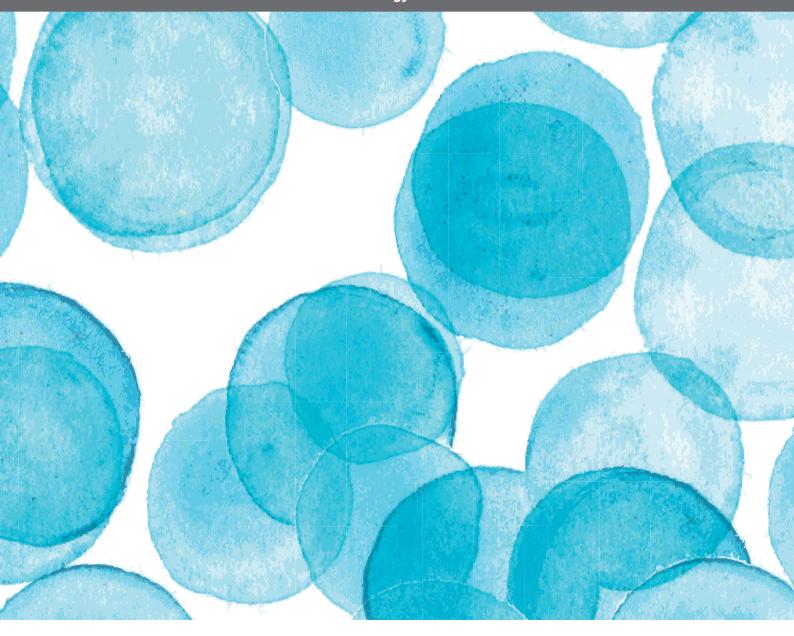
# HTLV-1: ADDRESSING UNMET RESEARCH NEEDS EDITED BY: Louis M. Mansky and Woi Thans

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# HTLV-1: ADDRESSING UNMET RESEARCH NEEDS

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# **Table of Contents**

05	Adult T-Cell Leukemia/Lymphoma-Related Ocular					
	Manifestations: Analysis of the First Large-Scale Nationwide Survey					
	Koju Kamoi, Akihiko Okayama, Shuji Izumo, Isao Hamaguchi,					
	Kaoru Uchimaru, Arinobu Tojo and Kyoko Ohno-Matsui					

12 Neuroimmunology of Human T-Lymphotropic Virus Type 1-Associated Myelopathy/Tropical Spastic Paraparesis

Satoshi Nozuma and Steven Jacobson

23 HTLV-1 HBZ Protein Resides Exclusively in the Cytoplasm of Infected Cells in Asymptomatic Carriers and HAM/TSP Patients

Greta Forlani, Marco Baratella, Alessandra Tedeschi, Claudine Pique, Steve Jacobson and Roberto S. Accolla

- **38 Mother-to-Child HTLV-1 Transmission: Unmet Research Needs**Carolina Rosadas and Graham P. Taylor
- 56 Evidence of New Endemic Clusters of Human T-Cell Leukemia Virus (HTLV) Infection in Bahia, Brazil

Felicidade Mota Pereira, Maria da Conceição Chagas de Almeida, Fred Luciano Neves Santos, Roberto Perez Carreiro, Carlos Gustavo Regis-Silva, Bernardo Galvão-Castro and Maria Fernanda Rios Grassi

- 68 Association Between HTLV-1 Genotypes and Risk of HAM/TSP
  Mineki Saito
- 76 TRAF3 Is Required for NF-κB Pathway Activation Mediated by HTLV Tax Proteins

Stefania Fochi, Elisa Bergamo, Michela Serena, Simona Mutascio, Chloé Journo, Renaud Mahieux, Vincenzo Ciminale, Umberto Bertazzoni, Donato Zipeto and Maria Grazia Romanelli

91 Inhibition of HTLV-1 Infection by HIV-1 First- and Second-Generation Integrase Strand Transfer Inhibitors

Michał S. Barski, Jordan J. Minnell and Goedele N. Maertens

102 Post-transcriptional Regulation of HTLV Gene Expression: Rex to the Rescue

Donna M. D'Agostino, Ilaria Cavallari, Maria Grazia Romanelli and Vincenzo Ciminale

111 Cerebrospinal Fluid CXCL10 as a Candidate Surrogate Marker for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

Keiko Tamaki, Tomoo Sato, Jun Tsugawa, Shinsuke Fujioka, Naoko Yagishita, Natsumi Araya, Junji Yamauchi, Ariella L. G. Coler-Reilly, Misako Nagasaka, Yasuhiro Hasegawa, Yoshihisa Yamano and Yoshio Tsuboi

123 Safety of Infliximab for the Eye Under Human T-Cell Leukemia Virus Type
1 Infectious Conditions in vitro

Minami Uchida, Koju Kamoi, Naoko Ando, Chenxi Wei, Hisako Karube and Kyoko Ohno-Matsui

