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Adult T-Cell Leukemia/ Lymphoma-Related Ocular Manifestations: Analysis of the First Large-Scale Nationwide Survey

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Adult T-cell leukemia/lymphoma (ATL) is a rare and aggressive T-cell malignancy with a high mortality rate, resulting in a lack of information among ophthalmologists. Here, we investigated the state of ophthalmic medical care for ATL and ATL-related ocular manifestations by conducting the first large-scale nationwide survey in Japan. A total of 115 facilities were surveyed, including all university hospitals in Japan that were members of the Japanese Ophthalmological Society and regional core facilities that were members of the Japanese Ocular Inflammation Society. The collected nationwide data on the state of medical care for ATL-related ocular manifestations and ATL-associated ocular findings were categorized, tallied, and analyzed. Of the 115 facilities, 69 (60%) responded. Overall, 28 facilities (43.0%) had experience in providing ophthalmic care to ATL patients. ATL-related ocular manifestations were most commonly diagnosed "based on blood tests and characteristic ophthalmic findings." By analyzing the 48 reported cases of ATL-related ocular manifestations, common ATL-related ocular lesions were intraocular infiltration (22 cases, 45.8%) and opportunistic infections (19 cases, 39.6%). All cases of opportunistic infection were cytomegalovirus retinitis. Dry eye (3 cases, 6.3%), scleritis (2 cases, 4.2%), uveitis (1 case, 2.1%), and anemic retinopathy (1 case, 2.1%) were also seen. In conclusion, intraocular infiltration and cytomegalovirus retinitis are common among ATL patients, and ophthalmologists should keep these findings in mind in their practice.

Keywords: adult T-cell leukemia, ocular manifestations, nationwide survey, intraocular infiltration, human T-cell leukemia virus type 1

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) was the first retrovirus found to infect and cause disease in humans (Hinuma et al., 1981). The routes of transmission are primarily through sexual contact to adults, and through breast milk to infants (Iwanaga et al., 2009). Such infections are prevalent in Melanesia, the Caribbean Islands, Central and South America, and Central Africa, as well as areas such as Kyushu and Okinawa in Southwestern Japan (Watanabe, 2011). Among developed nations, Japan is estimated to have the highest proportion of infected individuals, with approximately 1 million (Satake et al., 2012) out of a total population of 126 million.

HTLV-1 causes diseases such as adult T-cell leukemia/lymphoma (ATL) (Yoshida et al., 1984), HTLV-1-associated myelopathy (HAM) (Osame et al., 1986), and HTLV-1 uveitis (HU) (Mochizuki et al., 1992a,b; Kamoi and Mochizuki, 2012b). Ophthalmic care is required for HU and ATL-related ocular manifestations (Kamoi and Mochizuki, 2012a), but ATL-related ophthalmic manifestations remain relatively obscure among ophthalmologists (Kamoi and Mochizuki, 2012a).

ATL is a rare disease, and the annual rate of ATL developing among HTLV-1 carriers is estimated to be between 7.7 and 8.7 per 10,000 people (Satake et al., 2015). As for prognosis, median overall survival time was 7.7 months according to the simplified ATL prognostic index report (Katsuya et al., 2012). Subsequent central nervous system invasion of ATL occurs in 10–20% of cases (Bazarbachi et al., 2011). In treatment, many therapeutic agents have been used to improve this poor prognosis. Representative available therapies include intensive multi-agent chemotherapy (Tsukasaki et al., 2012), interferon-a combined with zidovudine (Gill et al., 1995; Hermine et al., 1995), and an anti-CCR4 antibody (mogamulizumab) (Ishida et al., 2012). Hematopoietic stem cell transplantation has recently been reported to achieve long-lasting remission (Zell et al., 2016) and the effectiveness of Tax peptide-pulsed dendritic cell vaccine has been reported (Suehiro et al., 2015).

As a result of its rarity and poor prognosis, very few reports have described ATL-related ocular manifestations (Kohno et al., 1993; Shibata et al., 1997; Kamoi et al., 2016; Hirano et al., 2017), and the details of ocular lesions remain unclear. Given this background of limited information, we conducted a large-scale survey on the state of ophthalmic practice for ATL patients in Japan, where there are a large number of patients with ATL caused by HTLV-1 infection, to analyze and assess ATL-related ocular manifestations.

MATERIALS AND METHODS

All study protocols for this investigation were approved by the ethics review committees of the Tokyo Medical and Dental University and the Institute of Medical Science at the University of Tokyo, in accordance with the tenets of the Declaration of Helsinki. In March 2015, a questionnaire survey regarding the state of ophthalmic medical care for ATL and ATL-related ocular manifestations was conducted on a total of 115 facilities, including all university hospitals throughout Japan that were members of the Japanese Ophthalmological Society and all ophthalmic facilities providing medical care for ocular inflammation that were members of the Japanese Ocular Inflammation Society.

Questions focused on facility locations, classification, experience with ophthalmic care for ATL patients, methods for diagnosing ocular manifestations of ATL, ocular findings observed with ATL-related ocular manifestations, and frequency of ATL-related ocular manifestations (**Figure 1** and **Table 1**). ATL-related ocular manifestations were defined as ocular disorders attributed to ATL. We suggested several expected ATL-related ocular manifestations in consideration of previous reports, and added a blank field to allow respondents to report other manifestations. Considering the rarity of the pathology, relatively few patients with ATL-related ocular lesions were expected to be registered by each facility, so we asked each facility to report all patients registered as showing ATL-related ocular manifestations as of March 2015.

Only valid responses to questions were included in the statistical analyses. If a respondent left a question blank, only that blank response was excluded from analysis. Data on categories pertaining to medical care for ATL-related ocular manifestations were tallied and analyzed.

RESULTS

Of the 115 facilities, 69 (60.0%) responded. Responses were received from facilities throughout Japan, with those in the Kanto region accounting for 39.1% of overall respondents and those in the high-prevalence Kyushu region accounting for 13.0% (**Table 2**). By type of facility, 55.0% of overall respondents were advanced treatment hospitals, 42.0% were hospitals, and 2.9% were clinics.

In terms of experience in providing ophthalmic care to ATL patients, 43.0% of facilities indicated that they have such experience throughout Japan (**Table 3A**). In particular, 88.9% of facilities in the Kyushu region of Southern Japan have experience with ophthalmic treatment for ATL patients, as do 80.0% of facilities in the Hokkaido/Tohoku region of Northern Japan. Looking at central/metropolitan areas, 36.4% of facilities in the Kinki region, 26.9% in the Kanto region, and 14.2% in the Chubu region have such experience.

The survey showed that ATL-related ocular manifestations are most commonly diagnosed "based on blood tests and characteristic ophthalmic findings" (65.0% of facilities), followed by "based on consulting hematologists and ophthalmic examination," which was a specific option provided in the questionnaire (18.3% of facilities), and "based on tests of intraocular fluid and characteristic ophthalmic findings" (16.7% of facilities) (**Table 3B**). Regarding the question of whether differential diagnosis is practiced by excluding other forms of uveitis when diagnosing ATL-related ocular manifestations, 90.6% responded "Yes," and the remaining 9.4% commented "No experience."

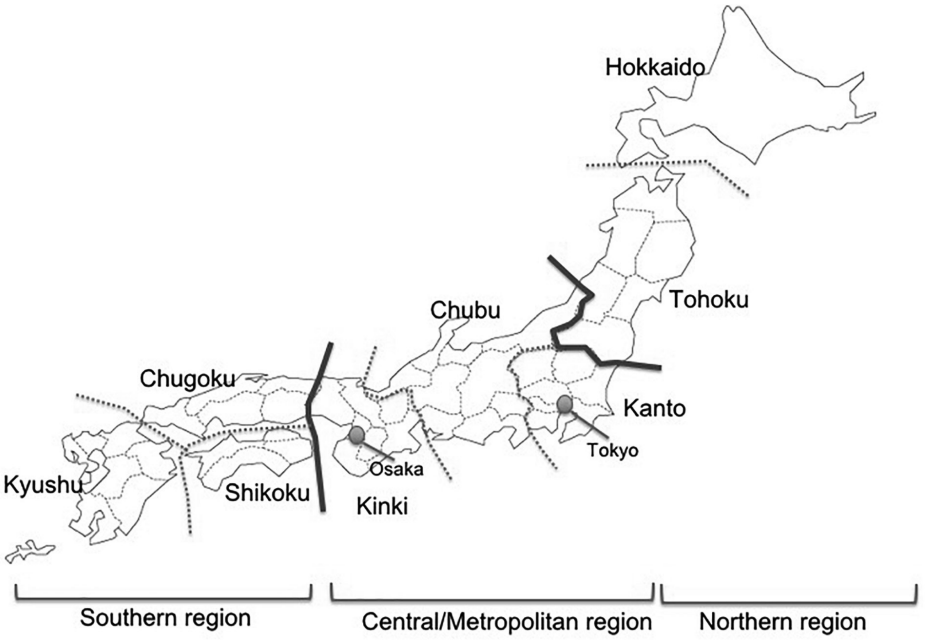

FIGURE 1 | A map showing the regions of Japan. The Kanto and Kinki regions are metropolitan areas in Japan with population growth in recent years due to population movements, and the Kyushu region has a high prevalence of HTLV-1.

TABLE 1 | Questionnaire used for assessing the state of medical care for ATL-related ocular manifestations and ATL-associated ocular findings.

Questionnaire	Answer
(1) Has your department ever provided ophthalmic care for ATL patients?	☐Yes ( ) cases ☐No
(2) How do you diagnose ATL-related ocular manifestations?	☐Based on a blood test (positive for HTLV-1 antibodies) and the characteristic ophthalmic findings ☐Based on a test of intraocular fluid (PCR test) and the characteristic ophthalmic findings ☐Other (Please provide the specifics:)
(3) Do you exclude other forms of uveitis when diagnosing an ATL- related ocular manifestation?	☐Yes ☐No ☐Other (Please provide the specifics:)
(4) What are the findings that have been observed in ATL-related ocular manifestations?	☐Intraocular infiltration ( ) cases ☐Opportunistic infection ( ) cases (Please provide the specifics:) ☐Dry eye ( ) cases ☐Other ( ) cases (Please provide the specifics:)
(5) Do you think the number of ATL-related ocular manifestations is on the rise in recent years?	☐On the rise ☐On the decline ☐Unchanged

Respondents reported 48 cases of ATL-related ocular manifestations. By region, the number of cases reported was highest in the Kanto region (22 cases), followed by the Kinki and Chugoku regions (**Table 4**). The most common type of ATL-related ocular manifestation was intraocular infiltration (22 patients, 45.8%), followed by opportunistic infection (19 patients, 39.6%) and dry eye (3 patients, 6.35%), with scleritis indicated in the blank field (2 patients, 4.2%). Additional responses included a case of uveitis that resolved after steroid treatment (2.1%) and a case of anemic retinopathy (2.1%), which is commonly seen with leukemia. All cases of opportunistic infection involved cytomegalovirus retinitis (CMVR). In addition to CMVR, one case of superinfection with *Toxoplasma* and two cases of herpesvirus were also reported (**Table 4**).

Among the responding facilities, 87.2% indicated that no changes in the number of cases of ATL-related ocular manifestations had been seen in recent years. Increases were reported by 4.3% of facilities, all from the Kanto region. On the other hand, 8.5% of facilities (from the Kyushu, Chugoku, and Kanto regions) reported decreases.

DISCUSSION

With ATL representing an extremely rare form of leukemia with a high mortality rate (Katsuya et al., 2012), most cases of ATL-related ocular manifestation have been reported as ocular lesions in the format of a case report, resulting in an extreme lack of systemic information (Kamoi and Mochizuki, 2012a). Given the large number of HTLV-1-infected individuals in Japan, presumably representing the largest number of ATL patients among advanced nations, we were able to collect information on 48 patients, representing an unprecedentedly large number, in the first large-scale survey conducted in Japan. Our survey focused particularly on investigating which regions of Japan have treated a large number of cases of ocular lesions in ATL patients and what types of ocular lesions are associated with ATL.

TABLE 2 | Number of facilities responding to the questionnaire.

Northern	Central/Metropol Itan			Southern		Total
Hokkaido/Tohoku	Kanto	Chubu	Kinki	Chugoku/Shikoku	Kyushu	
5 (7.2%)	48 (69.5%)			16 (23.2%)		69
5 (7.2%)	27 (39.1%)	9 (13.0%)	12 (17.4%)	7 (10.1%)	9 (13.0%)	

TABLE 3(A) | Experience with medical care for ATL patients.

	Northern (*n* = 5)	Central/Metropolitan (n = 44)			Southern (*n* = 16)		
Experience of medical care for ATL patients	Hokkaido/ Tohoku (*n* = 5)	Kanto (*n* = 26)	Chubu (*n* = 7)	Kinki (*n* = 11)	Chugoku/ Shikoku (*n* = 7)	Kyushu (*n* = 9)	Total
Yes	80.0%	29.7%			75.0%		43.0%
	80.0%	26.9%	14.2%	36.4%	57.1%	88.9%	
No/Un-identified	20.0%	70.3%			25.0%		57.0%
	20.0%	73.0%	85.7%	63.6%	42.9%	11.1%	

TABLE 3(B) | Diagnostic methods for ATL ocular manifestations.

Diagnostic Methods	Northern (*n* = 8)	Central/Metropolitan (*n* = 36)			Southern (*n* = 16)		
	Hokkaido/ Tohoku (*n* = 8)	Kanto (*n* = 20)	Chubu (*n* = 5)	Kinki (*n* = 11)	Chugoku/ Shikoku (n = 6)	Kyushu (*n* = 10)	Total
Blood test and ophthalmic examination	50.0%	69.4%			62.5%		65.0%
	50.0%	70.0%	80.0%	63.6%	83.3%	50.0%	
Intraocular fluid test and ophthalmic examination	37.5%	11.1%			18.8%		16.7%
	37.5%	10.0 %	0.0%	18.2%	0.0%	30.0%	
Consulting Hematologists and ophtalmic examination	12.5%	19.4%			18.8%		18.3%
	12.5%	20.0%	20.0%	18.2 %	16.7%	20.0%	

With regard to distribution, ATL-related ocular manifestations have been treated throughout Japan (**Tables 3A**, **4**), not just in regions with a high prevalence of HTLV-1. A large number of cases were seen in metropolitan areas such as the Kanto region (including Tokyo) and Kinki region (including Osaka). One possible interpretation is to attribute this finding to selection bias due to the nature of the questionnaire survey, as most ATL patients had been reported in southwestern Japan. However, the result might also reflect population movement-associated migration of HTLV-1-infected individuals to urban areas (Uchimaru et al., 2008).

With respect to diagnostic procedures, the survey revealed that a large number of facilities conduct blood tests that include testing for HTLV-1 antibodies to diagnose ATL, then render a diagnosis based on the characteristic ocular lesion. Approximately 20% of facilities conduct polymerase chain reaction (PCR) testing (Mochizuki et al., 2013) of intraocular fluid, representing a more precise method of diagnosis (**Table 3A**). This was attributed to the approval in Japan of PCR tests for intraocular fluid as advanced medical care in 2014.

As part of a measure to control such infections in Japan, pregnant women and blood donors have been screened for HTLV-1 antibodies in recent years (Iwanaga et al., 2009). Despite a decreasing trend in the number of infected individuals, 87.2% of responding facilities reported no changes in the number of cases of ATL-related ocular manifestation, and some responding facilities in the Kanto region have reported increases in the numbers of such cases. ATL develops after a long period of latency, with a high mean age of onset (around 60 years) (Iwanaga et al., 2012). Patients infected with HTLV-1 before the implementation of infection control measures have been

TABLE 4 | ATL-related ocular manifestations.

Manifestations	Number of patients						
	Northern	Central/Metropolitan			Southern		
	Hokkaido/Tohoku	Kanto	Chubu	Kinki	Chugoku/Shikoku	Kyushu	Total
Intraocular infiltration	2		13		7		22 (45.8%)
	2	4	1	8	5	2	
Opportunistic infection	1		15		3		19 (39.6%)
(Cytomegalovirus *)	(1)	(15)	(0)	(0)	(1)	(2)	(19)
(Herpesvirus**)	(0)	(0)	(0)	(0)	(0)	(2)	(2)
(Toxoplasma**)	(0)	(0)	(0)	(0)	(0)	(1)	(1)
Dry eye	0		2		1		3 (6.3%)
	0	2	0	0	0	1	
Scleritis	0		0		2		2 (4.2%)
	0	0	0	0	2	0	
Uveitis	0		0		1		1 (2.1%)
	0	0	0	0	0	1	
Anemic retinopathy	0		1		0		1 (2.1%)
	0	1	0	0	0	0	

**All cases of opportunistic infection were cytomegalovirus retinitis. **In addition to cytomegalovirus retinitis, one case of superinfection with toxoplasma and two cases of herpesvirus were reported.*

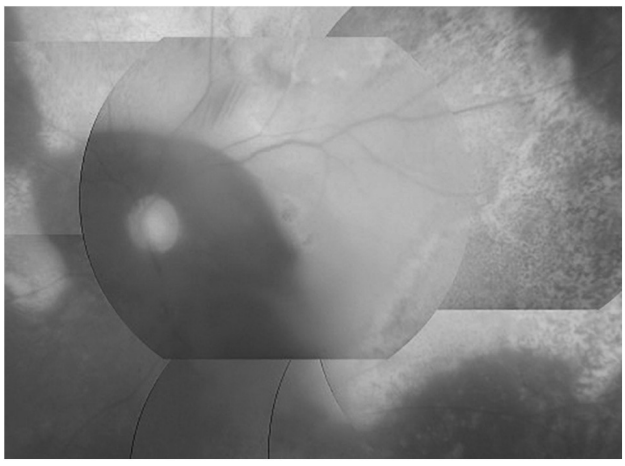

FIGURE 2 | Typical clinical picture of ATL infiltration. Color fundus photograph showing yellowish-white infiltrative foci associated with protrusions in the retina.

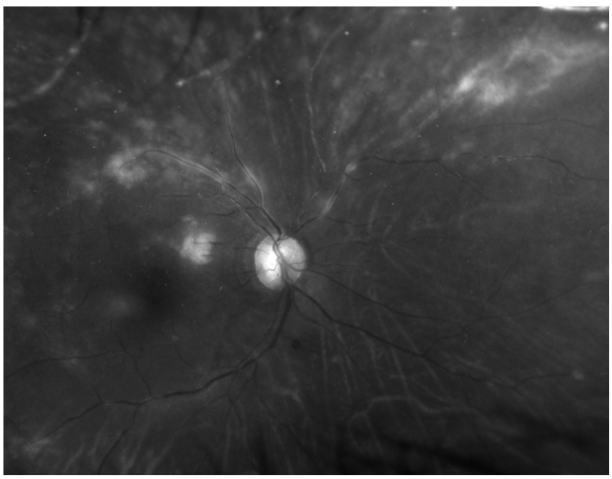

FIGURE 3 | Typical clinical picture of cytomegalovirus retinitis. Color fungus photograph showing cytomegalic cell infiltration and widespread retinal disorganization.

beginning to develop the disease in recent years, so no reduction in such cases is expected in the near future.

As for ocular manifestations of ATL, our review of the literature on ocular lesions revealed case reports of ATL causing intraocular infiltration of ATL cells, with retinal hemorrhage/white patches, optic disk redness, and vitreous opacity as the major symptoms, as well as opportunistic infections associated with immunosuppression (Liu et al., 2010). The majority of the literature comprises case reports, and the

types of ocular lesions that are common in ATL patients have thus remained unclear. The overwhelming majority of the 48 cases of ATL-related ocular lesions reported in this survey involved ocular infiltration (**Figure 2**) or opportunistic infection, with all cases of opportunistic infection being CMVR (**Figure 3**).

In large-scale surveys conducted in the past on acute myeloid leukemia, acute lymphatic leukemia, and chronic myeloid leukemia (Gordon et al., 2001; Russo et al., 2008; Khadka et al., 2014), the rate of ocular infiltration was not particularly high. In this survey, on the other hand, approximately half of the cases of ATL ocular lesions involved ocular infiltration. Compared with other forms of leukemia, ATL cells are thus much more adept at infiltrating the eyes. Recent studies have revealed that HTLV-1-infected T cells expressing C-C motif (CC) chemokine receptor 4 migrate to and infiltrate tissues such as the uvea, skin, parotid glands, and salivary glands, all of which express CC chemokine ligand (CCL) 17/CCL22 (Fukuda et al., 2003; Yoshie and Matsushima, 2015). This suggests that, through this mechanism, ATL cells migrate at a high rate to the uvea, resulting in ocular infiltration, and migrate to and infiltrate the lacrimal glands, causing dry eyes.

As for opportunistic infection, this study identified that all cases involved CMVR. CMV is a herpesvirus that infects 40–100% of adults. The clinical presentation of an active CMV infection often includes retinitis. CMV is well established as the most frequent pathogen of opportunistic infection in ATL patients (Suzumiya et al., 1993; Maeda et al., 2015) and CMV infection occurs more frequently in patients with ATL than in those with other leukemias (Funai et al., 1995). In ATL patients, HTLV-1-infected CD4-positive T cells can transform into malignant cells, losing the normal function of CD4-positive T cells. As a result, cellular immunity is severely impaired, resulting in frequent CMV infection.

The present results need to be considered in light of various limitations. This questionnaire survey asked questions regarding experience in providing medical care for ATL in major facilities throughout Japan. A more streamlined design may thus be needed to raise the response rate. Also, facilities without experience in treating ATL may well have been more likely to submit no response to the questionnaire. While the present results did not provide detailed information such as the main complaints, prognosis of visual acuity, sex ratio, or anatomical sites susceptible to infiltration, the results obtained provide valuable information regarding the medical care of such patients. We hope to work with each of the facilities in the future to further clarify the characteristics of ocular lesions in detail.

With the life prognosis of ATL patients improving in recent years due to improvements in treatment (Hermine et al., 2018; Marino-Merlo et al., 2018), ophthalmologists are increasingly likely to encounter opportunities to provide medical care to ATL patients. Ophthalmologists should keep in mind the high rates of intraocular infiltration and CMVR when examining patients with ATL.

AUTHOR CONTRIBUTIONS

KK designed the study and wrote the draft of the manuscript. AO designed the study. SI, IH, KU, AT, and KO-M contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All authors critically reviewed and approved the final manuscript.

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special article abstract

Revised Adult T-Cell Leukemia-Lymphoma International Consensus Meeting Report

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PURPOSE Adult T-cell leukemia-lymphoma (ATL) is a distinct mature T-cell malignancy caused by chronic infection with human T-lymphotropic virus type 1 with diverse clinical features and prognosis. ATL remains a challenging disease as a result of its diverse clinical features, multidrug resistance of malignant cells, frequent large tumor burden, hypercalcemia, and/or frequent opportunistic infection. In 2009, we published a consensus report to define prognostic factors, clinical subclassifications, treatment strategies, and response criteria. The 2009 consensus report has become the standard reference for clinical trials in ATL and a guide for clinical management. Since the last consensus there has been progress in the understanding of the molecular pathophysiology of ATL and risk-adapted treatment approaches.

METHODS Reflecting these advances, ATL researchers and clinicians joined together at the 18th International Conference on Human Retrovirology—Human T-Lymphotropic Virus and Related Retroviruses—in Tokyo, Japan, March, 2017, to review evidence for current clinical practice and to update the consensus with a new focus on the subtype classification of cutaneous ATL, CNS lesions in aggressive ATL, management of elderly or transplantation-ineligible patients, and treatment strategies that incorporate up-front allogeneic hematopoietic stem-cell transplantation and novel agents.

RESULTS As a result of lower-quality clinical evidence, a best practice approach was adopted and consensus statements agreed on by coauthors (> 90% agreement).

CONCLUSION This expert consensus highlights the need for additional clinical trials to develop novel standard therapies for the treatment of ATL

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INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is an intractable mature T-cell malignancy with diverse clinical features, etiologically associated with a retrovirus designated human T-cell leukemia virus type I or human T-lymphotropic virus type 1 (HTLV-1), which is endemic in several regions, including the southwest region of Japan, Central and South America, central Africa, the Middle East, Far East, central Australia, and Romania.[1,2] Because of population migration, sporadic cases are observed in North America, particularly in New York, NY, and Miami, FL; and Europe, mostly in France and the United Kingdom. Incidence of ATL is rising in nonendemic regions of the world.[3] In 2009, ATL researchers joined together and published an ATL consensus report that has been a standard reference for clinical trials of new agents for ATL and that focused on definition, prognostic factors, clinical subtype classification, treatment, and response criteria.[4]

Since publication, additional progress has been made in the molecular pathophysiology of ATL and risk-adapted treatment approaches.[5] The ATL clinical workshop held during the 18th International Conference on Human Retrovirology—HTLV and Related Viruses—held in Tokyo, Japan, March, 2017 focused on discussion and revision of the 2009 consensus report. Consensus methodology and its limitations are detailed in the Data Supplement.

Some therapeutic agents used in the treatment of ATL are not universally available and treatment strategies will therefore differ among countries, which is reflected in these recommendations (Table 1). For example, mogamulizumab and certain components of the vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP); doxorubicin, ranimustine, and prednisone (AMP); and vindesine, etoposide, carboplatin, and prednisone (VECP) chemotherapy regimen (modified LSG15) are presently unavailable outside of Japan, whereas zidovudine and interferon-alpha are not approved in Japan but can be used in other parts of the world. There is also variability in the availability of positron emission tomography/computed tomography

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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TABLE 1. Recommended Strategy for the Treatment of ATL

Recommendation: Consider All Patients for Inclusion in Prospective Clinical Trials
Asymptomatic smoldering (without skin lesions, opportunistic infections, and so on)
Outside clinical trial
Consider active monitoring
Symptomatic smoldering (skin lesions, opportunistic infections, and so on) and favorable chronic[6-20]
Outside clinical trial
Consider active monitoring or, if available, AZT/IFN-α with or without ATO with or without topical therapies/phototherapy
Where AZT/IFN-α is unavailable and tumorous skin lesions are present, consider chemotherapy* with or without topical therapies/phototherapy followed by allo-HSCT
Where AZT/IFN-α is unavailable and nontumorous skin lesions (patches, plaques, and so on) are present, consider skin-directed therapies and active monitoring
If progressive disease with tumorous skin lesions is present or patient experiences transformation into acute-lymphoma type during active monitoring or AZT/IFN, consider a switch to treatment strategy for aggressive ATL
Unfavorable chronic[10-21]
Outside clinical trial
Where available, consider AZT/IFN-α with or without ATO, continued indefinitely unless progressive disease
Where AZT/IFN-α is unavailable or if the patient experiences progressive disease on AZT/IFN-α, consider chemotherapy* followed by allo-HSCT
Acute[10-14,17-22]
Outside clinical trial
If there are nonbulky lymph nodes/tumors, consider either AZT/IFN-α or intensive chemotherapy* where AZT/IFN-α is available†
Where AZT/IFN-α is unavailable, the patient experiences progressive disease on AZT/IFN-α, or there are bulky acute subtypes, consider intensive chemotherapy*
Consider early up-front allo-HSCT for all eligible patients
Lymphoma-type ATL[10-14,17-22]
Outside clinical trials
Consider intensive chemotherapy* for all patients
Where available, consider concurrent or sequential low-dose AZT/IFN-α maintenance
Consider early up-front allo-HSCT for all eligible patients
Elderly and/or non–transplantation-suitable patients with aggressive ATL[10-12,14,16,22,23]
After first-line therapy (reduced dose of chemotherapy or AZT/IFN, if available), consider maintenance strategies (eg, with oral chemotherapy [etoposide, sobuzoxane, and so on]) or, if available, consider AZT/IFN with or without ATO
Options for relapsed/refractory disease[24-28]
Outside of clinical trials
Single agents or alternative combination chemotherapy regimens containing platinum, etoposide, and/or high-dose cytarabine should be considered.
In Japan, mogamulizumab and lenalidomide are both licensed for use in relapsed/refractory aggressive ATL
Mogamulizumab must be avoided within 50 days of allo-HSCT
Localized radiotherapy is effective for palliation in ATL
Recommended chemotherapy regimens in Japan*
VCAP-AMP-VECP (modified LSG15), which was established in a phase III study (JCOG9801)
EPOCH
Recommended chemotherapy regimens outside of Japan
CHOP, CHOEP, DA-EPOCH
Hyper-CVAD
A Miami version modified LSG15 omits ranimustine and uses vincristine instead of vindesine

Abbreviations: Allo-HSCT, allogeneic hematopoietic stem-cell transplantation; AMP, doxorubicin, ranimustine, and prednisolone; ATL, adult T-cell leukemia-lymphoma; ATO, arsenic trioxide; AZT/IFN, zidovudine/interferon; CHOP, cyclophosphamide, vincristine, doxorubicin, and prednisolone; CHOEP, cyclophosphamide, vincristine, doxorubicin, etoposide, and prednisolone; CVAD, cyclophosphamide, vincristine, doxorubicin, and dexamethasone, alternating with high-dose methotrexate and cytarabine; DA-EPOCH, dose-adjusted etoposide, prednisolone, vincristine, cyclophosphamide, and doxorubicin; VCAP, vincristine, cyclophosphamide, doxorubicin, and prednisolone; VECP, vindesine, etoposide, carboplatin, and prednisolone.

*Recommended chemotherapy options within Japan and outside Japan listed within Table 1.

†Where feasible, identify molecular prognostic markers (eg, p53 mutation, IRF4 expression, and so on).

(PET/CT) and various molecular diagnostic tools, although their usefulness remains mostly unproven. Whereas there is general consensus among experts that the treatments listed are appropriate (> 90% consensus), the level of evidence should be regarded as low or very low unless specifically listed—the equivalent of a GRADE evidence score of C or D, or National Comprehensive Cancer Network (NCCN) 2B—and the treatment recommendations (Table 1) reflect the best practice consensus of expert opinion. The current consensus report is not a guideline as in case of the 2009 consensus.[4] An aim of this report is to recommend good practice where there is a limited evidence base but for which a degree of consensus or uniformity is likely to benefit patient care and may be used as a tool to assist policymakers.

LYMPHOMA TYPE OF ATL, EXTRANODAL PRIMARY CUTANEOUS VARIANT

Cutaneous lesions of ATL are variable and may resemble those of mycosis fungoides (MF), with mostly an indolent course, but some are associated with a poor prognosis. Therefore, ATL should be distinguished from cutaneous T-cell lymphomas, including MF, and peripheral T-cell lymphoma (PTCL), especially in endemic areas, by HTLV-1 serology and genomic analysis as necessary. In a large Japanese retrospective study of ATL with cutaneous lesions, 5-year survival rate was 0% in nodulotumoral and erythrodermic types compared with more than 40% in multipapular, plaque, and patch types.[6] In the 2009 report, primary cutaneous tumoral (PCT) ATL without leukemic, lymph node, and other lesions was frequently included within smoldering ATL and was considered a poor prognostic factor by univariable analyses.[4,7] PCT-ATL is distinct, with cutaneous lesions appearing as tumors that grow rapidly and whose histology shows large, atypical cells with a high proliferative index[7] (Fig 1). In this revision, we agreed that watchful waiting is inappropriate in PCT-ATL as it frequently has a progressive and fatal clinical course that resembles aggressive ATL.[4,7] Recently, Japanese hematologists, dermatologists, and pathologists proposed the entity lymphoma type of ATL, extranodal primary cutaneous variant, which shows a poor prognosis and includes PCT-ATL. Macroscopic findings are mostly nodulotumoral and pathologic findings show high-grade T-cell lymphoma type (pleomorphic, medium, or large size cells) with prominent perivascular infiltration and scant epidermotropism.[8] Such cases could be considered for immediate treatment per

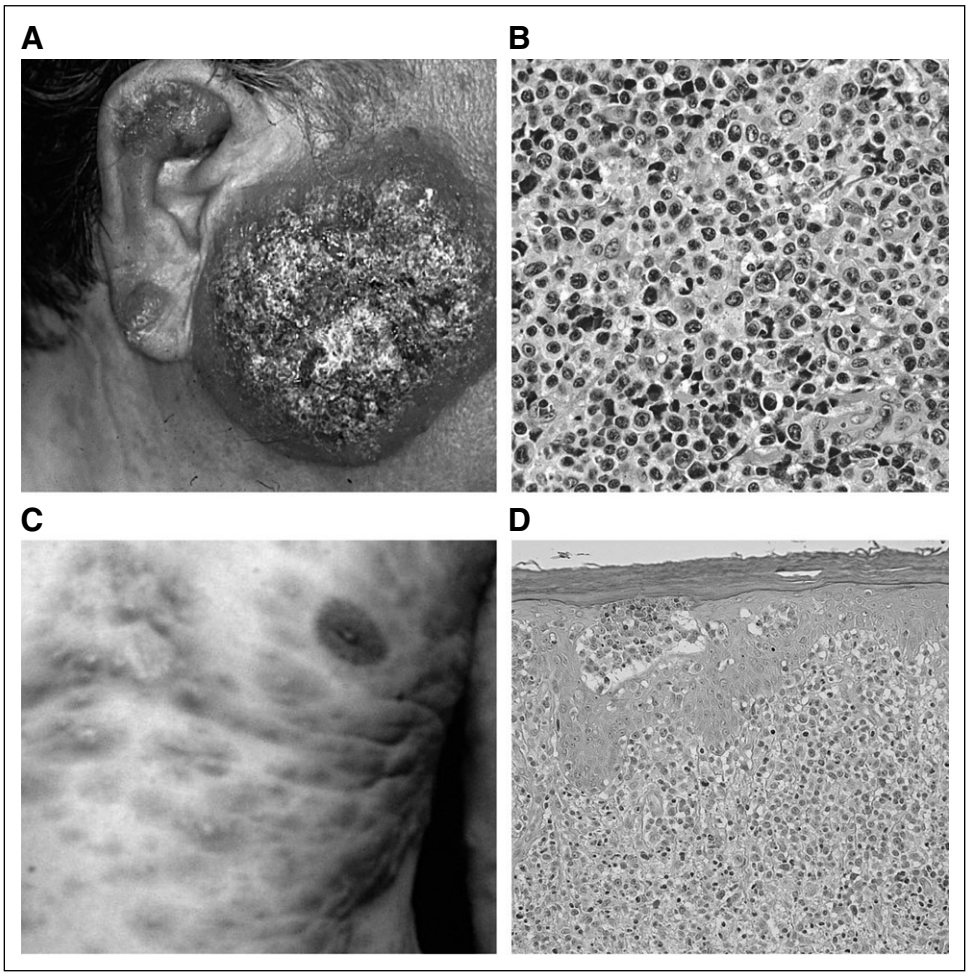

FIG 1. Primary cutaneous tumoral type (PCT) ATL. (A) Facial PCT with (B) histopathology that shows massive infiltration of pleomorphic lymphocytes in the dermis and subcutaneous tissue. (C) Nodulotumors of the chest with (D) histopathology that shows massive infiltration of atypical lymphocytes in the dermis and with epidermotropism.

aggressive ATL protocols with intensive chemotherapy with or without skin-directed therapies, including phototherapy or radiation followed by allogeneic hematopoietic stem-cell transplantation (allo-HSCT; see Allo-HSCT for Agressive ATL section) or interferon with zidovudine (IFN/AZT) with or without arsenic trioxide with or without skin-directed therapies. It should be noted, however, that precisely defining cutaneous tumoral type is difficult—size, height, number, with or without ulceration or subcutaneous extension, and so on—although the nodulotumoral type was defined as nodules or tumors with diameters $>$ 1 cm and the multipapular type as multiple papules with a diameter $<$ 1 cm.[6] Furthermore, other types of cutaneous lesions can be aggressive.[6] Papules, nodules, and tumors are considered as solid, palpable, and raised lesions with a diameter of $<$ 1 cm, $<$ 3 cm, and $\geq$ 3 , respectively. Papules usually occur as multiple lesions, whereas tumor (s) may be seen even as a solitary lesion. Recently, a prognostic index (PI) for chronic- and smoldering-type ATL identified soluble interleukin-2 receptor (sIL-2R) levels as an independent prognostic factor. sIL-2R may therefore be useful but requires additional prospective validation.[9] Additional clinicopathologic and molecular studies are warranted in cutaneous ATL, including the application of recent new agents for MF and PTCL.[8]

Consensus Statements

1. HTLV-1 serology should be undertaken in cases of cutaneous T-cell lymphoma and PTCL, particularly in HTLV-1 endemic regions.
2. Active monitoring is not appropriate for PCT-ATL, and intensive treatment should be considered.

