated by NFATs.

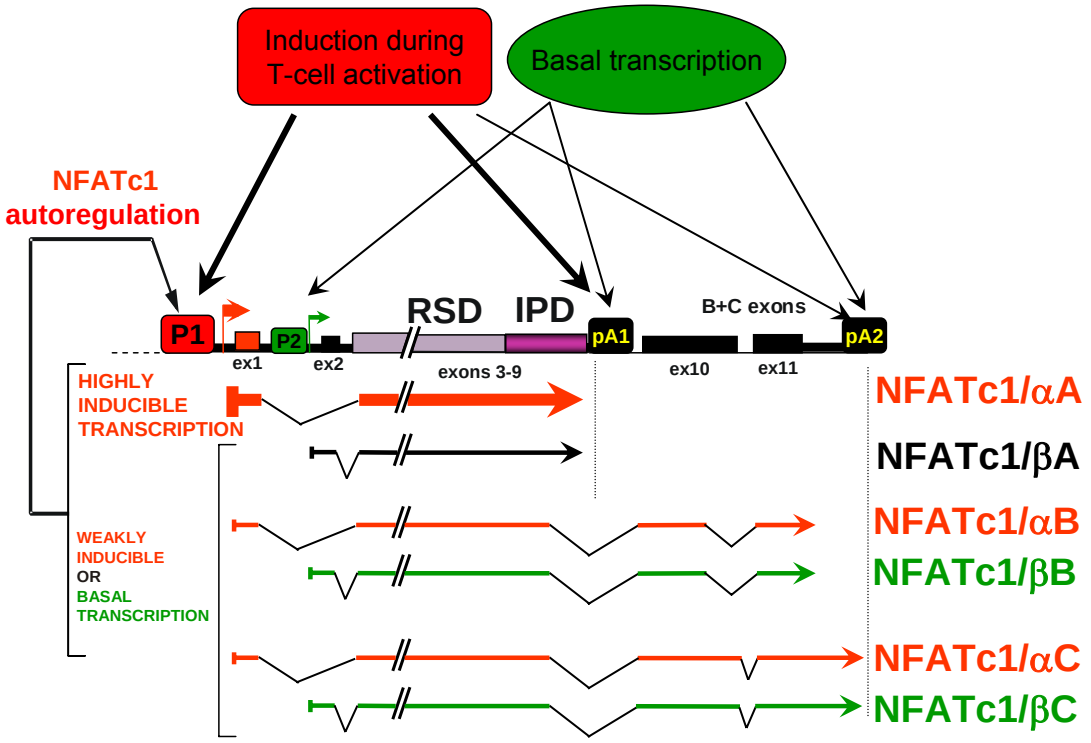


Fig.17 Transcription of murine NFATc1 gene in effector T cells
The transcription of the NFATc1 gene is controlled by two promoters, P1 (directing transcription of the alpha isoform) and P2 (beta isoform), and two poly A sites, pA1 (shorter isoform A) and pA2 (long isoforms B and C). Red color represents inducible and blue color represents constitutive transcription. Rel homology domain (RHD); Immunoglobulin-like fold, plexins, transcription factors like domain (IPD).

3.4 NFATc1 gene regulation

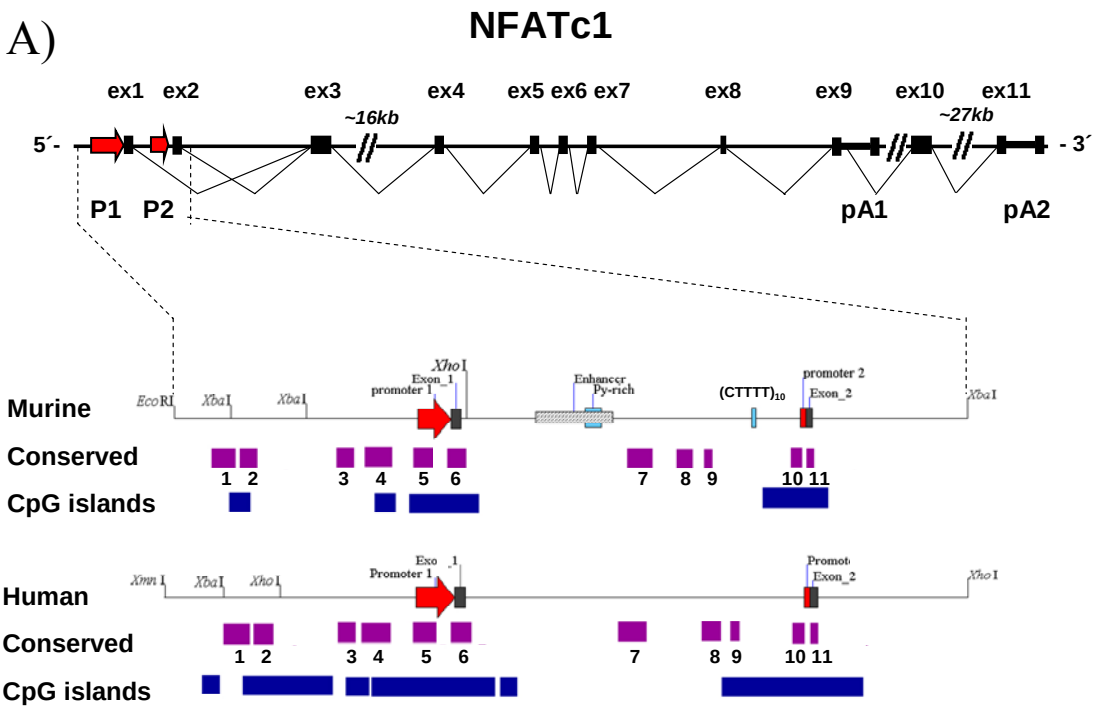
Despite of individual NFATc1 isoform induction properties in Th1, Th2, Tnp and T cell lymphoma, accumulated evidences indicate that P1 promoter is inducible and P2 promoter is involved in basic NFATc1 transcription. What causes such difference? To answer this question, we have to study the NFATc1 gene structure and regulatory elements located in or near by the promoter, and remote control elements.

The transcription of eukaryotic genes is controlled at many levels, such as at the level of transcription factors and co-factors, at the epigenetic level (e.g. by DNA methylation and histone modifications), by gene silencing of micro RNAs etc. For tissue specific transcription, DNA methylation plays a crucial role (Jaenisch and Bird 2003). DNA methylation results in methylation of cytosine (C) residues in CpG dinucleotides. In mammals, maintenance of the appropriate methylation patterns is

essential for normal development. Methylation protects DNA from harmful damage and keeps DNA intact for heritage. Methylated DNA often hinders transcription factors to bind to DNA. Most methylated cytosine residues are found in CpG dinucleotides that are located outside of CpG islands (CpG rich areas). In mammals, more than 50% of genes are characterized by CpG islands, especially at or near its regulatory and promoter regions. Sp1 binding sites occur very frequently within CpG islands, and in several reports it has been shown (or suggested) that Sp1 prevents methylation of CpG islands in the genome. So in mammals, CpG islands usually represent the important regulatory region of gene (Larsen, Gundersen et al. 1992; Antequera and Bird 1993).

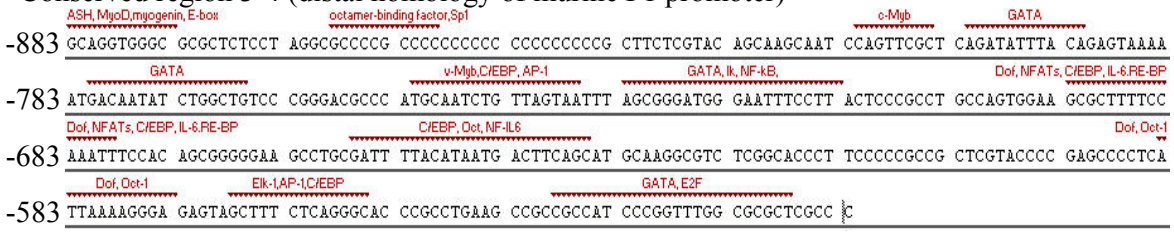
To search for CpG islands, the CpGPlot from EMBL was used. Although there are several definitions for CpG islands, we used a simple definition that was introduced by Gardiner-Garden *et al*.(Gardiner-Garden and Frommer 1987). In brief, observed/expected ratio > 0.60, percent C + percent G > 50.00, and Length > 200. In the murine NFATc1 gene, there are 4 CpG islands located in the 10 kb DNA 5' region as indicated, and there are 6 CpG islands in the 5' region from the human NFATc1 gene (Fig.18). In murine, almost all CpG islands (5 in total) exclusively locate near the two promoter regions, especially P1 promoter (Fig.S4). But in human, 34 CpG islands equally distribute over the whole NFATc1 gene locus (Fig.S4). A notable difference of CpG islands between murine and human NFATc1 gene, which may indicate human NFATc1 is under more sophisticated epigenetic control.

The same gene from different species is supposed to have similar regulation of transcription. This property is reflected by conserved DNA homology regions, and the conserved regions often harbor important regulatory regions. Near the promoter, regions conservation between the human and mouse NFATc1 gene were marked on comparable sites (Fig.18). The NFATc1 gene promoter from human and mouse, the exons 1 and 2, and most of conserved regions are located within the CpG islands. The conserved regions nearby the promoter and located in CpG islands were analysed on the Transcription Element Search System (TESS) and the results were indicated on Fig.18b. In the conserved regions 4 and 5, and in the regulatory region of promoter 1, NFAT binding sites are found, but not in conserved region 10, the candidate regulatory region of P2 promoter. This may explain the activity of P1 promoter is controlled by NFAT autoregulation (Fig.15B).

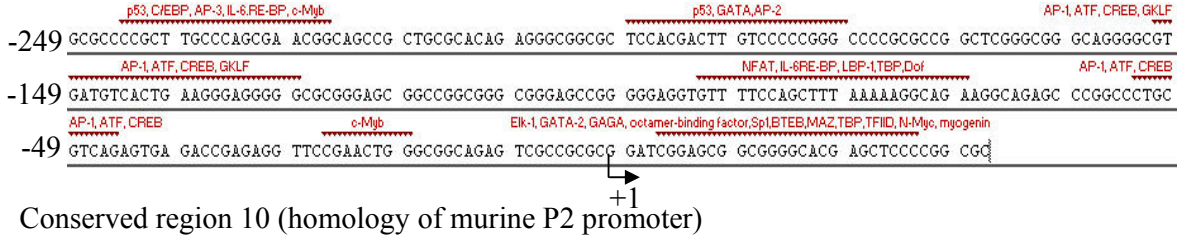


B)

Conserved region 3-4 (distal homology of murine P1 promoter)



Conserved region 5 (proximal homology of murine P1 promoter)



Conserved region 10 (homology of murine P2 promoter)

Fig.18 Schematic structure of the NFATc1 gene and its regulatory elements

A) As indicated, there are 11 exons, two promoters (P1 and P2), one enhancer and two poly A sites (pA1 and pA2) in the murine NFATc1 gene. To search for the potential regulatory elements, the promoter DNA region of mouse (*EcoR I*, *Xba I*) and human (*Xmn I*, *Xho I*) were used. Mouse CpG islands (blue boxes) of the length 288 (709..996), 291 (2607..2897), 924 (3064..3987), 868 (7679..8546) base pairs respectively, and human CpG island of a

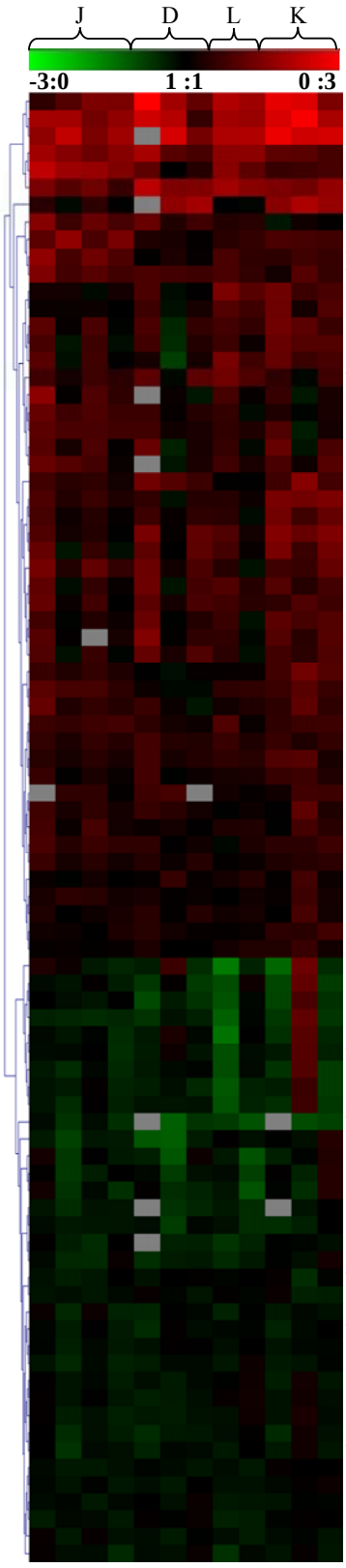
length 221 (433..653), 1067 (913..1979), 284 (2119..2402), 1461 (2420..3880), 210 (3936..4145), 1671 (6529..8199) base pairs, were. There are 11 conserved regions (purple boxes) larger than 100 bp. The conserved regions inside and near the CpG islands were considered as potential regulatory regions. B). Transcription factor and co-factor binding sites in the homology regions of promoter. Conserved regions near the promoters were searched for transcription factor binding sites (TESS - the Transcription Element Search System). Since there are many Sp1 binding sites in the CpG islands, the DNA binding sites for Sp1 are not indicated in this picture.

Comparing the regulatory regions of P1 and P2 promoters, there are more potential transcription factor binding sites in the P1 promoter. This might imply that the P1 promoter is regulated in a more complex and sophisticated way. Those transcription factors can be grouped into two types, i.e., in activators or repressors. NFAT, NF-κB, Elk-1, AP-1, EGR-1, CREB and ATF factors promote transcription (Tsai, Yie et al. 1996; Chuvpilo, Jankevics et al. 2002; Decker, Nehmann et al. 2003; Barlow, Rose et al. 2006; Serfling, Chuvpilo et al. 2006), whereas transcription factors p53 and GKLF are candidates for transcriptional repressors (Buckley, Kuo et al. 2001; Vousden and Lu 2002).

It should also be noted, that in addition to marked sites for transcription factors, there are many SP1 binding sites in conserved regions. As mentioned above, Sp1 prevents methylation of CpG islands in the genome. A second link between Sp1 and nuclear architecture is the observation that Sp1 may function in remodeling chromatin structures (Jongstra, Reudelhuber et al. 1984; Grosveld, van Assendelft et al. 1987). The activity of transcription factors and the DNA methylation in the regulatory regions will finally decide the mode of NFATc1 gene transcription.

3.5 Gene expression profiling of PKC activation in lymphocytes

Similar to the IL-2 promoter, the prototype promoter for which activity is specifically induced upon T-cell activation (Serfling, Avots et al. 1995), the inducible NFATc1 P1 promoter needs more than one signal for its optimal induction (Fig. 12, 13 and 15). One signal is provided by a persistent increase in free cytosolic Ca^{2+} induced by ionomycin, which stimulates the nuclear translocation and transcriptional activation of all NFATc factors (Serfling, Berberich-Siebelt et al. 2000; Hogan, Chen et al. 2003); another signal is provided by phorbol esters, which mainly activate protein kinase C-θ in T cells and PKC-β in B cells (Altman, Isakov et al. 2000; Su, Guo et al. 2002; Pfeifhofer, Kofler et al. 2003; Serfling, Chuvpilo et al. 2006).