#### 131 HTLV-1 Extracellular Vesicles Promote Cell-to-Cell Contact

Daniel O. Pinto, Catherine DeMarino, Michelle L. Pleet, Maria Cowen, Heather Branscome, Sarah Al Sharif, Jennifer Jones, Helene Dutartre, Benjamin Lepene, Lance A. Liotta, Renaud Mahieux and Fatah Kashanchi

152 Biomarkers and Preclinical Models for Adult T-Cell Leukemia-Lymphoma Treatment

Lee Ratner

159 Progress in Allogeneic Hematopoietic Cell Transplantation in Adult T-Cell Leukemia-Lymphoma

Atae Utsunomiya

- 175 Collagen IV (COL4A1, COL4A2), a Component of the Viral Biofilm, Is Induced by the HTLV-1 Oncoprotein Tax and Impacts Virus Transmission Sebastian Millen, Christine Gross, Norbert Donhauser, Melanie C. Mann, Jean-Marie Péloponèse Jr. and Andrea K. Thoma-Kress
- 197 In vitro Evaluation of the Safety of Adalimumab for the Eye Under HTLV-1 Infection Status: A Preliminary Study

Hisako Kurozumi-Karube, Koju Kamoi, Naoko Ando, Minami Uchida, Isao Hamaguchi and Kyoko Ohno-Matsui





## 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 ATLrelated 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

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#### 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.

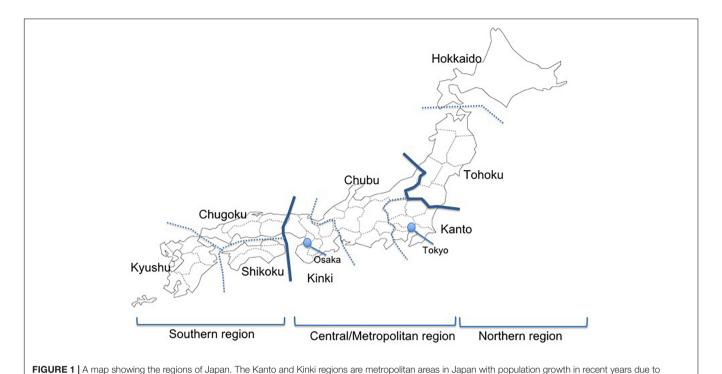
Only valid responses to questions were included in the statistical analyses. If a respondent left a question blank, only that blank response was excluded from analysis. Data on categories pertaining to medical care for ATL-related ocular manifestations were tallied and analyzed.

#### 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."



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 guestionnaire.

Northern		Central/Metropol Itar	ı	Souther	Total	
Hokkaido/Tohoku	Kanto Chubu		Kinki	Chugoku/Shikoku		Kyushu
5 (7.2%)	48 (69.5%)		16 (23.29	%)	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)	Tohoku		Kinki (n = 11)	Chugoku/ Shikoku (n = 7)	Kyushu (n = 9)	Total
Yes	80.0%		29.7%		7	'5.0%	43.0%
	80.0%	26.9%	14.2%	36.4%	57.1%	88.9%	
No/Un-identified	20.0%		70.3%		2	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)			Se (		
	Hokkaido/ Tohoku (n = 8)	Kanto (n = 20)	Chubu ( <i>n</i> = 5)	Kinki (n = 11)	Chugoku/ Shikoku (n = 6)	Kyushu (n = 10)	Total
Blood test and ophthalmic	50.0%		69.4%		62.5%		65.0%
examination	50.0%	70.0%	80.0%	63.6%	83.3%	50.0%	
Intraocular fluid test and	37.5%		11.1%		18.8%		16.7%
ophthalmic examination	37.5%	10.0 %	0.0%	18.2%	0.0%	30.0%	
Consulting Hematologists	12.5%		19.4%		18.8%		18.3%
and ophtalmic examination	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			1	lumber of pat	ients		
	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)	(O)	(O)	(1)	(2)	(19)
(Herpesvirus**)	(0)	(O)	(O)	(O)	(0)	(2)	(2)
(Toxoplasma**)	(O)	(O)	(O)	(O)	(O)	(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	

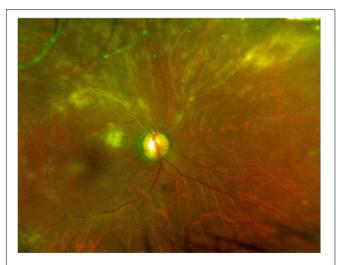
<sup>\*</sup>All cases of opportunistic infection were cytomegalovirus retinitis. \*\*In addition to cytomegalovirus retinitis, one case of superinfection with toxoplasma and two cases of herpesvirus were reported.



