T cell responses in feline immunodeficiency virus-infected cats

ネコ免疫不全ウイルス感染ネコにおけるT細胞応答

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CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
CD	cluster of differentiation
Con A	concanavalin A
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
CXCR4	chemokine, CXC motif, receptor 4
E	erythrocyte
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	envelope
E:T	effector:target
FCM	flow cytometry
FCS	fetal calf serum
FeLVC-R	feline leukemia virus subgroup C receptor
Fig.	figure
FITC	fluorescein-isothiocyanate
FIV	feline immunodeficiency virus
FSC	forward scatter
GFP	green fluorescence protein
HIV	human immunodeficiency virus
hr	hour
ICAM	intercellular adhesion molecule

IFA	indirect fluorescence assay
IL	interleukin
IL-2R	IL-2 receptor
kb	kilo base
kDa	kilo dalton
LFA	leukocyte function-associated antigen
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute
MLV	Moloney-murine leukemia virus
moi	multiplicity of infection
MW	molecular weight
N-CAM	neural cell adhesion molecule
NK	natural killer
nt	nucleotide
ORF	open reading frame
P3U1	P3X63Ag8U.1
PBS	phosphate-buffered saline
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythrin
pi	post infection

RBC	red blood cell
RFLP	restriction fragment length polymorphism
RT	reverse transcription
SD	standard deviation
SDF	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SIV	simian immunodeficiency virus
SPF	specific pathogen-free
SSC	side scatter
TCR	T cell receptor
$TCID_{50}$	median tissue culture infectious dose
T_h	T-helper
TRAF	tumor necrosis factor receptor-associated factor
UTR	untranslated region
VSV-G	vesicular stomatitis virus G glycoprotein
wk	week

PREFACE

Aims and scope of the thesis

Feline immunodeficiency virus (FIV) is a member of the genus *Lentivirus* of the family *Retroviridae*. FIV infection in domestic cats (*Felis catus*) causes AIDS-like diseases after a protracted asymptomatic phase of several years. Accompanied by vaccine-development trials for the virus, there is a dramatically increasing interest in the feline immune system. Cellular immunity has been suggested to be critical to control FIV infection, while humoral immunity including production of neutralizing antibodies is also induced in the FIV-infected cats. Therefore, more detailed information on the feline immune system, specifically on the T cell immune responses, are necessary to design rational and effective vaccines against the virus.

In the thesis, the author described biological and molecular biological studies on peripheral blood T lymphocytes of FIV-infected cats to understand T cell-mediated immune responses well and to help vaccine-development against FIV. In the section of GENERAL INTRODUCTION, molecular properties of CD8 antigen and immune responses of $CD8^+$ T lymphocytes in FIV-infected cats were overviewed for the background of the thesis. In PART I, the author identified cDNAs encoding feline T cell surface molecules and then generated mAbs to the molecules (CD2 in CHAPTER 1, and CD11a, TCRα, TCRδ, and CD122 in CHAPTER 2). In PART II, the author described the improvement of the expression cloning method to shorten its screening procedure

(CHAPTER 3) and its application to identify interactive molecule(s) with FIV (CHAPTER 4). In PART III, the author described functional (CHAPTER 5) and phenotypic (CHAPTER 6) analyses of T lymphocytes of FIV-infected cats by using the antibodies established in PART I. In PART IV, the author generated an mAb to feline CD56 and then analyzed CD56 expression in feline cells (CHAPTER 7) and correlation of CD56 expression and FIV infection in MYA-1 cells, a feline T-lymphoblastoid cell line (CHAPTER 8). In conclusion, the author discussed the data in the thesis collectively.

GENERAL INTRODUCTION

$CD8^+$ lymphocytes in FIV-infected cats

Abstract

T cell glycoprotein CD8 molecules are composed of two chains, α and β, and both $CD8\alpha^{+}\beta^{+}$ and $CD8\alpha^{+}\beta^{-}$ lymphocytes exist. Most of $CD8^{+}$ PBLs of normal cats are $CD8\alpha^{+}\beta^{high}$. However, two subpopulations with reduced or no expression level of β chain, $CD8\alpha^{+}\beta^{low}$ or $CD8\alpha^{+}\beta^{-}$ PBLs, each of which in uninfected cats is usually ≈2%, are increased in cats infected with FIV. In GENERAL INTRODUCTION, the molecular basis of CD8 in several animals and the characteristics of various $CD8^{+}$ lymphocytes in FIV-infected cats are summarized.

$CD8^+$ lymphocytes have very important roles in the regulation of infection with some viruses such as HIV [Yang & Walker, 1997]. The cells generally recognize MHC-restrictedly virus-infected cells and then lyse the cells. In the recognition step(s), CD8 molecules function as both an adhesion molecule and a signaling molecule [Devine & Kavathas, 1999]. However, because CD8 is composed of two polypeptides and the two seem to have different functions [Parnes, 1989], the distinction of various $CD8^+$ subpopulations ($CD8\alpha^+\beta^+$ cells, $CD8\alpha^+\beta^-$ cells, and so on) will be necessary for precise understanding of the cellular functions. $CD8\alpha^+\beta^+$ cells are cells expressing both α and β chains, and $CD8\alpha^+\beta^-$ cells expressing only α chains but not β chain.

CD8 molecules of lymphocytes

The antigen is a membrane glycoprotein composed of two disulfide-linked molecules, α and β chains (of 30-35 kDa MW), which are encoded by two closely linked genes [Gottlieb, 1974; Gorman *et al.*, 1988], and expressed as αα homodimer or αβ heterodimer [Baume *et al.*, 1990; Moebius *et al.*, 1991; Terry *et al.*, 1990]. The αβ heterodimer is mainly expressed in αβ T cells (the latter "αβ" indicates the usage of TCR components) and functions as a co-receptor for recognition of MHC-peptide complex presented by APC and as a following signal transducing molecule [Devine & Kavathas, 1999]. On the other hand, the αα

homodimer is expressed on NK cells and γδ T cells [Baume *et al.*, 1990; Moebius *et al.*, 1991]. CD8 molecule on NK cells dose not seem to be correlated with the cellular functions. Also in antigen recognition by human γδ T cells, CD8 molecule does not seem to be involved in the T cell-APC interaction [Kabelitz *et al.*, 2000]. The $CD8\alpha^{+}\beta^{+}$ cells also express αα homodimers on the same cells [Moebius *et al.*, 1991; Norment & Littman, 1988].

CD8 molecule binds to α_3 (and α_2, probably) domain of MHC class I via α chain of CD8 [Devine & Kavathas, 1999]. The binding not only enhances signal transduction mediated by TCR/CD3 complex which binds to the same MHC class I molecule as CD8, but also strengthens interaction between TCR and MHC [Renard *et al.*, 1996]. There is no major difference in affinity of αα homodimer and αβ heterodimer for MHC [Garcia *et al.*, 1996]. However, the heterodimer can function better (≈100-fold) than the homodimer in interaction with APC for displaying cytotoxicity or producing cytokines [Karaki *et al.*, 1992; Kern *et al.*, 1999; Renard *et al.*, 1996; Wheeler *et al.*, 1992; Witte *et al.*, 1999], because β chain enhances α chain-associated tyrosine kinase activity [Irie *et al.*, 1995, 1998]. Also in respect of direct association with TCR, CD8αβ heterodimer is superior to CD8αα homodimer [Kwan *et al.*, 1998]. CD8β chain is also required for thymic positive selection of CD8-lineage T cells [Nakayama *et al.*, 1994], and phosphorylation of CD3ζ by CD8α-associated tyrosine kinase

occurs in both development and activation of $CD8^+$ cells.

CD8 molecule on γδ T cells and NK cells is αα homodimers in humans and mice, however, only in rats almost CD8 molecule of γδ T splenocytes is αβ heterodimer [Straube & Herrmann, 2000, 2001]. CD8αβ molecules expressed by rat γδ T cells have potential to act as a co-receptor for MHC, but rat γδ T cells seem to show no MHC class I-restriction [Straube & Herrmann, 2000]. In chikens, ≈30% of intestinal intraepithelial lymphocytes with TCRγδ are expressing CD8αβ heterodimer [Tregaskes *et al.*, 1995].

Mouse has two CD8α forms with different cytoplasmic tail length occurred by alternative splicing [Parnes, 1989]. In human, there is a soluble form of CD8α lacking transmembrane and cytoplasmic tail regions arising from alternative splicing [Giblin *et al.*, 1989]. The soluble CD8 is secreted from activated $CD8^+$ T cells in vivo and in vitro [Nishanian *et al.*, 1991; Tomkinson *et al.*, 1989] and can inhibit CTL activity of $CD8^+$ cells [Sewell *et al.*, 1999].

Mouse CD8αα preferentially binds to thymus leukemia antigen (TL), one of non-classical MHC class I antigens, rather than classical MHC [Leishmann *et al.*, 2001]. Because CD8αβ heterodimer dose not bind to TL efficiently, TL/CD8αα interaction may cause efficient interaction of classical MHC/CD8αβ [Liu *et al.*, 2003].

Feline homologue of CD8

The antigen was first identified by Klotz & Cooper in 1986. They established an mAb, clone FT2, reactive to thymocytes. Based on analyses of $FT2^+$ cell distribution in lymphoid cells, function of $FT2^+$ or $FT2^-$ lymphocytes, MW of reactive antigen, FT2 was determined to be an mAb for feline homologue of CD8. In 1990 and 1991, two groups also reported anti-feline CD8 mAbs (clones 3.357 and vpg9), still based on antigen-distribution or antigen-MW [Tompkins *et al.*, 1990; Willett *et al.*, 1993]. However, there were differences between FT2- and 3.357-mAb reactivities against splenocytes (14% and 47%, respectively). In 1998, the author revealed epitopes recognized by the three mAbs by using molecularly cloned feline CD8α and CD8β as follows: CD8β for FT2, CD8α for 3.357, and CD8αβ-complex for vpg9 [Shimojima *et al.*, 1998b]. The reactivity-difference between FT2 and 3.357 might be due to the existence of CD8αα splenocytes. The relationship between CD8 dimer (αβ or αα) and TCR usage (αβ or γδ) on T lymphocytes is not yet revealed in cats due to the lack of available specific antibodies. Whether CD8αα splenocytes are NK cells is also unknown.

Reduced expression of CD8β on PBLs

Lehmann *et al.* [1992] and Willett *et al.* [1993] reported $CD8^+$ PBLs of FIV-infected cats which had a reduced surface

expression level of CD8 molecules. Later, the author elucidated such cells as "$CD8\alpha^{+}\beta^{low}$" cells expressing CD8α at a normal level but CD8β at a lower level (see below for details). Such phenotype of $CD8^{+}$ lymphocytes was further analyzed functionally and phenotypically in the field of feline immunity (FIV and Borna disease virus-infection [Berg *et al.*, 1999]), however in other animals including humans, no study concerning reduction of CD8β molecules was reported until recently.

In humans, quite recently, Schmitz *et al.* [1998] reported that $CD8^{+}$ TCRαβ PBLs having similar phenotype "$CD8\alpha^{+}\beta^{low}$" increased and exhibited strong antiviral activity in HIV-infected individuals. Also in healthy individuals, peripheral $CD8\alpha^{+}\beta^{low}$ T cells were reported to expand with advancing age [Konno *et al.*, 2002; Werwitzke *et al.*, 2003]. CD8β chain reduction associates with intracellular cytotoxic granules and TCR-origoclonality in $CD8^{+}$ T cells [Konno *et al.*, 2002]. $CD8\beta^{low}$ or $^{-}CD5^{-}$ cells are the major source of γ-chemokine lymphotaxin [Stievano *et al.*, 2003]; the loss of CD5 in $CD8^{+}$ T cells resembles CD5 down regulation in feline $CD8\alpha^{+}\beta^{-}$ cells [Shimojima *et al.*, 1998a]. Azuma *et al.* reported $CD8^{+}CD28^{-}$ αβ T cells as effectors [1993], and, later, Konno *et al.* [2002] and Werwitzke *et al.* [2003] reported that the cells expressed CD8β at a low level. In vitro activation of peripheral blood $CD8^{+}$ T cells by anti-CD3 mAb and IL-2 also resulted in reduced expression of CD8β chain

[Werwitzke *et al.*, 2003]. These reports suggest that CD8β chain expression reduces physiologically along with $CD8^+$ TCRαβ cell activation/differentiation.

In rat, expression levels of both CD8 α and β chains on TCRαβ splenic cells are transiently reduced by in vitro stimulation with anti-TCR mAb and IL-2 or CD28-mediated co-stimulation and then retun to the original levels [Straube & Herrmann, 2000, 2001]. In contrast, in case of TCRγδ cells, return to the original levels only occurs for CD8α after transient reduction, resulting in the phenotype $CD8\alpha^+\beta^{low}$ [Straube & Herrmann, 2000, 2001].

Surprisingly, peripheral blood $CD8^+$ cells in uninfected, normal African green monkeys have a population (≈50% of $CD8\beta^+$ cells) with reduced CD8β expression [Holznagel *et al.*, 2002].

$CD8^+$ lymphocyte responses in FIV infection

It is well known that FIV infection results in the increase of $CD8^+$ lymphocyte number in peripheral blood as early as 3 to 4 wk pi [Willett *et al.*, 1993; Bucci *et al.*, 1998b]. The $CD8^+$ PBLs in FIV-infected cats are composed of, at least, three subpopulations of $CD8\alpha^+$ cells; $CD8\alpha^+\beta^{high}$ cells, which are also the most part of $CD8^+$ cells in uninfected cats, $CD8\alpha^+\beta^{low}$ cells with reduced expression of CD8β chain, and $CD8\alpha^+\beta^-$ cells with no (or undetectable) expression of β chain [Shimojima *et al.*,

1998a]. The $CD8^+$ subpopulation with reduced CD8β expression can be observed in peripheral blood as early as 3 wk pi and increases up to 80 to 90% within $CD8\beta^+$ PBLs over 5 years pi [Bucci *et al.*, 1998b; Gebhard *et al.*, 1999]. Thus $CD8^+$ PBL increase by FIV infection is probably due to expansion of $CD8\beta^{low}$ cells [Willett *et al.*, 1993]. In contrast, $CD8\alpha^+\beta^-$ population is often observed in long term-infected cats [Gebhard *et al.*, 1999; Shimojima *et al.*, 1998a]. The increases of both $CD8\alpha^+\beta^{low}$ and $CD8\alpha^+\beta^-$ lymphocytes are also observed in thymus and lymph nodes [Crawford *et al.*, 2001; Gebhard *et al.*, 1999; Orandle *et al.*, 2000]. It is suggested that $CD8\alpha^+\beta^{low}$ cells are formed in thymus and later localized in lymph nodes after a transient expansion in peripheral blood [Crawford *et al.*, 2001]. $CD8\alpha^+\beta^{low}$ cells have the activation phenotype ($CD18^{high}$, $CD44^{high}$, $CD49d^{high}$, $CD62L^-$) compared to $CD8\alpha^+\beta^{high}$ cells ($CD18^{low}$, $CD44^{low}$, $CD49d^{low}$, $CD62L^+$) [Gebhard *et al.*, 1999]. MHC II expression in $CD8^+$ lymphocytes was reported to be enhanced by FIV infection [Bucci *et al.*, 1998b; Willett *et al.*, 1993]. $CD8\alpha^+\beta^-$ cells have reduced expression of CD5 [Shimojima *et al.*, 1998a].

Similar to HIV and SIV infection, FIV infection in cats cause virus-specific CTL response and antiviral non-CTL response in the host. CTL response against viral structural proteins Gag and Env [Flynn *et al.*, 1995; Li *et al.*, 1995] appears 2 to 9 wks pi in peripheral blood, lymph nodes, spleen, and vaginal

intraepithelium [Beatty *et al.*, 1996; Burkhard *et al.*, 2001; Flynn *et al.*, 1996, 2002], and are mainly mediated by $CD8^+$ cells [Flynn *et al.*, 1996; Song *et al.*, 1992, 1995]. The CTL response can also be induced by vaccination [Dunham *et al.*, 2002; Flynn *et al.*, 1994, 1995; Lockridge *et al.*, 2000; Pu *et al.*, 2001; Tellier *et al.*, 1997]. The distinct population(s) within the $CD8^+$ subpopulations mediating CTL response is yet to be defined. However, it is speculated that the population may exist in $CD8\beta^{high}$ cells or $CD8\beta^{low}$ cells rather than $CD8\beta^-$ cells because β chain of CD8 is suggested to be important for efficient interaction between TCR and MHC class I molecules [Arcaro *et al.*, 2000; Irie *et al.*, 1995].

Antiviral non-CTL activity appears 5 to 6 wk pi in peripheral blood, the same time in which humoral immune response also occurs [Bucci *et al.*, 1998a; Fevereiro *et al.*, 1991; Hohdatsu *et al.*, 2000]. Spleen and lymph node $CD8^+$ cells also have the activity [Flynn *et al.*, 1999, 2002; Hohdatsu *et al.*, 2000]. Viral load in thymus or blood or $CD4^+$ cell count in blood was correlated with the $CD8^+$ PBL-mediated antiviral activity [Bucci *et al.*, 1998a; Crawford *et al.*, 2001; Hohdatsu *et al.*, 2003]. The activity is shown to be mediated by unidentified soluble factor(s) [Choi *et al.*, 2000; Flynn *et al.*, 1999; Hohdatsu *et al.*, 1998b], not limited to the homologous FIV strain [Flynn *et al.*, 1999], not restricted to MHC [Choi *et al.*, 2000; Flynn *et al.*, 1999; Hohdatsu

et al., 1998b] and act at mRNA synthesis level [Hohdatsu *et al.*, 2000]. It was also reported that some cats with the strong $CD8^+$ antiviral activity did not seroconvert [Bucci *et al.*, 1998a]. These activities are detected in two subpopulations within peripheral $CD8\alpha^+$ lymphocytes, $CD8\alpha^+\beta^{high}$ [Flynn *et al.*, 2002] and $CD8\alpha^+\beta^{low}$ [Bucci *et al.*, 1998b; Gebhard *et al.*, 1999], however it is unclear whether the subpopulations have different functions or whether $CD8\alpha^+\beta^-$ lymphocytes have similar activities or not. Whether a single cell shows both cytotoxic and non-cytotoxic activities against FIV or not is also unclear at present.

Susceptibility of $CD8^+$ cells to FIV infection

Proviral DNA of FIV can be found from $CD4^+$ T cells and B cells of peripheral blood and lymph nodes of FIV-infected cats [English *et al.*, 1993; Dean *et al.*, 1996] and its burden was greatest in $CD4^+$ T cells during an early stage of infection and in B cells during a chronic stage of infection [Dean *et al.*, 1996]. $CD8^+$ T cells also have provirus and can produce FIV in vitro [English *et al.*, 1993; Dean *et al.*, 1996]. Susceptibility of $CD8^+$ T cell clones was also observed by in vitro-FIV infection [Brown *et al.*, 1991]. However $CD8^+$ T cell in FIV-infected cats have anti-viral activities as described above and, at present, it is not unclear whether the cells are productive reservoirs in infected cats.

Perspective

There are still many questions that should be addressed concerning CD8 molecule/$CD8^+$ cells. For example, what is the significance of CD8β chain reduction? What is the molecular mechanism for CD8β chain reduction without CD8α chain-reduction during immune response or by FIV infection? Or is there any relationship between β chain reduction and memory/effector phases of $CD8^+$ T cells? The author longs for them to be elucidated in the near future, and in addition, for the researchers in the field of FIV/cats to take a leadership in such studies.

PART I

Molecular cloning of feline T cell surface antigens

In many assays used for biological studies, antibodies including polyclonal antibodies and mAbs are the important, indispensable reagents. In the field of immunology, use of the antibodies makes it possible to (i) measure increase/decrease of immune cells absolutely/relatively (ii) induce active/apoptotic signals in cells (iii) sort cell populations and (iv) block intracellular/intercellular interactions, resulting in elucidation of immune status and functions of individual cells or molecules. However, for feline lymphoid cells, a few antibodies are available so far. To my knowledge, specific mAbs for feline CD1a, CD3ε, CD4, CD5, CD8, CD9, CD16, CD21, CD22, CD25, CD69 and MHC IIb, and cross-reactive mAbs for CD10, CD11b, CD11d, CD14, CD18, CD20, CD29, CD30, CD44, CD49b, CD49d, CD49f, CD57, CD61, CD62L, CD81, CD132, MHC I and MHC IIa are reported to date.

