

Evaluation of expression patterns of feline CD28 and CTLA-4 in feline immunodeficiency virus (FIV)-infected and FIV antigen-induced PBMC

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It is known that CD28, a positive costimulatory receptor, plays a very important role in inducing the optimal stimulation of T lymphocytes. CTLA-4 (CD152), however, acts as a negative regulator in T lymphocyte activation. The effect of an feline immunodeficiency virus (FIV) infection on the expression of feline CD28 and CTLA-4 was studied with FIV-infected and uninfected peripheral blood mononuclear cells (PBMC) using a competitive PCR assay. The nature of CD28 and CTLA-4 expression was also examined with fresh and antigen-stimulated PBMC. FIV infection induced a lower expression of CD28, but a higher expression of CTLA-4 in the infected PBMC than in the uninfected PBMC. Relatively high levels of CD28 expression were demonstrated in both the fresh and the antigen-stimulated PBMC. The expression level of CTLA-4 in the freshly isolated PBMC was rather low, however, FIV antigen stimulation induced a relatively high expression of CTLA-4 in feline PBMC.

Key words: CD28, CTLA-4, FIV infection, PBMC, competitive PCR.

Introduction

CD28, a T cell-specific glycoprotein, is expressed as a homodimer in most T lymphocytes [2, 31]. CD28 has also been identified as the major co-receptor for binding B7-1 [15]. It is known that the interaction of CD28 with B7 ligands in humans and mice provides a costimulatory signal inducing T cell proliferation, IL-2 production, and cytotoxicity [3, 14, 24, 27]. CD28 is expressed in approximately 95% of CD4⁺ and 70% of CD8⁺ human T cells [7].

CTLA-4 is also a T lymphocyte costimulatory receptor belonging to the immunoglobulin superfamily (Ig SF) [5]. CTLA-4 is expressed as a homodimer only in activated T lymphocytes [20, 26, 30, 34]. It has been suggested that CTLA-4 binds to B7-1 and B7-2 on antigen presenting cells (APC) with a higher avidity than CD28 [16, 29, 35]. However, CD28 and CTLA-4 deliver opposing signals in activated T cells [21, 37]. CTLA-4 has been shown to be a negative regulator of T cell activation, inhibiting CD28-mediated T cell proliferation [38].

Human immunodeficiency virus (HIV) infection induces a gradual decrease of CD28 expression in both CD4⁺ and CD8⁺ T lymphocytes [7, 10]. When CD4⁺ T cells from HIV-1 infected subjects are stimulated by alloantigen, the expression of CD28 is decreased [18]. This down-modulation of CD28 expression in CD4⁺ and CD8⁺ T cells has been suggested to correlate with reduced responsiveness to costimulation, the development of AIDS-related diseases, and increased apoptosis [4, 19, 25, 33, 36]. The down-regulation of CD28 expression in T lymphocytes has also been observed in patients infected with other pathogens, including human T lymphotropic virus (HTLV), *Bordetella pertussis*, and *Trypanosoma cruzi* [11, 22, 32].

Since CD28 and CTLA-4 function as positive and negative regulators in T cell activation, respectively, an evaluation and comparison of the expression patterns of these molecules could contribute to an understanding of the mechanisms involved in the induction of T cell-mediated immunity. However, it is still unknown whether or not an FIV infection modulates the expression of CD28 and CTLA-4 in lymphocytes. It is also unknown how *in vitro* stimulation using autologous APC affects the expression of CD28 and CTLA-4 in PBMC from infected cats. Accordingly, this study examined the expression of CD28 and CTLA-4 in FIV-infected and noninfected feline PBMC at the mRNA level. The differential expression of CD28 and CTLA-4 was also compared with freshly

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isolated and antigen-stimulated PBMC from an FIV-infected cat.

Materials and Methods

Virus

A virus stock of the FIV-PPR strain was prepared by collecting the cell culture supernatants from PBMC of infected cats after 12 days of culture. The presence of FIV in the collected supernatants was determined using an FIV p24 antigen detection kit (IDEXX, Portland, Maine). Those supernatants showing more than OD 3.5 were used as the virus stock to infect fresh PBMC.