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

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



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

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

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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 ATI

#### **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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## Neuroimmunology of Human T-Lymphotropic Virus Type 1-Associated Myelopathy/Tropical Spastic Paraparesis

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Human T-lymphotropic virus type 1 (HTLV-1) is the etiologic agent of both adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is clinically characterized by chronic progressive spastic paraparesis, urinary incontinence, and mild sensory disturbance. Given its well-characterized clinical presentation and pathophysiology, which is similar to the progressive forms of multiple sclerosis (MS), HAM/TSP is an ideal system to better understand other neuroimmunological disorders such as MS. Since the discovery of HAM/TSP, large numbers of clinical, virological, molecular, and immunological studies have been published. The host-virus interaction and host immune response play an important role for the development with HAM/TSP. HTLV-1-infected circulating T-cells invade the central nervous system (CNS) and cause an immunopathogenic response against virus and possibly components of the CNS. Neural damage and subsequent degeneration can cause severe disability in patients with HAM/TSP. Little progress has been made in the discovery of objective biomarkers for grading stages and predicting progression of disease and the development of molecular targeted therapy based on the underlying pathological mechanisms. We review the recent understanding of immunopathological mechanism of HAM/TSP and discuss the unmet need for research on this disease.

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#### INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) is the first human retrovirus discovered and infects 10–20 million people worldwide (Poiesz et al., 1980; de The and Bomford, 1993). Highly endemic areas include Southern Japan, the Caribbean, South America, Central and Southern Africa, Middle East, and Central Australia. Although the majority of infected individuals remain lifelong asymptomatic carriers (ACs), approximately 2–5% develop adult T-cell leukemia/lymphoma (ATLL) (Uchiyama et al., 1977) and another 0.25–3.8% develop HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). HTLV-1 has also been associated with other chronic inflammatory diseases including uveitis,

myositis, dermatitis, and pulmonary infiltrative pneumonitis (Gessain and Mahieux, 2012). HTLV-1 can be transmitted through sexual contact, intravenous drug use, and breastfeeding from mother to child. While ATLL is mainly associated with breastfeeding, HAM/TSP can manifest in infected individuals by all routes of transmission (Yamano and Sato, 2012). The mean age of onset is 40-50 years, and the frequency is higher in women than in men (Gessain and Mahieux, 2012). The interaction between the host immune system and HTLV-1infected cells regulates the development of HAM/TSP. In particular, HTLV-1-specific CD8-positive cytotoxic T lymphocytes (CTL) against HTLV-1 have been thought to play a pivotal role in the development of HAM/TSP (Matsuura et al., 2010; Saito, 2014). Two HTLV-1 viral regulatory proteins, Tax and HTLV-1 basic leucine zipper factor (HBZ), have been demonstrated to be involved in HTLV-1 infectivity and proliferation (Matsuoka and Jeang, 2011). Tax have been intensely studied because Tax induces the expression of many cellular genes and consequently contribute to cell activation and proliferation observed in HAM/TSP. HBZ has been recently recognized to play a critical role in inflammation and pathogenesis of HAM/TSP (Enose-Akahata et al., 2017). Although HAM/ TSP is not directly life-threatening, the disease severely impacts patients' quality of life (Olindo et al., 2006; Coler-Reilly et al., 2016), and there is no satisfactory curative treatment. Here, we review our recent understanding of the immunopathogenesis and the clinical features of HAM/TSP and discuss the need for continued basic and clinical research.

#### **Human T-Lymphotropic Virus Type 1**

HTLV-1 belongs to the Deltaretrovirus genus of the Orthoretrovirinae subfamily of retroviruses. HTLV-1 integrates a single copy of the provirus into the genome of the host cell (Cook et al., 2012). HTLV-1 proviral genome contains structural genes, gag, pol, and env flanked by long terminal repeat at the both the 5' and 3' ends. HTLV-1 genome also has a pX region encoded several accessory genes including tax, rex, p12, p21, p30, p13, and HTLV-1 basic leucine zipper factor (HBZ) (Matsuoka and Jeang, 2007). The viral genes are transcribed from the 5'LTR, but only HBZ encoded on the minus strand of the provirus is transcribed from the 3'LTR. Two of these accessory genes, tax and hbz, play a key role in HTLV-1 pathogenesis. HTLV-1 Tax is a transcriptional transactivator of virus replication and induces the expression of a variety of cellular genes by activation of the NF-κB and CREB/ATF pathways (Matsuoka and Jeang, 2011). Tax is associated with many of the characteristic immune abnormalities observed in HAM/TSP and is related to dysfunction in immune cells of HAM/TSP patients (Enose-Akahata et al., 2017). Ex vivo, Tax protein is spontaneously expressed in peripheral blood mononuclear cells (PBMCs) after culture without any exogenous stimulation (Hanon et al., 2000a), and the level of tax mRNA was significantly higher in HAM/TSP patients than in ACs (Yamano et al., 2002). Tax is an immunodominant antigen recognized by HTLV-1-specific cytotoxic CD8+ T-cells (CTLs)