Therefore, in PART I, the author identified cDNAs encoding feline T cell surface molecules and then generated mAbs to the molecules. The targeted molecules are CD2 (CHAPTER 1), CD11a, TCRα, TCRδ, and CD122 (CHAPTER 2).

CHAPTER 1

A feline CD2 homologue interacts with human RBCs

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Abstract

A cDNA encoding a feline CD2 was identified. Several aa important for ligand interaction, molecular folding or signal transduction found in other mammalian CD2, were highly conserved in the predicted feline CD2 aa sequence. Feline CD2-expressing COS cells or feline PBMCs were able to form rosettes with human RBCs (probably via human CD58), and the rosette formation was inhibited by an anti-feline CD2 mAb. These results are indicative of the similarity of feline and human CD2 structures. Feline CD2 was found to be expressed in feline peripheral blood T lymphocytes, monocytes and cultured lymphoid cells.

Introduction

T-lymphocyte surface CD2 antigen (also called LFA-2) is a glycoprotein (of ≈50 kDa MW) that is expressed on T cells, NK cells, monocyte lineage and thymocytes. On T cells, CD2 functions as an adhesion molecule to bind to target or APCs [Davis & van der Merwe, 1996]. In addition to this function, CD2 can also transduce several types of signals in T cells, namely activation [Boussiotis *et al.*, 1994; Gollob *et al.*, 1996; Meuer *et al.*, 1984; Wingren *et al.*, 1993] and negative [Palacios *et al.*, 1982; van Wauwe *et al.*, 1981] or apoptotic signals [Dumont *et al.*, 1998; Mourad *et al.*, 1997]. In NK cells, anti-CD2 mAbs can induce up-regulation of IL-2R, leading to the enhancement of cytotoxic activity [Schmidt *et al.*, 1985], and such effect via CD2 requires co-expression of CD16 [Anasetti *et al.*, 1987], whereas CD2-mediated activation of T cells requires CD3 co-expression for its signal transduction [Spruyt *et al.*, 1991]. CD2 expression levels on monocytes are lower than that of T or NK cells, and circulating $CD2^+$ and $CD2^-$ monocytes are thought to be dendritic cells and precursors of macrophages, respectively [Crawford *et al.*, 1999]. In the thymus, CD2 plays a role in pre-TCR function in $CD4^-CD8^-$ double-negative thymocytes and TCR selection events during thymocyte development [Sasada *et al.*, 2001]. CD2 expression on murine B cells [Yagita *et al.*, 1989] and human foetal thymic B cells [Punnonen & de Vries, 1993] has also been

reported, while its function on such cells is unclear [Keogh *et al.*, 1997].

The main ligand for CD2 is CD58 [Davis & van der Merwe, 1996; Wang *et al.*, 1999], which is broadly distributed, being found on non-hematopoietic as well as hematopoietic cells. E-rosette formation of sheep RBCs by human T cells [Bach *et al.*, 1969], a process widely used to identify human T cells prior to the advent of suitable antibodies, is mainly dependent on binding between CD2 on T cells and CD58 on sheep RBCs [Dustin *et al.*, 1987; Plunkett & Springer, 1986; Verbi *et al.*, 1982]. No rodent homologue of CD58 has been identified; instead, the structurally related molecule CD48 has been identified as a CD2 ligand both in mice and rats [Davis & van der Merwe, 1996].

CD2 belongs to the immunoglobulin superfamily [Driscoll *et al.*, 1991]. An extracellular region of CD2 contains two domains which are flexibly linked, and GFCC'C'' β-sheet of the first domain (domain 1) is a binding site for its ligands [Davis & van der Merwe, 1996; Wang *et al.*, 1999]. A cytoplasmic region contains proline-rich sequences [Clayton *et al.*, 1987; Sewell *et al.*, 1986, 1987; Tavernor *et al.*, 1994; Williams *et al.*, 1987]. Several cytoplasmic proteins ($p56^{lck}$, CD2AP, CD2BP1 and CD2BP2) have been shown to bind to the specific sequences of the CD2 cytoplasmic region, and they are considered to be involved in the signal transduction via CD2 [Bell *et al.*, 1996; Dustin *et*

al., 1998; Li *et al.*, 1998; Nishizawa *et al.*, 1998].

To investigate the feline immune system, especially related to FIV infection [Pedersen *et al.*, 1987; Shimojima *et al.*, 1998a], the author and co-workers have generated mAbs specific for feline immunological molecules [Masuoka *et al.*, 1992; Shimojima *et al.*, 1997, 1998b]. In this CHAPTER, the author cloned a cDNA encoding a feline CD2 and used it to generate mAbs reactive to CD2. Further, the author compared the feline CD2 aa sequence with other mammalian counterparts to predict its function in feline immune system. In addition, the author analyzed feline CD2 distribution in feline lymphoid cells.

Materials & Methods

Cells

Feline PBMCs were separated from heparinized peripheral blood of SPF cats by Ficoll-Paque™ (Amersham Pharmacia Biotech, Uppsala, Sweden). The feline PBMCs were washed with cold PBS and used for E-rosette formation and FCM analysis, or for extraction of RNA after 3 days of culture [Pecoraro *et al.*, 1994] in RPMI1640 growth medium containing 10% heat-inactivated FCS, 100 U/ml recombinant human IL-2, 50 μM 2-mercaptoethanol, 2 μg/ml polybrene, 10 μg/ml Con A (Amersham Pharmacia Biotech), and antibiotics. A feline T-lymphoblastoid cell line MYA-1 cells [Miyazawa *et al.*, 1989] were also cultured in the RPMI1640 growth medium without Con A. Human peripheral blood were mixed with the same volume of Alsever's solution (110 mM D(+)-glucose, 27 mM sodium citrate, 2.6 mM citric acid, and 72 mM sodium chloride) and preserved at 4°C until used for E-rosette formation. COS-7 cells and Crandell feline kidney (CRFK) cells [Crandell *et al.*, 1973] were maintained in DMEM supplemented with 10% FCS and antibiotics.

Preparation of cDNA

Poly A^+ mRNA was extracted from cultured feline PBMCs by Quick Prep™ micro mRNA purification kit (Amersham Pharmacia Biotech). The mRNA was annealed with oligo dT primer (Clontech,

Palo Alto, CA) at 70°C for 10 min, and then incubated with Super Script™ II reverse transcriptase (Gibco BRL, Gaithersburg, NJ) at 45°C for 2 hrs to synthesize cDNA.

Identification of feline CD2 cDNA

The homologue cloning method by PCR [Nishimura *et al.*, 1999c], using a feline PBMC cDNA library, was performed (Fig. 1). Briefly, a partial ORF of *feline CD2* cDNA (≈0.4 kb) was first amplified with a primer pair (2HMCD2F and HuMoCD2R) that was designed based on the highly conserved sequences between human [Sewell *et al.*, 1986] and murine [Sewell *et al.*, 1987] *CD2* cDNAs. Next, to analyze regions upstream and downstream of the partial *feline CD2* cDNA, double strand cDNA was synthesized from poly A^+ mRNA by Marathon™ cDNA Amplification Kit (Clontech) without adaptor ligation step and circularized by T4 ligase (Takara, Tokyo, Japan). PCR was performed with the circularized cDNA library and another primer pair (fCD2outR and fCD2outF) designed based on the partial *feline CD2* sequence. The amplified fragments were cloned into T vector pCR2.1 (Invitrogen, NV Leek, the Netherlands) and sequenced using the ABI PRIZM™ 377 auto sequencer (Perkin Elmer, Branchburg, NJ). For confirmation of the *feline CD2* cDNA sequence identified, the PCR and sequencing were performed three times independently.

Sequence analysis

The nucleotide and predicted aa sequences of the *feline CD2* cDNA were analyzed using the default settings of a genetic information processing software GENETYX-MAC ver. 9.0 (Software Development, Tokyo, Japan). Signal peptides and transmembrane regions of aa sequences were determined by the methods of Nielsen *et al.* [1997] and Sonnhammer *et al.* [1998], respectively. Human [Sewell *et al.*, 1986] (accession no. M14362), rat [Williams *et al.*, 1987] (NM 012830), murine [Sewell *et al.*, 1987] (NM 013486) and equine [Tavernor *et al.*, 1994] (X69884) *CD2* sequences were used for comparisons with the feline sequence identified.

Expression of feline CD2

For expression of feline CD2 protein, a primer pair was designed based on the *feline CD2* cDNA sequence (Fig. 1); fCD2exF (5′-AACTAATCCCAAAGATG-3′) is upstream of the start codon, and fCD2exR (5′-CGATTTCTATCGCTTTTA-3′) is downstream of the stop codon. The PCR product amplified by the primer pair was cloned into pCR2.1, then digested with *Eco*RI, and the resultant *Eco*RI fragment was cloned into the *Eco*RI site of pcDNA3.1/Myc-His(+) A (Invitrogen) to produce the expression plasmid pcfCD2. COS-7 cells were transfected with pcfCD2 by electroporation and cultured for 3 days. An irrelevant expression plasmid, pcfCD80 [Nishimura *et al.*, 2000], was used as a negative control.

E-rosette formation

Transfected COS-7 cells or fresh feline PBMCs were suspended in PBS supplemented with 50% heat-inactivated FCS, mixed with human RBCs [Taylor *et al.*, 1975; Kuramochi *et al.*, 1987] and incubated for 20 min at 37°C. The mixture was then centrifuged and incubated on ice for 2 hr. The pellet was gently resuspended and observed by microscopy. To analyze the effect of anti-fCD2 mAb on E-rosette formation, cells were incubated with mAb before mixing with human RBCs.

mAb to feline CD2

Hybridomas producing anti-feline PBMC antibodies were generated by fusion of P3U1 myeloma and splenocytes of BALB/c mice which had been immunized with feline PBMCs [Shimojima *et al.*, 1998b]. Culture supernatants of the cells were screened using IFA against pcfCD2-transfected COS-7 cells. Isotypes of mAbs were determined by a mouse monoclonal typing kit (The Binding Site, Birmingham, UK).

IFA

Cells were washed with PBS, smeared on glass slides and then fixed with cold acetone. The cells were incubated with antibodies for 30 min at 37°C. After wash with PBS, cells were

incubated with FITC-labeled secondary antibodies for 30 min at 37°C. After further wash, cells were observed by UV microscopy.

FCM analysis

Single-color FCM analysis was performed as described previously [Shimojima *et al.*, 1998b]. Cells were washed with cold wash buffer (PBS containing 2% FCS and 0.1% sodium azide) and incubated with antibodies for 20 min on ice. After washing with the wash buffer, the cells were incubated with FITC-labeled secondary antibodies for 20 min on ice and washed with the buffer. Then the cells were analyzed by FACScan flow cytometer with Cell Quest soft wear (Becton Dickinson, San Jose, CA).

For two-color analysis, anti-feline CD4 mAb 45B4 (IgG2a) [Shimojima *et al.*, 1997], anti-feline CD8α mAb 2D7 (IgG2a) [Shimojima *et al.*, 1998b], FITC-labeled anti-feline CD3ε mAb NZM1 (IgG3) (Y. Nishimura *et al.*, unpublished), PE-labeled anti-feline CD5 mAb f43 (Southern Biotechnology Associates, Birmingham, AL, USA), PE-labeled anti-mouse IgG1 antibody (Exalpha, Boston, MA) and PE-labeled anti-mouse IgG2a antibody (Zymed laboratories, San Francisco, CA) were used. In CD2/CD4 or CD2/CD8α-staining, cells were incubated with antibodies in the following order: anti-CD2 mAb, FITC-labeled secondary antibodies, anti-CD2 mAb (for blocking), anti-CD4 or CD8α mAb (mouse IgG2a), and PE-labeled anti-mouse IgG2a antibody. In

CD2/CD5-staining: anti-CD2 mAb, FITC-labeled secondary antibodies, anti-CD2 mAb (for blocking), and PE-labeled anti-CD5 mAb. In CD2/CD3ε-staining: anti-CD2 mAb simultaneously with FITC-labeled anti-CD3ε mAb, and PE-labeled anti-mouse IgG1 antibody. Washings were performed after each incubation for 2 or 3 times and then cells were analyzed.

Northern blot analysis

Total RNA was isolated from CRFK, cultured feline PBMCs and MYA-1 cells using ISOGEN-LS (Nippon Gene, Tokyo, Japan). Five micrograms of total RNA were electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde, blotted onto Biodyne B Membrane (Pall BioSupport, East Hills, NY) and then hybridized with a *Hin*fI fragment of *feline CD2* cDNA (Fig. 1) which was radiolabeled with [α-^{32}P]dCTP by Nick Translation Kit (Boehringer Mannheim, GmbH, Germany). After washing with 2 x SSC (0.3M sodium chloride, 0.03M sodium citrate) containing 0.5% SDS, the membrane was subjected to autoradiography for 4 days [Izumiya *et al.*, 1999].

Results

Cloning and sequence analysis of feline CD2

A cDNA containing a *feline CD2* ORF was cloned (see Materials & Methods) and sequenced (≈1.5 kb, DDBJ accession number AB062551). Sequence analysis of the *feline CD2* cDNA revealed that it contains an ORF of 1008 nt encoding 336 aa residues (Fig. 2). The nucleotide sequence of *feline CD2* ORF has 64 - 72% identity with human, rat, murine and equine *CD2* sequences. The predicted aa sequence (Fig. 2) consists of a signal peptide of 19 residues, an extracellular region of 203 residues, a transmembrane region of 23 residues and a cytoplasmic region of 110 residues. Six potential *N*-linked glycosylation sites in the extracellular region and nine cystein residues are present (Fig. 2). The predicted aa sequence shares 57%, 48%, 46% and 57% identity with human, rat, murine and equine CD2 sequences, respectively, and the highest degree of identity (62 - 71%) is found in the cytoplasmic region.

Interspecies comparison of CD2 sequences

The CD2 aa sequences of five species (cats, humans, rats, mice and horses) were aligned (Fig. 3). One hundred and fifteen aa are identical among the five species. Six regions are probably important for ligand interaction, molecular folding or signal transduction [Arulanandams *et al.*, 1993; Bell *et al.*, 1996;

Dustin *et al.*, 1998; Jones *et al.*, 1992; Li *et al.*, 1996, 1998; Peterson & Seed, 1987; Tavernor *et al.*, 1994; Withka *et al.*, 1993], which are completely or highly conserved in the feline sequence (Fig. 3). They are:

(1) L37[h19] (h corresponds to the aa residue of mature human CD2), W52[h35], A60[h45], L83[h68], I85[h70] and Y96[h81], which are the residues important for contact between two β-sheets (GFCC'C'' and BED) of CD2 domain 1 [these residues are shown as (1) in Fig. 3];

(2) Y101[h86] and G105[h90], which are two residues located within the GFCC'C'' β-sheet of the binding site to CD58 [these residues are shown as (2) in Fig. 3];

(3) the linker region which gives flexibility between domains 1 and 2 [these residues are shown as (3) in Fig. 3];

(4) four (the third to the sixth) of nine cystein residues present in the extracellular domain 2-corresponding region (the first cystein residue is also conserved but positioned in the signal peptide), that would form two disulfide bonds [these residues are shown as (4) in Fig. 3];

(5) the C-terminal stalk that links the extracellular region to the transmembrane region [these residues are shown as (5) in Fig. 3]; and

(6) fifteen contiguous aa residues QQKGPPLPRPRVQPK towards the C-terminus of cytoplasmic domain [these residues are shown

as (6) in Fig. 3].

The three dimensional positions of residues (1), (2) and (4) described above were confirmed by computer modeling (data not shown).

Human and rodent CD2 cytoplasmic regions have been extensively investigated and several regions responsible for signal transduction or binding of cytoplasmic proteins have been determined [Bell *et al.*, 1996; Chang *et al.*, 1990; Dustin *et al.*, 1998; Li *et al.*, 1998; Nishizawa *et al.*, 1998]. Two PPPGHR repeats are important for human CD2 signal transduction [Chang *et al.*, 1990] and are the binding sites for the cytoplasmic CD2-binding protein, CD2BP2 [Nishizawa *et al.*, 1998]. Two peptide sequences called 2 (PGHRPLPPSHR) and 4 (QQKGPPLPRPRV) from rat CD2 sequence have the potential to bind to $p56^{lck}$ [Bell *et al.*, 1996], a protein involved in signal transduction. The peptide sequence 4 is part of the contiguous 15 aa residues described above (Fig. 3) and overlaps the binding sites (PPLPRPR and KGPPLPRPRV) for the CD2-associated protein (CD2AP) and CD2BP1, respectively [Dustin *et al.*, 1998; Li *et al.*, 1998]. The cytoplasmic region of feline CD2 was aligned along with those of human and rodent CD2, and it was revealed that the cytoplasmic sequences described above are highly conserved in the feline sequence (Fig. 4).

Expression of feline CD2 and generation of anti-feline CD2 mAb

Twenty-seven and 22% of COS-7 cells transfected with the feline CD2 expression plasmid pcfCD2 (see Materials & Methods) formed rosettes with human RBCs in two independent experiments (Fig. 5b). Using the transfected COS-7 cells for screening antigens, one mAb (SKR2, IgG1)-producing hybridoma was generated. This mAb blocked the rosette formation completely between human RBCs and fCD2-transfected COS-7 cells (Fig. 5c) or feline PBMCs (data not shown).

Feline CD2 distribution

To investigate feline CD2 distribution on freshly isolated feline PBMCs in single-color FCM analysis, $CD2^+$ (Fig. 6b), $CD3\varepsilon^+$ (Fig. 6c), and $CD5^+$ (Fig. 6d) PBMCs were gated and then light scatters (FSC and SSC) of the gated cells were shown. Two populations of different fluorescence intensities, $CD2^{high}$ and $CD2^{low}$, were observed (Fig. 6b); most of $CD2^{high}$ PBMCs were positioned in the lymphocyte area (Fig. 6f) whereas most of $CD2^{low}$ were in a different area (Fig. 6e). In addition most of the feline $CD3\varepsilon^+$ or $CD5^+$ PBMCs were positioned in the lymphocyte area (Fig. 6g, h).

Two-color FCM analysis of fresh PBLs, which were gated based on the light scatters revealed that most of $CD5^+$ or $CD3\varepsilon^+$ PBLs corresponded with $CD2^+$ PBLs (Fig. 7a). Almost all $CD4^+$ or

$CD8\alpha^+$ PBLs were positive for CD2 (Fig. 7a). Similar results were obtained in PBLs of FIV-infected cats (Fig. 7b).

In Northern blot analysis, a single band at ≈1.5 kb was detected from feline PBMCs cultured for 3 days and the T-lymphoid cell line MYA-1, but not from the renal fibroblast cell line CRFK (Fig. 8). The detected size of 1.5 kb was in agreement with the nt length of cloned *feline CD2* cDNA (AB062551).

Discussion

Crystallographic, mutational and nuclear magnetic resonance analyses [Arulanandam *et al.*, 1993; Driscoll *et al.*, 1991; Jones *et al.*, 1992; Li *et al.*, 1996; Peterson *et al.*, 1987; Withka *et al.*, 1993] have revealed several sequences within the extracellular region of CD2 that are responsible for binding to its ligand or its intramolecular bonds. Several regions important for signal transduction present within the CD2 cytoplasmic domain have also been determined [Bell *et al.*, 1996; Chang *et al.*, 1990; Dustin *et al.*, 1998; Li *et al.*, 1998; Nishizawa *et al.*, 1998]. The author found that such regions are highly conserved in feline CD2 (Figs. 3, 4). Identity of feline CD2 with human CD2 (57% aa sequence identity) is higher than with rodents (46 - 48%); furthermore, feline CD2 has an *N*-linked glycosylation site at N80[h65] [Wyss *et al.*, 1995] and lacks two membrane-proximal cystein residues, as does human CD2 [Yang & Reinherz, 2001] (Fig. 3). Moreover feline CD2 can bind to human RBCs probably via human CD58 (Fig. 5), and conserves 10 (D49[h32], K56[h41], K58[h43], K97[h82], Y101[h86], D102[h87], G105[h90] - V108[h93], Fig. 3) out of 12 aa residues critical for human CD2 binding to human CD58 [Li *et al.*, 1996; Withka *et al.*, 1993]. Taken together, these findings indicate that feline CD2 would behave like other mammalian CD2, especially like human CD2, both in receptor-ligand interaction and in signal transduction.

