

CHAPTER 5

T cell subpopulations mediating inhibition of FIV replication  
in mucosally infected cats

Microbes and Infection in press

## Abstract

FIV infection induces an increase of two subpopulations ( $CD8\alpha^+\beta^{low}$  and  $CD8\alpha^+\beta^-$ ) within  $CD8^+$  PBLs of cats. It is known that depletion of  $CD8^+$  cells often results in augmentation of FIV proliferation in PBL culture, similar to the case of HIV. In this CHAPTER, the author attempted to define PBL subpopulations mediating antiviral activity in five cats intravaginally infected with a molecularly cloned FIV isolate. Several subpopulations ( $CD8\alpha^+\beta^+$ ,  $CD8\alpha^+\beta^-$ , and  $CD4^+$  cells) were shown to participate in inhibition of the FIV replication, at least in part, by an MHC-unrestricted manner. Interestingly, the subpopulations showing anti-FIV activity were different among the individual cats. These results indicate that anti-FIV factors, if any, can be secreted from a variety of subpopulations of PBLs.

## Introduction

FIV [Pedersen *et al.*, 1987], a member of the genus *Lentivirus*, infects domestic cats and causes an AIDS-like disease after a protracted asymptomatic phase of several years [Ackley *et al.*, 1990; Yamamoto *et al.*, 1988]. Both virus-specific cytotoxic T cell [Beatty *et al.*, 1996; Burkhard *et al.*, 2001; Flynn *et al.*, 2002; Li *et al.*, 1995; Song *et al.*, 1992, 1995] and non-cytotoxic antiviral activities [Bucci *et al.*, 1998a, b; Choi *et al.*, 2000; Crawford *et al.*, 2001; Flynn *et al.*, 1999, 2002; Gebhard *et al.*, 1999; Hohdatsu *et al.*, 1998b, 2000; Jeng *et al.*, 1996] are observed within CD8<sup>+</sup> PBLs of the infected cats in early through asymptomatic phase. These findings resemble human immunity in HIV infection [Yang & Walker, 1997]. Furthermore, FIV infection in cats can be achieved via genital and rectal mucosa [Burkhard *et al.*, 2001; Finerty *et al.*, 2000, 2002; Jordan *et al.*, 1998; Kohmoto *et al.*, 2003; Matteucci *et al.*, 2000; Obert & Hoover, 2002]. Therefore, the FIV/cat system is a useful animal model to examine immunological responses in mucosal infection and develop vaccines or antiviral therapies.

Previously the author reported an increase of two subpopulations, CD8 $\alpha^+\beta^{\text{low}}$  and CD8 $\alpha^+\beta^-$  cells, in CD8<sup>+</sup> PBLs of FIV-infected cats [Shimojima, *et al.*, 1998a]. The CD8 $\alpha^+\beta^{\text{low}}$  cells increase as early as 3 to 4 wks post infection [Bucci *et al.*, 1998b; Willett *et al.*, 1993] and are maintained through the

asymptomatic phase. Bucci *et al.* [1998b] and Flynn *et al.* [2002] reported the strong anti-FIV activity within CD8 $\alpha^+\beta^{low}$  cells of both acute and chronic infections. A time of CD8 $\alpha^+\beta^-$  cell increasing after infection is yet to be elucidated, however the cells are often observed in relatively long-term infected cats [Gebhard *et al.*, 1999, Hohdatsu *et al.*, 2003; Shimojima *et al.*, 1998a]. Their role in FIV infection or correlation with disease progression has not been investigated, due to insufficient expansion of the cells in a short term after infection [Gebhard *et al.*, 1999].

In this CHAPTER, to better understand the cellular immunity in the mucosal infection, the author investigated the antiviral activities of these CD8 $^+$  subpopulations or other cells (CD4 $^+$  lymphocytes) of cats mucosally infected with a molecularly cloned FIV isolate by two means, "depletion" and "reconstitution" assays.

## Materials & Methods

### *Experimental animals*

The procedures used for inoculating SPF cats with FIV were reported previously [Kohmoto *et al.*, 1998a, 2003]. Six female SPF cats (Cats 301-306) 5 to 7 months old were used: Cats 301 and 304, Cats 302 and 305, and Cats 303 and 306 were from the same litters. Briefly the cats were inoculated via the vagina with MYA-1 cells (a feline T-lymphoblastoid cell line) [Miyazawa *et al.*, 1989] infected with FIV strain TM2. Consequently five cats (301-305) became positive for proviral DNA in PBMCs within 8 weeks post-inoculation. Four years after infection, anti-FIV antibodies were observed in these cats at a high level as observed in the early phase of infection and these cats were asymptomatic. Plasma samples of these cats were inoculated onto  $2 \times 10^5$  MYA-1 cells at dilutions of 1:5 and 1:50 and the cells were cultured for 16 days, however no evidence of viral infection was confirmed by IFA of the cells [Kawaguchi *et al.*, 1990] nor by ELISA of the culture supernatants in any cases, indicating no or very low viral titers in plasma [Kohmoto *et al.*, 1998b]. One cat (Cat 306) did not become positive for provirus or antibody, indicating no establishment of infection in this animal.

### *Depletion and culture of PBMCs (depletion assay)*

PBMCs were isolated from heparin-treated peripheral blood

with Ficoll-paque (Amersham Pharmacia Biotech). Aliquots were used to analyse the expression of two surface molecules, CD8 $\alpha$  and CD8 $\beta$ , on PBLs by two-color FCM as described previously [Shimajima *et al.*, 1998a]. For depletion by panning, isolated PBMCs were divided into three and then incubated with no antibody (mock), anti-CD8 $\alpha\beta$  vpg9 (to deplete CD8 $\beta^+$  but not CD8 $\alpha^+\beta^-$  cells) or anti-CD8 $\alpha$  12A3 (to deplete all CD8 $^+$  cells), respectively. After wash, the cells were seeded on a Petri dish (Bio-Bik) which had been pre-treated with goat anti-mouse IgG antibodies (Rockland) (see CHAPTER 3 for details). Non-adherent cells were harvested by gentle washing of the dish, and aliquots were analysed by FCM to estimate depletion efficiencies. The harvested PBMCs ( $1.5 \times 10^5$  cells) were mixed with MYA-1 cells ( $1 \times 10^5$  cells) as indicator cells, stimulated with Con A for 3 days, and cultured for a further 9 days in the presence of IL-2. Culture supernatants were harvested at days 6, 9 and 12 for measurement of p24 FIV antigen by ELISA.

#### *Reconstitution of PBMCs (reconstitution assay)*

Isolated PBMCs were directly seeded on pre-treated Petri dishes to remove non-specifically adhered cells (most granulocytes and monocytes, Fig. 1). Then non-adherent cells (lymphocytes, Fig. 1) were harvested, labeled with adequate antibodies, and then panned as described above. In addition to

non-adherent (target) cells, specific adherent (effector) cells were also harvested with cell scrapers and used in the cell culture. These effector and target cells were co-cultured at concentrations of  $1 \times 10^5$  effector,  $1.5 \times 10^5$  target, and  $1 \times 10^5$  indicator cells per 1 ml, and then cultured as described for the depletion assay. When infected MYA-1 was used as the target, indicator cells were not added. The measurement of p24 was performed only at day 12 though for FIV-14-infected MYA-1, it was made at day 9.

#### *Measurement of FIV p24 antigen*

p24 antigen in culture supernatant was detected using a commercial kit (FIV Antigen Test Kit) (IDEXX). In the depletion assay, an  $OD_{655}$  of more than 0.5 was regarded as positive for the proliferation of FIV. In the reconstitution assay, % inhibition was calculated as follows:  $(\text{p24 of target cells} - \text{p24 of target cells co-cultured with effector cells}) / (\text{p24 of target cells} - \text{p24 of effector cells without target cells}) \times 100 (\%)$ . In co-culture with infected MYA-1 as target cells, effector cells co-cultured with uninfected MYA-1 were used as the "target-absent effector". Antiviral activity was regarded as significantly positive when the % inhibition was more than 50.

## *Antibodies*

To deplete subpopulations of PBMCs by panning, anti-CD8 $\alpha\beta$  vpg9 (specific for  $\alpha\beta$  heterodimer) [Willetts *et al.*, 1993], anti-CD8 $\alpha$ 12A3 [Shimojima *et al.*, 1998b], anti-CD3 $\epsilon$  [Y. Nishimura *et al.*, unpublished], anti-CD4 44A8 [Shimojima *et al.*, 1997] and anti-CD16 [Y. Nishimura *et al.*, unpublished] were used. For surface Ig<sup>+</sup> cell depletion, Petri dishes which were coated with rabbit anti-cat IgG (Rockland) were used. For FCM analysis, FITC-labeled anti-CD8 $\alpha$  antibodies 2D7, 10C7, 12A3 and anti-CD4 4D9 [Shimojima *et al.*, 1997, 1998b] and PE-labeled anti-CD8 $\beta$  antibody FT2 (Southern Biotechnology Associates, Birmingham, AL) were used.

To estimate epitope properties of vpg9 and FT2 mAbs, PBLs of Cats 301 and 302 were incubated with vpg9, being followed by incubation with PE-labeled FT2 mAb. For 2D7, 10C7, and 12A3 epitopes, feline PBLs were incubated with three unlabeled mAbs separately, then incubated with either of FITC-labeled mAbs. After incubation with second mAbs, cells were washed with wash buffer (CHAPTER 1) and analyzed by FACScan.

## *Preparation of infected MYA-1 cells*

Two infectious molecular clones, pTM219 (strain TM2) [Maki *et al.*, 1992] and pFIV-14 (strain Petaluma) [Olmsted *et al.*, 1989], were transfected into CRFK cells by an electroporation

method. Two days after transfection, each culture supernatant was inoculated onto MYA-1 cell culture. Fourteen days pi, the supernatants were harvested and stocked in aliquots at -80 °C until use for infection of fresh MYA-1 cells at a multiplicity of infection of 0.01. The titers of virus stocks were determined as described previously [Kawaguchi *et al.*, 1990]. In some experiments, these infected MYA-1 cells were used for co-culture with isolated PBMCs.

## Results & Discussion

### *Subpopulations of CD8<sup>+</sup> PBLs in intravaginally infected cats*

For this study, five FIV-intravaginally infected cats (Cats 301-305) and one uninfected control cat (Cat 306) were used. Four years passed after FIV inoculation. In FCM, the author observed reduced or diminished expression levels of CD8  $\beta$  chain in CD8<sup>+</sup> PBLs in these infected cats, a unique characteristic of PBLs in FIV-intraperitoneally infected cats [Shimojima *et al.*, 1998a]. Borderlines between high and low levels of CD8 $\beta$  were set arbitrarily to separate the two peaks, and then percentages were calculated. The percentages of CD8 $\alpha^+\beta^{\text{high}}/\beta^{\text{low}}/\beta^-$  within total PBLs were as follows: Cat 301, 16%/15%/10%; Cat 302, 25%/11%/16%; Cat 303, 16%/19%/38%; Cat 304, 10%/14%/4%; Cat 305, 18%/12%/6%. In contrast, the control Cat 306 exhibited a PBL composition of 21%/2%/2% which was characteristic of uninfected cats [Shimojima *et al.*, 1998a]. No remarkable changes of these percentages were observed during this study. This observation together with previous reports [Bucci *et al.*, 1998b; Flynn *et al.*, 2002; Gebhard *et al.*, 1999; Lehmann *et al.*, 1992; Orandle *et al.*, 2000; Shimojima *et al.*, 1998a; Willett *et al.*, 1993] revealed that FIV infection can be characterized by the reduction in the expression level of the CD8  $\beta$  chain on peripheral blood CD8<sup>+</sup> lymphocytes irrespective of the infection route (intravaginal, intravenous or intraperitoneal) or viral strain

used (Japanese, Swiss, British and American isolates). The reduction of  $\beta$  chain, but not  $\alpha$  chain, has not been well documented in other viral infections in other animals, however to the author's knowledge, a similar phenotype is reported in only two studies; Borna disease virus-infected cats [Berg *et al.*, 1999] and HIV-infected patients [Schmitz *et al.*, 1998]. The  $\beta$  chain reduction is not identical to a transient reduction of both  $\alpha$  and  $\beta$  chains along with TCR, which usually occurs after T cell interaction with a peptide-presenting MHC class I molecule [Kambayashi *et al.*, 2001]. Recently, rat splenic  $\gamma\delta$  T cells were reported to reduce the expression of CD8 $\beta$ , but not CD8 $\alpha$ , after *in vitro* co-stimulation through TCR and CD28 [Straube & Herrmann, 2001]. Unfortunately, no analysis of TCR expression on feline lymphocytes has been reported. The phenomenon of CD8  $\beta$  chain reduction should be well analyzed also in various species.

*CD8 $\alpha^+\beta^+$  and CD8 $\alpha^+\beta^-$  lymphocytes are involved in the anti-FIV activity*

To examine the anti-FIV activity of the CD8<sup>+</sup> subpopulations, depletions of the subpopulations from PBLs were performed by panning. Representative FCM-results of CD8 $\beta^-$  or CD8 $\alpha^-$ -depleted PBLs in the depletion assay are shown in Figure 1. Incubation of mock-treated PBMCs (Fig. 1a) had negligible effects on ratios of the CD8<sup>+</sup> subpopulations (data not shown). In each cat, cell

populations positive for CD8 $\beta$  and CD8 $\alpha$  after depletion of CD8 $\beta$  and CD8 $\alpha$  were <1% (Fig. 1b) and <3% (Fig. 1c), respectively. While anti-CD8 $\alpha\beta$  vpg9 (used for depletion) slightly blocked the binding of anti-CD8 $\beta$  FT2 (used for FCM, Fig. 2), vpg9 antibody was not detected in the depleted PBMCs (data not shown). Anti-CD8 $\alpha$  12A3 used for depletion did not block the binding of anti-CD8 $\alpha$  10C7 for FCM analysis (Fig. 3). From these facts, the author concluded that the depletion of the cell populations observed in the FCM analysis was not due to epitope masking and that the depletion of the CD8 $\beta$  or CD8 $\alpha$  population by the panning was properly performed. Further, the author's panning method used for the depletion also removed non-lymphoid cells such as monocytes and granulocytes that adhere to plastic dishes non-specifically (Fig. 4). Thus, the present study can be interpreted as an analysis of lymphocytes rather than mononuclear cells.

The author co-cultured the depleted cells with indicator cells (MYA-1) and measured p24 antigen in the supernatants as described in Materials & Methods. As shown in Figure 5, a striking increase in p24 was observed at day 12 in several cases. In Cat 301, neither mock- nor CD8 $\beta$ -depletion resulted in an increase of p24 antigen, however CD8 $\alpha$ -depletion did. In Cat 302, an increase in p24 was observed irrespective of depletions. In Cat 303, no increase of p24 was observed even in the CD8 $\alpha$ -depleted

cell culture. In Cats 304 and 305, mock-treatment did not result in increase in p24, while both CD8 $\beta$ <sup>-</sup> and CD8 $\alpha$ <sup>-</sup> depletion did. These data indicate that anti-FIV activities are present within CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> in Cats 304 and 305, and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> lymphocytes in Cat 301. These findings do not exclude the possibility that CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> lymphocytes in Cats 301 and 303 have antiviral activity, because if other populations had enough antiviral activity, the author should not have detected the effect caused by the depletion of CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> cells. While several groups reported suppressive activities within CD8<sup>+</sup> lymphocytes in FIV-infected cats, there are few studies mentioning the relationships between the different expression levels of the CD8  $\beta$  chain and the suppressive activities. Two groups reported suppressive activities in purified CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>low</sup> cells [Bucci *et al.*, 1998b; Flynn *et al.*, 2002], and Gebhard *et al.* [1999] obtained a similar result by use of anti-CD8 $\beta$  and CD62L antibodies to sort the subpopulation. Concerning the CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>high</sup> cells, the results by Flynn *et al.* [2002] were inconsistent with those by others [Bucci *et al.*, 1998b; Gebhard *et al.*, 1999]; the former detected antiviral activities in the subpopulation, while the latter did not. The author did not elucidate the distinct populations within CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>+</sup> lymphocytes that had antiviral activity, because separation of CD8 $\beta$ <sup>high</sup> and CD8 $\beta$ <sup>low</sup> cells by sorting was difficult due to the unclear borderline between high and low levels of  $\beta$ -chain expression

as shown in Fig. 1. As performed by Gebhard *et al.* [1999], use of the CD62L (L-selectin) marker may be more suitable for the subdivision of responsible cells than use of the CD8 $\beta$  molecule.