(Jacobson et al., 1990). The number of Tax-specific CTLs is greatly elevated and these CTLs produce proinflammatory cytokines (Kubota et al., 1998) and show degranulation activity in HAM/TSP patients that is comparable to that in ACs (Abdelbary et al., 2011). Though Tax is usually undetected in vivo, recent analysis shows that Tax protein is expressed in intermittent but intense bursts at the single-cell level (Billman et al., 2017; Mahgoub et al., 2018). HBZ plays a key role in the growth and survival of leukemic cells; however, little is known about its role in the immunopathogenesis of HAM/TSP (Enose-Akahata et al., 2017). As HBZ closely cooperates with Tax, HBZ has opposing functions to Tax and modify transcription of various host genes (Matsuoka and Jeang, 2011). HBZ is persistently expressed in infected cells, maintains viral latency (Philip et al., 2014), and promotes proliferation of ATLL cells (Satou et al., 2006; Arnold et al., 2008). HBZ interacts with CREB/ATF pathway, suppresses Tax-mediated transactivation, and selectively inhibits the classical NF-κB pathway (Matsuoka and Jeang, 2011). The level of HBZ mRNA that was detected in HAM/TSP patients was significantly lower than that in ATLL patients but higher than in ACs. Furthermore, HBZ mRNA expression was associated with proviral load and increased disease severity in HAM/TSP patients (Saito et al., 2009). HBZ is also an immunogenic protein recognized by HBZ-specific CTL clones; however, HBZ is considered to be a weaker immunogen for CTLs then Tax. HBZ-specific CTL clones could not lyse ATLL cells (Suemori et al., 2009) and HBZ-specific CTL clones killed significantly fewer infected cells than were killed by Tax-specific CTL clones (Rowan et al., 2014). Antibody response against HBZ was detected in HTLV-1-infected subjects, but the antibody test could not distinguish between different clinical outcomes (Enose-Akahata et al., 2013). The lower immunogenicity of HBZ could allow HTLV-1-infected cells to escape from the host immune response.

HTLV-1 proviral load, which is strongly related to the risk of developing HAM/TSP, remains relatively stable within each subject while HTLV-1 drives a strong proliferation of infected T-cells (Bangham et al., 2015). The genomic location of the provirus is identical in every cell within an individual infected clone but differs between clones. Integration of HTLV-1 appeared to occur in genes associated with transcriptional start sites, and CpG island (Doi et al., 2005; Derse et al., 2007). Analysis of proviral integration sites between HTLV-1-infected individuals demonstrated that frequent integration into transcriptionally active sites was associated with an elevated rate of Tax expression (Meekings et al., 2008). Furthermore, a larger number of distinct HTLV-infected T-cell clones was detected in HAM/TSP patients than in ACs (Gillet et al., 2011). The frequency of spontaneous Tax expressing cells is considerably higher in clones of low abundance than in those of high abundance (Melamed et al., 2013). These results indicate that oligoclonal proliferation of HTLV-1-infected cells does not account for the development of HAM/TSP and clonal expansion of infected cells might be controlled by host immune response to Tax or by other viral factor such as HBZ in HAM/TSP patients.

# Current Topics: Extraordinary High Prevalence in Central Australia

One of the hot topics in HTLV-1 is the high prevalence in Central Australia, where more than 40% of Indigenous adults in some remote communities are HTLV-1c infected (Einsiedel et al., 2016b). HTLV-1 infection in the Australo-Melanesian region was observed in the early 1990s (Gessain and Cassar, 2012), but high prevalence rates in Central Australia has not been recognized until recently. As discussed below, HTLV-1c is one of the genetic subtypes of HTLV-1, which is found only in Oceania. HTLV-1 sequence in subtype c that infect the indigenous Australians reveals the high genetic diversity, while the sequence variability within subtype a, which is the most common worldwide, is very low (Cassar et al., 2013). High sequence diversity in HTLV-1c is considered to be due to a long period of evolution in isolated populations living on different islands of the Pacific area (Gessain and Cassar, 2012). Several studies have reported that common clinical manifestations of HTLV-1 infection in Indigenous Australians are bronchiectasis and blood stream infections, which are associated with higher HTLV-1c proviral load (Einsiedel et al., 2014a, 2016a). These findings are consistent with the proposed immunopathology of HTLV-1-related inflammatory diseases, in which the higher proviral load induces various immunologic abnormalities. Little is known about more common disease caused by HTLV-1 including HAM/TSP and ATLL, and only few cases have been described among the Indigenous population of Central Australia (Einsiedel et al., 2014b). Our knowledge of the global prevalence of HTLV-1 is still insufficient, and the risk of the retrovirus to the public health worldwide is underestimated. Future analysis is apparently needed for the clinical and basic research to clarify the pathogenetic, immunological and oncogenic mechanisms of this unknown variant of HTLV-1.