EGR1	early growth response 1
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
SLC15A2	solute carrier family 15 (H+/peptide transporter), member 2
JUNB	jun B proto-oncogene
GADD45A	growth arrest and DNA-damage-inducible, alpha
TIEG	TGFB inducible early growth response
PCF11	pre-mRNA cleavage complex II protein Pcf11
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
TXNRD1	thioredoxin reductase 1
TRIP8	thyroid hormone receptor interactor 8
RBBP6	retinoblastoma binding protein 6
CENTB2	centaurin, beta 2
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)
PTP4A1	protein tyrosine phosphatase type IVA, member 1
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked
TFPI2	tissue factor pathway inhibitor 2
RCOR	REST corepressor
CLECSF2	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
SFRS1	splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)
PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome)
STCH	stress 70 protein chaperone, microsome-associated, 60kDa
NXF1	nuclear RNA export factor 1
CUL3	cullin 3
GTF2B	general transcription factor IIB
GARS	glycyl-tRNA synthetase
ZNF216	zinc finger protein 216
IDI1	isopentenyl-diphosphate delta isomerase
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
RPL13A	ribosomal protein L13a
RAB1A	RAB1A, member RAS oncogene family
IMPACT	hypothetical protein IMPACT
GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)
XBP1	X-box binding protein 1
HSPH1	heat shock 105kDa/110kDa protein 1
IMPA2	inositol(myo)-1(or 4)-monophosphatase 2
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta
CDK7	cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)
RAB5A	RAB5A, member RAS oncogene family
BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2
ADAM9	a disintegrin and metalloproteinase domain 9 (meltrin gamma)
KIAA0005	KIAA0005 gene product
EIF2C2	eukaryotic translation initiation factor 2C, 2
FACL3	fatty-acid-Coenzyme A ligase, long-chain 3
DHX36	DEAH (Asp-Glu-Ala-His) box polypeptide 36
SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
SDC2	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)
KNS2	Homo sapiens cDNA FLJ42496 fis, clone BRACE2035003
ANXA5	annexin A5
KCNS1	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
KIAA0233	KIAA0233 gene product
GAMT	guanidinoacetate N-methyltransferase
ARHGEF5	Homo sapiens FKSG35 (FKSG35) mRNA, complete cds
STRN4	striatin, calmodulin binding protein 4
TMP21	transmembrane trafficking protein
SMARCD2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d
UROD	uroporphyrinogen decarboxylase
ZNF75	zinc finger protein 75 (D8C6)
TIA1	TIA1 cytotoxic granule-associated RNA binding protein
SNAPC2	small nuclear RNA activating complex, polypeptide 2, 45kDa
USP13	ubiquitin specific protease 13 (isopeptidase T-3)
SLC35D2	solute carrier family 35, member D2
RUNX1	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)
SFRS11	splicing factor, arginine/serine-rich 11
PAI-RBP1	PAI-1 mRNA-binding protein
GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)
SLC11A2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
TTL	tubulin-tyrosine ligase
E2F5	E2F transcription factor 5, p130-binding
NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha
TAL1	T-cell acute lymphocytic leukemia 1
C1orf16	chromosome 1 open reading frame 16
RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4
GAS2L1	growth arrest-specific 2 like 1
MGC15875	hypothetical protein MGC15875
PRKCSH	protein kinase C substrate 80K-H
FLJ10747	hypothetical protein FLJ10747
THAP11	THAP domain containing 11
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
LU	Lutheran blood group (Auberger b antigen included)
UBE2L6	ubiquitin-conjugating enzyme E2L 6
GSTM2	glutathione S-transferase M2 (muscle)
AKT1	v-akt murine thymoma viral oncogene homolog 1
DCTN1	dynactin 1 (p150, glued homolog, Drosophila)

Fig.19 Gene profiling of PKC activation in human malignant lymphocytes

To see the common pathways of PKC activation in lymphocytes, we use 3 different human lymphocytes – Jurkat (T-cell leukemia), DOHH2 (B-cell lymphoma), L428 and KMH-2

(Hodgkin's lymphoma) for microarray analysis. The cell lines are described in abbreviation letters: Jurkat (J), DOHH2 (D), L428 (L), and KMH-2 (K). Cells were treated with TPA for 4 h, untreated cells as control. This array represents approximately 4,551 human cDNA genes and most of them belong to oncogene and oncogene-related genes. The similar regulated genes (up and down regulated) were chosen by Self-Organizing Maps (SOM) or Self Organizing Tree Algorithm (SOTA) combining with visual inspection in TIGR Multiple Array Views and the data was expressed in Hierarchical Clustering (HCL).

Unlike calcineurin which have a specific target - NFAT, PKC activation have many down-stream effects, such as induce NF-κB and NFAT activation (Sun, Arendt et al. 2000; Pfeifhofer, Kofler et al. 2003), etc. Previous experiments showed that PKC activation through TPA enhanced the inducible short isoform NFATc1/αA transcription which controlled by P1 promoter (Fig. 12, 13 and 15). These indicated that PKC were not only involved in NFAT activation but also transcriptional control. A global view of PKC activation in lymphocytes will provide a frame work in understanding PKC activation related NFAT activity and gene transcription. In the experiments, we used microarray to analyse the PKC-involving gene transcriptions in 4 different malignant lymphocyte cell lines (Fig.19).

After PKC activation, transcriptions of NF-κB (NFkB1) and AP-1 (Fos, FosB and JunB) was found to be upregulated (Fig.19). Both are prominent TFs binding to the P1 promoter of NFATc1 (Fig.19). In addition, the increasing transcription of members RAS oncogene family (RAB1A and RAB5A), which upstream of MAPK kinase, also provide a potential ways in AP1 activation (Deng and Karin 1994) and stable mRNA by MAPK kinase (Wu, Hsu et al. 2003). A further prominent PKC target which appeared in our DNA microarray analyses was nuclear RNA export factor 1 (NXF1), which might be involved nuclear export of NFATc1. In sum of these, PKC activation could affect the NFATc1 gene transcription in multiple ways, such as by inducing TFs (NF-κB and AP-1) and general TFs (TFIIB and CDK7), by enhancing mRNA export proteins and, possibly, by the RNA stabilization of MAPKs.

As already mentioned before, NFAT and NF-κB proteins are the prominent transcription factors whose activity plays a crucial role in the activation, proliferation and apoptosis of lymphocyte (Serfling, Berberich-Siebelt et al. 2004). To consider their contribution to transcription of genes controlling cytokine and apoptosis, we checked the gene transcriptions level of NFAT and NF-κB family in microarray (Fig.20). For NFAT members, a high transcription level for NFATc3 and NFATc4 was detected, whereas human NFATc1 was at a low level transcription even upon

TPA induction alone. For NF-κB family, the transcription of Rel A and B was found to be highly constitive, especially for Rel A. A comparison between NFAT and NF-κB family members revealed that NF-κB had a dominant transcription in transformed human lymphocytes (Fig.20). The highly constitutive NFAT and NF-κB transcription indicated that those two family members were important regulator in lymphomagenesis (Rabson and Weissmann 2005).

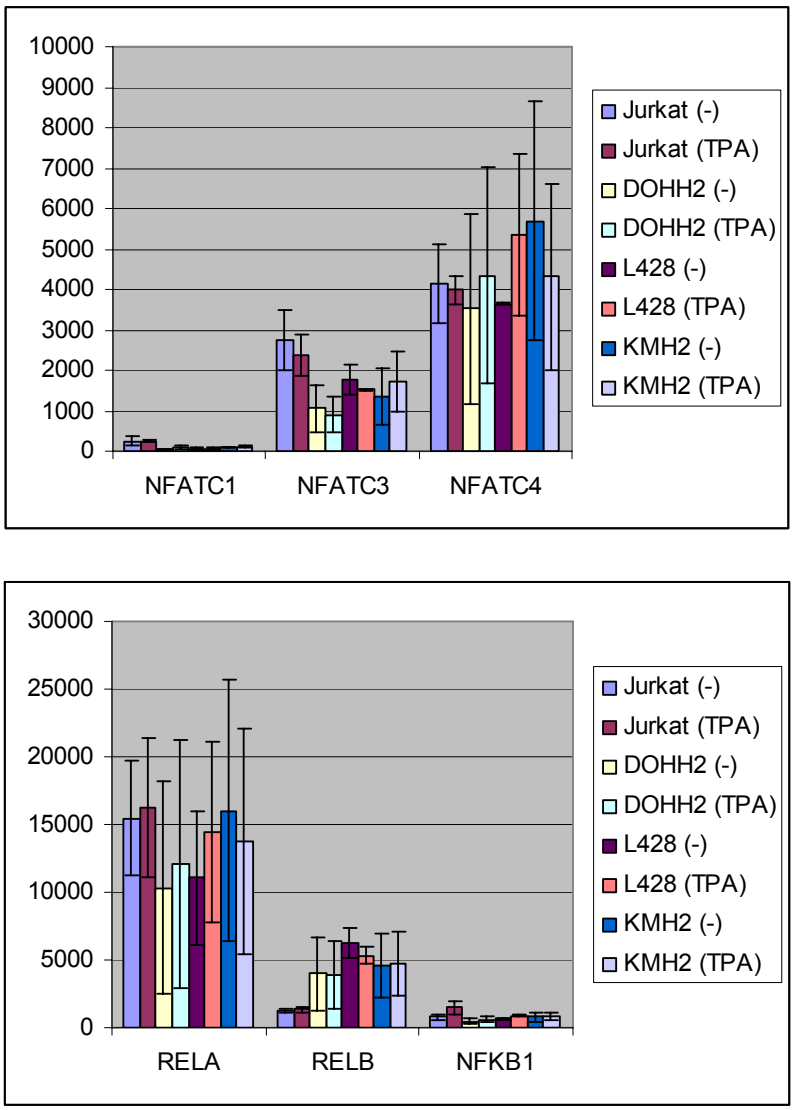


Fig.20 Transcriptional levels of NFAT and NF-κB in malignant lymphocytes
The gene Transcription levels are represented by C3 or C5 intensity from the microarray. We used ScanAlyze software to read the fluoresce intensity, and data was normalized before comparison (chapter 2.2.17). Data shown represent 2-4 individual experiment per condition – such as Jurkat (n=4), DOHH2 (n=3), L428 (n=2) and KMH2 (n=3).

3.6 NFATs and p53 in lymphomas

Several reports indicated that NFATs control the expression of pro-apoptotic genes, such as the CD95 ligand, nur77 and TNF-α gene. Unpublished data of our laboratory also revealed that NFATc2 suppresses anti-apoptotic Bcl-2 gene. The pro-apoptotic activity of NFATc may favor tumor suppression of lymphoid cells (Serfling, Berberich-Siebelt et al. 2004). NFATs also control early immune response genes such as cytokines. In the peripheral T cells, NFATc1/A expression induced upon lymphocyte activation. These may favor tumorigenesis. Thus, EL-4 lymphoma T cells showed a constitutive expression of inducible NFATc1 α/A without any stimulation (Fig.16).

In the proximal region of the P1 promoter, there are two p53 binding sites in the conserved region 5 (proximal homology of P1 promoter) (Fig.18B). *p53* deficient mice showed an increase in tumor incidence, the most common type of tumor is T-cell lymphoma (Donehower, Harvey et al. 1992; Harvey, McArthur et al. 1993; Philipp-Staheli, Kim et al. 2004). In *p53* and *decorin* double knockout mice, 95% of double homozygotes developed thymic lymphomas (Iozzo, Chakrani et al. 1999). We assume that p53 is a master control factor in T cell lymphomagenesis. To establish a good model for $p53^{-/-}$ T cells lymphomas, a new T cell lymphoma line was established from fresh lymphomas of *p53*-deficient mice in our laboratory, designated as p53T. According to the reports, a 20 kb of sequence that included exons 1-6 and a large undefined upstream region was deleted in *p53*-deficient mice (Donehower, Harvey et al. 1992). The established cell line was homogeneous according to the cell and DNA content (Fig.21A and B). The cell phenotype was $CD8^+$, $TCR\beta^+$ and $CD3^-$, and the cells showed an immature T cell phenotype (Fig.21C and D).

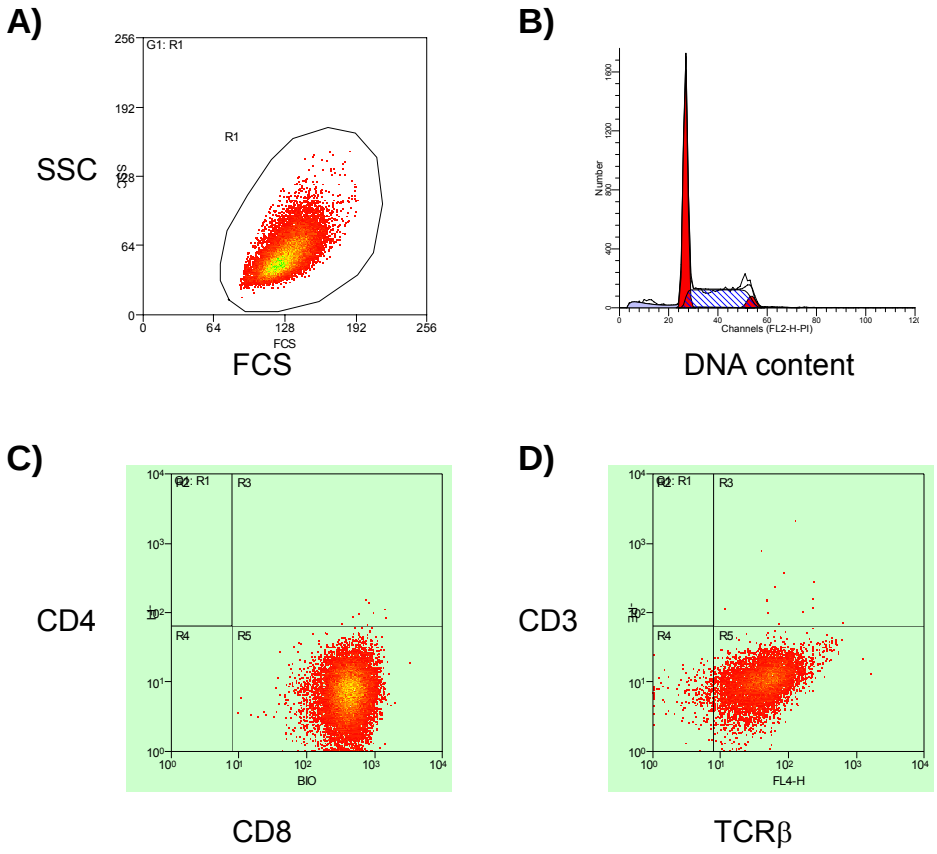


Fig.21 Characteristics of p53T T-cell lymphoma
A) The cell content was measured with FCS and SSC by flow cytometry. B) The DNA content of p53T cells was measured by flow cytometry, and propidium iodide was used for this purpose. The cell phenotype was identified by using antibodies: C) CD4-APC and CD8-PE, D) CD3-APC and TCRβ-PE respectively.

To uncover a possible role of NFATc factors in T cells lymphomas, NFATc protein expression in T-cell lymphomas and leukemia were investigated by western blotting. In murine T lymphoma cells (p53T) there was a high NFATc1 protein expression and activation, as manifested in a predominant of NFATc1 localization in the nucleus (Fig.22A). When we studied NFATc1 concentration in the same amount of human Jurkat T leukemia cells as in murine EL-4 and p53T lymphoma cells, we were unable to detect any NFATc1 expression. In contrast, NFATc2 in Jurkat cells was expressed at the same level as in EL-4 and p53T cells.

In considering two p53 binding sites within the P1 promoter region of NFATc1 (Fig 18), it is interesting to gain more insights into a role of p53 in NFATc1 transcription. Various P1-luciferase constructs containing p53 binding sites were co-

transfected with a vector expressing p53 into EL-4 lymphoma cells. As shown in Fig.22B, expression of p53 exerted a suppressive effect on the TPA+ionomycin mediated induction of luciferase reporter genes driven either by a short P1 (0.8 kb) or a long P1 (5.5 kb) NFATc1 promoter. This indicated that p53 has a suppression effects on the activity of the murine NFATc1 P1 promoter. Further experiments showed that p53 also inhibited the induction of a luciferase gene driven by multiple NF-κB sites (Figs.22C and 22D), whereas the induction of a reporter gene (IL-2) driven by multiple NFAT binding sites remained unaffected (data unshown).

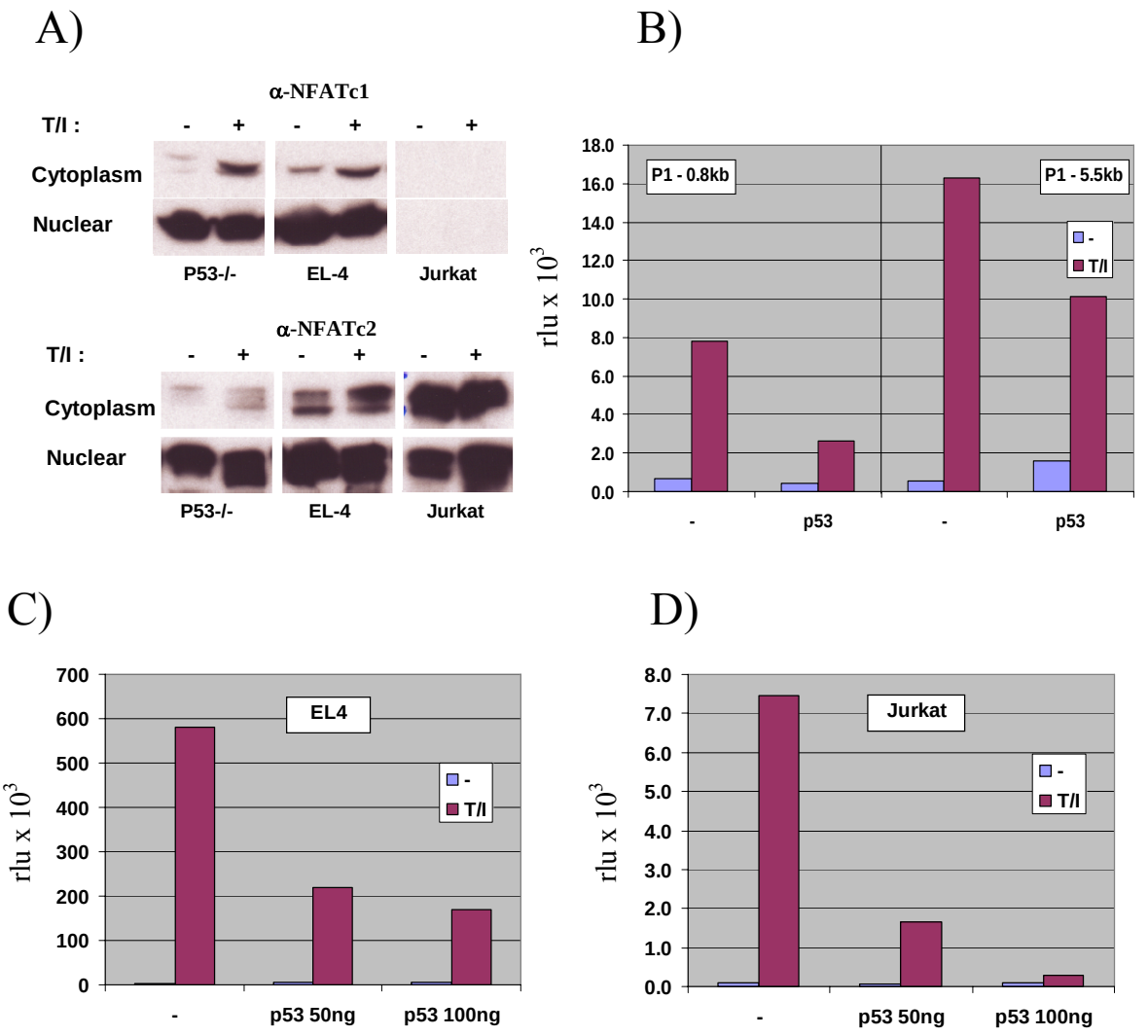


Fig.22 p53 suppress NFATs activation in T-cell lymphomas
A). Western blotting indicating NFAT expression levels in lymphoma and leukemia cells. As indicated, 3 different cell lines were used. A *p53*$^{-/-}$ cell line was derived from lymphoma of *p53*-deficient mice. After T/I induction for 4 h, cytoplasmic and nuclear proteins were isolated for western blotting. Two antibodies were used in this experiment for detection; α-NFATc1 (top panel), α-NFATc2 (lower panel). B). p53 inhibits the murine NFATc1 P1

promoter. Luciferase vectors driven by P1 promoter of different size were co-transfected in EL4 T cells with a p53wt expression vector (100ng), T/I induction for 4 h. C) and D). p53 inhibit NF-κB activity. A luciferase vector driven by 7 x NF-κB binding segment was co-transfected with 50 ng and 100ng DNA of p53 expression vector. Two cell lines, EL4 and Jurkat cells, were used as indicated. One representative experiment out of three was shown here.

