The results in this study suggested that molecular mechanisms of FIV-associated lymphocyte apoptosis and antiapoptosis might be similar as in the HIV infections.

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NOTE *Virology*

Prevalence of Canine Distemper Virus, Feline Immunodeficiency Virus and Feline Leukemia Virus in Captive African Lions (*Panthera leo*) in Japan

Yasuyuki ENDO[1,2], Masashi UEMA[1], Ryuichi MIURA[1], Kyoko TSUKIYAMA-KOHARA[1], Hajime TSUJIMOTO[3], Kumiko YONEDA[4] and Chieko KAI[1]*

[1]Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, 4–6–1 Shirokanedai, Minato-ku, Tokyo 108–8639, [2]Department of Veterinary Internal Medicine, Faculty of Agriculture, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0056, [3]Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, and [4]Japan Wildlife Research Center, 2–18–18 Sakuragaoka, Setagaya-ku, Tokyo156–0054, Japan

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ABSTRACT. Sero-prevalences of canine distemper virus (CDV), feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) were evaluated in 20 captive lions in two Japanese zoos. Anti-CDV antibody was detected in 13 of 20 lions. We could pursue antibody responses against CDV in three lions back to 1996. Sera collected in 1996 were negative for anti-CDV antibody, therefore, all of them showed sero-conversion in 2000. This result suggested that the epidemic of CDV infection in this zoo might have happened between 1996 and 2000. The lions were also examined for FIV and FeLV infections. We had no evidence for FeLV infection but eight lions were sero-positive for anti-FIV antibody.

KEY WORDS: CDV, Lion, Retroviruses.

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Recently, many cases of interspecies transmission of viral diseases between humans and animals between animal species have been reported [8, 13, 25–27] and wildlife is no exception. An incident that threatened the lives of African lions (*Panthera leo*) occurred at Serengeti National Park of Tanzania in 1994. Roelke-Parker *et al.* and others finally clarified that canine distemper virus (CDV) had infected the lions by breaking through the species barrier, and induced the death of infected lions in this area [14, 23]. In addition, it was also reported that the captive large felids, including leopards (*Panthera pardus*), tigers (*Panthera tigris*), lions and jaguars (*Panthera onca*), were affected with CDV and died [1, 3, 11]. On the other hand, some feline retrovirus infections were also detected in large felids. The cross-reactive antibodies to feline immunodeficiency virus (FIV) have been found in wild felids including lion, cheetah (*Acinonyx jubatus*), puma (*Felis concolor*), bobcat (*Felis rufus*) and Florida panther (*Panthera concolor coryi*) [2, 7, 17, 21]. However, the lentiviruses isolated from these felids were highly adapted in each species and genetically distant from FIV in domestic cats. These viruses were designated as lion lentivirus (LLV) or puma lentivirus (PLV) [16, 17, 21]. The infection of feline leukemia virus (FeLV) was found in puma, clouded leopard (*Neofelis nebulosa*), bobcat, European wildcat (*Felis silvestris*) and cheetah [4, 9, 18, 19, 24]. The fundamental features of the retroviruses described above has poorly understood in wild felids and it will be required the further analyses considering the potential of retroviruses for mutation and difficulties of virus elimination. Research on viral infections of wild animals has advanced in the last few decades. Continuous efforts in this field are indispensable for prevention of the spread of lethal infectious diseases and the preservation of wild animal species. Most of the large felids in Japan are housed in zoos. It is important to understand the status of virus infections in them from the epidemiological, virological and economical points of views. In the present study, we evaluated the status of CDV, FIV (or LLV) and FeLV infections of 20 lions in two Japanese zoos.

Details of the 20 lions examined in this study are shown in Table 1. These lions ranged from 3-months to 11-years-old in age, except an unknown case (lion B1). Eleven were male and the others were female. All lions housed in zoo A and six of these in zoo B were born at the respective zoo, but three (lions B1, B2 and B3) were transferred from other zoos (zoo C or D) to B zoo at 1 or 3 years before the sample collection. All twenty lions were healthy and showed no obvious clinical signs at the time of sampling. In addition, they have no history of vaccinations for CDV, FIV and FeLV infections. Blood samples were collected from these 20 lions of two zoos under anesthesia with xylazine and ketamine in a squeeze cage. Serum and peripheral blood mononuclear cells (PBMC) were prepared and subjected to the following serological and molecular analyses, respectively.

Anti-CDV humoral immune responses were examined by enzyme-linked immunosorbent assay (ELISA) as described previously [12]. This assay showed that 12 of the 20 lions possessed the anti-CDV antibodies (Table 1). Nine out of eleven (81.8%) and three out of nine (33.3%) lions were positive for CDV antibody in zoo A and zoo B, respectively. We also examined the existence of neutralizing antibody against CDV in the same samples as described previously [15]. Neutralizing antibody titers were well correlated with the results of ELISA (Table 1). One exceptional case (B5)

* CORRESPONDENCE TO: KAI, C., Laboratory Animal Research Center, Institute of Medical Science, The University of Tokyo, 4–6–1 Shirokanedai, Minato-ku, Tokyo 108–8639, Japan.

Table 1. Clinical profile and Serological status for CDV, FIV and FeLV infections of lions examined in this study

Animal ID	Zoo	Sex[1)]	Age	Origin	Transfer date	CDV Ab (ELISA)		CDV neutralization Ab titer (2000)	FIV Ab	FeLV Ag
						2000	1996			
A1	A	M	9Y	A	–	+	ND[2)]	905	–	–
A2	A	F	7Y	A	–	+	ND	320	–	–
A3	A	N	3M	A	–	–	ND	<10	–	–
A4	A	F	5Y	A	–	+	ND	1522	–	–
A5	A	F	3Y	A	–	+	ND	381	–	–
A6	A	M	6Y	A	–	+	ND	95	–	–
A7	A	F	6Y	A	–	+	–	1280	–	–
A8	A	F	4M	A	–	–	ND	<10	–	–
A9	A	M	5Y	A	–	+	ND	320	–	–
A10	A	F	6Y	A	–	+	–	113	–	–
A11	A	M	6Y	A	–	+	–	113	–	–
B1	B	F	Unknown	C	11/21/97	–	ND	<10	+	–
B2	B	M	2Y	D	3/15/99	–	ND	<10	+	–
B3	B	M	3Y	D	3/15/99	–	ND	<10	–	–
B4	B	M	11Y	B	–	+	ND	190	+	–
B5	B	F	8Y	B	–	–	ND	95	+	–
B6	B	F	8Y	B	–	+	ND	113	+	–
B7	B	M	8Y	B	–	+	ND	320	+	–
B8	B	M	5Y	B	–	–	ND	<10	+	–
B9	B	M	2Y	B	–	–	ND	<10	+	–

M, male; F, female; ND, not done.

was judged as negative for anti-CDV antibody in ELISA but possessed neutralizing antibody. Neutralizing antibody responses are generally stricter with each infectious agent than ELISA. Thus, thirteen lions in zoo A and B were diagnosed as having been infected with CDV from the findings in ELISA and the virus neutralizing assay. Fortunately, we could review the status of CDV infection in the sera from three lions (lions A7, A10 and A11) housed in zoo A back to 1996. In 1996, sera from all three lions were negative for anti-CDV antibody. However, all of them showed sero-conversion against CDV in 2000 (Table 1). These results suggested that an epidemic of CDV infection in zoo A might have occurred between 1996 and 2000. Additionally, the lack of infection in cubs indicates that the prevalence occurred before birth. The lions in zoo A were shown to be highly affected with CDV. This may be due to keeping style of lions. In zoo A, the lions are able to contact each other in the display field. Although how the first infection occurred is still unclear, the explosive expansion of the infection might have happened from the first affected lion through the direct contact. In zoo B, transferred lions (lions B1, B2 and B3) and young lions (B8 and B9 were less than 6 years old) were sero-negative for CDV. This finding also suggests that active infection occurred more than 5 years ago in B zoo.

Although exact infectious route cannot be determined, two possible routes are considered for CDV infection. One is that the domestic dogs or free ranging dogs around the zoo might have been a source of the virus as described previously [6, 10, 23]. In this case, however, it is difficult to suppose that there could be a direct contact of lions with dogs because of complete separation of lions from outside. Therefore, it is conceivable that some agents (including human and foods) have played a role as a vector or carrier for CDV infection in lions. It is also conceivable that exchanges of lions between zoos to avoid inbreeding might have spread the virus from one zoo to other zoos. On the pathogenicity of the CDV in lions, there is a major difference between the previous epidemics in Africa and the United States, and current ones in Japanese zoos. It has been reported no cases of large felids which showed typical symptoms of CDV infection in Japan. Although we could not molecularly characterize these viruses, this inconsistency may be due to the differences in the CDV strains. Further molecular analyses of the viruses are required to clarify this point.

We next analyzed the status of FIV and FeLV infections by detecting the anti-FIV antibody against p24 gag protein or FeLV p27 antigen in serum using a commercial kit (Idex Laboratories, Portland, ME). All lions housed in zoo A were negative for anti-FIV antibody, whereas eight of nine lions in zoo B were positive for it. FeLV p27 antigen was not detectable in all lions examined in the present study. Since FIV or LLV infection was strongly suspected from the findings of serological survey, PCR was performed to detect viral RNA genome in sera or provirus in PBMC from two lions (B2 and B3). In the PCR analyses, three primer sets were used for FIV *env* or LLV *pol* region. Nested primers 5'-GAG TAG ATA C(A/T)T GGT T(G/A)C AAG-3' (VE-1S), 5'-CAT CCT AAT TCT TGC ATA GC-3' (VE-1R), 5'-CAA AAT GTG GAT GGT GGA A(T/C)-3' (VE-2S) and 5'-ACC ATT CC(A/T) ATA GCA GT(G/A) GC-3' (VE-2R) were designed based on the sequences conserved among a number of FIV isolates [20, 22]. For amplification of LLV genome, two primer sets, 5'-CCT ATA TTT TGC

ATT AAA AAG-3' (6635F) and 5'-ACC CCA TAT GAT ATC ATC C-3' (6637R), and 5'-AAA GAA TCA GGA AAA TAT A-3' (6636F) and 5'-GAT ATC ATC CAT ATA TTG ATA T-3' (6638R), were used [5]. However, neither viral RNA nor proviral DNA was detected in any case (data not shown). The negative results in the detection of viral RNA or proviral DNA might be due to the elimination of the virus from the serum by host humoral immune responses or the low viral replication efficacy. In general, it is recognized that FIV is transmitted among hosts in a blood borne manner via injury, through fighting or bites. Therefore, it is conceivable that direct contact of lions with FIV-infected cats or transference from infected lions is a major possible route. However, such events do not commonly happened in Japanese zoos. Although the virus detection was unsuccessful in this study, the virus isolation from captive lions in Japanese zoos followed by genetic analyses of the viruses should clarify the infection route. The lions showing typical symptoms of FIV infection, including stomatitis, lymphadenopathy, opportunistic infections and immunodeficiency, were not observed in the present surveillance. However, FIV and other lentiviruses frequently have mutations, especially in the *env* region. Therefore, the appearance of lion-adapted and highly pathogenic strains of FIV or LLV for Lions can not be excluded. Careful monitoring of the lions with respect to FIV or LLV infection as well as CDV infection will be also required.