#### **RISK FACTORS**

#### **Proviral Load**

An elevated HTLV-1 proviral load (PVL) is the main risk factor for developing HAM/TSP in HTLV-1 infected subjects. The HTLV-1 PVL is measured as the number of HTLV-1 DNA copies per PBMCs and is usually expressed as the percentage of infected PBMCs. The PVL is 16-fold higher in HAM/TSP patients than in carriers and associated with an increased risk of progression to disease (Nagai et al., 1998). This high PVL is associated with multiple immunologic abnormalities that contribute to the development of HAM/TSP. When compared to ACs, HAM/TSP patients have higher levels of spontaneous lymphocyte proliferation (Sakai et al., 2001; Pinto et al., 2011), increased HTLV-1 expression, and importantly, a higher frequency of HTLV-1 specific CD8+ T-cells (Kubota et al., 2000; Yamano et al., 2002), which are thought to be critical mediators of central nervous system (CNS) injury. PVL is significantly elevated in cerebrospinal fluid (CSF) of HAM/TSP patients than in ACs and HTLV-1-infected patients with other neurological disorders (Puccioni-Sohler et al., 2007). Furthermore, higher PVL is also detected in CSF than in PBMCs of HAM/TSP (Nagai et al., 2001; Brunetto et al., 2014) and correlated with spinal cord atrophy (Azodi et al., 2017). The PVL of HTLV-1 reaches a stable level in each individual (Matsuzaki et al., 2001), which is maintained by the equilibrium between the proliferation of infected cells and their elimination by activated CTLs (Bangham, 2003). Although higher PVL is clearly associated with developing HAM/TSP, an elevated PVL alone is an incomplete requirement since a small minority of AC with a high proviral load can also develop HAM/TSP. Given that HTLV-1 PVLs are higher in ACs genetically related to HAM/TSP patients than in non-HAM/TSP-related ACs (Nagai et al., 1998), genetic factors might contribute to the replication of HTLV-1.

#### **Host Genetic Factors**

Several host genetic factors, including human leukocyte antigen (HLA) and non-HLA gene polymorphisms affect the occurrence of HAM/TSP (Saito and Bangham, 2012). The HLA class I genotype determines the specificity and the efficacy of their CD8+ T-cell response and consequently has a large impact on the proviral load and the risk of developing HAM/TSP. Possession of the HLA-class I gene HLA-A\*02 and Cw\*08 was associated with a significant reduction in both HTLV-1 PVL and the risk of HAM/TSP (Jeffery et al., 1999, 2000; Catalan-Soares et al., 2009), while individuals with HLA-class I HLA-B\*5401 and class II HLA-DRB1\*0101 was indicated to have the susceptibility of developing with HAM/TSP (Jeffery et al., 1999, 2000). Analysis of non-HLA host genetic factors showed that single nucleotide polymorphisms (SNPs) in a small number of candidate genes affect the risk of developing HAM/TSP. Tumor necrosis factor (TNF)-α promoter -863 A (Vine et al., 2002) and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter (Kodama et al., 2004) were associated with disease susceptibility, whereas interleukin (IL)-10 -592 A (Sabouri et al., 2004), stromal-derived factor-1 + 801 A, and IL-15 + 191°C alleles (Vine et al., 2002) were related to protective effect. IL6 promoter -634C allele frequencies were higher among HAM/TSP patients than among ACs in Brazil (Gadelha et al., 2008). IL28B gene SNP rs8099917 was reported to be associated with HAM/TSP in Brazil (Assone et al., 2014). Familial clusters of HAM/TSP were reported, but genetic analysis was not able to detect any disease-associated genes due to small number of cases (Nozuma et al., 2014, 2017). Comprehensive analysis of host genetics in a larger sample size and cases focusing on familial clustering might be useful to detect the susceptibility genes of HAM/TSP.

#### **HTLV-1** Genotype

HTLV-1 is segregated into seven major genetic subtypes (1a-1g) based on the nucleotide diversity of its LTR region, including a cosmopolitan subtype (subtype 1a), a Melanesian/ Australian subtype (subtype 1c), and five African subtypes (subtype 1b, 1d, 1e, 1f, and 1g) (Verdonck et al., 2007). The cosmopolitan subtype is further divided into five

sub-subtypes (Proietti et al., 2005), and globally the most common HTLV-1 genotype is transcontinental subtype (Gessain and Cassar, 2012). HTLV-1 has remarkably low genetic variability, although minor variations exist between geographical isolates (Komurian et al., 1991). Most studies of HTLV-1 genotype have reported no correlation between nucleotide substitutions and the risk of HAM/TSP (Mahieux et al., 1995), and the recent analysis of complete HTLV-1 sequence could not detect any HAM/TSP-specific mutations (Pessoa et al., 2014; Nozuma et al., 2017). However, the transcontinental HTLV-1 subtype is reported to be more frequently detected in HAM/TSP patients than in ACs in southern Japan, where the transcontinental and Japanese HTLV-1 subtypes co-exist (Furukawa et al., 2000; Nozuma et al., 2017). HAM/TSP patients with transcontinental subtype showed lower levels of HBZ mRNA expression (Yasuma et al., 2016) and higher levels of CXCL10, which has been proposed to be a prognostic biomarker for HAM/TSP (Naito et al., 2018). Different HTLV-1 subgroups revealed different patterns of host gene expression (Naito et al., 2018), which also might contribute to the higher incidence of HAM/TSP infected with transcontinental subtype.