Cell culture and cell viability

Feline PBMC were cultured in complete RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 50mg/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 100 units/ml of human recombinant IL-2. After six days of infection with FIV, the cell viability was determined by the trypan blue exclusion method.

Preparation of cDNA

Con-A stimulated PBMC obtained from an FIV-uninfected cat were divided into two aliquots. One aliquot of cells was cultured without virus, whereas the other was infected *in vitro* with FIV-PPR. After 7 days of culture, the total RNA was extracted from equal numbers of both aliquots of cells, and single stranded cDNA was made with a First Strand cDNA Synthesis Kit (Gibco BRL, Gaithersburg, MD). In order to compare the expression patterns of CD28 and CTLA-4 before and after antigen stimulation, the total RNA was prepared from freshly isolated PBMC and PBMC stimulated with autologous irradiated APC for 10 days. Single stranded cDNA was made from the RNA and then used in PCR reactions for examining modulations of CD28 and CTLA-4.

Competitive PCR of CD28

In order to perform a competitive PCR assay, three kinds of primers, forward (primer A), backward (primer C), and 3' linker (primer B + C) primers, as listed in Table 1, were made by modifying a previously described method [8]. The 3' linker primer was composed of the primer B sequence at the 3' end of the primer that corresponded to the target strand, and the primer C sequence at the 5' end of the primer. The internal standard DNA for CD28 was

made with primer A and primer B + C (Table 1) by following the scheme illustrated in Fig. 1A. The following PCR amplification for 30 cycles was used for the synthesis of the internal standard DNA: 94°C for 30 sec, 55°C for 30 sec and, 72°C for 40 sec. The PCR product was analyzed on an agarose gel and the DNA band of the correct size was cut out of the gel. The DNA was extracted using a Microseparator (Amicon, Beverly, MA) and purified with a PCR purification kit (Qiagen, Valencia, CA). The DNA was then eluted in 50 μ l of dH₂O and diluted 10,000-fold before being used in the competitive PCR as the internal standard DNA. CD28 competitive PCR was performed, as illustrated in Fig. 1B, using cDNA synthesized with mRNA from noninfected and FIV-infected PBMC, the internal standard DNA, primer A, and primer C (Table 1) according to the following 30 cycles: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. The PCR products were analyzed on an 1.5% agarose gel and the densities of the bands were determined by an NIH Image Documentation Program.

Competitive PCR of CTLA-4

The three kinds of primers used for the production of the internal standard DNA and in the CTLA-4 competitive PCR were synthesized by methods similar to those described in the competitive PCR of CD28 (Table 2). The PCR for the synthesis of the CTLA-4 internal standard DNA included the following conditions for 30 cycles: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. After analyzing the PCR product on an agarose gel, the DNA band was cut out of the gel. It was then purified, and diluted 10,000-fold before being used in the competitive PCR. The following PCR amplification for 30 cycles was used for the competitive PCR of CTLA-4: 94°C for 30 sec, 55°C for 30 sec and, 72°C for 40 sec. The densities of the CTLA-4 and the internal standard DNA bands were measured using an NIH Image Documentation Program after analyzing the PCR products on an 1.5% agarose gel.

PCR for detection of CD28 and CTLA-4

PCR reactions were performed to examine the modulation of CD28 and CTLA-4 expression before and after antigenic stimulation. cDNA made from the mRNA of fresh PBMC and antigen-stimulated PBMC from an FIV infected cat was used as the template in the PCR. The CD28 forward primer was 5'-ATG ATC CTC AGG CTG

Table 1. Primers used in competitive PCR for CD28

Primer A:	5' ATGATCCTCAGGCTGCTTCTGG 3'
Primer B+C:	5'CGGGGGGTCATGTTTCATATAGTCATG GCTTGGAAGATTCAGGAGAC 3'
Primer C:	5' CGGGGGGTCATGTTTCATATAGTC 3'