No functional studies have been done for CD8 $\alpha^+\beta^-$ , probably due to insufficient expansion of the population in a relatively short time (1 to 3 years) after FIV infection [Gebhard *et al.*, 1999], while differential tissue dynamics of CD8 $\alpha^+\beta^{\text{high}}$ ,  $\beta^{\text{low}}$  and  $\beta^-$  cells were reported in neonatally infected cats [Crawford *et al.*, 2001]. However, at least regarding the suppression of the p24 increase in culture supernatants, the author observed that CD8 $\alpha^+\beta^-$  cells in one cat (Cat 301) had suppressive activity as CD8 $\alpha^+\beta^+$  cells did in the other cats. Similar results were also observed using cryopreserved PBMCs of the cat (Fig. 6). More detailed studies will be required for these subpopulations in relation to a mechanism of the antiviral activity and maintenance of the asymptomatic phase or acquisition of immunodeficiency.

Thus, both CD8 $\alpha^+\beta^+$  and CD8 $\alpha^+\beta^-$  PBLs were shown to have antiviral activity. However, in the case of Cat 302 that had a typical characteristic of CD8 $^+$  PBLs of FIV-infected cats (Fig. 1a), there was no antiviral activity observed (Fig. 5). This means that CD8 $\alpha^+\beta^{\text{low}}$  and CD8 $\alpha^+\beta^-$  PBLs observed in asymptomatic phase of FIV infection do not always show such activity *in vitro*. Considering that FIV could not be isolated from plasma

of the animal (described in Materials & Methods) and that the animal is asymptomatic like the others, neutralizing antibodies or other mechanisms may be responsible for the regulation of FIV proliferation in this cat in vivo. Jeng *et al.* [1996] and Hohdatsu *et al.* [1998b] also reported viral replication in undepleted PBMCs of infected cats, but without analysis for detailed CD8 phenotypes or viremia.

*CD4<sup>+</sup> lymphocytes also can be responsible for anti-FIV activity*

In the case of Cat 303, the CD8-depletion from PBLs did not result in FIV replication (Fig. 5). Similar results were observed using cryopreserved PBMCs of the cat (Fig. 6). There are two possibilities to explain this phenomenon: this cat no longer had infected cells in the isolated PBMCs or FIV could not replicate in the depleted PBLs for some reason. To determine which is correct, the author co-cultured FIV TM219- or FIV-14-infected MYA-1 cells with CD8-depleted PBLs of Cat 303. No increase of p24 was observed in the co-culture, although the peak of p24 production was observed at day 12 for TM2- or day 9 for FIV-14-infected MYA-1 cells (data not shown). The result indicated that anti-viral activity was present in CD8 negative cells in this animal. Next the author depleted other subpopulations (CD3 $\epsilon$ <sup>+</sup>, surface Ig<sup>+</sup>, or CD16<sup>+</sup> cells) from the PBLs of Cat 303, and then conducted co-cultures with FIV-infected

MYA-1 cells. As shown in Figure 7a, CD3 $\epsilon$ -depletion induced an increase in p24 in the FIV-14-infected MYA-1 cell culture. Because the CD3 $\epsilon$ <sup>+</sup> population is mostly composed of CD4<sup>+</sup> and CD8<sup>+</sup> cells (CHAPTER 1), depletion of CD4<sup>+</sup> or CD8<sup>+</sup> cells was also carried out. As shown in Figure 7b, a p24 increase was observed in both CD3 $\epsilon$ - and CD4-depleted, but not CD8 $\alpha$ -depleted, PBLs co-cultured with FIV-14-infected MYA-1. In the last experiment, uninfected MYA-1 was also used for the co-culture, and a significant increase in endogenous p24 was observed in the CD3 $\epsilon$ -depleted cell culture (Fig. 7c). In addition, though not significant (OD<sub>655</sub><0.5), an increase in p24 was observed in the CD4-depleted cell culture compared with the mock-treated cell culture. Hence, this cat certainly had infected cells in the periphery, and CD4<sup>+</sup> lymphocytes were the principal effector hindering FIV replication in PBLs. The non-proliferation of endogenous or exogenous virus in cultures of CD8-depleted PBLs was due to this potent antiviral activity of CD4<sup>+</sup> lymphocytes. However, CD8<sup>+</sup> lymphocytes also might have weak antiviral activities in this cat, because CD3 $\epsilon$ -depletion induced more viral replication from an FIV-14-infected cell line than CD4-depletion (Fig. 7b, endogenous FIV replication was negligible, data not shown). These results suggest that more than two distinct populations in one individual can be involved in the suppression of FIV-replication.

No replication of virus in CD8-depleted PBMCs of infected

individuals like in the case of Cat 303 was occasionally reported in FIV [Bucci *et al.*, 1998a; Jeng *et al.*, 1996] and HIV [Walker *et al.*, 1986] studies. Several reasons for the phenomenon could be proposed, however detailed immunological analyses for these individuals have not been done. The author consider that CD4<sup>+</sup> PBLs, although less frequent than CD8<sup>+</sup> PBLs, can control lentivirus replication and that the infected individuals described above might control FIV or HIV replication by CD4<sup>+</sup> lymphocyte-mediated cytotoxicity [Curiel *et al.*, 1993; Kundu *et al.*, 1992, Kundu & Merigan, 1992; Siliciano *et al.*, 1988], secretion of interferon- $\gamma$  and  $\beta$  chemokines [Furci *et al.*, 1997; Rosenberg *et al.*, 1997] or other unidentified mechanisms. The lack of a correlation between CD8<sup>+</sup> cell numbers and non-cytolytic activities [Bucci *et al.*, 1998a; Flynn *et al.*, 2002; Hohdatsu *et al.*, 2003; Jeng *et al.*, 1996] may be explained by the activity of these CD8-negative lymphocytes. Studies with larger numbers of cats will be needed to test this hypothesis.

#### *CD4:CD8 ratios*

FIV infection induces the decrease of CD4:CD8 ratios of PBLs of the infected cats and the decreases are often used to estimate immunological disorders [Ackley *et al.*, 1990; Beatty *et al.*, 1996; Kohmoto *et al.*, 1998b; Willett *et al.*, 1993]. The CD4:CD8 $\alpha$  ratios of the cats used in this study were as follows:

Cat 301, 0.77; Cat 302, 0.59; Cat 303, 0.33; Cat 304, 0.95; Cat 305, 0.59; Cat 306, 0.77. Three out of five FIV-infected cats showed lower ratios than that of uninfected one (Cat 306). There seemed to be no apparent correlation between the CD4:CD8 $\alpha$  ratios and the increases of CD8 $\beta$ -decreased subpopulations within CD8 $^+$  PBLs or lymphocyte-phenotypes responsible for inhibition of FIV replication. Surprisingly, Cat 303, whose CD4 $^+$  PBLs showed the potent antiviral activity (Fig. 7), had the lowest CD4:CD8 $\alpha$  ratio among the cats.

#### *Reconstitution assay*

To further analyze the antiviral activities, a reconstitution assay was performed based on the results obtained from the depletion assay. CD8 $\alpha^+$  cells from Cats 301, 304 and 305, and CD4 $^+$  cells from Cat 303 were isolated by panning from each animal as effector cells that were regarded to suppress FIV replication. The purity of the effector cells for CD8 $\alpha$  or CD4 was over 80 % (data not shown). PBMCs depleted of the effector cells served as target cells where FIV could proliferate. Mock-treated PBMCs of Cat 302 and FIV TM2- or FIV-14-infected MYA-1 cells were also used as target cells. These effector and target cells were mixed in all combinations, cultured, and then measured for p24 antigens in culture at day 12 or 9 (Table 1). Effector cells from Cats 301, 303 and 305 inhibited p24 production

from self-PBMCs significantly (> 50% inhibition), confirming the results in the depletion assay, while those from Cat 304 did not. The failure of inhibition in Cat 304 might be due to effector cellular damage. Effectors from Cats 301, 303 and 305 also inhibited FIV proliferation in non-self targets and in homologous (TM2) or heterologous (FIV-14) FIV strain-infected MYA-1 cells, although not in all combinations. These results suggest that the antiviral activity of CD4<sup>+</sup> as well as CD8<sup>+</sup> lymphocytes was mediated in a non-restricted manner by MHC, and possibly in an antigen-non specific manner. No restriction of the activity to homologous isolates was reported [Flynn *et al.*, 1999]. The reason for the absence of suppressive activity of effectors against viral replication in Cat 302 PBLs is unclear.

In conclusion, the author has demonstrated that various subpopulations of PBLs in FIV-intravaginally infected cats (CD8 $\alpha^+\beta^+$ , CD8 $\alpha^+\beta^-$ , and CD4<sup>+</sup> phenotypes) are involved in the suppression of FIV replication, at least in part, in a MHC-non-restricted manner. A detailed understanding of immune responses in mucosal infection may help with vaccine- or antiviral drug-development against both FIV and HIV, therefore further analysis of these cells carrying antiviral activities is important.

## Figure legends

### Fig. 1

Depletion of CD8 $\beta$ <sup>+</sup> or CD8 $\alpha$ <sup>+</sup> cells from PBMCs of FIV-infected cats. Representative FCM results of Cat 302 PBLs are shown. PBMCs were labeled with no mAb (mock), anti-CD8 $\alpha\beta$  or anti-CD8 $\alpha$ , and depleted of the intended cell populations by the panning method. Then non-adherent cells were harvested as mock-treated (a), CD8 $\beta$ -depleted (b) and CD8 $\alpha$ -depleted (c) PBLs and used for the depletion assay (Fig. 5).

### Fig. 2

Blocking effects of vpg9 mAb (anti-CD8 $\alpha\beta$  complex) on FT2 mAb (anti-CD8 $\beta$ ) binding. PBMCs of Cat 301 and 302 were pretreated with unlabeled control mouse serum (thin lines) or vpg9 (bold lines), and incubated with PE-labeled FT2.

### Fig. 3

Epitopes of three anti-CD8 $\alpha$  mAbs: 2D7, 10C7, and 12A3 [Shimajima *et al.*, 1998b]. PBMCs were pretreated with unlabeled mAbs, and then further treated with FITC-labeled mAbs in all combinations. White histograms were the results of pretreatment with control mouse serum, and black histograms were the results of 2D7-, 10C7-, or 12A3-pretreatment. Note that 12A3 mAb did not block binding of 10C7 mAb.

Fig. 4

Light scatters in FCM for PBMCs of Cats 301 (a, b) and 302 (c, d). Isolated PBMCs (a, c) were seeded on anti-mouse IgG-coated Petri dishes, and then non-adherent cells were harvested (b, d).

Fig. 5

Depletion assay: FIV replication in depleted PBLs. Mock-treated ( $\circ$ ), CD8 $\beta$ -depleted ( $\triangle$ ) and CD8 $\alpha$ -depleted ( $\square$ ) PBLs of FIV-infected cats (Cats 301-305) were mixed with FIV-highly sensitive T-lymphoblastoid cell line (MYA-1 cells), stimulated by Con A for 3 days and cultured for a total of 12 days. Culture supernatants were harvested on the days indicated and measured for the FIV p24 Gag antigens by ELISA. Experiments were performed in duplicate and the averages of OD<sub>655</sub> values are shown. A value greater than 0.5 was regarded as indicating positivity for FIV-proliferation.

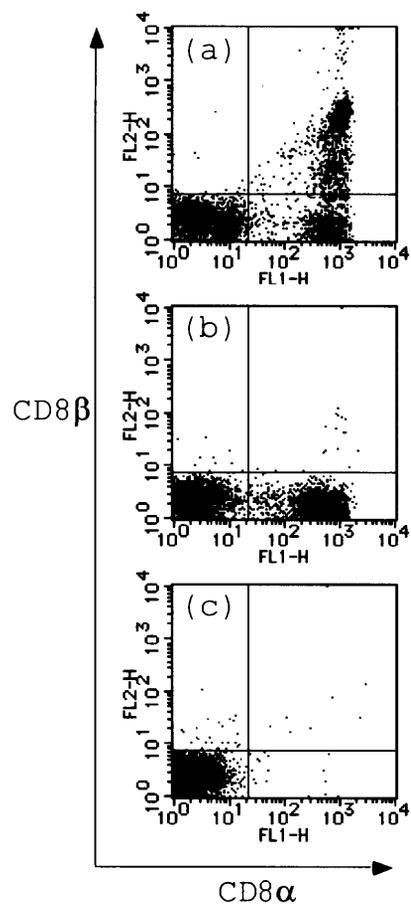
Fig. 6

Depletion assay using cryopreserved PBMCs of Cat 301 and 303. ELISA for culture supernatants were performed only at day 12. " $\Delta$ CD8beta" means CD8 $\beta$ -depleted PBLs, and "mock" means PBLs labeled with no mAb for panning. Results are shown as the mean

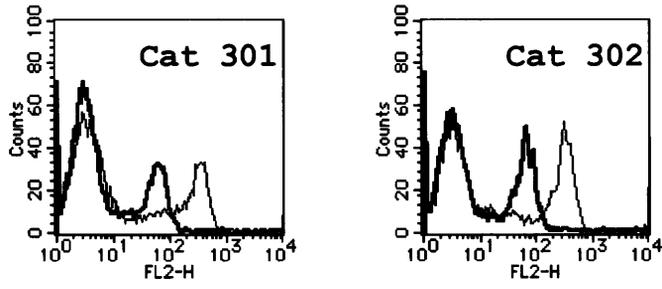
± SD of quadruplicate data. White bars are results in MYA-1 cell culture without mixing of the depleted cells.

Fig. 7

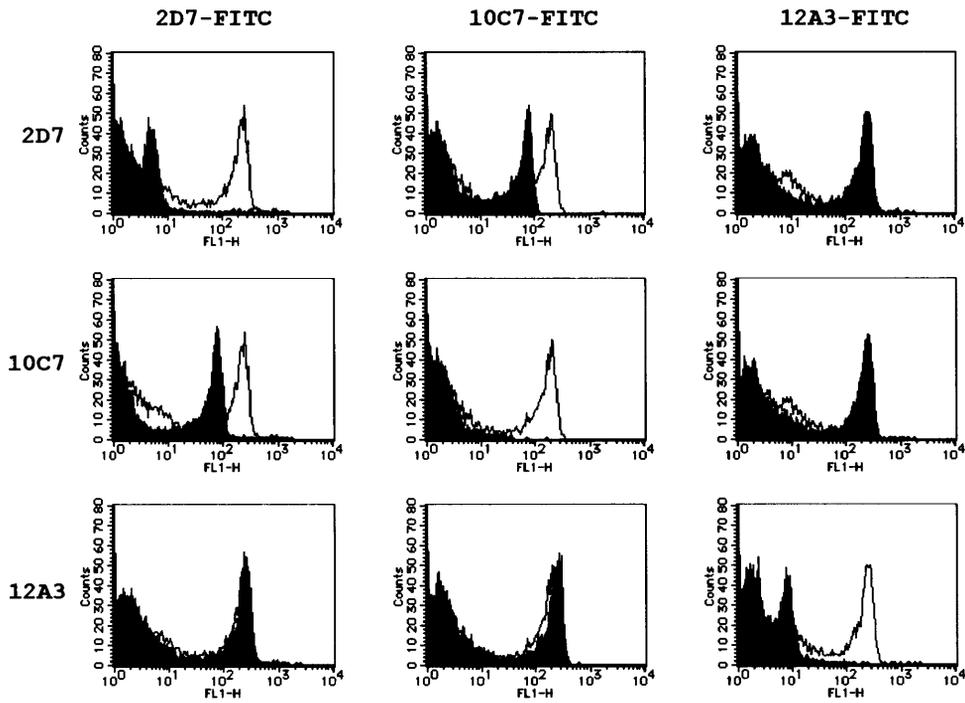
Effects of depletion of other subpopulations in Cat 303 PBLs on FIV replication. By the panning method, the PBMC-subpopulations indicated were depleted from PBLs of Cat 303 (for example, "ΔCD3" means CD3ε-depleted PBLs, and "mock" means PBLs labeled with no mAb for panning). These depleted cells were mixed with FIV-14-infected (a, b) or uninfected (c) MYA-1 cells and cultured. p24 antigens in culture supernatants were measured by ELISA at day 9 (a, b) or at day 12 (c). Black bars are results in infected MYA-1 cell culture without mixing of the depleted cells. Results are shown as the mean ± SD of quadruplicate data. The background (uninfected MYA-1 cell culture alone) is approximately 0.1 OD<sub>655</sub>.