#### **HOST IMMUNE RESPONESE TO HTLV-1**

#### Neuropathology

The main pathological feature in HAM/TSP is a chronic inflammation with diffuse degeneration throughout the central nervous system (Izumo et al., 2000). The spinal cord shows loss of myelin and axons symmetrically in the lateral and posterior column of the thoracic cord with the inflammation of grey and white matter, most notably at the thoracic level (Iwasaki, 1990; Yoshioka et al., 1993). These lesions are associated with perivascular and parenchymal lymphocytic infiltration with associated axonal loss, demyelination, reactive astrocytosis and fibrillary gliosis (Umehara et al., 1993). CD4+ and CD8+ cells were evenly distributed in active inflammatory lesions, while the predominance of CD8+ cells and high levels of interferon (IFN)-γ were observed in the chronic stage of disease (Umehara et al., 1993; Ave et al., 2000). A recent radiological study also demonstrated that a more atrophic spinal cord in HAM/TSP was associated with higher percentage of inflammatory CD8+ T-cells and HTLV-1 PVL in CSF (Azodi et al., 2017). HTLV-1-infected cells were determined to be CD4+ lymphocytes in the central nervous system (CNS) (Matsuoka et al., 1998), and HTLV-1 Tax-specific CTLs could be detected in the spinal cord (Matsuura et al., 2015). HTLV-1 has not been shown to actively infect neurons, oligodendrocytes, or microglia in vivo (Lepoutre et al., 2009). Alteration in tight junctions between endothelial cells in the vasculature causes blood-brain barrier (BBB) disruption with subsequent T-cell transmigration into the CNS in HAM/TSP (Afonso et al., 2007, 2008). Higher levels of MMP-2 and MMP-9 were detected in the CSF and in infiltrating perivascular mononuclear cells in active lesions in the CNS, indicating that MMP-2 and MMP-9 may play a key role in the BBB breakdown in HAM/TSP patients (Umehara et al., 1998). Cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and cell adhesion molecule 1 (CADM1/TSLC1), and activated leukocyte cell adhesion molecule (ALCAM/CD166) have shown to be modulated by Tax (Valentin et al., 1997; Nejmeddine et al., 2009; Curis et al., 2016; Manivannan et al., 2016). Increased adhesion molecule expression might therefore facilitate the recruitment and migration of HTLV-1 infected lymphocytes through the BBB endothelium.

#### **Cytotoxic T Lymphocytes**

The CD8+ cytotoxic T-cells (CTLs) response has been considered to play an important role in the development of HAM/TSP. One of the most prominent features of the cellular immune response in HAM/TSP patients is that the number of HTLV-1-specific CTLs is greatly elevated in PBMCs compared with ACs (Jacobson et al., 1990; Kubota et al., 2003). The immunodominant antigen recognized by HTLV-1-specific CTLs is the Tax protein particularly in association with HLA-A\*0201 (Jacobson et al., 1990). These virus-specific CTLs, infiltrating CNS lesions, produce proinflammatory cytokines and show degranulation activity (Kubota et al., 1998; Yamano et al., 2002; Abdelbary et al., 2011). The frequency of Tax specific CD8+ T-cells is also higher in CSF than in PBMCs and is proportional to HTLV-1 PVL (Greten et al., 1998; Nagai et al., 2001). Tax-specific CTLs were also detected in spinal cord parenchyma (Matsuura et al., 2015). Strong Tax-specific CTL response has been considered to control the viral replication and play key role in developing of HAM/TSP. While Tax expression is generally suppressed in infected cells in vivo (Hanon et al., 2000b), CTL response to Tax is chronically activated, implying frequent exposure to newly-synthesized Tax protein in vivo (Rende et al., 2011). Single cell analysis demonstrated that Tax protein is expressed in intermittent but intense bursts (Billman et al., 2017; Mahgoub et al., 2018). Recently, exosome containing Tax was detected in virus-free CSF of patients with HAM/TSP and may therefore also be the target of CTLs (Anderson et al., 2018). These mechanisms may help maintain persistently activated CTL responses to HTLV-1. HBZ is also an immunogenic protein recognized by HBZ-specific CTL clones and HBZ-specific CTLs are detected in ATLL or HAM/ TSP patients and ACs (Suemori et al., 2009; Macnamara et al., 2010). HTLV-1-infected individuals with HLA class I alleles strong binding the HBZ and the HBZ-specific CTL response was shown to be associated with a lower proviral load and a reduced risk of HAM/TSP (Macnamara et al., 2010; Hilburn et al., 2011). However, compared with Tax-specific CTLs, HBZ-specific CTLs are present at lower frequency in the peripheral blood and kill fewer HTLV-1-infected cells in vitro (Macnamara et al., 2010; Rowan et al., 2014). While HBZ is continuously expressed in HTLV-1 infected cells in vivo, the immunogenicity of HBZ is lower compared to tax, indicating that HTLV-1 could escape from host immune response and persistence of viral latency. Therapeutically, enhancing anti-HBZ immune responses or identifying HBZ epitopes that can induce a stronger CTL response in HAM/TSP may have clinical benefit.