Table 2. Primers used in competitive PCR for CTLA-4

Primer A:	5' TGAAGTCTGTGCTGCGACATACAC 3'
Primer B+C:	5'GCCTCAGCTCTTAGAAATTGGACAGT- GGGGGCATTTTCACATAGACC 3'
Primer C:	5' GCCTCAGCTCTTAGAAATTGGACA 3'

CTT CTG GC-3', whereas the backward primer was 5'-TCA GGA ACG GTA TGC CGC AAA GTC-3'. The following PCR conditions were used to amplify the CD28 for 30 cycles: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec. The forward primer of the CTLA-4 was 5'-AGC CAT GGC TTG CTT TGG ATT C-3', whereas the backward primer was 5'-TGA TGG GAA TAA AAT AAG GCT G-3'. The following PCR conditions were used to amplify the CTLA-4 for 30 cycles: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec.

Results

Effect of FIV-infection on cell viability

The % viability of the uninfected and the FIV-infected PBMC was examined before the isolation of the total RNA. After six days of *in vitro* infection, the FIV replication in the infected cells was confirmed by an FIV p24 ELISA using the culture supernatant, and showed an OD of 4.0. The % viability of the uninfected cells was 80.6% and that of the FIV-infected cells was 82.1% (Table 3). Therefore, the six-day FIV infection did not induce any decrease in cell viability.

Effect of FIV-infection on the expression of CD28

Based on the assumption of an approximately equivalent % viability, as shown in Table 3, the differences in the expression of CD28 between the FIV-infected and the uninfected PBMC were compared using a competitive

Table 3. Comparison of % viable cells after FIV infection

Cells	p24 antigen ^a	% viability ^b
FIV uninfected	OD 0.0	80.6
FIV infected	OD 4.0	82.1

^aThe expression level of FIV p24 antigen was determined using ELISA.

^bViable cells were determined by the trypan blue exclusion method.

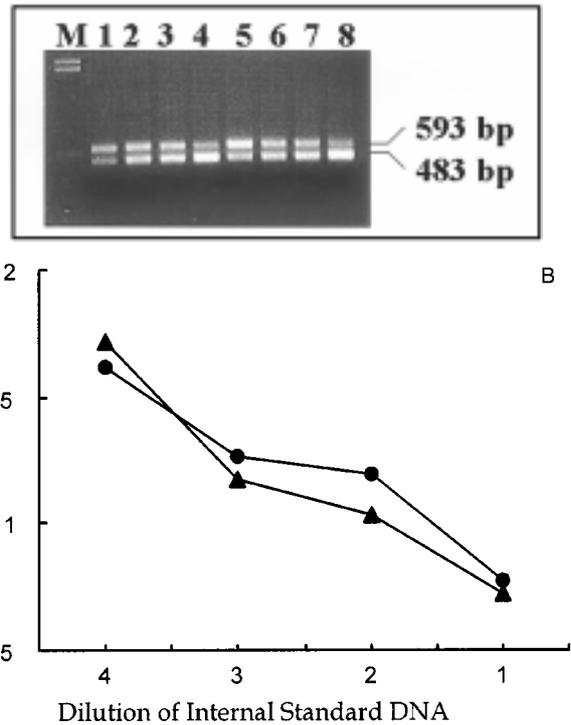


Fig. 2. (A) Competitive PCR of CD28. The expression of feline CD28 was compared with uninfected (lane 1-4) and FIV-infected (lane 5-8) PBMC. The 10,000-fold diluted internal standard DNA was serially diluted by a two-fold dilution, and 2⁴, 2³, 2² and 2¹-fold diluted internal standard DNA were added to the PCR reactions to compete with template DNA. The upper bands (593 bp) and the lower bands (483 bp) are CD28 and internal standard DNA-specific bands, respectively. Dilution of internal standard DNA was expressed as values of log2. (B) Competitive PCR results are represented by the density ratios of CD28/internal standard DNA bands in uninfected (circles) and FIV-infected (triangles) PBMC.