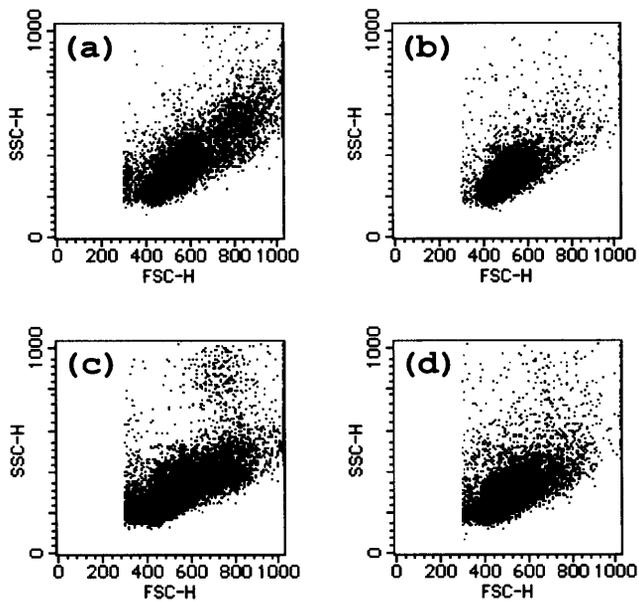


CHAPTER 5/Fig. 2

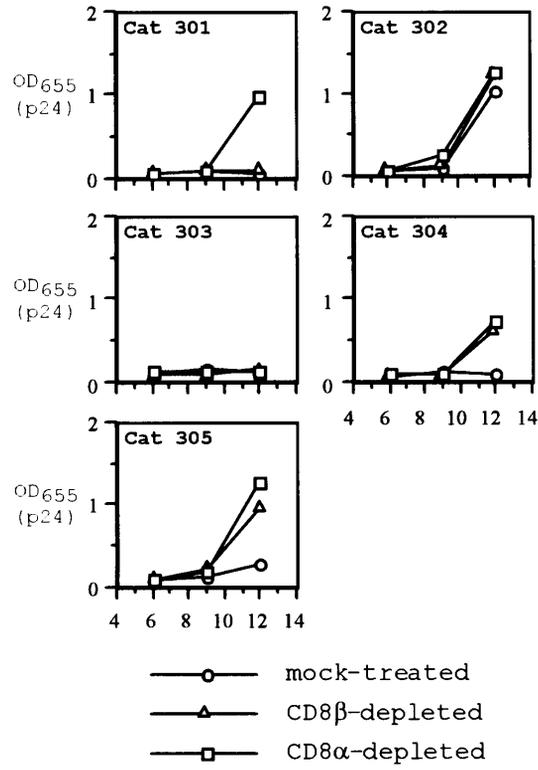


CHAPTER 5/Fig. 3



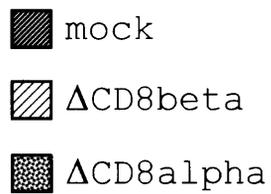
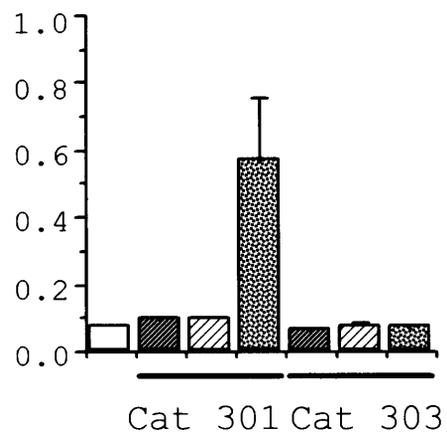


CHAPTER 5/Fig. 5

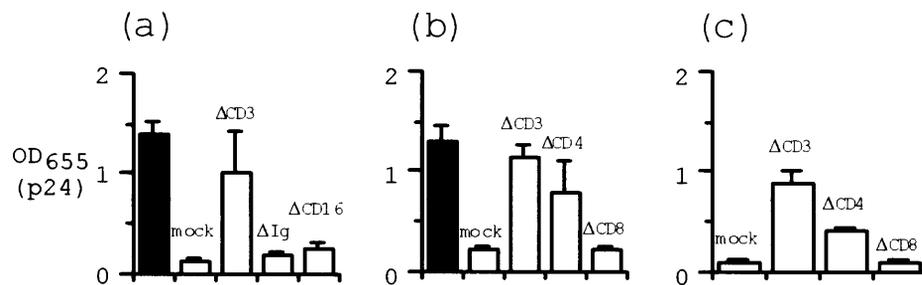


CHAPTER 5/Fig. 6

OD<sub>655</sub> (p24)



CHAPTER 5/Fig. 7



CHAPTER 5/Table 1

Reconstitution assay: co-culture of effector cells with self- or non-self-target cells from FIV-infected cats or with an *in vitro*-infected cell line (MYA-1).

		Inhibition % <sup>a</sup>						
		Target cell						
		Cat301	Cat302	Cat303	Cat304	Cat305	TM2- inf.	FIV14- inf.
Effector	$\Delta$ CD8 $\alpha^b$	whole	$\Delta$ CD4	$\Delta$ CD8 $\alpha$	$\Delta$ CD8 $\alpha$	MYA-1 <sup>c</sup>	MYA-1	
301	CD8 $\alpha^d$	74	15	99	21	95	61	16
303	CD4	97	8	98	100	95	54	84
304	CD8 $\alpha$	33	23	-19	46	43	29	19
305	CD8 $\alpha$	94	-7	32	84	71	48	46

<sup>a</sup>Inhibition percentages were calculated as described in Materials & Methods. More than 50% is regarded as significantly positive.

<sup>b</sup>CD8 $\alpha$ -depleted PBLs of Cat 301.

<sup>c</sup>MYA-1 cells infected with FIV strain TM2 at an moi of 0.01.

<sup>d</sup>CD8 $\alpha^+$  cells isolated from Cat 301 PBLs.

CHAPTER 6

Phenotypic changes in CD8<sup>+</sup> PBLs in cats infected with FIV

Microbes and Infection 2003; 5:1171-6

## Abstract

It is well documented that several cell surface molecules of T lymphocytes are altered by immune activation. The author previously reported that FIV infection induces a reduction in CD8  $\beta$  chain expression of PBLs in cats. In this CHAPTER, the author performed three-color FCM analyses for activation-associated cell surface molecules (CD2, CD11a, CD45RA-like and MHC II) and light scatters (cellular size and complexity) to examine whether phenotypic changes occurred also in CD4<sup>+</sup> PBLs, in addition to CD8<sup>+</sup> PBLs, of five FIV-infected cats and one uninfected cat. It was shown that (i) CD8 $\alpha$ <sup>+</sup> PBLs, but not CD4<sup>+</sup> PBLs, had a distinct subpopulation with increased CD11a expression accompanying a reduced CD8  $\beta$  chain and increased intracellular granules (ii) CD8 $\alpha$ <sup>+</sup> PBLs, but not CD4<sup>+</sup> PBLs, expressed CD45RA-like antigen with diverse expression levels and (iii) MHC II expression was greater in CD8 $\alpha$ <sup>+</sup> PBLs than CD4<sup>+</sup> PBLs and the CD8  $\beta$  chain reduction was correlated with the MHC II decrease within CD8 $\alpha$ <sup>+</sup> PBLs. These results suggest that FIV infection induces phenotypically heterogeneous subpopulations in CD8<sup>+</sup> PBLs, including activated phenotypes, rather than in CD4<sup>+</sup> PBLs.

## Introduction

The activation of T cells results in phenotypic changes of cell surface antigens such as co-stimulatory and adhesion molecules. The changes affect the interaction of T cells with other lymphocytes, APCs and vascular endothelial cells. Naïve and memory/effector T cells can now be discerned from the expression levels of these molecules. For example, CD8<sup>+</sup> T cells are distinguishable as CD11b<sup>-</sup>CD28<sup>+</sup>CD62L<sup>+</sup> or naïve, CD11b<sup>-</sup>CD28<sup>+</sup>CD62L<sup>low</sup> or memory, and CD11b<sup>+</sup>CD28<sup>-</sup>CD62L<sup>-</sup> or effector cells in humans [Hamann *et al.*, 1997] and mice [Zimmermann *et al.*, 1996].

FIV infections in domestic cats have an early phase characterized by viremia and rapid CD8<sup>+</sup> lymphocytosis in circulation, followed by an asymptomatic phase in which CD4<sup>+</sup> cell numbers decrease gradually, and then immunodeficiency-like syndromes are terminally induced [Ackley *et al.*, 1990; Kohmoto *et al.*, 1998b; Willett *et al.*, 1993; Yamamoto *et al.*, 1988]. Previously the author reported that the increase in CD8<sup>+</sup> PBLs includes unique subsets having reduced or no expression of the CD8  $\beta$  chain, but not  $\alpha$  chain [Shimojima *et al.*, 1998a]. The  $\beta$  chain reduction has not been well documented in other viral infections, but is a common phenomenon in HIV infection [Schmitz *et al.*, 1998]. Recently, the  $\beta$  chain-reduced CD8<sup>+</sup> PBLs were shown to have anti-viral effects in vitro [Bucci *et al.*, 1998b; Flynn

*et al.*, 2002]. However little is known about phenotypes of T lymphocytes in response to FIV-infection in cats. In the CHAPTER, the author analyzed CD4<sup>+</sup> PBLs in addition to CD8 $\alpha$ <sup>+</sup> PBLs of FIV-infected or uninfected cats by FCM for expression of cell surface molecules (CD2, CD11a, CD45RA-like and MHC II) and light scatters (cellular size and complexity), which are thought to be altered by immune activation [Hamann *et al.*, 1997; Sanders *et al.*, 1988; Zimmermann *et al.*, 1996].

## Materials & Methods

### *Experimental animals*

Five infected cats and one uninfected cat used were described in CHAPTER 5.

### *Antibodies & reagents*

Anti-feline CD4 4D9 [Shimojima *et al.*, 1997] and anti-feline CD8 $\alpha$  10C7 [Shimojima *et al.*, 1998b] antibodies were used after being labeled with FITC or biotin. Biotin-labeled anti-feline CD8 $\beta$  FT2 (Southern Biotechnology Associates), PE-labeled anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA), and streptavidin PerCP (Becton Dickinson) were purchased commercially. Anti-feline CD2 SKR2 (CHAPTER 1), anti-feline CD11a TMM11a (CHAPTER 2) and anti-feline MHC II vpg3 (kindly provided by Dr. B. J. Willett, University of Glasgow) [Willett *et al.*, 1991] were used without labeling. Previously, Masuoka *et al.* [1992] reported that 15B3 antibody recognized 220 kDa molecules of feline T lymphoma and were reactive with B cells and a population of T cells in lymph node but not with thymocytes in cats. The staining pattern was strikingly associated with the human CD45RA isoform [Pulido *et al.*, 1988], therefore the antibody was used as "anti-CD45RA-like" in the thesis.

### *Three-color FCM*

PBMCs were isolated as described in CHAPTER 1 and washed with cold wash buffer (CHAPTER 1). Cells were incubated on ice with antibodies or reagents in the following order: unlabeled antibodies (anti-CD2, anti-CD11a, anti-CD45RA-like, anti-MHC II or mouse serum), PE-labeled secondary antibody, FITC-labeled antibody (anti-CD4 or anti-CD8 $\alpha$ ) simultaneously with biotin-labeled antibody (anti-CD8 $\alpha$  or anti-CD8 $\beta$ ) and streptavidin PerCP. After incubation with secondary antibody, excess binding sites were blocked using mouse serum. The washing of cells with cold wash buffer was performed two or three times after each incubation. Labeled cells were analyzed after gating for lymphocytes based on FSC and SSC using FACScan flow cytometer with Cell Quest software (Becton Dickinson). FSC and SSC values were also used for analyses of cellular size and complexity, respectively.

## Results

### *Phenotypic comparison between CD4<sup>+</sup> and CD8α<sup>+</sup> PBLs*

PBMCs were labeled for three antigens, CD4, CD8α and molecules of interest (CD2, CD11a, CD45RA-like and MHC II), and then analyzed after gating for CD4-single positive and CD8α-single positive PBLs (Fig. 1a) by FCM. Representative results are shown in Fig. 1. CD2 expression was observed in almost all CD4<sup>+</sup> (> 97%) and CD8α<sup>+</sup> (> 96%) PBLs with a similar MFI (Fig. 1c). CD11a was also expressed constitutively in both populations (> 99%). However a distinct subpopulation of CD8α<sup>+</sup> PBLs, but not CD4<sup>+</sup> PBLs, showed increased expression of the antigen (Fig. 1d). While a substantial but diverse expression of CD45RA-like antigen was observed within CD8α<sup>+</sup> PBLs (7.4 - 44.4%), only a very small population of CD4<sup>+</sup> PBLs (0.8 - 2.3%) expressed the antigen (Fig. 1e). Almost all CD4<sup>+</sup> and CD8α<sup>+</sup> PBLs expressed MHC II antigen (> 99%), and MFI ratios (MFI in CD8α<sup>+</sup>/MFI in CD4<sup>+</sup> PBLs) were greater than 1 (Table 1), indicating that CD8α<sup>+</sup> PBLs expressed the antigen more than CD4<sup>+</sup> PBLs.

### *Increase of CD11a expression in CD8α<sup>+</sup> PBLs accompanies cellular complexity*

CD8α<sup>+</sup>CD11a<sup>+</sup> PBLs were further analyzed for cellular size and complexity after subdivision into two subpopulations depending on CD11a expression, CD11a<sup>low</sup> and CD11a<sup>high</sup>. As shown

in Table 1, mean SSC value ratios (SSC in  $CD8\alpha^+CD11a^{high}$ /SSC in  $CD8\alpha^+CD11a^{low}$  PBLs) were greater than 1, indicating that  $CD8\alpha^+CD11a^{high}$  PBLs had more cellular complexity. Similar analyses were performed for other combinations (Table 1). The SSC ratio of  $CD8\alpha^+/CD4^+$  in the uninfected cat (Cat 306) was 0.94, but values for infected cats were over 1. SSC ratios in  $CD8\alpha^+CD11a^{low}/CD4^+$  of cats except one infected animal (Cat 301) did not exceed 1. Taken together, with the fact that the uninfected cat did not have sufficient numbers of  $CD8\alpha^+CD11a^{high}$  PBLs (Fig. 1g), these results showed that  $CD8\alpha^+CD11a^{high}$  PBLs had more cellular complexity than  $CD8\alpha^+CD11a^{low}$  and  $CD4^+$  PBLs. No apparent tendency in FSC ratios among these populations was observed (ratios of 0.96 to 1.02).

#### *Relationships with CD8 $\beta$ chain expression*

To examine relationships among the expression of CD11a, CD45RA-like and MHC II antigens with CD8  $\beta$  chain expression, PBMCs were labeled for combinations of CD8 $\alpha$ , CD8 $\beta$  and molecules of interest and then analyzed after gating for  $CD8\alpha^+$  PBLs (Fig. 2a). As shown in Fig. 2, the CD11a increase was strictly limited in  $CD8\alpha^+\beta^{low}$  and  $CD8\alpha^+\beta^-$  but not in  $CD8\alpha^+\beta^{high}$  PBLs (Fig. 2c). However, very small subpopulations of  $CD8\alpha^+\beta^{low}$  and  $CD8\alpha^+\beta^-$  PBLs did not show such increases, but rather a slightly decreased expression, and similar subpopulations were also observed in the uninfected

cat (Fig. 2d). Both CD45RA-like positive and negative cells were observed in each subpopulation of CD8 $\alpha^+$  lymphocytes, and additionally, a median expression level of the antigen was also observed particularly within CD8 $\alpha^+\beta^{\text{high}}$  lymphocytes (Fig. 2e, f). CD8  $\beta$  chain reduction was accompanied by a reduction in MHC II MFI, but not in one infected cat (Cat 303) (Fig. 3). CD8  $\beta$ -negative cells (CD8 $\alpha^+\beta^-$ ) had a still lower MHC II MFI than CD8 $\alpha^+\beta^{\text{high}}$  or CD8 $\alpha^+\beta^{\text{low}}$  PBLs (Figs. 2g, 3).