3.7 Enhanced NFATc1 transcription in Gadd45α-deficient Jurkat T cells

After PKC activation, a prominent increasing molecular in all lymphocytes was Gadd45α (Fig.18). It has been reported before that Gadd45α was a negative regulator of T cell activation. Compared to wild-type cells, *Gadd45α*$^{-/-}$ T cells have a lower threshold of activation and proliferate to a greater extent following primary T cell receptor stimulation. In addition, *Gadd45α*$^{-/-}$ mice develop an autoimmune disease, similar to human systemic lupus erythematosus (SLE), characterized by high titers of anti-dsDNA, anti-ssDNA, and anti-histone autoantibodies, severe hematological disorders, autoimmune glomerulonephritis, and premature death (Salvador, Hollander et al. 2002). Combined with the facts that NFATs are gate keepers in T cell activation (Serfling, Berberich-Siebelt et al. 2000; Peng, Gerth et al. 2001), Gadd45α may regulate T cells activation through NFAT activation or transcription.

To elucidate a possible relation between Gadd45α and NFATc1 upon T cell activation, we established Jurkat T cells (JSRG7) in which Gadd45α expression was knocked down by stably transfected Gadd45α siRNA expression vector and JRG cells (transfected with emptor vector pSUPER.retro.GFP) as control. Results indicated that more than 80% of transfected Jurkat T cells expressed the Gadd45α siRNA (Fig.23A). Northern blotting proved that TPA-induced increase of Gadd45α transcription is abolished in the established Jsrg7 cell line, whereas no effect was seen in Jurkat T cells transfected with the emptor vector control (Fig.23B). Since p53 deficient cells still showed an increasing Gadd45α transcription (Fig.23B), it indicated that PKC activation induced Gadd45α transcription in a p53-independent pathway. Without induction, Jsrg7 show an increasing of constituting long NFATc1/B

or C isoform transcription compared to JSR control cells. When stimulated by TPA/ionomycin, the inducible short NFATc1α/A isoform was strongly induced in Gadd45α-siRNA Jurkat T cells. >100 times increase in NFATc1/A induction was observed (Fig.22c, d). This result indicates that Gadd45α have a suppressing effect on NFATc1 transcription.

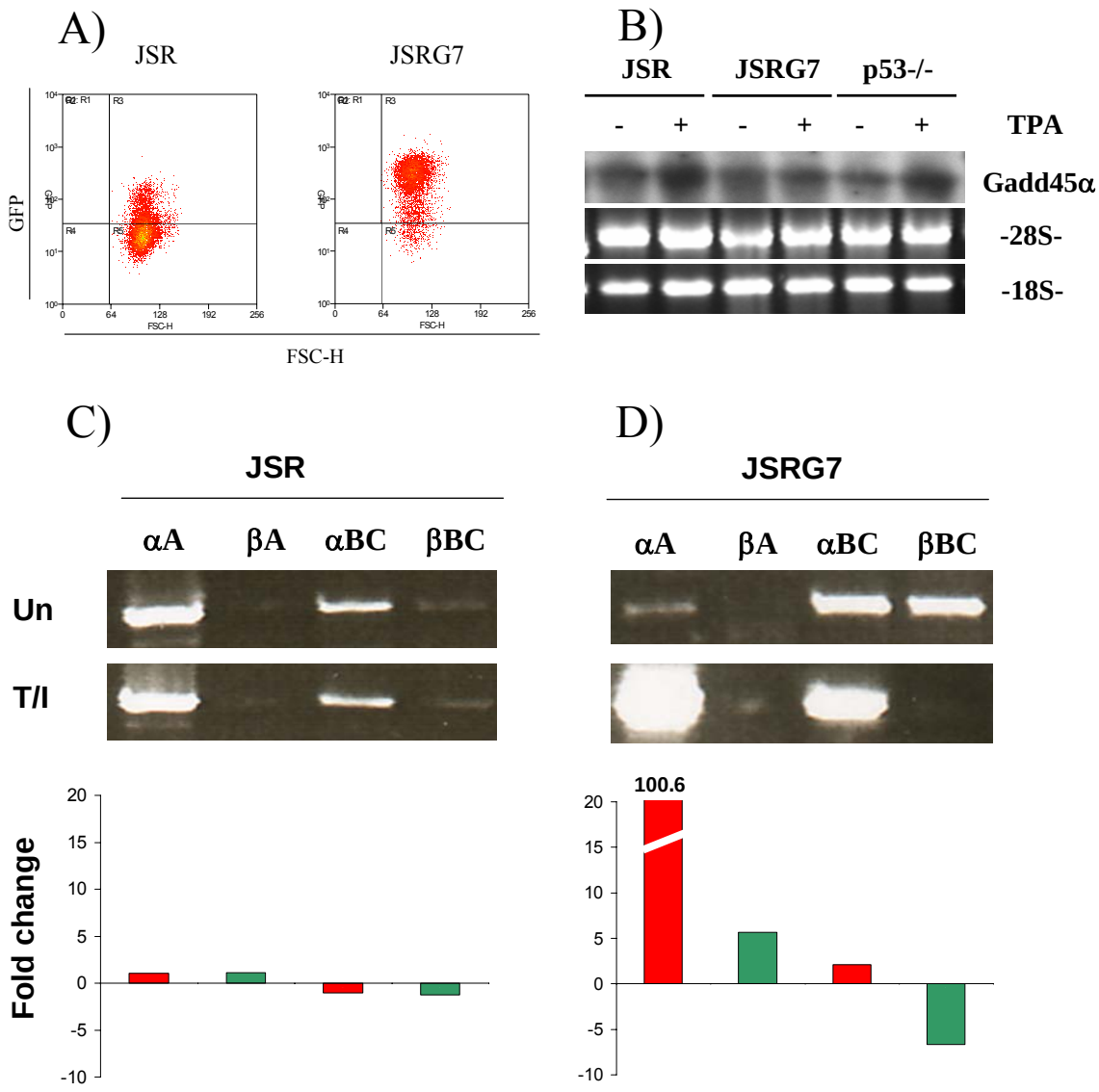


Fig.23 Increasing NFATc1 transcription in Gadd45α deficiency T cells
a) Gadd45α-siRNA T cells were established by stable transfection with the pSRG vector (Gadd45α siRNA expression vector), and an empty vector pSUPER.retro.GFP (pSR) as control, transfection efficiencies were indicated by GFP expression. b) Northern blotting of Gadd45α expression. Gadd45α transcription induced by TPA was suppressed in Jurkat T

cells with stable expression pSRG (pSRG7 clone), and this effect was not seen Jurkat T cells transfected with an empty vector (pSR clone). c) And d) NFATc1 transcriptions were checked by semi-quantitative long-distance PCR, and fold change of different NFATc1 isoform transcriptions represented by intensity of T/I stimulation in comparison to intensity of un-stimulation. Red boxes represent isoforms controlled by the inducible P1 promoter (α), blue box correspond to P2 promoter directed isoforms (β).One representive result was shown here.

Before the Gadd45 family proteins were established as regulators of T cell function and immunity (Lu, Yu et al. 2001; Nakanishi 2001; Morinobu, Gadina et al. 2002; Salvador, Hollander et al. 2002; Amanullah, Azam et al. 2003; Chi, Lu et al. 2004), several lines of evidence indicated that Gadd45 members were a p53-target genes involved in cell cycle regulation and DNA-damage repair (Kastan, Zhan et al. 1992; Smith, Chen et al. 1994; Hollander, Sheikh et al. 1999; Li, Ting et al. 2000; Tran, Brunet et al. 2002). Besides suppressing NFATc1 transcription, the question arises whether Gadd45α also regulates T cell activation and T cell cycle progression. In wild type Jurkat T cells, PKC activation mildly induces cell cycle arrest in G2 phase, and no sign of apoptosis was observed before/or after stimulation (Fig.24). For the Gadd45α-deficient T cells (JSRG7), cell cycle arrest in G2 phase by TPA was not observed any more, it imply Gadd45α are correspond to G2 arrest induced by PKC activation. Unexpectedly, a higher sub-G1 phase was observed in JSRG7 cells comparing to wild type Jurkat cells, and that PKC activation suppressed the formation of sub-G1 phase (Fig.24). These findings establish Gadd45α may also take part in T cells activation and proliferation though cell cycle regulation and apoptosis control, which coordinated with the level of NFATc1 transcription suppressed by Gadd45α.

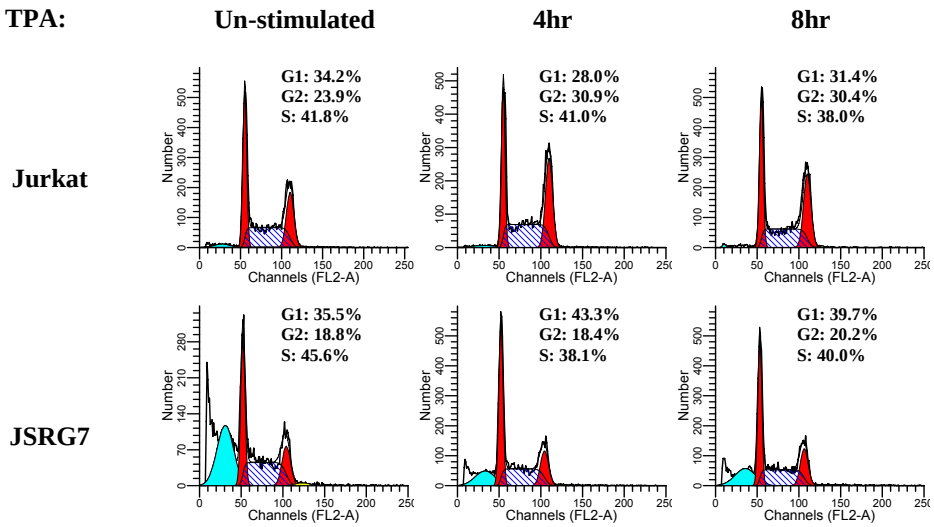


Fig.24 Cell cycle progression of Jurkat T cells regulated by Gadd45α
To check cell cycle regulation, TPA-treated or untreated cells at a given time were stained with propidium iodide; ModFit LT^{TM} was used for cell cycle analysis. JSRG7 – a Gadd45α deficiency Jurkat T cells. One representative result is shown.

3.8 Targeted disruption of the NFATc1 Gene

The 6 different isoforms controlled by two promoters and poly A sites can be divided into two groups, inducible isoforms governed by the P1 promoter, and the constitute isoforms governed by the P2 promoter. Many lines of evidence support that the NFATc1 α and β have different functional roles in T cells. NFATc1-deficient mice show defects in the development of cardiac valves and septa, and die in uterus before day 14 of gestation (de la Pompa, Timmerman et al. 1998; Ranger, Grusby et al. 1998), precluding analysis of the role of NFATc in lymphocyte activation. By using RAG-2-deficient blastocyst complementation, the generated NFATc1- “null” T cells are a good model to investigate NFATc1 function in T lymphocytes, but this pan-deletion of all isoforms of NFATc1 could not be used to study the function of specific isoforms in T cell maturation and activation. Also the degree of chimerism in nonlymphoid organs and tissues affected the results observed in vivo. In trying to elucidate the function of individual NFATc1 isoforms in T cells and its transcriptional regulatory properties, we designed conditional knockout vectors for the conditional inactivation of the NFATc1 gene. 3 different vectors were constructed, targeting the P1 promoter, P2 promoter and enhancer.

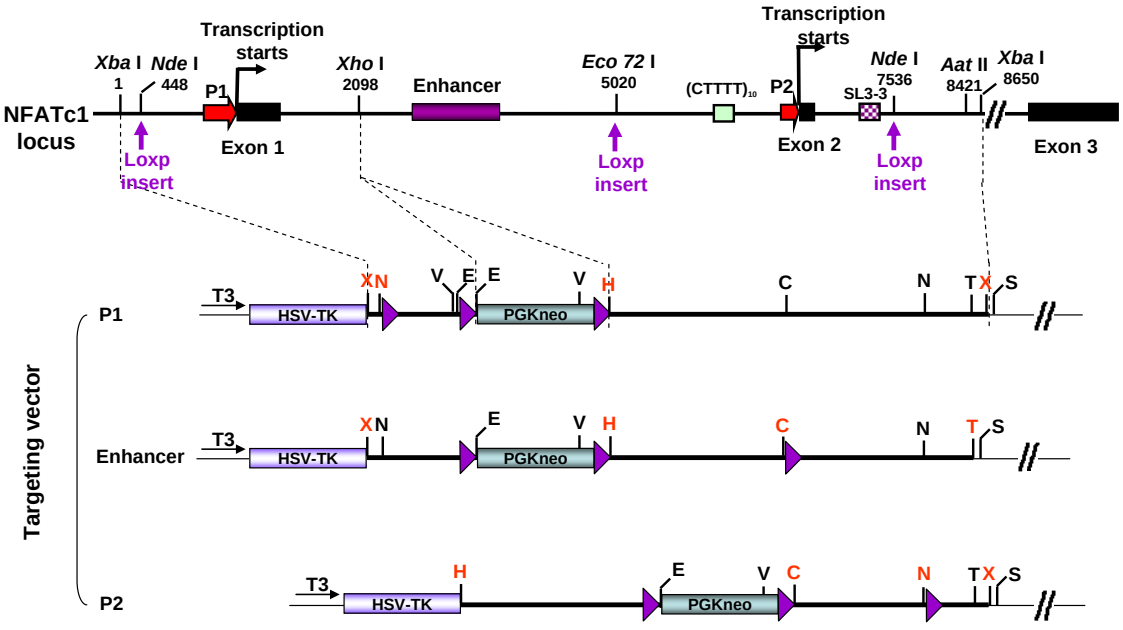


Fig.25 Targeting vectors for the conditional deletion of the murine NFATc1
The pGKTKNeoloxp plasmid containing two selection cassettes was used for constructing. The inserted left arms and right arms are taken from the 8.65Kb *Xba I* DNA fragment harboring the two promoters P1 and P2. According to the target deletion position, loxP sites (purple color) were inserted into 3 different positions as indicated. In the targeting vector, the rough dash represents the inserted DNA, the slim dash represents the plasmid DNA and triangles indicate loxP insertion. The following restriction enzymes for indicating position are described in abbreviation letters: *Xba I* (X), *EcoR V* (V), *EcoR I* (E), *Xho I* (H), *Eco72 I* (C), *Aat II* (T), *Sal I* (S). Since all inserted fragments were blunt ended, the red color shows positions of restriction enzymes only for indicating insertion.