PCR assay. The forward and backward primer set in the competitive PCR of CD28 produced upper CD28-specific bands (593 bp) and lower internal standard DNA-specific bands (483 bp) (Fig. 2A). An analysis of the CD28-specific and internal standard DNA-specific bands demonstrated that the expression of CD28 seemed to be lower in the FIV-infected PBMC than in the noninfected PBMC (Fig. 2B). This result indicated that the FIV-infection in the feline PBMC induced a downregulation of the CD28 expression.

Effect of FIV infection on the expression of CTLA-4

Similarly, the expression levels of CTLA-4 were compared

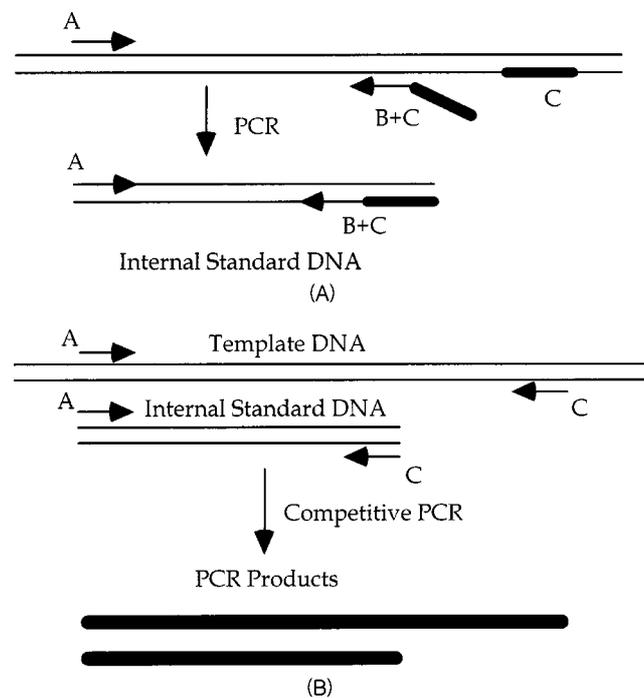


Fig. 1. Schematic procedures for the synthesis of internal standard DNA (A) and the competitive PCR (B).

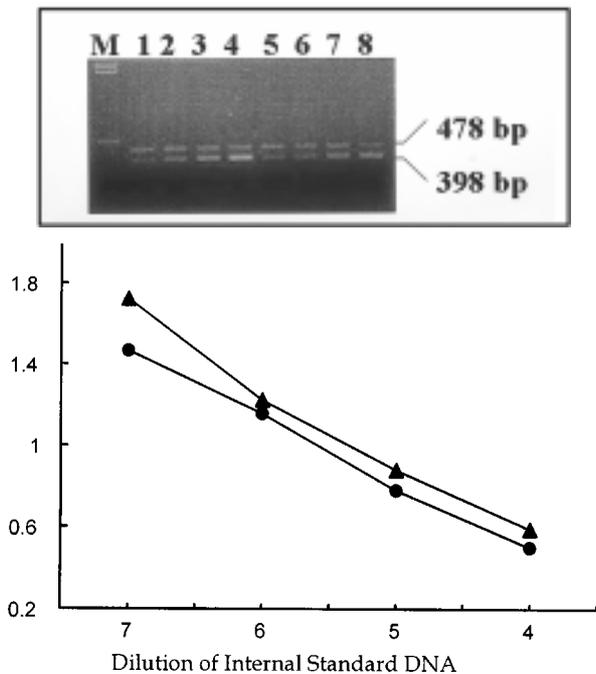


Fig. 3. (A) Competitive PCR of CTLA-4. The expression of feline CTLA-4 was compared with uninfected (lane 1-4) and FIV-infected (lane 5-8) PBMC. The already 10,000 fold-diluted internal standard DNA was serially diluted by a two-fold dilution, and 2^7 , 2^6 , 2^5 and 2^4 -fold diluted internal standard DNA were added to the PCR reactions to compete with the template DNA. The upper bands (478 bp) and the lower bands (398 bp) are CTLA-4 and internal standard DNA-specific bands, respectively. (B) The competitive PCR results are represented by the density ratios of CTLA-4/internal standard DNA bands in uninfected (circles) and FIV-infected (triangles) PBMC.