## Discussion

In HIV-infected individuals, a selective increase of activation antigens such as HLA-DR, CD11a and CD38 has been demonstrated in both CD4<sup>+</sup> and CD8<sup>+</sup> cells [Ho *et al.*, 1993; Kestens *et al.*, 1992, 1994; Scala *et al.*, 1995]. The author found various phenotypes within CD8 $\alpha$ <sup>+</sup> PBLs of FIV-infected cats, as well as CD8  $\beta$  chain reduction, that were previously reported [Shimojima *et al.*, 1998a]. Within CD4<sup>+</sup> PBLs, however, distinct subpopulation(s) were not found, and did not appear with infection. These results indicate that CD8<sup>+</sup> PBLs rather than CD4<sup>+</sup> PBLs profoundly respond to FIV infection in cats.

CD11a (an  $\alpha$ L chain of LFA-1, a member of the  $\beta$ 2 integrin family) is expressed in all leukocytes and functions as an adhesion molecule between the cells and target cells for cytotoxicity, vascular endothelial cells, and the cell itself via ICAMs. CD11a expression in CD8<sup>+</sup> memory/activated lymphocytes is up-regulated when compared with that in naïve cells, therefore the up-regulation can be used as a reliable marker for memory/activated lymphocytes in various mammals, including humans [Hviid *et al.*, 1993; Okumura *et al.*, 1993; Scala *et al.*, 1995], monkeys [Pitcher *et al.*, 2002], mice [Andersson *et al.*, 1995; Slifka *et al.*, 2000] and rats [Hedlund *et al.*, 1995]. It is reasonable that feline CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>low</sup> or CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup> PBLs, which increase with FIV infection and have anti-FIV activities [Bucci *et al.*,

1998b; Flynn *et al.*, 2002; CHAPTER 5], show the up-regulation of the CD11a molecule (Fig. 2c). Besides CD11a, the expression levels of several surface antigens were also reported to change within CD8 $\alpha^+$  PBLs of FIV-infected cats, however the use of them is shown to be difficult for division into different immune stages [Gebhard *et al.*, 1999; Kern *et al.*, 1994; Mobley *et al.*, 1994; Zimmermann *et al.*, 1996]. In the future, anti-CD11a antibody TMM11a will be a useful reagent with which to analyze immunological states in FIV-infection.

In humans and mice, memory/activated CD8 $^+$  PBLs show increased values of FSC as well as SSC [Hoflich *et al.*, 1998; Zimmermann *et al.*, 1996]. In FIV-infected cats, while the author could not detect such increased values in FSC of CD8 $\alpha^+$  PBLs, an increase of SSC was observed, suggesting that the cells actively synthesize cytokines such as perforin, IFN- $\gamma$  and TNF- $\alpha$  [Hamann *et al.*, 1997; Hoflich *et al.*, 1998] which would function as anti-viral factors [Bucci *et al.*, 1998b; Flynn *et al.*, 2002].

The CD45RA molecule is expressed in naïve CD4 $^+$  and CD8 $^+$  lymphocytes by nature, gradually down-regulated as the memory state after the first encounter with specific antigens, and re-up-regulated upon a re-encounter as the effector state of CD8 lymphocytes in humans [Okumura *et al.*, 1993]. In CD4 $^+$  lymphocytes, re-up-regulation of CD45RA does not occur in humans [Okumura *et al.*, 1993] and mice [Lee & Vitetta, 1991]. In the

study, the author observed a similar diverse expression (high, low and negative) of CD45RA-like antigen in CD8<sup>+</sup> T lymphocytes in cats (Fig. 1e). These results suggest that CD45RA<sup>high</sup> and CD45RA<sup>-</sup> cells within CD8 $\alpha^+\beta^{\text{low or }-}$ CD11a<sup>high</sup> PBLs (Fig. 2) are effector and memory T lymphocytes, respectively. However, the expression pattern within CD8 $\alpha^+\beta^{\text{high}}$  PBLs of cats is inconsistent with that of humans. In humans, the loss of CD45RA always accompanies an up-regulation of CD11a [Hoflich et al., 1998; Okumura et al., 1993]. As shown in Fig. 2, in cats, CD45RA-like antigen apparently decreases without this up-regulation. In addition, the uninfected cat, in which the immune system was not so activated, did not have enough CD4<sup>+</sup> PBLs expressing CD45RA-like antigens (2.0% in CD4<sup>+</sup> PBLs). It is yet to be defined whether the discrepancy is due to species differences or the 15B3 antibody used in this study does not exactly detect the feline CD45RA homologue. Further characterization of the antigen recognized by 15B3 antigen may be needed.

The author's results also showed that CD8<sup>+</sup> cells with increased CD11a expression and intracellular granules, namely activated CD8<sup>+</sup> PBLs, had down-regulated MHC II expression compared with CD8 $\alpha^+\beta^{\text{high}}$ , perhaps unprimed lymphocytes (Fig. 3). However this is not consistent with other reports in which MHC II expression was increased by FIV infection [Rideout et al., 1992; Willett et al., 1993]. The reason for the discrepancy is

unclear, but may be the difference in infection duration, infection route or virus strains used.

MHC II expression levels in CD4<sup>+</sup> PBLs may be down-regulated by FIV infection, consistent with a previous study using CD4<sup>+</sup> cell line [Willett *et al.*, 1991]. This speculation is based on the finding that the ratio of MHC II MFI (CD8 $\alpha$ <sup>+</sup> vs CD4<sup>+</sup>) was increased by FIV infection (Table 1) even though the infection resulted in CD8 $\alpha$ <sup>+</sup> subpopulations with decreased MHC II (Fig. 3). However, the possibility can not be excluded that FIV infection causes MHC II up-regulation of whole CD8 $\alpha$ <sup>+</sup> PBLs. Lerner *et al.* [1998] reported upregulation of MHC II in FIV-infected CD4<sup>+</sup>CD8<sup>+</sup> cell line MCH5-4. Further analyses of MHC II dynamics in both CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> PBLs will be necessary.

The author has shown that FIV infection causes phenotypically heterogenous subpopulations in CD8<sup>+</sup> PBLs. Although no apparent phenotypic changes were found within CD4<sup>+</sup> PBLs, analyses of other surface antigens (CD28, CD38, CD45RO, CD95, CCR7) or cytokines (IL-2, IL-4, IFN- $\gamma$ ) will be helpful to elucidate the dynamics of CD4<sup>+</sup> PBLs as well as CD8<sup>+</sup> PBLs in response to FIV infection.

## Figure legends

### Fig. 1

Three-color FCM of feline PBLs. Isolated PBMCs were labeled for combinations of CD4 (FL1), CD8 $\alpha$  (FL3) and other molecules (FL2) and CD4<sup>+</sup>CD8 $\alpha$ <sup>-</sup> and CD4<sup>-</sup>CD8 $\alpha$ <sup>+</sup> PBLs were gated (a) for analyses of the expression of molecules of interest (b-g). These molecules are control (b), CD2 (c), CD11a (d, g), CD45RA-like antigen (e), and MHC II (f). Fine and bold lines are results for gated CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> PBLs, respectively. PBMCs were isolated from FIV-infected (a-f) and uninfected (g) cats.

### Fig. 2

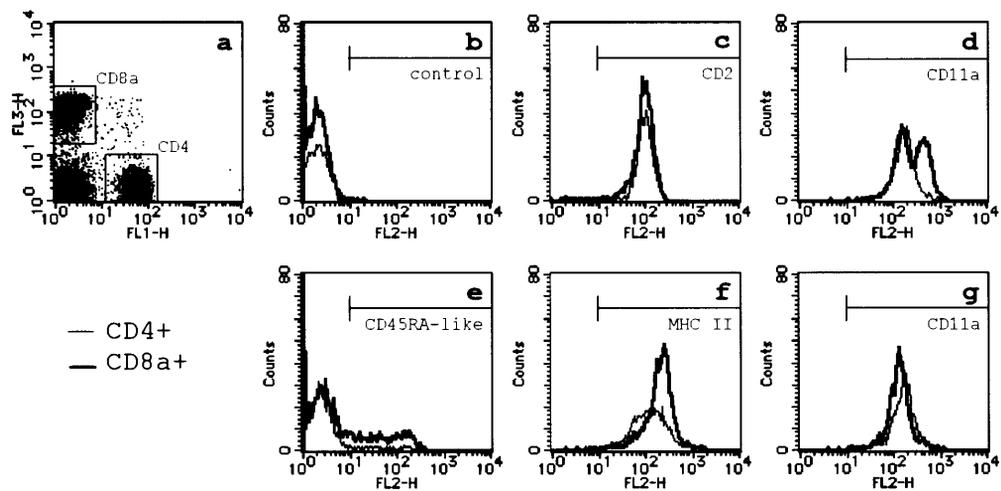
FCM analyses of CD8 $\alpha$ <sup>+</sup> PBLs in cats. PBMCs were labeled for combinations of CD8 $\alpha$  (FL1), CD8 $\beta$  (FL3) and other molecules (FL2). CD8 $\alpha$ <sup>+</sup> PBLs were gated (a) to analyze the correlation of the molecules of interest with the CD8  $\beta$  chain (b-g). These molecules are control (b), CD11a (c, d), CD45RA-like antigen (e, f) and MHC II (g). X and Y axes are fluorescence intensities for the molecules and CD8  $\beta$  chain, respectively (b-g). PBMCs were isolated from FIV-infected (a-c, e, g) and uninfected (d, f) cats.

### Fig. 3

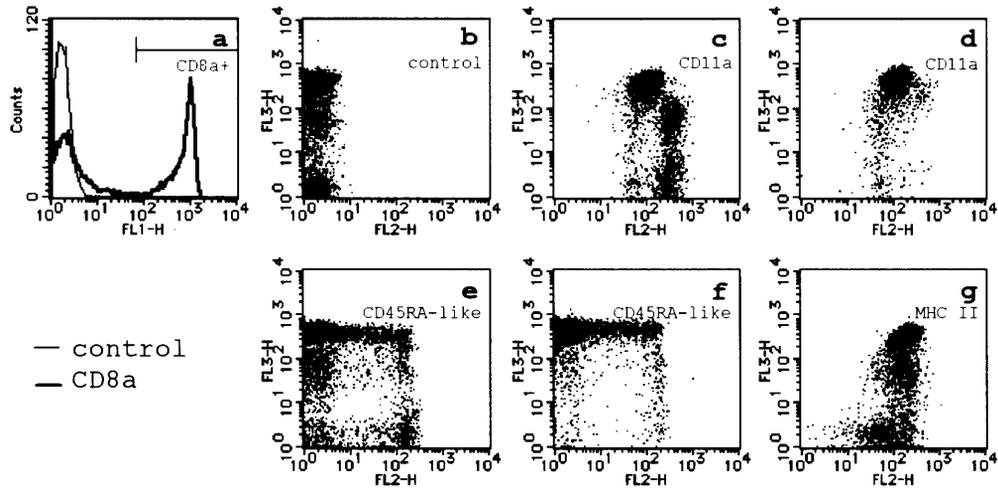
MFI of MHC II antigens in CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>high</sup>, CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>low</sup> and CD8 $\alpha$ <sup>+</sup> $\beta$ <sup>-</sup>

PBLs. The results obtained in Fig. 2g were re-analyzed for expression of MHC II in each subpopulation. ○, Cat 301; △, Cat 302; □, Cat 303; ●, Cat 304; ▲, Cat 305; ■, Cat 306.

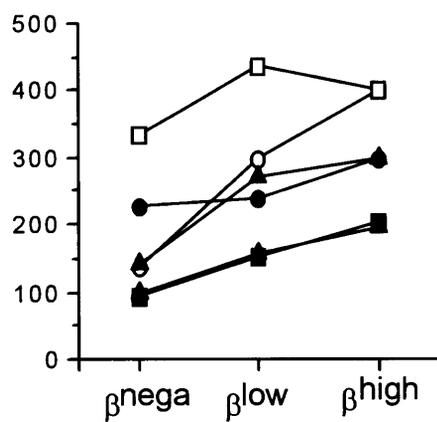
CHAPTER 6/Fig. 1



CHAPTER 6/Fig. 2



MFI of MHC II antigen



## CHAPTER 6/Table 1

Table 1. Ratios of MFI or SSC in FCM analysis

	MHC II-MFI ratio		SSC ratio	
	CD8 $\alpha^+$ vs CD4 $^+$	CD8 $\alpha^+$ CD11a <sup>high</sup> vs CD8 $\alpha^+$ CD11a <sup>low</sup>	CD8 $\alpha^+$ vs CD4 $^+$	CD8 $\alpha^+$ CD11a <sup>low</sup> vs CD4 $^+$
Infected				
Cat301	1.26	1.22	1.13	1.05
Cat302	1.23	1.22	1.01	0.95
Cat303	1.34	1.19	1.06	0.93
Cat304	1.78	1.20	1.13	1.00
Cat305	1.55	1.21	1.05	0.96
Uninfected				
Cat306	1.09	1.21	0.94	0.95

Antigen expression levels (MFI) and cellular complexity (SSC) were analyzed by FCM for each subpopulation indicated, and then ratios were calculated.

PART IV

Characterization of MYA-1 cells, a feline T-lymphoblastoid cell  
line

MYA-1 cells established by T. Miyazawa are a feline T-lymphoblastoid cell line which expresses CD4 and shows IL-2-dependent growth. The cell line is often used for FIV studies because of its high sensitivity to FIV infection.

In PART IV, to characterize MYA-1 cells more, the author generated an mAb to feline CD56 and then analyzed CD56 expression in feline cells as well as MYA-1 (CHAPTER 7) and correlation of its expression and FIV infection in MYA-1 cells (CHAPTER 8).

CHAPTER 7

CD56 expression in feline lymphoid cells

The Journal of Veterinary Medical Science 2003; **65**:769-73

## Abstract

The N-CAM consists of three major types of polypeptides (180, 140, and 120 kDa) whose predominant differences exist within the transmembrane and cytoplasmic domains. In this CHAPTER, the author generated an mAb, termed SZK1, reactive to feline CD56 molecules (140 kDa form of N-CAM) expressed by the baculovirus expression system and investigated CD56 expression in feline lymphoid cells. In FCM analysis, SZK1 was reactive to a feline T-lymphoblastoid cell line MYA-1. Further, SZK1 was reactive to a very small population (1.1-1.7%) of freshly isolated PBLs of three SPF cats, and the reactivity was increased by culturing of PBLs in the presence of IL-2 following Con A-stimulation (>10%). In immunoblotting analysis, SZK1 detected an approximately 160 kDa antigen from MYA-1 cells, while from RNA of the cells RT-PCR amplified the fragment resembling 140 kDa form of N-CAM. These findings suggest that feline CD56 has similar characteristics with human CD56.

## Introduction

The N-CAM is a member of the immunoglobulin superfamily that mediates homotypic adhesive interactions of cells in neural and muscle tissues [Cunningham *et al.*, 1987]. N-CAM consists of three major types of polypeptides (180, 140, and 120 kDa, estimated by SDS-PAGE) generated by alternative mRNA splicing from a single gene in mammals and avians [Barthels *et al.*, 1988; Hemperly *et al.*, 1990; Murray *et al.*, 1986a, b; Small *et al.*, 1987]. The predominant differences in these forms are within the transmembrane and cytoplasmic domains [Cunningham *et al.*, 1987]. The 140 kDa form lacks an insert of approximately 270 aa in the cytoplasmic region, which is present within the 180 kDa form [Goridis & Brunet, 1992]. The 120 kDa form lacks a membrane spanning domain and is linked to a glyco-phosphatidylinositol [Hemperly *et al.*, 1986]. In addition, the three forms have the optional sequences within extracellular domains that arise by the splicing of smaller RNA segments and correspond to 10-40 aa [Goridis & Brunet, 1992]. The form(s) of N-CAM that is expressed seems to be dependent on the developmental-stages and cell types. In brain, 180 kDa form are mainly expressed by postmitotic neuron and 140 kDa form are more widely distributed. 120 kDa form appears during nerve system development. In muscle, 140 and 120 kDa forms are distributed [Goridis & Brunet, 1992; Rutishauer & Goridis, 1986].