In the present study, we showed that captive lions in Japanese zoos possessed antibodies against CDV, FIV (LLV) or both, which are originally lethal pathogens for domestic dogs and cats. Continuous surveillance of them and studies to clarify their pathogenicity in lions and to determine the sources of infections are required.

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Identification of HIV-1 epitopes that induce the synthesis of a R5 HIV-1 suppression factor by human CD4$^+$ T cells isolated from HIV-1 immunized hu-PBL SCID mice

ATSUSHI YOSHIDA[1], REIKO TANAKA[1], AKIRA KODAMA[1], NAOKI YAMAMOTO[2], AFTAB A. ANSARI[3], & YUETSU TANAKA[1]

[1]*Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara, Okinawa 903-0215, Japan,* [2]*National AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan, and* [3]*Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA*

Abstract

We have previously reported that immunization of the severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood mononuclear cells (PBMC) (hu-PBL-SCID mice) with inactivated human immunodeficiency virus type-1 (HIV-1)-pulsed-autologous dendritic cells (HIV-DC) elicits HIV-1-reactive CD4$^+$ T cells that produce an as yet to be defined novel soluble factor *in vitro* with anti-viral properties against CCR5 tropic (R5) HIV-1 infection. These findings led us to perform studies designed to identify the lineage of the cell that synthesizes such a factor *in vivo* and define the epitopes of HIV-1 protein that have specificity for the induction of such anti-viral factor. Results of our studies show that this property is a function of CD4$^+$ but not CD8$^+$ T cells. Human CD4$^+$ T cells were thus recovered from the HIV-DC-immunized hu-PBL-SCID mice and were re-stimulated *in vitro* by co-culture for 2 days with autologous adherent PBMC as antigen presenting cells, APC previously pulsed with inactivated HIV in IL-2-containing medium to expand HIV-1-reactive CD4$^+$ T cells. Aliquots of these re-stimulated CD4$^+$ T cells were then co-cultured with similar APC's that were previously pulsed with 10 μg/ml of a panel of HIV peptides for an additional 2 days, and their culture supernatants were examined for the production of both the R5 HIV-1 suppression factor and IFN-γ. The data presented herein show that the HIV-1 primed CD4$^+$ T cells produced the R5 suppression factor in response to a wide variety of HIV-1 gag, env, pol, nef or vif peptides, depending on the donor of the CD4$^+$ T cells. Simultaneous production of human interferon (IFN)-γ was observed in some cases. These results indicate that human CD4$^+$ T cells in PBMC of HIV-1 naive donors have a wide variety of HIV-1 epitope-specific CD4$^+$ T cell precursors that are capable of producing the R5 HIV-1 suppression factor upon DC-based vaccination with whole inactivated HIV-1.

Keywords: *HIV-1, vaccination, dendritic cells, HIV-1 suppression*

Abbreviations: *HIV-1, human immunodeficiency virus type I; DC, dendric cells; HIV-DC, inactivated HIV-1-pulsed DC; Th, helper T; hu-PBL-SCID mouse, severe combined immunodeficiency mouse engrafted with human PBMC; AT-2, aldrithiol-2; 50% $TCID_{50}$, tissue culture infectious dose*

Introduction

Virus specific CD4$^+$ helper T (Th) cell responses have been shown to play an essential role in the maintenance of effective immune responses in a variety of animal models [3,12,20,32,34]. Human immunodeficiency virus type 1 (HIV-1) infection is associated with a progressive loss of total CD4$^+$ Th cells by both direct and indirect mechanisms [21,25,27,28,30]. In particular, memory CD4$^+$ Th cells become more susceptible to the cytopathic effects of HIV-1 than naive CD4$^+$ Th cells after activation [8]. Several lines of evidence strongly suggest that HIV-1-specific CD4$^+$ Th cells are critical for control

Correspondance: Dr Y. Tanaka, Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan. Tel: 81 98 895 1202. Fax: 81 98 895 1437. E-mail: yuetsu@ma.kcom.ne.jp

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of HIV-1 in part by maintaining HIV-1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses [13,17,22,23,26,28]. Highlighting the complexity of HIV-1 infection was the finding that HIV-1-specific $CD4^+$ Th cells were in fact preferentially infected by HIV-1 *in vivo* [9]. It was reasoned that such mechanisms may indeed contribute to an impairment in the control of not only HIV-1 but also opportunistic infections. While such $CD4^+$T cell depletion continues to occur with variable kinetics in HIV-1 infected patients, a significant frequency of HIV-1-specific $CD4^+$ Th cells are detectable in most of these individuals with a higher frequency in long-term non-progressors (LTNP) than in subjects during progressive disease [4,28,37,38]. In addition to the traditional function of $CD4^+$ T cells in facilitating the generation of HIV-1 specific $CD8^+$ T cells and the synthesis of antibodies, $CD4^+$ Th cells from HIV-1 infected patients have also been shown to exert anti-viral effects not only by direct lysis of HIV-1 infected target cells by HIV-1 gag specific $CD4^+$ T cells [18] but also by the secretion of a variety of HIV-1 suppression factors [1,15,31,41]. These findings together suggest that $CD4^+$ T cells from HIV-1 infected and/or immunized individuals have acquired a series of unique anti-viral activity which may contribute to the control of viremia and the detailed studies of the mechanisms by which such activity is acquired and induced thus appears warranted. We have previously reported that SCID mice engrafted with HIV-1-naive human PBMC together with autologous DC pulsed with inactivated whole HIV-1 virion became subsequently completely resistant to R5 HIV-1 challenge *in vivo*. The resistance to infection was specific since it was only seen in the hu-PBL-SCID mice immunized with inactivated R5 or X4 HIV-1 virions, but not OVA or KLH. Studies of the mechanisms by which these mice were protected led to the discovery of a novel soluble anti-viral factor produced in the serum of these HIV-DC immune mice. While this factor synthesized by human $CD4^+$ T cells from the hu-PBL-SCID HIV-1 immunized mice was unable to neutralize HIV-1 *in vitro*, it was capable of inhibiting R5, but not X4 HIV-1 infection of primary macrophages and activated PBMC which was not secondary to the down regulation of either CCR5 or CD4 and appeared to act prior to viral integration. Of interest was also the finding that the factor was not effective in controlling R5 HIV-1 infection of CCR5-expressing $CD4^+$T cell lines.

Since the generation of the suppressor factor appeared to be specifically induced by HIV-1 immunization of the hu-PBL-SCID mice, it was reasoned that the delineation of the epitopes of the HIV-1 encoded proteins that induce the generation of such factors would be appropriate and informative. Results of such studies are the basis of this report.

Materials and methods

Mice

SCID mice lacking functional T, B and natural killer cells (NK), BALB/c-rag2 ′ γc ′ [24] were used in the present study. The mice were kept in a SPF and BSL-3 animal facilities of the Laboratory Animal Center, University of the Ryukyus. The protocols for the care and use of hu-PBL-SCID mice have been approved by the committee on animal research of the University of the Ryukyus.

Reagents

RPMI 1640 medium (SIGMA, St Louis, MO) supplemented with 5% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (hereinafter called RPMI medium), serum free medium (AIM-V) and Iscove's medium (Lifetechnology, NY) supplemented with 10% FCS with the antibiotics (hereinafter called AIM-V medium and Iscove's medium) were utilized as sources of media. Soluble recombinant human IL-4 and GM-CSF were produced in 293 T cells transfected with pCMhIL4 and pCMhGM (RIKEN Gene Bank, Ibaraki, Japan), respectively, by the calcium phosphate method [33] and purified. Concentrations of human IL-4 and GM-CSF were determined using commercial ELISA kits (BioSource, Camarillo, CA). Human recombinant IL-2 was kindly supplied by NIH AIDS Research and Reference Program.

Blocking monoclonal antibodies (mAb) against human MIP-1α, human MIP-1β and human RANTES were purchased from R&D systems (Rockville, MD). To maintain their blocking activity, these antibodies in a lyophilized form were reconstituted in accordance with the manufacturer's instructions, and aliquots were kept at −80°C until use. Heparin–Sepharose (Pharmacia, Sweden) was used to absorb β-chemokines as described previously in Ref. [39].

HIV-1 peptides were obtained from the NIH AIDS Research and Reference Program, including the complete sets of the HIV-1 HXB2 gag peptides (Cat#5107 and Cat#3992), HIV-1 MN env peptides (Cat#6451), HIV-1 HXB2R pol peptides (Cat#4358), HIV-1 clade B nef peptides (Cat#5189), HIV-1 consensus B vif peptides (Cat#6445), HIV-1 consensus B rev peptides (Cat#6445), HIV-1 BRV nef peptides (Cat#6441) and HIV-1 consensus vpu peptides (Cat#6444). Each peptide was dissolved in DMSO at a concentration of 10 mg/ml, and then diluted in RPMI medium at 10 μg/ml prior to use.

Viruses

HIV-$1_{JR\text{-}CSF}$ [16] and HIV-$1_{NL4\text{-}3}$ [2] viral stocks were each produced in the 293T cells by transfection with

the appropriate HIV-1 infectious plasmid DNA utilizing the calcium phosphate method [33]. HIV-1_{IIIB} was harvested from Molt-4/IIIB cell cultures. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by an endpoint infectious assay using PBMC stimulated with anti-CD3/anti-CD28-mAb conjugated DynaBeads (Dynal, Oslo, Norway). HIV-1 preparations were inactivated with Aldrithiol-2 (AT-2), as originally described by Rossio et al. [29].

Generation of HIV-1 pulsed mature DC's from monocytes

Fresh PBMC at 5×10^6 cells/ml in RPMI medium were dispensed into individual wells of 12-well plates (1 ml/well), which had been precoated with autologous plasma for 30 min at 37°C. The PBMC cultures were allowed to incubate at 37°C for 1 h. The non-adherent cells were removed by gentle washing with serum-free RPMI 1640 medium and the remaining adherent cells were cultured in Iscove's medium (2 ml/well) containing human GM-CSF (500 ng/ml) and IL-4 (200 ng/ml) for 5 days. The resulting immature DC cultures were depleted of contaminating lymphocytes by using the monocyte-negative isolation kit (Dynal, Oslo, Norway), and the enriched population of DC's were further cultured at 2×10^6/ml in human IFN-β (1000 U/ml; Toray, Tokyo, Japan) in the presence of AT-2 inactivated whole HIV-1 (containing 50 ng/ml p24) for 2 days to obtain mature DC pulsed with HIV-1, as described previously in ref. [39].