#### **T Helper Cells**

CD4+ helper T-cells are necessary for both CTL response and B cell reactivity against HTLV-1. The inflammatory cells in the CNS in early stages of HAM/TSP are dominated by CD4+ T-cells with relatively high proviral loads and elevated Tax and IFN-γ expression (Umehara et al., 1993; Furuya et al., 1997; Moritoyo et al., 1999). The most common HTLV-1 antigen recognized by CD4+ T-cells is the Env protein (Kitze et al., 1998; Goon et al., 2004). The frequency of HTLV-1 specific CD4+ T-cells were higher in patients with HAM/TSP than in ACs with a similar PVL (Goon et al., 2004; Nose et al., 2007). Additionally, the antiviral Th1 phenotype is also dominant among HTLV-1-specific CD4+ T-cells in patients with HAM/ TSP (Goon et al., 2002), and higher frequency of IFN-γ, TNF-α, and IL-2 secreting CD4+ T-cells has been demonstrated in patients with HAM/TSP compared to ACs with a similar PVL (Goon et al., 2002, 2003). HTLV-1-infected CD4+ T-cells secrete IFN-γ (Hanon et al., 2000b; Araya et al., 2014), and the frequency of IFN-γ-secreting HTLV-1-specific CD4<sup>+</sup> T-cells has been shown to be higher in patients with HAM/TSP than in asymptomatic HTLV-1 carriers with a similar proviral load (Goon et al., 2002). Immunogenetic analysis also demonstrates that possession of HLA-DRB1\*0101 is associated with susceptibility to HAM/ TSP in Japan (Jeffery et al., 1999). It has been demonstrated that HTLV-1 infected T-cells which coexpressed the Th1 marker CXCR3 and produced T-bet and IFN-y were present in the CNS (Araya et al., 2014). When stimulated by IFN-y, the astrocytes produce the CXCL10, which recruits more CXCR3+ T-cells, including more infected cell, to the CNS (Ando et al., 2013). These cells also produce pro-inflammatory cytokines such as IFN-y, which stimulates astrocytes, further creating an inflammatory positive feedback loop with subsequent tissue damage in the CNS (Yamano and Coler-Reilly, 2017).

#### **Regulatory T-Cells**

HTLV-1 preferentially and continuously infects CD4+CD25+ T lymphocytes in vivo (Yamano et al., 2005). CD4+CD25+ T-cells, termed Tregs, contribute to the maintenance of immunologic self-tolerance by inhibiting the activation and proliferation of self-reactive lymphocytes (Sakaguchi et al., 2001). In HAM/TSP patients, CD4<sup>+</sup>CD25<sup>+</sup> T-cells contain higher amount of HTLV-1 PVL and levels of HTLV-1 tax mRNA expression than in CD4+CD25- cells and induces various cytokines including INF-y (Yamano et al., 2004). HTLV-1-infected CD4+CD25+ T-cells were not functionally suppressive but rather were shown to stimulate and expand HTLV-1 Tax-specific CD8+ T-cells (Yamano et al., 2004). In HAM/TPS patients, the levels of the forkhead box P3 (FoxP3) expression was decrease in CD4+CD25+ T-cells compared to ACs and healthy controls (Yamano et al., 2005; Oh et al., 2006). Furthermore, transduction of Tax reduced the FoxP3 mRNA expression and inhibited the suppressive function of the CD4+CD25+ T-cells isolated from healthy donors (Yamano et al., 2005). HTLV-1 preferentially infects cells expressing the C-C chemokine receptor 4 (CCR4) +CD4+ T-cells through the CCR4 ligand (CC chemokine ligand (CCL)22), which is produced by Tax (Hieshima et al., 2008). In HAM/TSP patients, the frequency of IFN- $\gamma$ -producing CD4+CD25+CCR4+ T-cells was increased and correlated with disease activity and severity (Yamano et al., 2009). Anti-CCR4 monoclonal antibody effectively reduced the proviral load and proinflammatory cytokines in PBMCs from patients with HAM/TSP (Araya et al., 2014; Yamauchi et al., 2015). Clinical trial of anti-CCR4 monoclonal antibody has been performed and showed promising results (Sato et al., 2018a).