In humans, CD56 (Leu19, NKH-1) antigen is expressed on approximately 15% of PBLs that are mainly composed of NK cells ( $\approx 10\%$ ) and CD3<sup>+</sup> T lymphocytes ( $< 5\%$ ) [Lanier *et al.*, 1986]. Both CD56<sup>+</sup> NK cells and CD56<sup>+</sup> T cells mediate non-MHC-restricted cytotoxicity [Hercend *et al.*, 1985; Lanier *et al.*, 1986; Tarazona *et al.*, 2000]. CD56 has been shown to be identical to the 140 kDa form of N-CAM [Lanier *et al.*, 1989], however the antigen as well as other forms of N-CAM undergoes posttranslational modifications including addition of polysialic acids in some cell-types such as lymphocytes, resulting in showing larger MW [Goridis & Brunet, 1992; Hercend *et al.*, 1985; Lanier *et al.*, 1986, 1989].

Little is known about feline NK cells or CD56 expression in feline T cells. Recently Nishimura *et al.* [1999b] cloned a cDNA encoding feline homologue of N-CAM from a feline thymus cDNA library, that corresponds to 140 kDa form at the level of the gene. In the present CHAPTER, the author generated an mAb to CD56, termed SZK1, and examined the expression and molecular size of the antigen recognized by SZK1 mAb in feline lymphoid cells by FCM, immunoblotting, and RT-PCR.

## Materials & Methods

### *Cell culture*

MYA-1 cells, an IL-2 dependent feline T-lymphoblastoid cell line [Miyazawa *et al.*, 1989], were cultured as described in CHAPTER 1. The culture was passaged every three or four days to achieve approximately  $5 \times 10^5$  cells/ml. PBMCs were separated as described in CHAPTER 1 from heparin-treated whole blood of three 11 to 13-years old, SPF cats (Cats 201, 102 and 202 obtained from Dr. K. Nakano, Kitasato University). PBMCs were stimulated by Con A (10  $\mu$ g/ml) for three days and maintained as described for MYA-1 cells.

### *mAb*

mAbs to CD56 were generated as described previously [Shimojima *et al.*, 1997]. Briefly, BALB/c mice were immunized with an insect cell line, High Five<sup>TM</sup> cells (Invitrogen), infected with the recombinant baculovirus rAcfCD56F140 [Nishimura *et al.*, 1999a], which contains feline CD56 cDNA (140 kDa form of N-CAM) under the control of a polyhedrin promoter. Hybridomas, which were generated by the fusion of mouse spleen cells with P3U1 myeloma, were screened with Con A-stimulated, cultured feline PBLs by IFA (CHAPTER 1). The isotype of antibody was determined by IsoDetect<sup>TM</sup> mouse mAb isotyping kit (Stratagene, La Jolla, CA).

### *Immunoblotting*

Immunoblotting was performed as described in CHAPTER 2. As the first antibodies, mouse ascitic fluids were used at 1:2000 dilution. Antibodies on membrane were detected using goat anti-mouse IgG + IgM conjugated with horse radish peroxidase together with 3, 3'-diaminobenzidine, tetra hydrochloride or enhanced chemiluminescence (Amersham Pharmacia Biotech).

### *FCM*

FCM analysis was performed as described in CHAPTER 1. It was confirmed that the IgG2a-specific antibody does not cross-react with SZK1 (IgG1) or with other IgG1 mAbs (data not shown).

### *Panning*

To purify CD56<sup>+</sup> or CD56<sup>-</sup> MYA-1 cells, panning using SZK1 mAb was performed (CHAPTER 3). MYA-1 cells ( $5 \times 10^6$  in 4 ml of culture medium) were seeded on the mAb-coated Petri dish and incubated at 4 °C for 90 min. Non-adherent cells were harvested as feline CD56<sup>-</sup>MYA-1. After the dish was washed well with 2FCS-PBS, adherent cells were harvested as feline CD56<sup>+</sup> MYA-1 by cell scrapers.

### *RT-PCR*

Total RNA was prepared from MYA-1 cells by ISOGEN-LS and cDNA was synthesized using an oligo (dT) primer and Super Script II RT as described in CHAPTER 1. For the subsequent PCR, the author used the primers fCD56/3'-2 5'-CAGGCACTACCTGGTCAAGTACCGAG-3' (nt position 2102-2127) and fCD56R2 5'-TCTGCTCATTCTTGTCACCCATCA-3' (nt position 2775-2751). Both primers have been used for the cloning of feline CD56 cDNA [Nishimura *et al.*, 1999b]. Amplified fragments obtained with the primer pair from the 140 kDa N-CAM would be approximately 650 bp. In the case of the 180 kDa form, the amplified fragments would be more than 1.4 kbp long due to an additional exon (approximately 800 bp [Goridis & Brunet, 1992]).

## Results

### *mAb to feline CD56*

From hybridomas obtained from mice immunized with rAcfCD56F140-infected insect cells, one clone SZK1 (IgG1) was established according to its reactivity with cultured feline PBLs. The mAb was reactive to rAcfCD56F140-infected cells, but not to control baculovirus-infected cells in IFA (data not shown). In the immunoblotting analysis, SZK1 mAb detected a broad band ranging from 130 to 140 kDa in the rAcfCD56F140-infected cells, but not in the control baculovirus-infected Sf9 cells (Fig. 1).

### *CD56 expression in feline lymphoid cells*

No apparent fCD56<sup>+</sup> population was found in feline PBLs freshly isolated from three SPF cats by FCM with SZK1 (data not shown). However, a feline T-lymphoblastoid MYA-1 cell line, which has been established by long term (over 11 months) culture of feline PBLs with IL-2 after Con A-stimulation [Miyazawa et al., 1989], showed approximately 20% reactivity to SZK1 mAb (Fig. 2a). To determine whether the culture increases CD56 expression, feline PBLs were stimulated and cultured as described for MYA-1 cells in the presence of IL-2, and then analyzed. As shown in Figure 2b, the cultured feline PBLs showed 5.6% reactivity to CD56 mAb as early as at day 7 of culture, and the percentage of CD56<sup>+</sup> cells increased up to 16% at day 38, while fresh PBLs

showed only 1.1-1.7% reactivities (day 0). In the indirect single-color FCM analysis, no binding of normal mouse-serum to MYA-1 cells, fresh PBMCs or cultured PBMCs (days 7 and 38) were observed (data not shown) when compared with the cells treated only with secondary antibodies. In two-color FCM at day 22, CD56 expression was observed in both CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> cells (6-20% in CD4<sup>+</sup> cells and 2-14% in CD8 $\alpha$ <sup>+</sup> cells, Fig. 3).

#### *Molecular size of feline CD56*

Immunoblotting analysis was performed for two types of cells, CD56<sup>+</sup> (>98% CD56<sup>+</sup> in FCM) and CD56<sup>-</sup> (<5% CD56<sup>+</sup>) MYA-1 cells. SZK1 detected approximately 160 kDa molecules from CD56<sup>+</sup> but not from CD56<sup>-</sup> MYA-1 cells (Fig. 4). Control mAb did not detect any specific bands (Fig. 4). In RT-PCR, an approximately 650 fragment was amplified from MYA-1 RNA (Fig. 5).

## Discussion

In humans, unprimed, normal individuals have CD56<sup>+</sup> cells in periphery that comprise 10-15% of PBLs and exhibit non-MHC-restricted cytotoxicity [Hercend *et al.*, 1985; Lanier *et al.*, 1986]. The author could not detect apparent CD56<sup>+</sup> cell population in freshly isolated PBLs of SPF cats in this CHAPTER (< 2%). Tompkins *et al.* [1983] reported that PBMCs of clinically normal, nonimmune cats (4 to 9 years old) which had been in an isolation ward for over 3 years showed natural cytotoxic cell activities against virus-infected cells (e.g. 35% cytotoxicity at E:T ratio of 50:1). Hanlon *et al.* [1993] reported that PBMCs of SPF cats (6 months old) showed NK-mediated cytotoxicities against hamster BHK cells (e.g. 10% cytotoxicity at E:T ratios of 50:1). CD56 expression is not essential to the cytotoxic function of human NK cells [Lanier *et al.*, 1987, 1991; Nitta *et al.*, 1989], and further, to my knowledge, CD56 expression on murine and rat lymphoid cells including NK cells has not been reported. Therefore, it is unlikely that only a small population (< 2%), but not other population, exhibits such NK activities, and cats may have a CD56<sup>-</sup> population as MHC-unrestricted cytotoxicity-exhibiting cells in addition to CD56<sup>+</sup> population. Identification of distinct population(s) exhibiting MHC-unrestricted activity might be possible by using other cell antigens such as NK markers [Ryan *et al.*, 2001] and CD57 [Zhao

*et al.*, 1995].

In addition to the MYA-1 cell line, both cultured CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> PBLs that have been stimulated by Con A and cultured in the presence of IL-2, expressed CD56 antigens. MYA-1 cells express CD3 $\epsilon$  (>98% in indirect FCM, data not shown) and are IL-2-dependent [Miyazawa *et al.*, 1989]. The cultured PBLs had also the same characteristics (data not shown). The significance of CD56 expression in MYA-1 cells or the cultured PBLs is yet to be clarified, however the finding is consistent with Lanier's report [1987] that CD56 is expressed on most CD4<sup>+</sup> and CD8<sup>+</sup> IL-2-dependent human T cell lines and clones that have been maintained in long term culture. Also Lerner *et al.* [1998] detected CD56 mRNA by PCR in both CD4<sup>+</sup> and CD8<sup>+</sup> IL-2-dependent feline T cell lines. However, it is unclear whether feline CD56<sup>+</sup> cells in cultured PBLs are the lineage of fresh CD56<sup>+</sup> PBLs or not. Further experiments using purified CD56<sup>+</sup> PBLs or CD56-depleted PBLs will be needed.

The molecular size of feline CD56 expressed in MYA-1 cells was larger than 140 kDa (Fig. 4), therefore, the author examined the cytoplasmic region of N-CAM expressed in MYA-1 cells by RT-PCR. While there are small variants (approximately 40 aa at most) within the extracellular domain, the changes of MW affected by them are slight compared with those occurred within cytoplasmic region (270 aa) in other animals. The author detected a 650 bp

fragment from the cDNA of MYA-1 cells, indicating that the amplified fragment corresponds to a partial sequence of the 140 kDa form which does not contain an insertion (exon 18 in other animals [Barthels *et al.*, 1988; Hemperly *et al.*, 1990; Murray *et al.*, 1986a; Ramos *et al.*, 1989]) specific for the 180 kDa form within the fragment. Thus, as in humans, feline CD56 molecules of MYA-1 cells might be structurally identical to the 140 kDa form of N-CAM but have a greater MW (160 kDa in appearance) because of a modification with, for example, abundant sialic acid residues [Lanier *et al.*, 1989]. This speculation is also supported by the result in the insect expression system in which proteins are not usually sialylated [Marchal *et al.*, 2001]; as shown in Fig. 1, "140 kDa form"-coding baculovirus produces approximately 140 kDa molecules in insect cells detected by the anti-feline CD56 mAb. The difference of MW between MYA-1 and insect cells was also observed when analyzed using the same gel for immunoblotting (Fig. 6).

## Figure legends

### Fig. 1.

Reactivity of anti-feline CD56 mAb SZK1 to control baculovirus (lane 1) - or rAcfCD56F140 (lane 2) - infected Sf9 cells on immunoblotting. Numbers on the left represent molecular size markers (kDa). The results are very similar to those for anti-human CD56 mAb (YLEM, Roma, Italy) [Nishimura *et al.*, 1999a].

### Fig. 2.

CD56 expression in a feline T-lymphoblastoid cell line MYA-1 (a) and cultured feline PBLs (b) in FCM. Feline PBLs were stimulated by Con A and cultured in the presence of IL-2, then CD56 expression was examined on the days indicated (b). Three SPF cats were used: Cat 201 (○), Cat 102 (△) and Cat 202 (□).

### Fig. 3.

Feline CD56 expression in CD4<sup>+</sup> and CD8α<sup>+</sup> PBLs. Two-color FCM was performed at day 22 (Cat 202).

### Fig. 4.

Immunoblotting of CD56 in MYA-1 cells. MYA-1 cells were separated into two types, CD56<sup>+</sup> (lanes 1 and 3) and CD56<sup>-</sup> (lanes 2 and 4), by a panning method and used with anti-feline CD56

(lanes 1 and 2) or irrelevant (lanes 3 and 4) mAbs. Numbers on the left represent molecular size markers.

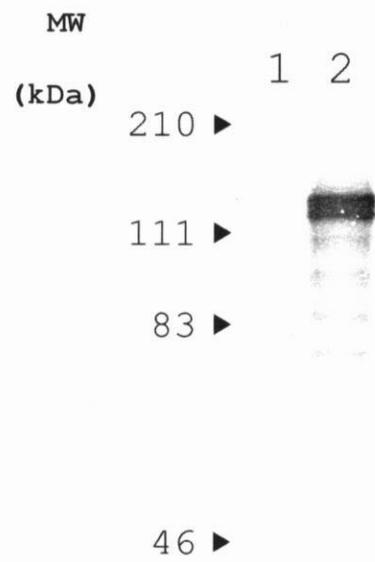
Fig. 5.

RT-PCR to amplify a partial fragment of *feline CD56* cDNA from total RNA of MYA-1 cells without (lane 1) or with (lane 2) RT. Numbers on the left represent molecular size markers.

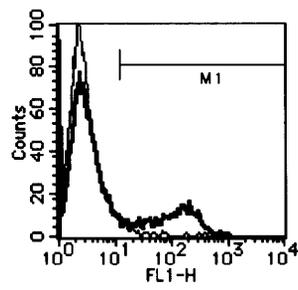
Fig. 6.

Immunoblotting of feline CD56 in insect cells (lane 1) and feline MYA-1 cells (lane 2).