The feline sequence PPPPSHRPQAPGHR in the cytoplasmic region is identical to its equine counterpart (Fig. 3). Tavernor *et al.* [1994] discussed this region and suggested that equine CD2 may contain a six aa-insertion (SHRPQA) in the first PPPGHR motif. However, this region of feline CD2 is very similar to those of humans and rats (Fig. 4). The author think that the inconsistent alignments (Figs. 3, 4) are caused by the existence of several repeats of similar sequence. In addition, the author suggest that a different interpretation may be possible and that the feline and human, and equine sequences have three and four tandem repeats of PPPPGHR motif in this region, respectively.

Cross-species interaction between feline CD2 and human CD58 (Fig. 5) may occur via the 10 conserved aa residues of CD2 described above, and the epitope recognized by anti-feline CD2 mAb SKR2, which can block interaction between feline CD2 and human CD58 (Fig. 3), might include one or more of the 10 residues. However, no cross-reactivity of the SKR2 mAb to human PBL was observed (data not shown), indicating that other residues in feline original sequence are also involved in the SKR2 epitope.

In FCM analysis using the mAb SKR2, the distribution of CD2 in feline PBLs were very similar to those of other species, as would be expected (Fig. 7). Therefore this mAb will be useful for analyzing feline CD2 distribution and function on T cells in various viral infections in cats [Pedersen *et al.*, 1987;

Shimojima *et al.*, 1998a]. A subpopulation of feline PBMCs, that is different from lymphocytes and has a low level of CD2 (Fig. 6b, e), is probably monocytes, judging from the expression level and the position on light scatters [Crawford *et al.*, 1999]. Therefore the mAb SKR2 will be also a useful tool for investigating feline monocytes. Although plural forms of human and murine *CD2* mRNA were reported [Sewell *et al.*, 1986, 1987], only one size (of 1.5 kb) was detected in cats. The discrepancy may be a result of sensitivities of the assay used or the origins of the cells from which RNA were prepared.

Figure legends

Fig. 1

Schematic structure of *CD2* cDNA and PCR cloning strategy for *feline CD2*. Location and direction of PCR primers are indicated by arrow heads. Amplified fragments are shown by solid lines. *Hin*fI fragment of *feline CD2* cDNA used as a probe for northern blot analysis is indicated by dotted line.

Fig. 2

nt and predicted aa sequences of feline CD2. Polyadenylation signal is in bold italics. Signal peptide is in italics and transmembrane region is underlined. Cystein residues are in bold and potential *N*-linked glycosylation sites are marked [CHO]. Stop codon is indicated by an asterisk (*).

Fig. 3

An interspecies comparison of CD2 molecules. The signal peptide and transmembrane region of feline sequence are in italics and underlined, respectively. Six potential *N*-linked glycosylation sites and nine cystein residues are shaded. Dots are residues that are identical to feline CD2, and the residues completely conserved among five species are indicated by asterisks (*). Completely or highly conserved regions/residues that are boxed have been shown to be important for molecular

folding [labeled (1) and (3)-(5)], ligand interaction [labeled (2)], or signal transduction [labeled (6)] [Arulanandam *et al.*, 1993; Bell *et al.*, 1996; Dustin *et al.*, 1998; Jones *et al.*, 1992; Li *et al.*, 1996, 1998; Peterson & Seed, 1987; Tavernor *et al.*, 1994; Withka *et al.*, 1993]. The feline CD2 sequence in bold is discussed in the text.

Fig. 4

Alignment of feline, human and rodent CD2 cytoplasmic regions. Dots are residues identical to feline CD2. Two repeats of the human PPPGHR motif [Chang *et al.*, 1990; Nishizawa *et al.*, 1998] are boxed. Two peptide sequences that are involved in rat $p56^{lck}$ [Bell *et al.*, 1996] are underlined. Binding sites for CD2AP in the murine sequence [Dustin *et al.*, 1998] and CD2BP1 in the human sequence [Li *et al.*, 1998] are shaded. The feline CD2 sequence in bold is discussed in the text.

Fig. 5

E-rosette formation of COS-7 cells transfected with the expression vector pcfCD80 (negative control, a) or pcfCD2 (feline CD2, b and c). The transfected COS-7 cells were reacted with anti-feline CD2 mAb SKR2 before mixing with human RBCs (c). COS-7 cells and human RBCs can be readily distinguish, the former being larger than the latter.

Fig. 6

FCM analysis of feline PBMCs. Feline PBMCs expressing CD2 (b), CD3ε (c), or CD5 (d) were gated and their light scatters are shown: (a) all feline PBMCs isolated by Ficoll-Paque™, (e) PBMCs weakly positive for CD2; (f) feline PBMCs strongly positive for CD2; (g) PBMCs positive for CD3ε; and (h) PBMCs positive for CD5.

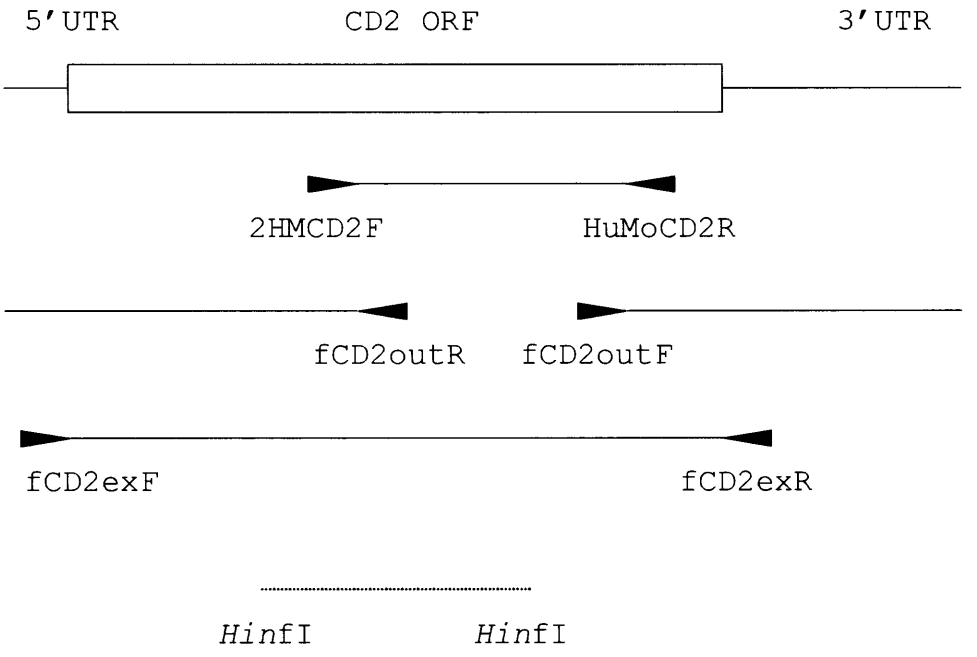
Fig. 7

Two-color FCM analysis of feline PBMCs. PBMCs were double-stained with antibodies indicated and analyzed for PBLs of normal (a) and FIV-infected (b) cats. The number in the corner of each panel indicates the percentage of cells in the area.

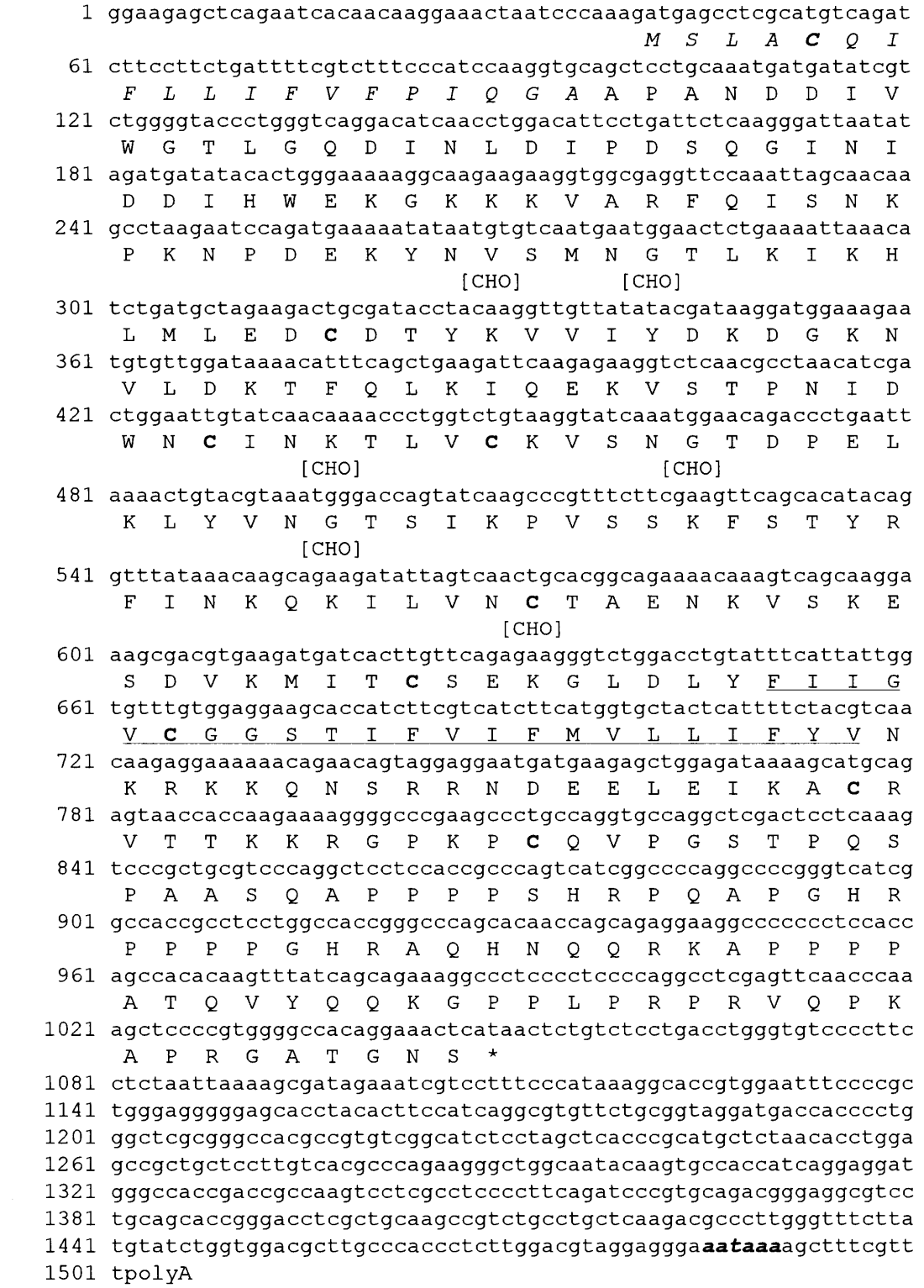
Fig. 8

Northern blot analysis of total RNA extracted from a feline renal cell line CRFK (lane 1), cultured PBMCs (lane 2) and a feline T-lymphoblastoid cell line MYA-1 (lane 3). *Hin*fI fragment of *feline CD2* cDNA (Fig. 1) was used as a probe. The numbers on the left are molecular size markers (kb).

CHAPTER 1/Fig. 1



CHAPTER 1/Fig. 2



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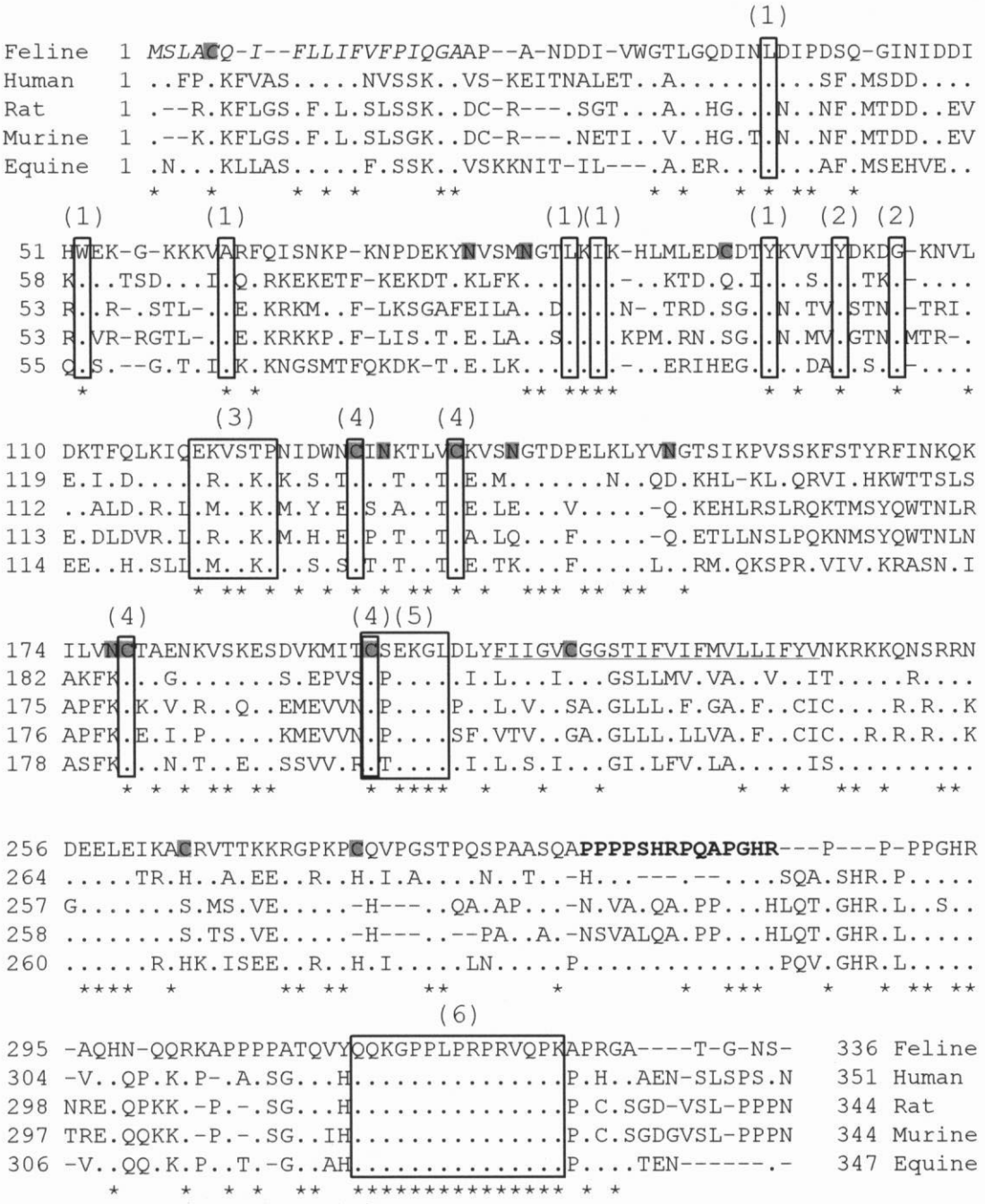
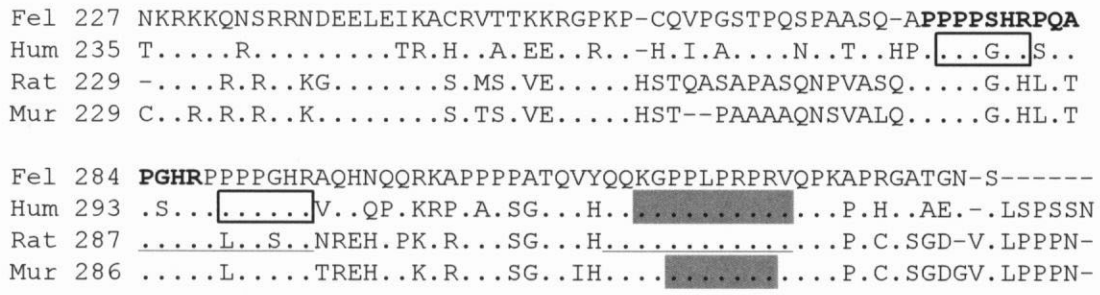
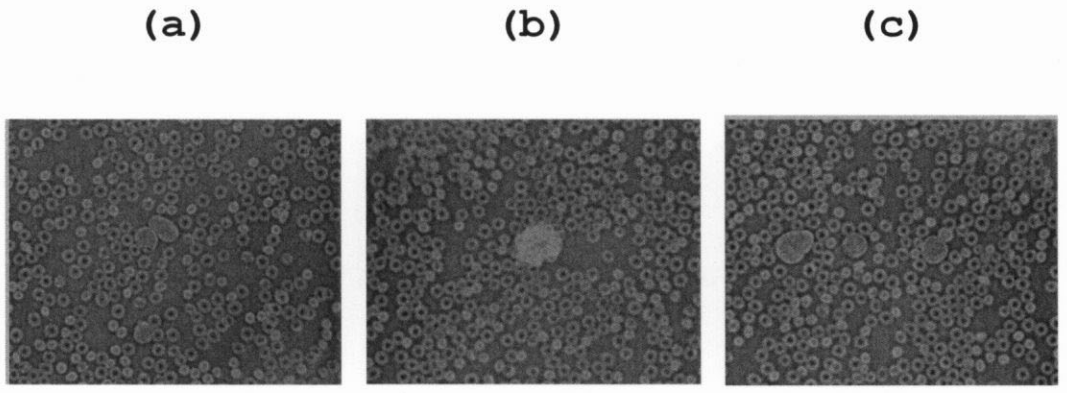