To construct NFATc1 targeting vectors, the 5´ end of the mouse NFATc1 cDNA was used to isolate genomic cosmid clones of the NFATc1 gene. This 8.5kb *Xba I* fragment harboring two promoters and one enhancer were characterized by DNase I hypersensitive sites (HSS) (Chuvpilo, Jankevics et al. 2002). The blunt left arm (from *Xba I* to *Xho I*) and right arm (from *Xho I* to *Xba I*) were subcloned into the pGL3 basic Sma I site. The vector containing in the left arm was designated as p16, the right arm vector was designated as p55 (chapter 2.1.7.3). To get the loxP

insertion within the left or right arm, loxP fragments cut from the pBS112 SX vector were inserted into the *Nde I* (448bp), *Eco72 I* (5020bp) and *Nde I* (7536bp) sites, respectively. The corresponding vectors were designated as p16N-loxp, p55E-loxp and p55N-loxp (chapter 2.1.7.4). The pTKNeoloxp plasmid containing the neo gene for positive selection and thymidine kinase gene (TK) for negative selection was used as basic vector in constructing the targeting vector (chapter 2.1.7.5). Before constructing the final targeting vector, the following pre-targeting vectors had to be constructed: pKN1, pKN2, pKN3 and pKN4 (chapter 2.1.7.7). To delete the P1, P2 promoter and enhancer from the NFATc1 gene, we constructed 3 different targeting vectors, which were designated as pKNF1, pKNF3 and pKNF4 respectively (chapter 2.1.7.8). The whole strategy is indicated in picture (Fig.25)

To screen for positive ES clones, southern blotting and PCR assays were used according to the following strategies as indicated (Fig.26). We designed 2 probes for the 5`- or 3´- termini that are specific for NFATc1 gene sequences outside of the targeting vector. This will detect insertions of sequences at the correct endogenous site rather than at random sites. In *EcoR I* and *EcoR V* digested genomic DNA, both probes hybridized with a 14.7 kb region in the wild type allele. For the 5' probe, the targeting event in P1 inserted two pink loxP flanking with the blue neo containing the restriction site, creating a 3.9 kb band (Fig.27). For the 3' probe, the targeting event created an 11-kb band. In P2 and enhancer targeting events, there are similar bands creating when hybridized with 5´- or 3`- probe, the new bands were indicated as shown (Fig.27).

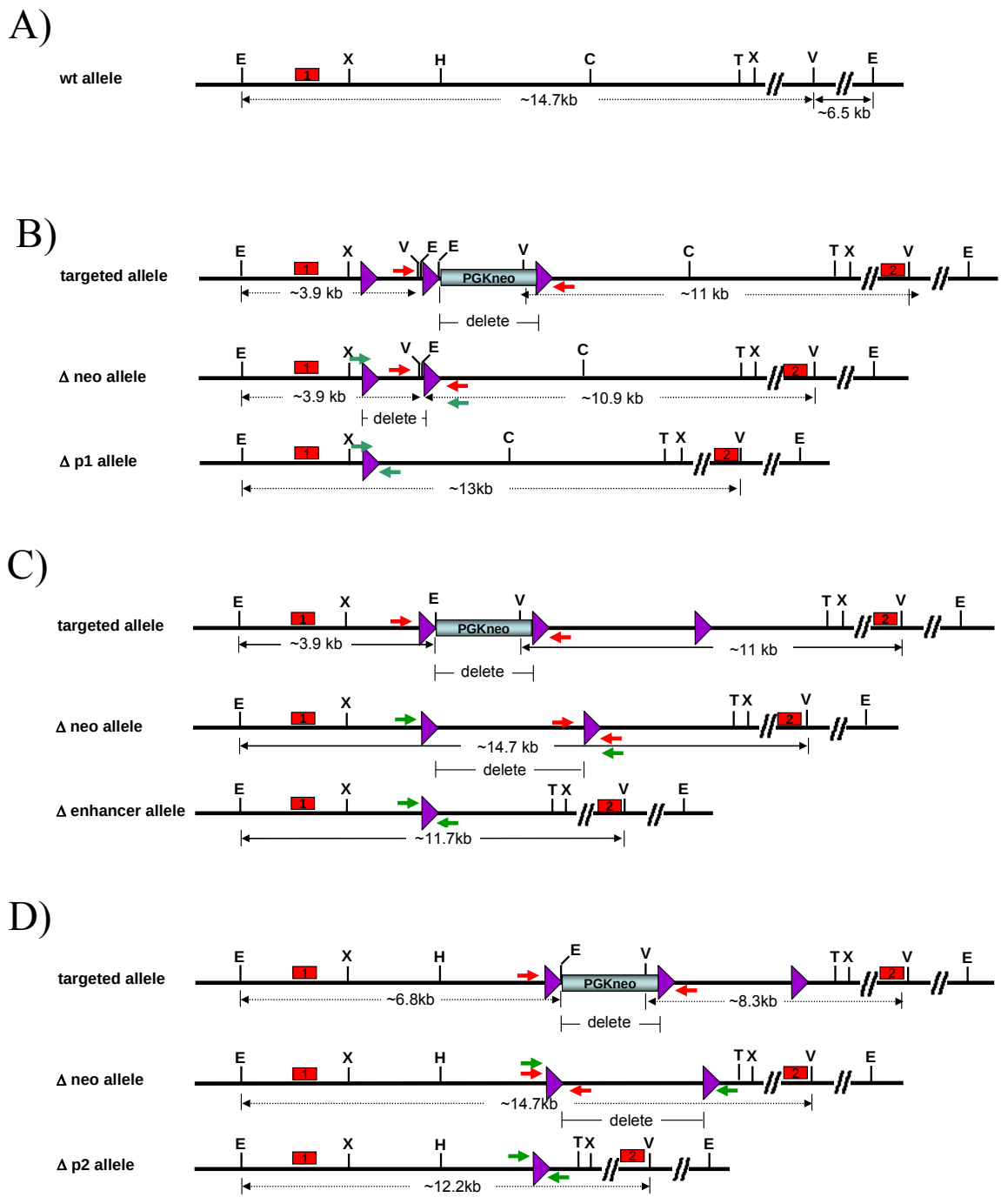


Fig.26 Strategy for conditional deletion of the murine NFATc1
A) wild type allele (wt allele), B) targeted alleles for the conditional deletion of promoter 1 and exon 1 region. C) Targeted alleles for the conditional deletion of enhancer region. D) Targeted alleles for the conditional deletion of promoter 2 and exon 2. To the conditional deletion of different NFATc1 regions, targeting vectors were transfected into ES cells, screening for the homology recombination clone. Triangle represent loxP insertion, the following restriction enzyme are marked as abbreviations in capital letters: *Xba I* (X), *EcoR V* (V), *EcoR I* (E), *Xho I* (H), *Eco72 I* (C), *Aat II* (T), *Sal I* (S).). All sites are shown for EcoR

I and EcoR V, the other restriction enzyme sites only for the indicated position. Targeting procedure will generate 3 different targeting allele for each targeting vector. First, the targeted allele coming from the insertion of targeting vector and wt allele homology recombination which contains 3 inserted loxP sites and neo DNA. Second, Cre-mediated recombination between the two loxP sites flanking the neo genes results in the generation of a neo deleted allele (Δ neo allele). Third, Cre-mediated recombination between the most 5' and 3' loxP sites creates the final targeted allele (Δ p1, Δ p2, Δ enhancer allele). In southern blot, EcoR I and (or) EcoR V – digested DNA was hybridized with the external probe (red box) and the sizes of the wild-type and targeted fragments are indicated. PCR primer pairs were indicated by same color arrows with different directions.

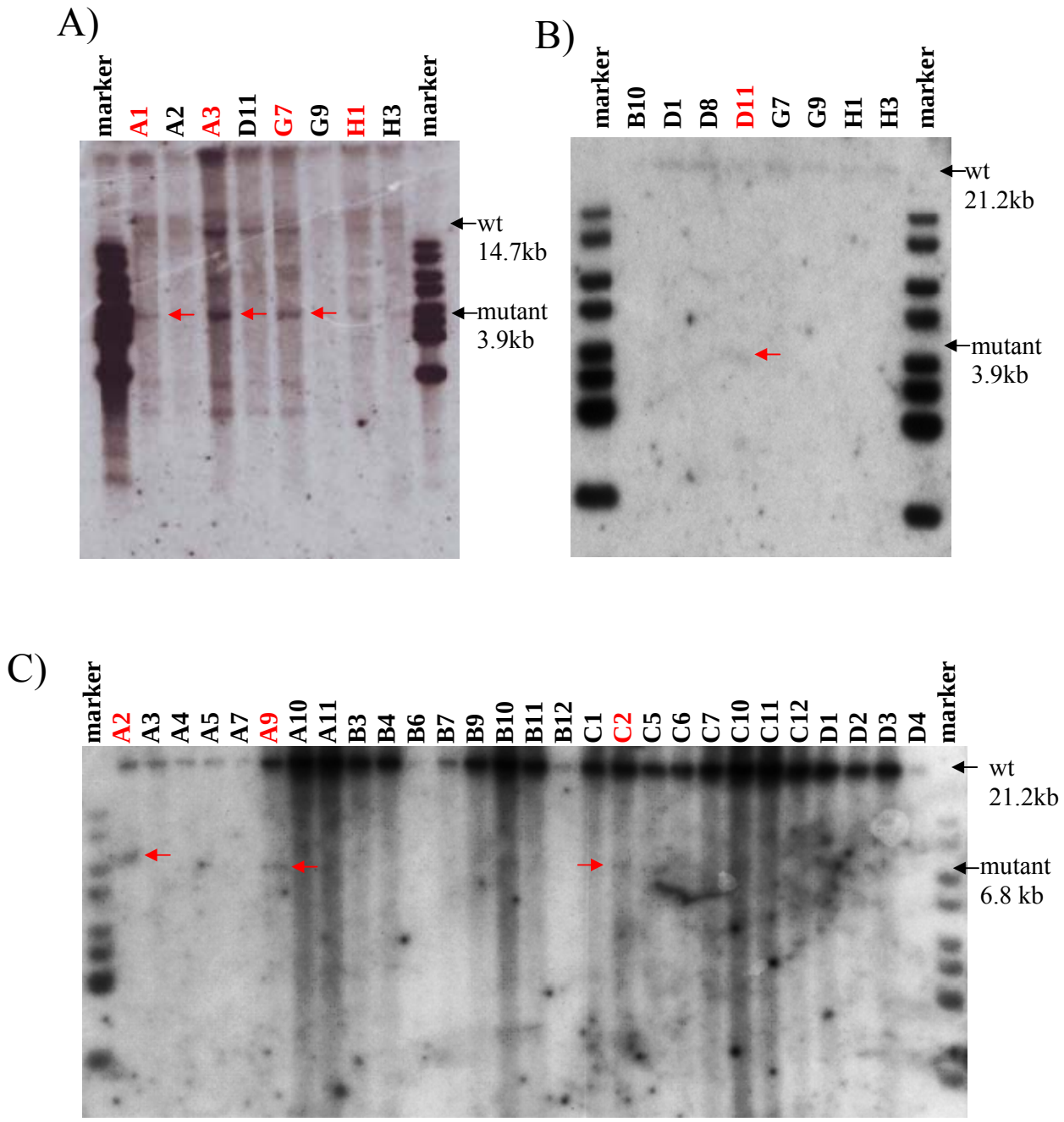


Fig.27 Southern blot analysis of the primary targeting event (5´- terminus)
For southern blotting, *EcoR* I and (or) *EcoR* V digested DNA was hybridized with an external (5') probe. 1kb marker (Fermentas) with 14 DNA fragments (in bp): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250. The sizes of wild-type and target fragments are indicated. The candidate clones were pointed out by arrows (red). The smaller (target) band is indicative for the presence of site-specific insertions. A) Promoter 1 and exon 1, genomic DNA digested by *EcoR* I and *EcoR* V. B) Enhancer, genomic DNA digested by *EcoR* I. C) Promoter 2 and exon 2, genomic DNA digested by *EcoR* I.

To further delete the neo gene, the positive ES cells were transfected with pMC-Cre expression vector (Gu, Zou et al. 1993), and clones with neo deletion were identified by southern blotting and PCR. To decrease the neo gene interfering NFATc1 gene transcription, we prepared neo deletion of P2 and enhancer targeted clone, the same strategy were used in screening ES cells (Fig.28). The neo-deletion (Δneo) targeted clones have two loxP flanking of targeted region, it were used for blastocyst injection. The generated chimera homozygous mice were confirmed by fur color and PCR. For clarity, we will refer to these deletions as Δp1, Δp2 and Δenhancer throughout this paper.

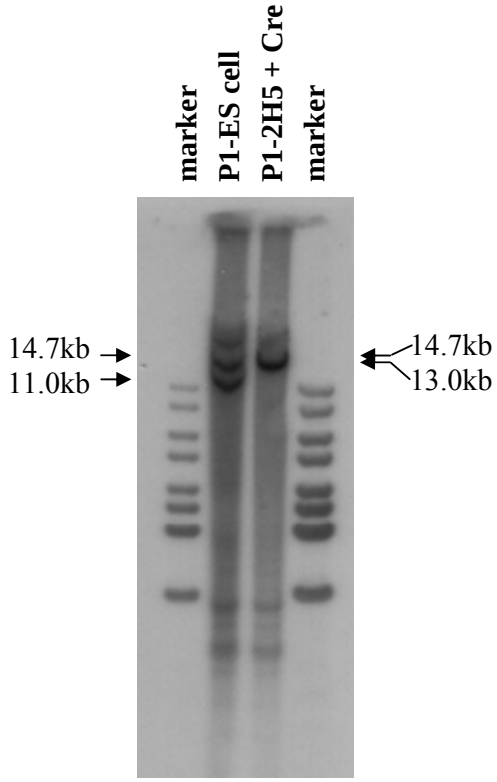


Fig.28 Southern blot analysis of the primary targeting event (3´- terminus)
For southern blotting, genomic DNA from promoter 1 targeting clone was digested by *EcoR* I and *EcoR* V and hybridized with an external (3') probe. 1kb marker (Fermentas) with 14 DNA fragments (in bp): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, and 250. The sizes of wild-type and target fragments are indicated as arrow. Lane 2) promoter 1 candidate clone. Lane 3) promoter 1 candidate clone treated by Cre.

CHAPTER IV - DISCUSSION

The triggering of lymphocytes through their TCR and BCR complexes plays a crucial role in the activation of NFAT and NF-κB factors which are involved in a variety of signaling responses. These two TFs synergistically play a crucial role in the activation, proliferation and apoptosis of lymphocytes (Serfling, Berberich-Siebelt et al. 2004). While NF-κBs controls both innate and adaptive immune responses, NFATs control mainly the adaptive immune system. The close link between the activity of NFATs and T cell function indicates the need for a detailed characterisation of NFAT gene regulation in individual T cell subsets.

Different members of a family of transcription factors often exert overlapping functions. This functional redundancy is important in evolution to guard against genetic accidents such as loss or mutation of essential genes. In mammals, the family of genuine NFATc transcription factors includes 4 proteins, NFATc1-c4 (Fig.6). NFAT factors appear to be synthesized in several isoforms which can differ in both their N- and C-terminal peptides (Serfling, Berberich-Siebelt et al. 2000). In particular, NFATc1 is synthesized in multiple isoforms which differ markedly in the length of their C-termini but also in the N-terminal peptides (Chuvpilo, Avots et al. 1999; Chuvpilo, Zimmer et al. 1999).

NFATc1 and NFATc2 are the most prominent NFAT factors in peripheral T cells. They overlap in their function but differ remarkably in the mode of expression (Serfling, Chuvpilo et al. 2006). A detailed understanding of the precise physiological roles served by the NFATc1 transcription factors in this process is complicated by functional redundancy and temporal changes in nuclear expression of different NFATc1 isoforms. For NFATc1, the generation of multiple isoforms is controled by two different promoters and two alternative polyadenylation sites. In searching for NFATc1 gene transcript variants and proteins from the NCBI library, we found six isoforms which correspond to our experimental data (Fig.11).

Our laboratory has shown before that in contrast to the constitutively expressed NFATc2 and NFATc1/βC, the inducible NFATc1/αA was unable to promote apoptosis of T effector cells (Chuvpilo, Jankevics et al. 2002; Serfling, Chuvpilo et al. 2006). This raises an intriguing question regarding the roles of multiple NFATc1 proteins in T cell function: how does NFATc1 transcription switches from the long isoforms to the short isoforms? Among the three NFATc proteins expressed in immune cells, the synthesis of NFATc1 is strongly induced by T cell receptor (TCR) and co-receptor signals in effector T cells. Regarding the central roles of NFATc1 in the control of T cell activation and function (Chow, Rincon et al. 1999; Serfling, Berberich-Siebelt et al. 2000; Peng, Gerth et al. 2001), in this thesis we tried to uncover gene regulatory mechanisms controling NFATc1 transcription in T cells.

In the present study, we have identified the induction properties of six NFATc1 isoforms in primary T cells and T lymphoma cells by using an improved semi-quantitative PCR technique. In line with the former results obtained in western blotting assays, we show that the generation of the short isoform NFATc1/αA is controlled by the promoter P1 and the proximal poly A site, pA1, and, therefore, highly inducible upon T cell activation. Whereas the constitutive transcription of the long isoforms NFATc1/βB and βC (and of the short ßA isoform) is controlled by the promoter P2 and the distal poly A site, pA2, their transcription becomes downregulated after induction (Fig.12,13,15). In searching for the putative TFs controling NFATc1 gene transcription, we found that the TFs binding to the NFATc1 promoters differ remarkably between the P1 and P2 promoters. Prominent TFs binding to the sequence motifs of P1 include CREB, ATF-2, Fos, Sp1/Sp3, NF-κB and NFAT factors, and probably more. In contrast, no inducible factors, like CREB, NF-κB or NFAT factors, were found to bind to the P2 promoter which is mainly recognized by Sp1/Sp3 and Octamer factors. These differences in factor binding might explain the marked differences in activity between both promoters in resting and activated T lymphocytes.

