

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⁺ 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⁺ 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 2×10^5 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 α and CD8 β , 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 β^+ but not CD8 $\alpha^+\beta^-$ cells) or anti-CD8 α 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 (1.5×10^5 cells) were mixed with MYA-1 cells (1×10^5 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 1×10^5 effector, 1.5×10^5 target, and 1×10^5 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 α 12A3 [Shimojima *et al.*, 1998b], anti-CD3 ϵ [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⁺ cell depletion, Petri dishes which were coated with rabbit anti-cat IgG (Rockland) were used. For FCM analysis, FITC-labeled anti-CD8 α antibodies 2D7, 10C7, 12A3 and anti-CD4 4D9 [Shimojima *et al.*, 1997, 1998b] and PE-labeled anti-CD8 β 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⁺ 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 β chain in CD8⁺ 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 β 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 β chain on peripheral blood CD8⁺ lymphocytes irrespective of the infection route (intravaginal, intravenous or intraperitoneal) or viral strain

used (Japanese, Swiss, British and American isolates). The reduction of β chain, but not α 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 β chain reduction is not identical to a transient reduction of both α and β 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 β , but not CD8 α , 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 β 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 $^+$ subpopulations, depletions of the subpopulations from PBLs were performed by panning. Representative FCM-results of CD8 β^- or CD8 α^- -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 $^+$ subpopulations (data not shown). In each cat, cell

populations positive for CD8 β and CD8 α after depletion of CD8 β and CD8 α 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 β FT2 (used for FCM, Fig. 2), vpg9 antibody was not detected in the depleted PBMCs (data not shown). Anti-CD8 α 12A3 used for depletion did not block the binding of anti-CD8 α 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 β or CD8 α 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 β -depletion resulted in an increase of p24 antigen, however CD8 α -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 α -depleted

cell culture. In Cats 304 and 305, mock-treatment did not result in increase in p24, while both CD8 β ⁻ and CD8 α ⁻ depletion did. These data indicate that anti-FIV activities are present within CD8 α ⁺ β ⁺ in Cats 304 and 305, and CD8 α ⁺ β ⁻ lymphocytes in Cat 301. These findings do not exclude the possibility that CD8 α ⁺ β ⁺ 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 α ⁺ β ⁺ cells. While several groups reported suppressive activities within CD8⁺ lymphocytes in FIV-infected cats, there are few studies mentioning the relationships between the different expression levels of the CD8 β chain and the suppressive activities. Two groups reported suppressive activities in purified CD8 α ⁺ β ^{low} cells [Bucci *et al.*, 1998b; Flynn *et al.*, 2002], and Gebhard *et al.* [1999] obtained a similar result by use of anti-CD8 β and CD62L antibodies to sort the subpopulation. Concerning the CD8 α ⁺ β ^{high} 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 α ⁺ β ⁺ lymphocytes that had antiviral activity, because separation of CD8 β ^{high} and CD8 β ^{low} cells by sorting was difficult due to the unclear borderline between high and low levels of β -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 β 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 $\alpha^+\beta^{\text{high}}$, β^{low} and β^- 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⁺ 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 ϵ ⁺, surface Ig⁺, or CD16⁺ cells) from the PBLs of Cat 303, and then conducted co-cultures with FIV-infected

MYA-1 cells. As shown in Figure 7a, CD3 ϵ -depletion induced an increase in p24 in the FIV-14-infected MYA-1 cell culture. Because the CD3 ϵ ⁺ population is mostly composed of CD4⁺ and CD8⁺ cells (CHAPTER 1), depletion of CD4⁺ or CD8⁺ cells was also carried out. As shown in Figure 7b, a p24 increase was observed in both CD3 ϵ - and CD4-depleted, but not CD8 α -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 ϵ -depleted cell culture (Fig. 7c). In addition, though not significant (OD₆₅₅<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⁺ 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⁺ lymphocytes. However, CD8⁺ lymphocytes also might have weak antiviral activities in this cat, because CD3 ϵ -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⁺ PBLs, although less frequent than CD8⁺ PBLs, can control lentivirus replication and that the infected individuals described above might control FIV or HIV replication by CD4⁺ lymphocyte-mediated cytotoxicity [Curiel *et al.*, 1993; Kundu *et al.*, 1992, Kundu & Merigan, 1992; Siliciano *et al.*, 1988], secretion of interferon- γ and β chemokines [Furci *et al.*, 1997; Rosenberg *et al.*, 1997] or other unidentified mechanisms. The lack of a correlation between CD8⁺ 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 α 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 α ratios and the increases of CD8 β -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 α 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 α^+ 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 α 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⁺ as well as CD8⁺ 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⁺ 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 β ⁺ or CD8 α ⁺ 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 α , and depleted of the intended cell populations by the panning method. Then non-adherent cells were harvested as mock-treated (a), CD8 β -depleted (b) and CD8 α -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 β) 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 α 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 β -depleted (\triangle) and CD8 α -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 OD₆₅₅ values are shown. A value greater than 0.5 was regarded as indicating positivity for FIV-proliferation.

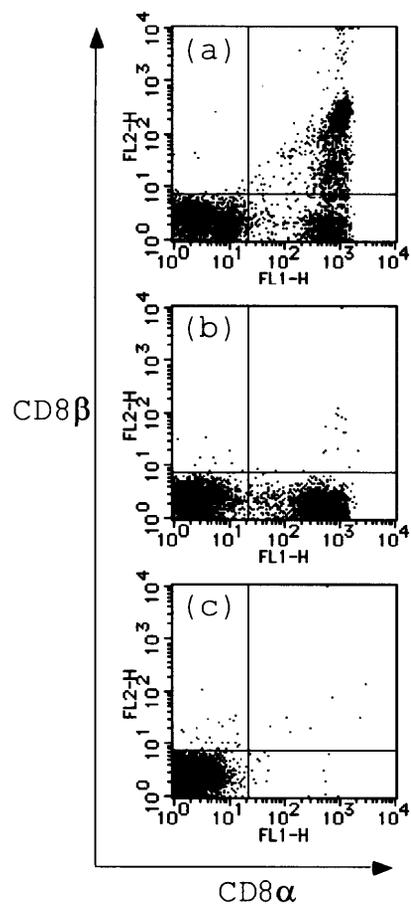
Fig. 6

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

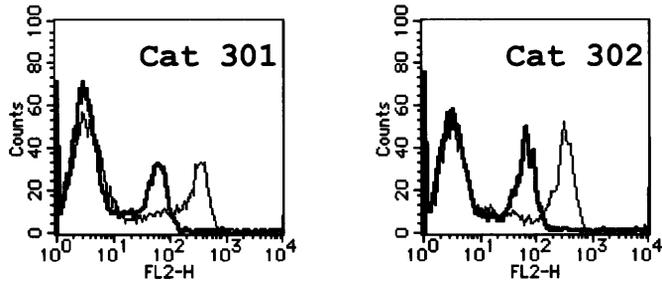
± SD of quadruplicate data. White bars are results in MYA-1 cell culture without mixing of the depleted cells.

Fig. 7

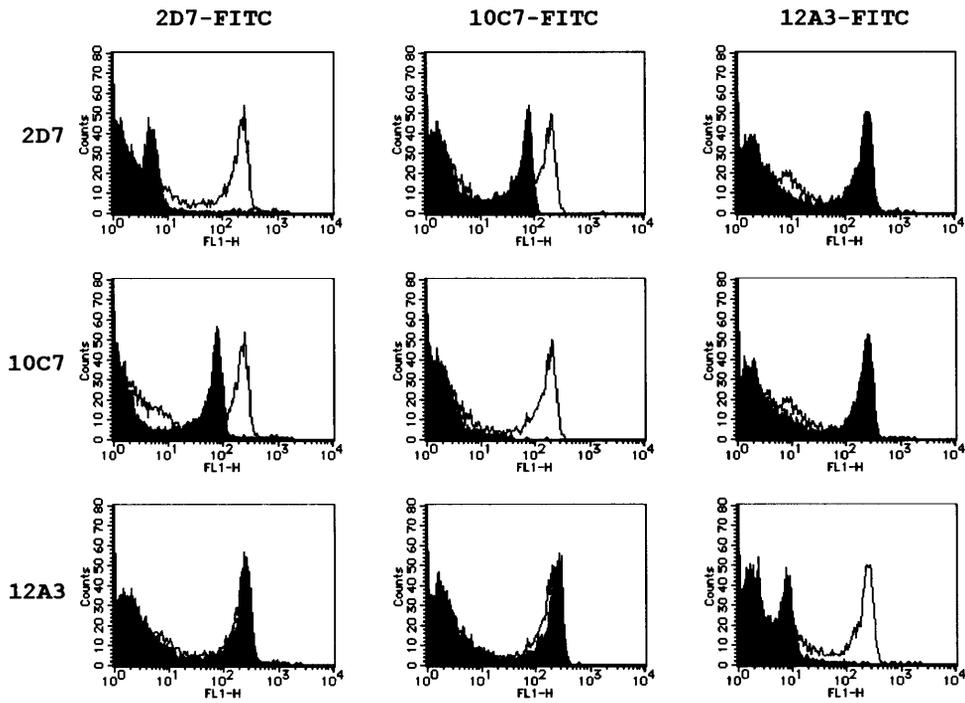
Effects of depletion of other subpopulations in Cat 303 PBLs on FIV replication. By the panning method, the PBMC-subpopulations indicated were depleted from PBLs of Cat 303 (for example, "ΔCD3" means CD3ε-depleted PBLs, and "mock" means PBLs labeled with no mAb for panning). These depleted cells were 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 OD₆₅₅.



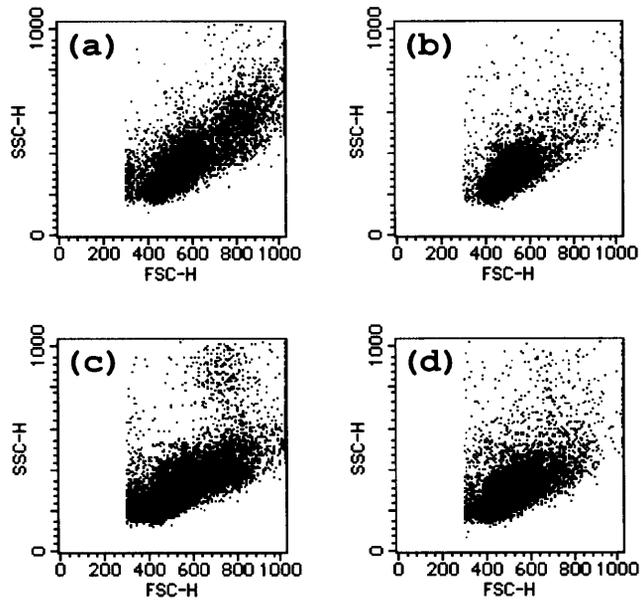
CHAPTER 5/Fig. 2



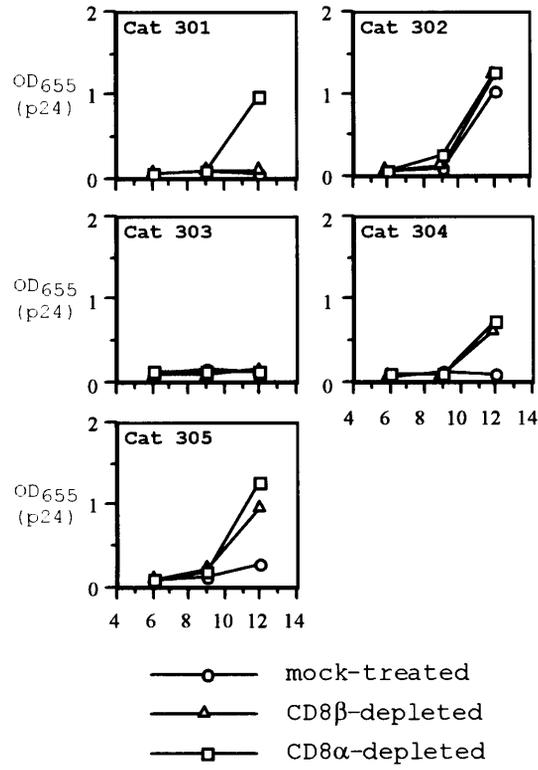
CHAPTER 5/Fig. 3



CHAPTER 5/ Fig. 4

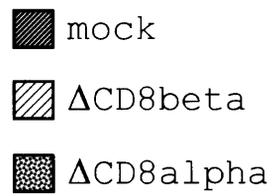
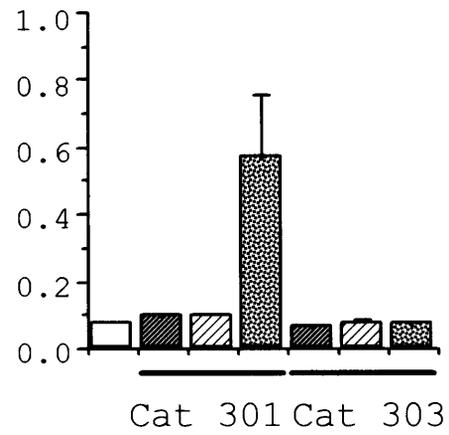


CHAPTER 5/Fig. 5

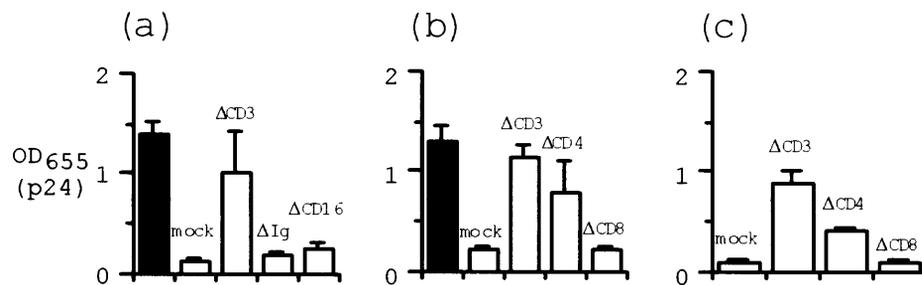


CHAPTER 5/Fig. 6

OD₆₅₅ (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 % ^a						
		Target cell						
		Cat301	Cat302	Cat303	Cat304	Cat305	TM2- inf.	FIV14- inf.
Effector	Δ CD8 α^b	whole	Δ CD4	Δ CD8 α	Δ CD8 α	MYA-1 ^c	MYA-1	
301	CD8 α^d	74	15	99	21	95	61	16
303	CD4	97	8	98	100	95	54	84
304	CD8 α	33	23	-19	46	43	29	19
305	CD8 α	94	-7	32	84	71	48	46

^aInhibition percentages were calculated as described in Materials & Methods. More than 50% is regarded as significantly positive.

^bCD8 α -depleted PBLs of Cat 301.

^cMYA-1 cells infected with FIV strain TM2 at an moi of 0.01.

^dCD8 α^+ cells isolated from Cat 301 PBLs.