Transplantation and immunization

HIV-1-pulsed mature DC (5×10^5 cells) mixed with autologous fresh PBMC (3×10^6 cells), or those depletes of $CD8^+$ T cells, or $CD4^+$ T cells by a magnetic isolation method (Dynal, Oslo, Norway) in a final volume of 100 μl in RPMI medium were injected into the spleen of SCID mice. Five days later, the same number of DC pulsed with the same dose of HIV-1 antigen were inoculated into the peritoneal cavity. Five days later, the mice were sacrificed and human lymphocytes were recovered from the spleen and the peritoneal cavity by lavage.

In vitro *re-stimulation of $CD4^+$ T cells*

The recovered human lymphocytes from the HIV-DC immune mice were depleted of human $CD8^+$ cells by the magnetic beads-negative selection method (Dynal). These enriched population of human $CD4^+$ T cells (2×10^6 cells) were co-cultured with freshly obtained 2×10^5 autologous mitomycin-C treated adherent PBMC as antigen presenting cells (APC), in the presence of AT-2 inactivated HIV-1 containing 50 ng p24 in a volume of 1 ml RPMI 1640 medium supplemented with 20 U/ml human IL-2 in individual wells of a 24-well plate (BD Pharmingen, San Diego, CA) at 37°C. Two days later, these $CD4^+$ T cells were assayed for HIV-1 peptide specific responses as follows. An initial screening was performed utilizing pools of 10 sequential overlapping peptides and once a pool of peptides was shown to lead to the synthesis of the HIV-1 suppression factor, a second screening was performed to identify the individual peptide. Thus, enriched population of 2×10^4 autologous APC were first dispensed in a volume of 50 μl into individual wells of a 96-well microtiter plate and triplicate wells incubated with 50 μl of media containing the various individual pools of 10 HIV-1 peptides for the primary screen (each at 10 μg/ml) or an individual HIV-1 peptide for the secondary screen (10 μg/m) for 1 h at 37°C. A total of 2×10^5 HIV-1 primed and *in vitro* re-stimulated $CD4^+$ T cells were then added to these cultures in a volume of 100 μl of media and the co-cultures incubated at 37°C in a 5% CO_2 humidified atmosphere. Two days later, the culture supernatants were harvested and aliquots assayed for HIV-1 inhibition activity and levels of IFN-γ.

HIV-1 inhibition and IFN-γ assays

As previously reported [39], PBMC were activated with anti-CD3/28 antibody-coated magnetic beads (Dynal) at cell to bead ratio of 1:1 in 20 U/ml IL-2 containing RPMI medium *in vitro* for 3 days. These activated PBMC's (5×10^5 cells/well) were pre-incubated at 37°C for 1 h with either various dilution of serum from the HIV-DC immune mice or culture supernatants obtained from the $CD4^+$ T cells that were stimulated with various HIV-1 peptides as described above. Five hundred $TCID_{50}$ of HIV-$1_{JR\text{-}CSF}$ or HIV-1 $_{NL4\text{-}3}$ was then added to these cultures for an additional 4 h. The microtiter plate was centrifuged and the supernatant fluid removed. The procedure repeated three times and the cultures then were incubated in 0.2 ml of 20 U/ml IL-2-containing RPMI medium for 5 days. The levels of HIV-1 p24 produced in the culture supernatants were determined by a commercial ELISA kit (Zepto Metrix, Buffalo, NY). Levels of human IFN-γ in the stimulated culture supernatants were assayed utilizing a commercial ELISA kit (R&D Systems Inc., Minneapolis, MN).

HLA typing

Donor PBMC were HLA typed by a DNA typing method.

Results

In order to identify the lineage of cells that was the major producer of the R5 HIV-1 suppression factor *in vivo*, SCID mice were engrafted with unfractionated

PBMC or PBMC depleted of either $CD4^+$ or $CD8^+$ cells, together with HIV-DC. Various dilutions of the immune sera from these mice were then examined for the R5 HIV-1 suppression activity *in vitro*. As seen in Figure 1, as little as 5% of the sera from the mice engrafted with either unfractionated PBMC or CD8-depleted PBMC significantly ($p < 0.05$) inhibited R5 HIV-1 infection. In contrast, up to 20% of the sera from the CD4-depleted PBMC-engrafted mice did not show any detectable R5 HIV-1 suppressive activity. Similar assays conducted using X4 HIV-1 failed to show any inhibition denoting that the inhibition was selective for R5 HIV-1 (data not shown). In these engrafted mice, the levels of human $CD4^+$ and $CD8^+$ T cells existed at similar levels as in the mice engrafted with unfractionated PBMC (data not shown). These data indicate that $CD8^+$ T cells are not required for the production of the R5 HIV-1 suppression factor *in vivo*, and that the HIV-immune human $CD4^+$T cell population is the major source for the R5 HIV-1 suppression factor *in vivo*.

In efforts to map the epitopes of HIV-1 antigens that lead to the activation of $CD4^+$ T cells and the subsequent release of the HIV-1 suppression factor, a large panel of 15–20 mer peptides spanning a wide range of HIV-1 proteins were screened using a re-stimulation assay as described in the methods section. In order to enrich for HIV-1 antigen-responding $CD4^+$ T cells *in vitro*, $CD8^+$ T cell-depleted lymphocytes obtained from three groups of HIV-DC immune hu-PBL-SCID mice, which had been engrafted with PBMC's from different individuals, were first stimulated with inactivated whole HIV-1 in the presence of autologous APC *in vitro*. These HIV-1-primed enriched $CD4^+$ T cells were then re-stimulated by co-culture in the presence of a pool of 10 HIV-1 peptides with fresh APC for primary screening. After 2 days, culture supernatants were harvested and examined for the R5 HIV-1 suppression factor at a final dilution of 50%. Supernatant fluids giving values of >50% reduction compared to controls (incubated with media alone) in p24 antigen synthesized in the R5 HIV-1 infected cells were considered positive and a secondary screen performed to identify the individual HIV peptide inducing such HIV-1 suppression factor activity. In order to make sure that the R5 HIV-1 suppression was not mediated by CCR5-binding β-chemokines, the culture supernatants to be tested were individually pre-absorbed by incubation with Heparin–Sepharose followed by incubation with a pool of blocking mAbs against human RANTES, MIP-1α and MIP-1β each a concentration of 10 μg/ml. Figure 2a–c shows representative data from a number of independent experiments conducted on cells from the three donors, respectively. These data suggest that the ability of HIV-1 peptides to induce the HIV-1 suppressor factor resides in multiple HIV-1 peptides from a number of HIV-1 proteins. As expected, there were variations noted in the HIV-1 proteins and peptides that induce the HIV-1 suppression factor in the three individual donors. The peptides recognized by donor 1 $CD4^+$ T cells consisted of peptides from HIV-1 pol, gag and env antigens, while those recognized by donor 2 were of gag, env, vif and rev antigens, and those recognized by donor 3 were of gag, env, vif and rev antigens. None of the supernatant fluids examined showed detectable X4 HIV-1 suppression activity in the assay employed (data not shown). There was specificity in the assay since the R5 HIV-1 suppression factor was not detected either in the culture supernatants from APC cultured with or without HIV-1 peptides alone or supernatant fluids from immune $CD4^+$ T cells cultured in the absence of APC or peptides (data not shown). The individual variations in the response to peptides may be due to HLA restriction of responding antigenic peptides, as the three donors were of different HLA class II type (Table I). It is interesting to note that the production of the R5 HIV-1 suppression factor by the bulk $CD4^+$ T cells was

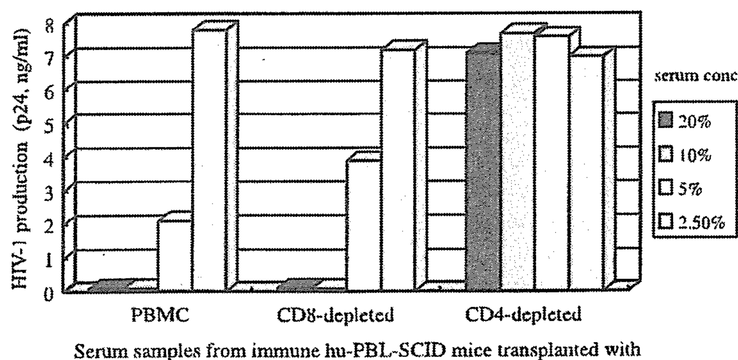

Figure 1. Requirement of human $CD4^+$ T cells, but not $CD8^+$ T cells, to generate the R5 HIV-1 suppression factor in the DC-HIV-immune hu-PBL-SCID mice. Together with HIV-DC, PBMC or those depleted of $CD8^+$ or $CD4^+$ cells were engrafted into the spleen of four SCID mice per group. After 5 days, mice were boosted with HIV-DC. After 5 days, serum samples from each group were pooled and tested for the R5 HIV-1 suppression activity on activated PBMC. Bars show the means of HIV-1 p24 values in the PBMC culture supernatants on day 5 post infection. HIV-1 proliferation in medium control was 7.7 ng/ml