Although PKC activation is well known to contribute to NFAT activation, the presice mechanisms and the possible roles of PKCs in NFATc1 gene transcription are still largely unknown (Rao, Luo et al. 1997; Serfling, Chuvpilo et al. 2006). To clarify the overall function of PKCs in NFATc1 transcription, we systematicly

evaluated TFs and other signaling molecules induced by PKC by using microarray assays. We found that PKC activation led to the induction of both positive and negative regulators of NFATc1 gene expression. Positive regulators are the NF-κB, AP-1 (Fos) and EGR factors. In addition to these promoting factors, we found several suppressor factors which are activated by PKC, such as Gadd45α. Moreover, we identified p53 as a suppressor of the NFATc1 gene transcription. So far, no suppressors of NFATc1 gene transcription have been identified. Since several of these factors, e.g. NF-κB, c-Fos and p53, play an important role in carcinogenesis, one may speculate that NFATc1 expression is involved in generation of T cell lymphomas.

4.1 Induction properties of NFATc1 in T cells

Our present work provides direct support for the synthesis of six NFATc1 RNAs which are differently expressed in specific T cell subsets and dependend on the activation state of cells.

To determine the induction properties of NFATc1 in primary T cells upon different activations, we have isolated naïve CD4+ T cells, which were treated (stimulated or re-stimulated) by a variety of specific stimuli. In agreement with the experimental data published previously by our laboratory (Chuvpilo et al., 1999, 2002), we also have shown that all isoforms directed by the P1 promoter were upregulated in effector T cells (Figs. 12b, 12c, 12d), i.e. in differentiated T cells. In contrast, all β isoforms whose synthesis is directed by the P2 promoter correspond to the most prominent NFATc1 transcripts in untreated naïve and resting T cells (Figs.12a, 12d and 13). These differences in induction properties between the P1(=α) and P2 (=ß) transcripts imply that these isoforms may play a different role in gene control in T lymphocytes.

The C termini of the short and long NFATc1 proteins comprise either a short C-terminal peptide of 19 aa (in A isoforms), or longer peptides of 128 aa (in the B isoforms) or 246 aa (in the C isoforms: see Fig. 11). The long C-terminal peptide of 246 aa which is missing in the short NFATc1/A proteins but present in the long NFATc1/C proteins harbors a transactivation domain, which we have designated as TAD-B (Chuvpilo, Avots et al. 1999). This TAD-B appears to be markedly weaker

than the strong N-terminal transactivation domain, TAD-A, that is common to all NFATs (Chuvpilo, Avots et al. 1999; Chuvpilo, Jankevics et al. 2002). This suggests different functional roles of individual isoforms in their transactivation capacity, such as in promoting the CD3 Ab-mediated AICD of murine T cells by NFATc1/C, whereas no effect on AICD was observed for the short NFATc1/A isoform missing TAD-B (Chuvpilo, Jankevics et al. 2002).

The identification of TAD-B in NF-Atc1/B+C raises the question of which roles these longer isoforms play in gene control in T lymphocytes. It has been reported that NFATc1/B proteins do not participate in transcriptional induction of the TNF-α gene, while they still activate IL-2 gene transcription in a CsA-sensitive manner (Park, Takeuchi et al. 1996). Chimeric RAG-$1^{-/-}$ mice bearing NFATc1-deficient lymphocytes showed reduced numbers of thymocytes and an impaired proliferation of peripheral lymphocytes suggesting a role for NFATc1 in regulating T cell growth and apoptosis, potentially through the regulation of the CD40L and FasL genes (Yoshida, Nishina et al. 1998). This implies that a constitutive expression of NFATc1 is required for maintaining T cell homeostasis. We observed a constitutive expression of all β isoforms in resting and naïve T cells (Figs.12, 15 and 16). In particular, the βB+C isoforms were found to be constitutely expressed at relatively high concentrations in resting T cells (Fig. 12) suggesting an important role for these β isoforms in maintaining T cell homeostasis.

Optimal T cell activation is determined by at least two signals, the primary signal provided by the antigen-receptor complex (TCR/CD3) and the second signal provided by costimulatory receptors (e.g. CD28). Engagement of the CD28 co-receptor, either by its ligand on APCs, B7-1 (CD80) or B7-2 (CD86), or by Abs can strongly enhance TCR signal responses. Importantly, blocking CD28 engagement inhibits T cell activation and may result in T cell anergy (Harding, McArthur et al. 1992; Turka, Linsley et al. 1992). Surprisingly, studies of mice that lack expression of CD28 demonstrated that significant residual T cell function exists in the absence of CD28 (Shahinian, Pfeffer et al. 1993; Green, Noel et al. 1994). Although T cells from CD28-deficient mice have decreased overall responses, they are still capable of mounting significant responses to mitogenic stimuli, and are capable of strong responses against transplanted tissues and some viral infections (Lin, Rathmell et al. 1998). Mice lacking CD2 or CD28 show only mild deficits, demonstrating that neither

protein is essential for T cell activation. In mice lacking both CD2 and CD28, T cells from these mice have a profound defect in activation by soluble anti-CD3 Ab and Ag, yet remain responsive to immobilized anti-CD3. This suggests that CD2 and CD28 may function together to facilitate interactions of the T cell and APC, allowing for efficient signal transduction through the TCR (Green, Karpitskiy et al. 2000). Thus, optimal T cell activation requires co-engagement of the TCR with accessory and costimulatory molecules, but CD28 is not absolutely required for T cell function. However, the exact mechanism of costimulatory function is still unknown.

Numerous data indicate that CD28 co-stimulation does not directly affect NFAT activation. However, it was believed that CD28 may enhance NFAT activity by increasing AP-1 activity, one of the nuclear components of NFAT transcription complexes. CD3 stimulation alone does not elicit AP-1 binding with NFAT to form productive NFAT+AP-1 transcription complexes. Instead, sole CD3 stimulation is one of the mechanisms leading to T cell anergy (Macian et al. 2002). Moreover, CD28 stimulation appears also to play a minor role in NFATc1 induction. Thus, no differences have been detected between immobilized CD3 Abs alone or in combination with CD28 Abs (Fig.13). This is in line with a view that CD28-mediated signals are not absolutely necessary for NFATc1 induction and, probably, T cell function.

The immunosuppressant CsA interferes with immune responses by inhibiting the evolutionary conserved calcineurin signal transduction pathway. CsA binds to its intracellular receptors, the immunophilins, thereby creating composite surfaces that block the activity of the specific target calcineurin. Inhibition of the action of calcineurin results in a complete block in the translocation of cytosolic NFAT proteins and in a failure to activate the genes regulated by NFAT factors. Does CsA also affect NFATc1 induction? In our former western blotting results, CsA showed a strong inhibition of NFATc1/A expression, but not of expression of long isoforms NFATc1/B+C (Chuvpilo, Jankevics et al. 2002). We show here that the induction of NFATc1/αA was completely inhibited by low doses of CsA when induced by ionomycin alone (or, to a lesser degree, together with TPA). But PKC activation, e.g. mediated by T+I co-stimulation, led to a strong induction of NFATc1/αA synthesis (Fig.15B). This suggests that PKC-mediated signals also contribute to P1 promoter induction. This view is supported by the composition of regulatory DNA sequence

elements within the P1 promoter which is recognized by several factors induced by PKC signals (Fig.18). In contrast, no inhibition of mRNA transcription by CsA was observed for the P2 promoter which controls the synthesis of NFATc1 β isoforms. This is in line with the regulatory sequence elements within the P2 promoter (Fig.18).

In addition to the alternative use of the promoters P1 and P2, alternative polyadenylation events also contribute to the induction of NFATc1 transcription (Chuvpilo, Zimmer et al. 1999). Does NFAT factors also play a role in the control of polyadenylation of NFATc1 RNAs ? In searching for conserved regions near the pA1 and pA2 sites, we found 5 NFAT binding sites just upstream of pA2 (see supplement Fig.S5), whereas only 1 candidate NFAT binding site was found near pA1. We observed that the synthesis of βA isoform controlled by the poly site pA1 and the P1 promoter remained unaffected by CsA, wherease αA transcription was inhibited by CsA. It is very likely that the inhibitory effect of CsA is due to the NFAT binding to the P1 promoter which harbors two NFAT binding sites.

The synthesis of NFATc1 isoforms controlled by pA2 appear not only dependend on promoter activity but also on NFATc1α/A levels. While the extent of transcription of αB/C and βB/C isoforms in unstimulated effector T cells seems to be caused by promoter activity, T cell stimulation exerted opposite effects on the long isoforms B and C, compared to the αA isoform. One might speculate that NFATc1αA suppresses the transcription of long isoforms mediated by pA2. This hypothesis implies that isoforms controlled by pA2 may be autoregulated by the generation of the short NFATc1/αA isoform. Both the activity of the P1 promoter and the pA2 poly A addition site appears to be controled by autoregulation, but with opposite outcomes. Unlike the P1 promoter, the proposed NFAT binding to pA2 could cause a suppressing effect on the transcription of the long isoforms. This might also explain why CsA exerts a strong inhibitory effect on the induction of NFATc1α/A whereas it exerts a mild, or no effect on transcripton of long isoforms βB+C in T cells (Fig. 15; Chuvpilo et al., 2002). The down regulation of NFATc1/βB+C might lead to an occupancy of the NFATc1 target gene promoters by NFATc1/αA. Such opposite effects – reflecting a negative feedback loop in T cell activation through NFATs - could play an important role in the autoregulation of NFATc1 gene induction by NFATc1 proteins. Simililar to electronic circuits, such biological circuits belong to the principle mechanism of gene control in all organisms.

Why do multiple NFATc isoforms exist? We speculate that the unique regions of the NFATc1 isoforms confer distinct functions to these proteins. Structure-function studies have demonstrated that the N-terminal domain of NF-ATc1/A, B and C and the C-terminal domains of NF-ATc1/B and C function as trans-activation domains. It is possible that unique domains act to attract unique coactivators. Proteins, such as c-Maf, NFAT-interacting protein-45 (NIP-45) and GATA-3, which have been described as essential co-factors for IL-4 gene expression in T cells, are also candidates for a role in cell type-specific co-activation. Cell type specific co-activators might stabilize specific NFATc1 isoform combinations bound to DNA. The existence of multiple isoforms that contain unique functional domains expands the potential repertoire of coactivators that can cooperate with NFATc1 isoforms to achieve selective gene expression in a given cell type. Different members or isoforms of NFATc1 may regulate inducible expression of different cytokine genes and maintain their transcriptional level.

It is intriguing in this regard that NF-ATc1/A is the NFATc1 isoform family expressed at the highest levels upon T helper cell activation. A comparison of the phenotypes of the NFATc1/A and NFATc1/B or C null mice will be interesting. To understand NFATc1 isoforms in T cell function, we have constructed different vectors for the conditional inactivation of individual murine NFATc1 isoforms. With the help of these vectors and the generation of mice for the conditional inactivation of individual isoforms we hope to reveal the functional role of different NFATc1 isoforms in T cell function in the future.

4.2 Structure and regulatory elements of the NFATc1 gene promoters

With regard to the different induction properties of NFATc1 isoforms one may ask how the transcription of the individual NFATc1 isoforms is controled in specific T lymphocyte subsets. Our former experiments indicated that the short NFATc1/αA protein was induced to high levels due to the autoregulation of the NFATc1 promoter P1 by NFATs (Chuvpilo, Jankevics et al. 2002). However the transcription of the short NFATc1/αA induced by T/I was partially inhibited by CsA (Fig.15). This implies that there are additional TFs - but not only NFATs - involved in P1 promoter activity,

and NFAT autoregulation might only be a part of the mechanism controlling P1 promoter activity. In addition, NFATc1 transcription showed a cell type and tissue-specific gene transcription. To gain more insight into the mechanisms of NFATc1 gene regulation, we tried to identify the regulatory regions outside (and within) the NFATc1 gene promoters P1 and P2, and we also compared their TF binding sites between them.

In mapping of DNase I hypersensitive chromatin sites, four DNase I hypersensitive chromatin sites (designated as HSS1-HSS4) were mapped over the 5'-region of the NFATc1 gene in EL-4 cells (Chuvpilo, Jankevics et al. 2002). HSS1 and HSS4 are located over the P1 and P2 promoters, respectively, and also are covered by the CpG islands 2, 3 and 4 (Fig. 17). The overlapping regions of CpG islands harbor two conserved regions in the P1 promoter, and one conserved region in the P2 promoter (Fig.18A). The conserved regions within the CpG islands and HSS sites might represent important regulatory regions for the transcription of the NFATc1 gene.

In addtion to two NFAT binding sites, we found several prominent TF binding sites in the conserved region of P1 (Fig.18B), such as for the binding of NF-κB and AP-1. Both TFs are activated by PKC in T lymphocytes (Sun, Arendt et al. 2000). This might explain that TPA (a PKC agonist) obviously enhanced NFATc1/αA transcription by ionomycin, and CsA (an NFAT inhibitor) exerts a mild effect on NFATc1/αA transcription by T/I. A comparison between the regulatory region of P1 promoter (i.e. the conserved regions 3, 4 and 5) and of P2 promoter (conserved region 10) shows that there are many more TF binding sites within the P1 promoter. The multiplicity of TF binding sites within P1 reflects its strong induction in effector T cells, and in many other cells.

In addition to these prominent TFs, the possible binding of GKLF to the NFATc1 promoters should attract our attention. In both the P1 and P2 promoters, tandemly arranged GKLF binding sites can be found. Recent studies have shown that quiescence of lymphocytes is an actively maintained state rather than a default pathway in the absence of TCR/co-receptor signals. Certain TFs appear to act as master regulators of gene expression patterns that enforce the quiescent phenotype (Yusuf and Fruman 2003). GKLF/LKLF family proteins have been shown to be important lymphocyte quiescence factors in memory and naïve T cells (Buckley, Kuo

et al. 2001). In one subtype of memory T cells, Tnp (TCM), there is no NFATc1 transcription (Fig.15B). As prominent TFs controlling T cell activation and proliferation, NFAT activity should be suppressed for maintaining a quiescent state. We speculate that tandemly arranged GKLF binding sites in the regulatory regions of NFATc1 promoters P1 and P2 (Fig.16) reflect a potential role for GKLF in quiescent memory T cells, may be by shutting down NFATc1 expression in Tnp cells (Fig.15).

4.3 NFATc1 gene transcription controlled by PKC pathways

Immune cells are in constant need of energy for both basic housekeeping and specific immune functions. Increased energy demand during lymphocyte stimulation is coordinated by signal transduction pathways. Quantitative aspects of signal transduction related to energy metabolism in T cells indicate that PKC-related pathways carry 54% and calcineurin-related pathways carry 30% of the signals (Krauss, Brand et al. 2001). Upon antigen stimulation, many of the molecules involved in T cell activation redistribute to the points of T cell-APC interaction and form the immunological synapse (Grakoui, Bromley et al. 1999; Bromley, Burack et al. 2001). A key molecule associated with the immunological synapse is PKC-θ, which is a novel Ca2+-independent PKC isoform (Baier, Telford et al. 1993). PKC-θ is specifically expressed in T cells and skeletal muscle cells (Baier, Telford et al. 1993). Through a mechanism involving Vav, Rac, and actin cytoskeleton reorganization, it localizes into the center of the immunological synapse and to lipid rafts after TCR stimulation and CD28 co-stimulation. In addition, studies using T cells from mice genetically deficient in PKC-θ have shown that signaling through PKC-θ is required for TCR-initiated induction of NF-κB, AP-1 and NFAT (Baier, Telford et al. 1993; Sun, Arendt et al. 2000; Pfeifhofer, Kofler et al. 2003). Similar to the IL-2 promoter region, there are tandemly arranged NFAT, AP-1 and NF-κB binding sites within the P1 promoter of NFATc1 (Fig.18). For optimal induction, PKC activation is required for IL-2 (Werlen, Jacinto et al. 1998) and NFATc1/αA induction (Fig.12 and 15) (Chuvpilo, Jankevics et al. 2002). According to those findings, we propose that PKC signals exert a synergic effect on NFATc1 gene transcription upon TCR-induction.

By using a well-characterized cDNA microarray representing 4,551 genes we systematically analyzed the PKC pathways in four different human lymphocyte cell lines. According to the results of these assays and the well-known signals activated by PKC, an overview of NFATc1 gene transcription controlled by PKC signals was drawn (see Fig.29).

After PKC activation, the transcription of NF-κB (NFkB1), AP-1 (Fos, FosB and JunB) and EGR1 was found to be upregulated (Fig.18). Those are prominent TFs binding to the P1 and P2 promoters of the NFATc1 gene (Fig.17). It implies that PKC may widely affect all NFATc1 isoform transcription by controlling transcription of multiple TFs. The transcription factors NF-κB and AP-1 are the primary physiological targets of PKCθ (Isakov and Altman 2002), and our results indicate that PKC activation also increased the transcription of both TFs (Fig.18). The intergrating roles of PKC activation may enhance both TFs binding to the regulatory regions of the NFATc1 P1 promoter (Fig.18). Finally it increased the NFATc1/αA transcription by synergy with Ca^{2+} signals (Fig.15 and 16).