CHAPTER 6

Phenotypic changes in CD8⁺ 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 β 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⁺ PBLs, in addition to CD8⁺ PBLs, of five FIV-infected cats and one uninfected cat. It was shown that (i) CD8 α ⁺ PBLs, but not CD4⁺ PBLs, had a distinct subpopulation with increased CD11a expression accompanying a reduced CD8 β chain and increased intracellular granules (ii) CD8 α ⁺ PBLs, but not CD4⁺ PBLs, expressed CD45RA-like antigen with diverse expression levels and (iii) MHC II expression was greater in CD8 α ⁺ PBLs than CD4⁺ PBLs and the CD8 β chain reduction was correlated with the MHC II decrease within CD8 α ⁺ PBLs. These results suggest that FIV infection induces phenotypically heterogeneous subpopulations in CD8⁺ PBLs, including activated phenotypes, rather than in CD4⁺ 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⁺ T cells are distinguishable as CD11b⁻CD28⁺CD62L⁺ or naïve, CD11b⁻CD28⁺CD62L^{low} or memory, and CD11b⁺CD28⁻CD62L⁻ 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⁺ lymphocytosis in circulation, followed by an asymptomatic phase in which CD4⁺ 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⁺ PBLs includes unique subsets having reduced or no expression of the CD8 β chain, but not α chain [Shimojima *et al.*, 1998a]. The β 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 β chain-reduced CD8⁺ 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⁺ PBLs in addition to CD8 α ⁺ 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 [Shimajima *et al.*, 1997] and anti-feline CD8 α 10C7 [Shimajima *et al.*, 1998b] antibodies were used after being labeled with FITC or biotin. Biotin-labeled anti-feline CD8 β 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 α) simultaneously with biotin-labeled antibody (anti-CD8 α or anti-CD8 β) 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⁺ and CD8 α ⁺ PBLs

PBMCs were labeled for three antigens, CD4, CD8 α and molecules of interest (CD2, CD11a, CD45RA-like and MHC II), and then analyzed after gating for CD4-single positive and CD8 α -single positive PBLs (Fig. 1a) by FCM. Representative results are shown in Fig. 1. CD2 expression was observed in almost all CD4⁺ (> 97%) and CD8 α ⁺ (> 96%) PBLs with a similar MFI (Fig. 1c). CD11a was also expressed constitutively in both populations (> 99%). However a distinct subpopulation of CD8 α ⁺ PBLs, but not CD4⁺ PBLs, showed increased expression of the antigen (Fig. 1d). While a substantial but diverse expression of CD45RA-like antigen was observed within CD8 α ⁺ PBLs (7.4 - 44.4%), only a very small population of CD4⁺ PBLs (0.8 - 2.3%) expressed the antigen (Fig. 1e). Almost all CD4⁺ and CD8 α ⁺ PBLs expressed MHC II antigen (> 99%), and MFI ratios (MFI in CD8 α ⁺/MFI in CD4⁺ PBLs) were greater than 1 (Table 1), indicating that CD8 α ⁺ PBLs expressed the antigen more than CD4⁺ PBLs.

Increase of CD11a expression in CD8 α ⁺ PBLs accompanies cellular complexity

CD8 α ⁺CD11a⁺ PBLs were further analyzed for cellular size and complexity after subdivision into two subpopulations depending on CD11a expression, CD11a^{low} and CD11a^{high}. As shown

in Table 1, mean SSC value ratios (SSC in CD8 α ⁺CD11a^{high}/SSC in CD8 α ⁺CD11a^{low} PBLs) were greater than 1, indicating that CD8 α ⁺CD11a^{high} PBLs had more cellular complexity. Similar analyses were performed for other combinations (Table 1). The SSC ratio of CD8 α ⁺/CD4⁺ in the uninfected cat (Cat 306) was 0.94, but values for infected cats were over 1. SSC ratios in CD8 α ⁺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 α ⁺CD11a^{high} PBLs (Fig. 1g), these results showed that CD8 α ⁺CD11a^{high} PBLs had more cellular complexity than CD8 α ⁺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 β chain expression

To examine relationships among the expression of CD11a, CD45RA-like and MHC II antigens with CD8 β chain expression, PBMCs were labeled for combinations of CD8 α , CD8 β and molecules of interest and then analyzed after gating for CD8 α ⁺ PBLs (Fig. 2a). As shown in Fig. 2, the CD11a increase was strictly limited in CD8 α ⁺ β ^{low} and CD8 α ⁺ β ⁻ but not in CD8 α ⁺ β ^{high} PBLs (Fig. 2c). However, very small subpopulations of CD8 α ⁺ β ^{low} and CD8 α ⁺ β ⁻ 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 α^+ 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 β chain reduction was accompanied by a reduction in MHC II MFI, but not in one infected cat (Cat 303) (Fig. 3). CD8 β -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⁺ and CD8⁺ cells [Ho *et al.*, 1993; Kestens *et al.*, 1992, 1994; Scala *et al.*, 1995]. The author found various phenotypes within CD8 α ⁺ PBLs of FIV-infected cats, as well as CD8 β chain reduction, that were previously reported [Shimojima *et al.*, 1998a]. Within CD4⁺ PBLs, however, distinct subpopulation(s) were not found, and did not appear with infection. These results indicate that CD8⁺ PBLs rather than CD4⁺ PBLs profoundly respond to FIV infection in cats.

CD11a (an α L chain of LFA-1, a member of the β 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⁺ 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 α ⁺ β ^{low} or CD8 α ⁺ β ⁻ 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 α^+ 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 α^+ PBLs, an increase of SSC was observed, suggesting that the cells actively synthesize cytokines such as perforin, IFN- γ and TNF- α [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⁺ T lymphocytes in cats (Fig. 1e). These results suggest that CD45RA^{high} and CD45RA⁻ cells within CD8 $\alpha^+\beta^{\text{low or }-}$ CD11a^{high} 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⁺ PBLs expressing CD45RA-like antigens (2.0% in CD4⁺ 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⁺ cells with increased CD11a expression and intracellular granules, namely activated CD8⁺ 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⁺ PBLs may be down-regulated by FIV infection, consistent with a previous study using CD4⁺ cell line [Willett *et al.*, 1991]. This speculation is based on the finding that the ratio of MHC II MFI (CD8 α ⁺ vs CD4⁺) was increased by FIV infection (Table 1) even though the infection resulted in CD8 α ⁺ 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 α ⁺ PBLs. Lerner *et al.* [1998] reported upregulation of MHC II in FIV-infected CD4⁺CD8⁺ cell line MCH5-4. Further analyses of MHC II dynamics in both CD4⁺ and CD8 α ⁺ PBLs will be necessary.

The author has shown that FIV infection causes phenotypically heterogenous subpopulations in CD8⁺ PBLs. Although no apparent phenotypic changes were found within CD4⁺ PBLs, analyses of other surface antigens (CD28, CD38, CD45RO, CD95, CCR7) or cytokines (IL-2, IL-4, IFN- γ) will be helpful to elucidate the dynamics of CD4⁺ PBLs as well as CD8⁺ 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 α (FL3) and other molecules (FL2) and CD4⁺CD8 α ⁻ and CD4⁻CD8 α ⁺ 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⁺ and CD8 α ⁺ PBLs, respectively. PBMCs were isolated from FIV-infected (a-f) and uninfected (g) cats.

Fig. 2

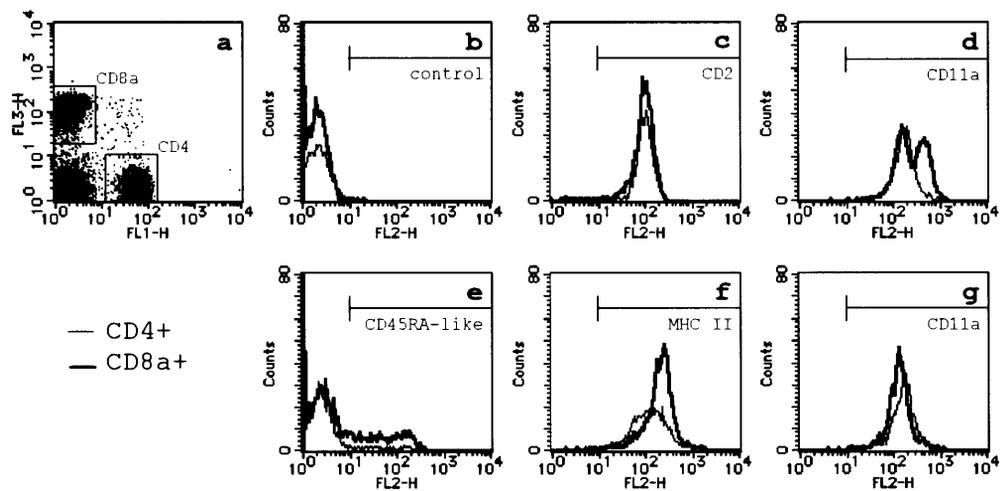
FCM analyses of CD8 α ⁺ PBLs in cats. PBMCs were labeled for combinations of CD8 α (FL1), CD8 β (FL3) and other molecules (FL2). CD8 α ⁺ PBLs were gated (a) to analyze the correlation of the molecules of interest with the CD8 β chain (b-g). These molecules are control (b), CD11a (c, d), CD45RA-like antigen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

Fig. 3

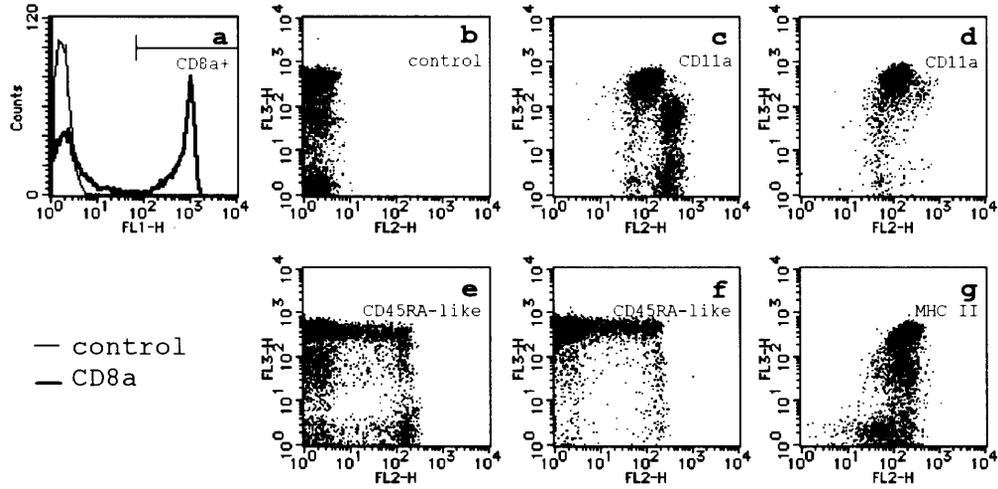
MFI of MHC II antigens in CD8 α ⁺ β ^{high}, CD8 α ⁺ β ^{low} and CD8 α ⁺ β ⁻

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.

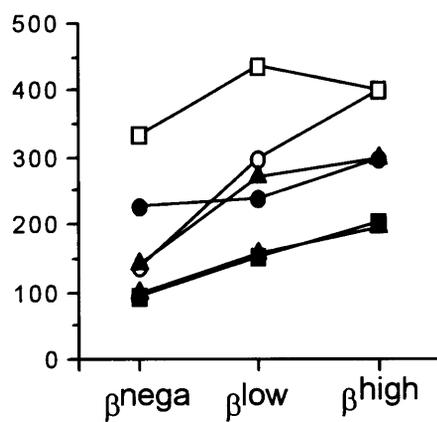
CHAPTER 6/Fig. 1



CHAPTER 6/Fig. 2



MFI of MHC II antigen



CHAPTER 6/Table 1

Table 1. Ratios of MFI or SSC in FCM analysis

	<u>MHC II-MFI ratio</u>		<u>SSC ratio</u>	
	CD8 α^+ vs CD4 $^+$	CD8 α^+ CD11a ^{high} vs CD8 α^+ CD11a ^{low}	CD8 α^+ vs CD4 $^+$	CD8 α^+ CD11a ^{low} 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⁺ T lymphocytes ($< 5\%$) [Lanier *et al.*, 1986]. Both CD56⁺ NK cells and CD56⁺ 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 5×10^5 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 μ 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™ 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™ 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⁺ or CD56⁻ MYA-1 cells, panning using SZK1 mAb was performed (CHAPTER 3). MYA-1 cells (5×10^6 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⁻MYA-1. After the dish was washed well with 2FCS-PBS, adherent cells were harvested as feline CD56⁺ 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'-TCTGCTCATTCTTGTACCCATCA-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⁺ 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⁺ 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⁺ and CD8 α ⁺ cells (6-20% in CD4⁺ cells and 2-14% in CD8 α ⁺ cells, Fig. 3).

Molecular size of feline CD56

Immunoblotting analysis was performed for two types of cells, CD56⁺ (>98% CD56⁺ in FCM) and CD56⁻ (<5% CD56⁺) MYA-1 cells. SZK1 detected approximately 160 kDa molecules from CD56⁺ but not from CD56⁻ 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⁺ 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⁺ 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⁻ population as MHC-unrestricted cytotoxicity-exhibiting cells in addition to CD56⁺ 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⁺ and CD8 α ⁺ PBLs that have been stimulated by Con A and cultured in the presence of IL-2, expressed CD56 antigens. MYA-1 cells express CD3 ϵ (>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⁺ and CD8⁺ 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⁺ and CD8⁺ IL-2-dependent feline T cell lines. However, it is unclear whether feline CD56⁺ cells in cultured PBLs are the lineage of fresh CD56⁺ PBLs or not. Further experiments using purified CD56⁺ 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⁺ and CD8α⁺ 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⁺ (lanes 1 and 3) and CD56⁻ (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.

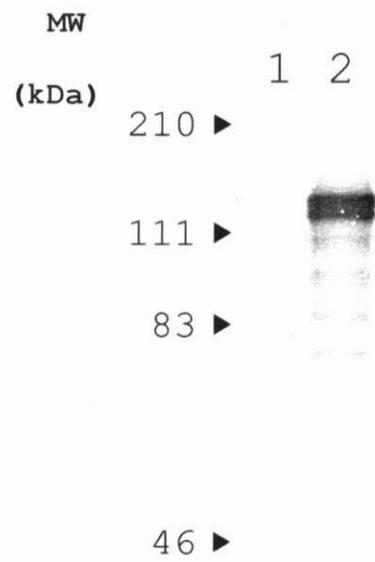
Fig. 5.

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.

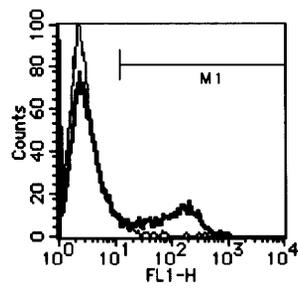
Fig. 6.