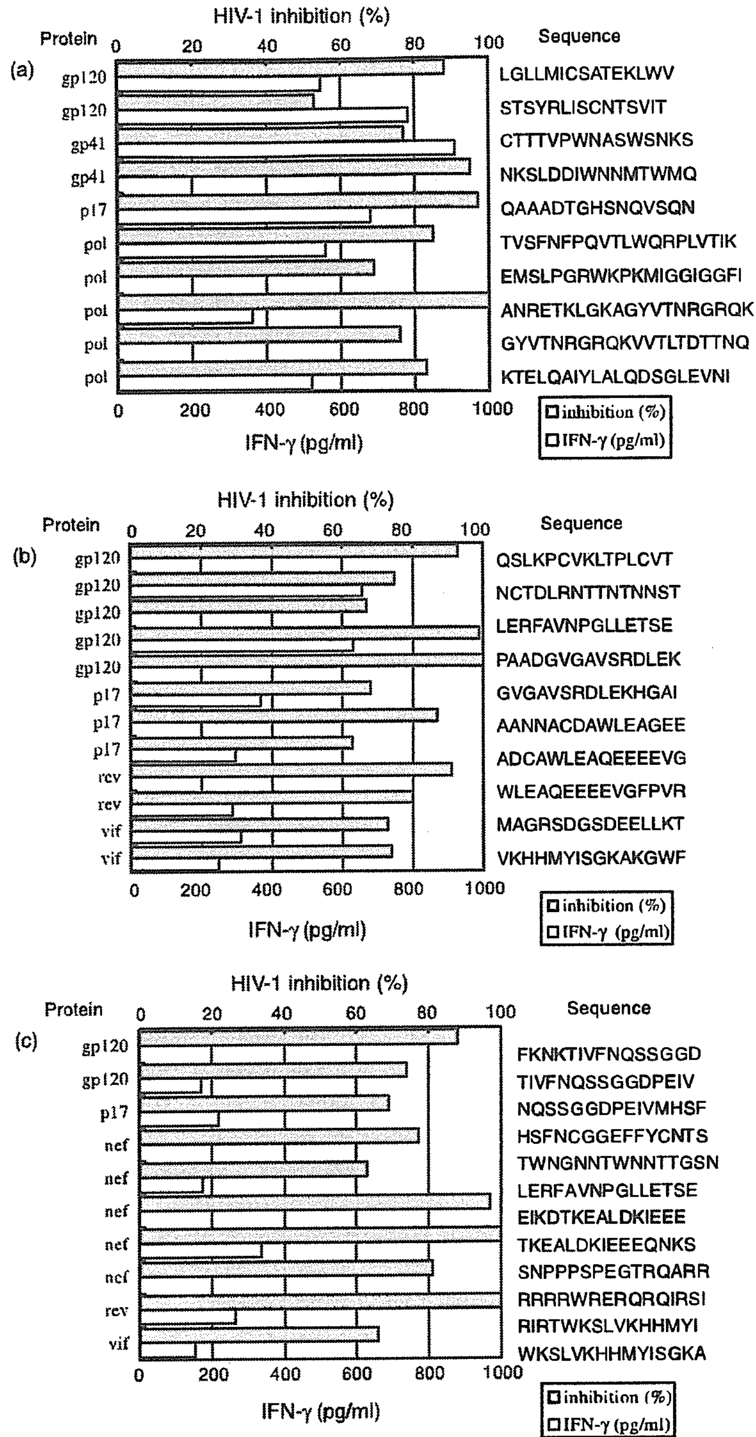

Figure 2. Recognition of various HIV-1 peptides by HIV-1-immune human CD4$^+$ T cells from three different donors generated in the HIV-DC-immune hu-PBL-SCID mice. HIV-DC immune CD4$^+$ T cells (2×10^5 cells) were stimulated with various peptides at a concentration of 10 μg/ml in the presence of 2×10^4 APC in a volume of 200 μl in individual wells of a 96-well plate. After 2 days, the culture supernatants were harvested and their activity of R5 HIV-1 inhibition and the levels of human IFN-γ were determined as described in the materials and methods. Percent inhibition of HIV-1 production was calculated using values obtained from HIV-1 infected PBMC that were pre-treated with the medium alone. Representative data from three independent experiments for donor 1 (a), donor 2 (b), and donor 3 (c) were shown.

Table I. HLA class II typing.

	DR	DQ
Donar 1	15/–	6/–
Donar 2	4/14	4/7
Donar 3	4/13	6/8

accompanied by the production of human IFN-γ but not in all the cases.

Discussion

Our laboratory has previously documented the synthesis of a soluble factor by $CD4^+$ T cells isolated from SCID mice previously engrafted with a mixture of human PBMC's and HIV-1-pulsed autologous DC's which suppressed infection of R5 HIV-1 *in vitro* [39]. In the present study we have identified the $CD4^+$T cell lineage as a requirement for the synthesis of this factor *in vivo*. In addition, we have attempted to identify the HIV-1 epitopes that are recognized by $CD4^+$ T cells that lead to the synthesis of this factor. There are a number of issues that need to be addressed in light of these findings. Thus, first of all, we submit that the anti-viral factor is distinct from the other anti-viral factors that have been previously identified. The fact that the suppressor activity was not neutralized by the addition of antibodies against the chemokines RANTES, MIP-1α and MIP-1β suggest that such suppression is not likely due to the synthesis of these chemokines which are known inhibitors of viral infection [7,36]. The factor described herein is also distinct from the cellular anti-viral factor (CAF) identified by the laboratory of Dr Jay Levy [19] since it is synthesized by $CD4^+$ T cells but not $CD8^+$ T cells and whereas Dr Levy's CAF is effective against all HIV-1, HIV-2 and select retroviruses. The factor reported herein appears to be specific for R5 but not X4 tropic HIV-1. In addition, it is not likely to be either lymphotactin [35], α-defensins [40], the heparin binding protein termed anti-thrombin III [10], the natural killer enhancing factors A and B [11], and some additional factors (reviewed by Levy et al. [5]) since all of these have been shown to be synthesized primarily by $CD8^+$ but not $CD4^+$ T cells. There has been an anti-viral factor that is synthesized following the interaction of $CD4^+$ T cells with APC's [6]. However, this factor is functionally distinct from our factor because the factor inhibits HIV-1 production at post-integration stage, and our factor inhibits R5 HIV-1 infection prior to integration [39]. It is clear though that there are a plethora of such anti-viral factors that have been and continue to be described. The biologic reasons for such a multitude of anti-viral factors is difficult to imagine except to state that these are likely due to redundancies that nature has bestowed on the vertebrate species to protest itself from such viral infections.

The second issue concerns the multiple epitopes of HIV-1 that induce the generation of this factor by $CD4^+$ T cells. These epitopes interestingly were not restricted to a single HIV-1 protein and included both viral structural and accessory proteins. The obvious individual variations in responding antigenic peptides suggests that this maybe due to HLA-restriction of peptide recognition by the immune $CD4^+$ T cells, although additional studies are necessary to confirm this issue. Furthermore, the present study suggests that the CD4 factor-producing HIV-1-immune $CD4^+$ T cells are heterogeneous Th clones. Since the peptides tested in this study were overlapping 15–20 mers, an additional study is required to more precisely define the specific HIV-1 epitope that has specificity for the $CD4^+$ T cells. It is also unclear whether the suppressor factor synthesized by $CD4^+$ T cells in response to distinct HIV peptides is identical or distinct. Biochemical identification of the factor(s) is needed to address this issue and such studies are in progress.

The next issue concerns the synthesis of IFN-γ by some but not all the $CD4^+$T cell in the assays reported herein. Since the cultures performed consisted of a mixture of $CD4^+$ T cells, it is not clear whether the synthesis of the anti-viral factor and IFN-γ was the result of a single clonal population of multiple clones of $CD4^+$ T cells. Clonal analysis needs to be performed to address this issue.

It is important to note that the HIV-1 gag p17 antigen peptide (*LERFAVNPGLLETSE*) recognized by the HIV-DC immune $CD4^+$ T cells from two out of the three donors shares amino acid sequence with another gag p17 peptide (*ERFAVNPGLLETSEGCR*) that is widely recognized by IFN-γ-producible $CD4^+$ T cells from at least 25% HIV-1-infected individuals [14]. These results strongly support the use of DC-based vaccination of hu-PBL-SCID mice with whole inactivated HIV-1 virion to stimulate and expand HIV-1-specific $CD4^+$ T cells in efforts to study the effectiveness of these cells for anti-viral control. More importantly, it should be noted that the CD4 factor producing $CD4^+$ T cells generated in the hu-PBL-SCID mice recognize multiple HIV-1 proteins similar the studies that have reported for HIV-1-specific IFN-γ producing $CD4^+$ T cells in HIV-1 infected individuals [39]. Although, the existence and clinical role of the CD4 factor in HIV-1-infected individuals remains unknown, the data reported herein suggest that HIV-1 vaccines containing multiple HIV-1 epitopes or proteins, rather than those with single HIV-1 protein or epitope, will be likely to be more effective in expanding a large number of multiple HIV-1-reactive $CD4^+$ T cells that are capable of producing the CD4 factor and other helper and HIV-1-suppresing cytokines.

As far as we know up to present, there have been no reports on cytokines that are identical to the CD4 anti-viral factor described herein. Attempts to identify the biochemical nature of this novel anti-viral factor have been hampered by both our inability to prepare sufficiently large quantities that are required for both biochemical identification and detailed characterization of this molecule coupled with the labile nature of the molecule. Fortunately, in our recent attempts, we have succeeded in immortalizing CD4 factor-producing $CD4^+$ T cells by HTLV-I-mediated transformation (Yoshida et al. unpublished). These cells will be helpful for the identification of not only the factor itself, but also its putative receptor.

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Engagement of specific T-cell surface molecules regulates cytoskeletal polarization in HTLV-1–infected lymphocytes

Amanda L. Barnard, Tadahiko Igakura, Yuetsu Tanaka, Graham P. Taylor, and Charles R.M. Bangham

Cell-cell contact is required for efficient transmission of human T-lymphotropic virus type 1 (HTLV-1). An HTLV-1–infected cell polarizes its microtubule-organizing center (MTOC) toward the cell-cell junction; HTLV-1 core (Gag) complexes and the HTLV-1 genome accumulate at the point of contact and are then transferred to the uninfected cell. However, the mechanisms involved in this cytoskeletal polarization and transport of HTLV-1 complexes are unknown. Here, we tested the hypothesis that engagement of a specific T-cell surface ligand is synergistic with HTLV-1 infection in causing polarization of the MTOC to the cell contact region. We show that antibodies to intercellular adhesion molecule-1 (ICAM-1; CD54) caused MTOC polarization at a higher frequency in HTLV-1–infected cells. ICAM-1 is up-regulated on HTLV-1–infected cells, and, in turn, ICAM-1 on the cell surface up-regulates HTLV-1 gene expression. We propose that a positive feedback loop involving ICAM-1 and HTLV-1 Tax protein facilitates the formation of the virologic synapse and contributes to the T-cell tropism of HTLV-1. In contrast, MTOC polarization induced in T cells by antibodies to CD3 or CD28 was significantly inhibited by HTLV-1 infection. (Blood. 2005;106:988-995)

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Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that infects 10 to 20 million people worldwide. The majority (95%) of HTLV-1–infected individuals remain asymptomatic, but about 3% develop adult T-cell leukemia or lymphoma and another 3% are affected by inflammatory disorders, of which HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the most commonly recognized.[1]

HTLV-1 transmission between individuals depends on cell-cell contact,[1] because virtually no cell-free infectious virions are produced during natural infection. Igakura et al[2] showed that cell contact rapidly induces an HTLV-1–infected T cell to orient its microtubule organizing center (MTOC) toward the cell-cell junction. HTLV-1 core (Gag) complexes and the HTLV-1 genome accumulate at the cell-cell junction. The structure of the resulting cell-cell contact has been termed a virologic (or viral) synapse (VS)[2] since it resembles the immunologic synapse (IS), the specialized contact made between a lymphocyte and an antigen-presenting cell.[3] A synapse is an organized structure that allows signaling and directional protein transfer between 2 cells. Identification of the mechanisms by which HTLV-1 induces formation of the VS will serve to elucidate how the virus persists and spreads, and it may provide new targets for interruption of HTLV-1 propagation.