CNS LESIONS OF AGGRESSIVE ATL

Ten percent to twenty percent of patients with aggressive ATL will experience CNS progression.[29] Thus, even in patients without a CNS lesion, it is important to incorporate CNS prophylaxis. Intrathecal prophylaxis was incorporated into sequential Japan Clinical Oncology Group (JCOG) studies for aggressive ATL (chemotherapy trials JCOG9303 and JCOG9801) following both the poor results of earlier study JCOG9109, which did not contain intrathecal prophylaxis,[10-13] and a previous retrospective analysis that revealed that more than one half of relapses that occurred at a new site after chemotherapy occurred in the CNS.[30] CNS involvement occurred in 1.6% of patients in JCOG9109 without intrathecal prophylaxis, in 6.3% of patients in JCOG9303 with intrathecal methotrexate (MTX)/prednisone, and in 3.5% of patients in the VCAP-AMP-VECP arm and 8.2% in the CHOP-14 arm (cyclophosphamide, doxorubicin, vincristine, prednisolone) of JCOG9801, both with intrathecal ara-C/MTX/prednisone(JCOG Prognostic Index and Characterization of Long-Term Survivors of Aggressive Adult T-Cell Leukaemia-Lymphoma [JCOG0902A]).[10-13] The reason for the higher incidence in the JCOG9303 trial compared with the JCOG9109 trial might be associated with simultaneous CSF examination in all patients even without symptoms associated with CNS disease after one cycle of chemotherapy.[10,11] In asymptomatic patients with aggressive ATL, diagnostic lumbar puncture/intrathecal chemotherapy should be performed at the end of the first chemotherapy or equivalent antiviral therapy (AZT/IFN) cycle upon successful disease control.

Options for treating active CNS disease at initial presentation include incorporating high-dose (HD) MTX into combination chemotherapy regimens (eg, CHOP-M or HD-MTX/HD cytarabine) for intracerebral tumors or adding intrathecal chemotherapy to the standard induction chemotherapy, as reported for other aggressive lymphomas.[31,32] However, the scantiness of reports for these options for ATL precludes a more specific recommendation.

Retrospective analysis revealed that allo-HSCT with local treatment of ATL with CNS involvement achieved long progression-free survival (PFS) in four of 10 patients ($>$ 2.5 years) despite high transplantation-related mortality (TRM).[7,33] Numbers of such cases are small and the indication of allo-HSCT for ATL with CNS involvement remains controversial. No specific transplantation conditioning regimen can be recommended, but those that incorporate drugs that can cross the blood-brain barrier (eg, thiotepa) should be considered.

Consensus Statement

1. Prophylactic CNS therapy should be considered for all patients with aggressive ATL.

ALLO-HSCT FOR AGGRESSIVE ATL

The prognosis of aggressive ATL remains dismal with nontransplantation treatments.[1,6-16,21-26,29,34-38] Nontransplantation treatment regimens alone are suboptimal for the majority of patients, although a proportion of patients with aggressive ATL who achieve complete remission after intensive chemotherapy or AZT/IFN therapy may achieve a long ($>$ 5 years) PFS.[13,14] After several promising case series were published,[17-19] several large retrospective analyses of allo-HSCT in Japan reported favorable outcomes, with long-term survival achieved in approximately one third of allo-HSCT recipients after chemotherapy,[20,39] but with significant TRM. The European Society for Blood and Bone Marrow Transplantation registry also demonstrated similar outcomes.[40] Thus, treatment strategies that include allo-HSCT as consolidation are recommended in transplantation-eligible patients with aggressive ATL, although there have yet to be any prospective randomized trials to support this approach. Three PIs for aggressive ATL after chemotherapy have been reported as useful with validation cohorts: JCOG-PI, on the basis of corrected calcium and performance status (PS)[13]; ATL-PI, on the basis of age, serum albumin, sIL-2R,[41] Ann Arbor stage, and PS; and modified ATL-PI, on the basis of corrected calcium, clinical subtype, PS, C-reactive protein, and

sIL-2R level.[39] However, long-term overall survival (OS) in the low-risk group was still poor and a group for whom up-front allo-HSCT might not be recommended could not be clearly identified. A proportion of patients with ATL with localized lymphoma had a long PFS after chemotherapy as well as chronic and a subgroup of acute cases treated with IFN/AZT.[13,14,41] Several reports demonstrated a graft-versus-ATL effect which contributed to long-term relapse-free survival.[19,42,43] ATL with abnormalities in tumor suppressor genes, such as p53, was reportedly resistant to IFN/AZT therapy and chemotherapy.[44,45]

As responses to intensive chemotherapy in general are not durable and long-term continuation of intensive chemotherapy is not feasible because of complications and cumulative toxicities, early allo-HSCT is recommended after response to first-line therapy.[46] Thus, early referral to a transplantation center at diagnosis is strongly recommended, particularly in patients with high-risk features as described.[11,39,41] With progressive disease, clinical outcome after allo-HSCT is poor[47,48] and it is crucial to conduct an up-front allo-HSCT while ATL is controlled to maximize the cure rate. It is recommended that allo-HSCT for progressive disease be performed within the setting of a prospective clinical trial to investigate novel HSCT conditioning regimens or post-HSCT treatment strategies to improve current limited treatment results. When considering allo-HSCT, the standard approach involves searching for an HLA-matched related donor (MRD) or an HLA-matched unrelated donor (MUD) at diagnosis. However, only a proportion of patients will have an MRD. HTLV-1 seronegative donors are also preferred to avoid the risk of donor-derived ATL.[49] When only HTLV-1 seropositive related donors are available, it is recommended to exclude the presence of abnormally abundant HTLV-1–infected clones using Southern blotting or polymerase chain reaction on the basis of clonality methods.[50,51] Furthermore, outside of Japan, HTLV-1 infection often arises in minority immigrant populations, which makes it difficult to obtain a suitable MUD from registry panels.[52,53] In these cases, other possible approaches include cord blood transplantation (CBT) or haploidentical HSCT (haplo-HSCT). Early experience with haplo-HSCT or CBT was unsatisfactory; however, protocols for both CBT and haplo-HSCT have been modified in recent years, although the efficacy of these protocols in ATL is not known. Preliminary anecdotal experience suggests that the TRM remains high, although several reports have suggested that CBT provides results that are similar to other transplantation procedures for selected patients with ATL.[54-56] Although experimental in the ATL setting, the addition of post-transplantation cyclophosphamide to haplo-HSCT protocols has improved transplantation outcomes for other hematologic malignancies. HSCT from alternative donor sources earlier in the disease course with good disease control may increase the potential to achieve outcomes that are similar to MUD transplantation outcomes. Without additional evidence from the results of ongoing trials (Data Supplement), it remains difficult to prioritize one donor source over another when an MRD or MUD is unavailable.

Both myeloablative and reduced-intensity conditioning (RIC) have been used in patients with ATL; however, as the median age in Japan is $>$ 60 years, RIC regimens are increasingly used and sequential prospective trials have revealed the relative safety and promising efficacy of allo-HSCT with RIC.[57-59] Intensity of conditioning should be determined by attendant comorbidities and patient fitness at transplantation.

Although allo-HSCT has the potential to cure ATL, relapse/progression of ATL after allo-HSCT remains a major obstacle and conveys a poor prognosis. In patients with focal relapse (eg, solitary lymph node or skin) radiotherapy alone with or without the reduction of immune suppression or donor lymphocyte infusions can achieve durable disease control.[60] The roles of AZT/IFN in this setting or in prophylaxis to prevent relapse are yet to be determined.

In Japan, where mogamulizumab and lenalidomide are available for the treatment of relapsed/progressed ATL, reports after allo-HSCT remain limited. In patients with aggressive ATL who received mogamulizumab before allo-HSCT, there seems to be a significantly increased risk of severe and steroid-refractory graft-versus-host disease (GVHD).[48,61] In general, if up-front allo-HSCT is planned after induction chemotherapy, intensive chemotherapy without mogamulizumab should be considered as a result of the described risk of GVHD. For relapsed/refractory patients treated with mogamulizumab, the interval between the last salvage mogamulizumab and allo-HSCT should be long (at least 50 days) to decrease drug levels in vivo[48,61] and additional intensification of GVHD prophylaxis should be considered. Data on allo-HSCT after up-front IFN/AZT are limited, but allo-HSCT is generally feasible and recommended for patients with acute ATL.

Pre-emptive treatment after the detection of minimal residual disease post-transplantation should be considered, although methods of monitoring for minimal residual disease of ATL have not been well established after allo-HSCT. In patients in whom HTLV-1 proviral load (PVL) and/or sIL-2R levels begin to increase (no clear threshold exists to detect patients who are at high risk of relapse) chimerism analysis should be considered as well as early taper of immunosuppression and donor lymphocyte infusions.

Kinetic changes in PVL post–allo-HSCT are variable, but three patterns have been observed in patients with full donor chimerism. The first pattern, observed in recipients of allo-HSCT from infected or uninfected donors, was that changes became undetectable after allo-HSCT and remain so. The second pattern, from uninfected donors, was that PVL became undetectable but returned to detectable levels thereafter, usually 6 to 12 months post–allo-HSCT. The third

pattern was in those who had detectable PVL throughout.[59] Strategies have been undertaken in the United Kingdom and France to minimize neoinfection of donor stem cells after allo-HSCT, including the addition of integrase inhibitors, such as raltegravir at engraftment, or close PVL monitoring and the early addition of zidovudine, although there is no published evidence to support this approach.

Consensus Statements

1. Up-front allo-HSCT should be considered for all suitable patients with aggressive ATL.
2. Early referral to a transplantation center is recommended.
3. HTLV-1 seronegative donors are preferred to reduce the risk of donor-derived ATL.
4. Mogamulizumab should not be used within 50 days of allo-HSCT.

ELDERLY AND NON–TRANSPLANTATION-SUITABLE PATIENTS WITH AGGRESSIVE ATL

A retrospective study of elderly patients with aggressive ATL (age > 70 years) observed that elderly patients who were treated with the VCAP-AMP-VECP regimen as initial treatment demonstrated OS that was similar to that in the trial reports of patients age 56 to 70 years.[23] The regimen was modified with dose reductions that were typically between 50% and 80% in 31 (91%) of 34 patients , and overall response rate [complete response (CR) or partial response (PR)] was 75% after two or three cycles of VCAP-AMP-VECP treatment; however, the completion rate of planned chemotherapy was only 19%. Eleven (32%) of 34 patients who achieved objective responses to initial treatment were switched to an oral maintenance chemotherapy regimen that contained oral etoposide or sobuzoxane and/or prednisolone therapy. With the exception of platelet count, there were no significant differences in the background between patients who were treated with the maintenance treatment and those not. Median survival time and 2-year OS rate of those who received maintenance oral chemotherapy were 16.7 months and 33%, respectively, which suggests that such a treatment strategy is a viable option for elderly and/or non–transplantation-suitable patients, warranting prospective trials.[23]

Alternatively, dose-reduced CHOP-14–like regimens could be considered in elderly and/or non–transplantation-suitable patients, as the subgroup analysis of the JCOG9801 trial demonstrated that the OS rate was similar between VCAP-AMP-VECP and CHOP-14 regimens in patients age 56 to 70 years in contrast to the superiority of the VCAP-AMP-VECP regimen in patients younger than 56 years of age with a 45% OS rate at 1 year.[12]

Combination mogamulizumab and chemotherapy (eg, dose-reduced VCAP-AMP-VECP regimen or CHOP like) could be considered as initial therapy for such patients, although the combination was only evaluated in relatively nonelderly patients.[21] Despite the lack of published reports on AZT/IFN outcomes for elderly patients, as with younger patients, this combination should be considered as a first-line option followed by maintenance where available.[14,22]

Given the lack of a preferred or compelling treatment strategy for elderly patients with aggressive ATL, alternative options, such as the selection of less intensive regimens that can be used in a continuous fashion, maintenance strategies using oral agents (single-agent etoposide or cyclophosphamide, etoposide, prednisolone [CEP]), or AZT/IFN, may be appropriate. There may be future roles for monoclonal antibodies (eg, anti-CCR4, anti-CD30, or small molecules, such as lenalidomide) as a maintenance strategy, but these require additional evaluation before recommendations can be made.

Consensus Statement

1. Less intensive induction therapy with or without maintenance therapy with either oral chemotherapy or AZT/IFN may be appropriate for elderly and/or non–transplantation-suitable patients with aggressive ATL.

STRATEGY OF TARGETING THERAPY: PRECLINICAL DATA

Several antiretrovirals used for HIV have demonstrated proven efficacy against HTLV-1 in tissue culture, including the reverse transcription inhibitors zidovudine and tenofovir, as well as the integrase inhibitor raltegravir.[62,63] However, whether these agents work in vivo via an antiviral effect has remained controversial. Several studies have shown little, if any, viral structural, enzymatic, or regulatory gene expression in ATL, with the exception of the antisense gene *HBZ*,[5,64,65] and it remains controversial whether the continued expression of non-*HBZ* viral genes is required for tumor maintenance. However, recent data demonstrate that the extinction of Tax expression leads to total growth inhibition of ATL-derived cells even if Tax protein is undetectable at baseline.[66,67] A possible explanation for these contradictory results is the recent demonstration on the basis of single-cell analysis of transient bursts of Tax oncoprotein, and presumably other viral proteins, which has led to the suggestion that transient Tax expression is essential for ATL survival.[66,67] Thus, immunotherapy against Tax might be theoretically promising. A recent phase I study that used vaccination with Tax peptide-pulsed dendritic cells in three patients with aggressive ATL in PR or stable disease after chemotherapy demonstrated an induction of immune response against Tax with promisingly long-term remission.[68] This warrants larger studies to demonstrate clinical effectiveness.

Although the combination of AZT/IFN has shown activity, it is uncertain whether these drugs function via antiviral activity in cells in the tumor or microenvironment or through mechanisms other than antiviral activity.[69] Of note, doses of AZT used for the treatment of ATL—600 to 900 mg/d and up to 3,000 mg/d in certain reports—are generally higher than those used for HIV treatment (600 mg/d) although the

inhibitory concentrations of AZT for HTLV and HIV replication are similar.[70-72] It is also unclear whether reactivation of HTLV-1 expression in malignant or nonmalignant cells after induction therapy leads to chemotherapy resistance[73]; however, it is notable that a recent chemotherapy trial that included raltegravir demonstrated results that were similar to a trial that lacked antiviral agents during the same induction therapy.[37] Conversely, a recent report demonstrated that the combination AZT/IFN induced a significant inhibition of HTLV-I reverse transcription activity in responding ATL patients but not in resistant patients.[74] These results are in line with a direct antiviral effect, likely in the HTLV-1–infected nonmalignant cells, which may play a major role in the survival of ATL cells.

New insights into the molecular biology of ATL have provided ideas for future clinical trials. Although there was no detectable viral Tax expression in ATL samples, many of the cellular proteins affected by Tax (Tax interactome) were found to be mutated in ATL.[5] These include genes that are involved in T-cell receptor pathway activation, including phospholipase γ, protein kinase Cβ, caspase recruitment domain-containing protein 11, and interferon regulatory factor 4, affecting the nuclear factor κB (NF-κB) pathway, which is activated in ATL and contributes to cell proliferation and resistance to apoptosis. Inhibitors of these pathway mediators, including protein kinase C or NF-κB inhibitors, could be useful in ATL therapy; however, a trial that included bortezomib, as an NF-κB inhibitor with induction chemotherapy, did not provide significant benefit.[37] IRF4 expression could have oncogenic potential in ATL and may be associated with interferon resistance,[75] which warrants therapeutic targeting of its function. Of note, lenalidomide, an active agent in ATL, may function via the inactivation of IRF4 by enhancing the degradation of the IRF4 gene activators Ikaros and Aiolos.[76]

Genomic studies of ATL have revealed high rates of genetic damage compared with other hematopoietic malignancies.[5] In addition, genes that are involved in antigen presentation and immune surveillance are often mutated,[5] including aberrant programmed death ligand-1 expression.[77] These findings suggest that immune checkpoint therapy could play a role in ATL treatment. Currently, a trial of a programmed death-1 inhibitor, nivolumab, is underway in Japan (Data Supplement), although a study in the United States was recently halted as a result of concerns over accelerated disease progression.[38]

NEW AGENTS FOR ATL

Recently, two new agents, mogamulizumab, an anti-CCR4 monoclonal antibody, and lenalidomide, an immunomodulatory drug, have been approved for the treatment of ATL in Japan after pivotal trials for relapsed aggressive ATL[27,28] Mogamulizumab has been also approved for the treatment of newly diagnosed aggressive ATL in combination with intensive chemotherapy.[21]

CCR4 is expressed in neoplastic cells in approximately 90% of ATL cases and mutated in 26%. This expression has been associated with cutaneous manifestations and poor prognosis.[78] In phase I and II studies conducted in Japan, a response rate of approximately 50% was observed with manageable toxicities, including moderate-to-severe skin reactions and other immunopathology, even in nontransplantation patients, possibly by depleting nontumor regulatory T cells.[24,28] CR rates varied among target lesions—rates were high in peripheral blood, intermediate in skin, and low in lymph nodes. Median PFS and OS were 5.2 months and 13.7 months, respectively. The findings of a subsequent randomized phase II study of intensive chemotherapy (modified LSG15) with or without mogamulizumab for the treatment of untreated aggressive ATL has recently been reported.[21] The combination was well tolerated and produced a higher CR rate compared with chemotherapy alone (52% [95% CI, 33% to 71%] *v* 33% [95% CI, 16% to 55%]). However, PFS and OS were identical in both arms, although the sample size and duration of follow-up were relatively short and median OS was not reached in either arm. Recently, a phase II trial in Europe, the United States, and South America of single-agent mogamulizumab (1 mg/kg per week for 4 weeks, followed by 1 mg/kg every 2 weeks until progressive disease) versus investigator's choice of salvage chemotherapy for relapsed aggressive ATL revealed a 15% confirmed overall response rate in the mogamulizumab arm versus 0% for investigator's choice.[25]

A phase I study of lenalidomide demonstrated preliminary antitumor activity in patients with relapsed ATL or PTCL at a higher dose than that used for the treatment of multiple myeloma.[26] A subsequent phase II study evaluated the efficacy and safety of lenalidomide monotherapy at an oral dose of 25 mg/d continuously until progressive disease or unacceptable toxicity in patients with relapsed or recurrent aggressive ATL.[78] Objective responses were noted in 11 of 26 patients [objective response rate, 42% (95% CI, 23% to 63%)], including four patients with CR and one with unconfirmed CR. Rate of disease control, including overall response and stable disease, was 73%. Median time to response and duration of response were 1.9 months and not estimable, respectively. Median PFS and OS were 3.8 months and 20.3 months, respectively. The most frequent grade 3 or greater adverse events were neutropenia (65%), leukopenia (38%), lymphopenia (38%), and thrombocytopenia (23%), which were all manageable and reversible.[26] In conclusion, lenalidomide demonstrated clinically meaningful antitumor activity and an acceptable toxicity profile in patients with relapsed or recurrent aggressive ATL. Lenalidomide also demonstrated activity with CNS involvement by diffuse large B-cell lymphoma,[79] which could have implications for its use in ATL despite the lack of data in the previous trial as CNS involvement was an exclusion criterion.

Other US Food and Drug Administration (FDA)–approved drugs are now available for the treatment of relapsed/refractory PTCL and have been used for ATL in the United States, including the antifolate agent pralatrexate and the histone deacetylase inhibitors belinostat and romidepsin (listed alphabetically). Mogamulizumab has recently received FDA approval in MF or Sézary syndrome after at least one prior therapy (August 2018). Although not FDA approved, current NCCN guidelines support the use of brentuximab vedotin in CD30$^+$ cases (NCCN grade 2A). Pralatrexate was included as an investigator choice drug in the recently completed randomized phase II trial of single-agent mogamulizumab versus salvage chemotherapy for the treatment of relapsed aggressive ATL, but no confirmed responses were reported.[25] Histone deacetylase inhibitors are known to activate HTLV-1 expression; therefore, these drugs might be used with caution in ATL, with consideration given to the addition of an antiretroviral agent (ie, AZT) and under clinical trials desirably.[80] Epstein-Barr virus reactivation in patients with extranodal natural killer/T-cell lymphoma was reported as a previously unrecognized serious adverse event in a pilot study with romidepsin.[81]

Consensus Statement

1. All patients should be considered for entry into clinical trials where available.

PERSPECTIVES

Whereas the backbone of ATL treatment has remained largely unchanged since the 2009 consensus report, there have been several advances in the clinical management of these patients, particularly for patients treated in Japan. These include the increased role of allo-HSCT after first-line treatment and the use of mogamulizumab and lenalidomide as novel single agents, both licensed in Japan. The precise roles of these agents and others, such as brentuximab vedotin for CD30$^+$ ATL, remains incompletely understood. Clinical trials are required to understand the roles of these agents in the front-line setting, including indolent subtypes, in maintenance therapy for transplantation-ineligible patients, in maintenance therapy after allo-HSCT, and in the prevention or treatment of CNS disease. As ATL is characterized by early relapses, clinical trials of therapies that might eradicate minimal residual disease are warranted. Preliminary data from France have shown that combining arsenic trioxide with low-dose interferon is feasible and an effective consolidation therapy that is capable of selectively eliminating the malignant clone and restoring oligoclonal architecture. This has raised hopes that the extinction of viral replication (AZT) and Tax degradation (arsenic/IFN) may eradicate the disease.[15,16,82,83] Addition of arsenic trioxide to IFN/AZT was promising in a phase II study for the treatment of chronic ATL.[16]

Since 2009, there has been more widespread use of PET/CT in the diagnosis and follow-up of patients with non-Hodgkin lymphoma. ATL is frequently associated with extranodal disease, and it might be recommended that PET/CT be used at diagnosis, where available, and that PET/CT assessment should be incorporated in future studies. The significance of negative interim PET/CT should be evaluated as it is not yet clear how this might affect treatment strategy.

Reported prognostic factors at diagnosis (eg, IRF4 expression, TP53 mutation/deletion, and other genetic markers) require validation in the context of treatment with AZT/IFN, chemotherapy, or active monitoring to assist in tailored treatment decisions. New methodologies to detect clonal HTLV minimal residual disease have been established using next-generation high-throughput sequencing methods, and the challenge now remains to validate these observations within clinical trials and bring them into the clinical domain in a rapid, cost-effective manner.

ATL continues to have a dismal prognosis with current therapies, and clinical trials that incorporate molecular and prognostic factors will remain paramount to advances in ATL treatment. We have proposed a strategy for ATL treatment stratified by subtype classification, including an updated opinion on the management of patients with tumorous skin lesions. Future clinical trials should remain a priority to ensure that the consensus is continually updated to establish evidence-based practice guidelines.

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EQUAL CONTRIBUTION

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Revised Adult T-Cell Leukemia-Lymphoma International Consensus Meeting Report

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NOTE

Development of reference material with assigned value for human T-cell leukemia virus type 1 quantitative PCR in Japan

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ABSTRACT

Quantitative PCR (qPCR) of human T-cell leukemia virus type 1 (HTLV-1) provirus is used for HTLV-1 testing and for assessment of risk of HTLV-1-related diseases. In this study, a reference material was developed for standardizing HTLV-1 qPCR. Freeze-dried TL-Om1 cells diluted with Jurkat cells were prepared and an assigned value for proviral load (PVL) of 2.71 copies/100 cells was determined by digital PCR. Nine Japanese laboratories using their own methods evaluated the PVLs of this reference material as 1.08–3.49 copies/100 cells. The maximum difference between laboratories was 3.2-fold. Correcting measured PVLs by using a formula incorporating the assigned value of this reference material should minimize such discrepancies.

Key words human T-cell leukemia virus type 1, proviral load, quantitative PCR, standard.

Human T-cell leukemia virus type 1 was the first human retrovirus to be discovered (1, 2). It has a worldwide distribution with some endemic areas, including Japan (3, 4). HTLV-1 is transmitted through breastfeeding, sexual contact and blood contact, such as transfusion or injection of HTLV-1-positive blood. Some carriers of HTLV-1 develop ATL, HTLV-1-associated myelopathy/tropical spastic paraparesis or

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List of Abbreviations: ATL, adult T cell leukemia; HTLV-1, human T-cell leukemia virus type 1; PVL, proviral load'; qPCR, quantitative PCR.

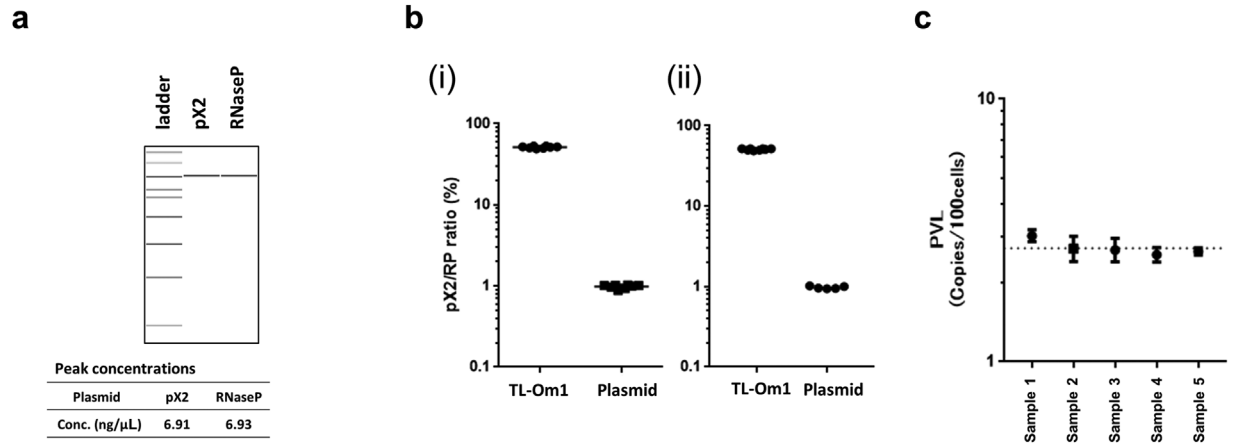

Fig. 1. Digital PCR analysis of reference for HTLV-1 qPCR. (a) Plasmid DNA containing HTLV-1 target sequence (pX2: middle lane) or internal control gene (RNaseP: right lane) were electrophoresed and the bands quantitated using Bioanalyzer. (b) Ratios of HTLV-1 (pX2) to RNaseP in TL-Om1 genomic DNA and 1% w/w plasmid mixture (pX2:RNaseP = 1:100) were analyzed using two types of digital PCR machines, (i) QX100 and (ii) QuantStudio 3D ($n = 8$). (c) PVLs of the reference were measured by droplet digital PCR. Genomic DNA extracted from five different tubes was subjected to droplet digital PCR ($n = 9$). PVLs (copies/100 cells) were calculated as follows: (pX2 copy number × 2)/(RNaseP copy number) × 100. The dotted line (PVL at 2.71 copies/100 cells) is the geometric mean of the five samples.

HTLV-1 uveitis/HTLV-1-associated uveitis after long incubation periods (5). qPCR, which detects the integrated provirus in the peripheral blood of carriers, is a conventional method for measuring the HTLV-1 PVL. Importantly, a high PVL is reportedly one of the risk factors for development of ATL and HTLV-1-associated myelopathy. Accurate PVL quantitation is therefore essential; however, current results differ by around five-fold between laboratories (6).

International standards for nucleic acid amplification tests for blood-borne pathogens such as hepatitis B and C viruses and HIV have been developed by the World Health Organization and made available worldwide by the National Institute for Biological Standards and Control (7–9). However, international standards for nucleic acid amplification tests for HTLV-1 have not yet been developed. Because Japan is one of the endemic areas of HTLV-1, reference material, even of a prototypic format, is urgently needed to enable accurate performance of HTLV-1 qPCR.

We have previously reported that TL-Om1, an HTLV-1 infected cell line derived from ATL, is suitable for creating a reference material for HTLV-1 qPCR and that it would be possible to standardize HTLV-1 qPCR using TL-Om1 (10). In this study, we prepared a freeze-dried cell reference material composed of TL-Om1 cells diluted with Jurkat cells to serve as a temporary reference for HTLV-1 qPCR and evaluated its quality with the collaboration of Japanese laboratories.

Because we had previously found that Jurkat cells are much more viable than frozen peripheral blood mononuclear cells, we selected Jurkat cells for preparation of the reference material (11). TL-Om1 cells were diluted with Jurkat cells at a concentration of approximately 1% and aliquots freeze-dried (see Supporting Materials and Methods). A value was assigned to the PVL of the reference material as determined by digital PCR. First, to confirm the accuracy of the digital PCR result, we measured a test sample made by mixing two plasmids, the first containing a HTLV-1 qPCR target sequence (pX region of HTLV-1 [pX2]) and the other an internal control gene target sequence (RNase P, [RP]). As shown in Figure 1a, the size and amount of the plasmids were confirmed to be the same. The plasmids were mixed at a concentration of 1.0% w/w (pX2/RP) (Fig. 1a). The concentration of the test sample was then measured by droplet digital PCR (QX-100) and chip-based digital PCR (QuantStudio 3D) as 0.98% and 0.99%, respectively (Fig. 1b and Table 1), confirming that the digital PCR results were extremely close to the absolute value. Under these conditions, the PVL of the reference material was determined to be 2.71 copies/100 cells (95% CI, 2.49–3.41) (Fig. 1c).

Table 1. Ratio of pX2 (HTLV-1) to RNaseP (internal control gene) (%) as analyzed by digital PCR

	QX100		QuantStudio 3D	
	Geometric mean	95% CI	Geometric mean	95% CI
TL-Om1	51.3	50.1–52.6	51.0	49.9–52.0
1:100 plasmid	0.98	0.94–1.03	0.97	0.93–1.02

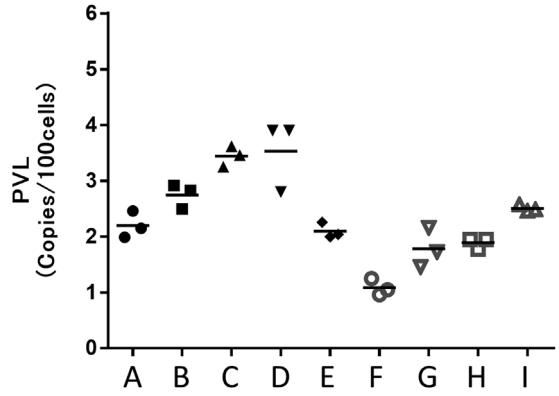

Fig. 2. Proviral loads of the reference measured collaboratively. qPCR was performed independently three times on different days. Horizontal axis: the letters A to I denote the laboratories that participated in this study. Bars, geometric means.

Next, the PVL of the reference material was measured by nine Japanese laboratories using their in-house methods. The results ranged from 1.08 to 3.49 copies/100 cells (Fig. 2 and Table 2), the maximum difference being 3.23-fold between laboratories and the geometric mean of the PVLs being 2.23 copies/100 cells. The ratio to assigned value ranged from 0.40 to 1.29, revealing a wide discrepancy among laboratories, as had been observed previously (11). However, correcting these PVLs by using a formula incorporating the assigned value of the reference material should minimize these discrepancies. To confirm this, PVLs of clinical samples were measured by different laboratories. The geometric coefficient of variation (%) between laboratories was less in most clinical samples after making this correction (Fig. 3 and Table 3). These results indicate that inter-laboratory variations can be minimized by correction with the formula incorporating the assigned value of the reference material. Finally, all participants agreed to

Table 2. PVL as measured by qPCR at nine Japanese laboratories

Laboratory	Geometric mean	95% CI of geometric mean	Ratio to assigned value
A	2.19	1.68–2.86	0.81
B	2.74	2.23–3.37	1.01
C	3.44	3.01–3.94	1.27
D	3.49	2.17–5.62	1.29
E	2.10	1.78–2.47	0.77
F	1.08	0.77–1.51	0.40
G	1.76	1.08–2.87	0.65
H	1.89	1.65–2.16	0.70
I	2.51	2.36–2.66	0.93

the formula and the assigned value (2.71 copies/100 cells) of the HTLV-1 qPCR reference material.

Unlike standards for hepatitis B and C viruses and HIV, the target for HTLV-1 qPCR is not viral particles within plasma, but provirus integrated into the genomes of host cells. Cell-based material rather than serum or plasma is therefore more suitable for use as a HTLV-1 qPCR reference. We used the Jurkat cell line for diluting TL-Om1 cells because, as we have reported previously, the karyotypes of internal control genes are close to 2N, which is useful for preventing discrepancies that may be generated by the use of different internal control genes in different laboratories (10).