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

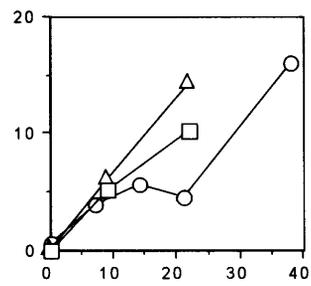
CHAPTER 7/Fig. 1

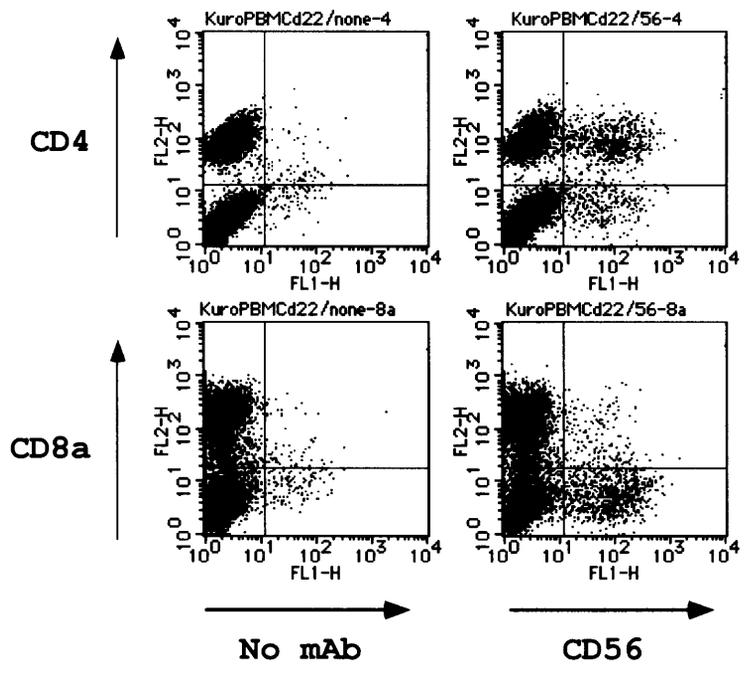


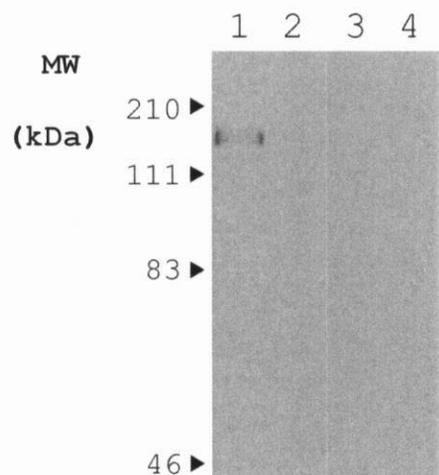
(a)



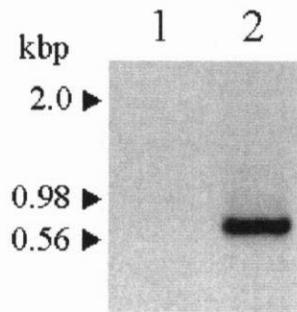
(b)

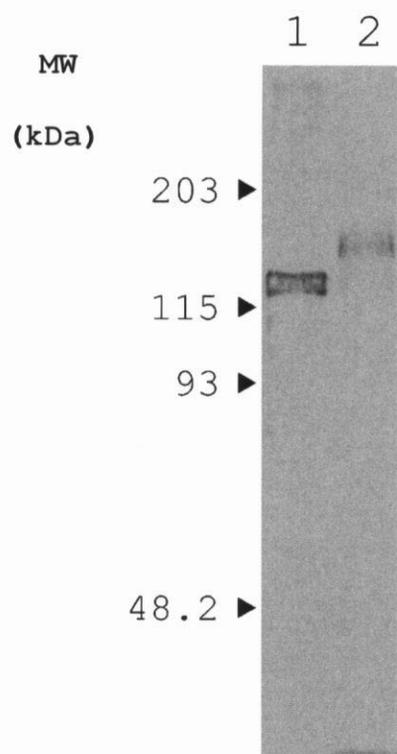






CHAPTER 7/Fig. 5





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⁺ (> 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⁺ 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⁺ and CD56⁻ 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 α 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 50×10^4 /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 μ l of the diluted virus was inoculated onto 1×10^5 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⁺ 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⁺ 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⁺ cells could proliferate more rapidly than CD56⁻ 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⁺ cells against CD56⁻ 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⁺ and CD56⁻ cells, CD56⁺ cells expressed FIV Env at a higher level than CD56⁻ cells after FIV infection (Fig. 5c).

FIV infection of CD4⁺ 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⁻ 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⁻ cells rather than CD4 reduction in CD4⁺ cells (Fig. 6 right panel).

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

CD4 reduction, more profoundly in CD56⁺ cells than in CD56⁻ 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⁺ 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⁻ population. Because CD56⁺ 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⁺ population within (the original) MYA-1 cells might be correlated with syncytium formation and CD4⁻ 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 50×10^4 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

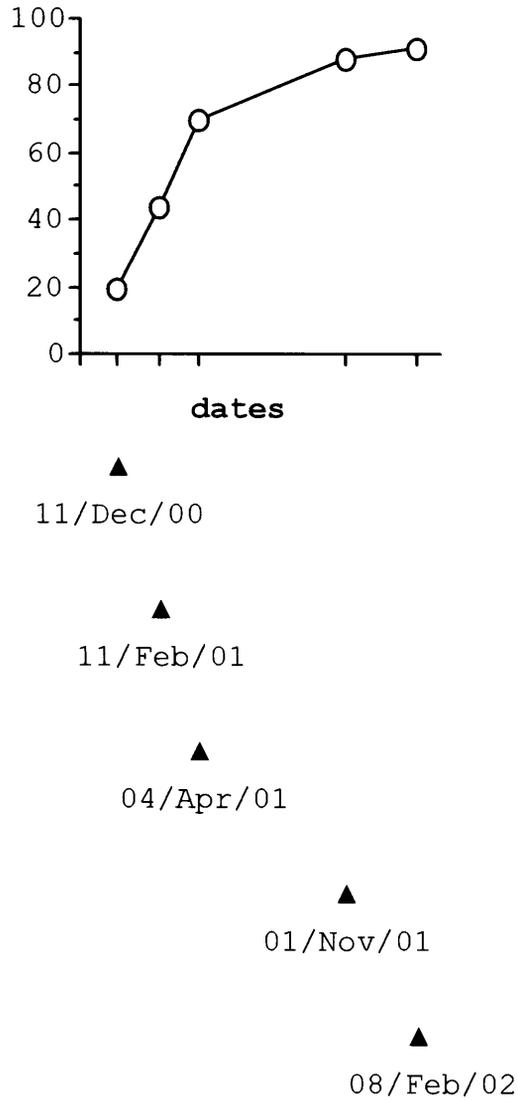
CPE by FIV infection observed in (a) MYA-156 and (b) the original MYA-1 cells. The cells were infected with FIV quadruplicately at the viral dilution of 10^{-1} and cultured for 10 days. Representative results are shown. Note that CPE such

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

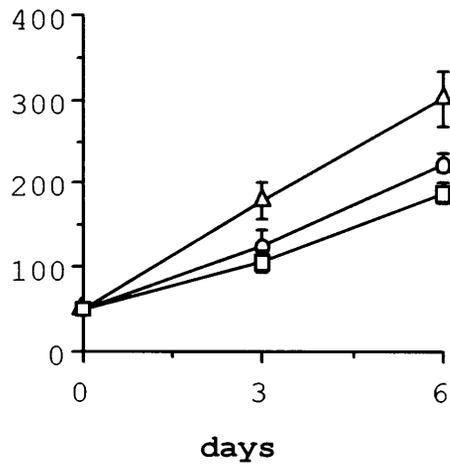
Fig. 8

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

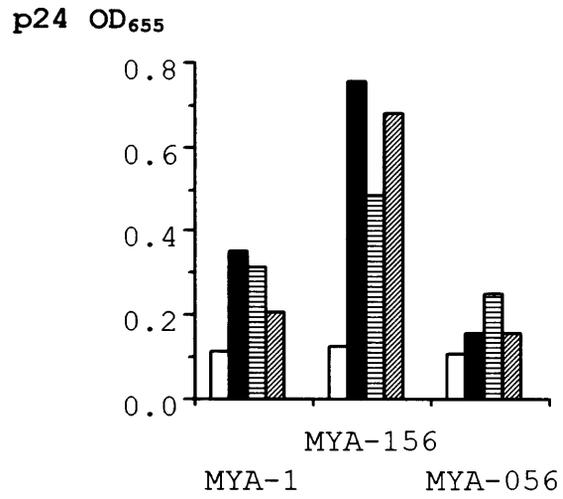


$\times 10^4$ cells/ml

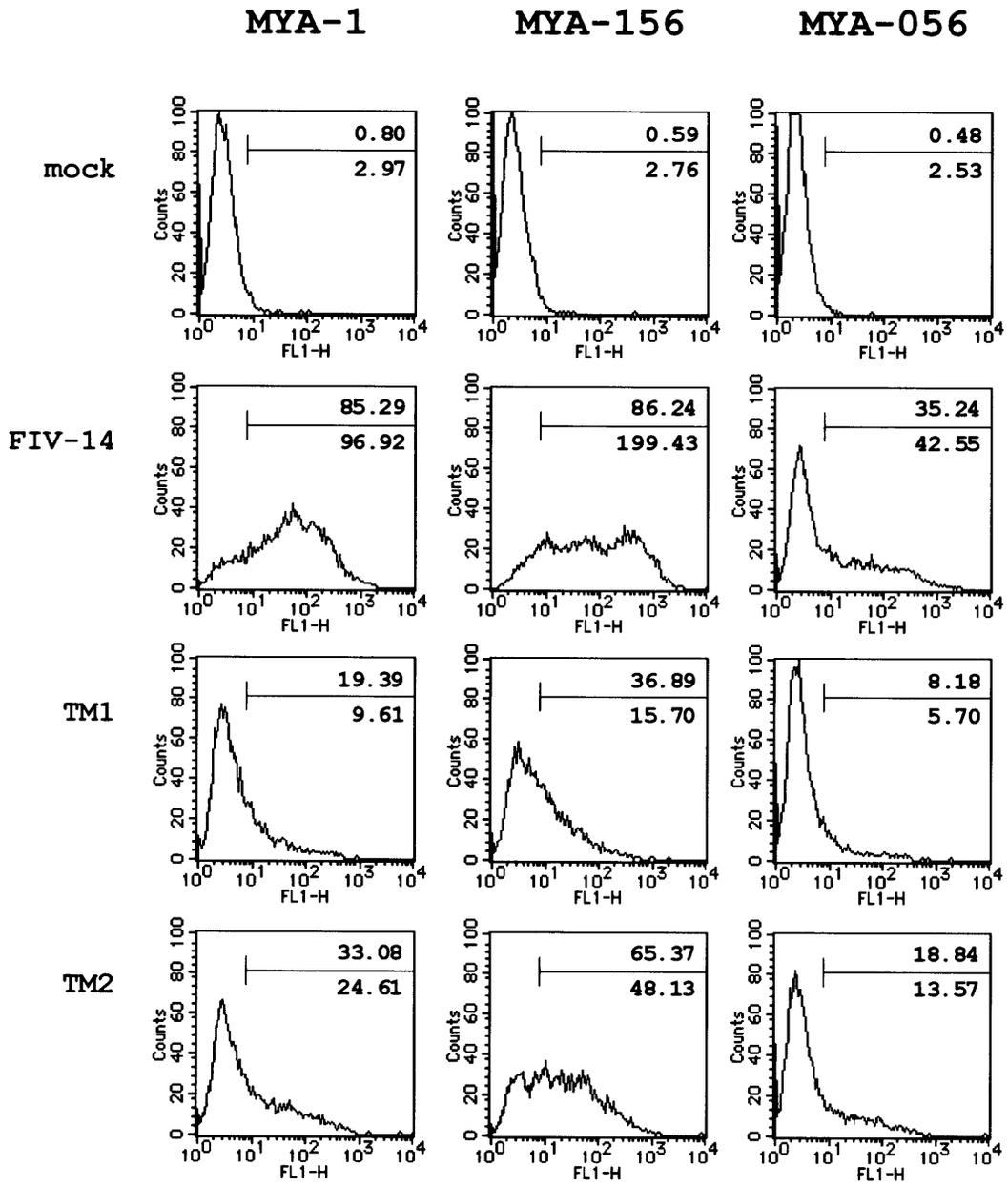


- MYA-1
- △— MYA-156
- MYA-056

CHAPTER 8/Fig. 3

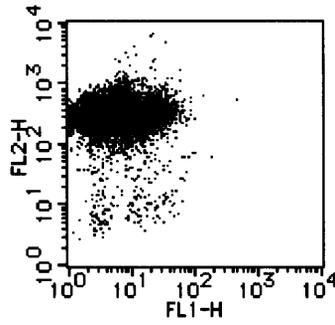


- mock
- FIV-14
- ▨ TM1
- ▩ TM2



(a)

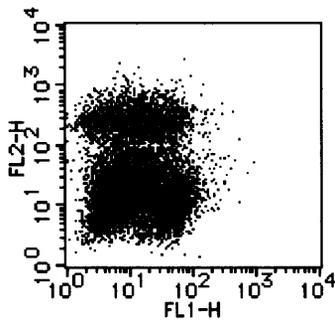
CD4



CD56

(b)

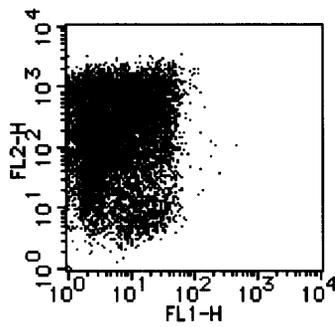
CD4



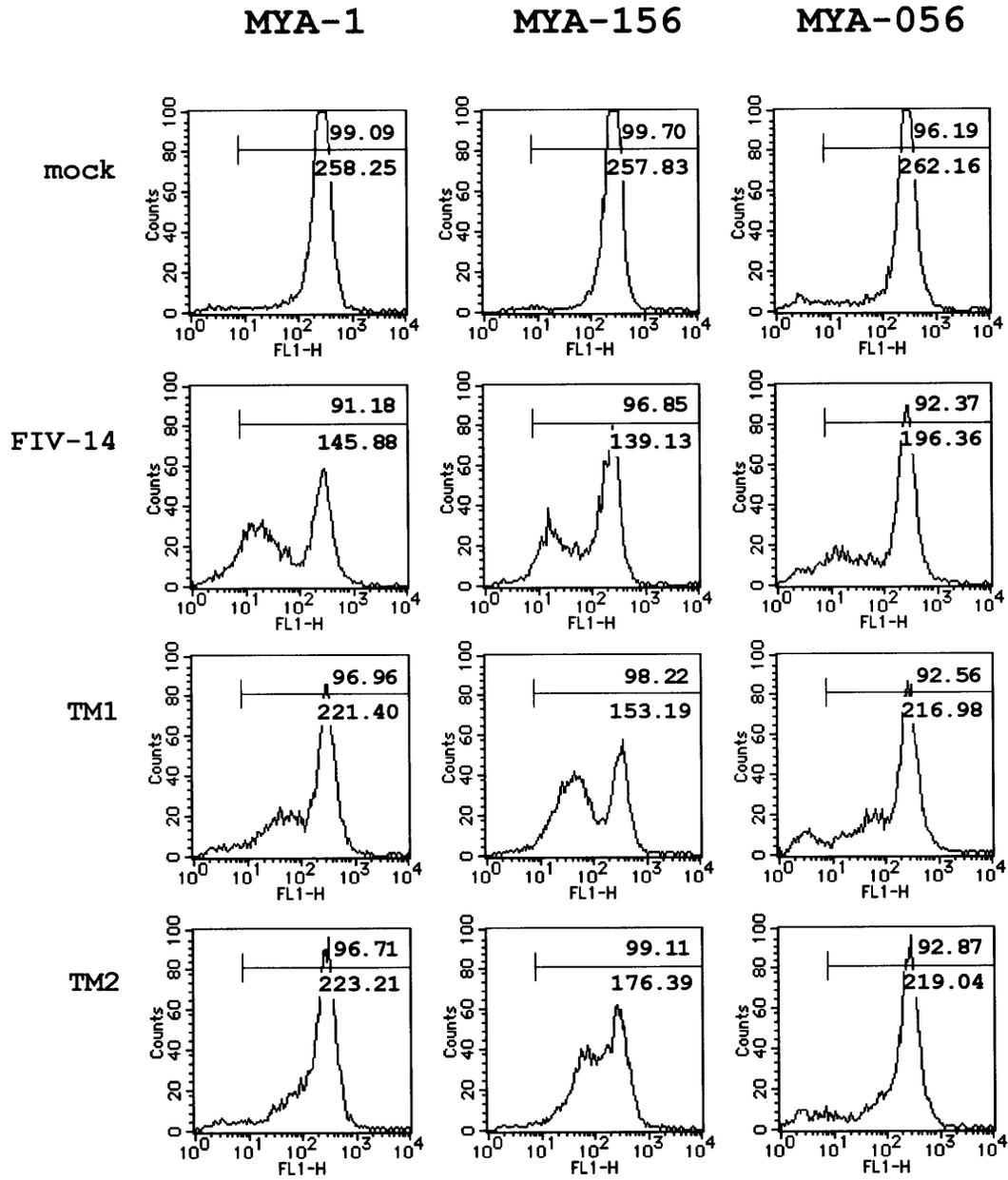
CD56

(c)