between the FIV-infected and the uninfected cells using a competitive PCR assay. The forward and backward primer set produced upper CTLA-4-specific bands (478 bp) and lower internal standard DNA-specific bands (398 bp) (Fig. 3A). A similar analysis of the competitive PCR products, performed by calculating the CTLA-4/internal standard DNA density ratios, demonstrated that the expression of CTLA-4 was slightly higher in the FIV-infected cells than in the uninfected cells (Fig. 3B). This result was consistent in all the competitive PCR samples and indicated that FIV-infection induced an upregulation of CTLA-4 expression in PBMC.

Modulation of CD28 and CTLA-4 expression after antigenic stimulation

The expression patterns of feline CD28 and CTLA-4 were measured using freshly isolated PBMC and autologous irradiated APC-stimulated PBMC from an FIV-infected cat. The PCR reaction produced CD28-specific 666 bp products (Fig. 4A). The expression of CD28 was detected in both the freshly isolated PBMC and the antigen-stimulated PBMC at almost the same level. Therefore, it

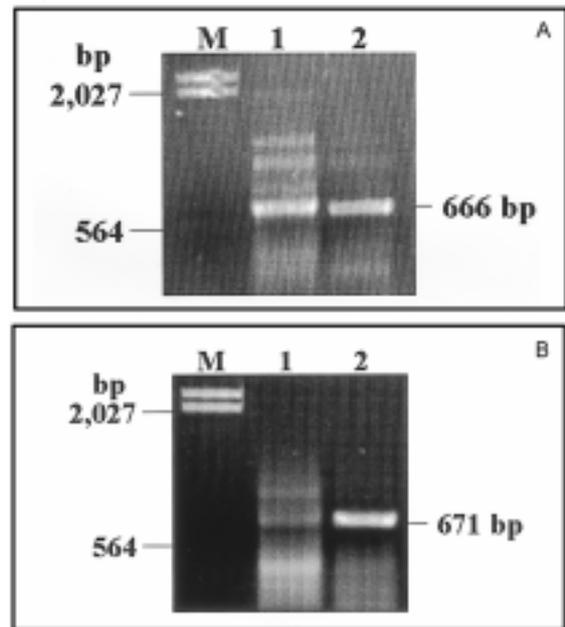


Fig. 4. (A) Modulation of CD28 after antigen stimulation. The expression of CD28 (666 bp) was determined by PCR with cDNA synthesized from the mRNA of freshly isolated PBMC (lane 1). PBMC were stimulated for 10 days with irradiated autologous APC and the mRNA expression of CD28 was examined by PCR (lane 2). (B) Modulation of CTLA-4 after antigen stimulation. The expression of CTLA-4 (671 bp) was determined by PCR with cDNA synthesized from the mRNA of freshly isolated PBMC (lane 1). PBMC were stimulated for 10 days with irradiated autologous APC and mRNA expression of CTLA-4 was examined by PCR (lane 2).

would appear that a relatively high level of feline CD28 was innately expressed in the resting PBMC without stimulation. Furthermore, antigen-specific stimulation did not induce any detectable change in the expression of CD28 in feline PBMC. On the other hand, the expression of feline CTLA-4 in the freshly isolated PBMC as measured by PCR was low (Fig. 4B). However, following the stimulation of the PBMC with autologous APC, the CTLA-4-specific 671 bp of the PCR product was readily detected (Fig. 4B). Accordingly, it was confirmed that the expression of CTLA-4 could be strongly induced by antigen-specific stimulation in feline PBMC.