In this study, we succeeded in preparing a reference material for HTLV-1 qPCR. Although we made relatively small amounts of this reference material using laboratory equipment, we believe we will be able to provide a continuous supply by renewing the lot periodically. We also plan to evaluate the long-term stability of the lyophilized reference material by regularly measuring its PVL using digital PCR. Inter-laboratory differences in qPCR should be dramatically decreased by correcting results by using the assigned value of the reference. The reference material could also be used to

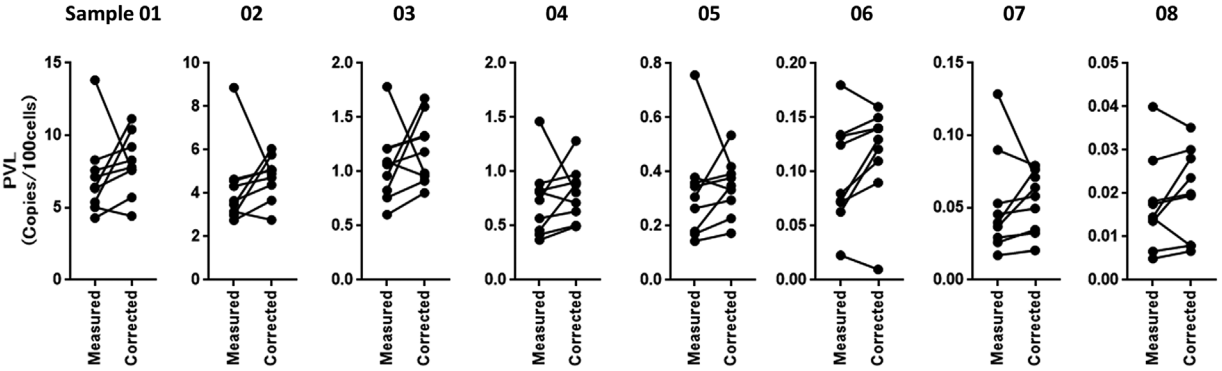

Fig. 3. Proviral loads of clinical samples before and after correction with the assigned value of the reference. Clinical samples with various PVLs were measured by nine laboratories. Dots show PVLs of measured values (left) and corrected values (right). Corrections were made by dividing by the ratio between the measured and assigned values of the reference.

Table 3. Geometric coefficient of variations of clinical samples among laboratories

Sample ID	01	02	03	04	05	06	07	08
Measured values								
PVL (geometric mean)	6.7	4.0	1.0	0.67	0.29	0.086	0.043	0.015
GCV (%)	35%	36%	32%	46%	54%	67%	68%	71%
Corrected values								
PVL (geometric mean)	7.7	4.6	1.2	0.77	0.33	0.097	0.050	0.017
GCV (%)	29%	24%	26%	32%	35%	93%	49%	71%

GCV, geometric coefficient of variations; PVL: copies/100 cells.

minimize discrepancies arising from the use of different methods, such as qPCR and digital PCR. Additionally, it will be useful for preparation of further nucleic acid amplification test standards for HTLV-1.

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DISCLOSURE

The authors declare they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supporting materials and methods. Materials and methods used in this report.
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REVIEW
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HTLV-1-Mediated Epigenetic Pathway to Adult T-Cell Leukemia–Lymphoma

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Human T-cell leukemia virus type 1 (HTLV-1), the first reported human oncogenic retrovirus, is the etiologic agent of highly aggressive, currently incurable diseases such as adult T-cell leukemia–lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 proteins, including Tax and HBZ, have been shown to have critical roles in HTLV-1 pathogenicity, yet the underlying mechanisms of HTLV-1-driven leukemogenesis are unclear. The frequent disruption of genetic and epigenetic gene regulation in various types of malignancy, including ATL, is evident. In this review, we illustrate a focused range of topics about the establishment of HTLV-1 memory: (1) genetic lesion in the Tax interactome pathway, (2) gene regulatory loop/switch, (3) disordered chromatin regulation, (4) epigenetic lock by the modulation of epigenetic factors, (5) the loss of gene fine-tuner microRNA, and (6) the alteration of chromatin regulation by HTLV-1 integration. We discuss the persistent influence of Tax-dependent epigenetic changes even after the disappearance of HTLV-1 gene expression due to the viral escape from the immune system, which is a remaining challenge in HTLV-1 research. The summarized evidence and conceptualized description may provide a better understanding of HTLV-1-mediated cellular transformation and the potential therapeutic strategies to combat HTLV-1-associated diseases.

Keywords: HTLV-1, ATLL, epigenetics, EZH2, gene expression, gene mutations

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INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) infection (Poiesz et al., 1980; Hinuma et al., 1981; Yoshida et al., 1982) is associated with the development of adult T-cell leukemia–lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), although most virus carriers remain asymptomatic throughout their lifespan. ATL is a highly aggressive T-cell malignancy refractory to the currently available combination chemotherapies (Uchiyama et al., 1977; Tsukasaki et al., 2007; Katsuya et al., 2012). HAM/TSP, a debilitating neuro-inflammatory disease, expresses chronic spinal cord inflammation and progressive myelopathic symptoms (Gessain et al., 1985; Osame et al., 1986).

Accumulating evidence has shown that HTLV-1 exhibits complicated involvement in the pathogenesis (Matsuoka and Jeang, 2007; Yamagishi and Watanabe, 2012). In particular, HTLV-1 Tax significantly affects host gene expression and interacts with multiple partner proteins (Boxus et al., 2008; Chevalier et al., 2012; Simonis et al., 2012). Moreover, Tax-transgenic mice develop malignant lymphoma, suggesting that Tax is an oncoprotein (Hasegawa et al., 2006; Ohsugi et al., 2007). The evolution of viral genes with virus expansion indicates that leukemogenesis by Tax is selectively advantageous for viral replication and cell proliferation. Transgenic expression of *HBZ* in $CD4^+$ T-cells also induces T-cell lymphomas and systemic inflammation in mice (Satou et al., 2011). Tax and HBZ certainly contribute to leukemogenesis in HTLV-1-infected T-cells. However, considering the low rate of incidence, clinical observation implies that HTLV-1 lacks a strong capacity to induce leukemogenesis, in contrast to other animal leukemia viruses.

Notably, most leukemic cells do not express viral genes, excluding HBZ (Gaudray et al., 2002; Taniguchi et al., 2005; Satou et al., 2006). Tax, a highly immunogenic protein, is not expressed in most aggressive-type ATL cases because HTLV-1 provirus is substantially silenced by proviral defect and/or an epigenetic mechanism (Tamiya et al., 1996; Koiwa et al., 2002; Taniguchi et al., 2005). It is assumed that this is one of the strategies that viruses use to evade host immune defense.

However, leukemic cells possess similar traits as Tax-expressing cells (Yamagishi and Watanabe, 2012). Although the reason for this seemingly paradoxical observation is yet to be determined, it is suggested that the acquired cellular characteristics, including promoting cell proliferation and apoptotic resistance, is conferred by viral genes in early-phase infected cells and by genetic/epigenetic abnormalities in late-phase, highly malignant ATL cells (**Figure 1**). Although Tax has already disappeared at the time of ATL onset, Tax and its interactome (described in later chapters) have already left multiple genetic and epigenetic memories, contributing ATL onset. This switch during leukemogenesis is indeed supported by transcriptome data; the changes in gene expression in infected cells are dominated by disordered homeostasis and the characteristics of ATL.

Although cancer is typically considered to be a genetic disease, chromatin and epigenetic aberrations as well as active roles of HBZ play important roles in tumor potentiation, initiation, and progression in ATL and HTLV-1-associated diseases. Based on recent findings, we introduce a hypothesis with important implications that might explain the underlying mechanism of the issue: the molecular memories inherited from HTLV-1.

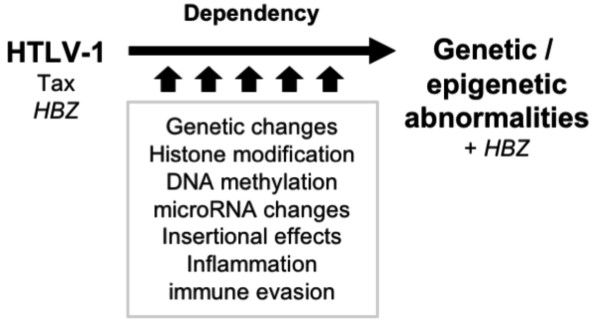

FIGURE 1 | Transition of the molecular characteristics during latent period. The acquired cellular characteristics such as promoting cell proliferation and apoptotic resistance are conferred by viral genes in early-phase infected cells and by genetic/epigenetic abnormalities in late-phase ATL cells. The aberrant characteristics are acquired and imprinted, nevertheless Tax disappears. The consequent genotype and epigenotype support the differential phenotypes and the disease entities.

TRANSCRIPTOME ABNORMALITY IN ATL

Cellular characteristics (i.e., phenotype) are strictly defined by the regulation of gene expression. HTLV-1 Tax directly affects host gene expression through multiple mechanisms, including the binding with host transcription factors and the perturbation of multiple signaling pathways (Ballard et al., 1988; Ruben et al., 1988; Kim et al., 1990; Migone et al., 1995; Good et al., 1996; Takemoto et al., 1997; Boxus et al., 2008). Intriguingly, the molecular hallmarks of aggressive ATL cells at the final stage of progression still comprise pronounced dysregulation of the signaling pathways that control the cell cycle, the resistance to apoptosis, and the proliferation of leukemic cells without Tax expression.

Cell cycle regulation is a typical example of the correlation between gene expression and phenotypic changes. The oncogenic function of Tax was first demonstrated in a study of cell cycle regulation. Tax inhibits cyclin-dependent kinase (CDK) inhibitor, *CDKN2A* ($p16^{INK4A}$), via physical interaction (Suzuki et al., 1996). The mitogenic activity of Tax is exerted through the stimulation of G_1-to-S-phase transition. Additionally, Tax affects a cohort of cell cycle-related proteins, including *CDKs*, *CDKN1A*, *CDKN1B*, and *CDKN2A*, via the regulation of their expression or physical interaction (Akagi et al., 1996; Neuveut et al., 1998; Schmitt et al., 1998; Santiago et al., 1999; Suzuki et al., 1999; de La Fuente et al., 2000; Iwanaga et al., 2001; Haller et al., 2002; Liang et al., 2002).

Comprehensive gene expression profiling revealed that several positive regulators of the cell cycle process are overexpressed in acute-type ATL, in most of which HTLV-1 sense-transcripts and the virus replication is silenced. Diverse abnormalities were also found in each of these comprehensive studies; however, several gene alterations and other critical events have been commonly implicated as the determinants of gene expression pattern. The abnormalities in the expression of different cytokines, their receptors, and various proteins that act as anti-apoptotic factors or proliferating agents are the cellular hallmarks responsible for malignant phenotypes (Tsukasaki et al., 2004; Sasaki et al., 2005; Watanabe et al., 2010; Yamagishi et al., 2012). These notable traits in the transcriptome may be genetically and epigenetically established during long-term latency periods.

ESTABLISHMENT OF HTLV-1 MEMORY

Genetic, metabolic, and environmental stimuli can induce overly restrictive or permissive epigenetic landscapes that contribute to the pathogenesis of cancer and other diseases. The restrictive chromatin states prevent the appropriate expression of tumor suppressors or block differentiation. In contrast, the permissive states allow the stochastic activation of oncogenic genes and stochastic silencing of tumor suppressor genes. The abnormal restriction or plasticity may also affect other processes mediated through factors such as chromatin–DNA repair and telomere maintenance.

Chromatin homeostasis, a basis of molecular memory (Flavahan et al., 2017), is disrupted by genetic and epigenetic stimuli (e.g., inflammation, aging, hypoxia, cell stress, developmental cues, metabolism, and pathogens). The heritable, selective adaptive changes are the hallmarks of cancers. Herein, we introduce the abnormality contributing to the molecular pathogenesis of HTLV-1 infection by tracing the function(s) of Tax and the characteristics of ATL cells.

Genetic Lesion in Tax Interactome Pathway

Tax directly participates in genetic damage (Jeang et al., 1990; Saggioro et al., 1994; Kao and Marriott, 1999; Haoudi et al., 2003). In parallel with this, persistent proliferation, which is boosted by cell cycle progression, may cause genetic instability and create stochastic genetic lesions; ≥ 1 lesions may then act as "drivers," allowing clonal evolution.

The recent advanced technology-based comprehensive characterization of genetic abnormalities delineated the spectrum of genetic alterations in ATL (Kataoka et al., 2015). Genomic data from a total of 426 patients with ATL identified 6,404 mainly age-related somatic mutations (2.3 mutations/Mb/sample) by whole-exome sequencing, including 6,096 single-nucleotide variants and 308 insertions–deletions, strongly suggesting that the clonal expansion of aggressive ATL cells is driven by multiple genetic abnormalities. One of the remarkable indications is that some of the somatically altered genes in ATL (mutation and copy number variation) encode the pivotal molecules that Tax physically interacts with and/or deregulates, including the components of TCR–NF-κB pathway [activated by Tax (Yamaoka et al., 1998; reviewed in Sun and Yamaoka, 2005)] and p53 and p16 tumor suppressors [inactivated by Tax (Suzuki et al., 1996; Grassmann et al., 2005)]; this strongly suggests that ATL cells still depend on the dysregulated Tax interactome even after the disappearance of Tax expression in most ATL cases, i.e., the influence of Tax is genetically imprinted in ATL cells.

Gene Regulatory Loop/Switch

Depending on the cellular status, a transient cue such as an inflammatory cytokine can induce stable malignant transformation through a positive feedback network that is normally held in check by a host defense mechanism (Iliopoulos et al., 2009; Barabási et al., 2011; Yosef and Regev, 2011). In this manner, network motifs, including a coherent feedforward, mutual negative feedback, and positive feedback loops, may switch the cell fate in some cases.

Tax can activate several signaling pathways and lead to an abnormal gene expression pattern. For instance, it can activate NF-κB and NFAT pathways responsible for the predominant expression of IL-2 and its receptor IL2R (Ballard et al., 1988; Ruben et al., 1988; Hoyos et al., 1989; McGuire et al., 1993; Good et al., 1996), whose activation leads to a positive feedback loop. The target transcriptome of NF-κB pathway includes the genes encoding the members of the Rel family, p100/p105, NF-κB-inducing kinase (NIK), and several cytokines that stimulate the same pathway.

Negative regulators within the network are critical for the homeostasis of the regulatory motif. In the developmental process of HTLV-1-infected cells, some NF-κB negative regulators are diminished or inactivated, leading to chronic activation of the signaling pathway. For example, miR-31, a new class of negative regulator of the non-canonical NF-κB pathway, acts by regulating NIK. One mechanism of NF-κB activation without Tax is the epigenetic silencing of miR-31 in HTLV-1-infected cells and aggressive ATL cells (Yamagishi et al., 2012).

Another NF-κB negative regulator, p47, which is essential for Golgi membrane fusion, associates with the NEMO subunit of IκB kinase (IKK) complex upon TNF-α or IL-1 stimulation and inhibits IKK activation. Tax inhibits the interaction between p47 and the IKK complex. In contrast, a significant reduction of p47 expression has been reported in ATL cells, which show a high-level constitutive NF-κB activation that protects ATL cells from apoptosis in a Tax-independent manner (Shibata et al., 2012). These findings indicate that defenseless signaling may cause automatically and chronically activated signaling pathways (Yamagishi et al., 2015), possibly even after the loss of Tax.

Chromatin Regulation

Chromatin is the fundamental medium through which transcription factors, signaling pathways, and various other cues influence gene activity. A dynamic change of the chromatin conformation reinforces regulatory activity or repression at each locus and causes reorganization in response to appropriate intrinsic and extrinsic stimuli.

Genes encoding epigenetic factors, including SWI/SNF complex members and DNA methylation modifiers, are among the most frequently mutated genes in human cancers (Lawrence et al., 2014). However, the genetic changes of such epigenetic factors are less common in ATL, although epigenetic dysregulation such as DNA methylation and histone acetylation is observed at each investigated locus (Nosaka et al., 2000; Tsuchiya et al., 2000; Hofmann et al., 2001; Yasunaga et al., 2004; Yoshida et al., 2004; Yang et al., 2005; Daibata et al., 2007; Taniguchi et al., 2008).

The ATL is also characterized by prominent CpG island DNA hypermethylation, leading to transcriptional silencing (Kataoka et al., 2015). Approximately 40% of the cases showed the CpG island methylator phenotype without any mutation at *TET2*,

IDH2, and *DNMT3A*. Additionally, C2H2-type zinc finger genes (implicated in the suppression of endogenous and exogenous retroviruses) were hypermethylated and silenced. Furthermore, the hypermethylation of MHC-I expression may contribute to immune evasion.

When we consider the chromatin aberrations that confer plasticity, the polycomb family and its substrate histone, H3K27, are of particular interest. EZH2 can repress a wide range of genes by catalyzing the trimethylation of H3K27 (H3K27me3). Regarding the cellular function, EZH2 and H3K27me3 act in a highly context-dependent manner. EZH2 gain-of-function mutations may be oncogenic in a B-cell lineage (Morin et al., 2010; Yap et al., 2011). In addition, an aberrant activation of polycomb repressive complex 2 (PRC2) mainly based on the overexpression of EZH2 is frequently observed in hematological malignancies and solid tumors (Yamagishi and Uchimaru, 2017). In contrast, EZH2 is genetically inactivated in myelodysplastic syndromes (Ernst et al., 2010) and T-cell acute lymphoblastic leukemia (Ntziachristos et al., 2012).

We recently analyzed the pattern of ATL histone modification and integrated it with the transcriptome from primary ATL cells to decipher the ATL-specific "epigenetic code" (Kobayashi et al., 2014; Fujikawa et al., 2016). PRC2-mediated H3K27me3 is significantly and frequently reprogrammed at half of genes in ATL cells. A large proportion of abnormal gene downregulation is observed at an early stage of disease progression, which is explained by H3K27me3 accumulation. Global H3K27me3 alterations involve ATL-specific gene expression changes that include several tumor suppressors, transcription factors, epigenetic modifiers, miRNAs, and developmental genes (Fujikawa et al., 2016), suggesting the diverse outcomes of the PRC2-dependent hierarchical regulation.

Importantly, the Tax-dependent immortalized cells also show significantly similar H3K27me3 reprogramming as that of ATL cells. A majority of the epigenetic silencing occurs in leukemic cells from indolent ATL and in HTLV-1-infected premalignant T-cells from asymptomatic HTLV-1 carriers.

The important implications for deciphering the triggers of the specific histone code are physical interaction and other influences on the host epigenetic machinery by Tax, including the key histone modifiers HDAC1 (Ego et al., 2002), SUV39H1 (Kamoi et al., 2006), SMYD3 (Yamamoto et al., 2011), and EZH2 (Fujikawa et al., 2016).

Epigenetic Lock by Modulation of Epigenetic Factors

The functional classification of genes has revealed that genes epigenetically suppressed by H3K27me3 are enriched in certain biological processes, including transcriptional regulation and histone modifiers, in ATL. Among these, the expression of *KDM6B*, encoding a JMJD3 demethylase of H3K27me3, is significantly downregulated upon H3K27me3 gain (Fujikawa et al., 2016). Because JMJD3 downregulation causes the global accumulation of H3K27me3, ATL cells seemingly acquire a coherent pattern that produces and maintains the systematic abnormality of H3K27me3.

Another coherent pattern is observed in EZH2 regulation. EZH2 is sensitive to promiscuous signaling networks, including NF-κB pathway. Upregulated EZH2 causes excessive PRC2 activity and suppresses multiple target genes such as NF-κB negative regulators (Yamagishi et al., 2012); this forms a positive feedback loop. HTLV-1 Tax is significantly involved in this motif by interacting with EZH2 and activating NF-κB pathway (Fujikawa et al., 2016). Regarding the chronic activation of PRC2 without Tax, an initial triggering event is unnecessary for the maintenance of epigenetic loop.

Loss of Gene Fine-Tuner microRNA

Among the regulators of gene expression, microRNAs are recognized as "buffers" and/or "fine-tuners." MicroRNA can reduce the noise in gene expression; thus, the loss of microRNA may create perturbed gene expression at the post-transcriptional level (Huntzinger and Izaurralde, 2011; Ebert and Sharp, 2012).

One of the key characteristics of ATL is the global downregulation of microRNA (Yamagishi et al., 2012). Although it has not been experimentally demonstrated, the loss of functional small RNA may cause disordered gene expression through transcriptional and post-transcriptional levels. Notably, this global loss is caused and imprinted by HTLV-1-induced H3K27me3 accumulation, suggesting that the global loss of microRNA is one of the processes required for the developmental pathway leading to ATL.

Alteration of Chromatin Regulation by HTLV-1 Integration

Recent advances regarding insertional effects by HTLV-1 have provided critical implications. Satou et al. (2016) found that CTCF (a key regulator of chromatin structure and function) binds to the provirus in the provirus pX region and acts as an enhancer blocker, leading to long-distance interactions with flanking host chromatin. Indeed, HTLV-1 was reported to alter local higher-order chromatin structure and gene expression in the host genome (Satou et al., 2016).

Rosewick et al. (2017) employed stranded RNA-seq data in combination with improved DNA-seq-based high-throughput mapping of integration sites and found that HTLV-1/BLV proviruses are integrated near cancer drivers, which they affect via provirus-dependent transcription termination or as a result of viral antisense RNA-dependent *cis*-perturbation. Remarkably, a similar result was observed at polyclonal non-malignant stages, indicating that provirus-dependent host gene perturbation contributes to the initial selection of the multiple clones characterizing the asymptomatic stage, requiring additional alterations in the clone that will evolve into aggressive leukemia/lymphoma. Although the hotspots of proviral integration sites and the influence of their insertion into the host genome/epigenome

are still being discussed, the previously unrecognized mechanisms may be complementary to viral gene products and the acquisition of somatic alterations in the host genome.

EPIGENETIC LANDSCAPE OF HTLV-1-INFECTED CELLS

The biologist Conrad Waddington first conceptualized developmental fate decision as an epigenetic landscape wherein differentiating cells proceed downhill along the branching canals separated by the walls that restrict cell identity (Waddington, 1957).

Decades of research have revealed that transcription factors are the predominant specifiers of cellular identity (Zaret and Mango, 2016; Bradner et al., 2017). However, the topography of this "hill" seems to be determined by the chromatin pattern, which is directly regulated by epigenetic mechanisms in response to the intrinsic and extrinsic (environmental) stimuli exemplified in this review.

Therefore, as a hypothesis, we propose, in agreement with the established developmental pathway of HTLV-1-infected cells, that disease progression fits with the epigenetic landscape, wherein the height of the walls between the canals is determined by several molecular events (**Figure 2**).

The initial trigger for restricting gene expression is HTLV-1 infection. This violent event significantly affects cell fate, primarily by Tax and HBZ. Then, the immortalized cells possibly undergo several molecular events, as described above (including genetic and epigenetic alterations). During a long period, several aberrant characteristics are acquired and fixed, nevertheless Tax disappears. The consequent genotype and epigenotype support the differential phenotypes and the disease entities of ATL and HAM/TSP.

HTLV-1 provirus is frequently defective or silenced in ATL. However, the lesions recurrently detected in ATL cells imitate the function of Tax and would be stably inherited in the progeny of the malignant cells. This raises several critical possibilities such as that the active imprinting of the viral function into the host genome and epigenome is one of the critical steps of leukemogenesis. Furthermore, the features of ATL cells are not accidental but are the products of HTLV-1 infection. In addition to the sustained roles of HBZ (reviewed in Ma et al., 2016), some crucial outcomes (including gene mutations in the components of TCR–NF-κB pathway and abnormal H3K27me3 accumulation) and many other stochastic events shape ATL cells and their characteristics.

FUTURE DIRECTION

At present, researchers and hematologists are sharing their findings on the characteristics of ATL cells. Additionally, the phenotypic characteristics of HAM/TSP have been studied.

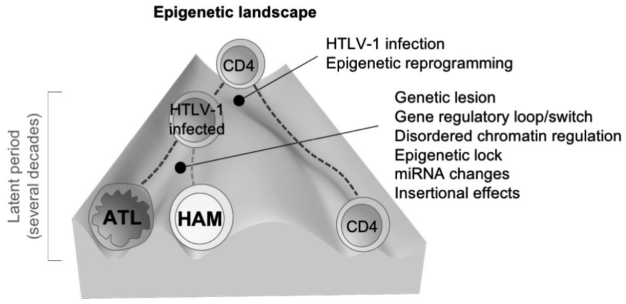

FIGURE 2 | A hypothetical model of developmental pathway in HTLV-1-associated diseases [adapted from Waddington (1957)]. The height of the walls between the valleys (or canals) is determined by several molecular events. The first HTLV-1 infection and the accompanied epigenetic alterations change the cell fate. The permissive state induced by HTLV-1-infection allows following stochastic perturbations such as genetic mutations and dysregulation of the signaling pathways and clonal selection, paralleled by a decrease in transcriptional noise, and the stabilization of cell states (deepening of the valleys).

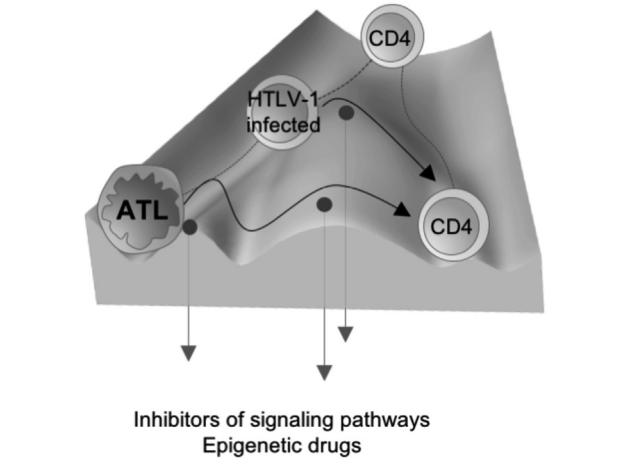

FIGURE 3 | The mechanism-based medicines such as epigenetic drugs and inhibitors of signaling pathways could reprogram the fate of HTLV-1-infected cells (conceptualized as a reduction or elevation of the walls (blue arrows), which promote crossing or bypassing within the epigenetic landscape (black curved arrows) into the normal state).

Considering the therapeutics for the HTLV-1-associated diseases and the need to eliminate the premalignant cell population, the establishment of a precise understanding of disease developmental pathways (routes, branch points, and the events that influence the landscape, as shown in **Figure 2**) is an urgent requirement. Therefore, there is a need to investigate the abnormalities contributing to the molecular pathogenesis, including those in master transcription factors and chromatin regulators. Furthermore, in addition to cellular traits, environmental parameters such as aging, cellular stress, and immune response should be integrated into our model of this process. The order of the molecular events is just a pathway of disease development. HTLV-1 infection and

following epigenetic reprogramming may be an initial step of fate changes.

Intentional regulation such as by inhibitor treatment will reprogram the fate of HTLV-1-infected cells, which can be conceptualized as a reduction or elevation of the walls between the canals in the epigenetic landscape, in line with the analogy mentioned above (**Figure 3**). Realizing the potential of such mechanism-based medicines and advanced diagnostic tools for the detection and evaluation of tumor stage and heterogeneity will require a deeper understanding of epigenetic plasticity and restriction. The road ahead is long but must be challenged to capture this major component of HTLV-1 biology and its associated diseases.

AUTHOR CONTRIBUTIONS

MY conceived and supervised the project, summarized and conceptualized the evidence, and wrote the paper. DF, TW, and KU discussed the new concept.

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Translational Cancer Mechanisms and Therapy

Clinical Cancer Research

CD30 Characterizes Polylobated Lymphocytes and Disease Progression in HTLV-1–Infected Individuals

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Abstract

Purpose: Although expression of CD30 is reported in a subset of adult T-cell leukemia/lymphoma cases, its clinicopathologic significance is poorly understood. We aimed to characterize CD30-positive cells and clarify their tumorigenic role in human T-cell lymphotropic virus type 1 (HTLV-1)–infected cells.

Experimental Design: CD30-positive peripheral blood mononuclear cells from individuals with differing HTLV-1 disease status were characterized, and the role of CD30 signaling was examined using HTLV-1–infected cell lines and primary cells.

Results: CD30-positive cells were detected in all samples examined, and the marker was coexpressed with both CD25 and CD4. This cell population expanded in accordance with disease progression. CD30-positive cells showed polylobation, with some possessing "flower cell" features, active cycling, and hyperploidy. CD30 stimulation of HTLV-1–infected cell lines induced these features and abnormal cell division, with polylobation found to be dependent on the activation of PI3K. The results thus link the expression of CD30, which serves as a marker for HTLV-1 disease status, to an active proliferating cell fraction featuring polylobation and chromosomal aberrations. In addition, brentuximab vedotin, an anti-CD30 monoclonal antibody conjugated with auristatin E, was found to reduce the CD30-positive cell fraction.

Conclusions: Our results indicate that CD30-positive cells act as a reservoir for tumorigenic transformation and clonal expansion during HTLV-1 infection. The CD30-positive fraction may thus be a potential molecular target for those with differing HTLV-1 disease status. *Clin Cancer Res; 24(21); 5445–57.* *©2018 AACR.*

Introduction

Adult T-cell leukemia/lymphoma (ATL) is a T-cell neoplasm with a poor prognosis that is caused by human T-cell leukemia/lymphoma virus type I (HTLV-1) infection. HTLV-1 is transmitted mainly through breast-feeding, and it is estimated that 20 million carriers exist worldwide. ATL develops after about 50 years of clinical latency in 2% to 5% of HTLV-1 carriers. The emergence of malignant cells with polylobated nuclei (the typical appearance is termed "flower cells") characterizes ATL; however, these cells are already present in HTLV-1 carriers without development of ATL (1).

CD30, a member of the tumor necrosis receptor superfamily, activates prosurvival signals by ligation of its ligand CD30L or by CD30 overexpression (2, 3). CD30 activates nuclear factor (NF)-κB, ERK MAPK, and p38 MAPK (4–6). Previous reports showed CD30 expression in some ATL cells (7, 8). However, the clinicopathologic significance of CD30 in HTLV-1–infected cells is unknown.

Transformation of HTLV-1–infected T cells *in vivo* is a multistage process, which reflects the status of HTLV-1 infection, i.e., asymptomatic, smoldering, chronic, or acute (9). These cells acquire a transformed phenotype independent of HTLV-1 during this process (1, 10), indicating the importance of molecular mechanisms that occur during this step for tumorigenesis.

In this report, we aimed to elucidate the clinicopathologic significance of CD30 in HTLV-1–infected cells. We present a series of supportive evidence suggesting that CD30 is involved in the generation of abnormal lymphocytes with "flower cell," which expands during the progression of carrier status and features an actively cycling virus-infected cell population, which causes chromosomal aberrations in HTLV-1–infected cells. We further discuss the significance of our finding in understanding the tumorigenic process of HTLV-1–infected cells and the therapeutic implication for the treatment of HTLV-1–infected individuals.

Materials and Methods

Cells

Primary cells, which were collected with informed consent as part of a collaborative study with the Joint Study on Predisposing

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Adult T-cell leukemia/lymphoma has a poor prognosis despite recent treatment strategies with new agents combined to conventional chemotherapies. Transformation of human T-cell leukemia/lymphoma virus type I (HTLV-1)–infected T cells is a multistage process that reflects the status of HTLV-1 infection, i.e., asymptomatic, smoldering, chronic, or acute. To develop new treatment strategies therefore, identification of the cellular fractions involved in disease progression is urgently required. We examined the significance of CD30 expression in the peripheral blood of individuals with differing HTLV-1 disease status and found that CD30 is responsible for the proliferation and expansion of HTLV-1–infected cells, characterized by polylobation and chromosomal aberrations. CD30-positive cells thus appear to act as a reservoir for transformation during HTLV-1 infection, and we found that brentuximab vedotin reduces this cell fraction. Our findings indicate that CD30-positive cells may be a therapeutic target for the prevention of disease progression in those with HTLV-1 infection.

Factors of ATL Development (JSPFAD), were used (11). The experiments and their analyses using the primary cells were performed at the University of Tokyo and were approved by its research ethics committee. Separation of mononuclear cells from the peripheral blood (PB) was performed as described (12). Clinical data of HTLV-1–infected individuals used are presented in Supplementary Table S1. Additional samples used for Supplementary Data were listed in Supplementary Table S2. All patients were categorized according to Shimoyama's criteria (9). MT-2 and HUT-102 are HTLV-1–infected T-cell lines (13, 14), and Jurkat and CEM are HTLV-1–uninfected T-cell lines. These cells were obtained from the RIKEN BioResource center. Karpas299, an anaplastic large cell lymphoma (ALCL) cell line, was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). Cells were cultured in RPMI1640 with 10% FCS, unless indicated. CHO cells were obtained from DS Pharma Biomedical and were cultured in Ham's F12 (Wako) with 10% FCS. TIG-1 cells and fetal lung fibroblast were obtained from Japanese Collection of Research Bioresources Cell Bank. These cells were used for the primary culture of HTLV-1–infected cells (15).

Chemicals

Inhibitors used to identify the pathway involved in the CD30-mediated generation of cells with polylobated nuclei were as follows: Ly294002 (Cell Signaling Technology) and duvelisib (Selleck Chemicals) for PI3K, UO126 for ERK kinase (MEK), SB203580 for p38MAPK (both from Cell Signaling Technology), and BMS-345541 for NF-κB (Calbiochem). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) prior to use. Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Laboratories and dissolved in DMSO.

Flow cytometry

For analysis of the expression of cell surface molecules, primary cells (3×10^4 cells) were analyzed as previously described after incubation with fluorochrome-conjugated primary antibodies for 30 minutes at 4°C, using FACS Aria or Verse (both from BD Biosciences) and Flowjo software (TreeStar; ref. 16). To detect the expression of Ki-67, the cells were fixed using IntraPrep (Beckman Coulter) according to the manufacturer's instructions before incubation with antibodies as described (17), and the cells (3×10^4 cells) were analyzed using FACS Verse. Cell-cycle and ploidy analyses of primary cells were performed as previously described, and the number of the cells analyzed by flow cytometry was indicated in the figure legend (18). The following fluorochrome-conjugated primary antibodies were used: anti–CD4-FITC, anti–CD4-Pacific Blue, and anti–CD25-APC (all from Biolegend), anti–CD30-PE, control IgG-FITC, and control IgG-PE (all from Beckman Coulter). For detection of Ki-67, anti–Ki-67 antibody (Santa Cruz Biotechnologies) and anti-rabbit secondary antibody conjugated with Alexa488 (Thermo Scientific) were used. For detection of HTLV-1–infected cells, anti-cell adhesion molecule 1 (CADM1) antibody (CM004-3, MBL) was used.

Measurement of $CD30^+$ cells in the PB of individuals with different status of HTLV-1 infection

The PB of randomly selected individuals with different status of HTLV-1 infection, i.e., asymptomatic (Asy#1-22), smoldering (Smo#1-8), chronic (Chr#1-7), or acute (Acu#1-7; $N = 44$; Supplementary Table S1), was examined by flow cytometry as described. Their clinical data are presented in Supplementary Table S1. The $CD30^+CD25^+CD4^+$ subpopulation was calculated as either a percentage of $CD4^+$ cells or as an absolute number per μL of blood. The values between the different statuses were statistically evaluated. The scatter plot was presented, and Pearson's product moment correlation coefficient was calculated.