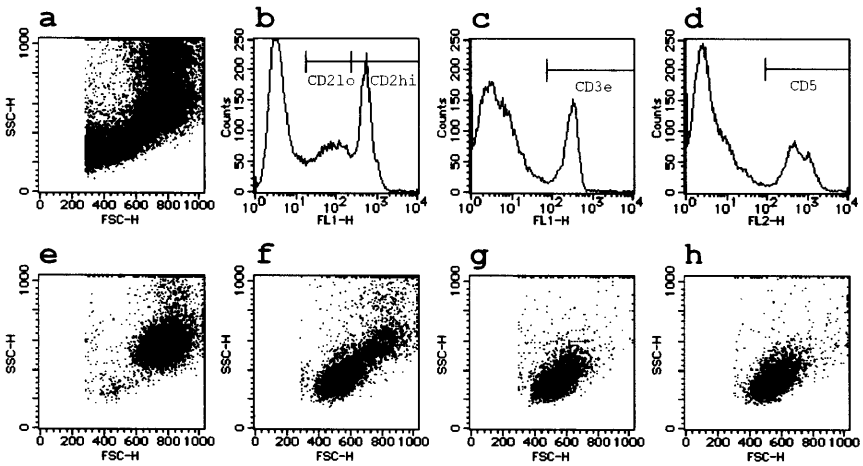
CHAPTER 1/Fig. 4



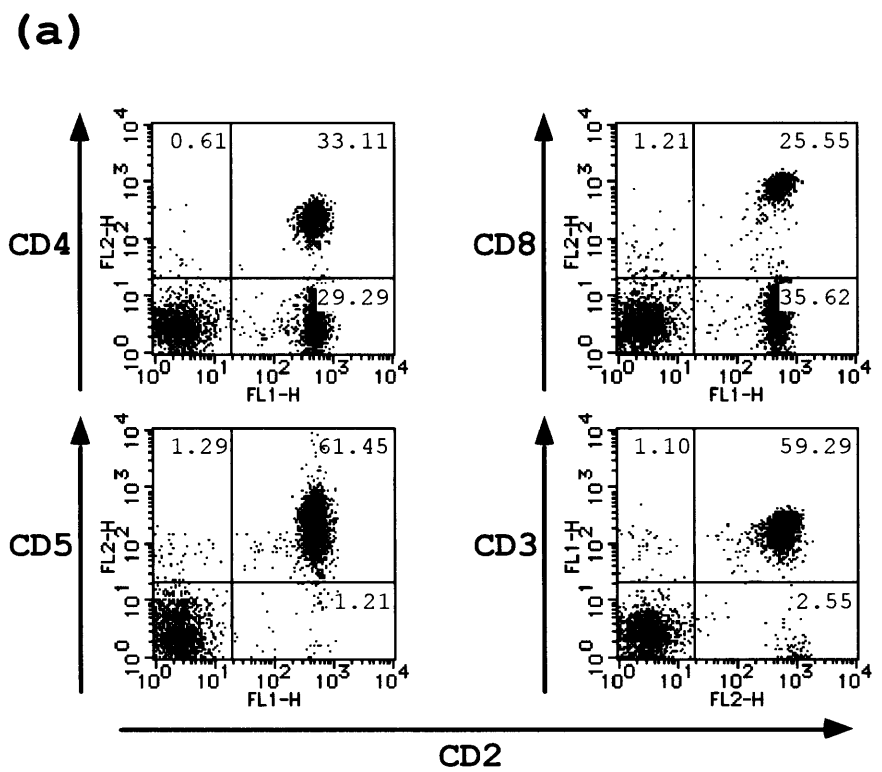
CHAPTER 1/Fig. 5

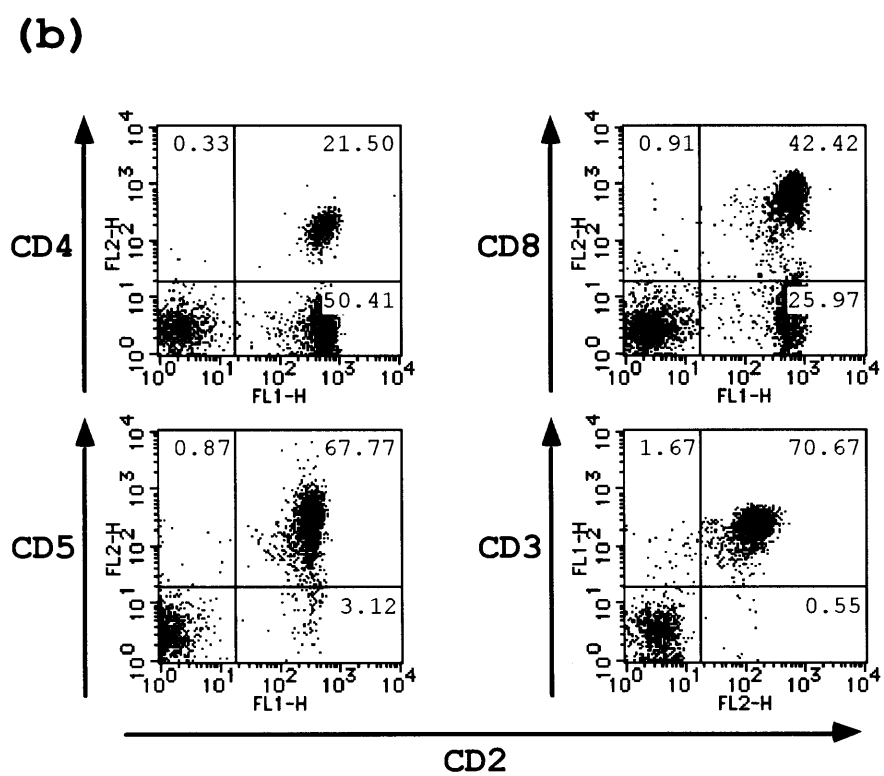


CHAPTER 1/Fig. 6

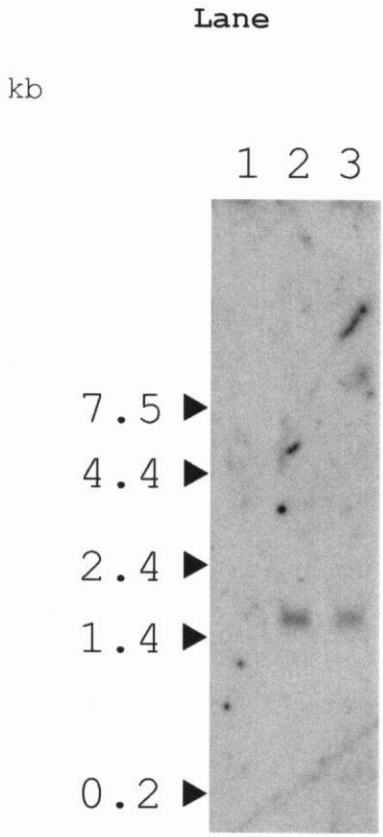


CHAPTER 1/Fig. 7





CHAPTER 1/Fig. 8



CHAPTER 2

Expression of feline CD11a, TCRα, TCRδ, and CD122 molecules

Abstract

The author generated an mAb, clone TMM11a, reactive to feline CD11a antigen expressed in insect cells by recombinant baculovirus. The mAb was also reactive to a feline T-lymphoblastoid cell line MYA-1 and freshly isolated feline PBLs. Based on the known sequences of feline TCRα and TCRδ [Cho *et al.*, 1998], the antigens were expressed in mammalian cells as fusion molecules with histidine tag (HHHHHH) from plasmids. Further, the author identified a partial cDNA of feline CD122 and expressed the antigen likewise. These basic investigations will help further molecular biological studies on the antigens.

Introduction

LFA-1 is a member of the β2 integrin subfamily and expressed on all leukocytes [Krensky *et al.*, 1984]. The ligands of LFA-1 are ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50) on endothelial cells, lymphocytes and APCs [Davignon *et al.*, 1981a, b; Dustin & Springer, 1988]. LFA-1 is composed of two chains, αL (CD11a, ≈180 kDa MW) and β2 (CD18, ≈95 kDa MW), the latter is a common chain in this subfamily including Mac-1, p150/95, and αdβ2. While LFA-1 functions as co-stimulatory molecules in concert with CD28 and CD2 [Abraham *et al.*, 1999; Ragazzo *et al.*, 2001], the interaction between LFA-1 and its ligands needs LFA-1 activation [Lub *et al.*, 1995]. Conformational changes of LFA-1 molecule following β2 phosphorylation by TCR/CD3 are thought to enable LFA-1/ligand interaction [Binnerts & van Kooyk, 1999]. Binding affinity of LFA-1/ICAM-3 is lower than those of LFA-1/other ligands, however, the low affinity LFA-1/ICAM-3 interaction augments LFA-1/ICAM-1-mediated T cell adhesion and signaling by LFA-1 distribution [Bleijs *et al.*, 2000]. It is known that CD11a expression are up-regulated on activated lymphocytes; therefore the up-regulation is a reliable marker for detection of memory/activated lymphocytes [Andersson *et al.*, 1995; Hedlund *et al.*, 1995; Hviid *et al.*, 1993; Okumura *et al.*, 1993; Pitcher *et al.*, 2002; Scala *et al.*, 1995; Slifka *et al.*, 2000].

TCR is composed in its minimal form of TCRαβ or TCRγδ, associated with CD3δε, γε/ζ2 complex [Borst *et al.*, 1996]. The TCRαβ/CD3 complex recognizes peptide-MHC presented by APCs, and the interaction plays important roles in signal transduction in αβ T cells (the cells expressing TCRαβ) [Gao *et al.*, 2002]. In contrast, γδ T cells recognize unconventional antigens that are not seen by αβ T cells, and further the recognition is a non-MHC-restricted fashion [Kabelitz *et al.*, 2000]. Both αβ and γδ T cells as well as NK cells are important in regulation of viral infection [Welsh *et al.*, 1997]. It is reported that a distinct population of γδ T cells was selectively activated and increased by HIV [De Maria *et al.*, 1992; Grottrup-Wolfers *et al.*, 1997; Rossol *et al.*, 1998] or SIV [Gan *et al.*, 1995] infection. Recently, the feline genes encoding constant regions of TCRα, γ, and δ were reported [Cho *et al.*, 1998].

The functional high-affinity receptor for IL-2 is composed of three subunits: CD25 (α), CD122 (β), and CD132 (γcommon) [Minami *et al.*, 1993]. However, CD122 and CD132-complex can form an IL-2R of low affinity in the absence of CD25 and function for IL-2-signal transduction [Theze *et al.*, 1996]. Either of the subunits can not form a functional receptor by itself [Minami & Taniguchi, 1995]. Furthermore, in contrast to $CD4^+$ T cells, freshly-isolated $CD8^+$ T cells express CD122, but not CD25, without in vitro stimulation [Jackson *et al.*, 1990; Johnson & Parkin,

1997; Sheldon *et al.*, 1993; Theze *et al.*, 1996; Zola *et al.*, 1989, 1991]. CD122 appears to be important for the maintenance of $CD8^+$ memory T cells [Ku *et al.*, 2000]. Recently, Ohno *et al.* [1992] identified feline homologue of CD25.

In this CHAPTER, the author expressed feline homologue of CD11a, TCRα, TCRδ, and CD122, and generated an mAb to feline CD11a.

Materials & Methods

Cells and recombinant baculovirus

293T cells were cultured in DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air. Sf9 cells (Invitrogen), an insect cell line, were cultured in TC100 insect medium (Gibco BRL, Gaithersburg, NJ) supplemented with 10% FCS, 0.26% tryptose phosphate broth, and antibiotics at 26°C. The recombinant baculovirus, rAcfCD11a, expressing feline CD11a under polyhedrin promoter, was kindly provided by Dr. Y. Nishimura (National Institute of Infectious Diseases).

Expression plasmid of feline TCRα (pfCD2-fTCRalphain3.1A)

Based on the sequence of the constant region of feline TCRα reported by Cho *et al.* [1998], two primers for PCR were designed: fTCRalphaBamATGver2 (5'-GGATCCATGAAATACATCTTTGGA-3') and XhofTcrAlpha (5'-CTCGAGAACCGCCTTCAGGAAGATGA-3'). The fragment amplified by PCR using the primers and a feline PBMC cDNA library (CHAPTER 1), was cloned in pCR2.1, digested with *Eco*RV and *Xho*I, and then cloned into the *Eco*RV-*Xho*I site of pcfCD2 plasmid (pcDNA3.1/Myc-His(+)A base, CHAPTER 1). The resultant plasmid, which was designated as pfCD2-fTCRalphain3.1A, was expected to express a fusion protein composed of 25 aa of feline CD2 N-terminus

(underlined in Fig. 1a), 6 aa ICRIRL derived from pCR2.1, the cloned feline TCRα (bold in Fig. 1a) and histidine tag (HHHHHH) sequences in frame in this order. The structural schema was shown in Fig. 1a.

Expression plasmid of feline TCRδ (pfCD2-fTCRdeltain3.1A)

Two primers, BamATGfTcrDelta (5'-GGATCCATGGATTTCTATCCTAAGGATAT-3') and XhofTcrDelta (5'-CTCGAGACTCTTGGCAAACACCATCC-3'), were designed based on the sequence of the constant region of feline TCRδ [Cho *et al.*, 1998]. Similar to TCRα, the expression plasmid of feline TCRδ, pfCD2-fTCRdeltain3.1A, was constructed. The structural schema was shown in Fig. 1b.

Identification of a partial feline CD122 cDNA

A partial ORF of *feline CD122* cDNA (≈0.85 kb) was first amplified by the homologue cloning method [Nishimura *et al.*, 1999c] using PCR with a primer pair (CD122F and 2CD122R, Fig. 2) that was designed based on the highly conserved sequences between human [M26062, Hatakeyama *et al.*, 1989], murine [M28052, Kono *et al.*, 1990], and rat [M55050, Page & Dallman, 1991] *CD122* cDNAs (see CHAPTER 1 for details). Next, to analyze a region upstream of the partial *feline CD122* cDNA, PCR was performed with a MYA-1 cDNA library constructed in pMX plasmid, another

primer (fCD122outR) designed based on the partial *feline CD122* sequence, and pMX5F primer (Fig. 2, see CHAPTER 3 for details). The amplified fragments were cloned and sequenced as described in CHAPTER 1.

Expression plasmid of feline CD122 (pCD122-5ATGin3.1A)

For expression of feline CD122 protein fused with histidine tag (HHHHHH), a primer was designed based on the partial *feline CD122* cDNA sequence; fCD122-5ATG (5'-AGGGCCCTGGGGTGATGGC-3') upstream of the start codon. The PCR product amplified by fCD122-5ATG and 2CD122R (existing in the cytoplasmic region of CD122) primers from the MYA-1 cDNA library was cloned into pCR2.1, then digested with *Eco*RI, and the resultant *Eco*RI fragment was cloned into the *Eco*RI site of pcDNA3.1/Myc-His(+) A (Invitrogen) to produce the expression plasmid pCD122-5ATGin3.1A that expresses a fusion protein of the partial feline CD122 with histidine tag (Fig. 1c). The *Eco*RI site within feline CD122 cytoplasmic region was indicated in bold in Fig. 5.

Transfection

Expression plasmids were transfected into 293T cells with MIRUS TransIT-LT1 reagent (Takara) according to the manufacturer's directions. A GFP-expression plasmid, pMX-GFP [Onishi *et al.*, 1996] was used as a control. After 3 days, the

cells were harvested by using 0.02% EDTA solution in PBS and subjected to IFA (see CHAPTER 1) and immunoblotting.

Immunoblotting

Cells were washed with PBS and then lysed with sample buffer (2% SDS, 0.0625M Tris-HCl pH6.8, 20% glycerol, and 5% 2-mercaptoethanol) and boiled for 3 min. The lysates were subjected to SDS-PAGE (12% gel) and transferred onto Hybond-C extra (Amersham Pharmacia Biotech). The membrane was incubated with Tetra-His mouse antibody (Qiagen K.K., Tokyo, Japan), and the antibodies on the membrane were detected using goat anti-mouse IgG + IgM conjugated with horse radish peroxidase (Biosource, Camarillo, CA) together with 3, 3'-diaminobenzidine, tetra hydrochloride.

Results & Discussion

Generation of mAb to feline CD11a

Hybridomas producing anti-feline PBMCs (CHAPTER 1) were screened by IFA with Sf9 cells that were infected with rAcfCD11a. One mAb, termed TMM11a (mouse IgG2b), was established, which was specifically interactive to rAcfCD11a-infected Sf9 cells (Fig. 3). TMM11a was also reactive to a feline T-lymphoblastoid cell line, MYA-1 [Miyazawa *et al.*, 1989], and freshly isolated PBLs of FIV-infected cats by FCM (Fig. 4), indicating that the mAb recognized the epitope existing the extracellular region of feline CD11a. It should be noted that the reactivity of TMM11a against FIV-infected cat PBLs was bimodal; the finding suggest that the mAb will be an important reagent to analyze immunological states of PBLs (see CHAPTER 6 for details).

Cloning and sequence analysis of feline CD122 cDNA

A cDNA containing a partial *feline CD122* ORF was cloned (see Materials & Methods) and sequenced (≈1.2 kb, Fig. 5). The nt sequence of *feline CD122* ORF has 69 - 79% identity with human, murine and rat *CD122* sequences. The predicted aa sequence (Fig. 5) consists of a signal peptide of 27 residues, an extracellular region of 244 residues, a transmembrane region of 23 residues and a partial cytoplasmic region of 94 residues. Three potential *N*-linked glycosylation sites in the extracellular region (shown

as [CHO]) and ten cystein residues (bold) are present (Fig. 5).

Interspecies comparison of CD122 sequences

The predicted aa sequence shares 70%, 61% and 61% identity with human, murine and rat CD122 sequences, respectively. The CD122 aa sequences of four species (cats, humans, mice and rats) were aligned (Fig. 6). One hundred and seventy five aa are identical among the four species. Nine of ten cystein residues observed in feline CD122 are conserved in all other species, while the first cystein exists in the signal peptide (Fig. 5). Three *N*-linked glycosylation sites, underlined in Fig. 6, are also conserved.

A number of cytokine receptors share significant homology and constitute the cytokine receptor superfamily. The extracellular domains contain two major regions of homology. One is a region containing four cystein (C) residues located in the N-terminal half of the domain "C-X_{9-20}-C-X-tryptophan (W)-X_{22-36}-C-X_{8-25}-C". It should be noted that feline CD122 also has this motif (W residue within the sequence is in bold in Fig. 6). Another is "W-serine (S)-X-W-S" motif located proximal to the transmambrane region. In the case of CD122, the motif plays a crucial role in proper folding of the molecule and in growth signal transduction [Miyazaki *et al.*, 1991]. Feline CD122 also conserves the motif (in italics, Fig. 6).

It is reported that CD122 by itself (and other IL-2R components) dose not have any catalytic motifs such as a kinase consensus sequence [Hanks *et al.*, 1988] or any apparent catalytic activity [Hatakeyama *et al.*, 1989], and that intracellular protein tyrosine kinases such as $p56^{lyn}$ [Hatakeyama *et al.*, 1991], $p59^{fyn}$ [Kobayashi *et al.*, 1993], Syk, and Janus kinase 1 (Jak1) [Minami & Taniguchi, 1995], couple to CD122 and mediate the IL-2-induced mitotic signal. A transmembrane-proximal serine-rich region of CD122 cytoplasmic domain is required for the mitotic signals by Syk and Jak1 [Minami *et al.*, 1993; Minami & Taniguchi, 1995]. In feline CD122, 16 of 94 cytoplasmic aa residues identified are serine (Figs. 5, 6).

Taken together, these findings indicate that feline CD122 would behave like other mammalian CD122 both in receptor-ligand interaction and in signal transduction.

Expression of feline TCRα, TCRδ, and CD122 molecules

As shown in Fig. 7, 293T cells transfected with pfCD2-fTCRalphain3.1A (for feline TCRα), pfCD2-fTCRalphain3.1A (feline TCRδ), or pCD122-5ATGin3.1A (feline CD122), were reactive to Tetra His antibody, but not to isotype-matched control mAb, in IFA assay. In immunoblotting (Fig. 8), 25-35 kDa band from TCRα-293 T cell-lysate, ≈28 kDa band from TCRδ lysate, and ≈45 kDa band from CD122 lysate were detected. No

band from pMX-GFP (GFP without no tag-expression plasmid)-transfected cells was detected. The predicted MW of TCRα and δ were ≈22 and ≈21 kDa, respectively, and the molecules had five and one potential *N*-glycosylation sites, respectively. The predicted MW of feline CD122 was ≈37 kDa and the molecule had three potential *N*-glycosylation sites as described above. Therefore, the results obtained by immunoblotting were reasonable.

Culture supernatants of mouse hybridomas (described also for CD11a and in CHAPTER 1) were screened by IFA with the 293T cells transfected with feline TCRα-, TCRδ-, or CD122-expression plasmid, however no positive clones were detected unfortunately. In the future experiment, these proteins would be helpful to generate mAbs and analyze their biological properties.

Figure legends

Fig. 1

Schematic structures of three fusion proteins: feline TCRα (a), TCRδ (b) and CD122 (c). Specific aa sequences of each molecules are in bold. The molecules are fused with N-terminus of feline CD2 (underlined) (CHAPTER 1) and histidine tag (HHHHHH) in C-terminus. Six aa sequence ICRIRL (a, b) is derived from T vector pCR2.1. Sequences existing between specific sequences (bold) and histidine tags are derived from pCDNA3.1/Myc-His(+) A.

Fig. 2

Schematic structure of *CD122* cDNA and PCR cloning strategy for *feline CD122*. Location and direction of PCR primers are indicated by arrow heads. Amplified fragments are shown by solid lines. Two primers CD122F and 2CD122R were designed based on the highly conserved sequence of human and rodent CD122. Other primers had specific sequences of corresponding regions.

Fig. 3

Reactivities of a TMM11a mAb, which was established by using Sf9 cells infected with rAcfCD11a as a screening antigen for IFA.

Fig. 4

FCM analysis with the established anti-feline CD11a mAb TMM11a in a feline T-lymphoblastoid cell line MYA-1 and PBLs of FIV-infected cat. Thin and bold lines are results for mouse serum and TMM11a mAb, respectively.

Fig. 5

nt and predicted aa sequences of partial feline CD122 cDNA. Signal peptide is in italics and transmembrane region is underlined. Cystein residues are in bold and potential *N*-linked glycosylation sites are marked [CHO]. An *Eco*RI site GAATTC in bold was used to subclone the partial cDNA in pCDNA3.1/Myc-His(+) A (details in Materials & Methods).

Fig. 6

An interspecies comparison of CD122 aa sequences. Dots are residues that are identical to feline CD122, and the residues completely conserved among four species are indicated by asterisks (*). A tryptophan (W) residue, which is one of characterictics for the cytokine receptor superfamily, is in bold. WSX(P in this case)WS residue in italics is another characteristic of the subfamily.

Fig. 7

IFA of 293T cells trasfected with expression plasmids for TCRα, TCRδ, and CD122 with control and Tetra His mAbs.

Fig. 8

Immunoblotting analysis of 293T cells trasfected with expression plasmids for GFP (without His tag, lane 1), TCRα (lane 2), TCRδ (lane 3), and CD122 (lane 4), the latter three including histidine tag sequence. The numbers on the left are MW markers (kDa).