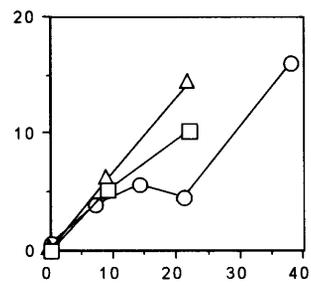
CHAPTER 7/Fig. 1

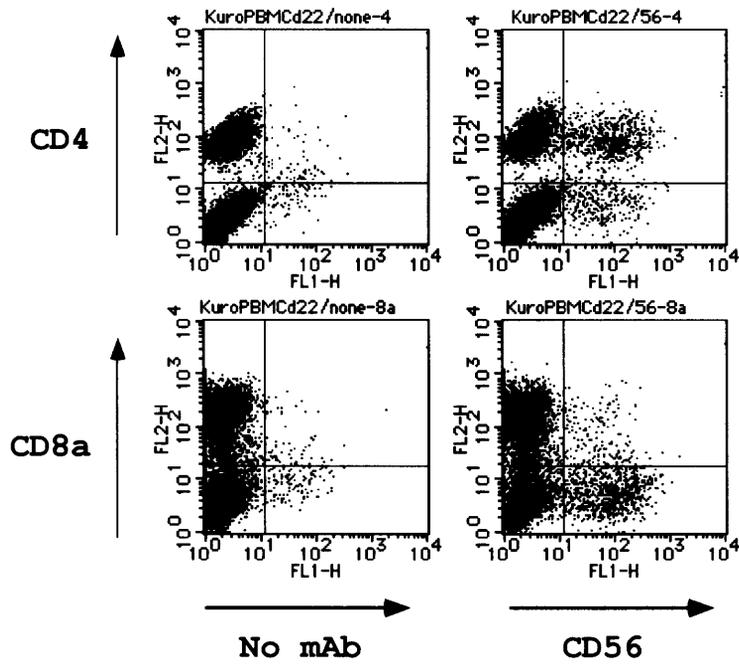


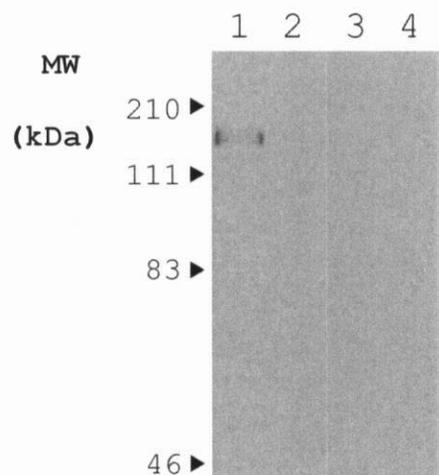
(a)



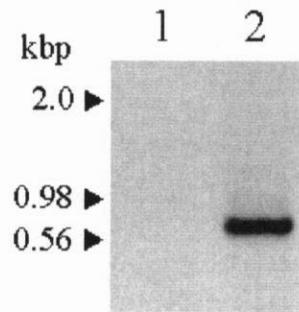
(b)

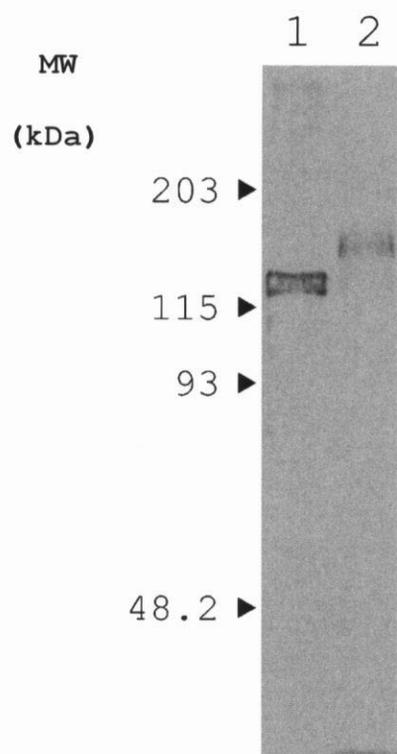






CHAPTER 7/Fig. 5





## CHAPTER 8

CD56 expression and FIV replication in MYA-1 cells

## Abstract

The author investigated correlations between CD56 expression and FIV infection in MYA-1 cells. Further, long term-culture effects of the cells on FIV infection were investigated. MYA-156 cells, which were obtained by panning from long-term cultured MYA-1 cells and were almost all CD56<sup>+</sup> (> 95%), were shown to be more useful than parental cells for FIV investigation in regard to viral propagation and analysis of CD4 reduction. MYA-156 cells showed more profound CPE (mainly syncytium formation) by FIV infection than the original MYA-1 cells with low passages, however viral productivities of MYA-156 were less than that of the original. Proper usage of MYA-1 sublines was suggested to be important according to aims of FIV studies.

## Introduction

MYA-1 cells are a feline T-lymphoblastoid cell line, which are CD4<sup>+</sup> and IL-2-dependent [Miyazawa *et al.*, 1989]. Because of high sensitivity for FIV infection [Miyazawa *et al.*, 1992], the cell line has been used for propagation and titration of FIV. Other feline viruses, feline herpes virus type 1 [Horimoto *et al.*, 1991], feline calicivirus [Kawaguchi *et al.*, 1994], and feline parvovirus [Miyazawa *et al.*, 1999], also can infect MYA-1 cell line.

In this CHAPTER, the author described unexpected increase of CD56 expression and correlation of the expression and FIV infection on long term-cultured MYA-1 cells. Further the author described comparison of the long term (> 2 years)-cultured MYA-156 and the original MYA-1 soon after the establishment in late 1980's, regarding to FIV infection.

## Materials & Methods

### *MYA-1 cells*

Culture of MYA-1 cells was as described in CHAPTER 1. Culture of all its sublines (below) was performed by the same method for MYA-1 cells. Division of MYA-1 cells into two parts was performed by panning (CHAPTERS 3) with anti-feline CD56 mAb (CHAPTER 7) to obtain CD56<sup>+</sup> and CD56<sup>-</sup> MYA-1 cells (see CHAPTER 7 for details).

### *FCM*

mAbs used for FCM were anti-feline CD4 4D9 [Shimojima *et al.*, 1997], anti-feline CD56 SZK1 (CHAPTER 7), and anti-FIV Env 5F7 (IDEXX) mAbs. All of these mAbs were mouse IgG1 isotype and an isotype-matched mAb anti-feline CD8 $\alpha$  10C7 [Shimojima *et al.*, 1998b] was used as a negative control. Single-color FCM was performed to examine positive % and MFI of molecules. For two-color FCM, cells were incubated with antibodies as following order; CD4 (4D9) or FIV Env (5F7) mAb, PE-labeled anti-mouse IgG1 antibody (Exalpha), CD4 or FIV Env mAb (for blocking), and FITC-labeled CD56 (SZK1) mAb. Cells were washed with wash buffer two or three times after each incubation and then analyzed as described in CHAPTER 1.

### *Cell proliferation*

To compare proliferation kinetics of MYA-1 sublines, cells were seeded at a concentration of  $50 \times 10^4$ /ml at day 0 and counted by use of Burker-Turk counters at days 3 and 6.

#### *FIV infection*

Preparation of three infectious molecular clones, pFIV-14 [Olmsted *et al.*, 1989], pFTM191CG [Miyazawa *et al.*, 1991], and pTM219 [Maki *et al.*, 1992], and infection of MYA-1 cells (and its sublines) were as described in CHAPTER 3. These cells and their supernatants were harvested at indicated days and used for FCM or CPE observation or p24 ELISA measurement (IDEXX), respectively.

To compare FIV-productivity or infection-sensitivity of cells, FIV stocks were serially diluted at  $10^{-1}$  to  $10^{-5}$  and then used for infection quadruplicately as reported by Kawaguchi *et al.* [1990]. Briefly, 100  $\mu$ l of the diluted virus was inoculated onto  $1 \times 10^5$  cells in 1 ml, and then incubated until use. IFA of the cells and p24 ELISA of culture supernatants were performed at 10 days pi.

## Results & Discussion

### *CD56<sup>+</sup> cells in MYA-1 cell culture*

The author already described CD56 expression in  $\approx 20\%$  of MYA-1 cells in CHAPTER 7. Surprisingly, the percentage of CD56 expression increased along with continuous cultivation of the cells (Fig. 1) and maintained over 90% for more than 6 months (data not shown). No apparent change of CD56 expression level (not percentage) was observed in CD56<sup>+</sup> cells by FCM (data not shown). Next the author divided MYA-1 cells by panning with CD56 mAb and compared their growth kinetics with that of parental MYA-1 cells. Two populations which were positively- (> 95% positive) or negatively- (< 10% positive) obtained from MYA-1 cells were designated MYA-156 and MYA-056 cells, respectively. As shown in Fig. 2, MYA-156 proliferated more than parental MYA-1, and MYA-056 less than parental MYA-1. Positive percentages for CD56 expression of parental MYA-1 cells were 40 to 70 during the proliferation study and following FIV-infection studies (below) (data not shown). These results suggested that CD56<sup>+</sup> cells could proliferate more rapidly than CD56<sup>-</sup> cells. By the way, CD56 percentage of MYA-056 tended to the increase, therefore repeats of panning were necessary to keep it under 10%, but not necessary for MYA-156 cells. CD56 percentage of MYA-156 would not decrease and keep a high percentage (98-99%) without additional panning. The facts were very likely consistent with

the rapid proliferation of CD56<sup>+</sup> cells against CD56<sup>-</sup> cells. Both of MYA-156 and MYA-056 cells conserved IL-2 dependency in proliferation (data not shown), similar to MYA-1 cells [Miyazawa *et al.*, 1989]. Parental MYA-1 cells often showed clusters of cells in its culture, and so did both of MYA-156 and MYA-056 cells (data not shown), suggesting that molecule(s) responsible for the cluster formation by MYA-1 cells, which is different from homophilic adhesion molecule CD56 [Nishimura *et al.*, 1999a; Nitta *et al.*, 1989; Pizzey *et al.*, 1981], such as CD2/LFA-3 or LFA-1/ICAM-1, must exist.

*FIV proliferation and cell surface antigens in MYA-156 and MYA-056 cells*

Many viral replication depends on activation status of the infected cells. The author infected parental MYA-1, MYA-156, and MYA-056 cells with three FIV molecular clones (FIV-14, TM1, and TM2) at moi of 0.01 and compared viral growth. Culture supernatants were harvested 7 days pi and p24 antigens were measured by ELISA. Results are shown in Fig. 3. Each FIV clones replicated more in MYA-156 and less in MYA-056 than in parental MYA-1 cells. The infected or mock-infected cells at the point were further investigated of their cell surface expression of FIV Env and CD4 by FCM. Results for FIV Env were shown in Fig. 4. Consistent with the results in p24 (Fig. 3), FIV Env antigens

were more expressed in MYA-156 and less in MYA-056 than in parental MYA-1 cells. Moreover, in MYA-1 cell population which includes CD56<sup>+</sup> and CD56<sup>-</sup> cells, CD56<sup>+</sup> cells expressed FIV Env at a higher level than CD56<sup>-</sup> cells after FIV infection (Fig. 5c).

FIV infection of CD4<sup>+</sup> lymphoid cells were reported to reduce surface CD4 expression on the cells [Johnson *et al.*, 1996; Willett *et al.*, 1991], similar to the case with HIV. However, the lymphoid cells used in their studies, FCD4-D [Johnson *et al.*, 1996] and Q201 [Willett *et al.*, 1991], contained CD4<sup>-</sup> cells (20 - 35 %), therefore reduction of CD4 expression by FIV infection could not be analyzed precisely. As shown in Fig. 6, almost all of each MYA-1 sublines expressed CD4 antigen; >99% for MYA-1 and MYA-156, >96% for MYA-056, suggesting MYA-1 and MYA-156 cells as useful cells for studies to analyze CD4 reduction. MFI of CD4 expression were reduced more severely by each FIV infection in MYA-156 than in MYA-1 cells, while CD4 % of infected cells were lower in MYA-1 than in MYA-156 cells (compare left and center panels in Fig. 6). FIV infection in MYA-056 cells seemed to induce increase of CD4<sup>-</sup> cells rather than CD4 reduction in CD4<sup>+</sup> cells (Fig. 6 right panel).

Taken together, it was strongly suggested that FIV could proliferate more productively in CD56<sup>+</sup> cells than in CD56<sup>-</sup> cells and, at the same time, FIV induced infection-effects, such as

CD4 reduction, more profoundly in CD56<sup>+</sup> cells than in CD56<sup>-</sup> cells. Namely, MYA-156 cells were suggested to be useful to propagate FIV and investigate infection with FIV.

#### *Comparison with original MYA-1 cells*

In the report concerning phenotypic analyses of MYA-1 cells [Miyazawa *et al.*, 1992], CD4 was only expressed on a subpopulation of the cells ( $\approx 50\%$ ), in contrast to long-term cultured MYA-1 cells (Fig. 6). Therefore, the author compared MYA-156 cells and the "original" MYA-1 cells in respect of FIV infection. The original MYA-1 cells used were stocked in liquid nitrogen in December 1988 until use in the study (in March 2002) and were used within 33 days after culture starting from the stock, indicating short term-cultured MYA-1 cells; the establishment of MYA-1 cells was reported in 1989 [Miyazawa *et al.*]. MYA-156 cells were propagated from the stocks in April 1997 and maintained for at least 2 years by the author, indicating with comparatively high passages. Isolation of MYA-156 from parental MYA-1 cells was described above.

Two FIV clones, FIV-14 and TM2, were serially diluted and co-cultured with MYA-156 or the original MYA-1 cells. Ten days later, many cells in MYA-156 culture showed severe CPE such as cell death and syncytium formation at the dilution of  $10^{-1}$  by either FIV-14 or TM2 (Fig 7a). In contrast, a few original MYA-1

cells showed CPE (Fig. 7b). Different patterns of IFA results for FIV antigen were observed for FIV-14 infection, but not for TM2 infection only at a dilution of  $10^{-2}$ ; in MYA-156 cells, 2/4 were positive and in the original MYA-1 cells, 3/4 were positive (Table 1). Culture supernatants were pooled within the same viral dilutions and measured for p24 antigens. MYA-156 cells produced lower amounts of p24 than the original MYA-1 cells at viral (either FIV-14 or TM2) dilutions of  $10^{-2}$  and  $10^{-1}$  (Fig. 8). Thus MYA-156 cells showed severe CPE by infection than the original MYA-1 cells, while the latter produced more FIV amounts than the former. Sensitivities to FIV infection were almost similar between the two cell populations, but the original MYA-1 might be slightly more sensitive than MYA-156 cells.

CD56<sup>+</sup> MYA-1 population with high passages were suggested to be useful for investigation of FIV infection, judging from productivities of FIV antigen and reduced degrees of CD4 expression, compared with CD56<sup>-</sup> population. Because CD56<sup>+</sup> cell ratio in MYA-1 cells increased by long term-culture itself, longer culture might confer more suited MYA-1 condition for the FIV investigation. But the original MYA-1 cells showed  $\approx 20\%$  reactivity for CD56 expression (data not shown), and a reason for the unexpected increase of CD56 expression from Dec 2000 are not clear. However, long term-cultured cells were apparently

inferior in FIV production to the original MYA-1 cells which were the ones soon after its establishment and with low passages. In other word, the original MYA-1 cells rather than MYA-156 cells were more suited for preparation of FIV itself. Change of MYA-1 subline usage was strongly suggested; for example, the original MYA-1 usage for propagation of FIV, MYA-156 usage for analyses of infection influence (viral antigen expression, CD4 reduction, CPE). CD4<sup>+</sup> population within (the original) MYA-1 cells might be correlated with syncytium formation and CD4<sup>-</sup> populations with FIV production. Further characterization of the original MYA-1 or MYA-156 cells will be necessary to elucidate mechanisms of FIV infection, propagation, and syncytium formation.

## Figure legends

### Fig. 1

MYA-1 cells were continuously cultured and expression of CD56 molecule was evaluated by FCM with anti-feline CD56 mAb SZK1 (CHAPTER 7) on indicated dates.

### Fig. 2

Growth kinetics of (parental) MYA-1 cells and two sublines MYA-156 and MYA-056, which were obtained from MYA-1 cells by panning. These cells were seeded at a concentration of  $50 \times 10^4$  cells/ml at day 0, cultured and then counted at indicated days by Burker-Turk counters. Results are shown as the mean  $\pm$  SD of triplicate data.

### Fig. 3

MYA-1, MYA-156, and MYA-056 cells were infected with FIV-14, TM1, and TM2, separately, at moi of 0.01 (CHAPTER 5) and p24 viral antigens in culture supernatant were measured by ELISA after 7 days pi. Results are shown as the mean of duplicate data.

### Fig. 4

FIV-infected cells after 7 days pi (the same cells for Fig. 3) were investigated for expression of FIV antigens by FCM with anti-FIV Env mAb. A gating bar was set to obtain  $< 1\%$  positive

to a negative control mAb against mock-infected MYA-1 cells (data not shown) and then the same setting was used for other cases. Numbers above bars are % positivities and numbers under bars are MFI for FIV Env staining. Representative of duplicate data are shown.

Fig. 5

Two-color FCM of (parental) MYA-1 cells. Mock-infected cells were stained for CD4/CD56 (a) and infected cells for CD4/CD56 (b) or FIV Env/CD56 (c).

Fig. 6

FIV-infected cells after 7 days pi (the same cells for Fig. 3) were investigated for expression of CD4 by FCM. A gating bar was set to obtain < 1% positive to a negative control mAb against mock-infected MYA-1 cells (data not shown) and then the same setting was used for other cases. Numbers above bars are % positivities and numbers under bars are MFI for CD4 staining.