Immunostaining

Peripheral blood mononuclear cells (PBMC) were separated using flow cytometry according to the expression of cell surface molecules and were sorted using FACS Aria as described (12). The primary cells or cell lines were cytospun onto a slide glass, fixed with 4% paraformaldehyde, and immunostained with anti–Ki-67 antibody (Santa Cruz Biotechnologies) and anti-rabbit secondary antibody conjugated with Alexa488 (Thermo Scientific) as previously described (12). The nuclei were stained with DAPI. The staining was analyzed using fluorescence microscopy (BX50F, Olympus).

Measurement of HTLV-1 provirus load

Measurement of the HTLV-1 provirus load was performed as described (12). Briefly, genomic DNA was extracted from sorted cells using a DNA blood mini kit (Qiagen), and multiplex real-time PCR was performed with TaqMan probes. Primer pairs for HTLV-1 pX and RNase P enzyme (control) (Applied Biosystems) were used. The calculated provirus loads represent copy numbers per 100 cells. pX-probe(FAM) 5′-CTGTGTACAAGGCGACTGG-TGCC-3′, pX2-sense 5′-CGGATACCCAGTCTACGTGTT-3′, pX2-antisense 5′-CAGTAGGGCGTGACGATGTA-3′, Taqman RNase P Control Reagents Kit (Product Number-4316844).

Preparation of stable cell transformants

CD30L cDNA was inserted into the CSII-EF1 vector (kindly provided by Dr. H. Miyoshi, RIKEN BioResource Center) for the expression of mCherry-CD30L fusion protein. This plasmid or its control without CD30L cDNA was transduced into CHO cells as described (12), and mCherry-positive cells were sorted using

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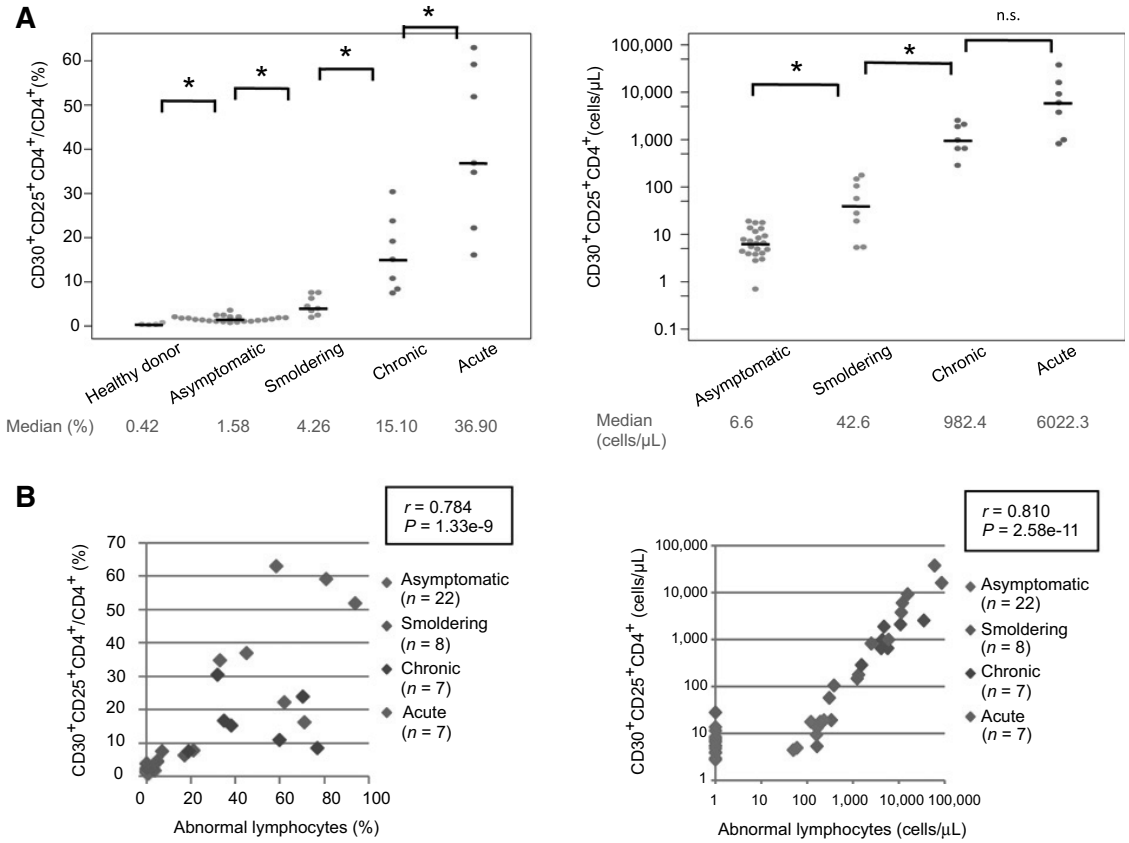

Figure 1.

CD30-positive cells in various statuses of HTLV-1 infection and their correlation with emergence of abnormal lymphocytes. **A,** Distribution map of $CD30^+CD25^+$ cells in the $CD4^+$ lymphocyte fraction of the PBMCs of HTLV-1–infected individuals with varying disease status. PBMCs isolated from the PB were analyzed using flow cytometry. Healthy donors served as control. The vertical and horizontal lines indicate the percentage of $CD30^+CD25^+$ cells in the $CD4^+$ population (left) or their absolute number (right) and the disease status of HTLV-1 infection, respectively. The black horizontal lines indicate the median number of $CD30^+CD25^+$ cells in the $CD4^+$ fraction, and their values are indicated in the red font below the map. Asterisks on the top indicate statistical significance ($P < 0.05$). n.s., not significant. **B,** Correlation of $CD30^+CD25^+CD4^+$ cells and abnormal lymphocytes in the PBMCs of HTLV-1–infected individuals with varying disease status. The vertical and horizontal lines indicate the percentage (left) or the absolute number (right) of $CD30^+CD25^+CD4^+$ cells and abnormal lymphocytes. *n* indicates the number of HTLV-1–infected individuals examined. Each plot is colored depending on the status of HTLV-1 infection as indicated on the right. Because cases with abnormal lymphocytes of 0/μL cannot be plotted on a logarithmic scale, these cases were plotted at the leftmost terminal on the horizontal axis for reference. The correlation coefficient and *P* value calculated by the statistical package R version 2.9.0 (available as a free download from http://www.r-project.org) are indicated on the top.

FACS Aria (BD Biosciences). The expression of transfected CD30L in CHO cells and endogenous CD30 in T-cell lines was confirmed using flow cytometry (Supplementary Fig. S1). The resultant transformants were indicated as CD30L/CHO and mock/CHO, respectively. For fluorescent detection of the nucleus, Histone H2B-GFP construct in the CSII-EF1 vector was transduced into HUT-102 and MT-2 cells as previously described (12), and GFP-positive cells were sorted using FACS Aria.

Stimulation of CD30-positive cells with CD30L

CHO cells with or without CD30L expression (5×10^4 cells each per well of a 12-well plate) were precultured in Ham's F12 (Wako Pure Chemical Industries) containing 10% FBS and were used for stimulation of CD30-expressing cells after 24 hours of culture. CD30-expressing cells (1.5×10^5 cells) were cultured with the CHO cells in media containing an equal volume of RPMI1640 plus Ham's F12. After incubation, the cells were harvested, washed with PBS, and were then further analyzed. Unless indicated otherwise, media were supplied with 10% FBS, and the stimulation time was 96 hours. When the inhibitors were used, they were added to media containing an equal volume of RPMI1640 plus Ham's F12 and cultured for 48 hours. CD30-expressing cells were harvested and reseeded into the new CHO cells already cultured for 24 hours, and fresh media with corresponding inhibitors were used for additional culture for 48 hours.

Treatment of primary cells with brentuximab vedotin

PBMCs (2.5×10^5) separated from HTLV-1–infected individuals were cultured with 200 μL of RPMI1641 containing 10% calf serum unless indicated, antibiotics, and 10 ng/mL recombinant human IL2 (R&D Systems, Inc.). The cells were treated

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with 10 μg/mL brentuximab vedotin (kindly obtained from Millennium Pharmaceuticals, Inc., an owned subsidiary of Takeda Pharmaceutical Company Limited) or vehicle (Milli-Q water) for 10 days. The harvested cells were analyzed as previously described, following incubation with fluorochrome-conjugated primary antibodies for 30 minutes at 4°C, using the FACS Aria or Verse (BD Biosciences) and FlowJo software (TreeStar; ref. 16).

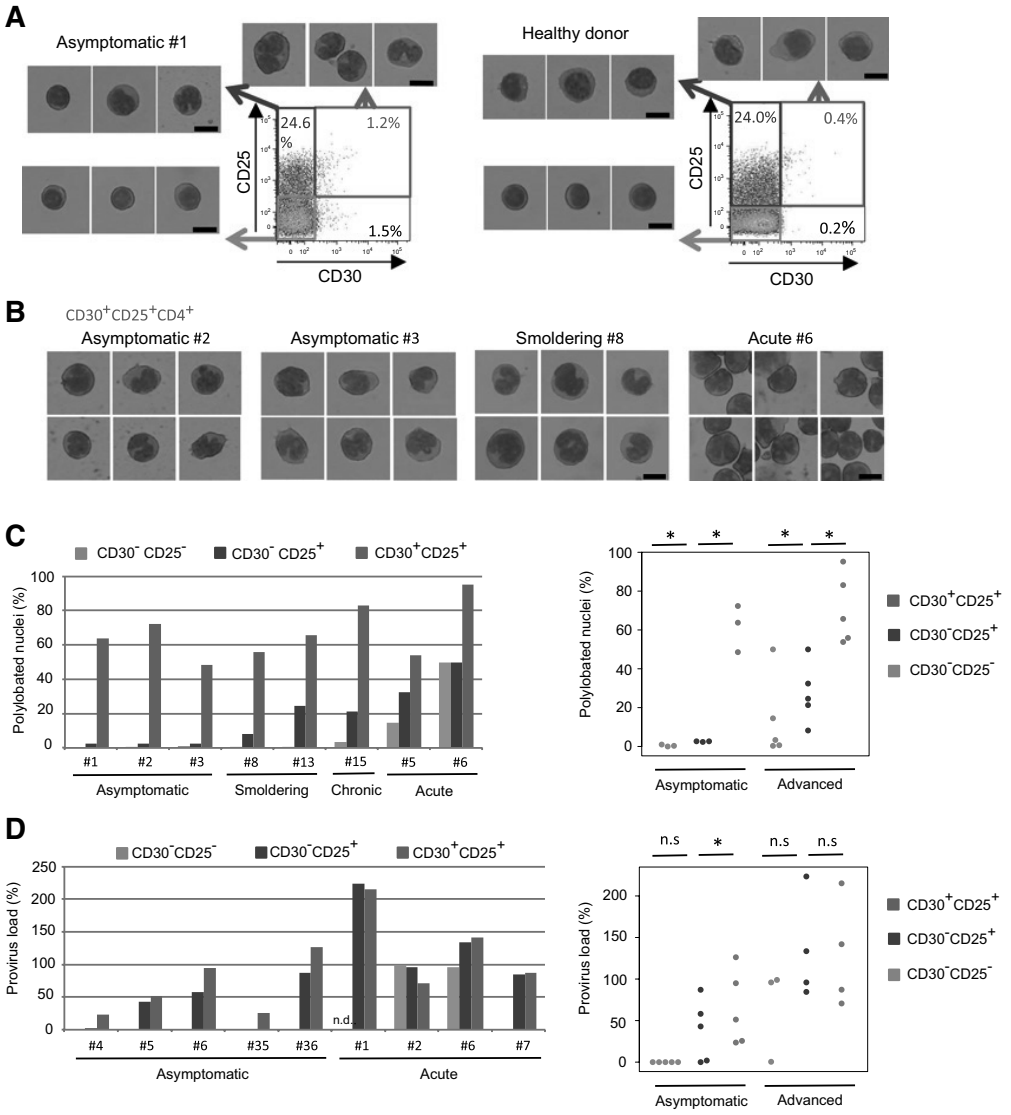

Figure 2.

Morphologic characteristics of CD30-positive cells and their HTLV-1 virus load. **A,** Morphologic appearance of representative $CD4^+$ lymphocytes from each of the lymphocyte fractions in an asymptomatic carrier (Asy#1) that were separated by CD30 and CD25 expression. Each fraction was analyzed by flow cytometry, sorted, cytospun, and stained using the Giemsa method. Analyses of Asy#1 (left) and a healthy donor (right) are shown. The percentage of cells in each fraction is shown in the graph. Black bars, 20 μm. **B,** The abnormal lymphocytes in $CD30^+CD25^+CD4^+$ cells in additional cases. Two asymptomatic carriers (Asy#2 and Asy#3), 1 smoldering case (Smo#8), and 1 acute case (Acu#6) were analyzed as described in Fig. 2A. The abnormal lymphocytes in $CD30^+CD25^+CD4^+$ cells are presented. Black bars, 20 μm. **C,** Percentage of polylobated cells in $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in the $CD4^+$ lymphocyte population of the PBMCs of three asymptomatic carriers (Asy#1-3), two smoldering (Smo#8 and 13), one chronic (Chr#15), and two acute (Acu#5 and 6). The bar graph and the beeswarm graph are presented on the left and right, respectively. The advanced stage includes smoldering, chronic, and acute cases. Asterisks indicate statistical significance ($P < 0.05$). **D,** Provirus load of $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in the $CD4^+$ population of 5 asymptomatic carriers (Asy#4, 5, 6, 35, and 36) and 4 acute cases (Acu#1, 2, 6, and 7). The bar graph and the beeswarm graph are presented on the left and right, respectively. The value indicates the number of infected cells per 100 cells in each fraction. The value more than 100% suggests existence of cells bearing plural copy number of HTLV-1. Provirus load of the $CD30^-CD25^-$ fraction of Acu#1 was not determined and indicated as n.d.

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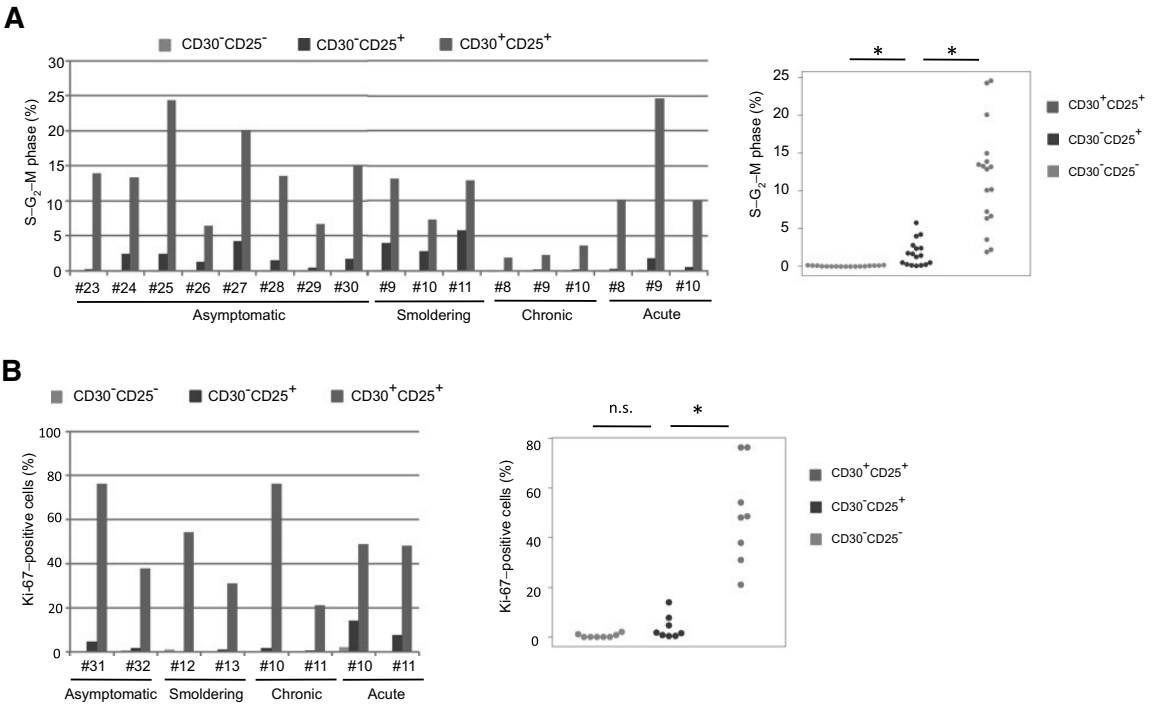

Figure 3.

Cell-cycle status of CD30-positive cells in HTLV-1–infected individuals. **A,** Cell-cycle analysis of $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in the $CD4^+$ population. The PBMCs of 8 asymptomatic carriers and of 3 each of smoldering, chronic, and acute cases were analyzed, and the percentage of cells in the S–G_2–M stage of each cell fraction is shown as a bar graph (left). The beeswarm graph of the S–G_2–M stage of each cell fraction is presented (right). Asterisks indicate statistical significance ($P < 0.05$). Original data of an asymptomatic carrier (Asy#23) and an acute case (Acu#8) obtained by flow cytometric analysis are presented as examples in Supplementary Fig. S5. **B,** Analysis of Ki-67 expression in $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in the $CD4^+$ population. Percentage of Ki-67–positive cells in $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in the $CD4^+$ lymphocytes of the PBMCs of 2 each of asymptomatic (Asy#31 and 32), smoldering (Smo#12 and 13), chronic (Chr#10 and 11), and acute (Acu#10 and 11) cases is shown as a bar graph (left). Each fraction was sorted by flow cytometry. The cells were then cytospun onto slides and stained with the anti–Ki-67 antibody. The nuclei were stained with DAPI, and the cells were analyzed using fluorescence microscopy (BX50F, Olympus). The bar graphs represent 3 independent counts of 200 cells in each fraction. The beeswarm graph of the Ki-67–positive cells in each cell fraction is presented (right). Asterisks indicate statistical significance ($P < 0.05$). n.s., not significant. Original data of a smoldering (Smo#12, left) and a chronic (Chr#10, right) case are presented as examples in Supplementary Fig. S6.

Statistical analysis

Differences between mean values were assessed using a two-tailed *t* test. A *P* value < 0.05 was considered to be statistically significant. Correlations were evaluated using Pearson correlation coefficient.

Results

The $CD30^+CD25^+CD4^+$ subpopulation expands during progression of HTLV-1 carrier status

HTLV-1 infects $CD4^+$ lymphocytes, and these cells express CD25. To elucidate the expression of CD30 in HTLV-1–infected individuals, we first examined $CD30^+$ cells in the PB of individuals with different status of HTLV-1 infection, i.e., asymptomatic (Asy#1-22), smoldering (Smo#1-8), chronic (Chr#1-7), or acute (Acu#1-7; $N = 44$; Supplementary Table S1). The $CD30^+CD25^+CD4^+$ subpopulation within PB, calculated as either a percentage of CD4-positive cells or as an absolute number per μL of blood, increased according to progression of the status of HTLV-1 infection (Fig. 1A). A previous report indicated that changes of the serum soluble interleukin-2 receptor (sIL2R) correlate with disease progression in HTLV-1–infected individuals (19). Therefore, we evaluated the relationship between the CD30 expression and sIL2R (Supplementary Fig. S2). The result indicated correlation between CD30 expression and sIL2R. The expansion of the $CD30^+CD25^+CD4^+$ subpopulation correlated with the expansion of abnormal lymphocytes according to progression of the status of HTLV-1 infection (Fig. 1B).

The $CD30^+CD25^+CD4^+$ subpopulation is characterized by abnormal lymphocytes with polylobated nuclei in HTLV-1–infected individuals

To characterize $CD30^+CD25^+CD4^+$ cells, we next examined the morphologic features of these cells in the HTLV-1–infected individuals shown in Supplementary Table S1 (Asy#1-3, Smo#8 and 13, Chr#15, and Acu#5 and 6). Cells with polylobated nuclei, some of which possessed flower-like features, were frequently found in the $CD30^+CD25^+CD4^+$ subpopulation of asymptomatic carriers. Although these cells were also found in the $CD30^-CD25^+CD4^+$ subpopulation, their frequency was very low. Representative cell morphology and the proportion of each

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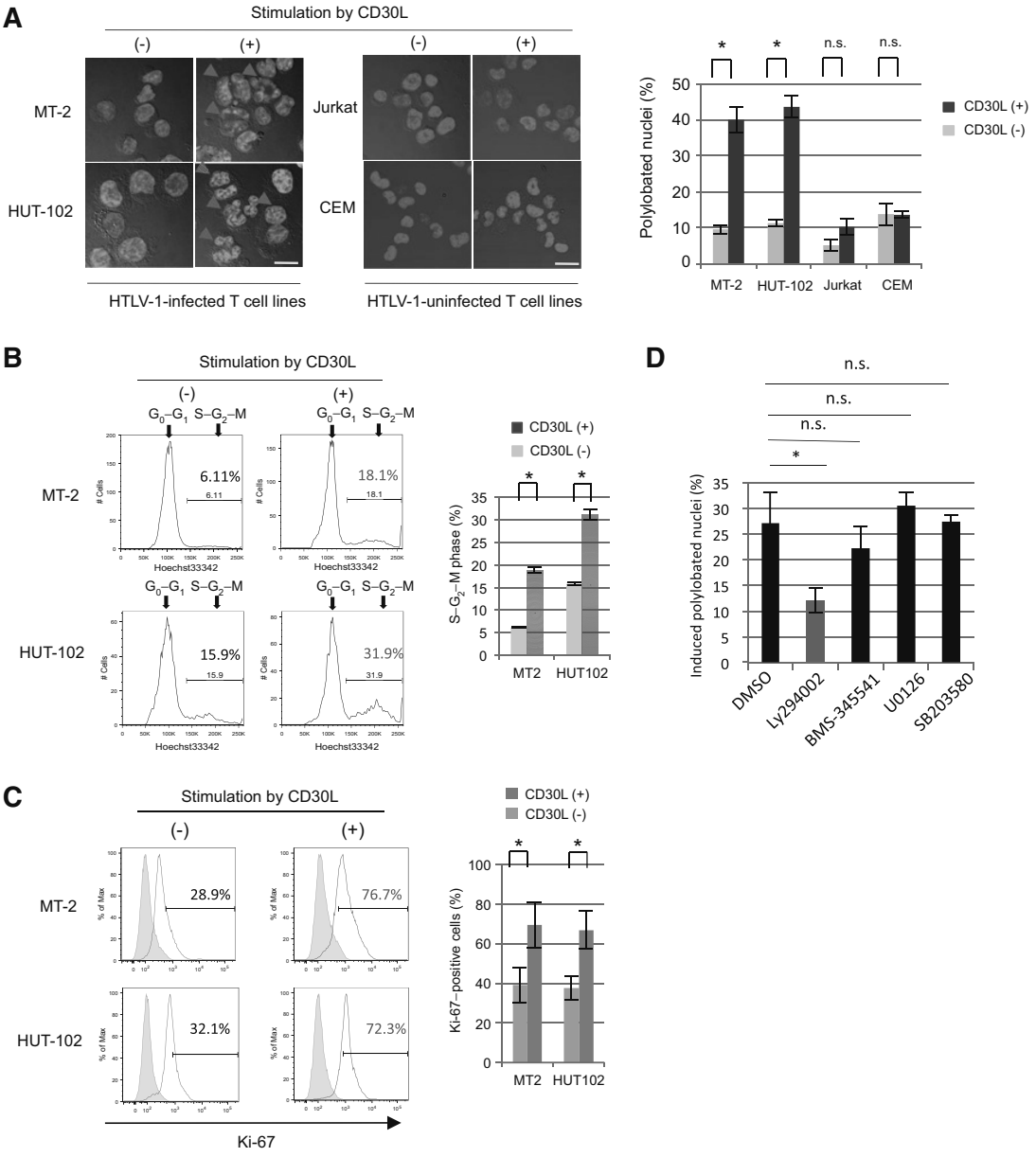

Figure 4.

Generation of cells with polylobated nuclei and induction of actively cycling cells by CD30 stimulation in HTLV-1–infected T-cell lines. **A,** Morphologic changes induced in the nuclei of $CD30^+$ T-cell lines with or without HTLV-1 infection after stimulation by CD30L. MT-2 and HUT-102 (HTLV-1–infected) or Jurkat and CEM (HTLV-1–uninfected) cells were stimulated by CD30L as described, cytospun onto slides, and fixed with 4% paraformaldehyde. The nuclei were stained with DAPI. The cells were analyzed using confocal microscopy (Confocal Microscopes A1, Nikon). Representative photographs are shown (left). Phase contrast images were merged with DAPI-stained images to better display cell morphology. Red arrows indicate polylobated cells. White bars, 80 μm. The percentage of polylobated cells in the total cell population with or without CD30L stimulation was calculated and is shown as a bar graph (right). Data shown are mean ± SD of three independent experiments. Three hundred cells were counted in each experiment. Asterisks indicate statistical significance ($P < 0.05$). n.s., not significant. CD30L(+) and CD30L(−) indicate the stimulation with CD30L/CHO and mock/CHO, respectively. **B,** Stimulation of CD30-positive T-cell lines with HTLV-1 infection increases the percentage of cells in the $S–G_2–M$ phase. MT-2 and HUT-102 cells were stimulated by CD30L, cultured for 120 hours under 1% FBS, and harvested as described. A DNA histogram of the cells was constructed based on flow cytometric analysis as described, and the representative results are shown (left). The percentage of the cells in the $S–G_2–M$ phase is indicated in the figure. The results are also presented as a bar graph, and data shown are mean ± SD of three independent experiments (right). The cells of 3×10^4 were counted in each experiment. Asterisks indicate statistical significance ($P < 0.05$). CD30L(+) and CD30L(−) indicate the stimulation with CD30L/CHO and mock/CHO, respectively. (*Continued on the following page.*)

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subpopulation in an asymptomatic carrier (Asy#1) are presented in Fig. 2A (left). In contrast, although there was a minor subpopulation of $CD30^+CD25^+CD4^+$ cells in healthy donors, polylobated nuclei were not observed in these cells (Fig. 2A, right). Representative cellular morphologies of $CD30^+CD25^+CD4^+$ cells in an additional 2 asymptomatic carriers (Asy#2 and 3) are shown in Fig. 2B (left most and second from the left). Such cells were also enriched in the $CD30^+CD25^+CD4^+$ subpopulation of smoldering (Smo#8) and acute (Acu#6) cases whose representative cellular morphologies are also shown in Fig. 2B (second from the right and right most). The percentage of cells with polylobated nuclei in each subpopulation of the three asymptomatic carriers (Asy#1–3) and six advanced stages (two smoldering: Smo#8 and 13, one chronic: Chr#15, and two acute: Acu#5 and 6) is shown in the bar graph and beeswarm graph in Fig. 2C (left and right). The results indicate that the cells with polylobated nuclei characterize $CD30^+CD25^+CD4^+$ subpopulation.

The $CD30^+CD25^+CD4^+$ subpopulation shows high virus load in PBMCs from HTLV-1–infected individuals

Although HTLV-1 infects $CD4^+$ cells and these cells express CD25, the $CD25^+CD4^+$ fraction is a mixture of infected and uninfected cells. We therefore examined the HTLV-1 provirus load of each subpopulation, and the result for 5 asymptomatic carriers (Asy#4-6, 35, and 36**)** and 4 advanced stages (Acu#1, 2, 6, and 7) listed in Supplementary Table S1 is shown as a bar graph and beeswarm graph (Fig. 2D, left and right). Because amount of sorted cells from $CD30^+CD25^+CD4^+$ fraction was limited, we could not measure virus load of samples used for morphological analyses in Fig. 2B and C except for Acu#6. Therefore, we used additional samples for further analyses. The result indicated that $CD25^+CD4^+$ cells bear a high virus load and that, within this cell population, $CD30^+$ cells have higher virus load than $CD30^-$ cells in asymptomatic carriers, whereas in acute phase, most $CD25^+CD4^+$ cells are virus-infected cells including $CD30^+$ cells. To obtain further support for this result, we examined the expression of CADM1, a marker for HTLV-1–infected cells (20). As shown in Supplementary Figs. S3 and S4, almost all of the $CD30^+$ cells are CADM1-positive and polylobated cells.

Because the $CD30^+CD25^+CD4^+$ subpopulation is highly enriched in cells with polylobated nuclei, this subpopulation appears to represent virus-infected cells with polylobated nuclei during the progression of the disease status.

The $CD30^+CD25^+CD4^+$ subpopulation of cells has an active cell-division cycle

To further confirm the biological significance of CD30 in HTLV-1–infected individuals, we used flow cytometric analysis to examine the state of the cell cycle in the $CD30^+CD25^+CD4^+$ subpopulation and in other cell subpopulations in the HTLV-1–infected individuals listed in Supplementary Table S1 (Asy#23-30, Smo#9-11, Chr#8-10, and Acu#8-10). Representative results for an asymptomatic carrier (Asy#23) and an acute case (Acu#8) are shown in Supplementary Fig. S5. The $CD30^+CD25^+CD4^+$ subpopulation was characterized by an increased proportion of $S–G_2–M$ phase cells. The percentages of $S–G_2–M$ phase cells within $CD4^+$ cell subpopulations of all the cases examined are shown in bar graphs in Fig. 3A (left). The beeswarm graph of each fraction is presented in Fig. 3A (right). To further confirm these results, we next examined the expression of the cell proliferation marker Ki-67 in the $CD30^+CD25^+CD4^+$ subpopulation and in other subpopulations of cells from individuals with different status of HTLV-1 infection. Representative results of a smoldering (Smo#12) and a chronic case (Chr#10) are shown in Supplementary Fig. S6. The results of eight cases (Asy#31 and 32, Smo#12 and 13, Chr#10 and 11, and Acu#10 and 11) are shown as a bar graph in Fig. 3B (left). The beeswarm graph of each fraction is presented in Fig. 3B (right). The $CD30^+CD25^+CD4^+$ fraction contains significantly higher amount of $Ki\text{-}67^+$ cells compared with other fractions, indicating Ki-67–positive cells are enriched in $CD30^+CD25^+CD4^+$ subpopulation. These results show that the $CD30^+CD25^+CD4^+$ subpopulation contains cells with active proliferation, whereas cells in other subpopulations without CD30 expression mostly stay in a resting state.

The results so far indicate that, among HTLV-1–infected cells, CD30 expression is closely related with polylobation of the cells and active cell cycling, whereas most other cells without CD30 expression do not. Because CD30-positive fraction increases according to the progression of the disease (Fig. 1A), the number of these actively cycling cells expands according to progression of the HTLV-1 carrier status.

CD30 signals induce polylobated nuclei that are coupled with cell-cycle promotion depending on HTLV-1 infection

To elucidate roles of CD30 in HTLV-1–infected cells, we examined the effect of CD30 signals using T-cell lines with HTLV-1 infection (MT-2 and HUT-102). The T-cell lines without HTLV-1 infection (Jurkat and CEM) served as control. We firstly examined the effect on the morphologic features of these cells. Stimulation of CD30 expressed on T-cell lines with HTLV-1 infection by CD30L expressed on CHO cells increased the number of T cells with polylobated nuclei, but this effect was not obvious in the cells without HTLV-1 infection (Fig. 4A, left). A bar graph of the percentage of polylobated cells induced by stimulation with CD30L clearly shows this difference between the response of HTLV-1–infected and –uninfected cells to CD30 stimulation (Fig. 4A, right). Furthermore, the stimulation of PBMCs from an HTLV-1–infected individual by CD30L actually induced polylobated cells (Supplementary Fig. S7). Conversely, the stimulation of Karpas299, an ALCL cell line with CD30 expression unrelated

(*Continued.*) **C,** Stimulation of $CD30^+$ T-cell lines with HTLV-1 infection with CD30L induces Ki-67–positive cells. MT-2 and HUT-102 cells were stimulated by CD30L, cultured for 96 hours under 1% FBS, harvested as described, and analyzed using flow cytometry as described in the Materials and Methods section. The representative results are shown in which the histogram of the control staining is shadowed (left). The results are also presented as a bar graph, and the data shown are mean ± SD of 3 independent experiments (right). Asterisks indicate statistical significance ($P < 0.05$). CD30L(+) and CD30L(−) indicate the stimulation with CD30L/CHO and mock/CHO, respectively. **D,** Inhibition of downstream CD30 signaling in HUT-102 cells. Cells were stimulated with or without CD30L for 96 hours, and inhibitors for each pathway (Ly294002 for PI3K, UO126 for MEK, and SB203580 for p38MAPK, and BMS-345541 for NF-κB) were added to the media and cultured as described in the Materials and Methods. Cells were cytospun, treated, and analyzed as described in Fig. 4A. Relatively higher concentrations, without loss of viability, were used (Ly294002, 10 μmol/L; UO126, 10 μmol/L; SB203580, 10 μmol/L; BMS-345541, 5 μmol/L). The percentage of polylobated cells with or without CD30L stimulation was calculated. Induction of polylobated cells by CD30 stimulation was calculated by subtracting the percentage of polylobated cells without CD30L stimulation from those with CD30L stimulation, and is shown as a bar graph. Data represent the mean ± SD of three independent counts of 300 cells. Asterisks indicate statistical significance ($P < 0.05$); n.s., not significant.

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to HTLV-1 by CD30L/CHO, did not generate polylobated cells, indicating the effect of CD30 stimulation in the generation of polylobated cells is different between ALCL cells and HTLV-1–infected T cells (Supplementary Fig. S8).

For clarification of CD30 signals in the cells with or without HTLV-1 infection, we examined the activation of known downstream pathways of CD30, i.e., NF-κB, JNK, p38 MAPK, ERK, and PI3K after CD30 stimulation in the cell lines used in this study by Western blot analysis (4–6, 21). The results indicated that the response for CD30 stimulation was generally undetected in the cells without HTLV-1 infection compared with those with HTLV-1 infection (Supplementary Fig. S9). These results suggest that cellular status might modify the responses of the cells for CD30 stimulation. JunB and IRF4 are considered to involve in CD30 induction (22, 23). In addition to JunB, IRF4, a molecule regulated by HTLV-1, is differently expressed between HTLV-1–uninfected cells and infected cells (Supplementary Fig. S10).

We next examined whether stimulation of CD30 with CD30L triggers promotion of the cell cycle in HTLV-1–infected cells. Stimulation of CD30 expressed on the HTLV-1–infected cell lines HUT-102 and MT-2 by CD30L promoted cell cycling, inducing an increase in the percentage of cells in S–G_2–M phase as shown in representative DNA histograms and in a bar graph (Fig. 4B, left and right). CD30 stimulation by CD30L also increased the percentage of Ki-67–positive cells in HTLV-1–infected cell lines (Fig. 4C, left and right). The expansion of $CD30^+$ cells in the PBMCs of HTLV-1–infected individuals by CD30 stimulation was also indicated in the experiments using primary cells (Supplementary Figs. S11–S13).

These results indicate that CD30 signals corroborating with HTLV-1 infection involve both the generation of polylobated nuclei and the induction of an actively cycling phenotype.