CHAPTER 2/Fig. 1

(a) TCRα

MSLACQIFLLIFVFPIQGAAPANDDICRIRL**GSMKYIFGEGTRLTVLPTIQNPDPAVYQL**
KSPESSNISVCLFTDFDSEVNVNPSTESIMIRLKSTSLDMKTMDSKSNGALAWSNSFDLG
CNSTFNYTFHSSSEFPCDANVVEKGFETDMNLNFDNLTVIMLRIIFLKAVLESRGPFEQK
LISEEDLNMHTGHHHHHH

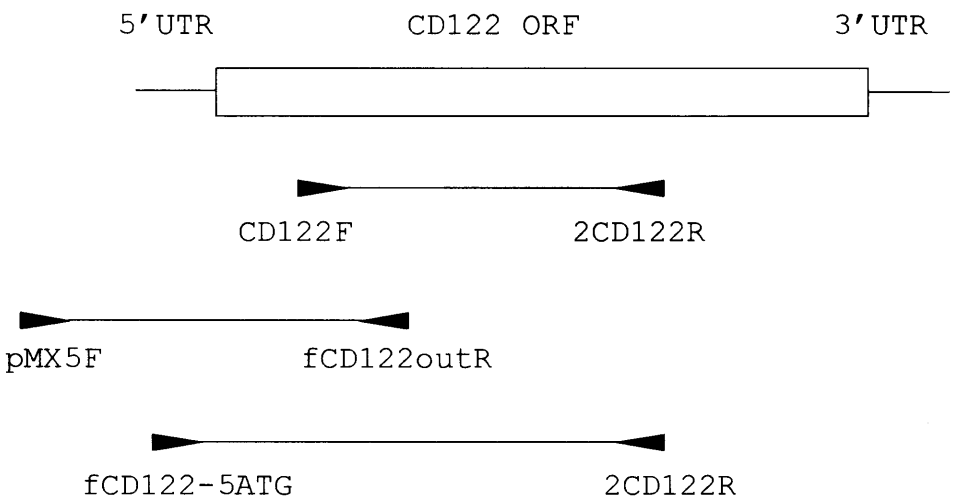
(b) TCRδ

MSLACQIFLLIFVFPIQGAAPANDDICRIRL**GSMDFYPKDINIKLESTKKLEEFDPAIVV**
SPSGKYSAVKLGRYEDPNSVTCSVEHNSEVVSSTNFEPRTNSSGTVKPKPTENENIKLTP
KSISEPKATVHHSSQFTAGNVNMLSLTVLGLRMVFAKSLESRGPFEQKLISEEDLNMHTG
HHHHHH

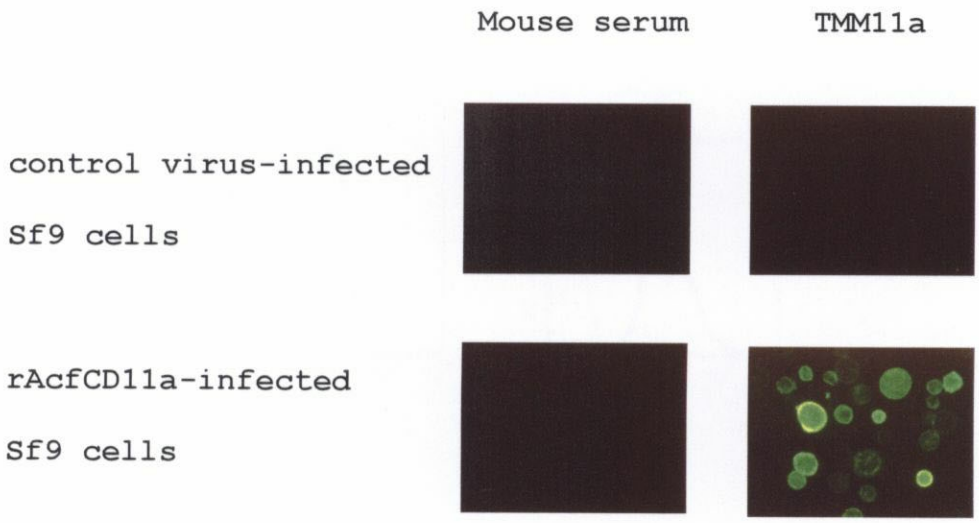
(c) CD122

MAAPGLLWCLSLLVLFLPLAIPRASTAVNDPSKLACFYNSKANISCVWSWDGDLQATSCR
IHAKPDQRSWNKSCELLPMGSASWACYLILGSPDTQSLTSADTVDMRVMCHEGGRWRVLM
TQKFKPFENLRLMAPDSLQVTHVGTHRCNITWKVRQSSHYIERYLEFEARKRSPGHSWEE
ASLLTLAQNQQWISLETLTPDTLYELQVRVRPQLGKNEAWSPWSQPLAFRTRPAAPGKEA
VPFPWLGHIIMGISSAFGFIISVYLLANCRYIGPWLKKVLKCHIPDPSEFCRYPAQWRPL
ESRGPFEQKLISEEDLNMHTGHHHHHH

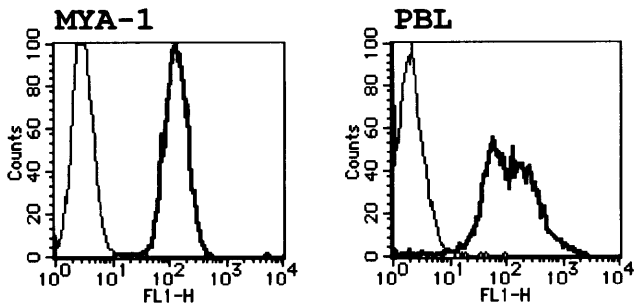
CHAPTER 2/Fig. 2



CHAPTER 2/Fig. 3



CHPTER 2/Fig. 4

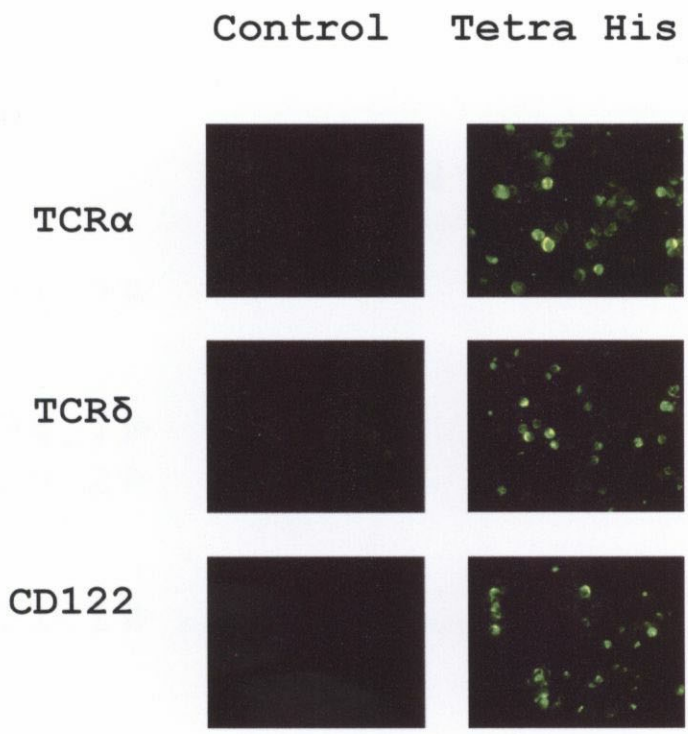


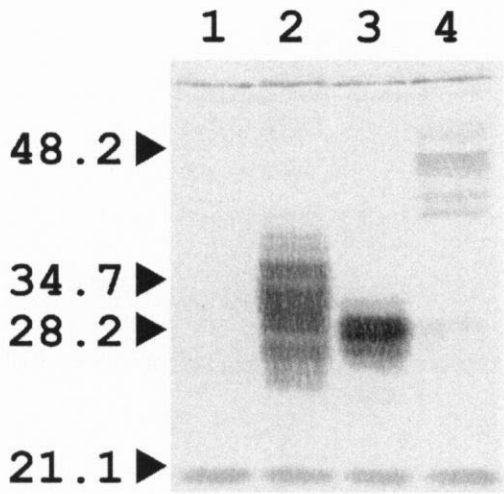
CHAPTER 2/Fig. 5

```
   1 GGGAGTCTGAGTCGGAGCAGGGCCCTGGGGTGATGGCTGTGGCCCCAGCAGCAGAGAGAT
  61 CCCGAGAGAGTGCGCCGCAACCCCATGTCTCAGCCAGGGCTCCCTTCCCTAGCTCCCACA
 121 CATGGATGTGATGGCGGCTCCTGGTCTGCTCTGGTGTCTGTCCCTCCTTGTCCTCTTCCT
               M  A  A  P  G  L  L  W  C  L  S  L  L  V  L  F  L
 181 GCCCCTGGCCATCCCTCGGGCATCCACAGCGGTGAACGACCCCTCCAAGCTCGCGTGTTT
      P  L  A  I  P  R  A  S  T  A  V  N  D  P  S  K  L  A  C  F
 241 CTACAACTCAAAAGCCAACATCTCCTGTGTCTGGAGCTGGGATGGGGACCTACAGGCCAC
      Y  N  S  K  A  N  I  S  C  V  W  S  W  D  G  D  L  Q  A  T
                     [CHO]
 301 ATCCTGCAGGATCCACGCTAAGCCGGATCAACGGTCTTGGAACAAATCCTGTGAGCTGCT
      S  C  R  I  H  A  K  P  D  Q  R  S  W  N  K  S  C  E  L  L
                                                  [CHO]
 361 TCCGATGGGATCGGCCTCCTGGGCCTGTTACCTGATCCTTGGATCCCCAGATACTCAGAG
      P  M  G  S  A  S  W  A  C  Y  L  I  L  G  S  P  D  T  Q  S
 421 TCTGACTTCGGCTGACACCGTGGACATGAGGGTGATGTGCCACGAAGGGGGGAGGTGGAG
      L  T  S  A  D  T  V  D  M  R  V  M  C  H  E  G  G  R  W  R
 481 GGTGCTGATGACCCAGAAATTCAAGCCCTTTGAGAACCTTCGCCTGATGGCCCCCGACTC
      V  L  M  T  Q  K  F  K  P  F  E  N  L  R  L  M  A  P  D  S
 541 CCTCCAAGTCACTCACGTAGGGACCCACAGATGCAACATAACCTGGAAAGTCCGCCAGTC
      L  Q  V  T  H  V  G  T  H  R  C  N  I  T  W  K  V  R  Q  S
                                       [CHO]
 601 CTCCCACTACATTGAAAGATACCTGGAGTTTGAGGCCCGGAAAAGGTCCCCAGGCCACAG
      S  H  Y  I  E  R  Y  L  E  F  E  A  R  K  R  S  P  G  H  S
 661 CTGGGAGGAGGCCTCCCTGCTGACCCTCGCGCAGAACCAGCAATGGATTTCCCTGGAGAC
      W  E  E  A  S  L  L  T  L  A  Q  N  Q  Q  W  I  S  L  E  T
 721 CCTCACTCCAGACACCCTGTATGAACTTCAAGTGCGGGTCAGGCCCCAGCTGGGCAAAAA
      L  T  P  D  T  L  Y  E  L  Q  V  R  V  R  P  Q  L  G  K  N
 781 CGAGGCTTGGAGCCCCTGGAGCCAGCCCCTGGCCTTCAGGACGAGGCCTGCAGCCCCAGG
      E  A  W  S  P  W  S  Q  P  L  A  F  R  T  R  P  A  A  P  G
 841 GAAGGAGGCTGTGCCCTTTCCTTGGCTGGGCCACATCATCATGGGCATCAGCAGTGCCTT
      K  E  A  V  P  F  P  W  L  G  H  I  I  M  G  I  S  S  A  F
 901 TGGCTTCATCATCTCGGTCTACTTGCTGGCCAACTGCCGGTACATCGGGCCATGGCTGAA
      G  F  I  I  S  V  Y  L  L  A  N  C  R  Y  I  G  P  W  L  K
 961 GAAAGTTCTGAAATGTCACATCCCAGATCCCTCAGAATTCTTCTCCCAGCTGAAGTCAGT
      K  V  L  K  C  H  I  P  D  P  S  E  F  F  S  Q  L  K  S  V
1021 GCATGGAGGAGATTTCCAGCAGAAGTGGCTCTCCTCTCCCTTCCCCTCGTCGTCCTTCAG
      H  G  G  D  F  Q  Q  K  W  L  S  S  P  F  P  S  S  S  F  S
1081 CCCCAACACGCCGGCCCCCGAGATCTCCCCGCTGGAGGTGCTGGACAGGGACGCCAAGGC
      P  N  T  P  A  P  E  I  S  P  L  E  V  L  D  R  D  A  K  A
1141 CACACAGTTGCTCCTGCTACAGCAGGAGAAGGTGCCCTCCTCGTCCTCAGCCGAGACCAG
      T  Q  L  L  L  L  Q  Q  E  K  V  P  S  S  S  S  A  E  T  S
1201 CGGCCACTCGCTGA
      G  H  S  L
```

```
Feline   1 MAAPGLLWCLSLLVLFLP-LAIPRASTAVNDPSKLACFYNSKANISCVWSWDG-DLQATS
Human    1 ....A.S.R.P..I-L.LP..TSW..A...GT.QFT.....R........-QDGA..D..
Murine   1 ..TIA.P.S...Y.FL.-L..T.W..A..KNC.H.E.....R..V..M..-HEEA.NV.T
Rat      1 ..TVD.S.R.P.YI.L.-L..TTWV.A....C.H.K.....R..V..M..-PEEA.NV..
             **   * * * *    *  **    * **   *   ***** ** ** **     *  *

          59 CRIHAKPDQRSWNKSCELLPMGSASWACYLILGSP-DTQSLTSADTVDMRVMCHEGGRWR
          59 .QV..W..R.R..QT.....VSQ.....N....A-P.S.K..TV.I.TL..L.R..V...
          59 .HV...SNL.H...T...TLVRQ.....N.....FPES.....V.LL.IN.V.W.EKG..
          59 .H....S.M.H...T...T.VRQ.....N....PLP.S.....V.LLSLS.V.W.EKG..
             *  **    * **  ***     ***** ****     * **  *     * * *   **

         118 VLMT-QKFKPFENLRLMAPDSLQVTHVGTHRCNITWKVRQSSHYIERYLEFEARKRS-PG
         118 -V.AI.D.............I....V..E......S.EIS.A...F..H......T-LS..
         119 RVK.C-D.H..D....V..H....L.ID.Q....S...S.V.....P........R.LLGH
         119 RVK.C-T.H..D....I..H....L.IE.R....S.E.S.V...VNP........R.LLDR
                    * ** **** ** **** *  * **** *   * ***    ******

         176 HSWEEASLLTLAQNQQWISLETLTPDTLYELQVRVRPQLGKNE-AWSPWSQPLAFRTRPA
         176 .T....P....K.K.E..C.........Q..F....K.LQ.EFT-T............K..
         178 -...D..V.S.K.R...LF..M.I.S.S..V....KA.RN-.TGT........T......
         178 -...D..VFS.K.R....F.........S.......IA.R.-KTRT.......M.......
               ** *    * * * *  ** * * * ** ****          *******  *** **

         235 APGKEAVPFP-WLGHIIMGISSAF-GFIIS-VYLLAN-CRYIGPWLKKVLKCHIPDPSEF
         235 .L..DTI.---....LLV.L.G..-...-IL..-.LIN..NT..........NT....K.
         236 D.M..IL.-MS..RYLLLVL-GC.S..FSC-..I.-VK...L.....T............
         236 D.-..IF.-LP..RCLLLVL-GC.F..LSC-.CV.-VK...L.....TL...........
                *   *   **          * **    *  *   **  *****  ***  **** *

         291 FSQLKSVHGGDFQQKWLSSPFPSSSFSPNTPAPEISPLEVLDRDAKATQLLLLQQEKVPS
         289 ....S.E....V.-.............GGL..........E..-.V..-.....D...E
         292 ....S.Q....L.-......V.L.F...SG............G.S..V...-.L.KDSAP
         291 ....S.Q....L.-......V.Q.F...TGS............S.TM.M.-.F.KEKA.
             ****.* **** * ****** * * ***   **********  * *  * * * *

         351 SSSAETSGHSL
         346 -PASLS.N...
         350 LP-SPSG-..Q
         349 .P-SPSG-..Q
                  **
```

CHAPTER 2/Fig. 7




lane
1
2
3
4
48.2
34.7
28.2
21.1

PART II

Improvement and application of expression cloning

There are many mAbs reported whose recognizing molecules have been unknown but show interisting characteristics such as specific tissue/cell-distributions or specific effects on cellular function or pathogen's life cycles. Several strategies can be possible to identify recognizing molecules. If such mAbs' interactions are blocked by another reagent(s) whose interactive molecule has been elucidated, the mAbs may recognize the molecule itself or it's associated one. The other strategy may be possible; in cases that characteristics of molecules recognized by the mAbs, such as MW, tissue distribution, and enzyme-sensitivity, are very similar to those of already known molecules. However the strategy needs such convenient reagents or information and can not reach the molecule-identification mostly. The other method that sequences aa of proteins purified by mAbs may be available. But the protein-purification is comparatively difficult, specifically for proteins with low quantity, and is also expensive. Expression cloning method recently established by Okayama & Berg [1983] is usable to isolate cDNAs encoding cell surface molecules recognized by mAbs and, in fact, the method has made a great contribution to the cloning of cell surface antigens. The method requires several repeats of screening to compensate its inefficiency to date.

In PART II, the author described the improvement of the expression cloning method to shorten its screening procedure

(CHAPTER 3). Furthermore, the author used FIV in place of mAbs and succeeded in identification of its primary receptor (CHAPTER 4).

CHAPTER 3

Usage of myeloma and panning in retrovirus-mediated expression cloning

Analytical Biochemistry 2003; **315**:138-40

Abstract

MLV-mediated expression cloning was improved to reduce the time needed for its screening step. By adoption of P3U1 myeloma and panning, the author could clone cells expressing feline cell surface molecule CD4 by anti-feline CD4 mAb within 1 wk.

Introduction

Expression cloning in mammalian cells is a powerful method for isolating cDNAs encoding cell surface proteins when the antibodies against the proteins are available [Okayama & Berg, 1983]. Two distinct methods have been developed to date, plasmid DNA-mediated [Seed & Aruffo, 1987] and retrovirus-mediated [Kitamura *et al.*, 1995] expression cloning, and have greatly contributed to the cloning of surface proteins. With both methods, however, several rounds of screening are unavoidable. In DNA-mediated cloning, a single cell is usually transduced with more than one cDNA clone by transfection. Therefore, the screening procedure must be repeated to obtain a single cDNA clone. In retrovirus-mediated cloning, although the induction of a single cDNA into one cell is achievable [Onishi *et al.*, 1996], positive cells can not be isolated with high purity from library-induced population by a single round of screening by FCM. Therefore, repeated screening is also required to purify the intended cells. The author report here a simple but powerful screening method that can be accomplished within 1 wk for obtaining cloned cells by panning using myeloma transduced with an expression retrovirus cDNA library.

Materials & Methods

cDNA library and retrovirus stock

A cDNA library of MYA-1 cells, a feline T-lymphoblastoid cell line positive for feline CD4 [Miyazawa *et al.*, 1989, 1992] was prepared and cloned into a retroviral vector, pMX, a derivative of an MLV-based vector [Onishi *et al.*, 1996] as described by Kitamura & Morikawa [2000]. Poly(A)$^+$ RNA of MYA-1 cells was prepared using FastTrack kit ver. 2.0 (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from the RNA with oligo dT_{12-18} primers, random hexamers (Invitrogen), Superscript II RT (Gibco BRL). Then double strand cDNA was synthesized by DNA ligase, DNA polymerase I, and RNaseH. Blunt end-cDNA was adapted with *Bst*XI adaptor (Invitrogen) and ligated to the *Bst*XI sites of pMX vector.The ligated DNA was amplified in DH10B competent cells (Gibco BRL) and plasmid DNA was extracted using Qiagen® plasmid midi kit (Qiagen K. K.). Amplified pMX harboring the MYA-1 cDNA library or pMX-GFP (an expression plasmid for GFP based on the pMX backbone) [Onishi *et al.*, 1996] was transfected into Plat-E packaging cells (expressing structural and enzymatic proteins of ecotropic MLV) [Morita *et al.*, 1996] with MIRUS TransIT-LT1 reagent (Takara) according to the manufacturer's directions. The culture supernatant was harvested 48 hr after transfection and used as retrovirus stock.