The IS and the VS share at least 2 structural features. First, the T cell's MTOC is oriented toward the cell-cell junction. Second, the lymphocyte function-associated antigen-1 (LFA-1)–associated molecule talin is organized in ring-shaped microdomains or patches in the cell contact area.[2] However, there is a crucial difference between the VS and the IS: in the IS, the MTOC is oriented toward the synapse inside the T cell that has recognized its cognate antigen,[4] whereas in the VS the MTOC is polarized toward the synapse inside the HTLV-1–infected cell. Therefore, while formation of the IS is initiated by signaling through the T-cell receptor,[5] the critical factor that drives VS formation is HTLV-1 infection, irrespective of the antigen specificity of the T cells involved.[2] It was concluded that at least 2 signals are necessary to trigger VS formation: one from HTLV-1 infection of the T cell and the second from contact with another cell. The aim of this study was to identify T-cell surface molecules involved in triggering the polarization of the microtubule cytoskeleton that is characteristic of the HTLV-1–associated virologic synapse. We adopted a simple antibody-coated bead-cell conjugate formation assay that others have used previously to dissect the mechanisms involved in T-cell activation.[6-10] Latex beads were coated with antibodies to T-cell surface molecules known to be involved in T-cell activation, such as CD2 and CD3, LFA-1 (CD11a and CD18), and CD28,[11,12] and also molecules known to be up-regulated on HTLV-1–infected T cells, such as intercellular adhesion molecule-1 (ICAM-1) and CD25.[13-15] The results were corroborated by 2 independent types of experiment, one using soluble cyclic peptides that block the interaction between ICAM-1 and LFA-1, the other using cell lines with a nonexpressing or constitutively activated *LFA1* gene. Evidence from all 3 types of experiments indicates that engagement of ICAM-1 on the surface of the HTLV-1–infected cell is sufficient to account for the signal from cell contact that triggers the observed preferential polarization of the microtubule cytoskeleton in the HTLV-1 virologic synapse.

From the Department of Immunology, Wright-Fleming Institute, Imperial College London, St Mary's Campus, London, United Kingdom; the Department of Genito-Urinary Medicine and Communicable Diseases, Wright-Fleming Institute, Imperial College London, St Mary's Campus, London, United Kingdom; and the Department of Infectious Disease and Immunology, Okinawa-Asia Research Center of Medical Science, Faculty of Medicine, University of the Ryukus, Nishihara, Okinawa, Japan.

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Reprints: Charles R. M. Bangham, Department of Immunology, Wright-Fleming Institute, Imperial College London, St Mary's Campus, London W2 1PG, United Kingdom; e-mail: c.bangham@imperial.ac.uk.

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Patients, materials, and methods

Patients and cells

The HTLV-1–immortalized cell line MS9 was a gift from Dr David Derse, National Cancer Institute, Rockville, MD. MS9 cells were derived by coculture of phorbol-12-myristate-13-acetate–activated human peripheral blood mononuclear cells (PBMCs) with DBS-FRhL (clone B5) cells that were infected with the HTLV-1 molecular clone, pHTLV-X1MT.[16] MS9 cells were cultured in RPMI 1640 (Sigma-Aldrich, Dorset, United Kingdom) supplemented with 2 mM glutamine (Invitrogen, Paisley, United Kingdom), 100 IU/mL penicillin (Invitrogen), 100 IU/mL streptomycin (Invitrogen), 20% heat-inactivated fetal calf serum (FCS; PAA Laboratories, Somerset, United Kingdom), and 100 U/mL recombinant interleukin 2 (IL-2; Sigma-Aldrich).

The Jβ2.7 cells were a gift from Dr Catarina Hioe, VA Medical Center Research Service, New York, NY. They were derived from Jurkat cells treated with ethyl methanesulfonate and selected for complete loss of cell surface LFA-1. The cells were then transfected with cDNA of the LFA-1 wild-type α chain subunit (CD11a) to restore LFA-1 cell surface expression (Jβ2.7/LFA-1 wt). A cell line expressing a constitutively active form of LFA-1 was created using a deletion mutant of the LFA-1 α subunit (Jβ2.7/LFA-1 Δ). A Jβ2.7 control line was generated by transfecting the vector alone (Jβ2.7/mock), which expressed no LFA-1 on the surface.[17,18] These cell lines were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM glutamine (Invitrogen), 100 IU/mL penicillin (Invitrogen), 100 IU/mL streptomycin (Invitrogen), 20% heat-inactivated FCS (PAA Laboratories), and 3 μg/mL puromycin (Sigma-Aldrich).

PBMCs were obtained from uninfected laboratory controls and HTLV-1 seropositive HAM/TSP patients with a high proviral load attending the National Centre for Human Retrovirology at St Mary's Hospital, London. All patients gave informed consent. PBMCs were isolated via density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich), washed twice with phosphate-buffered saline (PBS) and then once in PBS/10% FCS. $CD4^+$ cells were isolated using indirect magnetic labeling with the $CD4^+$ T Cell Isolation Kit from Miltenyi Biotech (Surrey, United Kingdom). The manufacturer's instructions were followed for negative selection of $CD4^+$ T cells from PBMCs. This procedure yielded $CD4^+$ cells at a purity of greater than 95%, ascertained by flow cytometry (data not shown). Before use, the isolated $CD4^+$ T cells were cultured overnight, widely dispersed in 10-cm diameter tissue culture dishes (10^6 cells/mL), to allow spontaneous expression of HTLV-1 proteins.[19] The culture medium used was RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM glutamine (Invitrogen), 100 IU/mL penicillin (Invitrogen), 100 IU/mL streptomycin (Invitrogen), and 20% heat-inactivated FCS (PAA Laboratories).

Antibodies

The following antibodies were used for staining of cells for microscopy: the mouse immunoglobulin G_{2b} (IgG_{2b}) anti–HTLV-1 Gag p19-specific monoclonal antibody (mAb), clone GIN7,[20] used at a concentration of 1 μg/mL; and the rat anti–tubulin α mAb (Chemicon Europe, Southampton, United Kingdom), used at a concentration of 3 μg/mL. Secondary antibodies for fluorescent staining with the anti-tubulin α and the anti-Gag p19 antibodies were Fluorescein (fluorescein isothiocyanate [FITC]) AffiniPure-labeled donkey anti–rat IgG (used at a concentration of 150 μg/mL; Stratech Scientific, Cambridgeshire, United Kingdom) and Alexa Fluor 568 goat anti–mouse IgG_{2b} (used at a concentration of 2μg/mL; Molecular Probes Europe BV, Leiden, The Netherlands), respectively.

Antibodies used to coat the polystyrene latex beads were as follows: anti-CD54 (ICAM-1), clone HA58; anti-CD2, clones 4B2, CLB-T11/1 and 4B2, CLB-T11.2/1; and anti-CD28, clone CLB-CD28/1, 15E8, all mouse IgG_1 antibodies (Research Diagnostics, Flanders, NJ); anti-CD3, clone HIT3a (mouse IgG_{2a}) from Bioscience (Insight Biotechnology Ltd., Wembley, Middlesex, United Kingdom); anti-CD11a clone G25.2 (mouse IgG_{2a}) from Becton Dickinson United Kingdom, Oxford, United Kingdom; anti-CD11a, clone mAb24 [21,22] and anti-CD54 (ICAM-1), clone 15.2[23,24] (both mouse IgG_1 antibodies), gifts from Nancy Hogg, Cancer Research United Kingdom London Research Institute, London, United Kingdom; anti-CD11a, clone 38 (mouse IgG_{2a}); anti-CD18 antibody, clone YFC118.3 (rat IgG2b), and anti-CD25, clone M-A251 (mouse IgG_1) from Serotec, Oxford, United Kingdom; anti-CD54 (ICAM-1), clone 84H10 (mouse IgG_1), anti-CD50 (ICAM-3), clone HP2/19 (mouse IgG_{2a}); and anti-CD58 (LFA-3), clone AICD58 (mouse IgG_{2a}), from Immunotech (Beckman Coulter United Kingdom, High Wycombe, United Kingdom). All isotype control antibodies were obtained from Serotec.

Antibody-coated latex beads

For each antibody, 80×10^6 surfactant-free sulfate white polystyrene latex 5-μm beads (Interfacial Dynamics, Portland, OR) were washed twice in 10 mL 0.025 M MES (2-[*N*-Morpholino] ethanesulfonic acid; buffer pH 6.1; Sigma-Aldrich). The beads were centrifuged for 20 minutes at 3000*g* and resuspended in 1 mL MES buffer in a 15-mL centrifuge tube. The respective antibody (100 μg) was added and incubated overnight at room temperature on a roller, with constant mixing. The beads were then washed twice in 10 mL PBS and resuspended in 1 mL filter-sterilized PBS/1% bovine serum albumin (BSA) (1 g BSA in 100 mL PBS). The antibody-coated beads were stored at 4°C. Flow cytometric analysis was used to verify that the beads were coated with antibody (results not shown).

Conjugate formation and immunofluorescence

Glass multiwell slides (Hendley-Essex, London, United Kingdom) were precoated with poly-L-lysine (Sigma-Aldrich). $CD4^+$ T cells that had been incubated overnight were then washed in serum and glutamine-free RPMI and were resuspended to a final concentration of approximately 5×10^6 cells/mL in RPMI. MS9 cells were used at a concentration of approximately 3×10^6 cells/mL and Jβ2.7 cells at approximately 5×10^6/mL.

For the cyclic peptide inhibition studies where only one half of the cells were treated, each sample of $CD4^+$ PBMCs was divided into 2 halves. One half was stained with CellTracker Blue CMAC, 7-amino-4-chloromethylcoumarin (CMAC; Cambridge Bioscience, Cambridge, United Kingdom) by incubating 2.5×10^6 cells/mL in a 30-μM CMAC solution for 30 minutes at 37°C, 5% CO_2. The cells were washed twice in RPMI/20% FCS, incubated at 37°C, 5% CO_2 for a further 30 minutes and washed again before use in cell conjugation experiments. In each experiment, either the CMAC-stained cells or the unstained cells were treated with the cyclic peptides. The frequency of MTOC polarization to the cell-cell junction was quantified only in unstained cells in conjugates formed between a CMAC-stained cell and an unstained cell.

To form cell-cell conjugates, the cells were placed in 5-mL polystyrene round-bottom Falcon tubes (Becton Dickinson United Kingdom) for 5 minutes to allow any large clumps to settle out. Suspended cells were then plated onto the precoated glass multiwell slides and incubated at 37°C for 40 minutes or 1 hour. For experiments involving antibody-coated beads, the cells were further diluted to give a final concentration of 5×10^5 cells/mL and 2.5×10^6/mL of beads (1:5 ratio of cells to beads). Beads and cells were left to conjugate for 1 hour or 2 hours. Samples were fixed with 100% methanol (precooled to −20°C) for 5 minutes, then washed extensively in PBS, blocked in PBS/1% BSA, and processed for immunofluorescence. Primary antibodies were added in the presence of PBS/1% BSA for 40 minutes and washed in PBS/1% BSA.[25] Secondary antibodies were added in the presence of PBS/1% BSA for 40 minutes, washed thoroughly in PBS and H_2O, then mounted in PBS containing 90% glycerol and 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich).