FIV Env

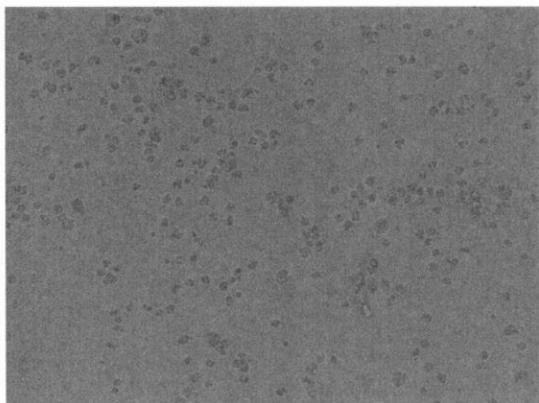


CD56

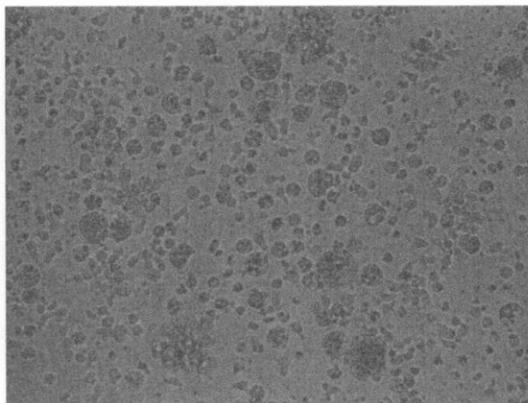


(a) MYA-156

FIV-14

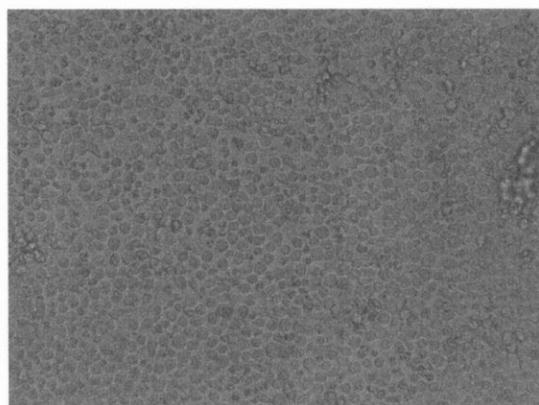


TM2

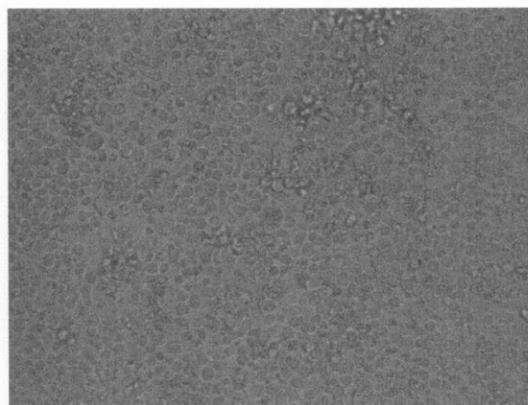


(b) original MYA-1

FIV-14



TM2



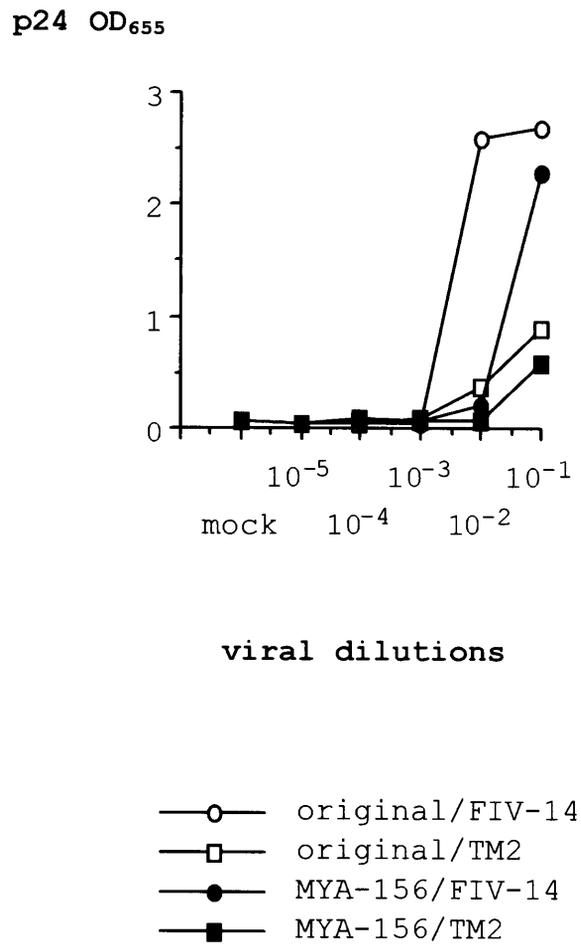


Table 1

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

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

^a+, 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⁺ and CD8⁺ 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 β chain) and expressed histidine tag-fusion proteins of feline TCR α , TCR δ , 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⁺ cells as well as CD8⁺ 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 α^+ PBLs had a distinct subpopulation

with increased CD11a expression accompanying a reduced CD8 β chain, increased intracellular granules, and MHC II decrease and that CD8 α^+ 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.

ACKNOWLEDGMENTS

The author would like to acknowledge the following people for their invaluable assistance:

- Dr. Hiroomi Akashi (The University of Tokyo)
- Dr. Yukinobu Tohya (The University of Tokyo)
- Dr. Takayuki Miyazawa (Obihiro University of Agriculture and Veterinary Medicine)
- Dr. Yorihiro Nishimura (National Institute of Infectious Diseases)
- Dr. Toshio Kitamura (The University of Tokyo)
- Dr. Brian Willett (University of Glasgow)
- Dr. Margaret Hosie (University of Glasgow)
- Dr. Yasuhiro Takeuchi (University College London)
- Dr. Yasuhiro Ikeda (University College London)
- Dr. George Rohrman (Oregon State University)
- Dr. Hideki Hatanaka (RIKEN Genomic Science Center)
- Dr. Yoshiharu Matsuura (Osaka University)
- Dr. Kentaro Kato (National Institute of Health)
- Dr. Yumiko Sakurai (The Ministry of Agriculture, Forestry and Fisheries of Japan)
- Dr. Kazuya Nakamura (Osaka University)
- Dr. Yoshihiro Izumiya (University of California Davis Cancer Center)
- Dr. Eiji Sato (Florida University)
- Dr. Eiji Takahashi (Teikyo University of Science and

Technology)

Dr. Takeshi Mikami (Food Safety Commission)

The author would also like to thank all the members of Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, for their encouragement.

Finally, the author would like to express special thanks to his family.



Mizuki Haduki Yukako
(son) (daughter) (wife)

REFERENCES

- Abraham C, Griffith J, Miller J. The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J Immunol* 1999; **162**:4399-405.
- Ackley C, Yamamoto J, Levy N, Pedersen N, Cooper M. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J Virol* 1990; **64**:5652-5.
- Al-Shamkhani A, Birkeland M, Puklavec M, Brown M, James W, Barclay A. OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand. *Eur J Immunol* 1996; **26**:1695-9.
- Alkhatib G, Combadiere C, Broder C, Feng Y, Kennedy P, Murphy P, Berger E. CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996; **272**:1955-8.
- Anasetti C, Martin P, June C *et al.* Induction of calcium flux and enhancement of cytolytic activity in natural killer cells by cross-linking of the sheep erythrocyte binding protein (CD2) and the Fc-receptor (CD16). *J Immunol* 1987; **139**:1772-9.
- Andersson E, Christensen J, Scheynius A, Marker O, Thomsen A. Lymphocytic choriomeningitis virus infection is associated with long-standing perturbation of LFA-1 expression on CD8⁺ T cells. *Scand J Immunol* 1995; **42**:110-8.
- Apostolopoulos J, McKenzie I, Sandrin M. Ly6d-L, a cell surface ligand for mouse Ly6d. *Immunity* 2000; **12**:223-32.
- Arcaro A, Gregoire C, Boucheron N, Stotz S, Palmer E, Malissen B, Luescher I. Essential role of CD8 palmitoylation in CD8 coreceptor function. *J Immunol* 2000; **165**:2068-76.
- Arulanandam A, Withka J, Wyss D, Wagner G, Kister A, Pallai P, Recny M, Reinherz E. The CD58 (LFA-3) binding site is a localized and highly charged surface area on the AGFCC' C'' face of the human CD2 adhesion domain. *Proc Natl Acad Sci USA* 1993; **90**:11613-7.
- Azuma M, Phillips J, Lanier L. CD28- T lymphocytes. Antigenic and functional properties. *J Immunol* 1993; **150**:1147-59.
- Bach J, Dormont J, Dardenne M, Balner H. In vitro rosette

- inhibition by antihuman antilymphocyte serum. *Transplantation* 1969; **8**:265-80.
- Bandres J, Wang Q, O'Leary J, Baleaux F, Amara A, Hoxie J, Zolla-Pazner S, Gorny M. Human immunodeficiency virus (HIV) envelope binds to CXCR4 independently of CD4, and binding can be enhanced by interaction with soluble CD4 or by HIV envelope deglycosylation. *J Virol* 1998; **72**:2500-4.
- Barthels D, Vopper G, Wille W. NCAM-180, the large isoform of the neural cell adhesion molecule of the mouse, is encoded by an alternatively spliced transcript. *Nucleic Acids Res* 1988; **16**:4217-25.
- Barton E, Forrest J, Connolly J *et al.* Junction adhesion molecule is a receptor for reovirus. *Cell* 2001; **104**:441-51.
- Baum P, Gayle III R, Ramsdell F *et al.* Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. *EMBO J* 1994; **13**:3992-4001.
- Baume D, Caligiuri M, Manley T, Daley J, Ritz J. Differential expression of CD8 alpha and CD8 beta associated with MHC-restricted and non-MHC-restricted cytolytic effector cells. *Cell Immunol* 1990; **131**:352-65.
- Beatty J, Willett B, Gault E, Jarrett O. A longitudinal study of feline immunodeficiency virus-specific cytotoxic T lymphocytes in experimentally infected cats, using antigen-specific induction. *J Virol* 1996; **70**:6199-206.
- Bell G, Fagnoli J, Bolen J, Kish L, Imboden J. The SH3 domain of p56^{lck} binds to proline-rich sequences in the cytoplasmic domain of CD2. *J Exp Med* 1996; **183**:169-178.
- Berg A, Johannisson A, Johansson M, Hein A, Berg M, Dorries R. Peripheral and intracerebral T cell immune response in cats naturally infected with Borna disease virus. *Vet Immunol Immunopathol* 1999; **68**:241-253.
- Berger E, Murphy P, Farber J. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999; **17**:657-700.
- Binnerts M, van Kooyk Y. How LFA-1 binds to different ligands.

- Immunol Today 1999; **20**:240-5.
- Bleijs D, Binnerts M, van Vliet S, Figdor C, van Kooyk Y. Low-affinity LFA-1/ICAM-3 interactions augment LFA-1/ICAM-1-mediated T cell adhesion and signaling by redistribution of LFA-1. J Cell Sci 2000; **113**:391-400.
- Bleul C, Wu L, Hoxie J, Springer T, Mackay C. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci USA 1997; **94**:1925-30.
- Borst J, Jacobs H, Brouns G. Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. Curr Opin Immunol 1996; **8**:181-90.
- Boussiotis V, Freeman G, Griffin J, Gray G, Gribben J, Nadler L. CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J Exp Med 1994; **180**:1665-73.
- Brown W, Bissey L, Logan K, Pedersen N, Elder J, Collisson E. Feline immunodeficiency virus infects both CD4⁺ and CD8⁺ T lymphocytes. J Virol 1991; **65**:3359-64.
- Brunner D, Pedersen N. Infection of peritoneal macrophages in vitro and in vivo with feline immunodeficiency virus. J Virol 1989; **63**:5483-8.
- Bucci J, English R, Jordan H, Childers T, Tompkins M, Tompkins W. Mucosally transmitted feline immunodeficiency virus induces a CD8⁺ antiviral response that correlates with reduction of cell-associated virus. J Infect Dis 1998a; **177**:18-25.
- Bucci J, Gebhard D, Childers T, English R, Tompkins M, Tompkins W. The CD8⁺ cell phenotype mediating antiviral activity in feline immunodeficiency virus-infected cats is characterized by reduced surface expression of the CD8 β chain. J Infect Dis 1998b; **178**:968-77.
- Burkhard M, Mathiason C, Bowdre T, Hoover E. Feline immunodeficiency virus Gag- and Env-specific immune responses after vaginal versus intravenous infection. AIDS Res Hum Retroviruses 2001; **17**:1767-78.
- Calderhead D, Buhlmann J, van den Eertwegh A, Claassen E, Noelle

- R, Fell H. Cloning of mouse OX40: A T cell activation marker that may mediate T-B cell interaction. *J Immunol* 1993; **151**:5261-71.
- Cerwenka A, Lanier L. Natural killer cells, viruses and cancer. *Nature Rev Immunol* 2001; **1**:41-9.
- Chang H-C, Moingeon P, Pedersen R, Lucich J, Stebbins C, Reinherz E. Involvement of the PPPGHR motif in T cell activation via CD2. *J Exp Med* 1990; **172**:351-5.
- Chen A, McAdam A, Buhlmann J *et al.* Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity* 1999; **11**:689-98.
- Cho K-W, Youn H-Y, Okuda M *et al.* Cloning and mapping of cat (*Felis catus*) immunoglobulin and T-cell receptor genes. *Immunogenet* 1998; **47**:226-33.
- Choi I, Hokanson R, Collisson E. Anti-feline immunodeficiency virus (FIV) soluble factor(s) produced from antigen-stimulated feline CD8⁺ T lymphocytes suppresses FIV replication. *J Virol* 2000; **74**:676-83.
- Clapham P, Reeves J, Simmons G, Dejucaq N, Hibbitts S, Mcknight A. HIV coreceptors, cell tropism and inhibition by chemokine receptors ligands. *Mol Membr Biol* 1999; **16**:49-55.
- Clayton L, Sayre P, Novotny J, Reinherz E. Murine and human T11 (CD2) cDNA sequences suggest a common signal transduction mechanism. *Eur J Immunol* 1987; **17**:1367-70.
- Crandell R, Fabricant C, Nelson-Rees W. Development, characterization, and viral susceptibility of a feline (*Felis catus*) renal cell line (CRFK). *In Vitro* 1973; **9**:176-85.
- Crawford K, Gabuzda D, Pantazopoulos V, Xu J, Clement C, Reinherz E, Alper C. Circulating CD2⁺ monocytes are dendritic cells. *J Immunol* 1999; **163**:5920-8.
- Crawford P, Papadi G, Levy J, Benson N, Mergia A, Johnson C. Tissue dynamics of CD8 lymphocytes that suppress viral replication in cats infected neonatally with feline immunodeficiency virus. *J Infect Dis* 2001; **184**:671-81.
- Croft M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* 2003; **3**:609-20.