Infection of target cells

Three mouse cell lines, embryonic fibroblast NIH3T3 cells, myeloma P3U1 cells and IL-2-dependent T cell line CTLL-2, were used. NIH3T3 cells were cultured in DMEM supplimented with 10% FCS and antibiotics. P3U1 cells were cultured in RPMI1640 growth medium with 10% FCS and antibiotics. CTLL-2 were cultured in RPMI1640 growth medium with 10% FCS, 50 U/ml recombinant human IL-2, and antibiotics. These cells were infected with the retrovirus stock obtained from pMX-GFP-transfected Plat-E cultures for comparison of infection efficiencies as described [Kitamura & Morikawa, 2000]. Briefly, cells were incubated with serially diluted retrovirus stock for 8 hrs in the presence of 8 μg/ml polybrene, washed with culture medium, and then cultured for another 24 hrs. The expression of GFP was measured by FCM.

Cloning of cells expressing feline CD4 molecules

Polystyrene Petri dish (90 mm diameter, Bio-Bik, Osaka, Japan) was incubated at 4 °C overnight with a goat anti-mouse IgG (Rockland, Gilbertsville, PA) prepared at 10 μg/ml in PBS. After being washed with PBS containing 2% heat-inactivated FCS (2FCS-PBS), the dish was further incubated at 37 °C for 30 min with 10 μl ascite of an anti-feline CD4 mAb 4D9 [Shimojima *et al.*, 1997] in 4 ml of 2FCS-PBS. The dish was washed with 2FCS-PBS

and then used for panning. No adhesion of P3U1 myeloma to untreated or antibody-coated dishes was observed. The P3U1 cells (1×10^7 in 4 ml of culture medium) infected with the expression retrovirus cDNA library (at moi of infection of about 0.2) were seeded on the mAb-coated Petri dish and incubated at 4 °C for 90 min. Non-adherent cells were removed gently as follows; tilt the dish for four to five times, aspirate medium well, and then add 4 ml of fresh culture medium slowly along the side of dish. After one more wash, the culture was incubated for 6 days with another wash at day 3. At day 6 the dish was washed 4 times and colony-forming P3U1 cells adherent to the dish were picked up with a penicillin cup.

Sequencing of the integrated retroviruses

From expanded P3U1 clones, genomic DNAs were extracted as follows. Two million cells were washed with PBS, lysed in 500 µl lysis buffer containing 50 mM NaCl, 10mM Tris-HCl (pH 7.4), 5mM EDTA (pH 8.0), and 0.2% SDS, and incubated with 100 µg/ml proteinase K for 5 hrs, followed by phenol-chloroform extraction. Isolated DNA was subjected to PCR using LA *taq* polymerase (Takara) and pMX primers [Kitamura & Morikawa, 2000]. Amplified fragments were cloned into T vector and sequenced using ABI PRISM 377 as described in CHAPTER 1.

Results & Discussion

High infection efficiency of P3U1 myeloma

On infection with the retrovirus to express the GFP gene as the genome, P3U1 myeloma showed higher positive percentages and MFI of GFP than NIH3T3 cells which are commonly used for retrovirus-mediated expression cloning (Table 1). CTLL-2 cells did not show any GFP expression. Therefore, P3U1 myeloma was used for further experiments as target cells for retrovirus-mediated expression cloning.

Expression cloning of feline CD4 cDNA

P3U1 cells were infected with retrovirus to express the MYA-1 cDNA library and then subjected to screening by the panning method with anti-feline CD4 mAb as described in Materials & Methods. Six days after infection, thirteen colonies of adherent P3U1, each of which consisted of 100-200 cells and was visible to the naked eye, appeared in the dish (Fig. 1). Five of them were removed and expanded separately. All five clones showed more than 80% reactivity to the anti-feline CD4 mAb by FCM analysis (Fig. 2): clone #1, 98.7%; clone #2, 83.8%; clone #3, 80.4%; clone #4, 87.0%; clone #5, 92.7%. From genomic DNAs of the cells, 2.5 (two clones) and 1.9 (three clones) kbp fragments were amplified by PCR with the pMX primers (Fig. 3). Each fragment essentially encoded the feline CD4 nt sequence [Norimine *et al.*,

1992], while in the shorter fragments (1.9 kbp), part of the cytoplasmic tail-corresponding region (68 of 114 bp) was missing. Nonetheless, the author succeeded in cloning the intended cDNA from the library by a single round-screening procedure taking less than 1 wk.

Vital to the method reported here is the use of non-adherent target cells (myeloma) and continuation of cell culture in the mAb-coated dish that is used for panning. Basically non-adherent cells such as lymphocytes are not very permissive for MLV-based expression libraries while expressing the receptor for the virus [Pizzato *et al.*, 1999]. This is due to the low efficiency for the Env-independent initial binding of the virus to the cells [Pizzato *et al.*, 1999]. In this study the author found that the P3U1 myeloma is highly sensitive to ecotropic MLV expressing GFP. Usually the P3U1 myeloma cells weakly adhere to "coated" dishes for tissue culture of adherent cells, however they do not adhere to uncoated normal Petri dishes for agar plates. Therefore the author could use the cells for the panning method. A cell expressing the intended molecule adheres to the dish and proliferates at a position where it is trapped, and becomes visible enough to be collected for further expansion (Fig. 1). Cells which do not express the intended molecule can not be eliminated entirely by the initial wash; however their daughter cells are efficiently removed by the wash at days 3 and 6 due

to non-adhesiveness (Fig. 1). Instead of mAbs, other reagents such as certain ligands [Apostolopoulos *et al.*, 2000; Waterhouse *et al.*, 2002] or viral particles [Barton *et al.*, 2001; Golovkina *et al.*, 1998] may be applicable to our method to identify interactive molecules or receptors present on the cell surface if the interactions are extensive enough for cell attachment and do not inhibit cell growth.

Figure legends

Fig. 1

A colony of P3U1 cells formed on an anti-feline CD4 mAb-coated petri dish at day 6 of culture. Before (A) and after (B) 4 washes of the dish. Non-adherent cells in clusters are indicated by arrow heads. White bar represents 1 mm.

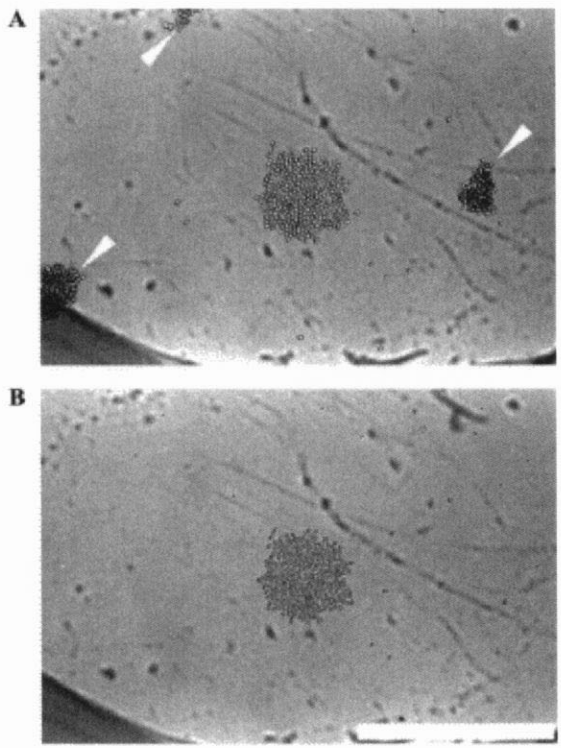
Fig. 2

FCM analysis of five cloned cells for expression of CD4. An irrelevant (thin) and anti-feline CD4 4D9 [Shimojima *et al.*, 1997] mAbs were used. Reactivities of the cells (clones #1, #2, #3, #4, and #5) to anti-feline CD4 mAb were more than 80%. Reactivities to control mAb were less than 1%.

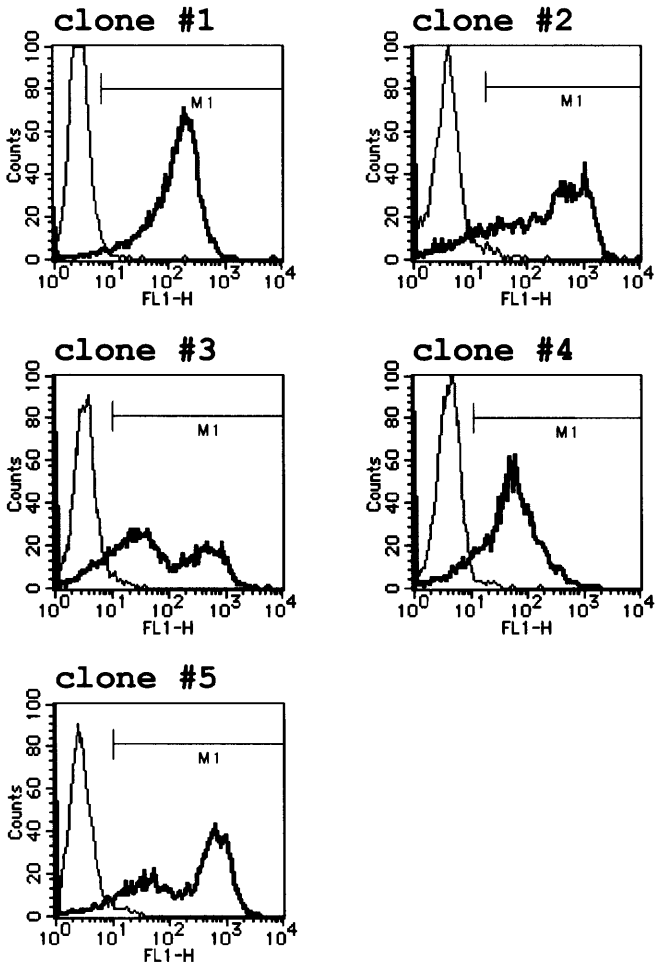
Fig. 3

Amplified fragments by PCR with pMX primers from genomic DNA of normal P3U1 cells (lane N) and five cloned cells (lanes 1 to 5) obtained by panning, corresponding to #1 to #5 in Fig. 2. Lane M is a molecular size marker used.

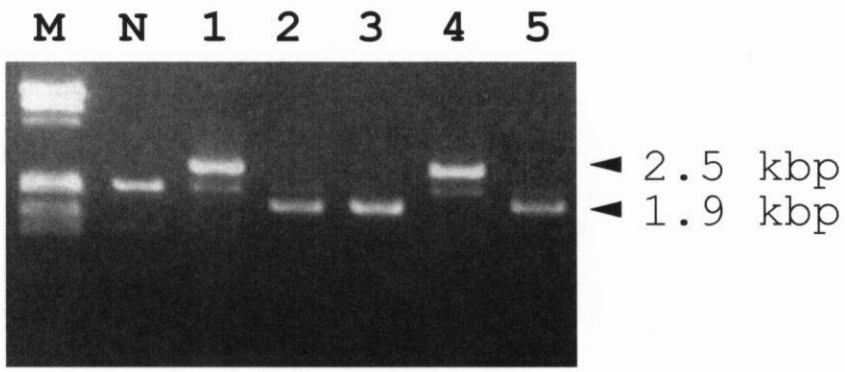
CHAPTER 3/Fig. 1



CHAPTER 3/Fig. 2



CHAPTER 3/Fig. 3



CHAPTER 3/Table 1

Infection efficiencies of mouse cell lines with the GFP-packaging retrovirus

	positive % (MFI)[a]				
	virus dilution				
cell line	control[b]	1:10000	1:1000	1:100	1:10
NIH3T3	1% (9)	1% (16)	1% (43)	6% (120)	36% (231)
P3U1	1% (21)	1% (94)	3% (336)	14% (685)	69% (1330)
CTLL-2	1% (7)	1% (7)	1% (7)	1% (7)	1% (7)

[a]Expression of GFP was determined by FCM.

[b]Culture medium was used as a negative control.

CHAPTER 4

OX40 is a primary receptor for an immunodeficiency-causing lentivirus

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Abstract

FIV induces a disease similar to AIDS in cats yet in contrast to HIV, it does not utilize CD4 as a cell surface receptor. Here, the author identified the primary receptor for FIV as OX40 (CD134), a T_h cell activation antigen and co-stimulatory molecule, by application of the improved expression cloning described in CHAPTER 3. OX40 expression promoted viral binding and rendered cells permissive for viral entry, productive infection and syncytium formation by FIV. OX40-dependent infection was species-specific with FIV displaying a strong preference for feline OX40 compared with human OX40. OX40-dependent infection required co-expression of the chemokine receptor CXCR4, analogous to infection with X4 strains of HIV. Thus, despite the evolutionary divergence of the feline and human lentiviruses, both viruses utilize cell surface molecules that target the virus to a subset of cells that is pivotal to the acquired immune response.

Introduction

As the initial stage of HIV infection, the viral Env interacts with T cell surface molecule, CD4 [Dalgleish *et al.*, 1984; Maddon *et al.*, 1986]. The interaction causes conformational changes of Env to promote fusion of the viral and cellular membranes [Berger *et al.*, 1999; Clapham *et al.*, 1999; Wyatt & Sodroski, 1998]. However, expression of CD4 alone is insufficient to HIV infection, and CC- [Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996] or CXC-chemokine [Feng *et al.*, 1996] receptor is required as a co-receptor for the second fusion step. While, in certain cases of HIV-1 [Bandres *et al.*, 1998; Dumonceaux *et al.*, 1998; Hesselgesser *et al.*, 1997], HIV-2 [Endres *et al.*, 1996; Potempa *et al.*, 1997; Reeves *et al.*, 1997; Reeves & Sculz, 1997], and SIV [Edinger *et al.*, 1997; Martin *et al.*, 1997; Potempa *et al.*, 1997], Env/co-receptor interactions have been reported in the absence of CD4, such viruses retain the ability to bind CD4 and CD4 binding enhances fusion or infection functionally.

Similar to the case of HIV, FIV infects preferentially $CD4^+$ cells and induces depletion of the cells in chronically-infected cat PBLs, often resulting in immunodeficiency [Ackley *et al.*, 1990; Beatty *et al.*, 1996; Kohmoto *et al.*, 1998b; Willett *et al.*, 1993; Yamamoto *et al.*, 1988]. However, CD4 molecule on lymphocytes dose not seem to be the primary receptor for FIV; CD4 expression in a feline kidney

cell line, CRFK, did not render them susceptibility to the viral infection [Norimine *et al.*, 1993], and antibodies recognizing diverse epitopes on CD4 could not inhibit FIV infection [Hosie *et al.*, 1993; Willett *et al.*, 1997b]. While mAbs for CD9 have been shown to inhibit FIV infection [Hosie *et al.*, 1993], the later studies suggest that the mAbs block postentry stage of viral life cycle [Willett *et al.*, 1997a], but not entry step. In contrast, chemokine receptor CXCR4 is profoundly involved in infection of laboratory adapted FIV strains; (i) ectopic expression of CXCR4 permits the viral infection in non-permitting cells or fusion between the cells and FIV-infected cells [Willett *et al.*, 1997b], (ii) antagonists or ligands (and their short peptides) for CXCR4 can inhibit infection, viral binding to cells, or the cell fusion [de Parseval & Elder, 2001; Endo *et al.*, 2000; Johnston & Power, 2002; Richardson *et al.*, 1999; Willett *et al.*, 1997b]. Recently, 42 kDa molecule, called gp95L by de Parseval & Elder [2001], but not CXCR4, is shown to interact directly with FIV Env-Fc fusion protein and may be necessary for infection of lymphotropic FIV strains.

In this CHAPTER, the author screened an interactive molecule(s) with FIV from MYA-1 cells by the improved expression cloning method described in CHAPTER 3 with some modifications. Feline homologue of OX40 (CD134) was identified as the primary receptor for FIV.

Materials & Methods

Cells and Viruses

Normal CRFK cells or the cell line persistently infected with FIV Petaluma strain (CRFK/Pet) was maintained in DMEM supplemented with 10% FCS and antibiotics. Cells (Plat-E, NIH3T3, P3U1), viruses (MLV), plasmids (retroviral vector pMX), and MYA-1 cell cDNA library used for expression cloning were as described in CHAPTER 3. Stable expression of feline CD2, FeLVC-R, or other molecules in CRFK, feline AH927, or human HeLa cells were obtained by G418 (Gibco BRL) selection in DMEM/10% FCS following transfection with a mammalian expression vector encoding both an appropriate cDNA and neomycin resistant gene in one plasmid.

Preparation of Petri dish for expression cloning of FIV-interactive molecule

Petri dishes coated with anti-mouse IgG (CHAPTER 3) were further incubated with anti-FIV Env mAb 5F7 (IgG1) (IDEXX, Westbrook, ME). After being washed, the dish was incubated with 10 ml culture supernatant of CRFK/Pet cells for 30 min at room temperature with occasional tilting. The culture supernatant was changed with fresh culture supernatant of CRFK/Pet and then incubated. After one more change and incubation, the dish was further incubated with 8 ml culture supernatant of CRFK/Pet cells pre-mixed with 2 ml of 5% paraformaldehyde. The dish was washed

with 2FCS-PBS for 5 times and used for screening in expression cloning, similar to the case of CD4 cDNA cloning (CHAPTER 3), to identify interactive molecules with FIV from MYA-1 cDNA library.

FCM analysis for virus binding

Adherent cells were detached by incubation with 0.02% EDTA solution in PBS at room temperature. Cells (1×10^5) were washed with PBS and incubated with 1 ml of prefiltered culture supernatant and incubated for 1 hr on ice. After being washed with cold wash buffer (CHAPTER 1), the cells were incubated with anti-FIV Env 5F7 antibody for 20 min on ice. After further incubation with PE-labeled anti-mouse IgG1 secondary antibody (Exalpha), the cells were analyzed with FACScan and Cell Quest.

Immunoblotting

Sample preparation, SDS-PAGE, and membrane-transfer were performed as described in CHAPTER 2. For detection of feline OX40 antigen, monoclonal anti-human CD134 (OX40) (Clone Ber ACT 35, Ancell, Bayport, MN) was used as a first antibody. An isotype-matched mAb (mouse IgG1, anti-feline CD8α, 10C7 [Shimojima *et al.*, 1998b]) was used as a negative control.

Membrane fusion assay

The F0556, F0827, F0425 and F1419 *envs* were amplified by PCR using the oligonucleotide primers 5′-GGGTCGACACCATGGCAGAAGGGTTTGCAGCA-3′ and 5′-GGGCGGCCGCCATCATTCCTCCTCTTTTTCAGAC-3′ from DNA prepared from PBMCs infected with primary field isolates of FIV from the United Kingdom. Env cDNAs (representing the entire *env* ORF) were cloned into *Sal*I and *Not*I sites of VR1012 (Vical Incorporated, San Diego, CA.) and their nt sequence confirmed. AH927 cells stably expressing FeLVC-R or OX40 were transfected with each *env* construct using Superfect (Qiagen) and incubated for 48 hrs at 37°C. The cells were then fixed and stained with 1% methylene blue/0.2% basic fuchsin in methanol and photographed using a Leica DMLB microscope (Leica Microsystems (UK) Ltd., Milton Keynes, UK) and Photometrics SenSys digital camera (Photometrics Ltd., Tucson, AZ).

Infectivity of OX40-expressing cells with FIV (RFLP)

To detect FIV provirus and analyze its genetic subtyping, PCR-RFLP analysis was performed as reported by Hohdatsu *et al.* [1998a] and Kurosawa *et al.* [1999] with some modifications. Using genomic DNAs of FIV-inoculated, then 14-day cultured CRFK cells (CHAPTER 3), a 329-bp fragment of *gag* gene (position 1036-1364 in Petaluma and TM2 strains) was amplified by PCR with a primer pair (5′-TATTCAAACAGTAAATGGAG-3′ and

5'-CTGCTTGTTGTTCTTGAGTT-3') and EX *taq* polymerase (Takara). Genomic DNAs of MYA-1 cells (harvested 3 days after inoculation) were also used for PCR templates. Amplification was for 4 min at 94°C as the first denaturation followed by 35 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C and synthesis for 1 min at 72°C. The PCR products were analyzed by electrophoresis directly or after being treated with either *Hin*dIII or *Pvu*II restriction enzyme.

Infectivity of OX40-expressing cells with FIV (IFA)

CRFK cells were harvested with EDTA solution at 1, 6, and 14 days pi and then analyzed by IFA as described in CHAPTER 1. FIV-infected cat serum, uninfected cat serum, and anti-FIV mAb 5F7 were used as first antibodies. Positive percentages were counted under UV microscope.

Infectivity of OX40-expressing cells with FIV (p24 ELISA)

Culture supernatants of CRFK were harvested at indicated days pi and p24 antigen was detected using a commercial kit (FIV Antigen Test Kit) (IDEXX) according to the manufacture's protocol.