One prominent factor we observed upon TPA induction is EGR-1. The gene encoding EGR-1 is rapidly induced in numerous cell types upon different stimuli, maintaining cell proliferation and activation. In numerous malignant lymphocytes, EGR-1 shows a constitutive expression (supplements Fig.S3). Although showing a high constitutive transcription, EGR-1 is still the most prominent TFs whose expression is induced upon PKC activation (Fig.19). A highly constitutive expression of EGR-1 could provide a signal for the constitute expression of NFATc1/βB or C in EL-4 cells (Fig.16), which is controlled by the P2 promoter harboring an EGR-1 binding site (Fig.18). EGR1 is known to interact with NFATs forming EGR-NFAT complexes, which control TNF and CD154 (CD40 ligand) transcription (Decker, Nehmann et al. 2003).

In addition, upon TPA stimulation an increase was detected in transcription of members of the RAS oncogene family (RAB1A and RAB5A). RAS proteins are located upstream of MAPK kinases. MAPK pathways provide potential ways for AP1 activation (Deng and Karin 1994) and stabilize mRNAs by MAPK kinase signals (Wu, Hsu et al. 2003).

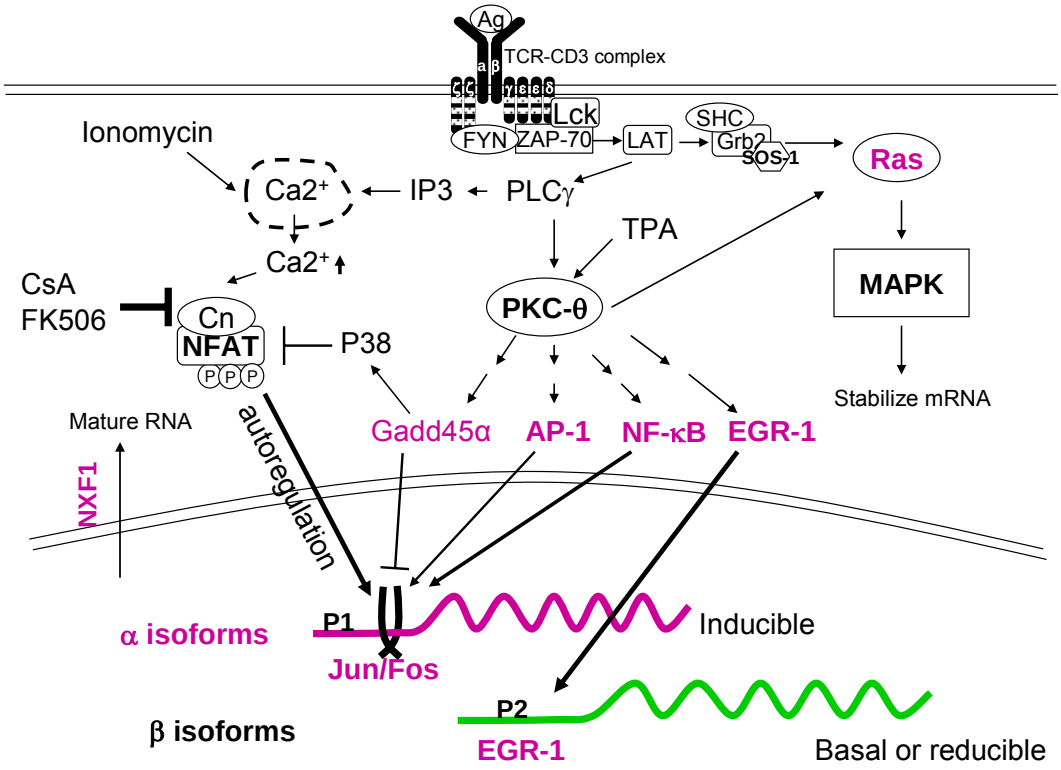


***Fig.29* PKC pathway in control NFATc1 gene transcription in T lymphocytes**
In addition to NFAT autoregulation, NFATc1 gene transcription is also modulated by PKC activation. PKC signals have no direct effect on the nuclear translocation of NFATc1, but they affect NFATc1 gene transcription by increasing the expression of several TF`s binding to the NFATc1 promoter region. The increasing transcription is marked by purple color. Promoting effects are indicated by arrows, and inhibition is also indicated.

4.4 NFATc1 gene transcription regulated by p53 and Gadd45α

In addition to the activation of apoptotic target genes, p53 can repress gene expression (Vousden and Lu 2002). Detailed studies of tumor-derived p53 mutants showed that the ability of p53 to repress gene expression is tightly linked to its ability to induce apoptosis (Ryan and Vousden 1998). The correlation between transcriptional suppression and apoptosis is also seen in the trimeric p53–MDM2–RB (retinoblastoma) complex that retains the ability to repress gene expression and to induce apoptosis, despite being defective for the transactivation of p53 target genes, such as CDKN1A (Hsieh, Chan et al. 1999). Several genes have been identified as in vivo targets of p53 repression, many of which have anti-apoptotic function. NFATs support activation-induced cell death (AICD) of T and B cells

(Serfling, Berberich-Siebelt et al. 2004). In line with p53 suppression, we observed a constitutive expression and activation of NFATc1 protein in murine *p53*$^{-/-}$ T cells lymphomas (Fig.22A). Further experiments indicated that p53 suppressed NFATc1 by interfering with the activity of the NFATc1 P1 promoter (Fig.22B). Searching for regulatory elements of NFATc1 P1 promoter, we detected two potential p53 binding sites within the proximal P1 promoter region. The identification of the transcriptional repressor protein SIN3A as an interacting protein of p53 provides a molecular explanation for how p53 might repress gene expression (Murphy, Ahn et al. 1999), although it remains unclear how p53 chooses its target genes for repression. It is also interesting to note that SIN3A binds to and stabilizes p53 through the proline-rich region of p53, a region that is required for apoptotic function. Overexpression of the wild-type p53 gene inhibits NF-κB activity (Shao, Fujiwara et al. 2000). In a similar way, we observed p53-mediated NF-κB suppression in a dose-dependent manner in both murine and human lymphocytes (Fig.22C and D). This might imply that p53 may indirectly inhibit NFATc1 gene transcription by suppressing NF-κB activity.

In addition to p53, we also found that one of its targeting gene, *Gadd45α*, which becomes activated by PKC (Fig.19). Abolish of GADD45α transcripton can obviously release the inducible NFATc1/αA gene transcription (Fig.23). The transcription of GADD45α, a small p38-binding molecule, is induced by environmental stress, including MMS treatment, UV light and gamma irradiation. Mice lacking *Gadd45α* develop a lupus-like autoimmune disease, T cell hyperproliferation and autoimmunity. These symptoms are also caused by constitutive activation of p38 in resting T cell. Resting T cells but not B cells from *Gadd45α*$^{-/-}$ mice had spontaneously increased p38 activity, in the absence of any 'upstream' MAPK kinase activation. The p38 kinase from resting *Gadd45α*$^{-/-}$ T cells was spontaneously phosphorylated on Tyr323, and its activity was specifically inhibited by recombinant Gadd45α in vitro (Salvador, Mittelstadt et al. 2005). *Gadd45α*$^{-/-}$*p53*$^{-/-}$ mice developed tumors with a latency similar to that of tumor-prone *p53*$^{-/-}$ mice. However, while *p53*$^{-/-}$ mice developed a variety of tumor types, nearly all *Gadd45α*$^{-/-}$*p53*$^{-/-}$ mice developed lymphoblastic lymphomas (LBL) (Hollander, Patterson et al. 2005). NFATc1 is a direct target of p38 MAPK. Inhibition of p38 MAPK led to the selective inactivation of NFATc1 in T cells, and the overall effect of

p38 MAPK on NFATc1 is activation (Wu, Hsu et al. 2003). Therefore, a constitutive p38 activation in *Gadd45*$\alpha^{-/-}$ T cells has a promoting effect on NFATc transcription (Fig.22).

For *Gadd45*$\alpha^{-/-}$ T cells, there is a constitutive activation of p38 in resting T cells (Salvador, Mittelstadt et al. 2005). It was revealed before that persistent activation of p38/JNK, especially in the absence of mitogenic stimuli, leads to an induction of apoptosis (Chen, Meyer et al. 1996). In line with those observations, a constitutive apoptosis induction was detected in human *Gadd45*α-deficient T cells without any stimulation (Fig.24). PKC activation lead to suppress apoptosis (Fig.24), which may be mediated by increasing Gadd45α expression in a p53-independent way (Fig.23) and by NF-κB activation.

Our findings suggest that during early stages of T cell activation, a regulatory hierarchy exists between the NFAT and the Gadd45α pathways, which is coordinated by PKC-θ. The integration of these two signaling pathways through NFAT transcription may offer an additional mechanism for controlling the expression of genes, such as the IL-2 gene.

4.5 Regulatory Mechanisms of NFATc1 gene transcription

We propose the following model of NFATc1 gene transcription (Fig.30). First, the transcription factors promote NFATc1 gene synthesis by binding to the regulatory regions of P1 and P2 promoter. Many of TFs are downstream targets of PKC signals, including those prominent factors as NFAT, NF-κB and AP-1. We observed little or no NFATc1/αA mRNA in untreated cells but a strong CsA-sensitive induction of its synthesis in ionomycin-treated cells. These results suggest that preexisting NFATc1/βB and C or NFATc2, which translocate to the nucleus following treatment of cells with ionomycin, might be important for the start of NFATc1/αA mRNA synthesis (Fig.15). Such positive autoregulation was achieved by binding of NFATs to the P1 promoter (Serfling, Chuvpilo et al. 2006).

Second, some TFs suppress NFATc1 transcription by binding to the regulatory regions of P1 promoter. Two well-known suppressors, p53 and GKLF, are candidate TFs in suppressing NFATc1 gene transcription.

Third, TGFβ and Gadd45α appear to repress indirectly NFATc1 transcription. TGFβ1 prevents thymocyte activation by self-MHC-TCR interactions through a Ca^{2+}-calcineurin pathway, and the hypersensitivity of *TGFβ1*$^{-/-}$ thymocytes is due to a lowered threshold for Ca^{2+}-dependent activation (Bommireddy, Ormsby et al. 2003). In TGFβ-treated Tnp cells, no NFATc1 transcription was observed (Fig.15). TGFβ may partially suppress NFATc1 transcription by inhibiting Tec kinase (Yang, Ghiotto et al. 2000; Chen, Seguin-Devaux et al. 2003). In contrast, Gadd45α regulates NFATc1 gene transcription by activation of p38 (Wu, Hsu et al. 2003; Salvador, Mittelstadt et al. 2005).

Fourth, we speculate that a negative autoregulation could control the activity of the distal poly A site, pA2. Activated NFATs could bind to the conserved and clustered five NFAT binding sites at pA2 wich could promote non-coding RNA transcription and, thereby, silencing the transcription of long NFATc1 isoforms.

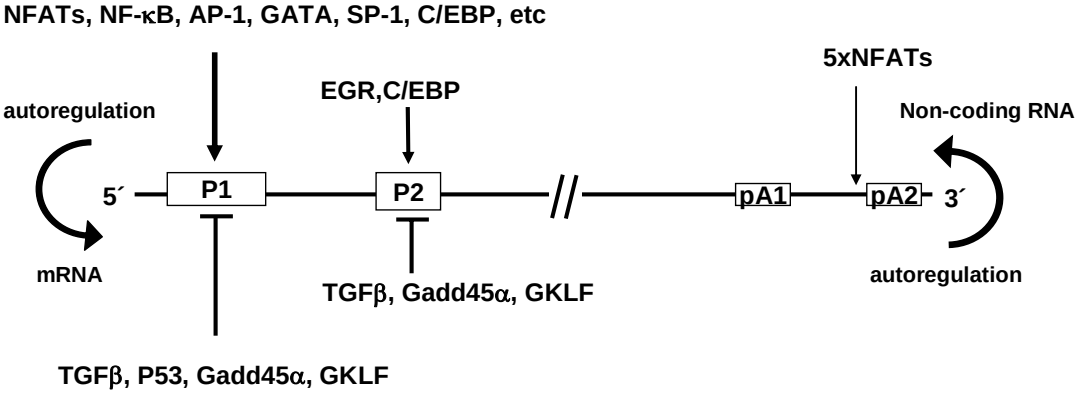


Fig.30 Regulatory mechanisms of NFATc1 gene transcription in murine T cells
Both positively and negatively acting TFs appear to be involved in NFAT transcription after T cell activation. NF-κB and NFAT binding to the promoter region promote NFAT transcription, whereas TGFβ signals, p53 and Gadd45α suppress NFAT transcription by unknown mechanisms. In addition to the NFATs binding to the P1 promoter which exert a positive autoregulation, NFATc1/αA could bind to pA2 sites and exerts a negative autoregulation by a non-coding RNA transcription. Without NF-κB and NFAT co-factors, such as AP-1, NFATs may drive an expression of intergenic RNA and silence NFATc1 transcription.

Fifth, NFATc1 transcription could be suppressed by the transcription of non-coding RNAs. TFs usually interact with their co-factors to form more composite factor complexes. In the absence of co-factors, TFs could drive expression of intergenic

RNAs, so-called non-coding RNAs. There is growing evidence that non-coding RNAs play a much larger functional role in the genome than originally anticipated (Mattick and Makunin 2006). However, in most of these cases, these RNAs impose a negative effect on gene expression either through microRNA-targeted mRNA degradation and inhibition of translation (Ambros 2004), through antisense (Sleutels, Zwart et al. 2002; Reis, Louro et al. 2005), or small interfering RNA-mediated DNA methylation and epigenetic silencing mechanisms (Lippman and Martienssen 2004). In addition, it has been reported that intergenic RNAs possess promoter activity in a Th2-specific manner. In the absence of NFATs, AP-1 drives an expression of intergenic RNA in the IL-10 gene locus. There are the five highly conserved noncoding sequences within the 40-kb IL-10 region which are transcribed in intergenic RNA. Four of these regions possess promoter activity (Jones and Flavell 2005). With respect to the many TFs binding sites within the NFATc1 P1 promoter, NFATs or NF-κB could drive the synthesis of non-coding RNA in the absence of AP-1 or other cofactors. Experiments have already shown that noncoding RNA transcripts (ncRNAs) are important regulators of NFATs in vivo (Mattick 2005; Willingham, Orth et al. 2005). In the light of these observations, it will be interesting to investigate whether intergenic RNA transcription controls the expression of NFATc1 locus.

4.6 NFATs and T cell lymphomas

A common feature of most T cell lymphomas is their constitutive, uncontroled activation showing high levels of TFs and protein kinase activities, a constitutive expression of numerous cytokines and chemokines, evading apoptosis and limitless replicative potential (Hanahan and Weinberg 2000; Abbott, Forrest et al. 2006). In lymphocytes, high activation of NFATs promotes expression of cytokines (Serfling, Berberich-Siebelt et al. 2000) and chemokine (Barlic, McDermott et al. 2004). In nonimmune cells, NFATs are expressed in breast cancer and colon carcinoma cells, playing a functional role in the regulation of target genes involved in the tumor phenotype and invasion, such as COX-2 (Duque, Fresno et al. 2005; Yiu and Toker 2006). Since NFATs control the transcription of many genes related to tumorigenesis - such as cytokine, chemokine and apoptosis-related genes, and genes controling

cell cycle and proliferation - we proposed that NFATs stand in the center of lymphomagenesis. In agreement with this assumption, we found a high constitutive expression of NFATc1 and NFATc2 in p53T (p53-/-) and EL-4 lymphoma by western blotting (Fig.21). High constitutive transcription of NFATc2 and NFATc3 was also observed in human lymphoid neoplasms by using microarrays (Fig.19). The redundant functions of NFATc1 and NFATc2 imply that NFATc2 may compensate for NFATc1 in human lymphomagenesis. Of particular interest is the finding that NFATc1/αA was found to be constitutively transcribed, and NFATc1 and NFATc2 were relocated to the nucleus in all of lymphoid neoplasms, reflecting a permanent activation of the NFAT pathway in those cells.

The TCR, a common site of dysfunction in T-cell malignancies, is the principal signal transduction moiety controlling both T cell activation and activation-induced apoptosis. A key event in T cell activation is the triggering of the nuclear import of the NFATs, which controls cytokine gene expression. How become NFATs constitutively activated in lymphomas? Cancer cells show often marked alterations in the methylation status of their genomes. Thus, defects in the epigenetic mechanisms controling NFAT activity might be one of the reasons for the hyper-activation and hyper-transcription of NFATs in lymphomas. Additionally, many human cancers, including aggressive T cell leukemias and lymphomas, have a high frequency of p53 mutations, particularly missense mutations. As a tumor suppressor gene, a prominent function of p53 is promoting apoptosis. In addition, a new role of mutant p53 has been described as an epigenetic mutator, bridging methylation and transcriptional silencing by p53 (Iwamoto, Mizuno et al. 1999). We have shown that wild type p53 could suppress NFATc1 (may be other NFATs also) and NF-κB activation (Fig.21). Mutant p53 may de-repress NFATc1 gene transcription. It would be of interest to correlate this feature with patterns of prominent oncogene expression, such as with the expression NF-κB, EGR1, NOTCH3, MAPK3 and DSCR3 (Fig.19 and supplements Fig.S3). All of them have a direct and indirect effect on NFAT activity (Decker, Skerka et al. 1998; Decker, Nehmann et al. 2003; Talora, Campese et al. 2003; Qin, Zhao et al. 2006). NF-κB binding to the P1 promoter enhances NFATc1 gene transcription (Fig.17). Another mechanism is that NF-κB drives the expression of pro-inflammatory cytokines IL-1 and TNF-α, which induce NFATc2 expression (Yang, Ung et al. 2006).