Cyclic peptides

Cyclic peptides cIBR (cyclo-1, 12-Pen-PRGGSVLVTGC) and cLAB.L (cyclo-1,12-Pen-ITDGEATDSGC) were gifts from Dr Teruna J. Siahaan, University of Kansas. The cIBR peptide is derived from the sequence of ICAM-1 and inhibits homotypic T-cell adhesion in vitro by blocking the interaction between ICAM-1 and LFA-1.[26] The introduction of a disulphide bond in the cyclic peptide stabilizes the secondary structure by imposing conformational rigidity on the peptide which results in stronger binding to ICAM-1 than that of linear peptides. The cLAB.L peptide is similarly

derived from the I-domain of α subunit of LFA-1 and is also able to inhibit ICAM-1/LFA-1–dependent adherence of T cells.[27]

Control cyclic peptides RD-LBEC (GGLKKVNRLD), RD-IBL (IVKSPSVSTQ), and R2BAL (GRQEGYFLPA) were also a gift from Dr Teruna J. Siahaan. Each control peptide is derived from the reversed amino acid sequence of the respective native protein: RD-LBEC from the β_2 subunit of LFA-1, R2BAL from the α subunit of LFA-1, and RD-IBL from ICAM-1.

$CD4^+$ T cells from HTLV-1–infected patients were obtained as described above (under "Patients and cells") and incubated overnight in Petri dishes. The cells were washed and resuspended at 5×10^5/mL in 5 mL-polystyrene round-bottom Falcon tubes. The cyclic peptides were then added at a final concentration of 200 μM. After 30 minutes excess peptide was washed out, and the cells were resuspended at 5×10^6/mL. The cultures were left for 5 minutes in the 5-mL polystyrene round-bottom Falcon tubes before incubating the cells on microscope slides for 1 to 2 hours at 37°C, to allow the cells to form conjugates.

Analysis of polarization and conjugation

A Laborlux 12 Leitz fluorescent microscope with a BGR filter system and a water-based objective with a numerical aperture of 1.0 (Leitz, Wetzlar, Germany) was used to observe the bead and cell conjugates at room temperature. Only cell-cell conjugates with 2 cells were counted. Cell-bead conjugates were counted where the cell was not in contact with another cell and where there was only one point of contact between the cell and a bead. Conjugates were examined by visually dividing the cell into 5 radial sectors; the MTOC was deemed polarized when it was orientated toward the conjugated bead or cell within the sector in contact with the bead or cell. Thus, a frequency of MTOC polarization of 20% implies random orientation of the microtubules (ie, no significant effect of cell contact etc). In each experiment a minimum of 300 conjugates were counted. An Optronics Magnafire cooled CCD camera (Optronics, Goleta, CA) on the Leitz fluorescence microscope was used to take photographs of conjugates; images were displayed using Magnafire software (Optronics).

Statistical analysis

The frequency of MTOC polarization in HTLV-1–infected and uninfected cells was compared by using the odds ratio (OR). To test whether a given OR differed significantly from 1.0 and to compare 2 different odds ratios, we applied normal theory to the distribution of ln(OR), the natural logarithm of OR.[28] To calculate the summary odds ratio for a given effect over a number of experiments, we used the inverse variance method of weighting individual values of ln(OR).[28] To calculate the statistical significance of a given effect over different experiments, we used Fisher chi-square method of combining probabilities:

$$\chi^2 = -2\sum_{i=1}^{k} \ln(p_i)$$

where p_i is the significance level obtained from the ith experiment, and k is the number of experiments; $-2\Sigma\ln(p_i)$ is distributed as a chi-square variate with 2k degrees of freedom.[29]

Results

Cross-linking of certain T-cell surface molecules leads to polarization of the MTOC

To test the ability of individual T-cell surface antigens to polarize the MTOC in uninfected $CD4^+$ T cells, we incubated $CD4^+$ T cells obtained from healthy HTLV-1-seronegative subjects with latex beads coated with antibodies to different T-cell surface molecules. T-cell surface molecules were chosen for study on the basis of their known importance in the formation of an IS or in T-cell signaling or costimulation. The frequency of MTOC polarization induced by the respective antibodies was compared with that induced by a matched isotype control antibody, to control for any nonspecific effects of immunoglobulin in contact with the T cell.

As previously described,[6,8,9] beads coated with anti-CD3 antibodies caused efficient polarization of the MTOC toward the bead in $CD4^+$ uninfected control T cells; the odds ratio of MTOC polarization induced by anti-CD3, compared with that induced by an isotype control antibody, was greater than 5 (Table 1). Beads coated with antibodies directed against CD2, LFA-1 (CD11a and CD18), CD28, and CD50 induced MTOC polarization with a frequency greater than 3 times that induced by their respective isotype control antibodies (Table 1); the polarization induced by anti-LFA-1 antibodies was greatest (ie, gave the highest odds ratio when compared with the isotype control). In contrast, relatively infrequent MTOC polarization (odds ratio, 1.0-2.0) was induced in uninfected $CD4^+$ cells by beads coated with antibodies directed against CD54 (ICAM-1), CD58, and CD25 (Table 1); that is, the frequency of polarized cells was close to the random expectation of 20% (see "Patients, materials, and methods").

Activation of T cells using antibodies cross-linking CD2 requires antibodies of 2 specificities: either 2 anti-CD2 antibodies, one of which is directed against a CD58-binding site and the other directed against an activation-related epitope known as CD2 R[30,31]; or one anti-CD2 and one anti-Ig antibody which cross-links the anti-CD2 antibody.[32] For this reason, 2 different antibodies to CD2 were chosen, clones 4B2, CLB-T11/1 and 4B2, CLB-T11.2/1. However, the frequency of MTOC polarization in $CD4^+$ control T

Table 1. Polarization of the MTOC in normal uninfected CD4+ PBMCs upon cross-linking with antibody to cell surface molecules

Antibody-coated bead	No. experiments	Odds ratio of MTOC polarization in antibody vs isotype control-stimulated cells (% polarization)	χ^2 (DF)	P
Anti-CD3 (TCR)	4	5.80 (60)	207 (8)	< .001
Anti-CD54 (ICAM-1)	7	1.21 (27)	53.2 (14)	< .001
Anti-CD11a (LFA-1)	5	7.80 (64)	61.0 (10)	< .001
Anti-CD58 (LFA-3)	3	1.76 (26)	23.0 (6)	< .001
Anti-CD28	3	3.27 (41)	60.0 (6)	< .001
Anti-CD18 (LFA-1)	3	10.86 (67)	168 (6)	< .001
Anti-CD2	6	5.39 (56)	175 (12)	< .001
Anti-CD50 (ICAM-3)	3	6.92 (60)	138 (6)	< .001
Anti-CD25 (IL-2Rα)	3	1.31 (25)	18.3 (6)	.006

Latex beads were coated with antibody of the respective specificity and mixed with $CD4^+$ PBMCs from HTLV-1 seronegative control subjects. Odds ratios were calculated by comparing the frequency of polarization of the MTOC toward beads coated with antibodies to each cell surface molecule with the frequency of polarization toward beads coated with isotype control antibodies. The summary odds ratios are reported (weighted mean odds ratio over the indicated number of experiments). DF indicates degrees of freedom.

cells toward beads coated with either of the 2 anti-CD2 antibodies alone was already high and there was no significant increase in polarization of the MTOC to beads coated with both antibodies (data not shown).

Three different antibodies to CD11a (LFA-1) were used because the LFA-1/ICAM-1 interaction plays an important role in the formation of the immunologic synapse, and because each of the antibodies used has a different action upon binding to LFA-1. The anti-CD11a antibody clone 38 blocks binding of CD11a to ICAM-1. However, this antibody is also a partial agonist; that is, its binding leads to signal transduction in the T cell through LFA-1. The antibody G25.2 cross-links CD11a but does not block or stimulate—that is, has no detected effect on—the function of LFA-1. Mab24 binds only to the active form of LFA-1.[22] Each of the 3 anti-CD11a antibodies caused a similar frequency of polarization of the MTOC.

Although all 3 anti–ICAM-1 antibodies used are described as blocking antibodies, more than one antibody was used because ICAM-1 is up-regulated in HTLV-1–infected cells[33] and is thought to be important in the initiation of formation of the virologic synapse. However, none of the antibodies used was effective in inducing polarization of the MTOC in the CD4+ T cells from uninfected control subjects. That is, each anti–ICAM-1 antibody induced less-frequent MTOC polarization than that induced by beads coated with antibodies to other cell surface molecules (Table 1). The anti–ICAM-1 antibody 84H10 blocks ligation of ICAM-1 to LFA-1 and reduces syncytium formation in HIV-1–infected cultures of a T-cell leukemic cell line, CEM.[34] The anti–ICAM-1 antibodies HA58[35] and 15.2[36] are also known to block ligation with LFA-1. Antibodies to ICAM-1 are not known to cause T-cell activation.

A significant increase in polarization of the MTOC is observed in HTLV-1–infected CD4+ T cells

The results reported in Table 1 showed that monoclonal antibodies against several different T-cell surface molecules each caused MTOC polarization in normal uninfected CD4+ T cells. Igakura et al[2] reported a strong association between MTOC polarization and HTLV-1 infection in T cells that make contact with another cell. We, therefore, wanted to test whether engagement of single specific T-cell surface molecules causes a higher frequency of MTOC polarization selectively in HTLV-1–infected T cells. To do this we examined conjugates formed between antibody-coated beads and PBMCs from HTLV-1–infected individuals.

We compared the frequency of polarization of the MTOC toward antibody-coated beads in infected and uninfected cells within the CD4+ PBMC population from HTLV-1–infected individuals. The results of these experiments are summarized in Table 2. There was a strong association between polarization of the MTOC and polarization of Gag p19, regardless of the antibody used. Figure 1 shows an example of polarization induced by a bead coated with anti-CD11a. HTLV-1 infection did not significantly alter the frequency of MTOC polarization induced by antibodies to CD2, CD18, CD50, or CD58 and isotype control antibodies (data not shown). However, cross-linking of each of 3 surface molecules caused a significantly higher frequency of MTOC polarization in HTLV-1–infected cells than in uninfected cells (Table 2): CD54 (ICAM-1), CD25 (IL-2Rα), and CD11a (a constituent chain of LFA-1). This synergistic effect of HTLV-1 infection and antibody cross-linking was both strongest (ie, gave the highest odds ratio) and most statistically significant in the case of cross-linking of ICAM-1. Interestingly, the MTOC polarization induced by cross-linking of either CD3 (T-cell receptor complex) or CD28 (T-cell costimulatory molecule) was in each case significantly and reproducibly reduced in frequency by HTLV-1 infection of the cell (Table 2).