- Cunningham B, Hemperly J, Murray B, Prediger E, Brackenbury R, Edelman G. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* 1987; **236**:799-806.
- Curiel T, Wong J, Gorczyca P, Schooley R, Walker B. CD4⁺ human immunodeficiency virus type 1 (HIV-1) envelope-specific cytotoxic T lymphocytes derived from the peripheral blood cells of an HIV-1-infected individual. *AIDS Res Hum Retroviruses* 1993; **9**:61-8.
- Dalgleish A, Beverley P, Clapham P, Crawford D, Greaves M, Weiss R. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 1984; **312**:763-7.
- Davignon D, Martz E, Reynolds T, Kurzinger K, Springer T. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2, 3 that participates in T lymphocyte-mediated killing. *Proc Natl Acad Sci USA* 1981a; **78**:4535-9.
- Davignon D, Martz E, Reynolds T, Kurzinger K, Springer T. Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. *J Immunol* 1981b; **127**:590-5.
- Davis S, van der Merwe P. The structure and ligand interaction of CD2: implications for T-cell function. *Immunol Today* 1996; **17**:177-87.
- De Maria A, Ferrazin A, Ferrini S, Ciccone E, Terragna A, Moretta L. Selective increase of a subset of T cell receptor $\gamma\delta$ T lymphocytes in the peripheral blood of patients with human immunodeficiency virus type 1 infection. *J Infect Dis* 1992; **165**:917-9. Erratum in: *J Infect Dis* 1992; **166**:950.
- de Parseval A, Elder J. Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. *J Virol* 2001; **75**:4528-39.
- Dean G, Reubel G, Moore P, Pedersen N. Proviral burden and infection kinetics of feline immunodeficiency virus in

- lymphocyte subsets of blood and lymph node. *J Virol* 1996; **70**:5165-9.
- Deng H, Liu R, Ellmeier W *et al.* Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996; **381**:661-6.
- Devine L, Kavathas P. Molecular analysis of protein interactions mediating the function of the cell surface protein CD8. *Immunol Res* 1999; **19**:201-10.
- Dow S, Poss M, Hoover E. Feline immunodeficiency virus: a neurotropic lentivirus. *J Acquired Immune Defic Syndr* 1990; **3**:658-68.
- Dragic T, Litwin V, Allaway G *et al.* HIV-1 entry into CD4⁺ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996; **381**:667-73.
- Driscoll P, Cyster J, Campbell I, Williams A. Structure of domain 1 of rat T lymphocyte CD2 antigen. *Nature* 1991; **353**:762-5.
- Dumonceaux J, Nisole S, Chanel C, Quivet L, Amara A, Baleux F, Briand P, Hazan U. Spontaneous mutations in the *env* gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype. *J Virol* 1998; **72**:512-9.
- Dumont C, Deas O, Mollereau B *et al.* Potent apoptotic signaling and subsequent unresponsiveness induced by a single CD2 mAb (BTI-322) in activated human peripheral T cells. *J Immunol* 1998; **160**:3797-804.
- Dunham S, Flynn J, Rigby M *et al.* Protection against feline immunodeficiency virus using replication defective proviral DNA vaccines with feline interleukin-12 and -18. *Vaccine* 2002; **20**:1483-96.
- Dustin M, Olszowy M, Holdorf A *et al.* A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* 1998; **94**:667-77.
- Dustin M, Sanders M, Shaw S, Springer T. Purified lymphocyte function-associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. *J Exp Med* 1987; **165**:677-92.
- Dustin M, Springer T. Lymphocyte function-associated antigen

- 1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol* 1988; **107**:321-31.
- Edinger A, Mankowski J, Doranz B *et al.* CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. *Proc Natl Acad Sci USA* 1997; **94**:14742-7.
- Endo Y, Goto Y, Nishimura Y *et al.* Inhibitory effect of stromal cell derived factor-1 on the replication of divergent strains of feline immunodeficiency virus in a feline T-lymphoid cell line. *Vet Immunol Immunopathol* 2000; **74**:303-14.
- Endres M, Clapham P, Marsh M *et al.* CD4-independent infection by HIV-2 is mediated by Fusin/CXCR4. *Cell* 1996; **87**:745-56.
- English R, Johnson C, Gebhard D, Tompkins M. In vivo lymphocyte tropism of feline immunodeficiency virus. *J Virol* 1993; **67**:5175-86.
- Feng Y, Broder C, Kennedy P, Berger E. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996; **272**:872-7.
- Feverheiro M, Roneker C, Laufs A, Tavares L, de Noronha F. Characterization of two monoclonal antibodies against feline immunodeficiency virus gag gene products and their application in an assay to evaluate neutralizing antibody activity. *J Gen Virol* 1991; **72**:617-22.
- Finerty S, Stokes C, Gruffydd-Jones T, Hillman T, Barr F, Harbour D. Targeted lymph node immunization can protect cats from a mucosal challenge with feline immunodeficiency virus. *Vaccine* 2002; **20**:49-58.
- Finerty S, Stokes C, Gruffydd-Jones T *et al.* Mucosal immunization with experimental feline immunodeficiency virus (FIV) vaccines induces both antibody and T cell responses but does not protect against rectal FIV challenge. *Vaccine* 2000; **18**:3254-65.
- Flynn J, Beatty J, Cannon C, Stephens E, Hosie M, Neil J, Jarrett O. Involvement of gag- and env-specific cytotoxic T lymphocytes

- in protective immunity to feline immunodeficiency virus. *AIDS Res Hum Retroviruses* 1995; **11**:1107-13.
- Flynn J, Cannon C, Beatty J, Mackett M, Rigby M, Neil J, Jarrett C. Induction of feline immunodeficiency virus-specific cytotoxic T cells in vivo with carrier-free synthetic peptide. *J Virol* 1994; **68**:5835-44.
- Flynn J, Cannon C, Sloan D, Neil J, Jarrett O. Suppression of feline immunodeficiency virus replication *in vitro* by a soluble factor secreted by CD8⁺ T lymphocytes. *Immunology* 1999; **96**:220-9.
- Flynn J, Dunham S, Mueller A, Cannon C, Jarrett O. Involvement of cytolytic and non-cytolytic T cells in the control of feline immunodeficiency virus infection. *Vet Immunol Immunopathol* 2002; **85**:159-70.
- Flynn J, Hosie M, Rigby M, Mackay N, Cannon C, Dunsford T, Neil J, Jarrett O. Factors influencing cellular immune responses to feline immunodeficiency virus induced by DNA vaccination. *Vaccine* 2000; **18**:1118-32.
- Flynn J, Keating P, Hosie M, Mackett M, Stephens E, Beatty J, Neil J, Jarrett O. Env-specific CTL predominate in cats protected from feline immunodeficiency virus infection by vaccination. *J Immunol* 1996; **157**:3658-65.
- Furci L, Scarlatti G, Burastero S *et al.* Antigen-driven C-C chemokine-mediated HIV-1 suppression by CD4⁺ T cells from exposed uninfected individuals expressing the wild-type CCR-5 allele. *J Exp Med* 1997; **186**:455-60.
- Gan Y, Pauza C, Malkovsky M. $\gamma\delta$ T cells in rhesus monkeys and their response to simian immunodeficiency virus (SIV) infection. *Clin Exp Immunol* 1995; **102**:251-5.
- Gao G, Rao Z, Bell J. Molecular coordination of $\alpha\beta$ T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. *Trends Immunol* 2002; **23**:408-13.
- Garcia K, Scott C, Brunmark A, Carbone F, Peterson P, Wilson I, Teyton L. CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature* 1996; **384**:577-81. Erratum in: *Nature* 1997; **387**:634.

- Gebhard D, Dow J, Childers T, Alvelo J, Tompkins M, Tompkins W. Progressive expansion of an L-selectin-negative CD8 cell with anti-feline immunodeficiency virus (FIV) suppressor function in the circulation of FIV-infected cats. *J Infect Dis* 1999; **180**:1503-13.
- Giblin P, Ledbetter J, Kavathas P. A secreted form of the human lymphocyte cell surface molecule CD8 arises from alternative splicing. *Proc Natl Acad Sci USA* 1989; **86**:998-1002
- Gollob J, Li J, Kawasaki H, Daley J, Groves C, Reinherz E, Ritz J. Molecular interaction between CD58 and CD2 counter-receptors mediates the ability of monocytes to augment T cell activation by IL-12. *J Immunol* 1996; **157**:1886-93.
- Golovkina T, Dzuris J, Hoogen B, Jaffe A, Wright P, Cofer S. M. A novel membrane protein is a mouse mammary tumor virus receptor. *J Virol* 1998; **72**:3066-71.
- Goridis C, Brunet J-F. NCAM: structural diversity, function and regulation of expression. *Sem Cell Biol* 1992; **3**:189-97.
- Gorman S, Sun Y, Zamoyska R, Parnes J. Molecular linkage of the Ly-3 and Ly-2 genes. Requirement of Ly-2 for Ly-3 surface expression. *J Immunol* 1988; **140**:3646-53.
- Gottlieb P. Genetic correlation of a mouse light chain variable region marker with a thymocyte surface antigen. *J Exp Med* 1974; **140**:1432-7.
- Gramaglia I, Jember A, Pippig S, Weinberg A, Killeen N, Croft M. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J Immunol* 2000; **165**:3043-50.
- Gramaglia I, Weinberg A, Lemon M, Croft M. Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 1998; **161**:6510-7.
- Grottrup-Wolfers E, Strzelecki R, Grunewald T, Schuler-Maue W, Ruf B. Enhanced expression of activation antigens HLA-DR and CD69 on $\gamma\delta$ T cells but impaired interleukin-2-induced upregulation in HIV-1 infection. *AIDS* 1997; **11**:838-9.
- Hamann D, Baars P, Rep M, Hooibrink B, Kerkhof-Garde S, Klein

- M, van Lier R. Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J Exp Med* 1997; **186**:1407-18.
- Hanks S, Quinn A, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* 1988; **241**:42-52.
- Hanlon M, Marr J, Hayes K, Mathes L, Stromberg P, Ringer S, Krakowka S, Lafrado L. Loss of neutrophil and natural killer cell function following feline immunodeficiency virus infection. *Viral Immunol* 1993; **6**:119-24.
- Hatakeyama M, Kono T, Kobayashi N, Kawahara A, Levin S, Perlmutter R, Taniguchi T. Interaction of the IL-2 receptor with the src-family kinase p56^{lck}: identification of novel intermolecular association. *Science* 1991; **252**:1523-8.
- Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M, Taniguchi T. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA's. *Science* 1989; **244**:551-6.
- Hedlund G, Hansson J, Ericsson P-O, Sjogren H-O, Dohlsten M. Expression of CD11a and CD45R isoforms defines distinct subsets of CD8⁺ TCR $\alpha\beta$ and TCR $\gamma\delta$ CTL *in vivo*. *Immunol Rev* 1995; **146**:81-94.
- Hemperly J, DeGuglielmo J, Reid, A. Characterization of cDNA clones defining variant forms of human neural cell adhesion molecule N-CAM. *J Mol Neurosci* 1990; **2**:71-8.
- Hemperly J, Edelman G, Cunningham B. cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate. *Proc Natl Acad Sci USA* 1986; **83**:9822-6.
- Hercend T, Griffin J, Bensussan A *et al*. Generation of monoclonal antibodies to a human natural killer clone. *J Clin Invest* 1985; **75**:932-43.
- Hesseltger J, Halks-Miller M, Del-Vecchio V, Peiper S, Hoxie J, Kolson D, Taub D, Horuk R. CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. *Curr Biol* 1997; **7**:112-21.

- Ho H-N, Hultin L, Mitsuyasu R *et al.* Circulating HIV-specific CD8⁺ cytotoxic T cells express CD38 and HLA-DR antigens. *J Immunol* 1993; **150**:3070-9.
- Hoflich C, Docke W-D, Busch A, Kern F, Volk H-D. CD45RA^{bright}/CD11a^{bright} CD8⁺ T cells: effector T cells. *Int Immunol* 1998; **12**:1837-45.
- Hohdatsu T, Miyagawa N, Okubo M, Kida K, Koyama H. Studies on feline CD8⁺ T cell non-cytolytic anti-feline immunodeficiency virus (FIV) activity. *Arch Virol* 2000; **145**:2525-38.
- Hohdatsu T, Motokawa K, Usami M, Amioka M, Okada S, Koyama H. Genetic subtyping and epidemiological study of feline immunodeficiency virus by nested polymerase chain reaction-restriction fragment length polymorphism analysis of the gag gene. *J Virol Methods* 1998a; **70**:107-11.
- Hohdatsu T, Okubo M, Koyama H. Feline CD8 T cell non-cytolytic anti-feline immunodeficiency virus activity mediated by a soluble factor(s). *J Gen Virol* 1998b; **79**:2729-35.
- Hohdatsu T, Yamazaki A, Yamada M, Kusuhara H, Kaneshima T, Koyama H. Ability of CD8⁺ T Cell anti-feline immunodeficiency virus activity correlated with peripheral CD4⁺ T cell counts and plasma viremia. *Microbiol Immunol* 2003; **47**:765-73.
- Holznapel E, Norley S, Holzammer S, Coulibaly C, Kurth R. Immunological changes in simian immunodeficiency virus (SIV_{agm})-infected African green monkeys (AGM): expanded cytotoxic T lymphocyte, natural killer and B cell subsets in the natural host of SIV_{agm}. *J Gen Virol* 2002; **83**:631-40.
- Horimoto T, Kawaguchi Y, Limcumpao J, Miyazawa T, Takahashi E, Mikami T. Replication of feline herpesvirus type 1 in feline T-lymphoblastoid cells. *J Vet Med Sci* 1991; **53**:503-5.
- Hosie M, Broere N, Hesselgesser J, Turner J, Hoxie J, Neil J, Willett B. Modulation of feline immunodeficiency virus infection by stromal cell-derived factor. *J Virol* 1998; **72**:2097-104.
- Hosie M, Flynn J. Feline immunodeficiency virus vaccination: characterization of the immune correlates of protection. *J Virol* 1996; **70**:7561-8.

- Hosie M, Osborne R, Reid G, Neil J, Jarrett O. Enhancement after feline immunodeficiency virus vaccination. *Vet Immunol Immunopathol* 1992; **35**:191-7.
- Hosie M, Willett J, Dunsford T, Jarrett O, Neil J. A monoclonal antibody which blocks infection with feline immunodeficiency virus identifies a possible non-CD4 receptor. *J Virol* 1993; **67**:1667-71.
- Hviid L, Odum N, Theander T. The relation between T-cell expression of LFA-1 and immunological memory. *Immunology* 1993; **78**:237-43.
- Ikeda Y, Tomonaga K, Kawaguchi Y *et al.* Feline immunodeficiency virus can infect a human cell line (MOLT-4) but establishes a state of latency in the cells. *J Gen Virol* 1996; **77**:1623-30.
- Irie H, Mong M, Itano A, Crooks M, Littman D, Burakoff S, Robey E. The cytoplasmic domain of CD8 β regulates Lck kinase activation and CD8 T cell development. *J Immunol* 1998; **161**:183-91.
- Irie H, Ravichandran K, Burakoff S. CD8 β chain influences CD8 α chain-associated Lck kinase activity. *J Exp Med* 1995; **181**:1267-73.
- Izumiya Y, Jang H-K, Sugawara M *et al.* Identification and transcriptional analysis of the homologues of the herpes simplex virus type 1 UL30 to UL40 genes in the genome of nononcogenic Marek's disease virus serotype 2. *J Gen Virol* 1999; **80**:2417-22.
- Jackson A, Matsumoto H, Janszen M, Maino V, Blidy A, Shye S. Restricted expression of p55 interleukin 2 receptor (CD25) on normal cells. *Clin Immunol Immunopathol* 1990; **54**:126-33.
- Jeng C, English R, Childers T, Tompkins M, Tompkins W. Evidence for CD8⁺ antiviral activity in cats infected with feline immunodeficiency virus. *J Virol* 1996; **70**:2474-80.
- Johnson C, Benson N, Papadi G. Apoptosis and CD4⁺ lymphocyte depletion following feline immunodeficiency virus infection of a T-lymphocyte cell line. *Vet Pathol* 1996; **33**:195-203
- Johnson N, Parkin J. Dysregulation of the interleukin-2 receptor α - and β -chain expression in CD4 and CD8 T cells in HIV infection.