Does the high activation and expression of NFATs in several lymphoid cells lines really imply that NFATs are controling tumorigenesis? The following evidence supports this assumption. By immunohistological stainings, a systematic evaluation of NFATc1 expression in almost 300 lymphomas showed that most neoplastic lymphoid cells express NFATc1 (Marafioti, Pozzobon et al. 2005). It is noteworthy that although NFATc1 is absent in classical Hodgkin's lymphomas and leukemic Jurkat T cells (Fig.19 and 21), a high expression of other NFATs may compensate with the role of NFATc1 in tumorigesis. NFATs are targets of integrin signaling and are involved in promoting carcinoma invasion (Jauliac, Lopez-Rodriguez et al. 2002), while NFAT inhibitors as FK506 inhibit TPA-induced tumor promotion (Jiang, Yamamoto et al. 1993; Yamamoto, Jiang et al. 1994).

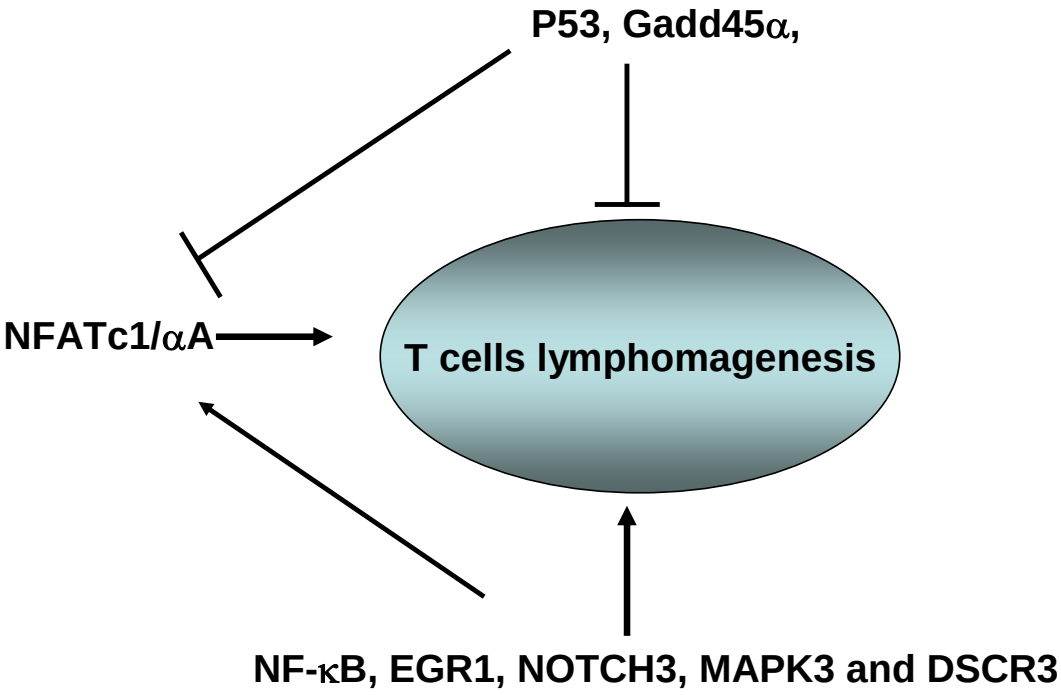


Fig.31 Model of control of T cell lymphomagenesis
NFATc1/αA (along with its controling molecules) promotes tumorigenesis of lymphomas through enhancing cytokine and chemokine expression. Wild type p53 and Gadd45α suppress lymphomagenesis, in part by inhibiting NFATc1 expression.

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4.7 Potential roles of NFATc1 in Th effector and central memory T cells

The polarization of T cell cytokine profiles toward Th1 or Th2 responses is one of the mechanisms by which the immune system responds to microbial challenges. The development of an inappropriate immune response can lead to ineffective immunity and immune pathology. Mice double deficient for NFATc1 and NFATc2 display a significantly impaired production of T cell-dependent effector functions. Double deficient T cells were unable to generate mature T effector cells. They are characterized by a loss of IFN-γ and IL-4 production. Such findings emphasize the importance of the NFATc transcription factors in the control of effector T cell and T_{CM} generation (Peng, Gerth et al. 2001). In line with this, the expression of all NFATc1 isoforms was found to be increased when naïve T cell differentiate into effector T cells, whereas no NFATc1 isoform was transcribed in T_{CM} cells which are characterized by a loss of IFN-γ and IL-4 production (Figs.12a and 15B).

Central memory T cells (T_{CM} or Tnp), a subtype of memory T cells are generated under non-polarized conditions by adding TGF-β. Comparing to polarized effector T cells, no NFATc1 transcription was detected in T_{CM} cells by RT-PCR assays. This might imply that silencing of NFATc1 is required for maintaining memory T cells. In agreement with this finding, the protein kinase GSK-3 is important for CD8 memory T cell survival (Zhang Y, etc. personal communications). GSK-3, which phosphorylates NFATs, controls the export of NFATs from the nucleus to the cytoplasm. Evading apoptosis is one of the important mechanisms to maintain T cell memory, and escape from AICD is required for effector T cells to differentiate into memory T cells. NFATs have been shown to bind to promoters of AICD related genes, such as the CD40L and CD95L promoters (Rengarajan, Mittelstadt et al. 2000; Lindgren, Axcrona et al. 2001). TGF-β inhibits T cell proliferation and differentiation. TGF-β has been shown to inhibit the expression of TFs, such as of GATA-3 and T-bet that play important roles in T cell differentiation. At a more proximal step, TGF-β inhibits an increase in intracellular Ca^{2+} levels and NFATc translocation. All together, these events suppress T cell differentiation into both the Th1 and Th2 lineages (Chen, Seguin-Devaux et al. 2003). In addition to TGFβ-

mediated suppression, quiescence factors of GKLF family may also be involved to suppress NFAT transcription in memory T cells (Buckley, Kuo et al. 2001). According to this, a model for NFAT function in forming memory T cells is proposed: In memory T cells, highly active GSK-3 phosphorylates NFATc1 and leads to the export of NFATc1 to the cytoplasm, in parallel, NFAT transcription is suppressed by TGFβ and GKLF factors. As a final consequence cytokine expression and AICD mediated by TCR activation are reduced. This reduction in apoptosis and cytokine synthesis supports the generation of memory T cells.

Although naïve and Tnp cells both are long living T cells, there is a complete difference in the expression of the long NFATc1/βB+C proteins between naïve and T_{CM} cells. It might impliy that both types of cells may utilize different mechanisms in the control in their long-living survival.

It should also be noted that the state of a memory quiescence T cell is different from T cell anergy. T cell anergy is induced by incomplete NFAT activation by missing co-factor binding. Unlike NFAT role in inducing T cell anergy, TGF-β induces Tnp “anergy” independent of NFATc1 expression.

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SUPPLEMENTS

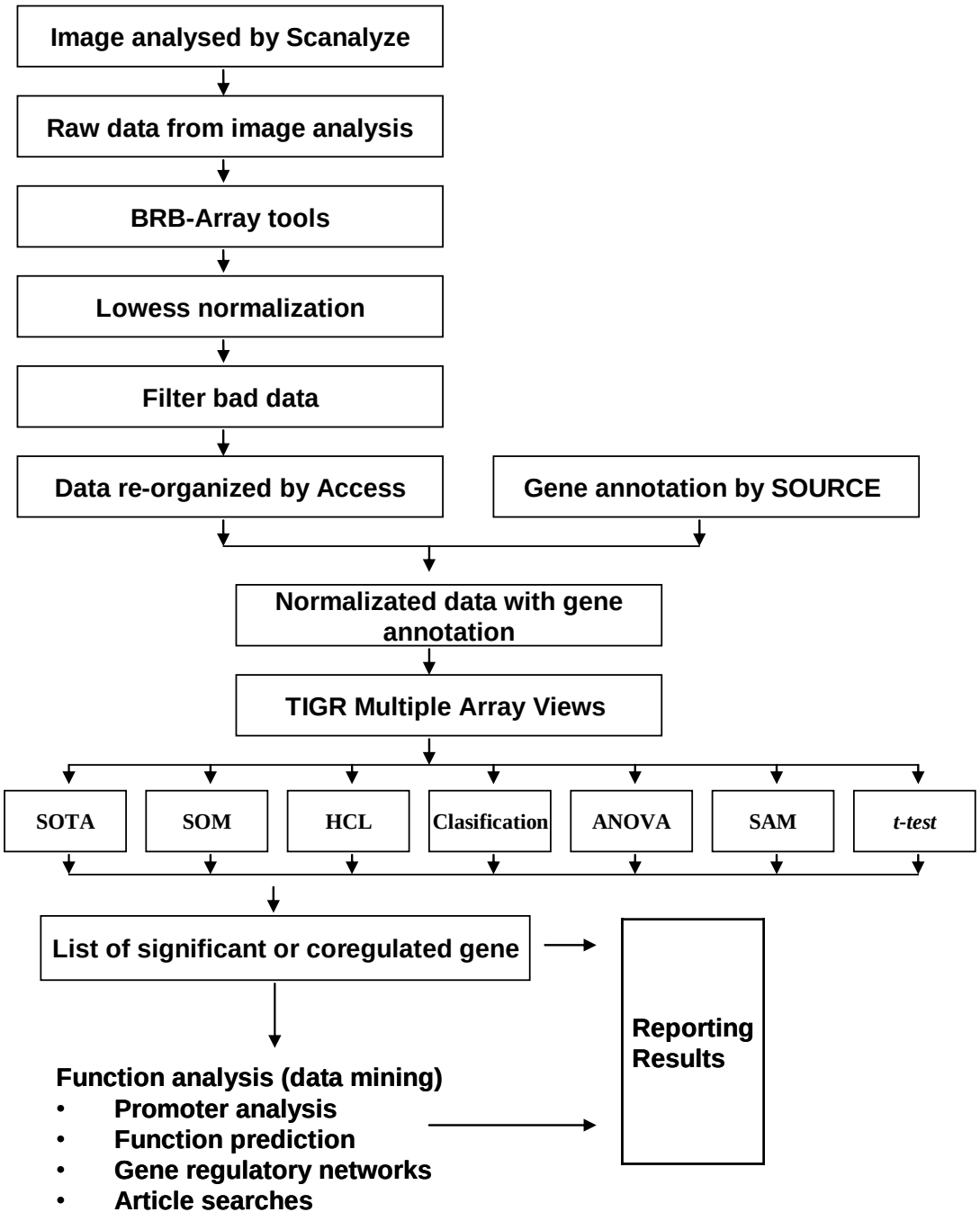


Fig.S1 Flowchart of data analysis in microarray

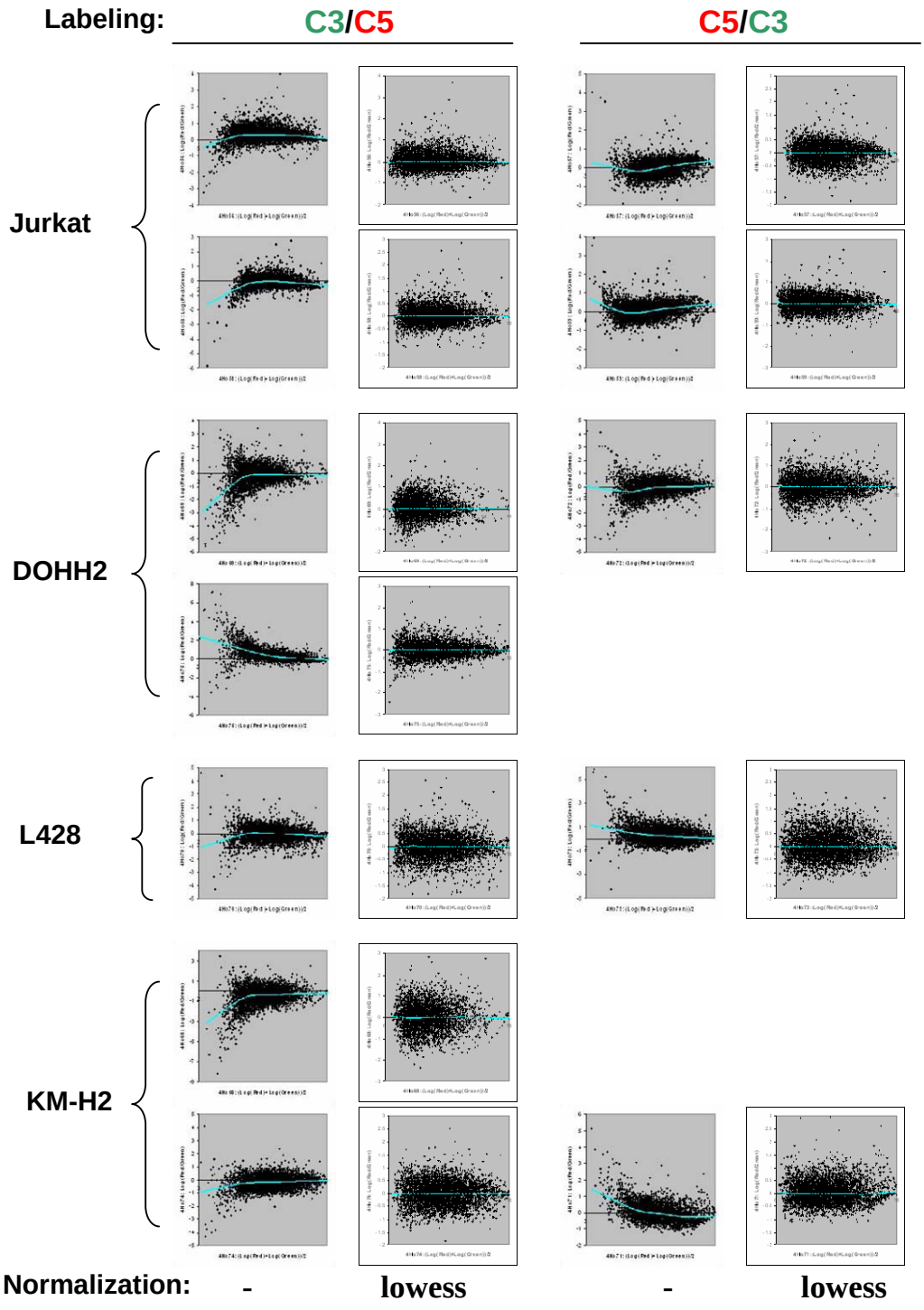


Fig.S2 Lowess normalization of data for microarray
Before further analysis, all data were normalized by Lowess smoother algorithm using BRB Array Tools software. Cut off value: IA and IB>100, Flag=1, 16%>1.5, percent missing>7.5%.

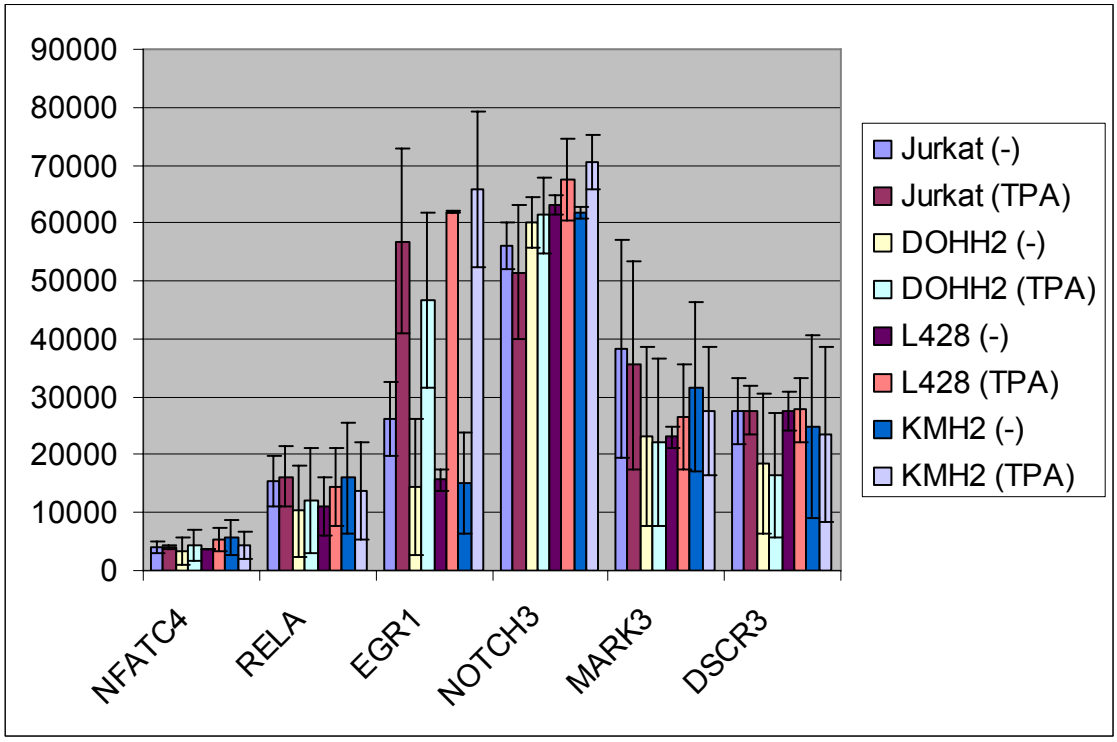


Fig.S3 Transcription level of potential tumorgenic genes in human malignant lymphocytes

The gene Transcription levels (Y-axis) are represented by Cy3 or Cy5 intensity from the microarray. ScanAlyze software was used in calculating the fluoresce intensity, and data was normalized before comparison. Intensity of each sample was calculated from the average intensity of the same condition experiments. The repeated conditions (n) for each cell line: Jurkat (n=4), DOHH2 (n=3), L428 (n=2) and KMH2 (n=3).