The PI3K pathway is involved in the CD30-mediated generation of cells with polylobated nuclei

To identify the signaling pathway involved in the CD30-mediated generation of cells with polylobated nuclei, we used inhibitors for known downstream effectors of CD30 signaling, i.e., NF-κB, ERK, MAPK, PI3K, and p38 MAPK (4–6, 21), and examined the effect on the generation of cells with polylobated nuclei in the HTLV-1–infected cell line HUT-102. The concentration of the inhibitors was optimized based on past studies (4–6, 21). As shown in Fig. 4D, inhibition of the PI3K pathway by Ly294002 showed significant inhibition of the generation of cells with polylobated nuclei, in the HTLV-1–infected cell line. Similar results were observed using another PI3K inhibitor duvelisib (Supplementary Fig. S14). Stimulation of PKC, downstream of PI3K by PMA, generated polylobated cells in HUT-102 cells (ref. 24; Supplementary Fig. S15). Furthermore, duvelisib inhibited polylobation by CD30L in primary cells from HTLV-1–infected individuals (Supplementary Fig. S7).

CD30 signals induce abnormal cell division and hyperploidy in HTLV-1–infected T cells

Because the results so far raised the possibility that CD30 signals provide impacts on chromosome by accelerating cell division, we next investigated the effect of CD30 signaling on this process. Flow cytometric analysis of DNA histogram indicated the possibility that the stimulation of T-cell lines with HTLV-1 infection by CD30L increases the fraction of 4N<. The representable result of HUT-102 is shown in Fig. 5A. Stimulation of T-cell lines with HTLV-1 infection (MT-2 and HUT-102) by CD30L increased 4N< fractions, whereas the stimulation of those without HTLV-1 infection (Jurkat and CEM) did not, suggesting CD30 signals increase hyperploid cells dependent on HTLV-1 infection (Fig. 5B).

To elucidate the cause of generation of hyperploid cells, we examined the effect for cell division by the stimulation of CD30 with its ligand in HTLV-1–infected cell lines. The nuclei of the T-cell lines with HTLV-1 infection were visualized using fluorescence microscopy following stable transfection of the cells with GFP fused to H2B. These cells were stimulated by CD30L expressed on CHO cells, and its effect on cell division was captured using time-lapse microscopy (Biostation IM-Q, Nikon). This analysis showed that CD30 stimulation frequently triggered abnormal cell division, most of which is abortive mitosis and cytokinesis. The representative cases of abnormal cell division observed in HUT-102 cells with stimulation by CD30L are presented in Fig. 5C (left and middle). Normal cell division observed in HUT-102 cells without stimulation by CD30L is presented in Fig. 5C (right). The original time-lapse videos of the cells are described in the legend. The percentage of abnormal cell division with CD30L stimulation of HUT-102 and MT-2 cells is shown as a bar graph in Fig. 5D.

Hyperploid cells are a feature of the $CD30^+CD25^+CD4^+$ subpopulation of HTLV-1–infected individuals

We next examined the ploidy of each $CD4^+$ cell fraction in the cells of individuals with different status of HTLV-1 infection, who are listed in Supplementary Table S1 (Asy#33 and 34, Smo#14 and 15, Chr#12 and 13, and Acu#12) by flow cytometric analysis of DNA histogram. Except for asymptomatic cases that did not contain a subpopulation with hyperploid cells (4N<), the $CD30^+CD25^+CD4^+$ subpopulation in all other cases showed increased hyperploid cells (4N<) compared with other CD4-positive fractions (Fig. 5E). These results support the notion that induction of abnormal cell division and hyperploidy by CD30 actually occurs in HTLV-1–infected individuals.

Brentuximab vedotin depletes CD30-positive cells in PBMCs samples from HTLV-1–infected individuals

The features of $CD30^+$ cells presented so far indicated that this fraction may play a role in the transformation of HTLV-1–infected cells. Brentuximab vedotin, a monoclonal antibody for CD30 conjugated with the tubulin inhibitor monomethyl auristatin E, shows a potent effect in classical Hodgkin lymphoma and anaplastic large-cell lymphoma (25). A recent study also showed the effectiveness of brentuximab vedotin on peripheral T-cell lymphoma (26). Therefore, we examined whether brentuximab vedotin can deplete the CD30-positive fraction of PBMCs isolated from HTLV-1–infected individuals. When PBMC samples were treated with brentuximab vedotin, the CD30-positive fraction ($CD30^+CD25^+CD4^+$) was shown to be reduced compared with those treated with vehicle alone (Fig. 6A). Examples of original flow cytometric data (Chr#18 and Acu#13) are presented in Supplementary Fig. S16. The reduction ranged from approximately 20%, to more than 90% (mean ± SD, 79.6 ± 21.79%; Fig. 6A). To clarify that the effect of brentuximab vedotin is selective for $CD30^+$ fraction, we compared reduction rate in the fractions of $CD30^+$ and $CD30^-$ in the cases treated by brentuximab vedotin. The result showed the significant reduction of the number of

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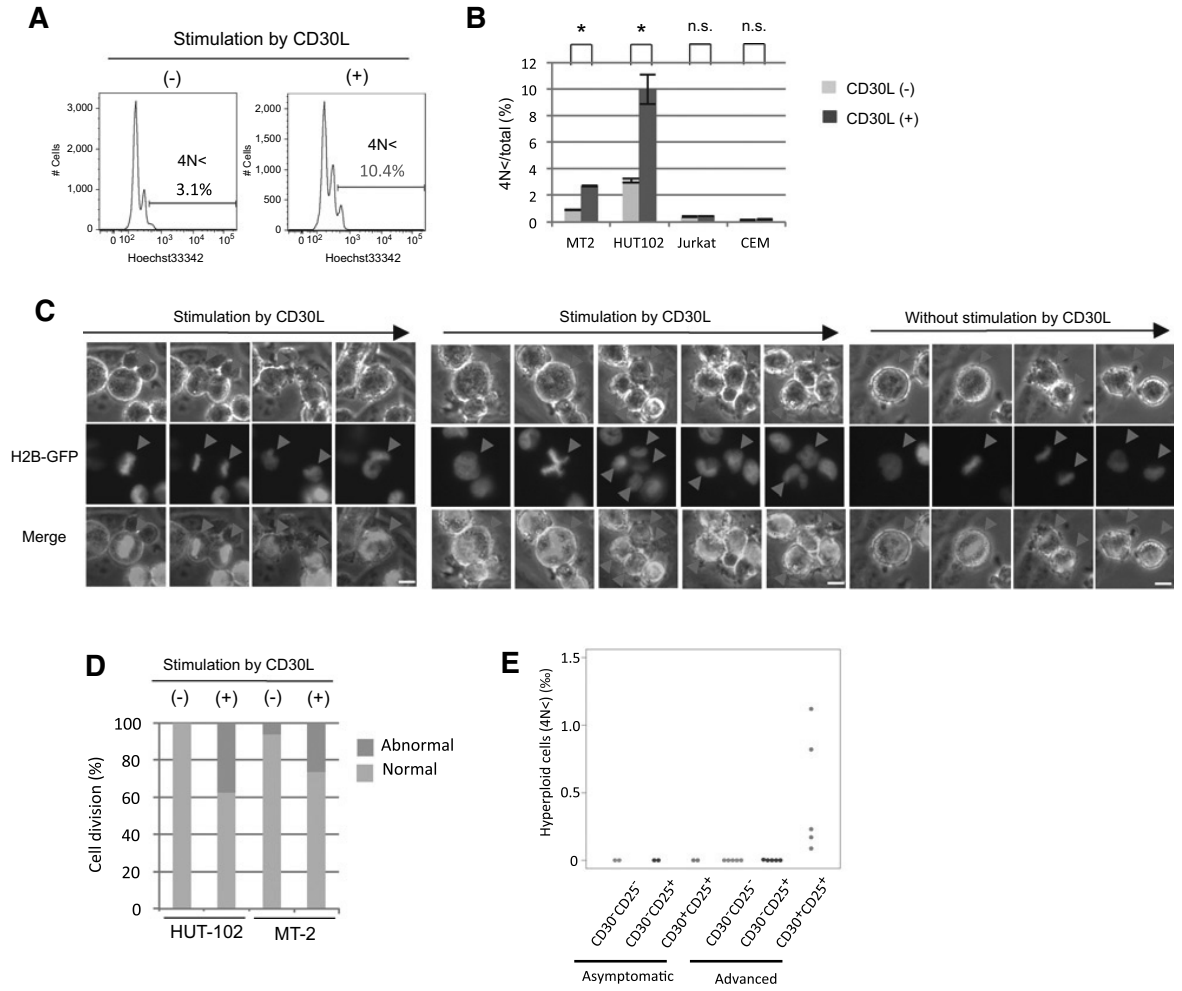

Figure 5.

CD30 stimulation induces abnormal mitosis and hyperploid cells in HTLV-1–infected T-cell lines. **A** and **B,** Increase in hyperploid cells after CD30 stimulation in MT-2 and HUT-102 cells. The cells were stimulated by CD30L as described. Ploidy of the cells (5×10^4) was analyzed using flow cytometry as described, and representative DNA histograms of HUT-102 are shown. The percent fraction of 4N< in the entire fraction is indicated in the graphs (**A**). The percent fraction of 4N< of cell lines with HTLV-1 infection (HUT-102 and MT-2) and without HTLV-1 infection (Jurkat and CEM) is calculated and expressed as bar graphs. The experiments were performed 3 times, and the results were also presented as bar graphs with means ± SD. Asterisks indicate statistical significance ($P < 0.05$; **B**). CD30L(+) and CD30L (−) indicate the stimulation with CD30L/CHO and mock/CHO, respectively. **C** and **D,** HUT-102 and MT-2 cells, whose nuclei were marked with GFP as described, were stimulated by CD30L as indicated, and more than 50 cells were observed with time-lapse microscopy. Representative photographs of abnormal cell division of HUT-102 cells are presented in the left and middle plots, respectively. Normal mitosis observed in the control (without stimulation by CD30L) is presented in the right. The arrowheads in the pictures indicate the cells of interest and their related cells. White bars, 10 μm. The original time-lapse videos of left, middle, and right are provided in Video 1 (mp4. 921 KB), Video 2 (mp4. 2 MB), and Video 3 (mp4. 794 KB), respectively (**C**). The percentage of normal and abnormal cell division in cells with or without CD30L stimulation in HUT-102 and MT-2 cells was calculated by integrating the results of more than 5 independent experiments (**D**). CD30L(+) and CD30L(−) indicate the stimulation with CD30L/CHO and mock/CHO, respectively. **E,** The ratio of hyperploid cells (4N<) in $CD30^+CD25^+$, $CD30^-CD25^+$, and $CD30^-CD25^-$ cells in HTLV-1–infected individuals. The ploidy of about 1×10^6 lymphocytes was analyzed using flow cytometry. The permil hyperploid cells (4N<) in the entire cells are measured using DNA histogram as described in Fig. 5A, and each value is plotted in the graph. Asymptomatic, two asymptomatic carriers (Asy#33 and 34); advanced, two smoldering (Smo#14 and 15), two chronic (Chr#12 and 13), and one acute (Acu#12).

viable cells in $CD30^+$ fraction, whereas $CD30^-$ fraction was relatively not affected (Supplementary Fig. S17). The results, which further support the effect of brentuximab vedotin on $CD30^+$ cells, were presented in Supplementary Figs. S18 and S19.

We also examined the relationship between the expression level of CD30 and the response to brentuximab vedotin (Supplementary Table S3; Supplementary Fig. S20). The result suggested that the reduction rate of $CD30^+$ cells by brentuximab vedotin was relatively low in cases with low CD30 expression (Asy#39 and Chr#15). These results suggest that brentuximab vedotin can selectively reduce the $CD30^+$ cell fraction of PBMCs from HTLV-1–infected individuals.

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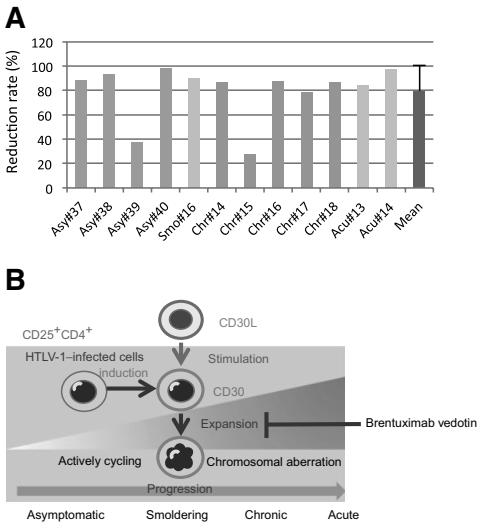

Figure 6.

Effect of brentuximab vedotin on CD30-positive cells in PBMCs of HTLV-1–infected individuals. **A,** PBMCs separated from HTLV-1–infected individuals (four asymptomatic, Asy#37-40; one smoldering, Smo#16; 5 chronic, Chr#14-18; and 2 acute, Acu#13 and 14) were cultured with 10% self-serum, treated as described, and reduction rate of $CD30^+CD25^+CD4^+$ cells in each case was calculated and is shown as bar graph. The reduction rate (%) was calculated as follows: ([Tc − Tbv]/Tc) × 100; here, Tbv and Tc indicate percent $CD30^+CD25^+CD4^+$ cells in $CD4^+$ fraction with or without the treatment by Brentuximab Vedotin respectively. Average of total 12 cases is presented as mean ± SD at the right end. **B,** Schematic representation of the role of CD30 in HTLV-1–infected individuals. CD30-positive HTLV-1–infected cells show polylobation of the nucleus and expansion in accordance with HTLV-1 disease progression. Stimulation of CD30-positive HTLV-1–infected cells with CD30L triggers polylobation and promotes cell cycling. These processes are accompanied by abnormal cell division and the generation of hyperploid cells. CD30-positive cells might thus serve as a reservoir for transformation and clonal expansion in HTLV-1–infected individuals. CD30L is expressed on granulocytes, and the increase in the CD30L-expressing cell fraction induced by parasitic diseases, such as strongyloidiasis, might promote such processes. Depletion of this cell reservoir might therefore contribute to the prevention of disease progression.

Discussion

Previous reports indicated the expression of CD30 in a part of ATL (7, 8). However, the clinicopathologic significance of $CD30^+$ cells in HTLV-1–infected individuals including acute phase is entirely unknown. This lack of knowledge might be due to differences in experimental designs for the evaluation of CD30 positivity such as high cutoff value for CD30 positivity. In addition, there have been no reports that have provided a detailed description of $CD30^+$ cells in the pre-ATL status. Roles of CD30 signals in HTLV-1–infected cells are also entirely unknown. Analyses of $CD30^+$ cells in the $CD25^+CD4^+$ subpopulation enabled us to more sensitively detect and evaluate $CD30^+$ cells. CD30 is induced in a portion of HTLV-1–infected cells, and these cells expand according to the progression of carrier status. A previous report indicated that sIL2R correlates with progression of the disease in HTLV-1–infected individuals (19). Therefore, we examined relationship between the expression of CD30 and the serum sIL2R. The result indicated that CD30 expression correlated with sIL2R (Supplementary Fig. S2).

The expansion of the $CD30^+CD25^+CD4^+$ subpopulation correlated with the expansion of abnormal lymphocytes according to the progression of HTLV-1 carrier status, suggesting impact of CD30 on the status of HTLV-1 infection and its progression. In support of this hypothesis, the experiments using HTLV-1–infected cell lines showed that CD30 is involved in the generation of abnormal lymphocytes with "flower cell" and features an actively cycling, which coincides with abnormal mitosis and chromosomal aberrations in corroboration with HTLV-1 infection. CD30 stimulation also triggered expansion and polylobation of primary cells. We could link these results to the characteristics of $CD30^+$ cells in HTLV-1–infected individuals and indicated involvement of CD30 in the progression of the disease status.

In this report, we indicated that the stimulation of HTLV-1–infected cell lines with CD30 could induce polylobation through the activation of PI3K. CD30 stimulation could not induce polylobation in HTLV-1–uninfected cell lines used in this study. This might be explained by the result that the effect of CD30 stimulation was different between HTLV-1–infected and –uninfected cells. Our finding is supported by those of a previous study, which indicated that activation-inducible lymphocyte immunomediatory molecule (AILIM/ICOS)–mediated activation of PI3K is responsible for the polylobation of lymphocytes in ATL (27).

PI3K regulates cytoskeletal rearrangements through the regulation of Rho-family cascades (28). However, we could not show the activation of these pathways by stimulation of CD30 in HTLV-1–infected cell lines (data not shown). The previous report indicated the involvement of microtubule rearrangement in polylobation (27). We could show that PMA, an activator of PKC, triggered polylobation in an HTLV-1–infected cell line, HUT-102 (Supplementary Fig. S15). PKC is a downstream molecule of PI3K and commits microtubule rearrangement (24, 29). Therefore, further study to clarify involvement of CD30 in PKC-mediated rearrangement of microtubule will provide important insights into mechanisms underlying polylobation in HTLV-1–infected cells.

A recent report demonstrated that AP-1 family transcription factors, including JunB, are responsible for the induction of activation-inducible lymphocyte immunomediatory molecule/inducible co-stimulator (AILIM/ICOS; ref. 30). Because CD30 can activate AP-1 by inducing JunB through the ERK pathway (31), it is possible that the expression of AILIM/ICOS is dependent on CD30 signaling and that CD30 can induce polylobated cells not only directly, but also indirectly via the induction of AILIM/ICOS, although further analyses are required to confirm this hypothesis.

Emergence of $CD30^-$ polylobated cells appears to increase according to the progression of disease status. Because most of these cells are HTLV-1–infected cells (Supplementary Fig. S4), alteration of the cellular status might mimic the signals for polylobation instead of CD30. Because CD30 is transiently downregulated after stimulation by CD30L, this process might partially be responsible for the existence of $CD30^-$ polylobated cells (32). Molecular mechanisms underlying the emergence of $CD30^-$ polylobated cells require further study.

We indicated that CD30 stimulation of HTLV-1–infected cells could induce active cycling. In fact, CD30 expression in HTLV-1–infected cells is also closely related with actively cycling cells, whereas most other cells without CD30 expression are quiescent in lymphocytes of HTLV-1–infected individuals. $CD30^+$ cells

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showed an increased number of cells in the S–G_2–M phase and with Ki-67 expression compared with $CD30^-$ cells. Ki-67 is a cell-cycle–related nuclear protein that is expressed by cells in a proliferative state, but not by cells in a quiescent state (33). Combined with the close relationship between CD30 expression and polylobated cells, these results imply that CD30 signals trigger cell-cycle promotion of HTLV-1–infected cells, which coincides with the generation of characteristic polylobated cells, and these cells expand according to the progression in HTLV-1–infected individuals.

We also indicated that CD30 stimulation triggers abnormal cell division and hyperploidy in HTLV-1–infected cell lines but does not in unrelated cell lines. Although further studies are required, cellular signaling status caused by HTLV-1 infection might also be responsible for this result (34). Based on the observations of time-lapse microscopy, abortive mitosis and its subsequent effects might involve the generation of hyperploid cells, which might be related with a possible cause of chromosomal instability and cellular transformation of ATL cells (35). We indicated that the $CD30^+$ cell fraction actually contains cells with chromosomal aberrations and that these cells expand according to progression of the status of HTLV-1 infection. A previous report indicated that changes in chromosome copy-number and gene expression profile occur according to progression of the disease status in ATL (36, 37). Ki-67 positivity correlates with high-grade malignancy (38). CD30-positive cells might be able to serve as a reservoir for malignant transformation and clonal expansion of HTLV-1–infected cells in HTLV-1–infected individuals. This notion is supported by very recent report, which identified CD30 as one of super enhancers and suggested its involvement of pathogenesis of ATL (39).

CD30 appears to be induced in a portion of HTLV-1–infected cells depending on their cellular status. CD30 expression appears not to be related to virus replication, because HTLV-1 is not replicating in PB (40). CD30 stimulation triggers growth-promoting effects, abnormal mitosis, and chromosomal aberration in HTLV-1–infected cell lines. Furthermore, it was shown that the stimulation of PBMCs from HTLV-1–infected individuals by CD30L actually expanded $CD30^+CD25^+CD4^+$ cells (Supplementary Figs. S11 and S12). These results suggest that the CD30 expression in HTLV-1–infected cells is important as a cause for the expansion of CD30-positive cells and tumorigenic process. However, further study is necessarily to clarify detailed mechanisms of involvement of the $CD30^+$ cells in these processes.

CD30L is present on T cells, B cells, granulocytes, and monocytes. These cells are widely distributed in the body, e.g., PB, bone mallow, and lymphoid system. Especially granulocytes, including eosinophils, are increased in ATL and modulate its prognosis (41, 42). Interaction between CD30L and CD30 triggers local gathering and subsequent internalization of this complex to CD30-expressing cells for signaling (32). ATL is closely associated with exteriorization of parasitic diseases such as strongyloidiasis (43). A previous report indicated that five steps are required for the final transformation of onset of ATL (44). We showed that CD30 expression correlated with sIL2R, a marker for disease progression in HTLV-1–infected individuals (19). Previous reports suggested serum-soluble CD30 is associated with poor prognosis in HTLV-1–infected individuals including ATL (45, 46). These results indicate the possibility that CD30 positivity is associated with poor prognosis in HTLV-1–infected individuals including ATL. CD30 signals might involve the expansion of HTLV-1–infected cells and trigger the transformation or clonal evolution of HTLV-1–infected cells that coincides with abortive mitosis, polylobation of the nuclei, and chromosomal aberrations. It is possible that CD30, which is induced in a portion of HTLV-1-infected cells, commits progression of asymptomatic, pre-ATL, and ATL status through clonal evolution of these cells.

We could show that brentuximab vedotin can purge $CD30^+$ cells in PBMCs from HTLV-1–infected individuals. This effect appears to depend on the expression level of CD30 in the cells (Supplementary Fig. S20). Our results by primary cells are almost in accordance with a previous report using HTLV-1–infected cell lines (47). *In vivo* effectiveness of brentuximab vedotin in mice bearing HTLV-1–infected cell lines was already reported (47). In this context, diminishing CD30-expressing cells with anti-CD30 monoclonal antibody therapies (48) might be considered for both pre-ATL status such as smoldering and chronic status, and ATL (Fig. 6B).

In conclusion, the results of this study indicate a biological link between the expression of CD30, which serves as a marker for HTLV-1 disease progression, and the actively proliferating fraction of HTLV-1–infected cells, which display chromosomal aberrations and polylobation. The findings also highlight the role of $CD30^+$ cells as a potential reservoir for transformation and clonal expansion in HTLV-1–infected individuals, and indicate that these cells may thus be a therapeutic target in this disease. Further examination of the $CD30^+$ cell fraction may improve our understanding of the tumorigenic processes of HTLV-1–infected cells.

Disclosure of Potential Conflicts of Interest

A. Utsunomiya reports receiving speakers bureau honoraria from Bristol-Myers Squibb, Celgene, Chugai Pharma, Daiichi Sankyo, Eisai, JIMRO, Kyowa Hakko Kirin, Mundi Pharma, Nippon Shinyaku, Novartis Pharma, Ono Pharmaceutical CO, Otsuka Pharmaceutical, Pfizer, and Siemens. R. Horie is a consultant/advisory board member for Takeda. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Watanabe, R. Horie
Development of methodology: M. Nakashima, M. Watanabe, R. Horie
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Nakashima, T. Yamochi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Nakashima
Writing, review, and/or revision of the manuscript: M. Nakashima, A. Utsunomiya, T. Watanabe, R. Horie
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Watanabe, K. Uchimaru, A. Utsunomiya, R. Horie
Study supervision: K. Uchimaru, M. Higashihara, T. Watanabe, R. Horie

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CD30 Characterizes Polylobated Lymphocytes and Disease Progression in HTLV-1–Infected Individuals

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CORRESPONDENCE

Chronic inflammatory demyelinating polyneuropathy in adult T-cell leukemia-lymphoma patients following allogeneic stem cell transplantation

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Adult T-cell leukemia-lymphoma (ATL) is a T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) [1, 2]. ATL is classified into four clinical subtypes proposed by the Japan Clinical Oncology Group: smoldering, chronic, lymphoma, and acute [1]. Acute- and lymphoma-type ATL have a poor prognosis. Allogeneic hematopoietic stem cell transplantation (allo-SCT) is being increasingly used as a curative therapy for ATL, and confers long-term survival in approximately 40% of ATL patients [2]. One of the major problems of allo-SCT is occurrence of severe complications. We observed ATL patients who developed peripheral neuropathy (PN) following allo-SCT. In this study, we analyzed their disease profiles and investigated the pathophysiology of PN.

We investigated the clinical features of 46 ATL patients who underwent allo-SCT after induction chemotherapy. The study consisted of 25 men and 21 women aged 28–71 (median age: 59) years. The ATL clinical subtypes observed in these patients included 33 (72%), 8 (17%), and 5 (11%) with the acute-, lymphoma-, and unfavorable chronic-types, respectively. Conditioning regimens consisting of myeloablative conditioning (MAC) and reduced intensity conditioning (RIC) were administered to 5 (11%) and 41 (89%) of the patients, respectively. Stem cell sources included the bone marrow for 35 (77%) of the cases, peripheral blood in 10 (21%), and cord blood in 1 (2%). PN was diagnosed by the symptoms of paresthesia, hyperesthesia, abnormal sensations, and muscle weakness. This included damage to sensory and motor nerves with axonal and/or demyelinating damage. We performed a full neurological examination, a nerve conduction study (motor or sensory conduction), as well as radiological procedures such as computed tomography and/or magnetic resonance imaging (MRI). We also performed cell counts, evaluated cerebrospinal fluid (CSF) biochemistry, and measured total protein levels, anti-HTLV-1 antibody titers, neopterin, and CXCL10 levels in CSF.

Three patients developed PN after allo-SCT. Profiles and CSF data of these patients are shown in Table 1. All patients had an Eastern Cooperative Oncology Group performance status (ECOG-PS) of grade 3 or 4. They achieved and maintained complete remission after allo-SCT; however, they developed acute graft-versus-host disease (aGVHD) and/or chronic graft-versus-host disease (cGVHD) involving multiple organs.

Patient 1 was a 57-year-old man who underwent allo-SCT. He had lymphoma-type ATL and achieved partial remission (PR) after induction chemotherapy. He received a human leukocyte antigen (HLA) 8/8 allele-matched unrelated bone marrow transplantation. Within a month after transplantation, he developed an abnormal sensation below the epigastrium, hypoesthesia, muscle weakness in the

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Table 1 Profiles and CSF data of the three patients who developed PN after allo-SCT

	Patient No.		
	1	2	3
Age (years)	57	60	68
Sex	Male	Male	Female
ATL type	Lymphoma	Lymphoma	Acute
DS before SCT	PR	PR	PR
Conditioning regimen	FLU/BU/TBI	FLU/BU	FLU/BU
Related/unrelated donor	Unrelated	Unrelated	Unrelated
Source	BM	PB	PB
Donor sex	Male	Male	Male
Blood type	$A^+ \rightarrow A^+$	$A^+ \rightarrow A^+$	$A^+ \rightarrow O^+$
HLA match	8/8	8/8	8/8
MAC or RIC	RIC	RIC	RIC
GVHD prophylaxis	Tac/MMF	Tac/MTX	CyA
DS after SCT	CR	CR	CR
aGVHD	Skin (grade I)	No	No
cGVHD	Skin, lungs	Skin, eyes, mouth, lungs	Skin, eyes, mouth, lungs, muscle
anti-GM1 antibody	Negative	N/A	Negative
Latency	1 month	11 months	7 years
Performance status	4	4	3
CSF data			
Cells/mL	5	1	2
Total protein (mg/dL)	62.0	51.0	233.0
Albuminocytologic dissociation	Yes	Yes	Yes
HTLV-1 provirus level (copies/100 cells)	4.48	[a]	1.45
Anti-HTLV-1 antibody titer	[a]	<2	4
Neopterin (pmol/mL)	16	2	3
CXCL10 (pg/mL)	2054.2	58.2	773.3

CSF cerebrospinal fluid, *PN* peripheral neuropathy, *SCT* stem cell transplantation, *ATL* adult T-cell leukemia-lymphoma, *HTLV-1* human T-cell leukemia virus type 1, *BU* busulfan, *CyA* cyclosporine A, *DS* disease status, *PR* partial remission *FLU* fludarabine, *BM* bone marrow, *PB* peripheral blood, *HLA* human leukocyte antigen, *MAC* myeloablative conditioning, *MMF* mycophenolate mofetil, *MTX* methotrexate, *RIC* reduced intensity conditioning, *Tac* tacrolimus, *TBI* total body irradiation, *aGVHD* acute graft-versus-host disease, *cGVHD* chronic graft-versus-host disease, *N/A* not available

[a]Below the detection limit

lower limbs, and absence of a tendon reflex. Concurrently, he also developed skin aGVHD (grade I). A nerve conduction study revealed no electrical conduction on the left median, fibular, and tibial nerves.

Patient 2 was a 60-year-old man who underwent allo-SCT. He was diagnosed with lymphoma-type ATL and achieved PR after induction chemotherapy. He received an HLA 8/8 matched unrelated peripheral blood stem cell transplantation (PBSCT). After 11 months, he developed lower limb-dominant muscle weakness, and sensory loss. He simultaneously developed cGVHD in his eyes, mouth, skin, and lungs. A nerve conduction study showed disappearance of electrical conduction on both sides of the peroneal and sural nerves.

Patient 3 was a 68-year-old woman who underwent allo-SCT. She had acute-type ATL and achieved PR after induction chemotherapy. She received an HLA 8/8 matched related PBSCT. She subsequently developed muscle weakness in her bilateral extremities after seven years. In addition, she developed hypoesthesia of the lower limb, and cGVHD in her eyes, mouth, skin, lungs, liver, and muscles. A nerve conduction study revealed absence of electrical conduction in the bilateral cervical nerve.

CSF anti-HTLV-1 antibody results were negative in two of these patients, while these results were weakly positive in the third patient who demonstrated a four-fold increase in the CSF anti-HTLV-1 titer levels. All patients showed albuminocytologic dissociation in their CSF.

All the three patients' symptoms persisted for more than 2 months, and diffuse demyelination of peripheral nerves was confirmed via a nerve conduction study. CSF examinations showed albuminocytologic dissociation, as mentioned earlier.

According to the diagnostic criteria (European Federation of Neurological Societies/Peripheral Nerve Society guidelines), all three patients met the chronic inflammatory demyelinating polyneuropathy (CIDP) criteria [3].

Some reports showed Guillain–Barre syndrome following allo-SCT [4–6]. However, Guillain–Barre syndrome was considered unlikely because of the absence of cranial nerve or respiratory muscle paralysis and autonomic dysfunction. In addition, prior infection usually found in Guillain–Barre syndrome was not present in all the patients. Although a previous report suggested that the onset of symptoms occurred immediately during the post-transplant period [5], the same was not observed in our patients. Two patients were found not to have any anti-GM antibody, a frequent finding in Guillain–Barre syndrome.

In HTLV-1-infected patients, it is important to consider human T-cell leukemia virus 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a differential diagnosis. As the deep tendon reflexes were reduced, and a pathological reflex was not apparent in these patients, results suggested that they did not have myelopathy. Based on the lack of myelopathy and CSF data, HAM/TSP was ruled out.

There was no increase in the number of cells in the CSF, and no mass lesion in the brain was detected using radiological procedures. ATL relapse in the central nervous system (CNS) was not considered.

Hence, we initiated the appropriate CIDP treatment, including corticosteroids, intravenous immunoglobulin, and plasmapheresis [7–10]. However, neither the corticosteroids nor the intravenous immunoglobulin was effective.

Previous studies have described neuropathy (including CIDP) following allo-SCT for various hematological diseases (Table 2) [6, 8–11]; however, to date the same has not been reported in ATL patients undergoing allo-SCT. To the best of our knowledge, this is the first report of CIDP in ATL patients who received allo-SCT.

The onset of CIDP in two patients occurred immediately after the onset of aGVHD or cGVHD. This suggests that CIDP may be one of the symptoms of GVHD. To support this, previous studies have reported of infiltrations of either CD4- or CD8-positive cells in nerve biopsy specimens obtained from patients with CIDP related to allo-SCT as histopathological evidence of GVHD in the peripheral nervous system [8, 10]. Although our patients preferred treatment intervention and did not undergo neurological biopsy, it may be beneficial to consider a biopsy. Other previous reports showed that cGVHD was associated with loss of self-tolerance and that its manifestation resembled an autoimmune disease. This suggests that cGVHD causes immune dysregulation and predisposes patients to develop CIDP following SCT [9]. A previous report investigating the incidence and characteristics of the peripheral nervous system manifestations occurring after allo-SCT in patients with hematological disease [12] found an association between RIC and PBSCT with specific peripheral nerve damage. In our study, all patients underwent RIC, and two of them underwent PBSCT.

Neopterin is a small molecule derived from guanosine triphosphate that is produced by macrophages and microglia. CXCL10 is an inflammatory chemokine that binds to CXCR3, expressed specifically on Th1 cells, and is produced by astrocytes. Neopterin and CXCL10 levels are reported to be elevated in the CSF of patients with neuroinflammatory disorders such as HAM/TSP and multiple sclerosis [13], and are indicators of CNS inflammation [14]. The expression of both neopterin and CXCL10 is stimulated

Table 2 Reports of patients with hematological disease who developed PN (including CIDP) after allo-SCT

	AML	ALL	MDS	CML	NHL	HL	AA	ATL
N	7	6	1	3	4	1	2	3
M/F	4/3	3/3	1/0	3/0	4/0	1/0	2/0	2/1
Age, years	17–71	29–66	66	31–44	26–43	21	29–50	57–68
Latency	58 d–16 m	8 d–2 y	21 m	7 d–8 m	1 m–5 y	16 d	2 w–1 y	1 m–7 y
Source	BM: 2 CB: 1 PB: 1 N/A: 3	BM: 2 CB: 1 PB: 3	PB: 1	BM: 3	BM: 2 N/A: 2	BM: 1	BM: 2	BM: 1 PB: 2
Outcome	R: 3 PR: 4	R: 1 PR: 4 N/A: 1	PR: 1	R: 2 D: 1	R: 1 PR: 3	D: 1	R: 2	U: 1 PR: 1 D: 1

AML acute myeloid leukemia, *ALL* acute lymphoblastic leukemia, *MDS* myelodysplastic syndromes, *CML* chronic myelogenous leukemia, *NHL* non-Hodgkin's lymphoma, *HL* Hodgkin's lymphoma, *AA* aplastic anemia, *ATL* adult T-cell leukemia-lymphoma, *PN* peripheral neuropathy, *CIDP* chronic inflammatory demyelinating polyneuropathy, *SCT* stem cell transplantation, *M* male, *F* female, *d* day, *w* week, *m* month, *y* year, *BM* bone marrow, *CB* cord blood, *PB* peripheral blood, *R* recovery, *PR* partial recovery, *D* death, *U* unchanged, *N/A* not available

by interferon-γ (IFN-γ) produced by T cells. Because all of our patients did not have HAM/TSP and CNS relapse of ATL, it was considered to be a nonspecific reaction. One of hypothesis is that aberrantly activated donor T cells produce IFN-γ, which leads to the elevation of the neopterin and CXCL10 levels. Thus, CIDP and GVHD seem to have similar mechanisms involving activated T cells.