- Cytometry 1997; **30**:289-95.
- Johnston J, Power C. Feline immunodeficiency virus xenoinfection: the role of chemokine receptors and envelope diversity. *J Virol* 2002; **76**:3626-36.
- Jones E, Davis S, Williams A, Harlos K, Stuart D. Crystal structure at 2.8Å resolution of a soluble form of the cell adhesion molecule CD2. *Nature* 1992; **360**:232-9.
- Jordan H, Howard J, Bucci J, Butterworth J, English R, Kennedy-Stoskopf S, Tompkins M, Tompkins W. Horizontal transmission of feline immunodeficiency virus with semen from seropositive cats. *J. Reprod. Immunol.* 1998; **41**:341-57.
- Kabelitz D, Glatzel A, Wesch D. Antigen recognition by human $\gamma\delta$ lymphocytes. *Int Arch Allergy Immunol* 2000; **122**:1-7.
- Kambayashi T, Assarsson E, Chambers B, Ljunggren H. IL-2 down-regulates the expression of TCR and TCR-associated surface molecules on CD8⁺ T cells. *Eur J Immunol* 2001; **31**:3248-54.
- Karaki S, Tanabe M, Nakauchi H, Takiguchi M. β chain broadens range of CD8 recognition for MHC class I molecule. *J Immunol*; **149**:1613-8
- Kawaguchi Y, Miyazawa T, Tohya Y, Takahashi E, Mikami T. Quantification of feline immunodeficiency virus in a newly established feline T-lymphoblastoid cell line (MYA-1 cells). *Arch Virol* 1990; **111**:269-73.
- Kawaguchi Y, Tohya Y, Horimoto T, Maeda K, Miyazawa T, Mikami T. Carrier-state infection of feline T-lymphoblastoid cells with feline calicivirus. *Vet Microbiol* 1994; **40**:379-86.
- Kawamata S, Hori T, Imura A, Takaori-Kondo A, Uchiyama T. Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor-associated factor (TRAF) 2- and TRAF5-mediated NF- κ B activation. *J Biol Chem* 1998; **273**:5808-14.
- Keogh M-C, Elliot J, Norton T, Lake R. A function for CD2 on murine B cells? *Immunol Cell Biol* 1997; **75**:333-9.
- Kern F, Docke WD, Reinke P, Volk HD. Discordant expression of LFA-1, VLA-4a, VLA- β 1, CD45RO and CD28 on T-cell subsets:

- evidence for multiple subsets of 'memory' T cells. *Int Arch Allergy Immunol* 1994; **104**:17-26.
- Kern P, Hussey R, Spoerl R, Reinherz E, Chang H. Expression, purification, and functional analysis of murine ectodomain fragments of CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ dimers. *J Biol Chem* 1999; **274**:27237-43.
- Kestens L, Vanham G, Gigase P, Young G, Hannel I, Vanlangendonck F, Hulstaert F, Bach BA. Expression of activation antigens, HLA-DR and CD38, on CD8 lymphocytes during HIV-1 infection. *AIDS* 1992; **6**:793-7.
- Kestens L, Vanham G, Vereecken C, Vandenbruaene M, Vercauteren G, Colebunders RL, Gigase PL. Selective increase of activation antigens HLA-DR and CD38 on CD4⁺CD45RO⁺ T lymphocytes during HIV-1 infection. *Clin Exp Immunol* 1994; **95**:436-41.
- Kitamura T, Morikawa Y. Isolation of T-cell antigens by retrovirus-mediated expression cloning. *Methods Mol Biol* 2000; **143**:143-52.
- Kitamura T, Onishi M, Kinoshita S, Shibuya A, Miyajima A, Nolan G. Efficient screening of retroviral cDNA expression libraries. *Proc Natl Acad Sci USA* 1995; **92**:9146-50.
- Kobayashi N, Kono T, Hatakeyama M, Minami Y, Miyazaki T, Perlmutter R, Taniguchi T. Functional coupling of the *src*-family protein tyrosine kinases p59^{fyn} and p53/56^{lyn} with the interleukin 2 receptor: Implications for redundancy and pleiotropism in cytokine signal transduction. *Proc Natl Acad Sci USA* 1993; **90**:4201-5.
- Kohmoto M, Ikeda Y, Sato E et al. Experimental mucosal infection with molecularly cloned feline immunodeficiency viruses. *Clin Diagn Lab Immunol* 2003; **10**:185-8.
- Kohmoto M, Miyazawa T, Sato E et al. Cats are protected against feline immunodeficiency virus infection following vaccination with a homologous AP-1 binding site-deleted mutant. *Arch Virol* 1998a; **143**:1839-45.
- Kohmoto M, Uetsuka K, Ikeda Y et al. Eight-year observation and comparative study of specific pathogen-free cats experimentally infected with feline immunodeficiency virus

- (FIV) subtypes A and B: terminal acquired immunodeficiency syndrome in a cat infected with FIV Petaluma strain. *J Vet Med Sci* 1998b; **60**:315-21.
- Konno A, Okada K, Mizuno K *et al.* CD8 $\alpha\alpha$ memory effector T cells descend directly from clonally expanded CD8 $\alpha^+\beta^{\text{high}}$ TCR $\alpha\beta$ T cells in vivo. *Blood* 2002; **100**:4090-7.
- Kono T, Doi T, Yamada G, Hatakeyama M *et al.* Murine interleukin 2 receptor β chain: dysregulated gene expression in lymphoma line EL-4 caused by a promoter insertion. *Proc Natl Acad Sci USA* 1990; **87**:1806-10.
- Kopf M, Ruedl C, Schmitz N, Gallimore A, Lefrang K, Ecabert B, Odermatt B, Bachmann M. OX40-deficient mice are defective in T_h cell proliferation but are competent in generating B cell and CTL Responses after virus infection. *Immunity* 1999; **11**:699-708.
- Krensky A, Robbins E, Springer T, Burakoff S. LFA-1, LFA-2, and LFA-3 antigens are involved in CTL-target conjugation. *J Immunol* 1984; **132**:2180-2.
- Ku C, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8⁺ memory T cells by opposing cytokines. *Science* 2000; **288**:675-8.
- Kurosawa K, Ikeda Y, Miyazawa T *et al.* Development of restriction fragment-length polymorphism method to differentiate five subtypes of feline immunodeficiency virus. *Microbiol Immunol* 1999; **43**:817-20.
- Kundu S, Katzenstein D, Moses L, Merigan T. Enhancement of human immunodeficiency virus (HIV)-specific CD4⁺ and CD8⁺ cytotoxic T-lymphocyte activities in HIV-infected asymptomatic patients given recombinant gp160 vaccine. *Proc Natl Acad Sci USA* 1992; **89**:11204-8.
- Kundu S, Merigan T. Equivalent recognition of HIV proteins, Env, Gag and Pol, by CD4⁺ and CD8⁺ cytotoxic T-lymphocytes. *AIDS* 1992; **6**:643-9.
- Kuramochi T, Takeishi M, Ishida T, Kato K, Ishida M. Characterization of feline T and B cells. *Am J Vet Res* 1987; **48**:183-5.

- Kwan L, McNeill L, Whitley K, Becker D, Zamoyska R. Co-capping studies reveal CD8/TCR interactions after capping CD8 β polypeptides and intracellular associations of CD8 with p56^{lck}. *Eur J Immunol* 1998; **28**:745-54.
- Lane P. Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (T_h)1 and T_h2 cells. *J Exp Med* 2000; **191**:201-6.
- Lanier L, Chang C, Azuma M, Ruitenberg J, Hemperly J, Phillips J. Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). *J Immunol* 1991; **146**:4421-6.
- Lanier L, Le A, Civin C, Loke, M, Phillips J. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* 1986; **136**:4480-6.
- Lanier L, Le A, Ding A, Evans E, Krensky A, Clayberger C, Phillips J. Expression of Leu-19 (NKH-1) antigen on IL 2-dependent cytotoxic and non-cytotoxic T cell lines. *J Immunol* 1987; **138**:2019-23.
- Lanier L, Test R, Bindl J, Phillips J. Identification of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med* 1989; **169**:2233-8.
- Latza U, Durkop H, Schnittger S, Ringeling J, Eitelbach F, Hummel M, Fonatsch C, Stein H. The human OX40 homologue: cDNA structure, expression and chromosomal assignment of the ACT35 antigen. *Eur J Immunol* 1994; **24**:677-83.
- Lee W, Vitetta E. The differential expression of homing and adhesion molecules on virgin and memory T cells in the mouse. *Cell Immunol* 1991; **132**:215-22.
- Lehmann R, von Beust B, Niederer E et al. Immunization-induced decrease of the CD4+:CD8+ ratio in cats experimentally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol* 1992; **35**:199-214.
- Leishman A, Naidenko O, Attinger A et al. T cell responses modulated through interaction between CD8 $\alpha\alpha$ and the nonclassical MHC class I molecule, TL. *Science* 2001;

294:1936-9.

- Lerner D, Grant C, de Parseval A, Elder J. FIV infection of IL-2-dependent and -independent feline lymphocyte lines: host cells range distinctions and specific cytokine upregulation. *Vet Immunol Immunopathol* 1998; **65**:277-97.
- Li J, Brown W, Song W *et al*. Retroviral vector-transduced cells expressing the core polyprotein induce feline immunodeficiency virus-specific cytolytic T-lymphocytes from infected cats. *Virus Res* 1995; **38**:93-109.
- Li J, Nishizawa K, An W, Hussey R, Lialios F, Salgia R, Sunder-Plassmann R, Reinherz E. A cdc 15-like adaptor protein (CD2BP1) interacts with the CD2 cytoplasmic domain and regulates CD2-triggered adhesion. *EMBO J* 1998; **17**:7320-36.
- Li J, Smolyar A, Sunder-Plassman R, Reinherz E. Ligand-induced conformational change within the CD2 ectodomain accompanies receptor clustering: implication for molecular lattice formation. *J Mol Biol* 1996; **263**:209-26.
- Liu Y, Xiong Y, Naidenko O *et al*. The crystal structure of a TL/CD8 α complex at 2.1 Å resolution: Implications for modulation of T cell activation and memory. *Immunity* 2003; **18**:205-15.
- Lockridge K, Chien M, Dean G, Stefano Cole K, Montelaro R, Luciw P, Sparger E. Protective immunity against feline immunodeficiency virus induced by inoculation with vif-deleted proviral DNA. *Virology* 2000; **273**:67-79.
- Lub M, van Kooyk Y, Figdor C. Ins and outs of LFA-1. *Immunol Today* 1995; **16**:479-83.
- Maddon P, Dalgleish A, Mcdougal J, Clapham P, Weiss R, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 1986; **47**:333-48.
- Maki N, Miyazawa T, Fukasawa M, Hasegawa A, Hayami M, Miki K, Mikami T. Molecular characterization and heterogeneity of feline immunodeficiency virus isolates. *Arch Virol* 1992; **123**:29-45.
- Mallett S, Fossum S, Barclay A. Characterization of the MRC OX40 antigen of activated CD4 positive T lymphocytes—a molecule

- related to nerve growth factor receptor. *EMBO J* 1990; **9**:1063-8.
- Marchal I, Jarvis D, Cacan R, Verbert A. Glycoproteins from insect cells: sialylated or not? *Biol Chem* 2001; **382**:151-9.
- Martin K, Wyatt R, Farzan M *et al.* CD4-independent binding of SIV gp120 to rhesus CCR5. *Science* 1997; **278**:1470-3.
- Masuoka K, Toyosaki T, Tohya Y, Norimine J, Kai C, Mikami T. Monoclonal antibodies to feline lymphocyte membranes recognize the leukocyte-common antigen (CD45R). *J Vet Med Sci* 1992; **54**:865-70.
- Matteucci D, Pistello M, Mazzetti P *et al.* AIDS vaccination studies using feline immunodeficiency virus as a model: immunisation with inactivated whole virus suppresses viraemia levels following intravaginal challenge with infected cells but not following intravenous challenge with cell-free virus. *Vaccine* 2000; **18**:119-30.
- Matteucci D, Pistello M, Mazzetti P *et al.* Vaccination protects against in vivo-grown feline immunodeficiency virus even in the absence of detectable neutralizing antibodies. *J Virol* 1996; **70**:617-22.
- Meuer S, Hussey R, Fabbi M *et al.* An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell* 1984; **36**:897-906.
- Minami Y, Kono T, Miazaki T, Taniguchi T. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* 1993; **11**:245-67.
- Minami Y, Taniguchi T. IL-2 signaling: recruitment and activation of multiple protein tyrosine kinases by the components of the IL-2 receptor. *Curr Opin Cell Biol* 1995; **7**:156-62.
- Miyazaki T, Maruyama M, Yamada G, Hatakeyama M, Taniguchi T. The integrity of the conserved 'WS motif' common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction. *EMBO J* 1991; **10**:3191-7.
- Miyazawa T, Fukasawa M, Hasegawa A, Maki N, Ikuta K, Takahashi E, Hayami M, Mikami T. Molecular cloning of a novel isolate of feline immunodeficiency virus biologically and genetically different from the original U.S. isolate. *J Virol* 1991;

65:1572-7.

- Miyazawa T, Furuya T, Itagaki S, Tohya Y, Takahashi E, Mikami T. Establishment of a feline T-lymphoblastoid cell line highly sensitive for replication of feline immunodeficiency virus. Arch Virol 1989; **108**:131-5
- Miyazawa T, Ikeda Y, Nakamura K *et al.* Isolation of feline parvovirus from peripheral blood mononuclear cells of cats in northern Vietnam. Microbiol Immunol 1999; **43**:609-12.
- Miyazawa T, Toyosaki T, Tomonaga K, Norimine J, Ohno K, Hasegawa A, Kai C, Mikami T. Further characterization of a feline T-lymphoblastoid cell line (MYA-1 cells) highly sensitive for feline immunodeficiency virus. J Vet Med Sci 1992; **54**:173-5.
- Mobley J, Rigby S, Dailey M. Regulation of adhesion molecule expression by CD8 T Cells *in vivo*: II. Expression of L-selection (CD62L) by memory cytolytic T cells responding to minor histocompatibility antigens. J Immunol 1994; **153**:5443-52.
- Moebius U, Kober G, Griscelli A, Hercend T, Meuer S. Expression of different CD8 isoforms on distinct human lymphocyte subpopulations. Eur J Immunol 1991; **21**:1793-800.
- Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Therapy 2000; **7**:1063-6.
- Mourad M, Besse T, Malaise J *et al.* BTI-322 for acute rejection after renal transplantation. Transplant Proc 1997; **29**:2353.
- Murray B, Hemperly J, Prediger E, Edelman G, Cunningham B. Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule (N-CAM). J Cell Biol 1986a; **102**:189-93.
- Murray B, Owens G, Prediger E, Crossin K, Cunningham B, Edelman G. Cell surface modulation of the neural cell adhesion molecule resulting from alternative mRNA splicing in a tissue-specific developmental sequence. J Cell Biol 1986b; **103**:1431-9.
- Nakayama K, Nakayama K, Negishi I, Kuida K, Louie M, Kanagawa O, Nakauchi H, Loh D. Requirement for CD8 β chain in positive selection of CD8-lineage T cells. Science 1994; **263**:1131-3.

- Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997; **10**:1-6.
- Nishanian P, Hofmann B, Wang Y, Jackson A, Detels R, Fahey J. Serum soluble CD8 molecule is a marker of CD8 T-cell activation in HIV-1 disease. *AIDS* 1991; **5**:805-12.
- Nishimura Y, Miyazawa T, Ikeda Y et al. Characterization of feline CD56 molecule expressed on insect cells by the baculovirus expression system. *J Vet Med Sci* 1999a; **61**:701-3.
- Nishimura Y, Miyazawa T, Ikeda Y et al. Molecular cloning and sequencing of feline CD56 (N-CAM). *Eur J Immunogenet* 1999b; **26**:29-32.
- Nishimura Y, Miyazawa T, Ikeda Y, Izumiya Y, Nakamura K, Sato E, Mikami T, Takahashi E. Molecular cloning and sequencing of a cDNA encoding the feline T-cell antigen CD28 homologue. *Immunogenetics* 1999c; **50**:369-70.
- Nishimura Y, Shimojima M, Miyazawa T et al. Molecular cloning of the cDNAs encoding the feline B-lymphocyte activation antigen B7-1 (CD80) and B7-2 (CD86) homologues which interact with human CTLA4-Ig. *Eur J Immunogenet* 2000; **27**:427-30.
- Nishizawa K, Freund C, Li J, Wagner G, Reinherz E. Identification of a proline-binding motif regulating CD2-triggered T lymphocyte activation. *Proc Natl Acad Sci USA* 1998; **95**:14897-902.
- Nitta T, Yagita H, Sato K, Okumura K. Involvement of CD56 (NKH-1/Leu19 antigen) as an adhesion molecules in natural killer-target cell interaction. *J Exp Med* 1989; **170**:1757-1761.
- Norimine J, Miyazawa T, Kawaguchi Y, Tohya Y, Kai C, Mikami T. A cDNA encoding feline CD4 has a unique repeat sequence downstream of the V-like region. *Immunology* 1992; **75**:74-9.
- Norimine J, Miyazawa T, Kawaguchi Y et al. Feline CD4 molecules on feline non-lymphoid cell lines are not enough for productive infection of highly lymphotropic feline immunodeficiency virus isolates. *Arch Virol* 1993; **130**:171-8.
- Norment A, Littman D. A second subunit of CD8 is expressed in

- human T cells. *EMBO J* 1988; **7**:3433-9.
- Obert L, Hoover E. Early pathogenesis of transmucosal feline immunodeficiency virus infection. *J Virol* 2002; **76**:6311-22.
- Ohno K, Goitsuka R, Kitamura K, Hasegawa A, Tokunaga T, Honda M. Production of a monoclonal antibody that defines the α -subunit of the feline IL-2 receptor. *Hybridoma* 1992; **11**:595-605.
- Okayama H, Berg P. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol Cell Biol* 1983; **3**:280-9.
- Okumura M, Fujii Y, Inada K, Nakahara K, Matsuda H. Both CD45RA⁺ and CD45RA⁻ subpopulations of CD8⁺ T cells contain cells with high levels of lymphocyte function-associated antigen-1 expression, a phenotype of primed T cells. *J Immunol* 1993; **150**:429-37.
- Olmsted R, Barnes A, Yamamoto J, Hirsch V, Purcell R, Johnson P. Molecular cloning of feline immunodeficiency virus. *Proc Natl Acad Sci USA* 1989; **86**:2448-52.
- Onishi M, Kinoshita S, Morikawa Y *et al.* Applications of retrovirus-mediated expression cloning. *Exp Hematol* 1996; **24**:324-9.
- Orandle M, Crawford P, Levy J, Udoji R, Papadi G, Ciccarone T, Mergia A, Johnson C. CD8⁺ thymic lymphocytes express reduced levels of CD8 β and increased interferon γ in cats perinatally infected with the JSY3 molecular clone of feline immunodeficiency virus. *AIDS Res Hum Retroviruses* 2000; **16**:1559-71.
- Page T, Dallman M. Molecular cloning of cDNAs for the rat interleukin 2 receptor α and β chain genes: differentially regulated gene activity in response to mitogenic stimulation. *Eur J Immunol* 1991; **21**:2133-8.
- Palacios R, Martinez-Maza O. Is the E receptor on human T lymphocytes a "negative signal receptor"? *J Immunol* 1982; **129**:2479-85.
- Parnes J. Molecular biology and function of CD4 and CD8. *Adv Immunol* 1989; **44**:265-311.
- Paterson D, Jefferies W, Green J, Brandon M, Corthesy P, Puklavec

- M, Williams A. Antigens of activated rat T lymphocytes including a molecule of 50,000 M_r detected only on CD4 positive T blasts. *Mol Immunol* 1987; **24**:1281-90.
- Pecoraro M, Kawaguchi Y, Miyazawa T et al. Isolation, sequence and expression of a cDNA encoding the α -chain of the feline CD8. *Immunology* 1994; **81**:127-31.
- Pedersen N, Ho E, Brown M, Yamamoto J. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* 1987; **235**:790-3.
- Peterson A, Seed B. Monoclonal antibody and ligand binding sites of the T cell erythrocyte receptor (CD2). *Nature* 1987; **329**:842-6.
- Pippig S, Pena-Rossi C, Long J et al. Robust B cell immunity but impaired T cell proliferation in the absence of CD134 (OX40). *J Immunol* 1999; **163**:6520-9.
- Pitcher C, Hagen S, Walker J, Lum R, Mitchell B, Maino V, Axthelm M, Picker L. Development and homeostasis of T cell memory in rhesus macaque. *J Immunol* 2002; **168**:29-43.
- Pizzato M, Marlow S, Blair E, Takeuchi Y. Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. *J Virol* 1999; **73**:8599-611.
- Plunkett M, Springer T. Purification and characterization of the lymphocyte function-associated-2 (LFA-2) molecule. *J Immunol* 1986; **136**:4181-7.
- Potempa S, Picard L, Reeves J. CD4-independent infection of human immunodeficiency virus type 2 strain ROD/B: the role of the N-terminal domain of CXCR-4 in fusion and entry. *J Virol* 1997; **71**:4419-24.
- Prell R, Evans D, Thalhoffer C, Shi T, Funatake C, Weinberg A. OX40-mediated memory T cell generation is TNF receptor-associated factor 2 dependent. *J Immunol* 2003; **171**:5997-6005.
- Pu R, Coleman J, Omori M, Arai M, Hohdatsu T, Huang C, Tanabe T, Yamamoto J. Dual-subtype FIV vaccine protects cats against in vivo swarms of both homologous and heterologous subtype

- FIV isolates. *AIDS* 2001; **15**:1225-37.
- Pulido R, Cebrian M, Acevedo A, Landazuri M, Sanchez-Madrid F. Comparative biochemical and tissue distribution study of four distinct CD45 antigen specificities. *J Immunol* 1988; **140**:3851-7.
- Punnonen J, de Vries J. Characterization of a novel CD2⁺ human thymic B cell subset. *J Immunol* 1993; **151**:100-10.
- Ragazzo J, Ozaki M, Karlsson L, Peterson P, Webb S. Costimulation via lymphocyte function-associated antigen 1 in the absence of CD28 ligation promotes anergy of naive CD4⁺ T cells. *Proc Natl Acad Sci USA* 2001; **98**:241-6.
- Ramos P, Safaei R, Kayalar C, Ellis L. Isolation and sequence of λ gt11 cDNA clones encoding the 5B4 antigen expressed on sprouting neurons. *Brain Res Mol Brain Res* 1989; **5**:297-303.
- Reeves J, Hibbitts S, Simmons G, McKnight A, Azevedo-Pereira J, Moniz-Pereira J, Clapham P. Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. *J Virol* 1999; **73**:7795-804.
- Reeves J, Mcknight A, Potempa S *et al.* CD4-independent infection by HIV-2 (ROD/B): use of the 7-transmembrane receptors CXCR-4, CCR-3, and V28 for entry. *Virology* 1997; **231**:130-4.
- Reeves J, Schulz T. The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion. *J Virol* 1997; **71**:1453-65.
- Renard V, Romero P, Vivier E, Malissen B, Luescher I. CD8 β increases CD8 coreceptor function and participation in TCR-ligand binding. *J Exp Med* 1996; **184**:2439-44.
- Richardson J, Morailon A, Baud S, Cuisinier A, Sonigo P, Pancino G. Enhancement of feline immunodeficiency virus (FIV) infection after DNA vaccination with the FIV envelope. *J Virol* 1997; **71**:9640-9.
- Richardson J, Pancino G, Merat R *et al.* Shared usage of the

- chemokine receptor CXCR4 by primary and laboratory-adapted strains of feline immunodeficiency virus. Dustin M, Springer T. *J Virol* 1999; **73**:3661-71.
- Rideout B, Moore P, Pedersen N. Persistent upregulation of MHC Class II antigen expression on T-lymphocytes from cats experimentally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol* 1992; **35**:71-81.
- Rogers P, Song J, Gramaglia I, Killeen N, Croft M. OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 2001; **15**:445-55.
- Rosenberg E, Billingsley J, Caliendo A, Boswell S, Sax P, Kalams S, Walker B. Vigorous HIV-1-specific CD4⁺ T cell responses associated with control of viremia. *Science* 1997; **278**:1447-50.
- Rossol R, Dobmeyer J, Dobmeyer T et al. Increase in V δ 1⁺ γ δ T cells in the peripheral blood and bone marrow as a selective feature of HIV-1 but not other virus infections. *Br J Haematol* 1998; **100**:728-34.
- Rutishauser U, Goridis C. NCAM - the molecule and its genetics *Trends Genet* 1986; **2**:72-6.
- Ryan J, Naper C, Hayashi S, Daws M. Physiologic functions of activating natural killer (NK) complex-encoded receptors on NK cells. *Immunol Rev* 2001; **181**:126-37.
- Sanders M, Makgoba M, Sharrow S, Stephany D, Springer T, Young H, Shaw S. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN- γ production. *J Immunol* 1988; **140**:1401-7.
- Sasada T, Reinherz E. A critical role for CD2 in both thymic selection events and mature T cell function. *J Immunol* 2001; **166**:2394-403.
- Scala E, Ansotegui IJ, Bellioni B, Guerra EC, Aiuti F, Paganelli R. Expansion of CD11a^{bright} cells in CD8⁺CD45RA⁺ from HIV-infected patients: a new early marker for disease progression? *AIDS Res Hum Retroviruses* 1995; **11**:1327-33.
- Schmidt R, Hercend T, Fox D et al. The role of interleukin 2

- and T11 E rosette antigen in activation and proliferation of human NK clones. *J Immunol* 1985; **135**:672-8.
- Schmitz J, Forman M, Lifton M et al. Expression of the CD8 $\alpha\beta$ -heterodimer on CD8⁺ T lymphocytes in peripheral blood lymphocytes of human immunodeficiency virus⁻ and human immunodeficiency virus⁺ individuals. *Blood* 1998; **92**:198-206.
- Seed B, Aruffo A. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc Natl Acad Sci USA* 1987; **84**:3365-9.
- Sewell W, Brown M, Dunne J, Owen M, Crumpton M. Molecular cloning of the human T-lymphocyte surface CD2 (T11) antigen. *Proc Natl Acad Sci USA* 1986; **83**:8718-22 (Correction in *Proc Natl Acad Sci USA* 1987; **84**:7256).
- Sewell W, Brown M, Owen M, Fink P, Kozak C, Crumpton M. The murine homologue of the T lymphocyte CD2 antigen: molecular cloning, chromosome assignment and cell surface expression. *Eur J Immunol* 1987; **17**:1015-20.
- Sewell A, Gerth U, Price D et al. Antagonism of cytotoxic T-lymphocyte activation by soluble CD8. *Nat Med* 1999; **5**:399-404.
- Sheldon A, Flego L, Zola H. Coexpression of IL-2 receptor p55 and p75 by circulating blood lymphocytes. *J Leukoc Biol* 1993; **54**:161-7.
- Shimajima M, Morikawa S, Maeda K, Tohya Y, Miyazawa T, Mikami T. Generation of monoclonal antibodies against a feline CD antigen (CD4) expressed by a recombinant baculovirus. *J Vet Med Sci* 1997; **59**:467-9.
- Shimajima M, Miyazawa T, Kohmoto M, Ikeda Y, Nishimura Y, Maeda K, Tohya Y, Mikami T. Expansion of CD8 $\alpha^+\beta^-$ cells in cats infected with feline immunodeficiency virus. *J Gen Virol* 1998a; **79**:91-4.
- Shimajima M, Pecoraro M, Maeda K, Tohya Y, Miyazawa T, Mikami T. Characterization of anti-feline CD8 monoclonal antibodies. *Vet Immunol Immunopathol* 1998b; **61**:17-23.
- Siebelink K, Tijhaar E, Huisman R et al. Enhancement of feline immunodeficiency virus infection after immunization with

- envelope glycoprotein subunit vaccines. *J Virol* 1995; **69**:3704-11.
- Siliciano R, Lawton T, Knall C, Karr R, Berman P, Gregory T, Reinherz E. Analysis of host-virus interactions in AIDS with anti-gp120 T cell clones: effect of HIV sequence variation and a mechanism for CD4⁺ cell depletion. *Cell* 1988; **54**:561-75.
- Slifka M, Whitton J. Activated and memory CD8⁺ T cells can be distinguished by their cytokine profiles and phenotypic markers. *J Immunol* 2000; **164**:208-16.
- Small S, Shull G, Santoni M-J, Akeson R. Identification of a cDNA clone that contains the complete coding sequence for a 140-kD rat NCAM polypeptide. *J Cell Biol* 1987; **105**:2335-45.
- Song W, Collisson E, Billingsley P, Brown W. Induction of feline immunodeficiency virus-specific cytolytic T-cell responses from experimentally infected cats. *J Virol* 1992; **66**:5409-17.
- Song W, Collisson E, Li J, Wolf A, Elder J, Grant C, Brown W. Feline immunodeficiency virus (FIV)-specific cytolytic T lymphocytes from chronically infected cats are induced *in vitro* by retroviral vector-transduced feline T cells expressing the FIV capsid protein. *Virology* 1995; **209**:390-9.
- Sonnhammer E, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. Glasgow J *et al.*, eds *Proc Sixth Int Conf on Intelligent Systems for Molecular Biology*, 175-182. AAAI Press, 1998:1-8.
- Spruyt L, Glennie M, Beyers A, Williams A. Signal transduction by the CD2 antigen in T cells and natural killer cells: requirement for expression of a functional T cell receptor or binding of antibody Fc to the Fc receptor, FcγRIIIA (CD16). *J Exp Med* 1991; **174**:1407-15.
- Stievano L, Tosello V, Marcato N, Rosato A, Sebelin A, Chieco-Bianchi L, Amadori A. CD8⁺αβ⁺ T cells that lack surface CD5 antigen expression are a major lymphotactin (XCL1) source in peripheral blood lymphocytes. *J Immunol* 2003; **171**:4528-38.
- Straube F, Herrmann T. Differential modulation of CD8β by rat γδ and αβ T cells after activation. *Immunology* 2001; **104**:252-8.
- Straube F, Herrmann T. Expression of functional CD8αβ heterodimer

- on rat $\gamma\delta$ T cells does not correlate with the CDR3 length of the TCR δ chain predicted for MHC class I-restricted antigen recognition. *Eur J Immunol* 2000; **30**:3562-8.
- Stuber E, Strober W. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J Exp Med* 1996; **183**:979-89.
- Takeuchi Y, Porter C, Strahan K, Preece A, Gustafsson K, Cosset F, Weiss R, Collins M. Sensitization of cells and retroviruses to human serum by (alpha 1-3) galactosyltransferase. *Nature* 1996; **379**:85-8.
- Tarazona R, DelaRosa O, Alonso C, Ostos B, Espejo J, Pena J, Solana R. Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells. *Mechanisms of Aging and Development* 2000; **121**:77-88.
- Tavernor A, Kydd J, Bodian D, Jones E, Stuart D, Davis S, Butcher G. Expression cloning of an equine T-lymphocyte glycoprotein CD2 cDNA: Structure-based analysis of conserved sequence elements. *Eur J Biochem* 1994; **219**:969-76.
- Taylor D, Hokama Y, Perri S. Differentiating feline T and B lymphocytes by rosette formation. *J Immunol* 1975; **115**:862-5.
- Tellier M, Soos J, Pu R, Pollock D, Yamamoto J. Development of FIV-specific cytolytic T-lymphocyte responses in cats upon immunisation with FIV vaccines. *Vet Microbiol* 1997; **57**:1-11.
- Terry L, DiSanto J, Small T, Flomenberg N. Differential expression and regulation of the human CD8 alpha and CD8 beta chains. *Tissue Antigens* 1990; **35**:82-91.
- Theze J, Alzari P, Bertoglio J. Interleukin 2 and its receptors: recent advances and new immunological functions. *Immunology Today* 1996; **17**:481-6.
- Tomkinson B, Brown M, Ip S, Carrabis S, Sullivan J. Soluble CD8 during T cell activation. *J Immunol* 1989; **142**:2230-6.
- Tompkins M, Gebhard D, Bingham H, Hamilton M, Davis W, Tompkins W. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. *Vet Immunol Immunopathol* 1990;

26:305-17.