Effect of HTLV-1 infection on microtubule polarization in an HTLV-1–infected T-cell line

In addition to testing the effects of antibody-coated beads on CD4+ T cells from HTLV-1–infected individuals and uninfected control subjects, we examined the effect of such beads on MTOC polarization in 2 human CD4+ T-cell lines: Jurkat cells and MS9 cells, which are continuously infected with HTLV-1.[16] The results are shown in Table 3. As in the experiments carried out on freshly isolated CD4+ T cells (data not shown), antibodies directed against CD50, CD58, and clone mAb24 against the CD11a chain of LFA-1 induced a similar frequency of MTOC polarization in the HTLV-1–infected MS9 cells as in the uninfected Jurkat cells. The strongest polarization in MS9 cells versus Jurkat cells was again induced by antibodies against CD25 and ICAM-1. However, the frequency of MTOC polarization induced by beads coated with anti-CD3 was greater than 30-fold lower (OR = 0.02) in the MS9 cells than in Jurkat cells. Similarly, the frequency of polarization induced by anti-CD28 was 6-fold lower (OR = 0.17) in the MS9 cells. These observations confirmed the inhibitory effect of HTLV-1 infection on cytoskeletal polarization induced by anti-CD3 or anti-CD28 that was observed in freshly isolated infected CD4+ T cells (Table 2). MTOC polarization in MS9 cells was also significantly less frequent than in Jurkat cells when mixed with beads coated in antibodies to CD18 or CD28. The anti-CD11a (clones 38 and

Table 2. Interaction between HTLV-1 infection and cross-linking of cell-surface molecules in causing polarization of the microtubule cytoskeleton

Cross-linking antibody	No. experiments	Odds ratio* of MTOC polarization in Gag p19+ cells vs Gag p19− cells	Polarization in Gag p19+ cells, %	χ^2 (DF)	P†
Anti-CD54 (ICAM-1)	8	2.58	56	166 (16)	< .001
Anti-CD25 (IL-2Rα)	3	2.18	47	34 (6)	< .001
Anti-CD11a (LFA-1)	8	1.14	70	42 (16)	< .001
Anti-CD3 (TCR complex)	3	0.84	63	17 (6)	.009
Anti-CD28 (costimulatory)	3	0.66	61	18 (6)	.006

Latex beads were coated with monoclonal antibodies to the respective T-cell surface molecule and incubated for 1 to 2 hours with PBMCs from individuals infected with HTLV-1. An odds ratio (OR) less than 1 indicates that HTLV-1 infection and cross-linking were synergistic in inducing MTOC polarization; OR greater than 1 indicates that HTLV-1 infection inhibited the antibody-induced polarization. TCR indicates T-cell receptor.

*Summary (weighted mean) odds ratio from indicated number of experiments.

†Significance level of odds ratio.

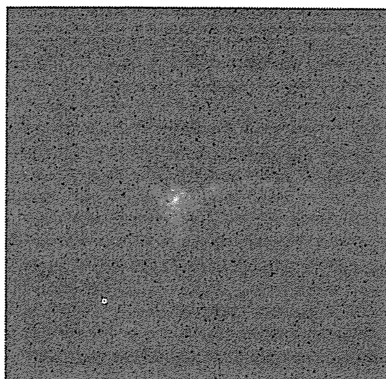

Figure 1. Polarization of the MTOC and Gag p19 toward an antibody-coated latex bead. The MTOC was polarized toward beads coated with anti-CD11a antibody in HTLV-1–infected and uninfected CD4+ T cells after 1 hour of incubation. Tubulin α is green, stained using Fluorescein (FITC), original magnification × 400. If the MTOC was polarized, then invariably Gag p19 (red, stained with Alexa Fluor 568) was also polarized toward an antibody-coated bead. Similar polarization of both the MTOC and Gag p19 was induced by latex beads coated with either anti-CD2, CD3, CD18 (LFA-1), CD25 (IL-2Rα), CD28, CD50 (ICAM-3), CD54 (ICAM-1), or CD58 (LFA-3)

G25.2)–coated beads induced significantly more frequent MTOC polarization in the HTLV-1–infected MS9 cells than in Jurkat cells; this effect was partly due to the weak polarization that these antibodies induced in the Jurkat cells. The frequency of polarization in freshly isolated uninfected CD4+ T cells with these same anti-CD11a–coated beads was also significantly higher than that seen in Jurkat cells. In summary, these observations confirmed the findings made in freshly isolated CD4+ PBMCs (Table 2); that is, HTLV-1 increased the frequency of MTOC polarization induced by cross-linking of ICAM-1, CD25, or CD11a but inhibited the polarization induced by antibodies to CD3 or CD28.

Cyclic peptides derived from ICAM-1 and LFA-1 abolished the preferential polarization of MTOC to the cell-cell junction in freshly isolated CD4+ T cells

Coculture of antibody-coated beads and CD4+ T cells showed (Table 2) that cross-linking of ICAM-1 leads to frequent polarization of the MTOC. The antibody-coated beads were used to identify which cell surface molecules were involved in the polarization event. However, antibody cross-linking may differ in important respects (eg, in affinity and kinetics) from engagement of ICAM-1 by its physiologic ligand LFA-1 on the opposing cell surface. To test whether the ICAM-1/LFA-1 interaction is indeed important in the MTOC polarization associated with formation of the virologic synapse between an HTLV-1–infected cell and another cell, we used cyclic peptides that are known to block this interaction.[26,27] The LFA-1–derived cyclic peptide (cLAB.L) and the ICAM-1–derived peptide (cIBR) were added to freshly isolated CD4+ T cells from HTLV-1–infected HAM/TSP patients to test the effect on polarization of the MTOC in CD4+:CD4+ T-cell conjugates.

As shown previously,[2] the odds ratio of MTOC polarization in a Gag p19+ cell, compared with a Gag p19− cell in conjugates formed between CD4+ PBMCs of an HTLV-1–infected subject, typically lies between 3.5 and 4.5. The results of experiments with the cyclic blocking peptides showed (Table 4) that treating the cells with either cLAB.L or cIBR reduced the odds ratio of MTOC polarization to the cell-cell junction in HTLV-1–infected T cells. Treatment of the CD4+ T cells simultaneously with both blocking peptides completely abolished the HTLV-1–associated MTOC polarization (Table 4): the OR of 1.25 was not significantly different from 1.0. To test the specificity of this inhibition, we used 3 different control cyclic peptides (see "Patients, materials, and methods"). When the control peptides RD-LBEC, RD-IBL, or R2BAL were added, in each case the odds ratio of MTOC polarization (2.85, 2.54, and 3.70, respectively) did not differ significantly from that seen in untreated cells (2.83), whereas the blocking peptides cIBR and cLABL, as before, significantly reduced the odds of polarization (ORs, 1.31 and 1.14, respectively). Simultaneous addition of all 3 control peptides similarly had no significant effect on the frequency of MTOC polarization (OR = 2.87).

In a second set of experiments with the cyclic peptides, only half the cells were pretreated with the cyclic peptides, then washed before being allowed to form conjugates with nonpeptide-treated cells. Washing neither removes the bound peptide nor abolishes its effect on cell-cell adhesion, because the peptide-ligand complex is rapidly internalized by the cell.[37] One half was stained with a fluorescent dye, CMAC, to distinguish the cells that had been treated with peptide. By examining conjugates formed between stained and unstained cells, we quantified the effect of blocking ICAM-1 or LFA-1 on either an infected cell or an uninfected cell.

The results of experiments with the cyclic blocking peptides showed (Table 5) that treating the cells with cIBR reduced the odds ratio of MTOC polarization to the cell-cell junction in HTLV-1–infected T cells by approximately 2-fold. However, treatment of the CD4+ T cells with cLAB.L, the blocking peptide that mimics LFA-1 and therefore blocks engagement of ICAM-1, completely

Table 3. Polarization of the MTOC in CD4+ T-cell lines

Monoclonal antibody	No. experiments	Polarization in Jurkat/MS9 cells, %	Odds ratio* of MTOC polarization in MS9 vs Jurkat cells	χ^2 (DF)	P†
Anti-CD3	2	89/15	0.02	147.0 (4)	< .001
Anti-CD54	4	31/68	3.97	24.5 (8)	.002
Anti-CD11a	4	40/55	1.68	37.0 (8)	< .001
Anti-CD58	1	25/30	1.25	0.63 (1)	.427
Anti-CD28	2	69/29	0.17	75.0 (4)	< .001
Anti-CD18	2	71/43	0.27	53.8 (4)	< .001
Anti-CD2	1	32/54	2.49	9.87 (1)	.002
Anti-CD50	1	72/69	0.87	0.21 (1)	.642
Anti-CD25	1	7/58	18.40	59.3 (1)	< .001

The CD4+ HTLV-1-infected cell line, MS9, or uninfected Jurkat cells were mixed with antibody-coated beads. Polarization of the MTOC toward the beads was observed, and the odds ratios were calculated when comparing polarization in MS9 versus Jurkat cells.

*Summary (weighted mean) odds ratio from indicated number of experiments.

†Significance level of odds ratio

Table 4. Treatment of CD4+ PBMCs with cyclic peptides derived from ICAM-1 (cIBR) or LFA-1 (cLABL)

Treatment	Odds ratio* of MTOC polarization in Gag p19+ PBMCs vs Gag p19− PBMCs	Polarization in Gag p19+ cells (%)	P†
Untreated control	3.68	69	
cIBR	1.74	42	<.001
cLAB.L	1.93	36	.004
cIBR + cLAB.L	1.25	36	< .001

Three independent experiments were performed with CD4+ PBMCs from 3 different HTLV-1-infected patients with HAM/TSP and high viral load. Where 2 cells formed a conjugate, the orientation of the MTOC was noted, and the odds ratio was calculated for polarization of the MTOC in infected versus uninfected cells.

*Summary (weighted mean) odds ratio from the 3 experiments.

†Significance level of reduction in odds ratio compared with untreated control.

abolished the HTLV-1–associated MTOC polarization (Table 4): the OR of 1.14 was not significantly different from 1.0.

Preferential polarization of the MTOC in Gag p19+ cells does not occur when conjugation is with a cell lacking LFA-1 expression

A Jurkat cell line selected for the loss of LFA-1 expression (Jβ2.7) was used as a further test of the effect of ICAM-1 cross-linking in an HTLV-1–infected cell on the triggering of polarization of the MTOC. The Jβ2.7 cell line had been transfected either with a vector encoding wild-type LFA-1 (Jβ2.7/wtLFA-1), or a mutant form of LFA-1 that is constitutively expressed in the active (high affinity) form (Jβ2.7/ΔLFA-1), or the empty vector (Jβ2.7/mock).[17] The 3 respective cell lines were used to form conjugates with the HTLV-1–infected cell line MS9. The odds ratio of polarization of the MTOC in Gag p19+ (MS9) cells versus Gag p19-(Jβ2.7) cells was lower than that induced in ex vivo CD4+ PBMCs (summary OR of 1.83; Table 6) by the wild-type LFA-1–expressing cell line. The cell line that constitutively expressed the active form of LFA-1 induced a higher frequency of MTOC polarization (summary OR = 2.50; Table 6). However, when there was no LFA-1 expression (Jβ2.7/mock cells), the summary OR was below 1.00, indicating that, without cross-linking of ICAM-1 or other LFA-1 ligands, the polarization of the MTOC was inhibited by HTLV-1 infection. The data in Table 7 confirm that the frequency of polarization was reduced in the infected (MS9) cells in contact with cells that lacked LFA-1 expression.