Fig. 7

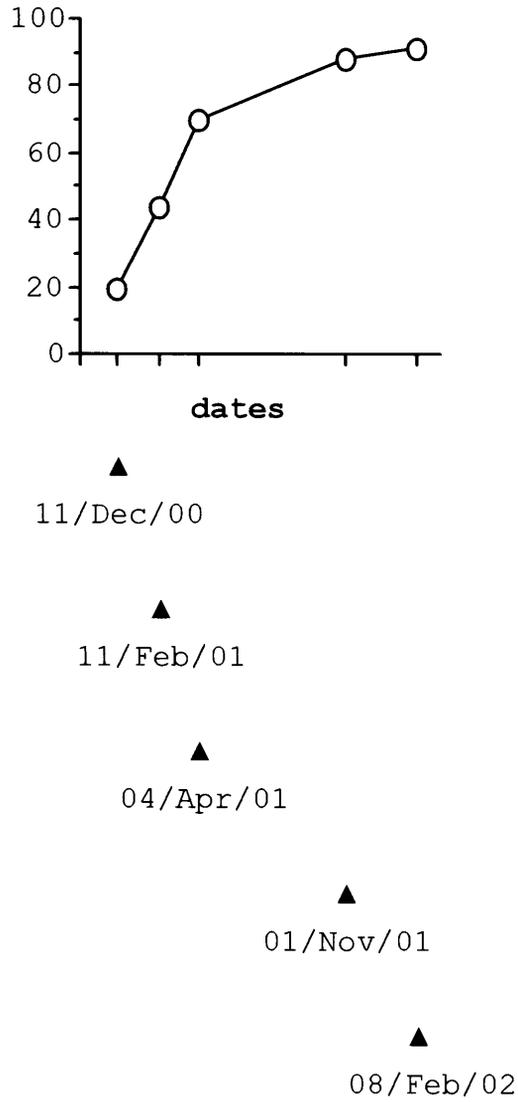
CPE by FIV infection observed in (a) MYA-156 and (b) the original MYA-1 cells. The cells were infected with FIV quadruplicately at the viral dilution of  $10^{-1}$  and cultured for 10 days. Representative results are shown. Note that CPE such

as syncytium and cell death are observed in MYA-156 cells more than the original MYA-1 cells.

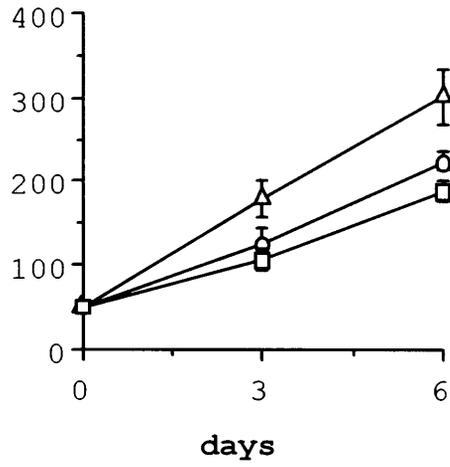
Fig. 8

MYA-156 and the original MYA-1 cells were infected quadruplicately with FIV at the indicated dilutions and cultured for 10 days. Culture supernatants were pooled and then measured for p24 antigen by ELISA.

% positive to feline CD56 mAb

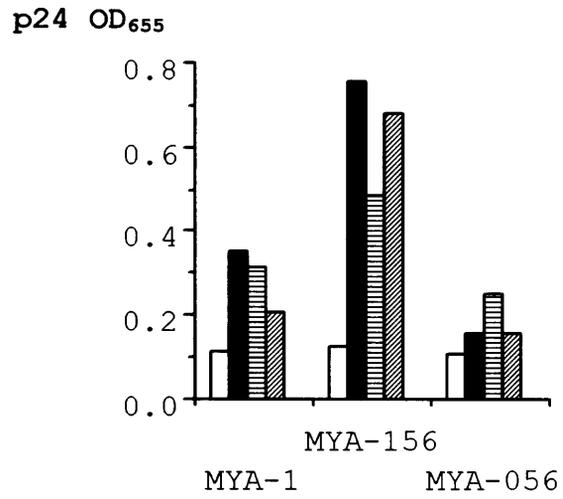


x 10<sup>4</sup> cells/ml

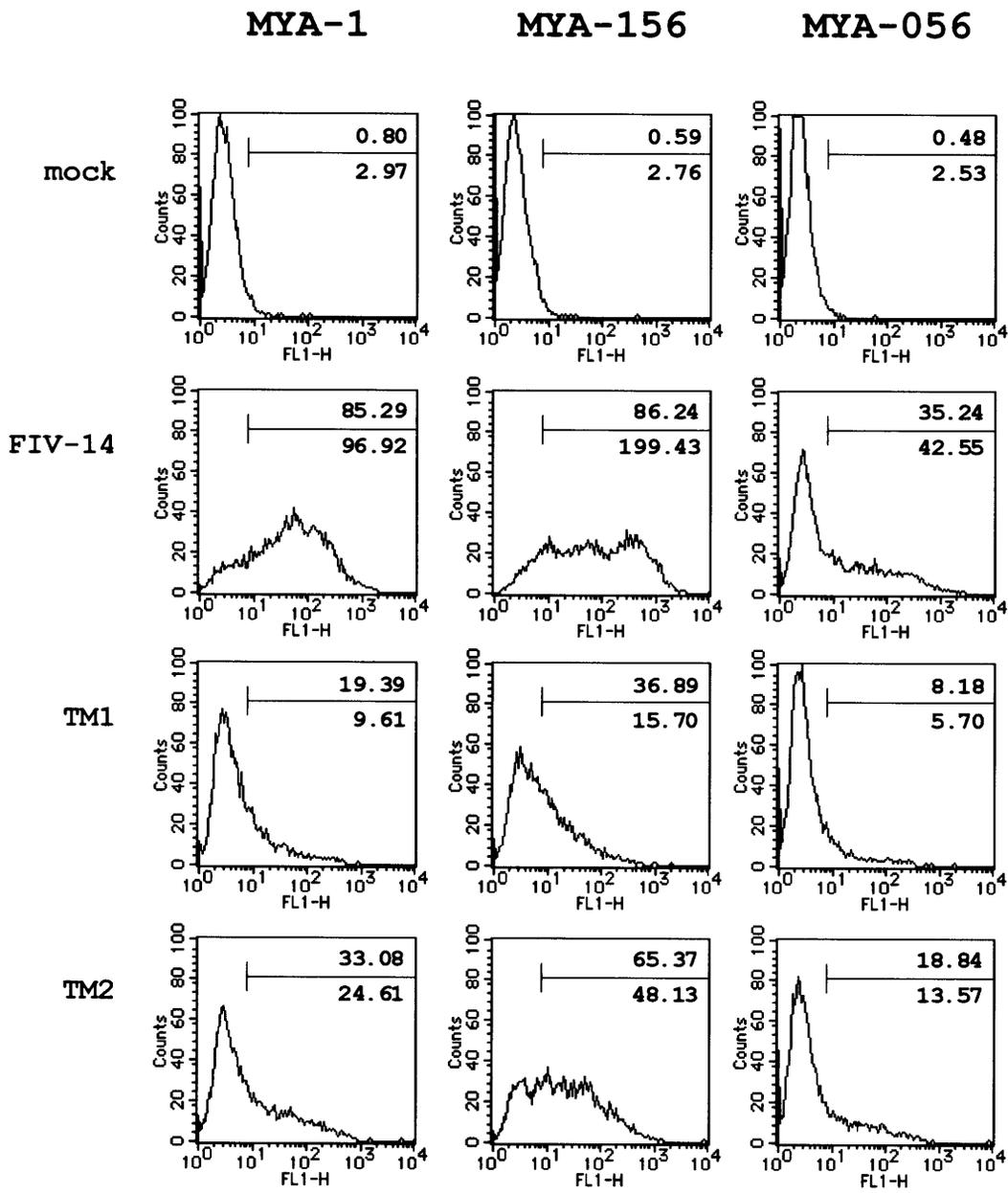


—○— MYA-1  
—△— MYA-156  
—□— MYA-056

CHAPTER 8/Fig. 3

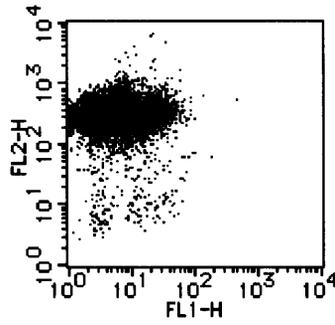


- mock
- FIV-14
- ▨ TM1
- ▩ TM2



**(a)**

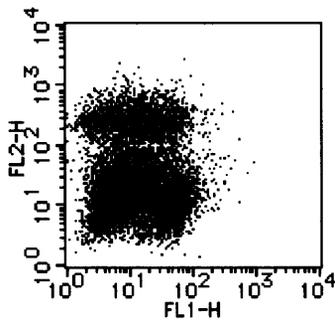
CD4



CD56

**(b)**

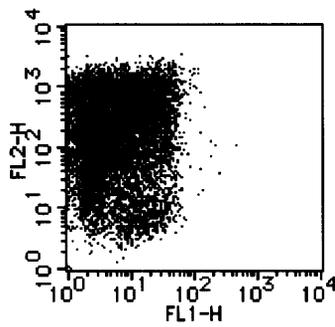
CD4



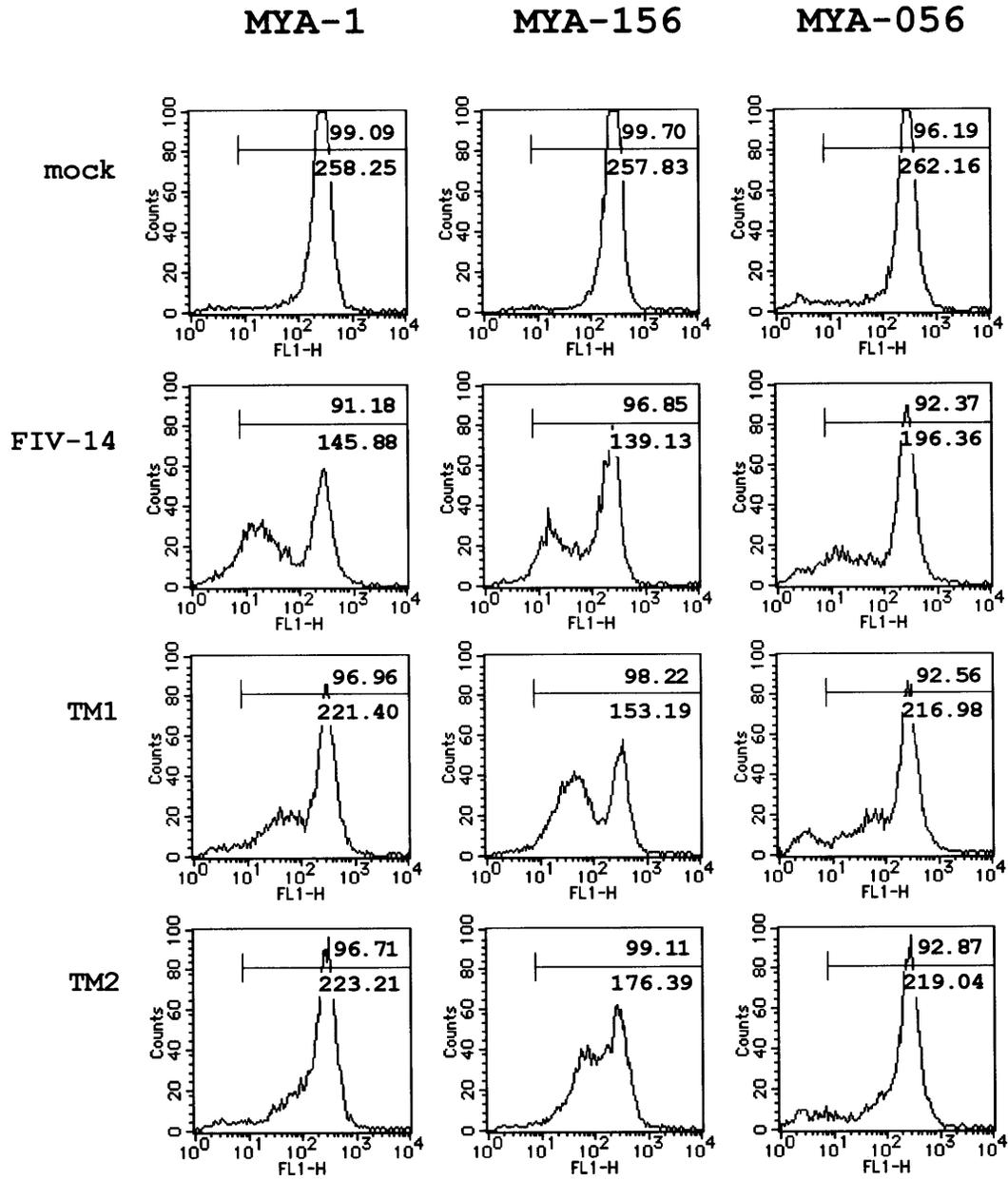
CD56

**(c)**

FIV Env

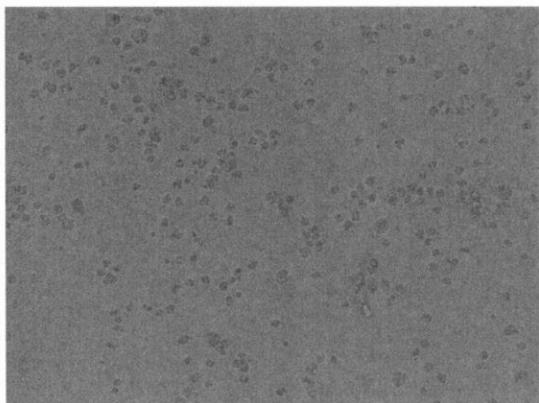


CD56

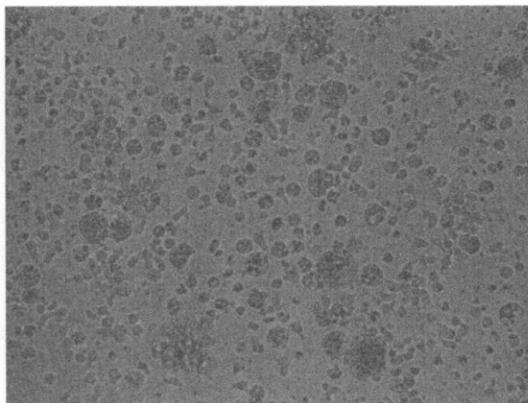


**(a) MYA-156**

FIV-14

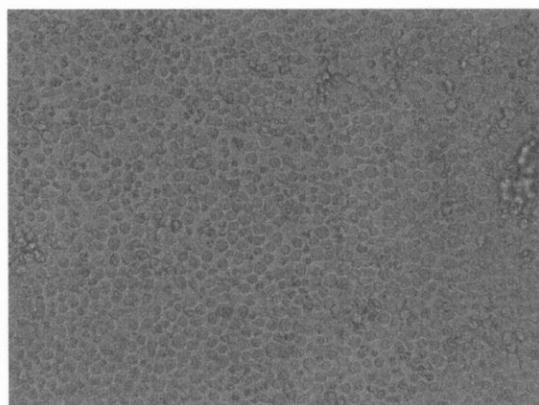


TM2

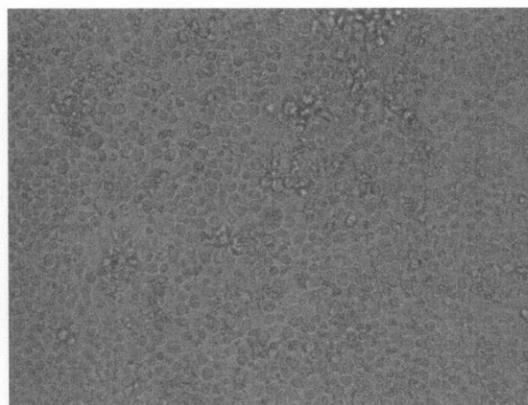


**(b) original MYA-1**

FIV-14



TM2



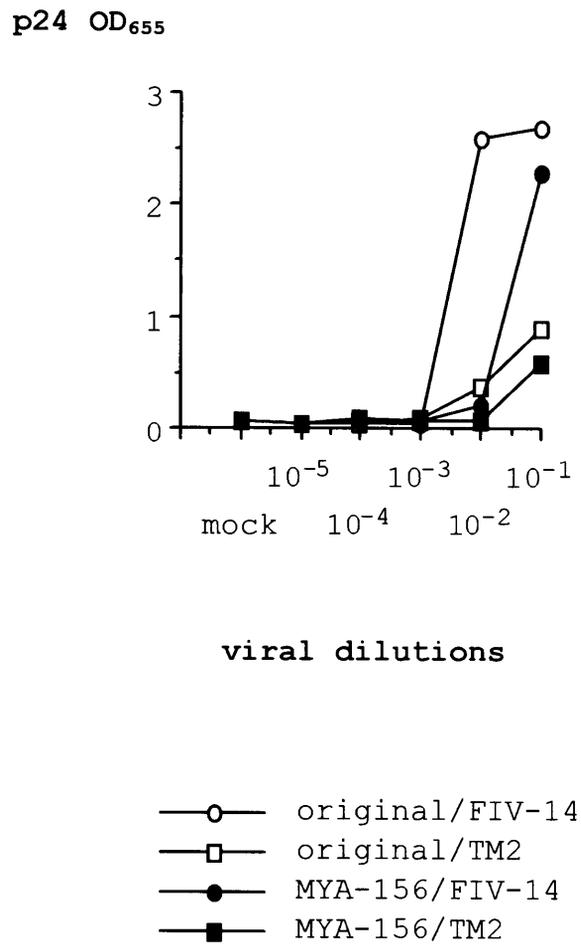


Table 1

Sensitivities of MYA-156 and original MYA-1 cells to FIV-14 and TM2 infection

Cells/virus	Results in IFA				
	Viral dilution				
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>
MYA-156/FIV-14					
	+ <sup>a</sup>	+	-	-	-
	+	-	-	-	-
	+	-	-	-	-
	+	+	-	-	-
MYA-156/TM2					
	+	+	-	-	-
	+	+	-	-	-
	+	+	-	-	-
	+	+	+	-	-
original/FIV-14					
	+	+	-	-	-
	+	-	-	-	-
	+	+	-	-	-
	+	+	-	-	-
original/TM2					
	+	+	-	-	-
	+	+	-	-	-
	+	+	+	-	-
	+	+	-	-	-

<sup>a</sup>+, positive; -, negative for viral antigen

## CONCLUSION

FIV is the pathogen which causes AIDS-like diseases for cats. At present, vaccines for the virus are needed and there is a dramatically increasing interest in feline immune system. Some studies have been reported the significance of T cells in regulation of the viral infection in vivo or dynamics of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes composing the T cells, but with insufficient analyses. Aims of the thesis are to elucidate T cell immune responses against FIV infection in order to support vaccine improvement or efficient vaccine development.