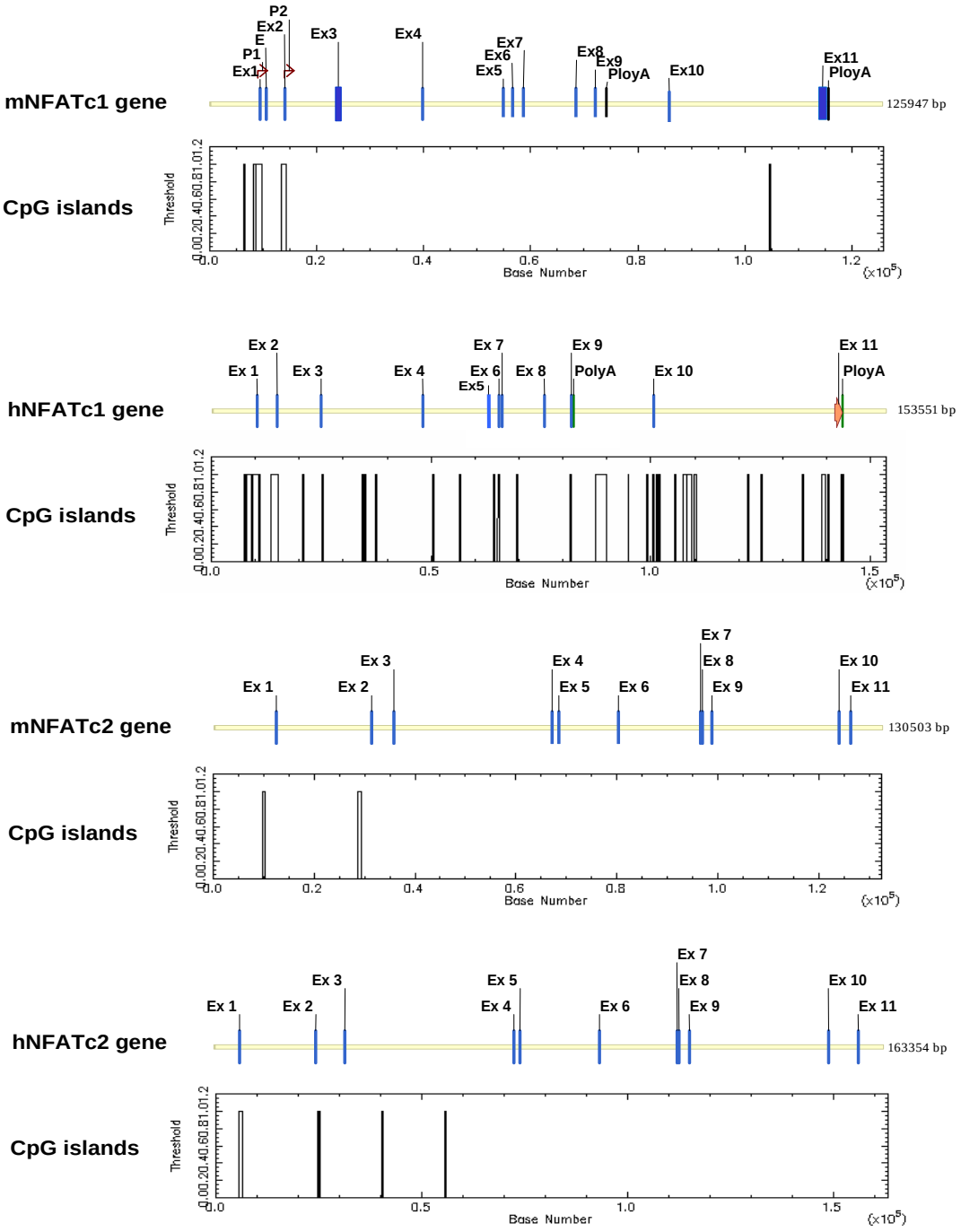
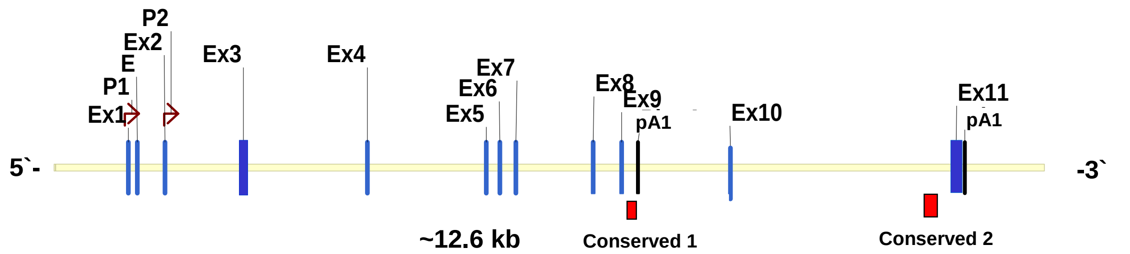


Fig.S4 Structure and CpG islands of NFATc1 and NFATc2 gene locus
CpG islands were analyzed by the CpGplot from EMBL. CpG islands were defined with observed/expected ration >0.60, percent C + percent G >50.00 and length >200. The blocks from top to bottom are murine NFATc1 (5 CpG islands), human NFATc1 (34 CpG islands), murine NFATc2 (2 CpG islands) and human NFATc2 (4 CpG islands), respectively.

A)



B)

```
                                                                      NF-kB-like
1    GGGAATGGCT TCTTTCATTC AGAAGTGATC GGCTCGCCTT TTCAACTTTC TAGGGTGTTT TATTTACGAA AATACCGTTT TTAACTGCTC CCCGCCCCGC
101  AAGCTTCTAG AAAGGTGTGT CCCAGGCGTC CAGGGTTTCC TGTGTGGTGC AGGCCATTCT CCTGCAGCAG GATGTATAAA CAGAGAGCAG AGTCGGTTGT
                                                                                  NFAT                  AP-1
201  TATCCTGAGT TCTATTGTAT TTTGAGTAAG CTAGGCTATG TCAACAACCT TTTTAAATTG CTACTTTTTT TTTCCTCTAA AAACTTAAGA TAGTCATGTA
             Elk-1
301  ATTTAAGAGG GAAGTTATAC AATAAATACT AGCCATGAAA GCAGCCATAT TGCTATCTTA GTAAAATCAA GGTGGTTTTG TTGTTGTTAT TTTGTTTTGT
```

C)

```
1    CAGGCGCCTC TTACAAGGAG GGCTGTGCTA GTGGAAGGGG AAGAAGAGCT AACCGCCTCT TAATGCCGGG TATCCCTGAC CTTTGCTGGG GAACCAAACA
101  TGAAGCTCAA GAGGTTGGCA AGAGACGAGT GAGAAACGCC GCGTGGACTC AGGAGGGCCT CAGGAGAGCC CTGTGACAGT GGCGTGTACT GCACAGAGCA
                                                                                                NFAT
201  GACAACCCTT GGTCCTCCTG GGGTCCCTGC TGCCTAGCTG CTTCTCAGGG CTGTGCAGAC GCCCCGCACC CACACTCCAG TATCATGGAA AATGCTGGTA
301  GCTAAGGAGC TCCCAGGGCG CACTCCAGGG ACAAGTTGAG TCCTGATTGC TGGTCCCAGA CCAGCCTGTG AGCACCTGGG TCTTGTTATT CCTCATTAGT
                                                    NF-kB like
                                                        NFAT
401  GGCTGAGAAG TCACAGGGCC AGAGGGAGCG TGCCTGCCTT CTGGTATTTT CCACACCAAA GGGTGGGCCC TCTCCACAGT GCGCTTGGTG CTGTGGTCAA
501  GGATCAATCC TGGAGCTCCA CAGAGGCGCC CAGGAGTGCC CGCTGCCCCA CCCCATCCCC AAGAAGCTGA CCAGGAAGCA GCCATGGTTC CTATCTGTAA
                                                                               NF-ATp
601  GTGTGACTTT TGAGGGAACA GTCCCAGGAG CAGCAAACCC ACCGGGGCGT CCTGGAGCGG TGGCCCTGGT ACAGGGAGCC TCGCGGTTAT AAGTCTCTTG
701  CCAGGCAGCC CTTTTGGGGC GAGCCGCCAG GCTTCCTCCT GCTATCAGTT CTCATGACAC AATTCCTGGT TCCCCCAACT GCGTTCTGTT ATTGTTATTC
                                                                                   NFAT
801  TTAAAACTAT TGCTGAGGAA GGGGTGTTGA AAGGCAGCTT AGGGAAGGGA GTGGCGATGA AGCGCACGCC CCCTGATGGC TCCAGGAAGC GTCCTCAGAC
                                                             NFAT
901  AGGCTGGGGC CTCCGAGGGT GAGCTCCAGG GGCTTATCTA GGTGCAGGCG GGGGCTCCAG CCCATGGGGA AGGGAAGCCC AGGCGGTTCC GTGACGTCAG
                                                                                              NF-kB
.001 CATGGGTGCC CTGCCTTCCA ACTCTGTTAG AAAAATGGGA CGTGGTGACA GCCACCCAGT GACATTTCCT ATCTCAGTGT GGATCAGGTG GCTGGGCAGA
.101 GAGAGGCAGG GTGGAGAGGG AGGGATGTTG TGCTGTTTGC TAGCTAGCTT CTCTCATTTC CTGTCCATCT GCCAAAAATC ACACGCTGAT AGTGGCCATC
.201 ACTGGCCTGT GGGGAACCAG TGTGACTTAA CCCTCTTCAG AAAAACAAAT GAAGTACCCC AAACAAAGGG AGGCCCACGG CCATGGTAGG TCTCAGTACC
```

Fig.S5 Conserved regions and TFs binding sites near the poly A site pA1 and pA2

A) A simplified view of the murine NFATc1 gene locus, there are 2 conserved regions near the poly A site pA1 and pA2. B) DNA sequence and TFs binding sites of the conserved region 1 (near the the poly A site pA1). C) DNA sequence and TFs binding sites of the conserved region 2 (near the the poly A site pA2).

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Since 4/2001	Scientific Assitant at the Institut für Pathologie, Universität Würzburg, leading to the qualification Doktor rer.nat. Germany
01/2000 - 09/2004	Lecturer and scientific researcher, Biotechnology Institute, Soochow University, PR.China
07/1995 - 12/2000	Assistant teacher and scientific researcher, Department of Immunology, Suzhou Medical College, PR.China
07/1993 – 05/1995	Undegraduate research, China Pharmaceutical University, PR.China

Research Grants (Participator):

1. NF-AT-Proteine und ihre transkriptionellen Co-Faktoren in Cardiomyocyten., DFG (CH 240 1-1), Germany (01.2000 - 12.2002).

2. Dendritic cell-based Tumor biotherapy: Expansion and Vaccination of tumor associated Ags, supported by National Health Ministry, P.R.China (1998-2001).

2. The mobilization effect of IL-8 on hematopoietic progenitors in vivo, supported by the Science and Technique Committee of Jiangsu Province, P.R.China (1996-1999). No. of the notice: H7410262

3. The effect of IL-8 on radiation injury and hematopoietic progenitor mobilization in vivo, supported by the Nuclear Industry Ministry, P.R.China (1996-1998). No. of the notice: BQ96016

Fellowships/Awards/Prizes:

1. The scientific prize for academic achievements in scientific research, awarded by the Science & Technology Committee of Jiangsu Province, PR.China (2001)

2. The Excellent-Young teacher for academic achievements in scientific research and teaching, awarded by Suzhou Medical College, PR.China (2000).

3. The Tang-Si prize for academic achievements in scientific research, awarded by Suzhou Medical College, PR.China (1999).

4. Undergraduate fellowship from China Pharmaceutical University for B.Sc. Pharmacy and Biotechnology program, PR.China (1994).

Publications and Other Scientific Activities

PUBLICATIONS

Publishing in English:

1) **Jiming Liu**, Sergei Chuvpilo, Dimitri Tyrsin, Alois Palmetshofer, Andris Avots, Edgar Serfling. Induciton properties and regulatory mechanisms of NFATc1 gene in T lymphocytes. (manuscript in preparation)

2) **Jiming Liu**, Sergei Chuvpilo, Andris Avots, Alois Palmetshofer, Edgar Serfling. Negative regulation of NFATc1 transcription by p53 and Gadd45α. (manuscript in preparation).

3) **Jiming Liu**, Susanne Kneitz, Alois Palmetshofer, Mithilesh Kumar Jha, Edgar Serfling. Comparison of non-polarized and polarized T cell subsets by microarray. (manuscript in preparation)

4) **Jiming Liu**, Susanne Kneitz, Sergei Chuvpilo, Alois Palmetshofer, Andris Avots, Edgar Serfling. Characteristics of PKC activation in malignant lymphocytes. (manuscript in preparation)

5) Edgar Serfling, Sergei Chuvpilo, **Jiming Liu**, Thomas Höfer, Alois Palmetshofer. NFATc1 autoregulation: a crucial step for cell-fate determination. Trends Immunol. 2006, 27(10):461-9.

6) **Jiming Liu**, Xueguang Zhang, Xiaoyan Sun, et al. Purification of IgM monoclonal antibodies from mouse ascites fluid by euglobin precipitation and gel filtration. U.S. Chinese Journal of Microbiology and Immunology 2000, 2(1): 43-46

Publishing in Chinese:

1) Caixia Li, Depei Wu, Yuedan Wang, Gehua Yu, **Jiming Liu**, Yumei Zhuang, Changshuo Yin, Xueguang Zhang. Expression of CXCR4 and its effect on the biological behavior of multiple myeloma cells. Zhonghua Xue Ye Xue Za Zhi (Chin J Hematol). 2003, 24(3):122-125

2) Huiling Yang, Hongtao Zhang, Tiansi Tang, Tongqi Yang, **Jiming Liu**. The experimental study of interleukin-8 expression during the spinal cord ischemia/reperfusion. Acta Academiae Medicinae Suzhou. 2002, (6): 22-24

3) Zhiliang Ding Fengqing Zhu Guangzhong Yin, **Jiming Liu**. Prevention of secondary brain injury after trauma by anti-IL-8 monoclonal antibodies. Chinese Journal of Stereotactic and Functional Neurosurgery. 2002, (2): 11-14

4) Li Ding, Fengqing Zhu, Xueguang Zhang, Changzao Xu, Youxin Zhou, **Jiming Liu**. Experimental studies on evaluation of protection by IL-8mAb to brain irradiation injury in rabbit. Chinese Journal of Stereotactic and Functional Neurosurgery. 2001, (1): 14-18

5) **Jiming Liu**, Xingguo Zhu, Yi Zhang, Huating Zhu, dechun Li, Suya Kang, Xueguang Zhang. Inhibition of neutrophil-mediated Ischemia/reperfusion injury in rabbit liver by anti-IL-8 mAbs. Current Immunology. 2001, 21(1): 8-11

6) **Jiming Liu**, Xueguang Zhang, Xiaoyan Sun, et al. Purification of IgM monoclonal antibodies from mouse ascites fluid. U.S. Chinese Journal of Microbiology and Immunology 2000, 2(1): 43-46

7) **Jiming Liu**, Yi Zhang, Huating Zhu, et al. Preparation of mouse monoclonal antibodies (mAbs) against human IL-8 and its biological function. Chin J Microbiol Immunol 2000, 20(6): 589-590

8) **Jiming Liu**, Xueguang Zhang, Qin Shi, et al. Technique in obtaining the high purity of recombinant human interleukin-8 from Escherichia coli. Acta Academiae Medicinae Suzhou 2000, 20(4): 340-343.

9) **Jiming Liu**, Xueguang Zhang, Yi Zhang, et al. Inhibition effect of ant-Interleukin-8 mAbs on LPS induced neutrophil infiltration in vivo. Acta Academiae Medicinae Suzhou 2000, 20(6): 12-15.

10) Qin Shi, **Jiming Liu**, Xueguang Zhang. Role of mitochondria in apoptosis and Bcl-2 family proteins. Current Immunology. 2000, (1): 60-62

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14) Yan Lu, **Jiming Liu,** Yue-dan Wang, et al. The negative impact on human dendritic cells: role of endothelial interleukin-8. Chin J Immunol 2000, 16(12): 32-35

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MEETINGS AND PUBLIC SEMINARS

1) Sergei Chuvpilo, Askar Akimzhanov, Friederike Berberich-Siebelt, Stefan Klein-Hessling, Rene Rost, **Jiming Liu**, Wen Chen, Alois Palmetshofer, Edgar Serfling. CSHL - Gene Expression & Signaling in the Immune System Meeting (Oral and poster presentation), April 26 - May 02, 2004, New York, USA.

2) **Jiming Liu**, Alois Palmetshofer, Susanne Kneitz, Edgar Serfling. Mitogenic pathways in malignant lymphoma. CSHL - Gene Expression & Signaling in the Immune System Meeting (Abstract), April 28 - May 02, 2004, New York, USA.

DECLARATION

I hereby declare all the above informations are true to the best of my knowledge.

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Place:……**Würzburg**…… Jiming Liu