Table 5. Treatment of only one half of the CD4+ PBMCs with cyclic peptides derived from ICAM-1 (cIBR) or LFA-1 (cLAB.L)

Treatment	Odds ratio* of MTOC polarization in Gag p19+ PBMCs vs Gag p19− PBMCs	P†
Untreated control	3.94	< .001
cIBR	1.84	.021
cLAB.L	1.14	.41
cIBR + cLAB.L	1.26	.39

One half of the cells were labeled with CMAC, so that it was possible to distinguish conjugates where only one of the cells was treated with peptide. Three independent experiments were performed with CD4+ PBMCs from 3 different HTLV-1-infected patients with HAM/TSP and high viral load. Where 2 cells formed a conjugate, the orientation of the MTOC was noted, and the odds ratio was calculated for polarization of the MTOC in infected versus uninfected cells.

*Summary (weighted mean) odds ratio from the 3 experiments.

†Significance level of odds ratio.

Table 6. Conjugation of a CD4+ HTLV-1-infected cell line with CD4+ cell lines with and without LFA-1

	Odds ratio* of MTOC polarization in MS9 versus Jβ2.7 cells*	χ^2 (DF = 6)	P†
Jβ2.7/ΔLFA-1	2.50	45.8	<.001
Jβ2.7/wtLFA-1	1.83	25.8	<.001
Jβ2.7/mock	0.85	15.3	.018

Three independent experiments were performed with the CD4+ cell lines. Conjugates were formed between the HTLV-1- infected cell line MS9 and the Jβ2.7 cell lines. Where 2 cells formed a conjugate, the orientation of the MTOC was noted, and the odds ratio was calculated for polarization of the MTOC in the infected MS9 cells versus the uninfected Jβ2.7 cells.

*Summary (weighted mean) odds ratio from the 3 experiments.

†Significance level of odds ratio.

Discussion

Polarization of the MTOC to the cell-cell junction is associated with formation of both the immunologic synapse[3] and the virologic synapse.[2] The role of MTOC polarization in the IS appears to be to direct the focal delivery of secreted proteins (lymphokines or lytic granules) to the appropriate antigen-presenting cell (APC), but not to nearby cells.[38] In the IS, polarization is triggered by engagement of the TCR[5] with the major histocompatibility complex (MHC)/peptide complex on the surface of the antigen-presenting cell. But in the HTLV-1–associated VS, polarization is strongly associated with HTLV-1 infection of the T cell; that is, the MTOC is polarized inside an infected T cell, not toward an infected cell. This observation implies that TCR-mediated recognition of HTLV-1 antigens plays no essential role in VS formation; rather, the VS must be triggered by a combination of 2 signals: HTLV-1 infection and cell contact. The present study was, therefore, designed to test the hypothesis that a ligand-receptor interaction between 2 CD4+ T cells is synergistic with HTLV-1 antigen expression in causing polarization of the infected T cell's MTOC toward the cell-cell junction. To test this hypothesis, we examined the effect of cross-linking individual T-cell surface molecules using monoclonal antibodies coated onto latex beads. Cross-linking by antibody can mimic physiologic stimuli: eg, anti-CD3 antibody delivers an activating signal through the T-cell receptor that mimics physiologic engagement with MHC/peptide complexes.[39]

The results of the antibody cross-linking experiments showed that cross-linking of several different T-cell surface molecules cause MTOC polarization. However, cross-linking of either of 2 molecules, ICAM-1 (CD54) or CD25, caused significantly more frequent polarization in an HTLV-1–infected cell than an uninfected cell, indicating a synergistic interaction between HTLV-1 infection and cross-linking of the respective surface molecule in triggering the cytoskeletal rearrangement. Similar results were obtained both in PBMCs from several individuals infected with HTLV-1 and in a CD4+ T-cell line (MS9) continuously infected with HTLV-1.

Table 7. Percentage polarization of the MTOC in cell-cell conjugates

	Infected cells, %	Uninfected cells, %
Jβ2.7/ΔLFA-1	87.6	32.8
Jβ2.7/wtLFA-1	53.7	29.3
Jβ2.7/mock	37.1	50.6

Three independent experiments were performed with the CD4+ cell lines. Conjugates were formed between the HTLV-1-infected cell line MS9 and the Jβ2.7 cell lines. The frequency of polarization in infected and uninfected cells is shown.

Cross-linking of any molecule that leads to a degree of polarization of MTOC and Gag has the potential to contribute to cell-cell spread of HTLV-1. However, the characteristics of the stimulus delivered by antibody cross-linking—that is, the strength and kinetics of the signal—may differ substantially from the physiologic stimulus. Therefore, any conclusions drawn from antibody-induced cross-linking experiments require corroborative evidence from experiments that involve a physiologic ligand-receptor interaction. To test the importance of the interaction between ICAM-1 and LFA-1 in the cytoskeletal polarization associated with the HTLV-1–induced VS in a more physiologic system than antibody-coated beads, we blocked this interaction in spontaneous conjugates formed between unstimulated $CD4^+$ T cells from HTLV-1–infected individuals. We used cyclic peptides (cIBR and cLABL) derived from either ICAM-1 or LFA-1. Each peptide alone reduced the odds of MTOC polarization in HTLV-1–infected cells; when both peptides were used together with the $CD4^+$ cells, there was no significant difference in the frequency of polarization in infected cells compared with uninfected cells. None of 3 control cyclic peptides significantly altered the frequency of MTOC polarization.

Since T cells express both ICAM-1 and LFA-1, we then wanted to test whether the direction of the interaction was important; that is, whether engagement of ICAM-1 or LFA-1 on the HTLV-1–infected cell was more effective in triggering the observed cytoskeletal polarization. Therefore, in the second set of experiments, only one of the cells within a conjugate was blocked with the cyclic peptides. The MTOC polarization that is strongly associated with the HTLV-1–infected T cell in the VS (Igakura et al[2]; Tables 4-5) was reduced in frequency by blocking LFA-1 with the ICAM-1–derived blocking peptide, but it was completely abolished by blocking ICAM-1 on an infected cell with the LFA-1–derived blocking peptide (OR not significantly > 1.0). These observations strongly imply that the ICAM-1/LFA-1 interaction indeed plays a necessary part in triggering the polarization of the cytoskeleton that is observed in the HTLV-1–associated VS. As a further independent test of the importance of cross-linking of ICAM-1 in an infected cell on polarization of the MTOC, the LFA-1–knock-out Jurkat cell lines were used. The results of these experiments showed that, in the absence of LFA-1 on a neighboring cell, the MTOC in an uninfected cell was polarized toward the neighboring cell with a higher frequency than in an infected cell. Thus, HTLV-1 infection inhibited cell-contact–induced MTOC polarization in the cell in the absence of ICAM-1 cross-linking. These results corroborate the conclusion that engagement of ICAM-1 on the surface of the HTLV-1–infected cell is particularly effective in triggering microtubule reorientation.

The ICAM-1/LFA-1 interaction is known to play a significant role in the formation of the immunologic synapse[3,11] and in reducing the threshold for T-cell activation.[40] LFA-1 is expressed chiefly on T cells,[41] whereas ICAM-1 is expressed on both T cells and many other nucleated cells.[42] Signal transduction from bound LFA-1 on the T cell has been well described.[43] But signaling into the T cell from ICAM-1, which has a very short cytoplasmic tail (28 amino acids), is less well understood.[44,45] The activated form of LFA-1 binds with high affinity to ICAM-1, which itself is up-regulated by HTLV-1 infection.[14] It remains possible that additional molecules on the HTLV-1–infected cell act to recruit or activate LFA-1 on the recipient cell. Intriguingly, there is evidence that the ICAM-1/LFA-1 interaction is also important in the spread of HIV-1 virions[46,47] and in HIV-1–induced syncytium formation.[48] However, the effects of blocking this interaction on the HIV-1 virologic synapse[49] have not yet been reported.

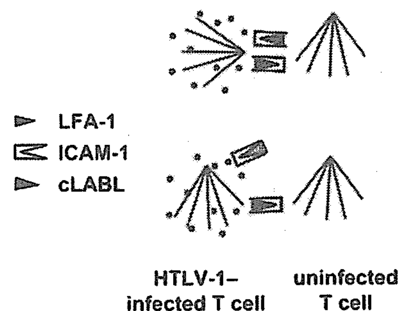

Figure 2. Blocking ICAM-1 on the HTLV-1–infected cell abolishes the preferential microtubule polarization induced by cell contact in an infected cell.

The effects on the cytoskeleton of cross-linking CD25 on the T-cell surface have not previously been reported. Since the ligand of CD25 (IL-2) is soluble and is not presented to the T cell bound to the surface of another cell, like the related cytokine IL-15,[50] the significance of the effect of CD25 cross-linking on cytoskeletal polarization in HTLV-1–infected T cells was at first unclear. However, it has been shown[51] that CD25 and ICAM-1 are physically associated in the plasma membrane; eg, they can be coimmunoprecipitated directly from solubilized cell membranes. Two inferences may be drawn: first, this observation corroborates the conclusion that ICAM-1 cross-linking is synergistic with HTLV-1 infection in inducing cytoskeletal polarization in the T cell. Second, it is likely that the effects of cross-linking either CD25 or ICAM-1 are mediated by a common pathway.

The transcriptional transactivator protein of HTLV-1, Tax, up-regulates expression of both ICAM-1[14] and CD25 (Figure 2).[13,15] Furthermore, Yamamoto et al[33] obtained evidence that cross-linking of ICAM-1 on the cell surface up-regulates *HTLV1* gene expression. As previously suggested,[2] this may constitute a positive feedback loop that increases the efficiency of cell-cell spread of HTLV-1.

In contrast to cross-linking with antibodies to ICAM-1 and CD25, cytoskeletal polarization induced by antibodies to either CD3 or CD28 was significantly inhibited by HTLV-1 infection; again, this effect was seen both in PBMCs and in the infected T-cell line, MS9. The effect of this inhibition may be to reduce the frequency of reinfection of a T cell that is already infected with HTLV-1.

We conclude that the interaction between LFA-1 and ICAM-1 plays an important part in the polarization of the T cell's cytoskeleton that is associated with the HTLV-1–induced virologic synapse. Engagement of either LFA-1 or ICAM-1 on an infected T cell caused strong polarization of the MTOC toward the cross-linked molecules. In particular, ICAM-1 engagement appeared to be synergistic with HTLV-1 infection in causing a higher frequency of cytoskeletal polarization. Since the physiologic ligand of ICAM-1 is LFA-1, which is expressed mainly on T cells, this synergistic interaction may contribute to the T-cell tropism of HTLV-1 observed in vivo.

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