Little availability of antibodies for feline cells prompted the author to generate antibodies against immunologically important molecules. In CHAPTERS 1, 2, & 7, the author performed cDNA-isolation, expression, and mAb-generation for several targeted molecules. In CHAPTER 3, the author performed improvement of expression cloning to identify molecule(s) recognized by mAbs showing some interesting characteristics such as inhibition/induction of certain interactions. In CHAPTER 4, the author performed application of the improved method (CHAPTER 3) with use of FIV instead of mAbs to identify cell surface molecule(s) interactive with FIV.

In CHAPTERS 5 & 6, the author analyzed immune T cells of FIV-infected cats functionally and phenotypically by using generated mAbs and already-reported mAbs. In CHAPTERS 7 & 8, the author performed further characterization of FIV-highly

sensitive MYA-1 cell lines in correlation with CD56 expression.

Abstract of CHAPTERS 1 to 8 are as followed:

In CHAPTER 1, the author identified *feline CD2* cDNA and generated an mAb (clone SKR2) to feline CD2 antigen expressed. Feline CD2 conserved several aa sequences important for human or other animal CD2 structure/signal transduction. Feline CD2-expressing COS cells as well as feline PBMCs could form rosette with human RBCs and the rosette formation were blocked by SKR2 mAb, being indicative of the similarity of feline and human CD2 structures. In addition, SKR2 mAb was shown to be useful to detect feline peripheral blood monocytes as well as T cells. SKR2 mAb was used also in CHAPTER 6.

In CHAPTER 2, the author generated TMM11a mAb to feline CD11a antigen expressed by baculovirus expression system, which would be useful for FCM analysis of the molecule. Further, the author identified partial cDNA encoding feline CD122 (IL-2R  $\beta$  chain) and expressed histidine tag-fusion proteins of feline TCR $\alpha$ , TCR $\delta$ , and CD122, either of which is thought to be a useful tool to investigate molecular-biologically the respective molecules. TMM11a mAb was used also in CHAPTER 6.

In CHAPTER 3, the author improved MLV-mediated expression cloning to reduce the time necessary for its screening step. Though limited in case of identification of cell surface antigens

by antibodies, the screening step could be accomplished within only 1 wk by the use of myeloma target cells and a panning selection method. The improved method could be thought to be applied to identify interactive molecules/receptors of ligands/virus.

In CHAPTER 4, the author applied the improved expression cloning method (CHAPTER 3) to identify the interactive cell surface receptor(s) with FIV particles. In the application, FIV was used as an alternative of antibodies to capture targeted cells. As results, helper T cell activation antigen OX40 was cloned and the molecule was shown to be a primary binding receptor for FIV. It was suggested that FIV pathogenesis is the result of the viral tropism specific to activated helper T cells.

In CHAPTER 5, the author functionally analyzed PBL subpopulations of FIV-infected cats to show antiviral effects of various subpopulations, CD4<sup>+</sup> cells as well as CD8<sup>+</sup> cells (both of CD8 $\alpha^+\beta^+$  and CD8 $\alpha^+\beta^-$ ). The antiviral effects of each subpopulation were thought to be mediated by non-MHC restricted manner, at least in part. Interestingly, the subpopulations showing anti-FIV activity were different among the individual cats and not likely correlated with CD4:CD8 ratios.

In CHAPTER 6, the author phenotypically (expression of CD2, CD11a, CD45RA-like and MHC II) analyzed FIV-infected cat PBLs in correlation with CD4/CD8 expression or light scatters in FCM. It was shown that CD8 $\alpha^+$  PBLs had a distinct subpopulation

with increased CD11a expression accompanying a reduced CD8  $\beta$  chain, increased intracellular granules, and MHC II decrease and that CD8 $\alpha^+$  PBLs expressed CD45RA-like antigen with diverse expression levels. These phenotypic varieties were not found in CD4 $^+$  population.

In CHAPTER 7, the author generated anti-feline CD56 mAb (SZK1) which could be used in IFA, FCM, and immunoblotting analysis for feline CD56 molecule. Feline CD56 was observed to be expressed in cultured feline T lymphoblasts as a 140 kDa form with highly sialylation probably, suggesting similar characteristics with human CD56. The mAb was used also in CHAPTER 8.

In CHAPTER 8, the author showed the differences between CD56 $^+$  and CD56 $^-$  MYA-1 cells and between long and short term-cultured MYA-1 cells in respect of FIV infection. The importance of MYA-1 cell culture period was suggested.

The studies in the thesis showed the significance of CD8 $^+$  T cells (and CD4 $^+$  T cells in some case) and the existence of many subpopulations in CD8 $^+$  T cells during the regulation of FIV infection. They also suggested requirement of further investigations such as identification of other molecules or ligands, cytokine quantification, and antigenicity analysis to clarify a mechanism(s) of FIV infection-regulation. Although

the author could not obtain results directly helpful to the vaccine development, many findings elucidated by the author as described above will be the firm foundations for FIV/cat investigations in the future. Moreover, the findings in the thesis will also contribute to the feline immunology including allergy and autoimmune diseases.

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(son)      (daughter)      (wife)

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**BIOGRAPHICAL SKETCH**

Masayuki Shimojima was born in Nagano, Japan on January 13, 1973, and graduated from Inakita High School, Nagano, Japan in 1991. In March, 1997, he graduated from Faculty of Agriculture, The University of Tokyo. Passed the Japanese National Veterinary Board Examination in March, 1997 and received the title of Doctor of Veterinary Medicine.

He entered into Graduate School of Agricultural and Life Sciences, the University of Tokyo in April, 2000 and started his doctoral experiments.



**SUMMARY IN JAPANESE**

## 論 文 の 内 容 の 要 旨

獣医学専攻

平成 12 年度博士課程入学

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論文題目: 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)

ネコ免疫不全ウイルス(以下 FIV)は、ネコに免疫不全様症状を引き起こす原因体である。このウイルスに対するワクチンが現在必要とされており、ネコ免疫系への関心が高まっている。しかし、感染制御における T 細胞の重要性や T 細胞を構成する  $CD4^+$ ・ $CD8^+$ リンパ球の動態に関する報告はいくつかあるものの、十分な解析がなされているとは言いがたい。本研究は、より良いワクチン作製やより効率的なワクチン開発に役立てるため、FIV 感染における T 細胞免疫応答を明らかにすることを目的とした。

実験を行なう上で有用なツールである抗体の種類がネコにおいては限られており、まずその充実化を試みた。第一・二・七章では、あらかじめ標的分子を設定し、その cDNA 同定・発現・抗体作製を行なった。第三章では、標的分子は不明であっても特徴ある性状(何かの反応の阻害や誘導等)を示す抗体が得られた場合を想定し、その標的分子を短時間かつ簡便に同定する方法を確立した。第四章では、第三章の方法が抗体以外の分子(ここでは FIV Env タンパク)にも

応用可能であることを示した。

第五・六章では、得られた抗体や既存の抗体を用い、FIV 感染ネコの末梢血 T 細胞の表面抗原および機能解析を行なった。第七・八章は細胞株における解析であるが、得られた抗体の一つ(抗 CD56)を用いて FIV 感染性について調べた。

各章の要約は以下の通りである。

第一章: T 細胞表面抗原 CD2 は、T 細胞と抗原提示細胞等との接着や T 細胞の活性化に重要な分子である。ネコ CD2 cDNA を、末梢血単核球由来 cDNA より PCR により新たに同定した。ネコ CD2 のアミノ酸配列中には、ヒトやその他の動物の CD2 分子の立体構造・細胞内シグナル伝達に重要な配列が高度に保存されていた。ネコ CD2 分子を発現させその単クローン抗体 (SKR2) を得た。SKR2 抗体はネコ CD2 発現細胞-ヒト赤血球間で認められるロゼット形成を阻害した。これらのことは、ネコと特にヒトの CD2 の構造および機能の類似性を示すものと考えられた。SKR2 抗体は、T 細胞に加え単球の検出にも有用であった。本抗体は第六章でも用いた。

第二章: インテグリン  $\alpha$ L 鎖 CD11a は、T 細胞と抗原提示細胞等との接着に重要な分子である。T 細胞受容体 (TCR) は、T 細胞の抗原特異的な応答を規定する分子である。CD122 は、IL-2 受容体を構成する  $\beta$  鎖で、IL-2 によるシグナル伝達に必須の分子である。昆虫細胞発現ネコ CD11a を用いて抗ネコ CD11a 単クローン抗体 TMM11a を得た。ネコ TCR $\alpha$  および TCR $\delta$  の定常領域に、ネコ CD2 (第一章) のシグナルペプチド領域を N 末に、ヒスタグ配列を C 末に付加して発現させた。ネコ CD122 の cDNA を PCR により新たに同定し、C 末にヒスタグ配列を付加して発現させた。これらの発現により、TCR や CD122 分子に対する抗体作製などが容易になると考えられた。TMM11a 抗体は第六章でも用いた。

第三章: 抗体が認識する細胞表面分子を同定する場合に発現クローニング法は極めて有効である。そのスクリーニングが短時間かつ簡便に行なえる方法を確認した。モデルとし

て、CD4<sup>+</sup> MYA-1 細胞の cDNA ライブラリーからの、抗 CD4 抗体による CD4 cDNA の同定を試みた。ライブラリー導入法としてレトロウイルスベクター、ライブラリー導入細胞としてミエローマ、選択法としてパンニングを用いた。その結果、わずか 6 日間の培養および 3 回の培養液交換のみでスクリーニングを終え、効率よく CD4 cDNA を得ることができた。

第四章： 第三章で確立した方法を、FIV と反応する細胞表面分子の同定に応用した。ライブラリー導入細胞の保持には、抗体ではなくウイルス液を用いた。その結果、FIV との結合性を有するヘルパー T 細胞活性化抗原 OX40 (CD134) を同定した。OX40 は単に FIV との結合性を有する分子であるだけでなくリンパ球指向性 FIV の感染に必要な分子 (受容体) であり、FIV 抗原特異的な CD4<sup>+</sup>細胞に FIV が感染することが FIV の病態の根底にあると考えられた。

第五章： FIV 感染により、感染ネコの末梢血リンパ球 (PBL) には CD8β 鎖の減少した CD8<sup>+</sup>細胞が増加し、一方 CD4<sup>+</sup>細胞は減少する。抗 CD8α・抗 CD8β・抗 CD4 抗体を用い、FIV TM2 株感染ネコの PBL の機能解析を行なった。CD8α<sup>+</sup>β<sup>+</sup>細胞のみでなく、CD8α<sup>+</sup>β<sup>-</sup>細胞および CD4<sup>+</sup>細胞も FIV 増殖抑制作用を持つことが明らかとなった。いずれの細胞集団による抑制作用も、少なくとも一部は MHC 非拘束性・抗原非特異的である可能性が示された。抗 FIV 活性を主に担う細胞は個体により異なり、病態進行の指標となりうる CD4:CD8 比との関連も認められなかった。

第六章： 白血球共通抗原 CD45 は、T 細胞の分化段階 (ナイーブやメモリー等) により発現型が変化する分子である。主要組織適合抗原複合体 (MHC) は、抗原提示を行なう分子である。FIV 感染ネコの PBL における CD2・CD11a・CD45RA 様および MHC II 分子の発現について、CD4 もしくは CD8 (α および β 鎖) 分子発現との関連性、または細胞サイズもしくは細胞内顆粒との関連性をフローサイトメトリーにより解析した。CD8α<sup>+</sup> PBL 中には、CD8β 鎖の発現減少を伴う CD11a 分子発現増加・細胞内顆粒増加・MHC II 分子減

少を示す亜群が存在した。CD8 $\alpha$ <sup>+</sup> PBL の CD45RA 様分子の発現量は様々であった。このような表現系の多様性は CD4<sup>+</sup> PBL では認められず、FIV 感染は主に CD8<sup>+</sup>細胞群に様々な変化を誘導するものと考えられた。

第七章: CD56 は神経細胞接着分子(N-CAM)の一つの型(140 kDa 型)で、NK 細胞や一部の T 細胞に発現する分子である。昆虫細胞発現ネコ CD56 を用いて抗ネコ CD56 単クローン抗体を得た。本抗体はフローサイトメトリーのみでなくイムノブロット解析にも用いることができた。ネコ CD56 分子は培養ネコ T 細胞(CD4<sup>+</sup>および CD8<sup>+</sup>) および MYA-1 細胞株に発現しており、N-CAM の 140 kDa 型ではあるが高度にシアル化されていると考えられた。これらのことは、ネコ CD56 がヒト CD56 と似た性状や分布を持つことを示すと考えられた。抗ネコ CD56 単クローン抗体は第八章でも用いた。

第八章: MYA-1 細胞は FIV に高感受性・IL-2 依存性のネコリンパ芽球細胞株である。MYA-1 細胞の CD56 発現・長期培養の FIV 感染性への影響を解析した。長期培養により MYA-1 細胞の CD56 陽性率は増加し、CD56<sup>+</sup> MYA-1 細胞は CD56<sup>-</sup> MYA-1 細胞に比べより多くの FIV(抗原)を産生(発現)し、また CD4 分子は FIV 感染によってより減少した。長期培養の MYA-1 細胞では、FIV による細胞変性効果の出現は起こりやすくなったが、FIV 産生量は減少した。FIV 感染の解析における、本細胞株の培養期間の重要性が示唆された。

本研究により、FIV 感染制御における CD8<sup>+</sup> T 細胞(時に CD4<sup>+</sup> T 細胞)の重要性や、CD8<sup>+</sup> T 細胞内に見られる多くの亜群の存在が示された。免疫応答機構の解明には、さらに多くのネコ分子の同定やリガンド同定・サイトカイン定量・抗原性解析等を行なう必要性が示唆された。ワクチン開発に直接役立つような結果は得られなかったが、著者の研究により明らかになった上述の多くの事実は、今後の FIV/ネコ研究の確固たる礎となるはずである。またアレルギーや自己免疫疾患等の分野にも貢献するものであると期待する。