Growth of FIV (reverse transcriptase activity)

The growth of FIV in vitro was also assessed in AH927 cells

expressing FeLVC-R or OX40. 1 x 10^5 cells were plated in 6 well culture plates and cultured overnight. The cells were then incubated with 0.45 mm-filtered culture supernatant from FIV infected MYA-1 cells containing approximately 10^4 $TCID_{50}$ (titered on MYA-1 cells) of virus for 2 hrs at 37°C. The medium was then aspirated and the cells washed twice with PBS and re-fed with culture medium. Supernatants were collected every 3 days and assayed for reverse transcriptase activity using Lenti-RT non-isotopic RT assay kit (Cavidi Tech., Uppsala, Sweden). Reverse transcriptase values were then calculated relative to purified HIV-1 reverse transcriptase standard.

HIV Pseudotype assay

Five µg of each VR1012-*env* construct (expression plasmid for FIV Env protein) or pCI-VSV-G (for VSV-G glycoprotein) and 7.5 µg of pNL4-3-Luc-E^-R^- (for HIV core protein and luciferase gene) were co-transfected into 293T cells using Superfect. Culture supernatants were collected at 48 hrs post-transfection, filtered at 0.45 mm and frozen at -70°C until required. AH927-FeLVC-R or AH927-OX40 were seeded at 1 x 10^4 cells per well of a CulturPlate™-96 assay plate (Packard Biosciences = PerkinElmer Life Sciences) and cultured overnight. The cells were then infected with 50 µl HIV (FIV) luciferase pseudotypes, cultured for 72 hrs and then luciferase activity quantified by

the addition of 50 µl of LucLite Plus™ (Packard) luciferase substrate and measurement by single photon counting on a TopCount luminometer (Packard).

CXCR4-dependence

CXCR4-dependence of OX40-mediated infection was evaluated by infecting AH927-OX40 cells with HIV (FIV) luciferase pseudotypes (described above) in the presence of the selective CXCR4 antagonist the bicyclam AMD3100 (Dominique Schols, Brussels, Belgium). Cells were seeded into 96-well plates as above and cultured overnight. The culture medium was then replaced with medium containing AMD3100 and incubated for 1 hr at 37°C. Luciferase pseudotypes were then added and incubated for a further 2 hrs at 37°C, at which time the medium was aspirated, the wells rinsed twice, and fed with fresh culture medium containing AMD3100. The cells were then cultured for a further 72 hrs and assayed for luciferase activity as above.

Results & Discussion

Adhesion of MYA-1 cells, a non-adherent lymphoblastoid cell line [Miyazawa *et al.*, 1989], to the FIV-coated Petri dish (Materials & Methods) was observed irrespective of formalin treatment of the dish used (data not shown). No adhesion of the cells to dishes that were coated with irrelevant mAbs instead of anti-FIV Env nor incubated with culture supernatant of normal CRFK cells instead of CRFK/Pet were observed (data not shown). Therefore the adhesion of MYA-1 cells to the FIV-coated dish was very likely the result of specific interaction between FIV particle and its receptor(s) on MYA-1 cells.

Using the FIV-coated dish for panning, MYA-1 cDNA library was induced into P3U1 cells, and screened as described in CHAPTER 3 and 14 cell clones (No. 1 to No. 14) were obtained. From the genome of clone No. 5, ≈1 kbp DNA was specifically amplified by PCR with pMX primers [Kitamura & Morikawa, 2000]. The fragments were cloned and sequenced in T vector. Sequence analysis revealed that the amplified fragment contains the ORF of 810 nt with 5'-untranslated 1 nt and 3'-untranslated 93 nt (Fig. 1, AB128982). The predicted 270 aa sequence (Fig. 1) has 67.6%, 64.4%, and 61.8% identity with human (NM 003327 [Latza *et al.*, 1994]), murine (NM 011659 [Calderhead *et al.*, 1993]), and rat OX40 (NM 013049 [Mallett *et al.*, 1990]) molecules (Fig. 2), respectively, also called CD134. The predicted sequence consists of a signal peptide

of 29 residues, an extracellular region of 213 residues, a transmembrane region of 23 residues and a cytoplasmic region of 34 residues (Fig. 1). One potential *N*-linked glycosylation site in the extracellular region and 17 cystein residues are present, and moreover, they are completely conserved in four animals of cats, humans, mice, and rats (Fig. 2). GGSFRTPI aa sequence (Fig. 2) in the cytoplasmic region of human OX40 was demonstrated to be required for TRAFs and NF-κB-associated activation [Kawamata *et al.*, 1998] and is highly conserved among the four animals (Fig. 2). TRAF2 activation seems to be linked to OX40-mediated memory T cell expansion and survival [Prell *et al.*, 2003].

To confirm binding of OX40 with FIV, the identified cDNA was subcloned into *Bst*XI site of pMX retroviral vector plasmid after digestion with the restriction enzyme. Resultant plasmid was designated as pMX-fOX40. The other plasmid pMX-fCD2 was also constructed from pcfCD2 (CHAPTER 1) to express feline CD2 molecule. pMX-fOX40 was transfected into the packaging cell line Plat-E, and culture supernatant, which was including MLV containing feline OX40 gene as its genome, was used for infection of P3U1 cells at a dilution of 1:10 (CHAPTER 3). The infected P3U1 expressing feline OX40, as well as MYA-1 cells (described above), specifically adhered to FIV-coated, but not to control, Petri dish (data not shown). Therefore, the author further

analyzed the interaction by FCM analysis using other cells, transfected Plat-E and infected NIH3T3 cells. As shown in Fig. 3a, the binding of FIV Petaluma to pMX-fOX40-, but not to control pMX-fCD2-, transfected Plat-E cells was observed. The viral binding was also observed against NIH3T3 cells infected with OX40-expressing MLV, but not with CD2-expressing MLV (Fig. 3b). As shown in Fig. 4a, these viral binding were also observed to OX40-expressing feline CRFK cells. A slight shift in FCM histogram for FIV/fCD2-CRFK (Fig. 4b) was due to the interaction between cell culture-adapted strain FIV Petaluma and heparin sulfate abundantly present in CRFK cells or CXCR4 directly [de parseval & Elder, 2001; Hosie *et al.*, 1998]. In addition, FIV TM2, which can infect lymphoid cells but not non-lymphoid cells [Miyazawa *et al.*, 1989; Norimine *et al.*, 1993], also bound OX40-expressing cells, though with a weak intensity (Fig. 4a), but not CD2-expressing CRFK (Fig. 4b). Similar weak bindings of FIV TM2 were also found to MYA-1 lymphoid cells (Fig. 4c), suggesting the physiological significance of the weak intensity. These results confirmed the specific binding of FIV particle and cell surface molecule OX40.

Antibody cross-reactivity of anti-human OX40 to feline OX40 was observed by FCM and OX40 expression intensities on OX40-CRFK and MYA-1 cells were well correlated with FIV binding (Fig. 4). The cross-reactivity of the mAb could also be confirmed

by IFA (data not shown) and immunoblotting (Fig. 5). The mAb did not inhibit OX40-FIV interaction (data not shown); it might recognize different epitopes from viral binding site on OX40.

Next, to test the ability of OX40 molecule to support FIV Env-mediated fusion, AH927 cells were transfected with FIV Env-expression constructs and examined for syncytium formation (Fig. 6). Transfection of AH927-OX40 cells with constructs bearing the *env* genes from primary isolate GL8 led to the development of syncytia whereas no syncytia were observed in the control cultures expressing the FeLVC-R (Fig. 6). Similar results were obtained in the case of other primary isolates F045, F0556, F0827, F1419 (data not shown). In contrast, PET *env* induced syncytium formation in both the OX40- and FeLVC-R-expressing cells (Fig. 6), consistent with this Env interacting with CXCR4 in the absence of a primary receptor [Hosie *et al.*, 1998] (analogous to CD4-independent strains of HIV-2 [Endres *et al.*, 1996]). Thus, the ability of feline OX40 to support fusion of Envs from primary FIV isolates was demonstrated.

FIV TM2 infection in fCD2-CRFK could not be yet detected even in cellular genomic PCR (Fig. 7), consistent with previous study [Norimine *et al.*, 1993]. In OX40-CRFK cells, a clear band at approximately 330 bp was detected in the PCR (Fig. 7, lane 6). The band could not be distinguishable from those both in TM2-infected MYA-1 cells (lane 2) and in Petaluma-infected CRFK

cells (lane 7) in respective of molecular size, suggesting the infectivity of TM2 in OX40-CRFK cells. In addition, in RFLP the patterns of lane 6 band were the same of TM2 (lane 2) but not Petaluma (lane 7). These results meant that OX40 permitted infection of the lymphotrophic FIV strain in non-lymphoid CRFK cells. The fact was further supported by detection of FIV antigens in cells and culture supernatants. As shown in Fig. 8, 2.2, 4.0, and 4.1 % of OX40-CRFK cells which had been inoculated with FIV TM2, were positive in IFA using FIV-infected (but not normal) cat serum at days 1, 6, and 14 days post inoculation, respectively. Similar results were obtained using an anti-FIV Env mAb (data not shown). In the assay, none of CD2-CRFK cells was negative irrespective of virus inoculation (data not shown). As shown in Fig. 9, p24 capsid antigens in culture supernatants were also detected from TM2-inoculated OX40 cells but not from CD2 cells. Thus the results indicated that OX40 permitted productive infection of the lymphotrophic FIV strain in non-lymphoid CRFK cells.

Cell culture adapted strains of FIV can entry human cells via a high affinity interaction with human CXCR4, although human cells remain refractory to entry of primary FIV strains. The author therefore examined the species specificity of the interaction between FIV and OX40. The human cell line HeLa, which expresses high levels of CXCR4, was stably transduced with either

feline or human OX40 and surface expression of OX40 was confirmed by FCM (data not shown). Fig. 10 shows that while ectopic expression of feline OX40 rendered HeLa cells permissive for infection with primary FIV strain GL8 or TM2 pseudotypes, human OX40 expression had little effect. In contrast, the susceptibility of the feline and human OX40-expressing cells to HIV pseudotypes with VSV-G envelope was broadly similar to the control HeLa cells. The results suggest that the specificity of the interaction between the virus and its primary receptor may be a major determinant of the species specificity of FIV.

HIV infection is mediated by the interaction between the virus and its primary receptor (CD4) followed by an interaction with a co-receptor, CXCR4 or CCR5, both of which are members of the seven transmembrane domain superfamily of molecules. Infection with both primary and cell culture-adapted strains of FIV are CXCR4-dependent [Richardson *et al.*, 1999; Willett *et al.*, 1997b]. The author therefore determined whether OX40-dependent infection required co-expression of CXCR4. HeLa-OX40 cells were infected with HIV (FIV) luciferase pseudotypes in the presence of increasing concentrations of the selective CXCR4 antagonist AMD3100 (Fig. 11). Infection with GL8 and TM2 pseudotypes were inhibited efficiently by AMD3100 while infection with control HIV (VSV) pseudotypes was not affected. The inhibition was dose-dependent with almost complete

inhibition at 100 ng/ml antagonist. Thus, although ectopic expression of feline OX40 rendered HeLa cells permissive to infection with FIV, there was an absolute dependence on the co-expression of CXCR4. Similar results were obtained with feline AH927 cells expressing OX40 (AMD3100 binds feline CXCR4) and pseudotypes bearing a range of FIV Envs (data not shown).

The author has shown that OX40 functions as a novel primary receptor for an immunodeficiency-causing lentivirus. Ectopic expression of feline OX40 promotes binding of FIV to human, murine, and feline cells, renders non-permissive cells permissive for viral entry and productive infection with primary strains of FIV and supports Env mediated syncytium formation. Further, the author has demonstrated that OX40-mediated infection requires co-expression of CXCR4, consistent with conservation of the receptor/co-receptor mechanism of viral entry between the feline and the primate lentiviruses. In rats and humans, OX40 expression is largely restricted to $CD4^+$ T lymphocytes, where expression typically peaks at 3-4 days post-activation [Al Shamkhani *et al.*, 1996; Gramaglia *et al.*, 1998; Paterson *et al.*, 1987; Stuber & Strober, 1996]. Signalling through OX40 is thought to be the primary co-stimulatory pathway for T cells that have encountered antigen and OX40-knockout mice display defective T cell responses to protein antigens [Gramaglia *et al.*, 1998; Kopf *et al.*, 1999; Pippig *et al.*, 1999]. Further, studies with OX40L-knockout mice

demonstrated that OX40L plays a critical co-stimulatory role in dendritic cell-T cell interactions since mice lacking OX40L exhibited impaired T cell priming and cytokine production [Chen *et al.*, 1999]. In addition, OX40 was reported to be an important co-stimulatory molecule, as well as CD28, for selection and differentiation of $CD4^+$ T cells [Croft ,2003; Lanes, 2000; Walker *et al.*, 2000]. Furthermore, for long-term survival of $CD4^+$ T cell memory, OX40-mediated signaling seems to be essential, probably through anti-apoptosis induction [Gramaglia *et al.*, 1998, 2000; Rogers *et al.*, 2001]. Thus it can be inferred that by targeting OX40-expressing cells, FIV would selectively deplete a subset of $CD4^+$ T cells that is integral to the development of antigen-specific T cell responses. In contrast, by utilizing CD4 as a primary receptor, HIV has the potential to infect all $CD4^+$ T cells and induce a more profound immune defect. However, the cell tropism of HIV is restricted by the expression of the viral co-receptor which, for the majority of strains that are transmitted, is CCR5. CCR5 expression on $CD4^+$ T cells is restricted to an effector/memory T cell subset [Bleul *et al.*, 1997; Wu *et al.*, 1997]. Thus despite the use of distinct primary binding receptors, both the human and feline viruses selectively impair antigen-specific helper T cell responses.

Previous studies have suggested that infection with primary strains of FIV have a restricted cell- and

species-tropism while cell culture adapted strains of virus interact directly with CXCR4 of several species. Our data are consistent with the tropism of primary FIVs being restricted at the viral entry step in that human OX40 did not support viral entry efficiently. In addition, the presence of post-entry blocks to viral replication in human cells [Ikeda *et al.*, 1996] and the neutralization of retroviruses grown in non-human cells by antibodies in human serum against Gal(alpha 1-3)Gal terminal carbohydrates expressed on the viral envelope [Takeuchi *et al.*, 1996] make the transmission of primary isolates of FIV to human beings extremely unlikely.

Several studies reported that FIV can infect $CD4^-$ cells, such as $CD8^+$ cells, B cells [Dean *et al.*, 1996; English *et al.*, 1993], macrophages/monocytes [Brunner & Pedersen, 1989], and neuronal cells [Dow *et al.*, 1990], while the cells would not express OX40. However, the results were obtained from chronic, but not acute, stages of infection, and cats in chronic stage of FIV infection must have FIV that have lost the requirement for a primary receptor, suggested by the observation that symptomatic cats harbour viruses capable of infecting cells using CXCR4 alone [B. Willett, personal communication]. Namely, in the acute infection stage, FIV must infect preferentially $CD4^+$ cells, similar to the case of HIV. The author thinks that OX40-dependency-lacking FIV such as Petaluma strain still

preferentially infects OX40-expressing cells, because Petaluma maintains reactivity with OX40 as shown in Fig. 3 and such interaction increases the fusion step between CXCR4 and FIV Env, similar to the case of HIV [Reeves *et al.*, 1999].

The phenomenon of vaccine-induced enhancement of infection in the feline model of AIDS has been well-documented [Hosie *et al.*, 1992; Richardson *et al.*, 1997; Siebelink *et al.*, 1995]. Our new data may shed new light on the mechanism of enhancement, since OX40 is a T cell activation antigen with expression in vivo restricted predominately to $CD4^+$ T cells. Vaccination may induce an expansion of a population of cells expressing the viral receptor so that if sterilizing immunity is not achieved, vaccination may prove counter-productive.

That two lentiviruses with host species as divergent as human beings and the domestic cat should use distinct primary receptors to target the similar T cell subsets underlines the central role of $CD4^+$ T lymphocyte infection in the pathogenesis of AIDS. Whether the feline and human lentiviruses evolved from a common ancestor, such as a CD4 or OX40-independent virus, is an intriguing question for the development of viral virulence, and this study represents the first step towards providing a solution.

Figure legends

Fig. 1

nt and predicted aa sequences of feline OX40 (AB128982) that was identified by expression cloning. Signal peptide is in italics and transmembrane region is underlined. Cystein residues are in bold and a potential *N*-linked glycosylation site is marked [CHO]. Stop codon is indicated by an asterisk (*).

Fig. 2

Interspecies comparison of OX40 aa sequences. Dots are residues that are identical to feline OX40, and the residues completely conserved among four species are indicated by asterisks (*). GGSFRTPI aa sequence in human OX40, which is underlined, is discussed in the text. One potential *N*-linked glycosylation site and seventeen cystein residues are shaded. GGSFRTPI aa sequence underlined is discussed in the text.

Fig. 3

FCM analyses for FIV binding to human and murine cells. (a) Human Plat-E cells transfected with pMX-fCD2 or pMX-fOX40 and (b) murine NIH3T3 cells infected with MLV packaging the gene for feline CD2- or OX40 were used. Cells were mixed with culture supernatant of normal CRFK (fine line) or CRFK/Pet (bold line) cells, and then cell surface-FIV antigens were detected using

anti-FIV Env mAb and secondary antibody.

Fig. 4

FCM analyses for FIV (Petaluma and TM2) binding on feline cells and OX40/CD2 expression. CRFK cells stably expressing and OX40 (a) or CD2 (b) and MYA-1 cells (c) were incubated with control medium (fine) or virus (bold) and then FIV antigen were detected by anti-FIV Env mAb. In addition, OX40 expression in OX40/CRFK cells and in MYA-1 cells and CD2 expression in CD2/CRFK cells were analyzed: fine, irrelevant mAb; bold, OX40 or CD2 antibody.

Fig. 5

Immunoblotting of feline CD2- (lanes 1 and 3) or OX40- (lanes 2 and 4) expressing CRFK cells under reduced condition. Anti-human OX40 mAb (lanes 1 and 2) or irrelevant mAb (lanes 3 and 4) were used. A specific band of ≈48 kDa was observed in lane 2. The numbers on the left are molecular size marker (kDa).

Fig. 6

OX40 expression renders AH927 cells permissive for syncytia formation mediated by primay FIV Env. FeLVC-R- or OX40-expressing cells were transfected with pVL1012 expressing the GL8 or Petaluma *env* genes. Envs of other primary isolates F045, F0556, F0827, and F1419 induced the similar results with

GL8 Env.

Fig. 7

PCR (upper) and RFLP (lower) analyses of cellular genomic DNAs. Upper: PCR was performed to amplify a 329 bp fragment of *gag* gene of FIV. Genomic DNAs were extracted from mock- (1) or TM2- (2) infected MYA-1 cells, mock-infected CRFK cells expressing CD2 (3) or OX40 (4), TM2-infected CRFK cells expressing CD2 (5) or OX40 (6), and Petaluma-infected CRFK cells expressing CD2 (7). Lower: RFLP following upper PCR. Two restriction enzymes *Hin*dIII (left) and *Pvu*II (right) were used. Lane numbers are identical to the case of upper PCR. M, 100 bp marker.

Fig. 8

IFA of OX40 expressing CRFK cells inoculated with a primay FIV TM2. Cells were harvested indicated days after inoculation and analyzed using FIV-infected cat serum and FITC-labeled anti-cat IgG.

Fig. 9

Production of p24 capsid antigen in culture supernatant. CRFK cells expressing feline OX40 or CD2 were infected with lymphotropic FIV strain TM2, culture supernatant were harvested

as indicated days, and then p24 antigen were measured by ELISA. ○, CD2-expressing, mock infected; △, OX40-expressing, mock infected; ●, CD2-expressing, TM2-infected; ▲, OX40-expressing, TM2-infected.

Fig. 10

Species specificity of OX40-mediated FIV infection. HeLa cells expressing feline or human OX40 were infected with HIV luciferase pseudotypes enveloped by lymphotropic FIV Env (GL8 and TM2) or VSV-G (VSV) and luciferase activities were measured. Results are shown as the mean ± SE of quadruplicated data.

Fig. 11

Dependency of OX40-mediated FIV infection. HeLa cells expressing feline OX40 were infected with HIV luciferase pseudotypes enveloped by lymphotropic FIV Env (GL8 and TM2) or VSV-G (VSV) in the presence of indicated concentration of CXCR4 antagonist AMD3100 (mean ± SE, n = 4) and luciferase activities were measured.