- Tompkins M, Huber K, Tompkins W. Natural cell-mediated cytotoxicity in the domestic cat: properties and specificity of effector cells. *Am J Vet Res* 1983; **44**:1525-9.
- Tregaskes C, Kong F-K, Paramithiotis E, Chen C, Ratcliffe M, Davison T, Young J. Identification and analysis of the expression of CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ isoforms in chickens reveals a major TCR- $\gamma\delta$ CD8 $\alpha\beta$ subset of intestinal intraepithelial lymphocytes. *J Immunol* 1995; **154**:4485-94.
- vanWauwe J, Goossens J, Decock W, Kung P, Goldstein G. Suppression of human T-cell mitogenesis and E-rosette formation by the monoclonal antibody OKT11A. *Immunology* 1981; **44**:865-71.
- Verbi W, Greaves M, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G. Monoclonal antibodies OKT 11 and OKT 11A have pan-T reactivity and block sheep erythrocyte "receptors". *Eur J Immunol* 1982; **12**:81-6.
- Walker C, Moody D, Stites D, Levy J. CD8⁺ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 1986; **234**:1563-6.
- Walker L, Gulbranson-Judge A, Flynn S, Brocker T, Lane P. Co-stimulation and selection for T-cell help for germinal centres: the role of CD28 and OX40. *Immunol Today* 2000; **21**:333-7.
- Wang J-H, Smolyar A, Tan K, Liu J-H, Kim M, Sun Z-Y, Wagner G, Reinherz E. Structure of a heterophilic adhesion complex between the human CD2 and CD58 (LFA-3) counterreceptors. *Cell* 1999; **97**:791-803.
- Waterhouse R, Ha C, Dveksler G. Murine CD9 is the receptor for pregnancy-specific glycoprotein 17. *J Exp Med* 2000; **195**:277-82.
- Welsh R, Lin M, Lohman B, Varga S, Zarozinski C, Selin L. Alpha beta and gamma delta T-cell networks and their roles in natural resistance to viral infections. *Immunol Rev* 1997; **159**:79-93.
- Werwitzke S, Tiede A, Drescher B, Schmidt R, Witte T. CD8 β /CD28 expression defines functionally distinct populations of peripheral blood T lymphocytes. *Clin Exp Immunol* 2003;

- 133:334-43.**
- Wheeler C, von Hoegen, Parnes J. An immunological role for the CD8 β -chain. *Nature* 1992; **357:247-9.**
- Willett B, Hosie M, Callanan J, Neil J, Jarrett O. Infection with feline immunodeficiency virus is followed by the rapid expansion of a CD8⁺ lymphocyte subset. *Immunology* 1993; **78:1-6.**
- Willett B, Hosie M, Dunsford T, Neil J, Jarrett O. Productive infection of T-helper lymphocytes with feline immunodeficiency virus is accompanied by reduced expression of CD4. *AIDS* 1991; **5:1469-75.**
- Willett B, Hosie M, Shaw A, Neil J. Inhibition of feline immunodeficiency virus infection by CD9 antibody operates after virus entry and is independent of virus tropism. *J Gen Virol* 1997a; **78:611-8.**
- Willett B, Picard L, Hosie M, Turner J, Adema K, Clapham P. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. *J Virol* 1997b; **71:6407-15.**
- Williams A, Barclay A, Clark S, Paterson D, Willis A. Similarities in sequences and cellular expression between rat CD2 and CD4 antigens. *J Exp Med* 1987; **165:368-80.**
- Wingren A, Dahlenborg K, Bjorklund M *et al.* Monocyte-regulated IFN- γ production in human T cells involves CD2 signaling. *J Immunol* 1993; **151:1328-36.**
- Withka J, Wyss D, Wagner G, Arulanandam A, Reinherz E, Recny M. Structure of the glycosylated adhesion domain of human T lymphocyte glycoprotein CD2. *Structure* 1993; **1:69-81.**
- Witte T, Spoerl R, Chang H. The CD8 β ectodomain contributes to the augmented coreceptor function of CD8 $\alpha\beta$ heterodimers relative to CD8 $\alpha\alpha$ homodimers. *Cell Immunol* 1999; **191:90-6.**
- Wu L, Paxton W, Kassam N *et al.* CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, *in vitro.* *J Exp Med* 1997; **185:1681-91.**
- Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1998; **280:1884-8.**
- Wyss D, Choi J, Li J *et al.* Conformation and function of the

- N-linked glycan in the adhesion domain of human CD2. *Science* 1995; **269**:1273-8.
- Yagita H, Nakamura T, Karasuyama H, Okumura K. Monoclonal antibodies specific for murine CD2 reveal its presence on B as well as T cells. *Proc Natl Acad Sci USA* 1989; **86**:645-9.
- Yamamoto J, Sparger E, Ho E *et al.* Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *Am J Vet Res* 1988; **49**:1246-58.
- Yang H, Reinherz E. Dynamic recruitment of human CD2 into lipid rafts. *J Biol Chem* 2001; **276**:18775-85.
- Yang O, Walker B. CD8⁺ cells in human immunodeficiency virus type I pathogenesis: cytolytic and noncytolytic inhibition of viral replication. *Adv Immunol* 1997; **66**:273-311.
- Zhao Y, Gebhard D, English R, Sellon R, Tompkins M, Tompkins W. Enhanced expression of novel CD57⁺CD8⁺ LAK cells from cats infected with feline immunodeficiency virus. *J Leukoc Biol* 1995; **58**:423-31.
- Zimmermann C, Brduscha-Riem K, Blaser C, Zinkernagel RM, Pircher H. Visualization, characterization, and turnover of CD8⁺ memory T cells in virus-infected hosts. *J Exp Med* 1996; **183**:1367-75.
- Zola H, Koh L, Mantzioris B, Rhodes D. Patients with HIV infection have a reduced proportion of lymphocytes expressing the IL 2 receptor p55 chain (TAC, CD25). *Clin Immunol Immunopathol* 1991; **59**:16-25.
- Zola H, Mantzioris B, Webster J, Kette F. Circulating human T and B lymphocytes express the p55 interleukin-2 receptor molecule (TAC, CD25). *Immunol Cell Biol* 1989; **67**:233-7.

LIST OF PUBLICATION

Shimojima M

- Kawaguchi Y, Maeda K, Pecoraro M, Inoshima Y, Jang H, Kohmoto M, Iwatsuki K, Ikeda Y, **Shimojima M**, Tohya Y, Kai C, Mikami T. The feline herpesvirus type 1 ICP4 down-regulates feline immunodeficiency virus long terminal repeat (LTR)-directed gene expression via the C/EBP site in the LTR. *J Vet Med Sci* 1995; 57:1129-31.
- Pecoraro M, **Shimojima M**, Maeda K, Inoshima Y, Kawaguchi Y, Kai C, Mikami T. Molecular cloning of the feline CD8 β -chain. *Immunology* 1996; 89:84-8.
- Inoshima Y, Ikeda Y, Kohmoto M, Pecoraro M, **Shimojima M**, Shimojima Y, Inada G, Kawaguchi Y, Tomonaga K, Miyazawa T, Mikami T. Persistence of high virus neutralizing antibody titers in cats experimentally infected with feline immunodeficiency virus. *J Vet Med Sci* 1996; 58:925-7.
- Shimojima M**, Morikawa S, Maeda K, Tohya Y, Miyazawa T, Mikami T. Generation of monoclonal antibodies against a feline CD antigen (CD4) expressed by a recombinant baculovirus. *J Vet Med Sci* 1997; 59:467-9.
- Ikeda Y, Kawaguchi Y, Inoshima Y, Kohmoto M, **Shimojima M**, Inada G, Sato E, Kai C, Miyazawa T, Mikami T. The effects of treatment with chemical agents or infection with feline viruses on protein-binding properties of the feline immunodeficiency virus long terminal repeat. *Virus Res* 1997; 51:203-12.
- Shimojima M**, Miyazawa T, Kohmoto M, Ikeda Y, Nishimura Y, Maeda K, Tohya Y, Mikami T. Expansion of CD8 $\alpha^+\beta^-$ cells in cats infected with feline immunodeficiency virus. *J Gen Virol* 1998; 79:91-4.
- Shimojima M**, Pecoraro M, Maeda K, Tohya Y, Miyazawa T, Mikami T. Characterization of anti-feline CD8 monoclonal antibodies. *Vet Immunol Immunopathol* 1998; 61:17-23.
- Kohmoto M, Uetsuka K, Ikeda Y, Inoshima Y, **Shimojima M**, Sato E, Inada G, Toyosaki T, Miyazawa T, Doi K, Mikami T. Eight-year observation and comparative study of specific pathogen-free cats experimentally infected with feline immunodeficiency virus (FIV) subtypes A and B: terminal acquired immunodeficiency syndrome in a cat infected with FIV petaluma strain. *J Vet Med Sci* 1998; 60:315-21.

- Satoh S, **Shimojima M**. セレクチン阻害剤としての Aryl C-glycosides の合成と評価 Medchem News 1999; 4:17-23.
- Kuribayashi T, Gohya S, Mizuno Y, **Shimojima M**, Ito K, Satoh S. Bis C-glycosylated diphenylmethanes for stable glycoepitope mimetics. Synlett 1999; 6: 737-40.
- Nishimura Y, **Shimojima M**, Miyazawa T, Sato E, Nakamura K, Izumiya Y, Ikeda Y, Mikami T, Takahashi E. Molecular cloning of the cDNAs encoding the feline B-lymphocyte activation antigen B7-1 (CD80) and B7-2 (CD86) homologues which interact with human CTLA4-Ig. Eur J Immunogenet 2000; 27:427-30.
- Kato K, Kawaguchi Y, Tanaka M, Igarashi M, Yokoyama A, Matsuda G, Kanamori M, Nakajima K, Nishimura Y, **Shimojima M**, Phung H, Takahashi E, Hirai K. Epstein-Barr virus-encoded protein kinase BGLF4 mediates hyperphosphorylation of cellular elongation factor 1δ (EF-1δ): EF-1δ is universally modified by conserved protein kinases of herpesviruses in mammalian cells. J Gen Virol 2001; 82:1457-63.
- Shimojima M**, Nishimura Y, Miyazawa T, Kato K, Nakamura K, Izumiya Y, Akashi H, Tohya Y. A feline CD2 homologue interacts with human red blood cells. Immunology 2002; 105:360-6.
- Matsuura Y, Tohya Y, Nakamura K, **Shimojima M**, Roerink F, Mochizuki M, Takase K, Akashi H, Sugimura T. Complete nucleotide sequence, genome organization and phylogenetic analysis of the canine calicivirus. Virus Genes 2002; 25:67-73.
- Hosie M, Willett B, Klein D, Dunsford T, Cannon C, **Shimojima M**, Neil J, Jarrett O. Evolution of replication efficiency following infection with a molecularly cloned feline immunodeficiency virus of low virulence. J Virol 2002; 76:6062-72.
- Shimojima M**, Miyazawa T, Sakurai Y, Nishimura Y, Tohya Y, Matsuura Y, Akashi H. Usage of myeloma and panning in retrovirus-mediated expression cloning. Anal Biochem 2003; 315:138-40.
- Shimojima M**, Nishimura Y, Miyazawa T, Kato K, Tohya Y, Akashi H. CD56 expression in feline lymphoid cells. J Vet Med Sci 2003; 65:769-73.
- Shimojima M**, Nishimura Y, Miyazawa T, Tohya Y, Akashi H.

- Phenotypic changes in CD8⁺ peripheral blood lymphocytes in cats infected with feline immunodeficiency virus. *Microbes Infect* 2003; 5:1171-6.
- Phung H, Tohya Y, **Shimojima M**, Kato K, Miyazawa T, Akashi H. Establishment of a GFP-based indicator cell line to quantitate feline foamy virus. *J Virol Methods* 2003; 109:125-31.
- Kohmoto M, Ikeda Y, Sato E, Nishimura Y, Inoshima Y, **Shimojima M**, Tohya Y, Mikami T, Miyazawa T. Experimental mucosal infection with molecularly cloned feline immunodeficiency viruses. *Clin Diagn Lab Immunol* 2003; 10:185-8.
- Shimojima M**, Kawaoka Y. SARSの基礎知識 ウイルス・細菌と感染症がわかる 2003; WJ18:93-8.
- Shimojima M**, Miyazawa T, Ikeda Y, McMonagle E, Haining H, Akashi H, Takeuchi Y, Hosie M, Willett B. Utilization of CD134 as a primary receptor by the feline immunodeficiency virus. *Science* 2004; 303:1192-5.
- Shimojima M**, Nishimura Y, Miyazawa T, Tohya Y, Akashi H. T cell subpopulations mediating inhibition of feline immunodeficiency virus replication in mucosally infected cats. *Microbes Infect* In press.
- Shimojima M**, Miyazawa T. ネコ免疫不全ウイルスのプライマリーレセプター: レンチウイルスはどこから来たのか? 細胞工学 In press.
- Sakurai Y, **Shimojima M**, Miyazawa T, Masuoka K, Tohya Y, Akashi H. Identification of the feline CD63 homologue using retrovirus-mediated expression cloning. *Vet Immunol Immunopathol* In press.
- Shimojima M**, Nishimura Y, Miyazawa T, Tohya Y, Akashi H. Molecular cloning and expression of feline T cell surface antigens. In preparation.
- Shimojima M**, Nishimura Y, Miyazawa T, Tohya Y, Akashi H. CD56 expression and feline immunodeficiency virus infection in MYA-1 cells. In preparation.

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

氏 名: 下島 昌幸

指導教官: 明石 博臣

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 α 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 β 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 α および TCR δ 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⁺ 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⁺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⁺細胞が増加し、一方 CD4⁺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⁺β⁺細胞のみでなく、CD8α⁺β⁻細胞および CD4⁺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⁺ 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 α^+ 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 $^+$ PBL では認められず、FIV 感染は主に CD8 $^+$ 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 $^+$ および CD8 $^+$) 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 $^+$ MYA-1 細胞は CD56 $^-$ 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 $^+$ T 細胞(時に CD4 $^+$ T 細胞)の重要性や、CD8 $^+$ 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。