Drug-induced CIDP cannot be completely ruled out. For example, tacrolimus has been reported to induce CIDP in patients who received liver transplantation [15]. Although our two patients were receiving tacrolimus, the onset of CIDP in our patients was much later than that observed in other case reports.

No structural disorder was detected on the MRI scans of the spinal cord, vertebrae, and discs in patients 1 and 2. Patient 3 had cervical spondylosis which was thought to be one of the causes of PN. However, subsequent findings, including the elevation of CXCL10 in CSF, indicated the possibility of other causes of PN in patient 3. Vincristine (VCR) was administered to all patients at induction chemotherapy before allo-SCT. If the cause of neuropathy is VCR, the neuropathy usually recovers after discontinuation of administration. However, it did not recover and VCR may not be main reason. None of them had diabetes mellitus, and diabetic neuropathy was not considered. Vitamin B_{12} levels in patients 1 and 2 were in the normal range. In patient 3, vitamin B_{12} was not checked; however, there were no history or background characteristics that could be regarded as a risk for vitamin B_{12} deficiency.

Transplant physicians should be aware of these clinical features in ATL patients. Further study is warranted to reveal the relationship between ATL and PN following allo-SCT and the underlying mechanism.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Research article

Trogocytosis of ligand–receptor complex and its intracellular transport in CD30 signalling

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Background Information. CD30, which is characteristically expressed in classical Hodgkin lymphoma (cHL), is thought to transduce signals by ligation of trimerised CD30 ligand (CD30L) on the surface of surrounding cells and recruitment of downstream molecules. In this report, we propose a new mechanism for CD30 signalling by its ligand. We prepared two stable transformants, CHO cells expressing CD30L fused to mCherry and HeLa cells expressing CD30 fused to GFP.

Results. Co-culture of these cells triggered clustering of CD30 and CD30L at the cellular interface, formation of multiple CD30L–CD30 complexes, internalisation of these complexes with a portion of the plasma membrane into the HeLa cells, and intracellular transport to the lysosomal compartment. The internalisation process was significantly inhibited by actin polymerisation inhibitors. The CD30L–CD30 interaction was found to trigger active signalling processes, as measured by Ca^{2+} influx, and similar mechanisms were observed using cHL cell lines.

Conclusions. These results suggest that CD30 extracts CD30L from CD30L-expressing cells by actin-mediated trogocytosis, resulting in the generation of signalosomes, intracellular signalling, lysosomal degradation and a subsequent refractory phase. We postulate that similar processes may operate in tumours endogenously expressing CD30. These observations thus provide new insights into our understanding of the biological roles of CD30 in normal and malignant cells and, in particular, in cHL.

Significance. This study suggests a novel model of CD30 signalling that provides new insights into the biological roles of CD30 and other members of this family in normal and malignant cells.

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Introduction

CD30, a member of the tumour necrosis factor receptor (TNFR) superfamily, is a type I single transmembrane protein consisting of 595 amino acids, whose molecular weight is 105–120 kDa [Durkop et al., 1992]. CD30 was initially found to be strongly expressed on Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma (cHL) [Schwab et al., 1982]. Soon after however, it was revealed that CD30 is also strongly expressed in a rare subtype of non-Hodgkin lymphoma, called anaplastic large cell lymphoma [Stein et al., 1985]. Later studies showed the expression of CD30 in other pathological, as well as normal, conditions. However, since its expression is limited and mainly

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Key words: CD30, CD30 ligand, Classical Hodgkin lymphoma, Signal transduction, Trogocytosis.

Abbreviations: CD30L, CD30 ligand; cHL, classical Hodgkin lymphoma; MFI, mean fluorescence intensity; siRNA, small interfering RNA; TNF, tumour necrosis factor; TNFR, tumour necrosis factor receptor; TRAF, TNFR-associated factor.

induced in activated lymphocytes, CD30 is referred to as activation-associated antigen [Croager et al., 1998; Horie and Watanabe, 1998].

The ligand for CD30 (CD30L), a member of the tumour necrosis factor (TNF) superfamily, is a type II single transmembrane protein consisting of 234 amino acids, whose molecular weight is 26–40 kDa [Smith et al., 1993]. Unlike CD30, CD30L is expressed relatively broadly, including in granulocytes, monocytes, macrophages, mast cells, and activated lymphocytes. In terms of pathological conditions, this protein is expressed in a subset of myeloid and lymphoid leukemias, and in Burkitt lymphoma [Croager and Abraham, 1997; Horie and Watanabe, 1998]. Stimulation of CD30-expressing cells by CD30L triggers various cellular signalling responses, including proliferation, survival, cytokine secretion, and cell death, depending on the type and differentiation status of the cells involved. These signals are triggered after CD30 becomes ligated to trimerised CD30L expressed on the surface of surrounding cells. The ligation of CD30L to CD30 triggers recruitment of intracellular adaptor proteins, such as TNFR-associated factor (TRAF) proteins, to the TRAF binding domain of the cytoplasmic tail of trimerised CD30, and resulting in further modification of downstream signalling molecules [Horie and Watanabe, 1998). CD30 is strongly expressed in cHL cells, and previous reports have shown that stimulation of CD30 by antibodies or CD30L triggers signalling events that may play a key role in anti-apoptosis and the expression of cytokines, characteristic features of cHL biology [Gruss et al., 1995; Wendtner et al., 1995; Zheng et al., 2003]. Other researchers, however, have argued against a functional role for CD30 in cHL [Hirsch et al., 2008].

Trimerised CD30 is thought to be located on the cell membrane during signalling processing and works as an initiator of signals by recruitment of downstream molecules [Kuppers et al., 2012; Brenner et al., 2015]. Recent studies using the chimeric anti-CD30 monoclonal antibody cAC10 linked to the antimitotic agent monomethyl auristatin E, an antibody–drug conjugate known as brentuximab vedotin, revealed that brentuximab vedotin–CD30 complexes are internalised into cells [Sutherland et al., 2006] and that this has shown to be an effective treatment approach for CD30-expressing lymphomas [Younes et al., 2010]. Although this observation clearly suggests that modification of CD30 can trigger trafficking of CD30 into cells, it is not clear whether this process mimics the endogenous process of CD30 signal transduction triggered by CD30L.

In the present study, we have addressed this question by examining CD30L–CD30 interactions between stably transfected CHO and HeLa cells using time-lapse microscopy. We show that the process of CD30 signalling is more dynamic than previously thought, and that ligation of CD30L triggers internalisation of the CD30L–CD30 complexes into cells expressing CD30. We have examined the molecular mechanisms underpinning this observation and performed additional experiments to clarify whether the process also operates in cHL cells, a haematological malignancy that is characterised by the overexpression of CD30. We thus propose a new model for CD30 signalling, and discuss the significance of our findings.

Results

Ligation of CD30L and internalisation of CD30L–CD30 complexes in CD30-expressing cells

To observe the intercellular transfer of CD30 after its ligation by CD30L, we prepared two stable cell line transformants, CHO cells expressing CD30L fused to mCherry on its N-terminus (mCherry-CD30L/CHO), and HeLa cells expressing CD30 fused to GFP at the C-terminus (CD30-GFP/HeLa) for time-lapse imaging (Figure 1A). Co-culture of these cells triggered gathering of GFP and mCherry signals at the cellular interface, formation of multiple complex clusters (indicated by co-localisation of GFP and mCherry), internalisation of the complex into the HeLa cells, and an intracellular transit path that disappeared around the nucleus (Figure 1B top and Supplementary Video S1). This phenomenon was not observed when we used either CHO cells expressing mCherry alone, or CHO cells expressing mCherry-Mem (containing the N-terminal 20 residues of neuromodulin to label the plasma membrane), as control cells in co-culture with CD30-GFP/HeLa (Figure 1B middle and bottom, Supplementary Videos S2 and S3). These results suggest that the ligation of CD30L to CD30 triggers the internalisation of the complex into HeLa cells and the subsequent intracellular migration.

Figure 1 | **See Legend on next page**

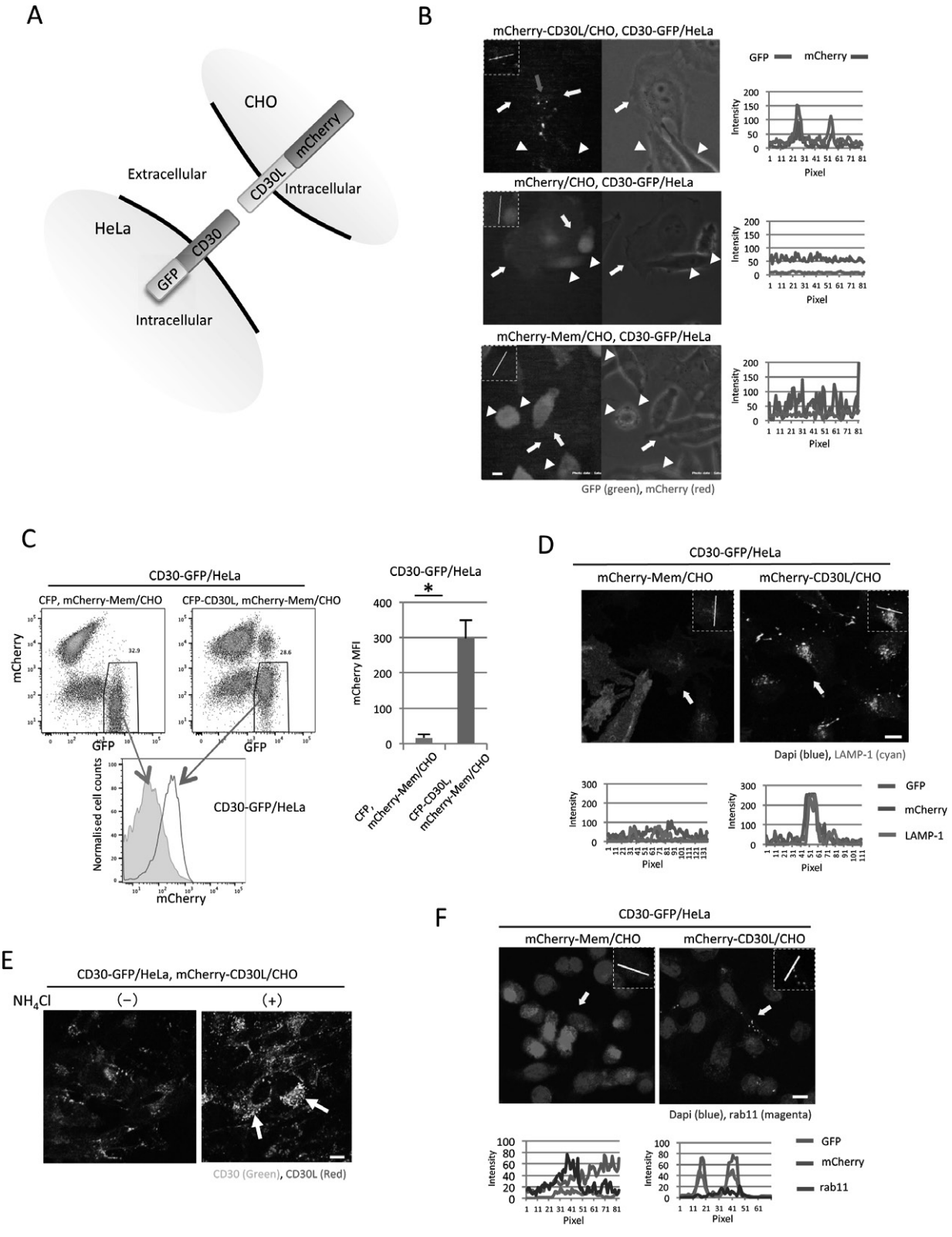


Figure 1 | Ligation of CD30L to CD30 and internalisation of CD30L–CD30 complexes in CD30-expressing cells
(**A**) The CD30 and CD30L constructs used to monitor their interaction. The C-terminus of CD30 (type I single transmembrane protein) and the N-terminus of CD30L (type II single transmembrane protein) were fused to GFP and mCherry, respectively. These constructs contain fluorescent proteins in their cytoplasmic regions, to avoid interference with interactions at the cell membrane. The expression of these proteins in transfected cell lines was confirmed by immunostaining. (**B**) Co-culture of mCherry-CD30L/CHO and CD30-GFP/HeLa cells (top). Control experiments using CHO cells expressing mCherry-Mem (mCherry-Mem/CHO), or CHO cells expressing mCherry alone (mCherry/CHO), and CD30-GFP/HeLa cells are also shown (middle and bottom panels, respectively). Co-localisation of the signals by mCherry and GFP was analysed by ImageJ ver1.0. The analysis position is indicated by the yellow line shown in each inset with the corresponding original area in the fluorescent image indicated by the yellow arrow. The results are presented on the right. The results were reproducible and representative of more than 10 cells analysed in each experiment. The images were captured from time-lapse microscopy, with the original time-lapse videos corresponding to the top, middle and bottom panels are presented as video 1, video 2 and video 3, respectively. Corresponding phase-contrast images are presented to the right of each fluorescent image. The white arrowheads and white arrows indicate CHO cells and CD30-GFP/HeLa cells, respectively. The red arrow indicates co-localisation of the signals by mCherry and GFP. Scale bar, 10 μm. (**C**) The plasma membrane of CFP-CD30L/CHO and CFP/CHO were labelled with the expression of mCherry-Mem as described in the *Materials and Method*. These cells were co-cultured with CD30-GFP/HeLa cells for 6 h at 37°C. The subsequent fluorescence intensity of mCherry in CD30-GFP/HeLa cells was measured by FACS Verse. The results are shown in the left. The mean $\pm$ S.D. of the mean fluorescence intensity (MFI) from three independent experiments, after subtraction of the corresponding mCherry background signal from HeLa cells only, is shown in the accompanying bar graph (*, $P < 0.05$; right). (**D**) The localisation of CD30L–CD30 complexes and lysosomes. CD30-GFP/HeLa and mCherry-CD30L/CHO cells were co-cultured for 3 h and lysosome compartment of the cells stained with anti-LAMP-1 antibody and anti-rabbit secondary antibody conjugated with Alexa647. These cells were observed by confocal fluorescence microscopy (right panel). The experiment using mCherry-Mem/CHO and CD30-GFP/HeLa cells served as control (left panel). Scale bar, 10 μm. Co-localisation of mCherry and GFP was analysed as described in the legend for Figure 1B and the results are shown below the fluorescent images. The results were reproducible and representative of more than 10 cells analysed in each experiment. (**E**) Lysosomal inhibition and detection of the fluorescent signal for CD30L–CD30 complexes. CD30-GFP/HeLa cells were co-cultured with CD30L-mCherry/CHO cells for 24 h before incubation with 10 mM of the lysosome inhibitor NH_4Cl for 4 h and subsequent evaluation by confocal fluorescence microscopy, under live cell conditions (right panel). The experiment without NH_4Cl served as control (left panel). The intracellular signals for the co-localised mCherry and GFP in CD30-GFP/HeLa cells (appearing as a yellowish aggregate) with NH_4Cl treatment are indicated by the white arrows. Results were confirmed in independent experiments. Scale bar, 10 μm. (**F**) The localisation of the fluorescent signals for CD30L–CD30 complexes and Rab11. CD30-GFP/HeLa and mCherry-CD30L/CHO cells were co-cultured for 3 h. The cells were stained with anti-Rab11 antibody and anti-rabbit secondary antibody conjugated with Alexa 647. These cells were observed by confocal fluorescence microscopy (right panel). The experiment using mCherry-Mem/CHO and CD30-GFP/HeLa cells served as control (left panel). Scale bar, 10 μm. Co-localisation of mCherry, GFP and Alexa 647 was analysed as described in the legend for Figure 1B and the results are shown below the fluorescent images. The results were reproducible and representative of more than 10 cells analysed in each experiment. Scale bar, 10 μm.

Since CD30L was fused to mCherry at its cytoplasmic N-terminus, CD30 appears to extract CD30L from CD30L-expressing cells along with a portion of the cell membrane. To assess this, we prepared CHO cells expressing CD30L fused to CFP on its N-terminus (CFP-CD30L/CHO) and labelled the plasma membrane with mCherry-Mem. We then performed co-culture and examined the subsequent presence of mCherry in CD30-GFP/HeLa cells. We found that the mCherry signal in CD30-GFP/HeLa cells was stronger than in the control experiment using CFP/CHO cells expressing mCherry-Mem in co-culture with CD30-GFP/HeLa cells (Figure 1C). We also labelled the membrane of mCherry-CD30L/CHO cells with a fluorescent dye (CellVue® Maroon; eBioscience), then repeated the co-culture experiment and examined the subsequent presence of the fluorescent dye in the CD30-GFP/HeLa cells. The results showed that the CellVue® Maroon signal in CD30-GFP/HeLa cells was stronger than in the control experiment using mCherry/CHO and CD30-GFP/HeLa cells (Supplementary Figure S1). These

results indicate that CD30L is transferred to the recipient HeLa cells along with a portion of the plasma membrane.

Next, we examined the intracellular transport of the CD30L–CD30 complex in CD30-GFP/HeLa cells. As discussed above, time-lapse imaging indicated that the complex is transferred around the nucleus before the fluorescent signal disappears, suggesting breakdown of the complex in the lysosome. Therefore, we labelled the lysosomal compartment with anti-LAMP-1 and a secondary antibody conjugated with Alexa 647. When we co-cultured CD30-GFP/HeLa and mCherry-CD30L/CHO cells, the fluorescent signal for the CD30L–CD30 complex in CD30-GFP/HeLa cells appeared to merge with the lysosomal compartment, but this was not observed during co-culture of CD30-GFP/HeLa and mCherry-Mem/CHO cells (Figure 1D). To obtain further support for this finding, we treated co-culture of CD30-GFP/HeLa and mCherry-CD30L/CHO cells with a lysosome inhibitor (NH_4Cl). This led to an increase in the intracellular signal for the CD30L–CD30 complex, in comparison with cells co-cultured without the lysosome inhibitor (Figure 1E). The signal for the CD30L–CD30 complex did not co-localise with the signal for Rab11, which is a marker for endocytic protein recycling (Figure 1F). Collectively, these results indicate that, upon ligation of CD30L, CD30 extracts CD30L and a portion of the plasma membrane from CD30L-expressing cells, triggering internalisation of the complex into the CD30-expressing cells and transport to the lysosome.

Internalisation of the CD30L–CD30 complex into CD30-expressing cells and its dependency on actin assembly

Internalisation of molecules from the plasma membrane is mediated by both dynamin- and non-dynamin-mediated pathways, with clathrin-mediated internalisation known to be regulated by the former [Harper et al., 2013]. To examine the mechanisms controlling internalisation of the CD30L–CD30 complex, we evaluated this process in HeLa cells after treatment with various inhibitors. We used sucrose, Pitstop® 2 and dynamin as inhibitors for clathrin-mediated internalisation [Suzuki et al., 2001; Macia et al., 2006] and the actin polymerisation inhibitors cytochalasin B [Rotsch and Radmacher, 2000] and latrunculin A [Lakshminarayan et al., 2014], and the microtubule depolymerising agent nocodazole [Vasquez et al., 1997], to evaluate the involvement of clathrin-independent pathways.

Following the co-culture of mCherry-CD30L/CHO, mCherry-Mem/CHO, mCherry/CHO or CHO cells, with CD30-GFP/HeLa cells, we used flow cytometry to measure the intensity of mCherry in cells co-expressing GFP. In the absence of inhibitors, the intensity of mCherry in CD30-GFP/HeLa cells was significantly higher following culture with mCherry-CD30L/CHO cells than after culture with mCherry-Mem/CHO, mCherry/CHO or CHO cells (Figures 2A and 2B). There was no significant difference between mCherry-Mem/CHO, mCherry/CHO or CHO cells in regard to the intensity of mCherry measured following their co-culture with CD30-GFP/HeLa cells (Figure 2B and Supplementary Figure S2). Using this experimental system, we wanted to address the mechanisms controlling internalisation of the CD30L–CD30 complex. When we used sucrose (an inhibitor of clathrin-dependent internalisation), Pitstop® 2 (a clathrin inhibitor) and dynasore (an inhibitor of dynamin-dependent internalisation), the internalisation of the CD30L–CD30 complex by CD30-GFP/HeLa cells, as measured by the intensity of the mCherry signal was not significantly affected. However, both cytochalasin B and latrunculin A significantly decreased complex internalisation (Figure 2C). Representative histograms and flow cytometry plots for cells treated with cytochalasin B are shown in Figure 2D and Supplementary Figure S3, respectively. Cytochalasin B and latrunculin A did not affect the viability of CD30-GFP/HeLa cells at the concentration used (Supplementary Figure S4).

In order to confirm that CD30L–CD30 complexes are internalised in a clathrin-independent manner, CD30-GFP/HeLa cells were treated with small interfering RNAs (siRNAs) for clathrin (or control siRNA), prior to co-culture with mCherry-CD30L/CHO. Representative flow cytometry data for this experiment are presented in Supplementary Figure S5. We observed no change in mCherry intensity in CD30-GFP/HeLa cells following clathrin knockdown (Figure 2E, left), indicating that internalisation of the CD30L–CD30 complex may be mediated by a non-clathrin, actin-dependent pathway.

Figure 2 | See Legend on next page

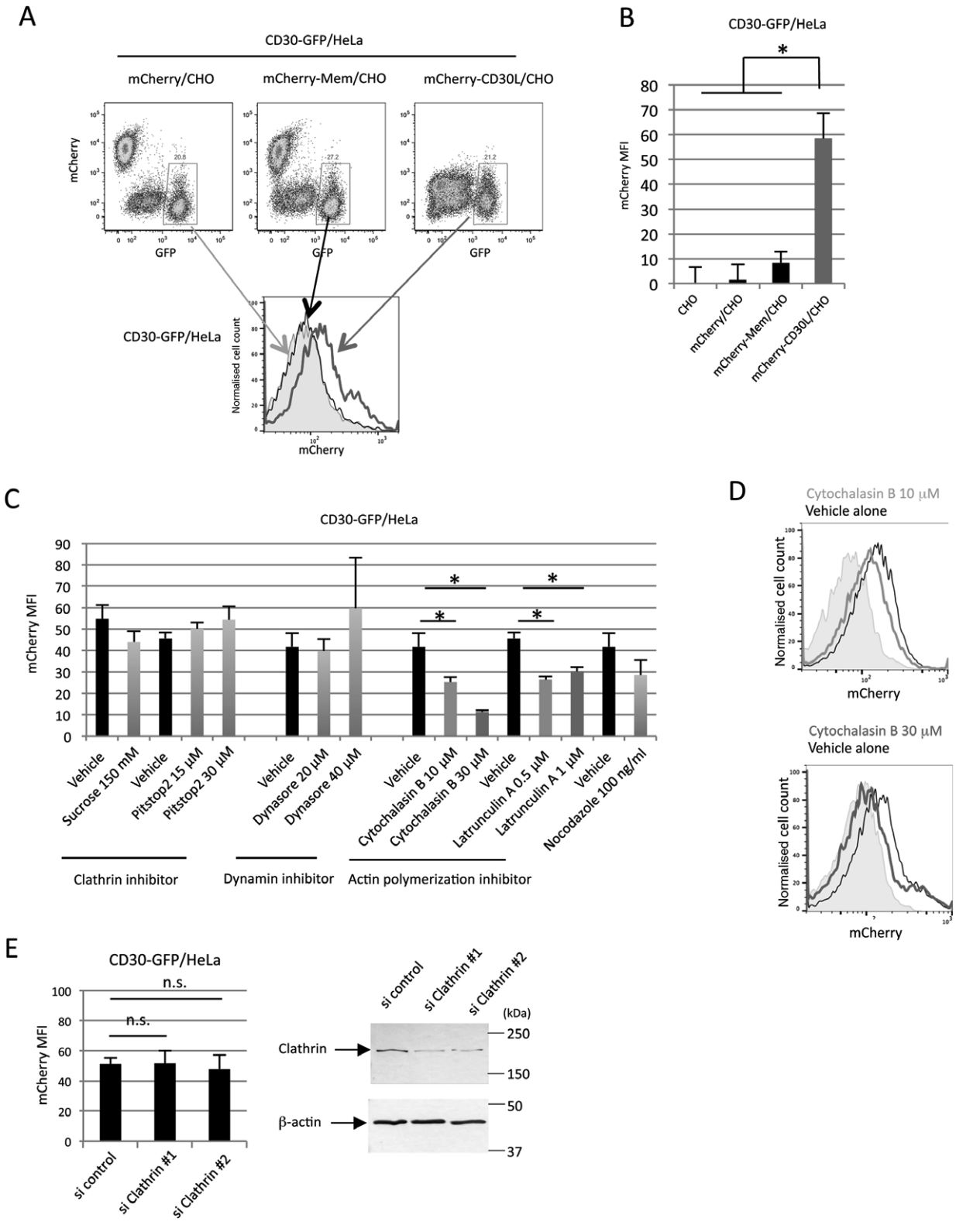


Figure 2 | Analyses of the molecular mechanisms governing the internalisation of the CD30L–CD30 complex into CD30-expressing cells

(A) CD30-GFP/HeLa cells, pre-cultured for 24 h, were co-cultured with mCherry-CD30L/CHO, mCherry-Mem/CHO, mCherry/CHO or CHO cells for 1 h. Following cell harvest, the HeLa cell mCherry signal was measured by FACS Verse to detect internalised CD30L. Representative results from the combination of CD30-GFP/HeLa cells with either mCherry-CD30L/CHO, mCherry-Mem/CHO or mCherry/CHO cells are indicated in the histogram with a red line, a black line, or as a solid grey area, respectively. **(B)** The experiment shown in Figure 2A was performed three times independently and the results presented as mean ± S.D of the MFI, after subtraction of the corresponding mCherry background signal of HeLa cells only (*, $P < 0.05$). Data without background subtraction were presented in Supporting Information Figure S2. **(C)** The effect of inhibitors on the internalisation of CD30L–CD30 complexes. Following pre-culture of CD30-GFP/HeLa cells in DMEM medium containing 10% FBS for 24 h, they were co-cultured for 1 h with mCherry/CHO or mCherry-CD30L/CHO cells in the presence of various inhibitors, then harvested. The mCherry fluorescent signal was detected by flow cytometry, with mCherry/CHO cells used for the control experiment. The inhibitors used were sucrose and Pitstop® 2 (clathrin inhibitors), dynasore (a dynamin inhibitor), nocodazole (a microtuble depolymerising agent) and cytochalasin B and latrunculin A (both actin polymerisation inhibitors). The data represent the summarised fluorescent mCherry signal in CD30-GFP/HeLa cells from each experiment, after subtraction of the background signal from experiments using control mCherry/CHO cells. The experiment was performed three times independently, and the results presented as the mean ± S.D. of the MFI (*, $P < 0.05$). The same results for the control (vehicle) are presented in the experiments carried out at the same time, namely pitstop®2 and latrunculin A, and dynasore, cytochalasin B and nocodazol. **(D)** Representative histograms of the experiments using cytochalasin B in Figure 2C are presented (black lines, no inhibitor; orange and red lines, cytochalasin B at different doses). Experiments using mCherry/CHO cells instead of mCherry-CD30L/CHO cells, and vehicle alone, are indicated by the solid-grey histograms. **(E)** The effect of clathrin knockdown on the internalisation of CD30L–CD30 complexes. siRNAs for the clathrin heavy chain (si Clathrin #1 or #2) or control siRNA (si-control) were transduced into CD30-GFP/HeLa cells and cultured for 48 h. The cells were then co-cultured with mCherry-CD30L/CHO cells for 1 h and harvested. Data represent the summarised fluorescent mCherry signal in CD30-GFP/HeLa cells from each experiment, after subtraction of the background CD30-GFP/HeLa mCherry signal from the control experiment by CD30-GFP/HeLa and mCherry/CHO. Experiments were performed three times independently, and the results were presented as the mean ± S.D. of the MFI (left, n.s. not significant). For confirmation of clathrin knockdown, immunoblotting was performed (right) with β-actin serving as loading control.

The knockdown of clathrin expression by siRNA was confirmed by immunoblot (Figure 2E right).

Ligation of CD30L to CD30 and internalisation of CD30L–CD30 complexes in cHL cells

We next examined whether the internalisation of the CD30L–CD30 complexes that we observed in transfectant HeLa cells might operate in tumours endogenously expressing CD30. Since CD30 is a characteristic marker for cHL and previous reports have indicated a significant biological role for the protein in this disease, CD30 signalling may be important as a molecular target for cHL [Gruss et al., 1995; Wendtner et al., 1995; Zheng et al., 2003; Kumar and Younes, 2014]. We therefore examined whether the CD30L-stimulation of CD30 expressed by cHL lines by CD30L, similarly triggered internalisation of the CD30L–CD30 complexes. To do this, mCherry-CD30L/CHO cells were co-cultured with the cHL cell lines, L428 or KMH2. Following harvest of cHL cells alone, CD30 was immunostained using an FITC- conjugated antibody and assessed by fluorescence microscopy. This experiment showed both co-localisation of mCherry and FITC, and a decreased surface intensity of FITC (Figure 3A). In contrast, when mCherry/CHO cells were co-cultured with cHL cells, co-localisation was not observed, and the surface signal for FITC remained strong (Figure 3A). This finding was supported by flow cytometry analysis (Figure 3B). These results suggest a down-regulation of surface CD30 after ligation to CD30L, and subsequent internalisation of the CD30L–CD30 complex in cHL cells.

Next we used CFP-CD30L/CHO cell labelled with mCherry-Mem, repeated the co-culture experiment, and then examined the presence of the mCherry signal in cHL cells fluorescently labelled with iRFP720 (iRFP720/cHL). This experiment showed a higher iRFP720/cHL mCherry intensity than the control experiment using CFP/CHO cells expressing

Figure 3 | **See Legend on next page**

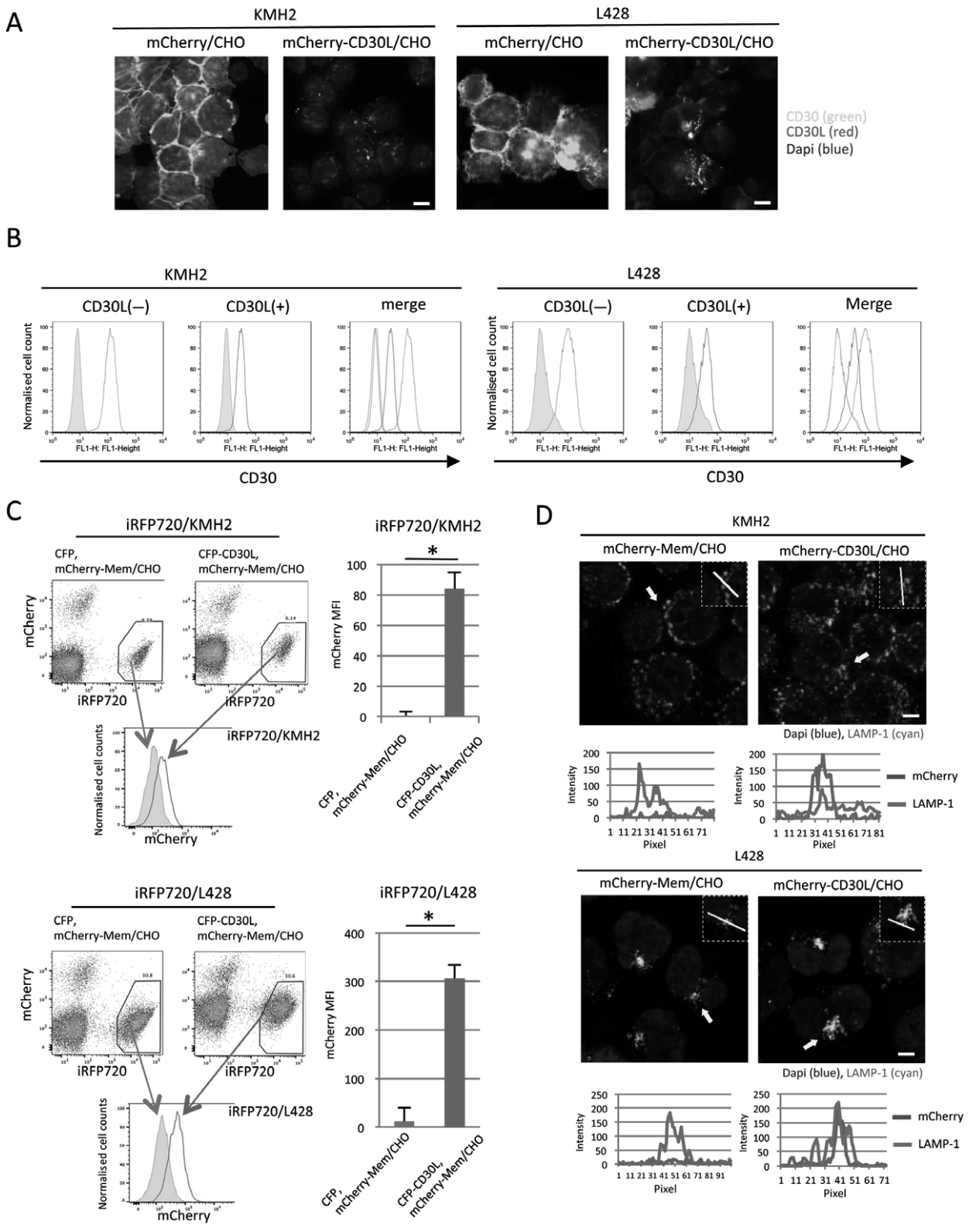


Figure 3 | Localisation of CD30L–CD30 complexes after co-culture with CD30L-expressing cells and cHL cell lines
(**A**) Representative images of the localisation of CD30 in cHL KMH2 and L428 cell lines after co-culture with CD30L-expressing cells. After pre-culture of mCherry-CD30L/CHO or control mCherry/CHO cells for 4 h, cHL cells were co-cultured with these cells and then cHL cells alone harvested. The harvested cHL cells were cytospun onto glass slides and stained with anti-CD30 antibody conjugated with FITC and DAPI, and imaged by fluorescence microscopy. Scale bar, 20 μm. (B) Measurement of CD30 expression on the surface of cHL cells after co-culture with mCherry-CD30L /CHO (+) or control mCherry/CHO cells (−). cHL cells were treated as described in Figure 3A, and the cell surface expression of CD30 measured using the FACSCalibur. Control staining with anti-IgG1 antibody conjugated with FITC is indicated by the grey histogram. (**C**) The plasma membrane of CFP-CD30L/CHO and CFP/CHO were labelled with the expression of mCherry-Mem as described in the *Materials and Method*. These cells were co-cultured with KMH2 or L428 cells labelled with iRFP720 for 6 h at 37°C. Then the fluorescence intensity of mCherry in KMH2 and L428 cells was measured by FACS Verse and the results were shown. KMH2 and L428 labelled with iRFP720 are indicated as iRFP720/KMH2 and iRFP720/L428, respectively. The mean ± S.D. of the MFI from three independent experiments, after subtraction of the background mCherry signal in cHL cells, is shown in the accompanying bar graph (*, $P < 0.05$). (**D**) Localisation of the fluorescent signals for CD30L and lysosomal compartment. mCherry-CD30L/CHO or mCherry-Mem/CHO cells, and cHL cells were co-cultured for 3 h, and cHL cells alone were harvested. The cHL cells were cytospun onto glass slides and stained with anti-LAMP-1 antibody, anti-rabbit secondary antibody conjugated with Alexa 647, and DAPI. The cells were imaged by confocal fluorescence microscopy. Co-localisation of fluorescent signals for mCherry and Alexa 647 was analysed using ImageJ ver1.0., and the results are presented below the photographs. The analysis position is indicated by the yellow line shown in each inset, with the corresponding area in the fluorescent image indicated by the yellow arrow. The results were reproducible and representative of more than 10 cells analysed in each experiment. Scale bar, 10 μm.

mCherry-Mem (Figure 3C). Labelling mCherry-CD30L/CHO cells using CellVue® Maroon and co-culturing with cHL cell lines, again showed an increased fluorescent signal for the dye in cHL cells (Supplementary Figure S6). These results indicate that cHL cell lines acquire signal for the CHO cell plasma membrane following co-culture. Finally, when we labelled the lysosomal compartment of cHL cells with anti-LAMP-1 and a secondary antibody conjugated with Alexa 647, the mCherry CD30L-CD30 signal again tracked around the nucleus and appeared to merge with the lysosomal compartment (Alexa 647 staining, cyan; Figure 3D). Collectively, these results indicate that the internalisation of the CD30L–CD30 complex occurs in similar manner in cHL cells as observed in CD30-GFP/HeLa cells.