CHAPTER 4/Fig. 1

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   1  GATGAGGGTGGTTGTGGGGGCTCAGCGGCCCAGGGCGCCTCACTCAGCTGTCCAGCTCCT
       M  R  V  V  V  G  A  Q  R  P  R  A  P  H  S  A  V  Q  L  L

  61  GGGGCTTGTGCTGGGCACCGCGGCCGCGCTCCACTGTGTCGGGAACACCTACCCCAAAGA
       G  L  V  L  G  T  A  A  A  L  H  C  V  G  N  T  Y  P  K  D

 121  CGGCAAGTGCTGTAGCGAGTGCCCACCAGGTTATGGGATGGAGAGTCGCTGCAGCGGTGA
       G  K  C  C  S  E  C  P  P  G  Y  G  M  E  S  R  C  S  G  D

 181  CCAGGACACCAAGTGCCTCCAGTGCGCGTCCGGCTTCTACAACGAGGCCGTGAACTACGA
       Q  D  T  K  C  L  Q  C  A  S  G  F  Y  N  E  A  V  N  Y  E

 241  GCCTTGCAAGCCCTGCACACAGTGCAACCAGAGAAGTGGGAGCGAGCCCAAGCAGAGATG
       P  C  K  P  C  T  Q  C  N  Q  R  S  G  S  E  P  K  Q  R  C

 301  CACACCCACGCAGGACACCGTCTGCCGCTGCAGGCCAGGCACTGAGCCCCAGGACGGTTA
       T  P  T  Q  D  T  V  C  R  C  R  P  G  T  E  P  Q  D  G  Y

 361  CGATCGTGGAGTCGACTGTGCCCCGTGCCCACCGGGACATTTCTCCCCAGGTGATGACCA
       D  R  G  V  D  C  A  P  C  P  P  G  H  F  S  P  G  D  D  Q

 421  GGCTTGCAAGCCCTGGACCAACTGCACCTTGGCGGGAAAGCGCACGCTGAGGCCGGCCAG
       A  C  K  P  W  T  N  C  T  L  A  G  K  R  T  L  R  P  A  S
                         [CHO]

 481  CCAAGGCTCAGATGCCGTGTGTGAGGACAGGAGCCCCCCAGCCACCACGCCTTGGGAGAC
       Q  G  S  D  A  V  C  E  D  R  S  P  P  A  T  T  P  W  E  T

 541  CCAGGGCCCCCCAGTCCGGCCCCCTACCACCCAGCCCACCACAGCCTGGCCTAGGACCTC
       Q  G  P  P  V  R  P  P  T  T  Q  P  T  T  A  W  P  R  T  S

 601  ACAGGAGCCCTTCACACCCCCTGCAGAGCCCCCCAGGGGTCCCCAGCTGGCCGCGGTCCT
       Q  E  P  F  T  P  P  A  E  P  P  R  G  P  Q  L  A  A  V  L

 661  GGGCCTGGGCCTGGGCCTGCTTGCCCCGGTGGCGGCCGCACTGGCCCTGCTCCTGCACCA
       G  L  G  L  G  L  L  A  P  V  A  A  A  L  A  L  L  L  H  H

 721  CAGAGCCTGGCGGCTACCCCCCGGTGGAAATAGCTTCCGGACCCCCATCCAAGAGGAGCA
       R  A  W  R  L  P  P  G  G  N  S  F  R  T  P  I  Q  E  E  H

 781  TGCCGATGCCAACTCCACCCTGGCCAAGATCTGAGCGGCCAGGCCAGGGGCCCAGAGGGC
       A  D  A  N  S  T  L  A  K  I  *

 841  CTGCTAGACATTGAGGGGCCGTGGGGCACCCCCAAGCACGGGCCGCCACATCCCACCTTC

 901  CCAC
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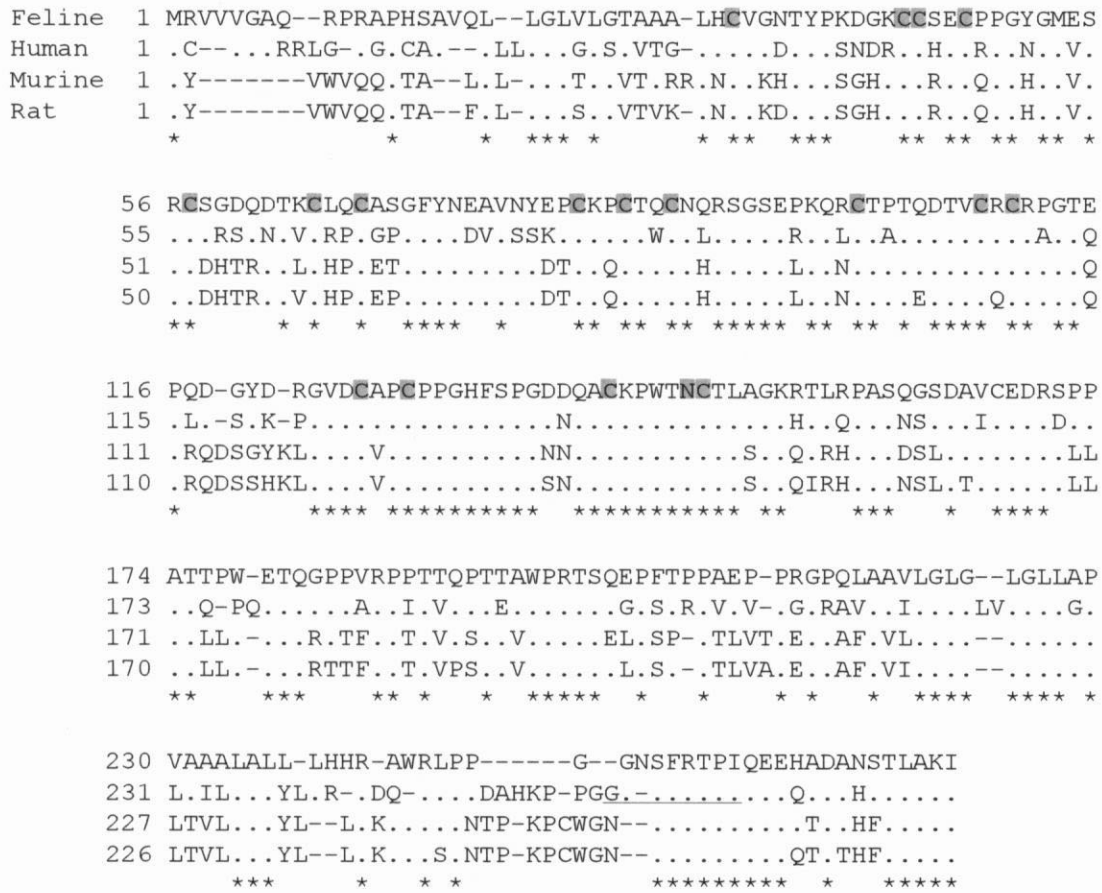
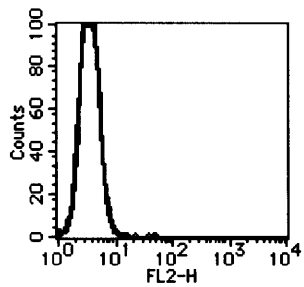
CHAPTER 4/Fig. 2



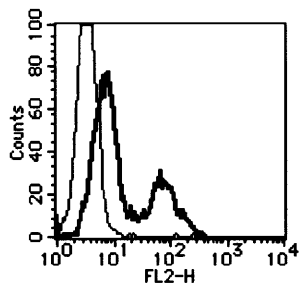
CHAPTER 4/Fig. 3

(a) Plat-E cells

pMX-fCD2 transfected

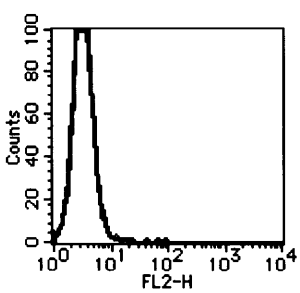


pMX-fOX40 transfected

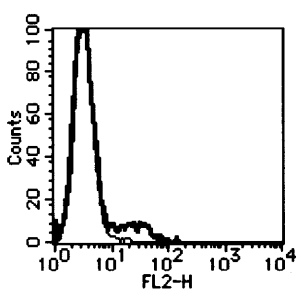


(b) NIH3T3 cells

fCD2 infected

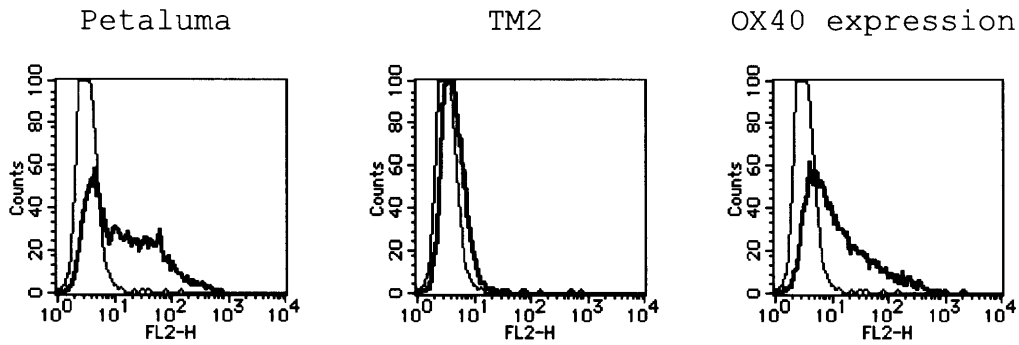


fOX40 infected

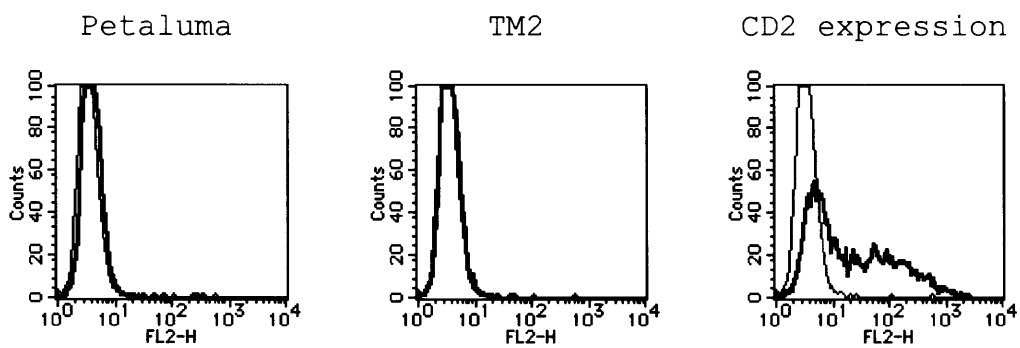


CHAPTER 4/Fig. 4

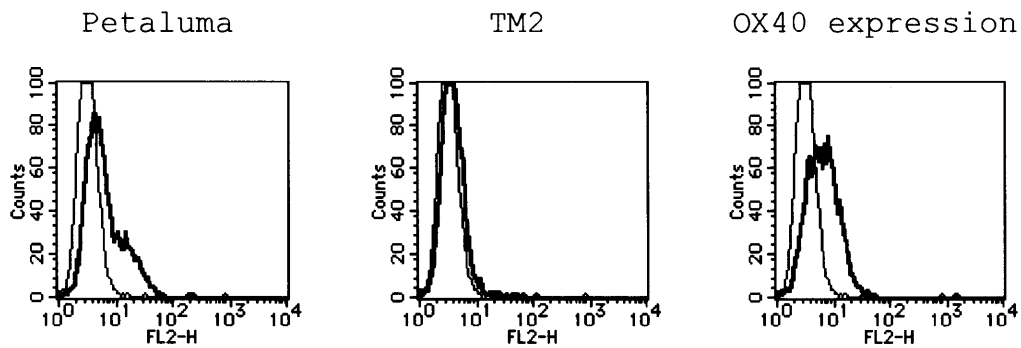
(a) OX40-CRFK cells



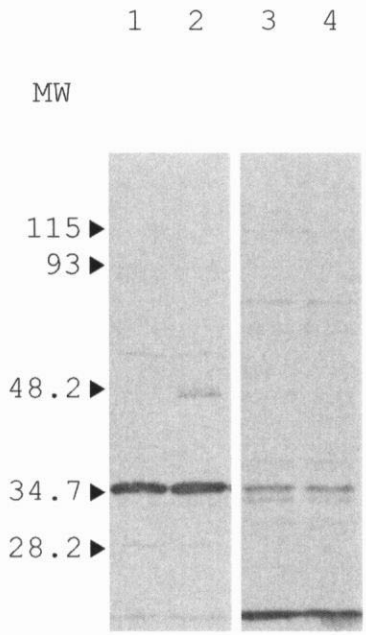
(b) CD2-CRFK cells



(c) MYA-1 cells



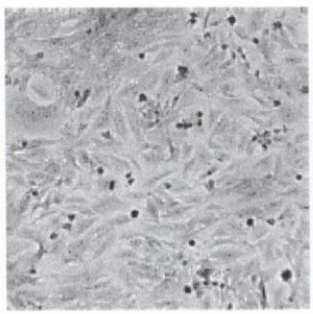
CHAPTER 4/Fig. 5



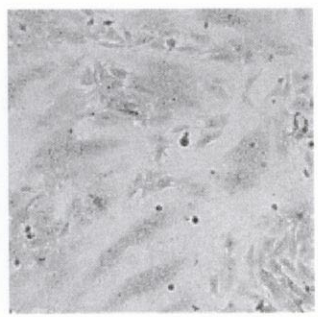
CHAPTER 4/Fig. 6

FeLVC-R/AH927 cells

GL8

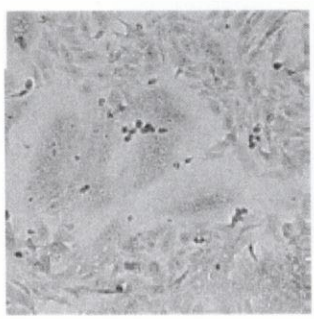


Pet

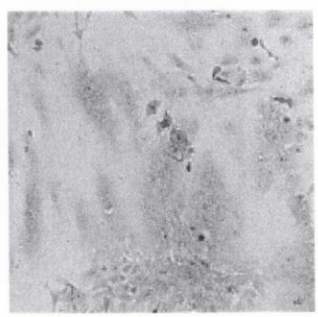


OX40/AH927 cells

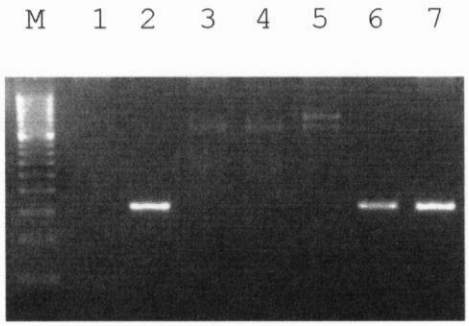
GL8



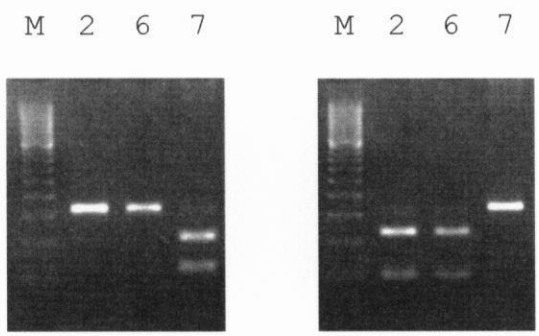
Pet



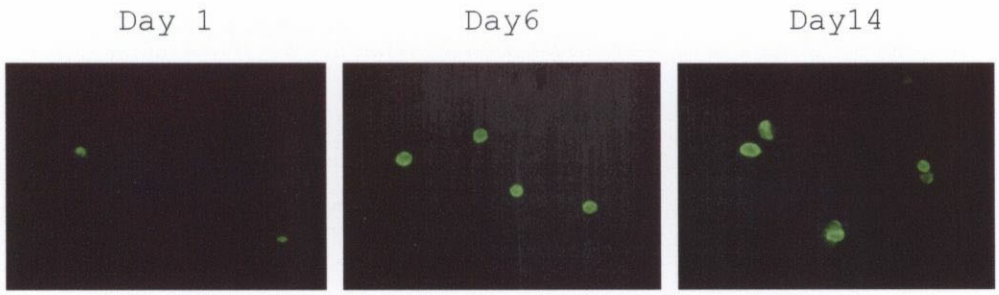
PCR



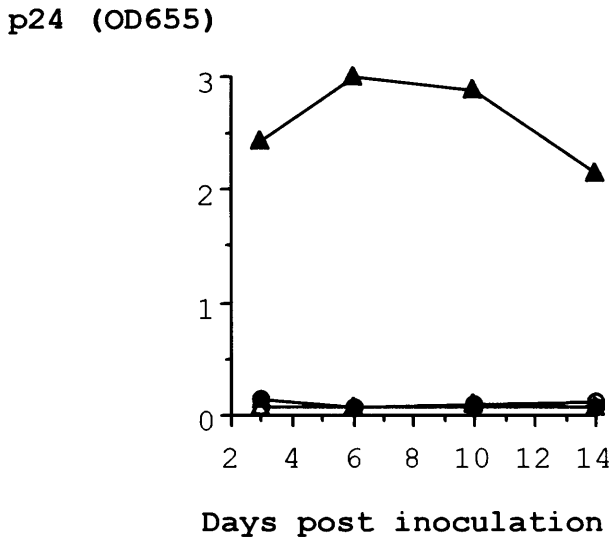
RFLP



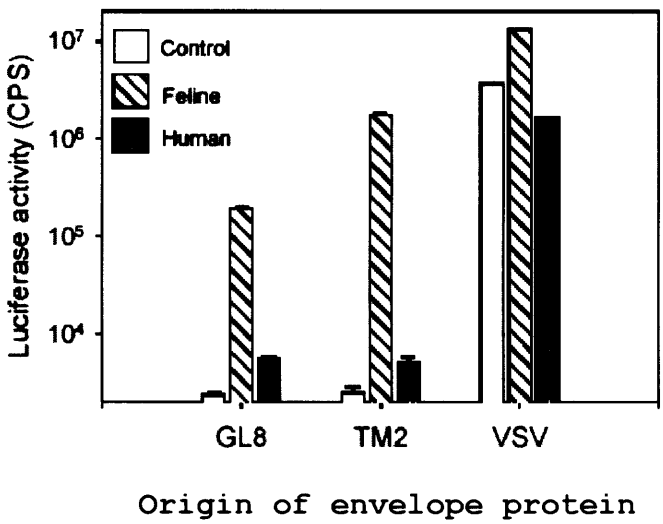
CHAPTER 4/Fig. 8



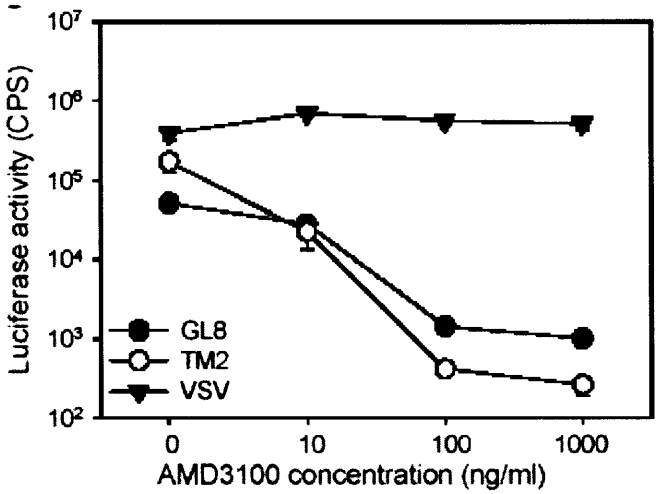
CHAPTER 4/Fig. 9



CHAPTER 4/Fig. 10



CHAPTER 4/Fig. 11



PART III

Characterization of T lymphocytes in FIV-infected cats

FIV infection results in induction of $CD8^+$ subpopulations in PBLs which show interesting characteristics in CD8β chain expression levels (details in GENERAL INTRODUCTION). The subpopulations with various CD8β expression were first reported from the feline field and later similar characteristics were also reported in normal or virus-infected rat or human lymphocytes. However, probably due to the lack of useful antibodies as described in PART I, detailed analyses of feline $CD8^+$ T cells in respect to viral control had not yet performed.

In PART III, the author described functional (CHAPTER 5) and phenotypic (CHAPTER 6) analyses of T lymphocytes of FIV-infected cats by using the antibodies established in PART I.