Discussion

It has been previously suggested that HIV-infection induces downregulation of CD28 in CD4⁺ and CD8⁺ T lymphocytes, which may be a part of the reason for the abnormal immune responses in HIV-infected individuals [7, 10, 18].

In this study, it was demonstrated that FIV-infection induced a slight downregulation of feline CD28 in feline PBMC. The reasons for the reduced expression of CD28 in

FIV-infected PBMC may be partly explained by two identified phenomena in HIV-infected patients [7, 10, 25, 36]. One of which is the reduction of CD28-bearing CD4⁺ and CD8⁺ T cells in HIV-infected individuals and the other is the concurrent expansion of CD28⁻ CD8⁺ T cells. Therefore, both events mentioned above or similar mechanisms may produce the decrease of CD28 expression in FIV-infected PBMC. The CD28⁻ CD8⁺ T cell subset has been suggested to be responsible for HIV-specific cytotoxic activity [12, 36]. However, the CD28⁺ CD8⁺ T cell subset exhibits potent noncytolytic anti-HIV activity [23]. CD28-mediated costimulation induces a HIV-resistant phenotype and prevents the apoptosis of CD4⁺ T cells in HIV-infected patients [6, 17]. It has also been demonstrated that anti-HIV therapy increases the expression of CD28 in CD8⁺ T cells [1]. Therefore, the CD28-mediated costimulatory signal would seem to play an important role in the development of an antiviral immune response. It would also appear that the FIV infection-induced downregulation of CD28 expression might be a helpful way developed by FIV to evade the antiviral immune response of the host.

This study also showed that the expression of feline CTLA-4 increased in FIV-infected PBMC. Although the difference of CTLA-4 expression in noninfected and FIV-infected cells seemed to be small, the increase was consistent in all the competitive PCR experiments. Haffar et al. [18] showed that the expression of human CTLA-4 was either unchanged or increased in primary HIV-infected CD4⁺ T cell lines after alloantigen stimulation. Therefore, it has been suggested that the enhanced expression of CTLA-4 compensates for the decreased expression of CD28 in HIV-infected T cells [18]. Some HTLV-I-transformed and virus secreting T cells express a high level of CTLA-4 without any expression of CD28 [13]. Therefore, it seems that FIV, HIV or HTLV may directly induce the elevated expression of CTLA-4 in infected cells, which concurrently decreases the anti-viral immune responses in the hosts.

It has been shown that CD28 is expressed in human and mouse resting T cells [26, 28]. The expression pattern of the CD28 molecule may also be applicable to its feline counterpart. CD28 expression was readily detected in the resting feline PBMC. After the identification of the expression pattern of CD28 in fresh PBMC, PBMC from an FIV-infected cat were stimulated with irradiated autologous APC to examine the effect on the expression of the costimulatory receptor. However, it appeared that antigen-specific stimulation had no modulatory effect on the expression of feline CD28 in PBMC.

It has been previously shown that CTLA-4 is not detected in human or mouse resting T lymphocytes [26, 28]. The expression of feline CTLA-4 was slight in freshly isolated PBMC. However, the expression of CTLA-4 was

increased in the antigen-stimulated cells. This enhanced expression of CTLA-4 in the antigen-stimulated PBMC could be explained by the following hypothesis. During the initial period of antigen stimulation, the T lymphocytes appear to be activated and proliferated by the engagement of both TCR and CD28. Thereafter, CTLA-4 may be induced to maintain cell homeostasis. However, feline CTLA-4 expressed in the APC-stimulated PBMC does not appear to result in a CTLA-4-directed shut down of activated T cells, since the same stimulation induces the production of anti-FIV soluble factor(s) and FIV-specific CTL activities [9]. As a result, these findings contribute in part to an understanding of the very delicate cellular immune responses induced by viral antigen-specific stimulation. The interaction between CD28 and CTLA-4 in CD8⁺ T cells to induce an optimal antiviral immune response should be addressed more specifically in future studies.

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