Internalisation of the CD30L–CD30 complex into cHL cells and its dependency on actin assembly

To address whether the internalisation of the CD30L–CD30 complex into cHL cells is dependent on actin, as we observed in HeLa cells, we used a similar experimental system, which was performed in HeLa cells (Figure 2A). In the absence of inhibitors, the intensity of mCherry in cHL cell lines labelled with iRFP720 (iRFP720/cHL) was higher following co-culture with mCherry-CD30L/CHO cells than after co-culture with control mCherry/CHO cells, as expected (Figures 4A and 4B). This result indicates that the CD30L–CD30 complex is internalised by cHL cells. Representative flow cytometry data for this experiment are presented in Supplementary Figure S7. When we treated cells with sucrose, Pitstop® 2, or dynasore, the signal intensity for mCherry was not significantly affected, however, cytochalasin B and latrunculin A again reduced the mCherry signal in iRFP720/cHL cells significantly (Figure 4C). Representative histograms and flow cytometry data for the experiments using cytochalasin B and sucrose are shown in Figure 4D and Supplementary Figure S8, respectively. Cytochalasin B and latrunculin A did not affect the viability of iRFP720/cHL cells (Supplementary Figure S9). These results indicate that internalisation of the CD30L–CD30 complex into cHL cells is also mediated by a non-clathrin, actin-dependent pathway, as was observed for CD30-GFP/HeLa cells.

CD30 stimulation induces Ca^{2+} flux

Finally, we examined whether the observed interaction between CD30L and CD30 in our experimental system was associated with a signalling events. A previous report showed that CD30 stimulation by an agonistic antibody in Jurkat cells triggers Ca^{2+} flux [Ellis et al., 1993]. Ca^{2+} is an important signalling messenger [Feske, 2007] and we reasoned it might

be mobilised by CD30L–CD30 internalisation. To address this question, we firstly measured the intracellular Ca^{2+} concentration of HeLa cells following co-culture of CFP-CD30L/CHO or CFP/CHO cells with CD30-GFP/HeLa cells. As shown in Figure 5A, we found that co-culture of CFP-CD30L/CHO and CD30-GFP/HeLa cells resulted in a significant increase in HeLa cell intracellular Ca^{2+}, compared to the co-culture of CFP/CHO and CD30-GFP/HeLa cells. Similar results were also obtained when we cultured CFP-CD30L/CHO or CFP/CHO cells with cHL cells (Figure 5B). Addition of the actin polymerisation inhibitor latrunculin A, which inhibits internalisation of the CD30L–CD30 complex, abrogated the Ca^{2+} flux in CD30-GFP/HeLa cells triggered by co-culture with CFP-CD30L/CHO cells (Figure 5C). We have previously reported that CD30 signalling can trigger aggregation of TRAF and formation of signalosomes [Horie et al., 2002]. Accordingly, we examined whether internalised CD30L–CD30 complexes co-localise with TRAF. When we co-cultured CD30-GFP/HeLa and mCherry-CD30L/CHO cells, the fluorescent signal for the CD30L–CD30 complex in CD30-GFP/HeLa cells appeared to merge with the

Figure 4 | **See Legend on next page**

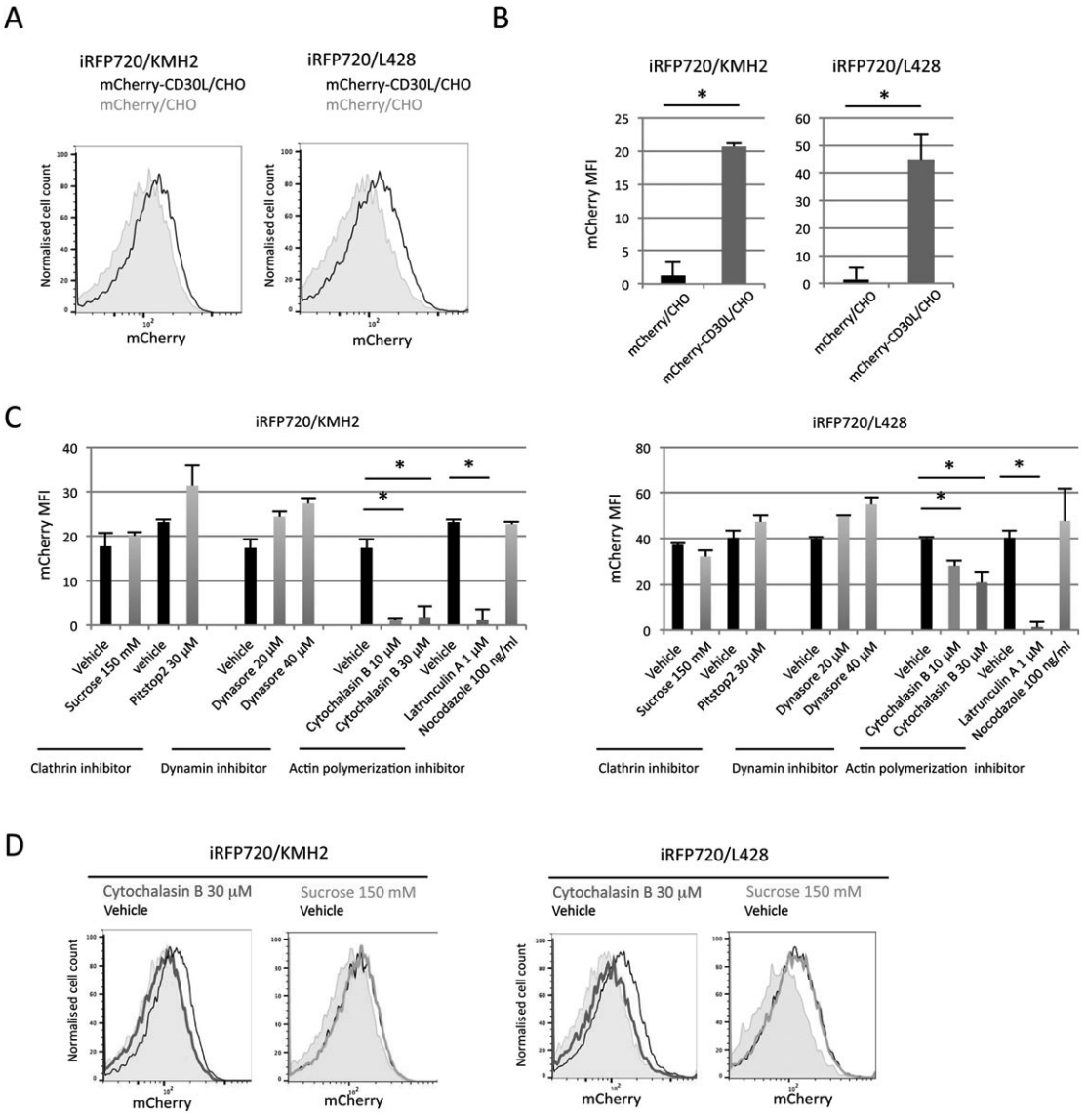


Figure 4 | Analyses of the molecular mechanisms governing the internalisation of the CD30L–CD30 complex into cHL cells

(**A**) Flowcytometric analyses of mCherry-CD30L internalisation into iRFP720/cHL cells. After pre-culture of mCherry-CD30L/CHO or mCherry/CHO cells in Ham's F12 medium containing 10% FBS for 24 h, they were then co-cultured for 3 h with iRFP720/cHL as described. The mCherry signal of the iRFP720/cHL cells was measured by flow cytometry and the resultant histograms are shown. The combination of iRFP720/cHL and mCherry-CD30L/CHO, or iRFP720/cHL and mCherry/CHO, is indicated by the black lines and the solid-grey histograms, respectively. The data are representative of three independent experiments. (**B**) Quantification of mCherry-CD30L internalisation into cHL cells. The experiment shown in Figure 4A was performed three times independently and the results presented as mean ± S.D. of mean mCherry fluorescent intensity (*, $P < 0.05$), after subtraction of the corresponding background mCherry signal for iRFP720 /cHL cells. (**C**) Inhibition of CD30L–CD30 complex internalisation in cHL cell lines. mCherry-CD30L/CHO or mCherry/CHO cells were pre-cultured in Ham's F12 medium containing 10% FBS. iRFP720/cHL cells were then co-cultured with these CHO cells in an equal volume of Ham's F12 and RPMI 1640 with the indicated concentration of inhibitors for 3 h, and then harvested. The fluorescent signal of mCherry, which represents internalised CD30L in cHL cells marked with iRFP720, was detected by flow cytometry. Experiments were performed three times independently and the results presented as mean fluorescence ± S.D. (*, $P < 0.05$), with the corresponding background signal of control experiments subtracted. The same results for the control (vehicle) are presented in the experiments carried out at the same time, namely pitstop®2 and latrunculin A, and dynasore and cytochalasin B. The control for the nocodazol experiments is identical to that for cytochalasin B. (**D**) Representative histograms of the experiments using cytochalasin B and sucrose from Figure 4C are presented (black line, no inhibitor; red line, cytochalasin B; blue line, sucrose). The experiment using mCherry/CHO cells instead of mCherry-CD30L/CHO cells and vehicle alone is indicated by the solid-grey histograms.

signal for TRAF (Figure 5D). These results suggest that the interaction of CD30L–CD30 complexes is coupled with intracellular signalling processes.

Discussion

Although CD30 ligated by CD30L is currently thought to work as a signal initiator that remains on the cell surface [Kuppers et al., 2012; Brenner et al., 2015], our results suggest that the process is in fact more dynamic. On the contact surface of CD30L and CD30-expressing cells, CD30L and CD30 form huge clusters, with CD30 extracting CD30L from the adjoining cell, along with part of their plasma membrane, and triggering internalisation of the clustered complex. Subsequently, the CD30L–CD30 complex appears to be transported to the lysosomal compartment without recycling. Our results indicate that this process is accompanied by signalling events, lysosomal degradation of the CD30L–CD30 complex, and down-regulation of cell surface CD30 expression that may initiate a refractory phase against further stimulation by CD30L (Figure 6).

We previously reported that CD30 signal transduction features cytoplasmic aggregates called signalosomes, consisting of molecules such as inhibitor of κB kinase and TRAF [Horie et al., 2002]. We and others have also shown that overexpression of the CD30 cytoplasmic tail is sufficient for signal transduction [Horie et al., 1996; Thakar et al., 2015]. These results indicate that CD30 can transduce signals during its cytoplasmic transport. Previously, Ca^{2+} influx has been demonstrated in response to antibody stimulation of CD30 in T-cells [Ellis et al., 1993], and in the present study, we have similarly shown that the interaction of CD30L and CD30 is accompanied by Ca^{2+} influx. Ca^{2+} serves as a messenger to trigger many cellular signalling pathways, including those that regulate the activation and proliferation of cells [Feske, 2007]. Collectively, our results suggest the interaction of CD30L and CD30, and their subsequent intracellular transport, is coupled with an active signalling process.

Signal transduction during the internalisation of TNFR superfamily members has been documented for the TNFR type I and Fas [Schutze et al., 2008; Schneider-Brachert et al., 2013]. Their respective ligands, TNF and FasL, exist as membrane-bound type II proteins that are released in a soluble form when cleaved just above the transmembrane region by metalloproteases [Aggarwal, 2003]. Experiments performed using these soluble forms [Schutze et al., 2008; Schneider-Brachert et al., 2013] have shown that the internalisation of TNFR type I and Fas after interaction with their soluble ligands is mediated by clathrin [Schutze et al., 2008; Schneider-Brachert

et al., 2013]. Similarly, the internalisation of CD30 by brentuximab vedotin has also been reported to be mediated by clathrin, but with a dependence on actin [Sutherland et al., 2006]. Since CD30L normally exists as membrane-bound form, we used physiological membrane-bound CD30L for the stimulation of CD30 in this study, and showed that internalisation of the CD30L–CD30 complex is dependent on actin rather than clathrin. These results indicate a difference in the internalisation process triggered by the stimulation of TNFR family members with either soluble ligands or membrane–bound ligands. The results also suggest that the internalisation of TNFR superfamily members after interaction with their membrane-bound ligands is not dependent on clathrin.

Trogocytosis represents the intercellular transfer of intact proteins through close cell-to-cell interaction and, in immune cells, is an active process coupled with receptor signalling and actin remodelling [Dopfer et al., 2011; Martinez-Martin et al., 2011]. Among the TNFR superfamily members, transfer of CD137 by trogocytosis has been reported in relation to the suppression of cytotoxic T-cell responses

Figure 5 | See Legend on next page

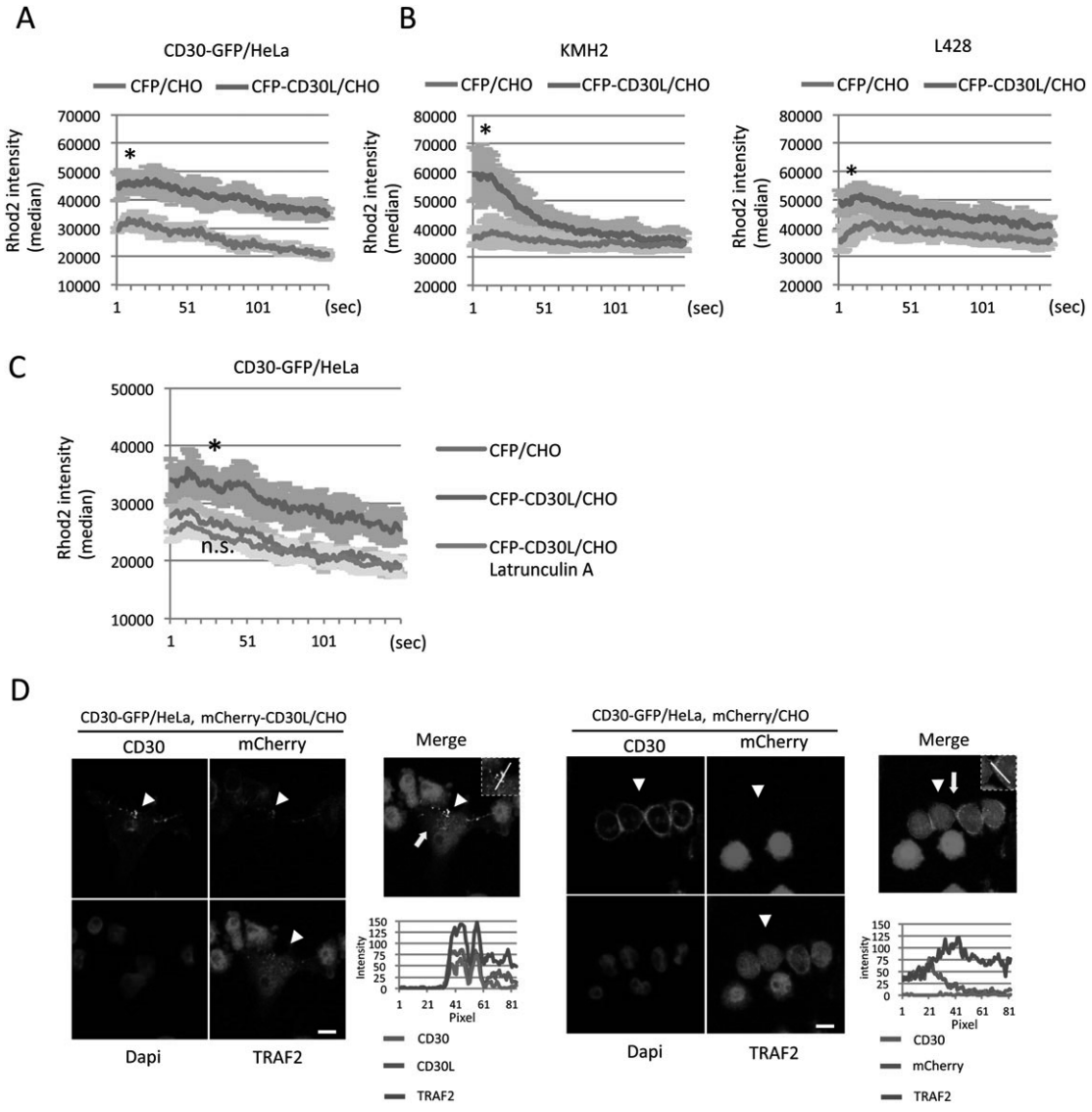


Figure 5 | Stimulation of CD30 by CD30L and intracellular signalling
(**A**) Co-culture of CFP-CD30L/CHO with CD30-GFP/HeLa cells and intracellular Ca^{2+}. Intracellular Ca^{2+} in CD30-GFP/HeLa cells was measured by Rhod2-AM binding using flow cytometry as described. The median intensity of Rhod2 over time, with and without CD30 stimulation, is presented by the thick red and grey lines, respectively. Data represent the mean ± S.D. of three independent counts (*, $P < 0.05$). (**B**) Co-culture of CFP-CD30L/CHO with cHL cell lines and intracellular Ca^{2+}. Intracellular Ca^{2+} in cHL cells was measured by Rhod2-AM binding using flow cytometry as described. The median intensity of Rhod2 over time, with and without CD30 stimulation, is presented by the thick red and grey lines, respectively. Data represent the mean ± S.D. of three independent counts (*, $P < 0.05$). (**C**) The effect of actin polymerisation inhibitor on the increase of intracellular Ca^{2+} in CD30-GFP/HeLa cells co-cultured with CD30L-expressing cells. Intracellular Ca^{2+} in CD30-GFP/HeLa cells was measured by Rhod2-AM binding using flow cytometry as described. Latrunculin A (1 μM) was added to co-culture of CD30-GFP/HeLa with CD30L-expressing cells for 30 min, after which the cells were harvested and analysed. The median intensity of the Rhod2 signal over time, with and without CD30 stimulation, is presented by the thick red and grey lines, respectively (*, $P < 0.05$). The median intensity of the Rhod2 signal after CD30 stimulation, in the presence of latrunculin A treatment is presented by the thick green line and was compared to intracellular Ca^{2+} without CD30 stimulation (n.s., not significant). (**D**) The localisation of internalised CD30L–CD30 complexes and TRAF2. CD30-GFP/HeLa and mCherry-CD30L/CHO cells were co-cultured for 3 h as described and fixed with 4% paraformaldehyde. The cells were stained with anti-TRAF2 antibody and secondary antibody conjugated with Alexa 647, then imaged by confocal fluorescence microscopy. The experiment using CD30-GFP/HeLa and mCherry/CHO cells served as control. The white arrowheads indicate CD30-GFP/Hela cells. Co-localisation of the fluorescent signals for mCherry, GFP and Alexa 647 was analysed by ImageJ ver1.0. The analysis position is indicated by the yellow line shown in each inset, with the corresponding area in the fluorescent image indicated by the yellow arrow. The results are representative of more than 10 cells analysed in each experiment and shown below the fluorescent images. The results are reproducible and representative of more than 10 cells analysed in each experiment. Scale bar, 10 μm.

against tumours by elimination of its ligand [Ho et al., 2013; Shao et al., 2015]. However, involvement of trogocytosis in the signalling of TNFR superfamily members has not been well documented to date. Here, we have shown that the internalisation of the CD30L–CD30 complex into CD30-expressing cells is associated with the transfer of plasma membrane containing CD30L, and is dependent on actin. These results suggest that trogocytosis of CD30 and other TNFR superfamily members takes place during cell signalling. It is important to note, however, that exosome-mediated transfer of cell membrane components can occur without the need for direct cell contact [Rechavi et al., 2009], and experimental discrimination between the processes of trogocytosis and exosome-mediated transfer can be difficult [Gutierrez-Vazquez et al., 2013]. Thus, while we postulate that the internalisation of CD30L into CD30-expressing cells occurs via trogocytosis, the potential involvement of exosomal transfer in this process cannot be excluded. A detailed study of the mechanism by which CD30L is taken up by CD30-expressing cells is needed to resolve this question.

Our results also indicate that CD30 signalling via trogocytosis similarly takes place in cHL cells. While CD30 signals associated with cell proliferation and activation in cHL cells have been previously described [Gruss et al., 1995; Wendtner et al., 1995; Zheng et al., 2003; Watanabe et al., 2011], there is one report that has argued the absence of such signals in cHL [Hirsch et al., 2008]. Our results clearly show that the mechanism of CD30L–CD30 internalisation and the associated intracellular signalling that we observed between CHO cells and HeLa cells expressing these molecules ectopically, also operates endogenously in cHL cells. In summary, the results of this study suggest a novel mode of signalling for TNFR superfamily members, and provide new insights into the biological roles of CD30 in normal and malignant cells and, in particular, in cHL.

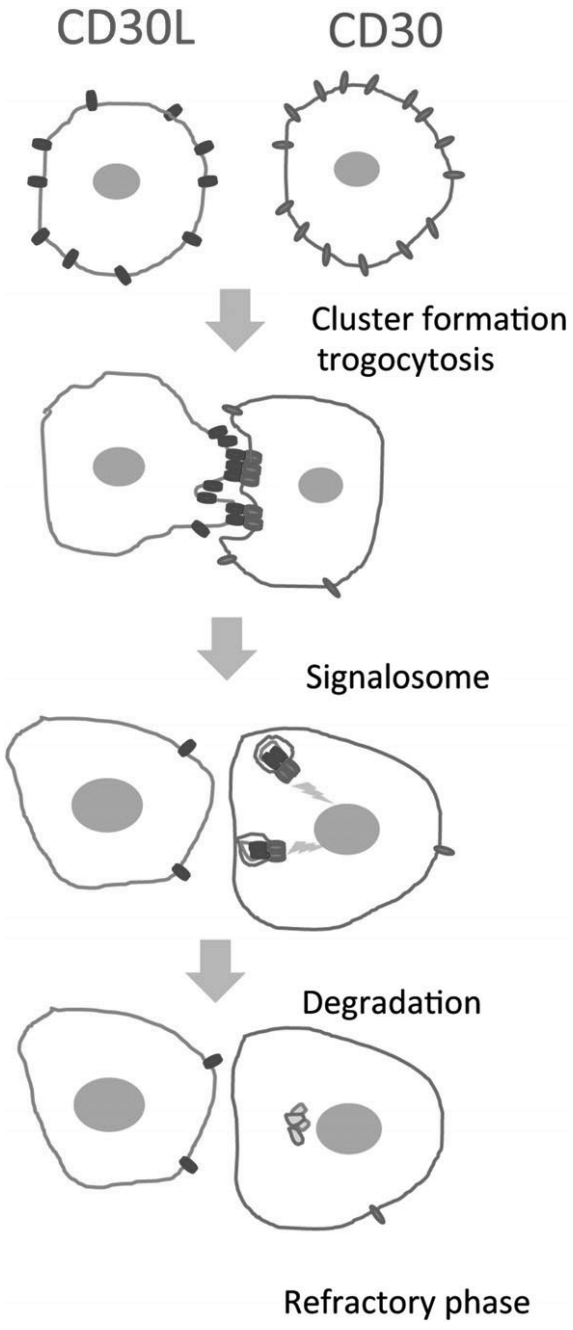
Materials and methods

Cell lines and cell cultures

cHL cell lines (L428, KMH2) were purchased from the German Collection of Microorganisms and Cell Cultures. HeLa and CHO cell lines were obtained from the Japanese Cancer Research Resources Bank and the DS Pharma Biomedical, respectively. cHL, HeLa and CHO cell lines were cultured in RPMI 1640 medium, Dulbecco's modified Eagle's medium and Ham's F12 (Wako) medium, respectively, with 10% foetal bovine serum (FBS) and antibiotics.

Figure 6 | Schematic representation of the formation of the CD30–CD30L ligand–receptor complex at the cell membrane, its intracellular transport and the status of cells during CD30 signalling

CD30 extracts CD30L from the adjacent cell in a trogocytic manner, resulting in actin-mediated internalisation of the complex, generation of signalosomes, intracellular signalling, lysosomal degradation and a subsequent refractory phase.



Chemicals

Sucrose (a clathrin-dependent internalisation inhibitor; Wako), Pitstop® 2 (a novel, selective cell-permeable clathrin inhibitor; Abcam), dynasore (a dynamin-dependent internalisation inhibitor; Sigma–Aldrich), cytochalasin B and latrunculin A (an actin polymerisation inhibitor; Sigma–Aldrich and Adipogen Life Sciences, respectively), and nocodazole (a microtubule depolymerising agent; Sigma–Aldrich) were used to examine internalisation of CD30L–CD30 complexes. The inhibitors were dissolved with DMSO except for sucrose, which was dissolved with H_2O and their conventional concentrations were used for the experiments. Rhod2-AM (PromoKine) was used as a Ca^{2+} indicator. CellVue® Maroon Cell labelling Kit (eBioscience) was used to label cell membranes.

Preparation of stable cell transformants

Full-length CD30 or CD30L cDNA was inserted into the CSII-EF1 vector (kindly provided by Dr. H. Miyoshi, RIKEN BioResource Center) for the expression of CD30-GFP, mCherry-CD30L and CFP-CD30L fusion protein. These plasmids, or corresponding empty vectors, were transduced into HeLa cells or CHO cells as described [Fujikawa et al., 2016], generating HeLa cells expressing CD30-GFP (CD30-GFP/HeLa), CHO cells expressing mCherry-CD30L (mCherry-CD30L/CHO) or CFP-CD30L (CFP-CD30L/CHO), as well as the corresponding control vector transformants (*i.e.* GFP/HeLa, mCherry/CHO and CFP/CHO). The mCherry-Mem construct (a gift from Catherine Berlot, Addgene plasmid # 55779; Addgene), containing the N-terminal 20 residues of neuromodulin fused to the N-terminus of mCherry vector, which labels the plasma membrane [Yost et al., 2007], was cloned into the CS-EF1 vector and stably transduced into CFP-CD30L/CHO or CFP/CHO, generating CHO cells expressing CFP-CD30L and mCherry-Mem (CFP-CD30L, mCherry-Mem/CHO), as well as its control transformant (CFP, mCherry-Mem/CHO). Cell lines were further enriched by sorting for fluorescence using the FACS Aria (BD Biosciences), which also served to confirm the expression of CD30 or CD30L in the transformed lines. For fluorescent detection of cHL cells, the iRFP720 construct (Addgene) in the CSII-EF1 vector was transduced into the L428 and KMH2 cell lines (iRFP720/L428 and iRFP720/KMH2) as previously described [Fujikawa et al., 2016], and the iRFP720 positive cells again sorted using the FACS Aria (BD Biosciences).

Immunostaining

Cells grown on poly-L-lysine-coated glass slides (Matunami Glass) or captured by Cytospin (Thermo Shandon) were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) or cold methanol for 10 min, and permeabilised with 0.1% tritonX-100 for 3 min. After washing with 0.01 M PBS, samples were incubated with the indicated antibody at room temperature for 1 h, washed with 0.01 M PBS with 1% bovine serum albumin (Roche) and if necessary, further incubated with an Alexa Fluor 488 conjugated secondary antibody for 1 h. Primary antibodies were: Anti-TRAF2 antibody (sc-7187) and anti-Clathrin HC antibody (sc-12734) (both from Santa Cruz Biotechnology), anti-LAMP-1 antibody (ab24170; Abcam), anti-CD30L antibody (AF1028; R&D Systems) and anti-Rab11 antibody (71-5300; Thermo Fisher Scientific). Secondary antibodies used

were anti-mouse IgG (H&L) AP conjugate antibody (S3728; Promega) and Alexa Fluor® 647 goat anti-rabbit (H+L) IgG antibody (A21245; Thermo Fisher Scientific). Nuclear staining was performed with DAPI solution (4',6-diamidino-2-phenylindole hydrochloride; Sigma–Aldrich). Fluorescence signals were detected using a Nikon A1 confocal immunofluorescence microscope (Nikon).

Time lapse imaging

For the observation of CD30L–CD30 complex trogocytosis, CD30-GFP/HeLa cells and mCherry-CD30L/CHO or mCherry/CHO cells were co-cultured in an equal volume of DMEM and Ham's F12 media and monitored by time-lapse microscopy (BioStation IM-Q; Nikon) at 3 min intervals.

Detection of the internalisation of CD30L–CD30 complexes by flow cytometry

CD30-GFP/HeLa cells (8×10^4) were pre-cultured in DMEM containing 10% FBS for 24 h and thereafter stimulated with the overlay of mCherry-CD30L/CHO cells (1×10^5), or control CHO cells expressing just mCherry-Mem or mCherry in an equal volume of DMEM and Ham's F12 media for 1 h. The cells were harvested, washed with PBS, and analysed by FACS Verse (BD Biosciences). For the stimulation of cHL cell lines, mCherry-CD30L/CHO or control mCherry/CHO cells (7×10^4) were pre-cultured for 24 h in Ham's F12 containing 10% FBS. cHL cells (1×10^5) were then overlaid on these CHO cells in an equal volume of RPMI1640 and Ham's F12 media containing 10% FBS. After incubation for 3 h, cells were harvested, washed with PBS, and analysed by FACS Verse (BD Biosciences).

RNA interference experiments

For RNA interference experiments, siRNAs were transfected into CD30-GFP/HeLa cells (8×10^4/mL) using Lipofectamine 2000 (Thermo Fischer Scientific) and cultured for 48 h. The siRNAs used were clathrin heavy chain (Ambion® # 107565 and s223262; both from Thermo Fischer Scientific) and AllStars Negative Control (1027280; Qiagen)

Immunoblot

Total cellular protein was isolated using cell lysis buffer [10 mM Tris–HCl (pH 7.4), 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA]. Whole cell lysates (30 μg) were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with the primary antibodies and alkaline phosphatase-conjugated secondary antibody. Antibodies were anti-Clathrin HC antibody (sc-12734; Santa Cruz Biotechnology), anti-β-actin antibody (sc-69879; Santa Cruz Biotechnology) and anti-mouse IgG (H+L) AP Conjugate secondary antibody (S3728; Promega). Immunoreactive proteins were visualised using BCIP/NBT Color Development Substrate. (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; Promega)

Measurement of Ca^{2+} flux

CFP-CD30L/CHO or control CFP/CHO cells (7×10^4/mL) were pre-cultured in Ham's F12 containing 10% FBS and were used for stimulation of CD30-GFP/HeLa and cHL cell lines after 24 h of culture. CD30-GFP/HeLa (1.5×10^5) and cHL cells (2×10^5) were loaded with Rhod2-AM (PromoCell) in 1 mL of DMEM and RPMI1640, respectively, for 40 min at 37°C. Then these cells were harvested, washed with PBS and overlaid on CFP-CD30L/CHO or control CFP/CHO cells in an equal volume of DMEM or RPMI 1640 and Ham's F12 media with 1 mM $CaCl_2$ at 37°C. After incubation of CD30-GFP/HeLa and cHL cells for 30 min or 1 h, respectively, these cells alone were collected and immediately analysed using FACS Verse (BD Biosciences) and FlowJo (TreeStar) [Ellis et al., 1993].

Statistical analysis

Differences between mean values were assessed using two-tailed *t*-tests, with $P < 0.05$ considered to be statistically significant.

Author contribution

M.N. performed the experiments with the support of M.W. and wrote a part of the paper. R.H. designed the study and wrote the paper with comments from K.U. All authors reviewed and approved the final version of the manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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特別講演

▷第63回中国・四国支部総会 特別講演(1)◁

成人T細胞白血病リンパ腫における フローサイトメトリー検査と応用

内丸 薫

日本臨床検査医学会

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臨床検査医学分野）

特別講演

▷第 63 回中国・四国支部総会　特別講演（1）◁

成人 T 細胞白血病リンパ腫における フローサイトメトリー検査と応用

内丸　　薫*

Use and Application of Flow Cytometry for Adult T-cell Leukemia-Lymphoma

Kaoru UCHIMARU, MD, PhD*

Adult T-cell leukemia is one of the most intractable hematological diseases, which is caused by HTLV-1 infection. Kyushu and Okinawa are the most endemic areas in Japan but the distribution of HTLV-1 carriers is changing because of migration to megalopolises such as Tokyo and Osaka. ATL is divided into 4 subtypes: smoldering, chronic, lymphoma, and acute types, and the latter two are called aggressive types. Treatment outcomes of chemotherapies showed little improvement for more than 40 years. Recently, hematopoietic stem cell transplantation was shown to improve treatment outcomes for ATL. Morphological estimation of tumor cells in the peripheral blood is very important for sub-classification or evaluation of the treatment outcome for ATL, but it is often difficult to discriminate tumor cells from normal lymphocytes because morphological abnormalities are ambiguous. We developed a new flow cytometric analysis system to detect ATL cells, which we named HAS-Flow (HTLV-1 Analysis System). HAS-flow detects ATL cells as $CD3^{dim}$/CD7-negative populations, which revealed the presence of an intermediate population. This system could not separate normal CD4 T lymphocytes from these intermediate cells, so we introduced analysis of CADM1 expression in this system. The new flow cytometric system successfully separated $CADM1^{-}/CD7^{+}$ (P) normal CD4 T cells, $CADM1^{+}/CD7^{-}$ (N) ATL cells, and an intermediate population (D). The CADM1/CD7 expression pattern reflects the progression of HTLV-1-infected cells into ATL cells. The D and N populations detected in the peripheral blood share many characteristics with aggressive ATL cells, which means basically these cells have features in common with ATL cells even in the carrier state. HAS flow is useful for monitoring the clinical course of ATL and HTLV-1 carriers. 【Review】

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【Key Words】 human T-cell leukemia virus type 1: HTLV-1(ヒトT細胞白血病ウイルス1型)、adult T-cell leukemia-lymphoma: ATL(成人T細胞白血病リンパ腫)、cell adhesion molecule 1: CADM1、flow cytometry(フローサイトメトリー)、HAS(HTLV-1 analysis system)-flow(HAS-フロー)

成人T細胞白血病リンパ腫(Adult T-cell leukemia-lymphoma: ATL)はヒトT細胞白血病ウイルス1型(Human T-cell leukemia virus type 1: HTLV-1)の感染によって起こる末梢性T細胞腫瘍である。ATLは1977年、当時京都大学のUchiyama、Takatsukiらにより新しいclinical entityとして提唱された[1]。ATLの疾患概念の提唱までは、皮膚病変を伴うT細胞性腫瘍としてSézary症候群や皮膚T細胞リンパ腫(CTCL)と診断されたり、ATLのうちリンパ節病理組織がHodgkin likeの組織像を示すものはHodgkinリンパ腫と診断されていたとみられ、これらの疾患が九州に多いのはなぜかという疑問がもたれていた。また、通常B細胞性である慢性リンパ性白血病に似たT細胞性の腫瘍が見られ、予後が悪いことが知られていた。Uchiyama、Takatsukiらはこれらの症例の、核に深い切れ込みが見られる形態的に特異な腫瘍細胞を認めること、高率に皮膚病変を認めること、患者の出身地が九州、南西諸島に大きく偏った特異な分布を示す、などの特徴からATLという新しい疾患概念として提唱した。ATLの発見には有名なエピソードがある。当時、現在でいうATLにあたる症例を診療していた高月は、患者の顔貌が似ている、いわゆる縄文人系の顔貌であることに気づき、病歴を確認すると全員九州出身であったことから出身地の特異な分布に気づいたというものであるが、このエピソードは臨床において患者をよく観察することの重要性に改めて気づかせてくれる。ATLの地域集積性は、この疾患の原因として環境的要因が関わることを示唆しており、その中の一つとして感染症があげられ、ATLの報告の当初から何らかのウイルス感染の関与が疑われた[1]。その後Miyoshi、Hinuma、GalloらによりATLがウイルス感染によるものであること、その原因ウイルスの分離などが報告され[2~4]、1982年にはYoshidaらにより約9 kBのレトロウイルスの全ゲノム配列が決定された[5]。これが現在のHTLV-1である。

HTLV-1感染者の本邦における分布は当初想定されたように偏りがあり、九州、南西諸島、四国太平洋岸から豊後水道寄りにかけての地域、隠岐、紀伊半島沿岸部、伊豆半島、東北地方太平洋沿岸地域などをあげることができる。世界的に見ても高浸淫地域(endemic area)とそうでない地域があり、赤道アフリカ、南アフリカ、ニューギニア、オーストラリアの一部、カリブ海沿岸諸国、南米、そして日本などがendemic areaである[6]。本邦におけるHTLV-1感染者は1988年の献血データをもとにした推定では約120万人、そのうち九州、沖縄地方在住者の比率は50.9%、関東地方在住者の比率は10.8%であったが[7]、2007年の同様の調査では全国で108万人と微減していたにも拘らず、関東地区在住者の比率は17.3%と増加していた。これはHTLV-1感染者が首都圏へ移住することにより国内の分布が変わってきていることを示唆し、endemic areaのみではなく、むしろこれまであまり認識されてこなかった、non-endemic areaの大都市における対策が重要であることを示している。2011年から国によるHTLV-1総合対策が開始され、妊婦は原則として全例公費負担で抗HTLV-1抗体のスクリーニングを受けている。2012年の妊婦検診における抗HTLV-1抗体陽性者の都道府県別推定によれば、大阪が鹿児島に次いで全国第3位、東京も長崎について第5位であり、大都市圏における対策が必要であることを物語っている。2016年に行われた推定では全国で約82万人となっており、漸減傾向にはあるが、今後とも全国レベルでの対策が必要となる。

I. ATLとHTLV-1

ATLはHTLV-1の発見の契機となった疾患であるが、HTLV-1は他にもHTLV-1関連脊髄症(HTLV-1 associated myelopathy: HAM)、HTLV-1ぶどう膜炎

(HTLV-1 uveitis: HU)など、炎症性疾患も惹き起こす。これらの疾患を発症するのは感染者のうちのごく一部であり、ATLの感染者における生涯発症率は5%程度、HAMについては0.3%程度と推定されている。HTLV-1の感染ルートはほとんどが母乳を介した母児感染、性交渉による感染と考えられている。かつては輸血感染があったと考えられるが、1986年以降日赤で抗HTLV-1抗体検査が導入されて以降は皆無と考えられる。

ATLを発症するのは母児感染例と考えられているが、ATL患者の年齢中央値は最新の第11次全国調査では68.8歳であり[8]、感染からATLの発症まで約70年かかることになる。HTLV-1感染によりポリクローナルに増殖し不死化した感染細胞に次第に遺伝子異常、エピジェネティックな異常などが積み重なり多段階発がんを惹き起こしていくと考えられる。この過程の、どこからを腫瘍と呼ぶかは、腫瘍というものの定義の仕方によると考えられる。このことはATL、特にindolent ATLをどのように診断するかと密接に関連する問題である。少なくともHTLV-1感染者の末梢血中に異常リンパ球が見られるというだけではATLという診断にはならない点に留意すべきである。ATLは1992年に提唱された下山分類(**Table 1**)が現在でもスタンダードである。下山分類ではATLは、くすぶり型、慢性型、リンパ腫型、急性型、の4病型に分けられるが、そのうち前2者は比較的緩徐な経過を取ることからindolent ATL、一方後2者は急激な経過を取ることからaggressive ATLにさらに分類される。それぞれの頻度は前出の第11次調査によれば、くすぶり型10.6%、慢性型14.2%、リンパ腫型25.7%、急性型49.5%でindolent ATLは比較的少なく、典型的なATLはaggressive ATLであるということができる[8]。このうち、くすぶり型は末梢血中の異常リンパ球が5%以上認められ、一部皮膚病変ないし肺病変を認める症例以外は臓器浸潤を伴わず、ほかの検査データも正常、ないし軽微な異常にとどまるものである。上述の、腫瘍とは何か、という観点からは、改めて、くすぶり型ATLと無症候性キャリアとの境界をどう考えるかは検討の余地があると思われる。この点については改めて議論する。慢性型は末梢血中のリンパ球の絶対数が4,000/mm^{3}以上に増加しているタイプで、ATLにおける慢性リンパ性白血病のcounterpartに相当するものである。慢性型は中枢神経、骨、消化管浸潤および胸腹水はあってはならないが、リンパ節腫脹の有無については規定がないことに注意を要する。リンパ節腫脹があるだけではリンパ腫型、急性型にはならない。

Table 1　ATLの下山分類

		くすぶり型	慢性型	リンパ腫型	急性型
抗HTLV-1抗体		＋	＋	＋	＋
リンパ球数(×10^9/L)		＜4	≧4	＜4	
異常リンパ球		≧5%(a)	＋(b)	≦1%	＋
花細胞(flower cell)		時々	時々	－	＋
LDH		≦1.5N	≦2N		
補正カルシウム(mEq/L)		＜5.5	＜5.5		
組織診のあるリンパ節腫大		no		yes	
腫瘍病変	肝腫大	no			
	脾腫大	no			
	中枢神経	no	no		
	骨	no	no		
	腹水	no	no		
	胸水	no	no		
	消化管	no	no		
	皮膚	(b)			
	肺	(b)			

(a)異常Tリンパ球が5%未満の場合，皮膚や肺に腫瘍性病変があることが組織診で証明されていること。
(b)異常Tリンパ球が5%未満の場合，組織診で証明された腫瘍病変が必要。

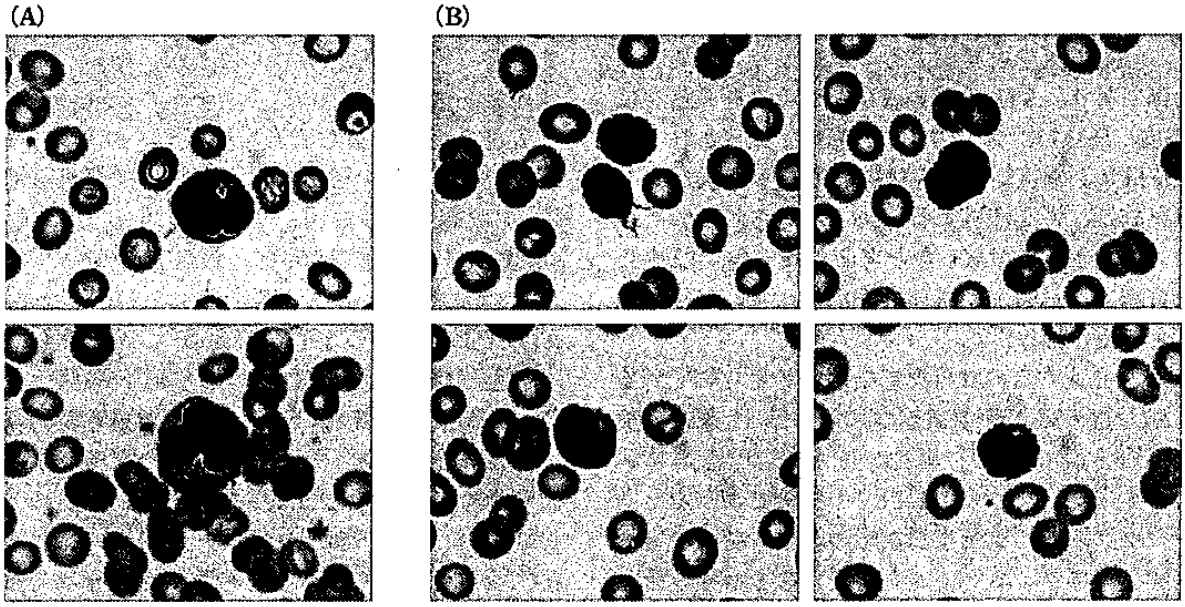

Figure 1 ATL 患者末梢血に見られる異常リンパ球
(A) 比較的典型的な flower cell。(B) 異型性が軽度な異常リンパ球：境界領域の異型性の場合、どこからを異型リンパ球と診断するか判断に迷うこともあり、鏡検者によって結果が異なることがありうる。

Aggressive ATL の化学療法の成績は、種々の臨床試験が行われてきたにも拘らず大きな改善はみられておらず、第 11 次全国調査の予後調査の結果では生存期間中央値は 9 ヵ月前後と、下山分類が発表された時代と比べてほとんど改善がみられていない。2000 年代に入って ATL に対しても適応のある症例を対象に造血細胞移植が積極的に行われるようになってきており、HLA 一致血縁、非血縁移植で 40%程度の長期生存が得られている[9]。また、おもに化学療法難治、再発例を対象に抗 CCR4 抗体 (mogamulizumab)[10]、レナリドマイド[11]などが導入され、治療成績の改善が期待されている。

II. HAS-Flow の開発

下山分類に従った病型分類において、また、ATL の治療効果を判定するにあたり、末梢血中異常リンパ球の定量は非常に重要な意味を持つ。典型的な ATL における異常リンパ球(腫瘍細胞)は Flower cell(花細胞)として大変有名な核に深い切れ込みのある特徴的な形態をとるが、急性型などにおいても異型性の軽い非典型的な腫瘍細胞も多く、くすぶり型、慢性型などの indolent ATL においては、むしろそういった細胞が普通であり、熟練した鏡検者でないと、しばしばその鑑別が困難である (Fig. 1)。そこで我々は急性白血病における blast gating のような、Flow cytometry による ATL 細胞の定量的評価を試みた。ATL 細胞は CD3 の発現が down-regulate されており、ほとんどの症例で CD7 が陰性であることから、まず CD4 陽性細胞における CD3/7 の発現により $CD4^+/CD3^{dim}/CD7^-$ の細胞集団として検出する系を開発し HAS(HTLV-1 Analysis System)-flow 法と命名した (Fig. 2)[12]。$CD4^+/CD3^{dim}/CD7^-$ の集団をソーティングしてサイトスピン標本で形態を観察すると、この分画は異常リンパ球の集団であり、inverse PCR による HTLV-1 プロウイルスの組み込み部位の解析、フローサイトメトリーを用いた TCR のレパトア解析の結果、クローナルに増殖している集団であることが示され、ATL の腫瘍細胞を検出しているものと考えられた。ATL 患者末梢血標本を熟練した技師が鏡検して計測した末梢血中異常リンパ球比率から算出した異常リンパ球数と、本法により算出した異常リンパ球数は、linear regression 1.034、Pearson's R=0.963 と極めて良い相関を示した[13]。

aggressive ATL 症例の末梢血を HAS 解析すると、$CD4^+/CD3^{dim}/CD7^-$ の腫瘍細胞、および $CD4^+/CD3^+/CD7^+$ の正常 CD4 陽性 T 細胞集団の間に $CD4^+/CD3^{dim}/CD7^{dim}$ という中間的な phenotype を示す細胞集団が存在する症例が一部認められた。この集団は両者の中間的な phenotype であることから腫瘍化の過程の中間段階の細胞集団を検出している可能性が

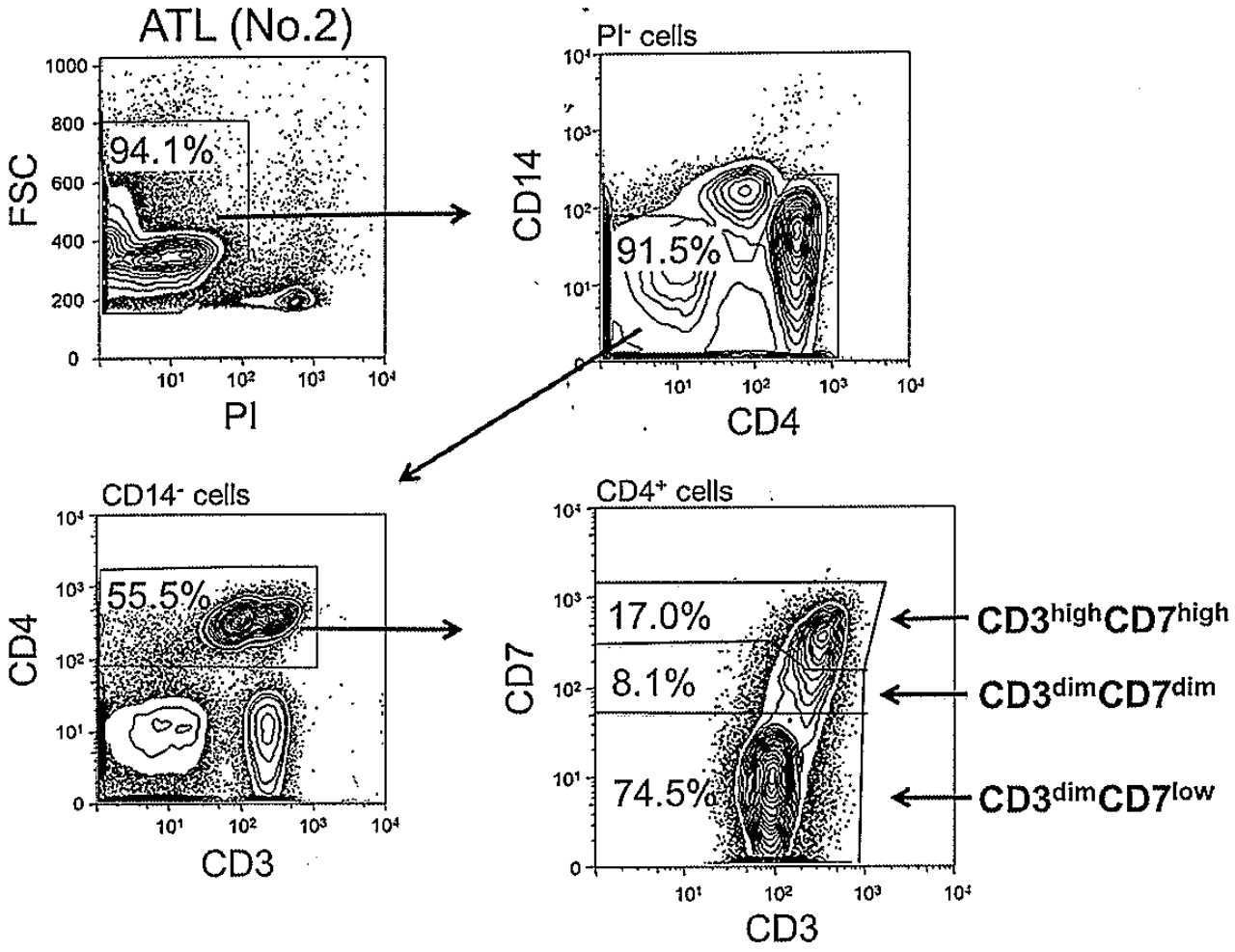

Figure 2　HAS(HTLV-1 Analysis System)-flow 法(第1世代)[12]

PI 陽性の死細胞をゲートアウトしたのち $CD4^{dim}/CD14^{+}$ の単球をゲートアウトし、CD3/CD4 陽性細胞にゲートをかけて CD3/CD7 の発現レベルで展開したものである。後述の CADM1 を組み込んだ HAS と区別するため第1世代(first generation: 1G)と呼ぶ。

想定された。実際、各集団をソーティングして細胞形態を確認すると $CD3^{dim}/CD7^{-}$ の集団が異型性の強い典型的な ATL 細胞であり、$CD3^{dim}/CD7^{dim}$ の集団は比較的異型性の軽い集団であった。また、無症候性キャリアから indolent ATL、aggressive ATL の各病型の ATL 症例まで末梢血を本法により解析した結果、末梢血プロウイルス量(PVL)の少ない症例から次第に PVL が増大し、くすぶり型、慢性型、急性型と病期が進展するにつれて、$CD3^{+}/CD7^{+}$ から次第に $CD3^{dim}/CD7^{dim}$ 集団が増加し、さらに $CD3^{dim}/CD7^{-}$ へと phenotype が変化していくと考えられ、本法は HTLV-1 感染細胞の腫瘍化過程をモニターするのに優れた方法と考えられた[14]。

HAS-flow 法(1st generation)の一つの問題点は $CD3^{+}/CD7^{+}$ の集団と $CD3^{dim}/CD7^{dim}$ の集団の分離が悪い症例が多いことであった。CADM1(Cell Adhesion Molecule 1)は TSLC1(Tumor Suppressor of Lung Cancer 1)とも呼ばれるがん抑制遺伝子であり、非小細胞肺がんにおいて 1998 年 Murakami らにより最初に同定された[15]。CADM1 は免疫グロブリンスーパーファミリーに属する接着分子であり、多くの組織で発現しているが、血球細胞では赤血球に弱陽性、制御性 T 細胞で弱陽性であるが、その他には好中球、単球でわずかに発現が見られるのみで、基本的に発現していない。2005 年、宮崎大学の Morishita らは網羅的発現アレイ解析によって ATL で過剰発現している分子の一つとして CADM1 を同定した[16]。さらに彼らは HTLV-1 キャリア末梢血中の CD4 陽性細胞

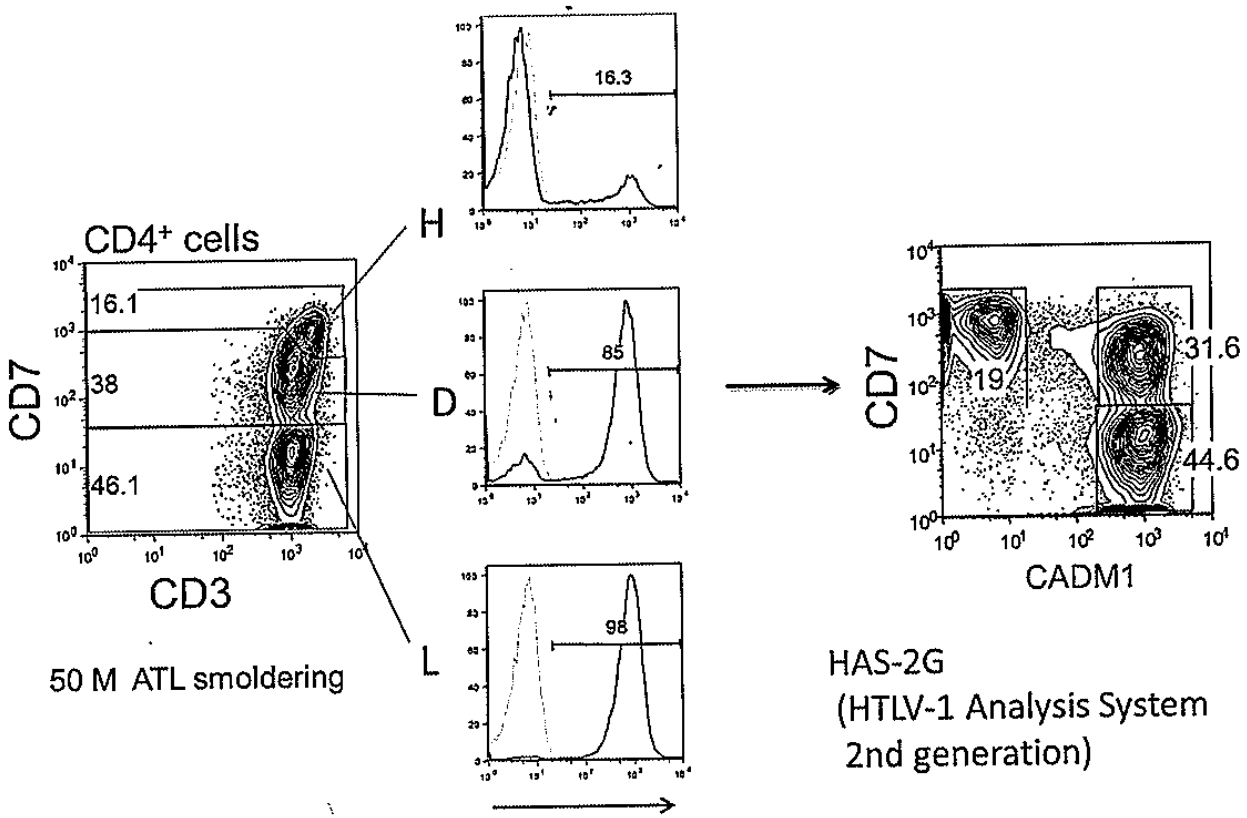

Figure 3 HAS(HTLV-1 Analysis System)-flow 法(第 2 世代)
HAS-flow 1G 法に CADM1 発現解析を加え、CD7/CADM1 で展開した。

中の CADM1 陽性細胞の比率が末梢血 PVL と相関することを報告した[17]が、このことは CADM1 が HTLV-1 感染細胞の腫瘍化過程のかなり早期から発現することを示唆し HTLV-1 感染細胞の腫瘍化過程のモニター、ATL 腫瘍細胞の検出に有用であることが期待された。そこで HAS-flow 法(1st generation)に CADM1 の発現解析を追加し、CD4 陽性 T 細胞を CD7、CADM1 で展開したところ、$CD7^{+}/CADM1^{-}$(P)、$CD7^{dim}/CADM1^{+}$(D)、$CD7^{-}/CADM1^{+}$(N)の 3 つの集団に分かれることがわかり、本法を HAS-flow 2G(2nd generation)と命名した(Fig. 3)[18]。HAS-2G により HTLV-1 キャリアから indolent ATL、aggressive ATL の各症例の末梢血を解析すると、HTLV-1 キャリアにおいて、PVL が増加して病態が進行するにつれて P の集団から次第に D の集団が増加していき、さらに N の集団へ移行していくことがわかる(Fig. 4)[18]。N の集団は inverse PCR、フローサイトメトリーによる TCR レパトア解析でモノクローナルに増殖している集団であることが示された。

HAS-flow 2G により解析した HTLV-1 感染細胞を上記の P、D、N の 3 つの集団に分画して、その特徴の解析を行った。無症候性キャリア、くすぶり型、慢性型、急性型症例、および非感染者の末梢血を HAS-flow 2G で P、D、N 分画にソーティングして遺伝子発現アレイ解析を行ってクラスタリング解析を行うと、非感染細胞とキャリア、indolent ATL の P、キャリアおよび indolent ATL の D と N、急性型 N にそれぞれクラスタリングされた[18]。また、急性型 ATL 細胞で発現が強く抑制されていることが報告されている miR-31[19]の発現レベルを検討すると、各病型の P 分画では抑制は見られないが、キャリアにおいても D 分画の集団は既に強くその発現が抑制されており、N の集団では急性型に匹敵する程度の強い発現抑制を示した。miR-31 の発現抑制により急性型 ATL ではポリコーム複合体を構成する EZH2 の発現が亢進していると考えられるが[19]、キャリアにおいても D、N の集団では EZH2 の発現レベルが上昇していた。さらに、急性型 ATL 細胞に見られる IKZF2(Helios)のスプライシング異常もこれらの D や N の集団で既に認められた[18]。これらの結果から HTLV-1 感染細胞は多段階発がんの過程で、$CD7^{+}/CADM1^{-}$から $CD7^{dim}/CADM1^{+}$の段階を経て $CD7^{-}$

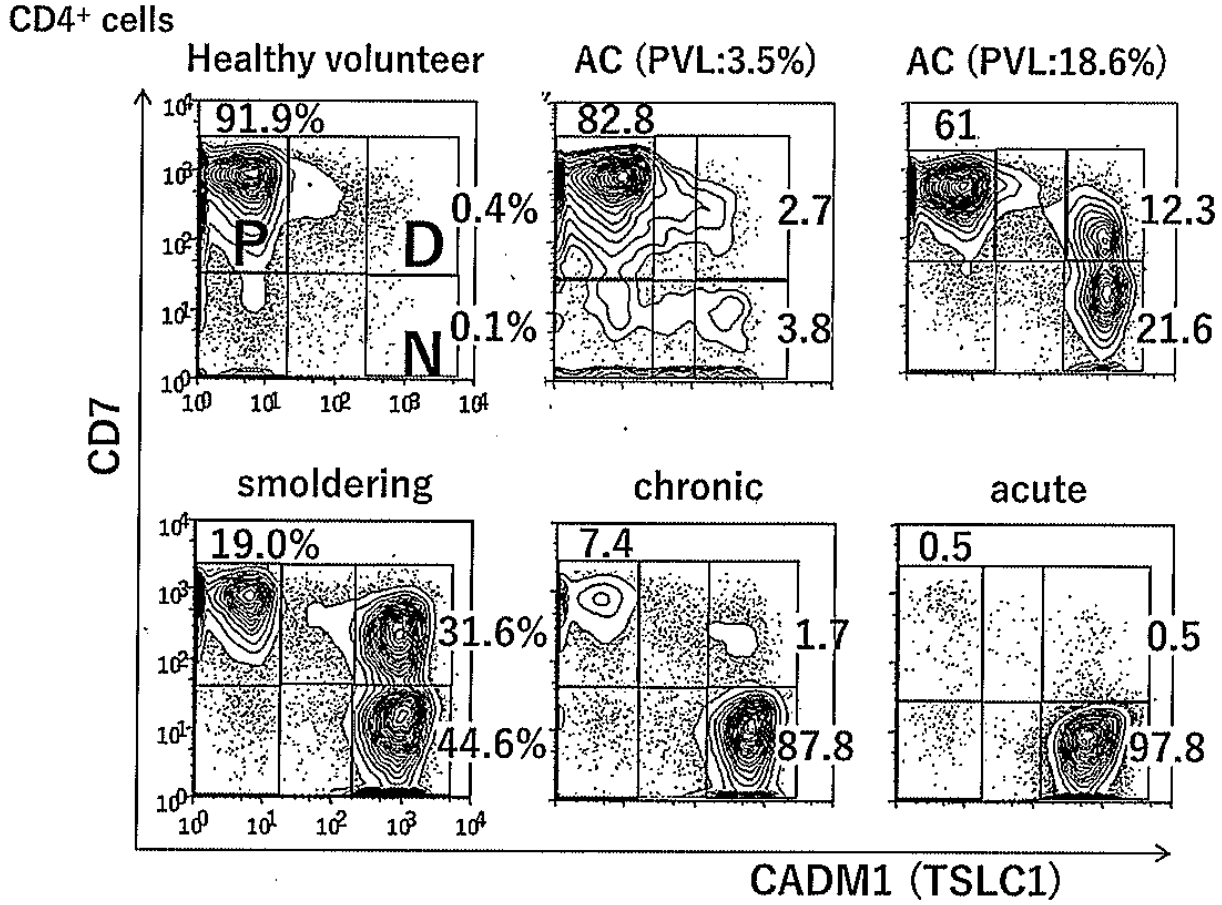

Figure 4　HAS-flow 2G による解析（第 2 世代）
無症候性キャリアから HAS 病期の進展に伴い次第に CADM1 が陽性となり CD7 の発現レベルが低下していく。

/CADM1$^+$へと phenotype を変えていくこと、CD7^{dim}/CADM1$^+$の段階で既に ATL としての基本的な性格を獲得していること、これらの細胞集団は既に無症候性キャリアの段階で病態の進行に伴って末梢血中に存在すること、indolent ATL の CD7$^-$/CADM1$^+$の集団に最終ヒットが加わって急性型へ進展していくことが推定される。したがってキャリアの段階から末梢血中の CD4$^+$/CD7^{dim}/CADM1$^+$および CD4$^+$/CD7$^-$/CADM1$^+$の集団の出現、およびその増加をモニターすることにより、HTLV-1 キャリアから ATL の発症に向けての進行を評価できると考えられる。

III. HAS-Flow の応用

HAS-flow 2G は様々な応用が考えられる。まず HAS-flow の開発にあたった当初の目的である末梢血中の腫瘍細胞を定量的に評価することにより、治療効果をより客観的に評価することに利用することが考えられる。現在 ATL に対して唯一根治が期待できる治療法と考えられる造血細胞移植を目指した治療方針を考えると、最近の後方視的研究によると移植までの日数が 100 日未満の症例が、100 日以上の症例に比べて有意に生存率が高いことが報告されている[20]。ATL は非常に抗がん剤耐性を獲得しやすい腫瘍であり、初回寛解導入療法中にいったん治療に反応していても再度病勢が悪化するということをしばしば経験する。したがって ATL に対して造血細胞移植を目指す方針を取る場合には、化学療法開始とともにドナーの準備を行い、ドナーの準備ができ次第、部分寛解（PR）以上の治療効果が得られていれば造血細胞移植を実施するという方針が必要であるが、ATL の場合患者が比較的高齢者であることもあって、血縁ドナーを得られる可能性は低い。したがって骨髄バンクを介した非血縁移植を行う症例が多くなるが、適合ドナーが得られて移植の準備に時

間がかかることもあり、その場合臍帯血などの代替ソースを用いざるを得ない。HAS-flow 2G は治療反応性を敏感に反映するので、化学療法の治療効果をモニターし、耐性を疑わせる動きがあった場合には骨髄バンクドナーを待たずに代替ソースによる移植に切り替えていくという応用が考えられる。現在 ATL に対する HLA 半合致移植の安全性と有効性を検討する臨床試験も進行中であり、今後 ATL に対する造血細胞移植ソースの代替ソースへの切り替えの判断は益々重要になると想定される。また、HAS-flow 2G は微小残存病変の検出にも有用であると考えられる。骨髄移植後の末梢血を HAS-flow 2G でモニターすると、再発例の多くで臨床的な再発の前にいったん消失していた $CD7^-/CADM1^+$ の集団が出現しており、今後症例を重ねて、その有用性を評価することにより、血液学的再発の前に再発の兆候を感知して、早期に免疫抑制剤の減量などの介入が可能になることが期待される。

HAS-flow 2G のパターンは無症候性キャリアから ATL の発症に向けて段階的に変化して行くことから、HAS-flow 2G のパターンにより発症過程をモニターすることが可能であるとともに、発症リスクグループの分類に有用であることが期待される。我々は 74 例の HTLV-1 キャリア、indolent ATL 症例を対象に HAS-flow 2G による解析を行い、これらの症例の予後を検討した。$CD7^{dim}/CADM1^+$ (D) および $CD7^-/CADM1^+$ (N) の合計が 10%以下の G1、10%から 25%の G2、25%から 50%の G3、50%以上の G4 に分類すると G1～G3 グループと比較して G4 グループは高度の有意差で早期に急性転化した。また、無症候性キャリアの症例のみで indolent ATL を含む ATL の発症を検討すると G3＋G4 グループは有意に高率に ATL を発症していた(論文準備中)。G3 グループには下山分類上くすぶり型 ATL に分類される症例と無症候性キャリアに分類される症例が混在しており、これらの症例は D および N の集団の比率に違いがなく、これらの D、N の集団は上記の通り遺伝子発現なども含めて基本的に相違がないことから分子生物学的には G3 に属する症例は同一の集団とみなすのが妥当であろう[21]。HTLV-1 感染細胞の腫瘍化過程は典型的な多段階発がんであり、これらの G3 集団を腫瘍ととらえるのかどうか、どこからを腫瘍と考えるのか、さらには、そもそも腫瘍とは何か、という問題を改めて提起していると考えられる。

HAS-flow 2G により検出される D の集団は基本的に ATL としての分子生物学的異常を兼ね備えている。無症候性キャリアの段階からこれらの D、N の集団が認められ、これらに種々の遺伝子異常、エピゲノム異常などが蓄積して aggressive ATL へと進展していくと考えられる。最近、多数例の ATL 細胞の解析により ATL の遺伝子異常の全貌が明らかにされた[22]が、HAS-flow 2G によって抽出される HTLV-1 感染細胞の腫瘍化の中間段階の細胞の解析により、これらの異常の中で、ATL の病態形成の本質に関わる初期の異常と、腫瘍化の進展に関わる異常を区別して検討することが可能になることが期待される。また HTLV-1 感染は ATL という腫瘍性疾患と HTLV-1 関連脊髄症(HTLV-1 associated myelopathy: HAM) という炎症性疾患を起こすが、HTLV-1 感染細胞が腫瘍化に向かう初期過程を解析することにより、同じ HTLV-1 感染細胞が、ATL という腫瘍性疾患を発症する一方で HAM という炎症性疾患を発症する疾患発症の制御のメカニズムの解明につながることも期待される。

Disclosure

開示すべき COI は以下の通りです。

・第一三共株式会社

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