#### **Antibody Response**

Robust humoral immune responses against HTLV-1 antigens have been reported (Osame et al., 1986; Kitze et al., 1996; Enose-Akahata et al., 2012), and it has been suggested that antibody syntheses might be related to both protective and pathogenic functions in HAM/TSP. Intrathecal antibody response to HTLV-1 inversely correlates with higher PVL and a worse prognosis (Puccioni-Sohler et al., 1999). HAM/TSP patients generally have a higher anti-HTLV-1 Ab titer than ACs with a similar HTLV-1 proviral load (Nagasato et al., 1991; Kira et al., 1992; Ishihara et al., 1994), and ATLL patients (Enose-Akahata et al., 2012). HTLV-1-Tax-specific antibody is elevated in HAM/TSP patients compared with both ACs and ATLL patients (Lal et al., 1994; Chen et al., 1997), and HTLV-1 specific antibody could distinguish HAM/TSP from ACs and ATLL (Enose-Akahata et al., 2012). However, the pathogenic role of these antibodies in HAM/TSP remains unclear. Antibodies against Tax and Gag p24, were reported to cross-react with host antigens, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and peroxiredosin-1 (PrX-1), respectively, suggesting a role for molecular mimicry in the autoimmune pathogenesis of HAM/TSP (Levin et al., 2002; Lee et al., 2008). However, subsequent analysis reported that the presence of hnRNPA1specific antibodies in the CSF was not specific to this disease (Yukitake et al., 2008). Recent analysis shows intrathecal HTLV-1 specific antibody is significantly elevated in HAM/TSP patients compared to ACs, which is significantly correlated with antibody secreting B cells (ASCs) (Enose-Akahata et al., 2018). These results suggest that elevated ASCs may contribute to antibody production in the CSF and importance of the B cell compartment in the pathogenesis of developing of HAM/TSP. A more detailed analysis of B cell response and development including B cell/T-cell interactions will improve our understanding of the pathological mechanism of antibody secretion in HAM/TSP.

#### **Cytokine Expression**

Elevated cytokine expression and production have been demonstrated in the peripheral blood and CNS lesion of patients with HAM/TSP (Jacobson, 2002). Inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  were expressed by macrophages, astrocytes, and microglia in the active inflammatory lesions (Umehara et al., 1993). Tax has been shown to transactivate a number of the common  $\gamma$  chain family of cytokines and the receptors, such as IL-2/IL2R, IL-9, IL-15/IL-15R, and IL-21/IL-21R (Enose-Akahata et al., 2017). IL-2/IL-2R and IL-15/IL-15R cytokine loops, which induce the proliferation and the elevated cytolytic activity of CD8+ T-cells, are deregulated in HAM/TSP and contribute to a variety of immunological abnormalities

characteristic of this disorder (Azimi et al., 1999). HTLV-1 Tax-specific CD8<sup>+</sup> T-cells expressed high IL-15Rα (Azimi et al., 2001) and induced degranulation and IFN-y expression when stimulated by IL-15 (Enose-Akahata et al., 2008). Elevated levels of IL-2, IL-9, and IL-15 driven by Tax lead to activate the Jak3/STAT5 pathway, and Jak3 inhibitor was shown to reduce in vitro immune activation in PBMCs from HAM/TSP patients (Ju et al., 2011). Monoclonal antibody therapy in HAM/TSP using a humanized antibody to IL-2 Ra showed reduction in the proviral load and spontaneous proliferation (Lehky et al., 1998), and a clinical trial of humanized-Mik-β-1, monoclonal antibody against IL-2/IL-15 Rβ, is currently underway, with promising early results (Waldmann, 2015). A selective inhibitor of IL-2 and IL-15 reduce the granulation and frequency of HTLV-1-specific CTLs (Massoud et al., 2015). The increase of the common y chain family of cytokines and receptors may therefore be associated with the pathogenesis of HAM/TSP where anti-cytokine strategy maybe a promising therapeutic approach for HAM/TSP.

#### **Exosome**

Exosomes are unilamellar extracellular vesicles originated from the endosomal compartment in various cell types (van der Pol et al., 2012). Several viruses take advantage of this mode of intracellular communication for incorporating viral components into exosomes (Anderson et al., 2016). Non cell-free virus has been detected in serum and CSF in HTLV-1-infected subjects (Pique and Jones, 2012). Exosomes derived from HTLV-1-infected cells appears to contain Tax protein and proinflammatory mediators as well as viral mRNA transcripts, including Tax, HBZ, and Env in vitro (Jaworski et al., 2014). Tax appears to be targeted for exosome entry by ubiquitination (Shembade and Harhaj, 2010; Jaworski et al., 2014), which was noted earlier to be an important target for endosomal sorting complex required for the transport pathway. Indeed, prior studies have shown Tax colocalized with organelles undergoing exocytosis (Alefantis et al., 2005a,b). Recent analysis showed that Tax protein was detected in exosomes isolated from CSF previously found to be negative for free HTLV-1 virus in HAM/TSP patients (Anderson et al., 2018). Exosomal Tax may therefore contribute to inflammatory cytokine production through packaging of these cytokines. Furthermore, HAM/TSP exosomes were shown to sensitize uninfected target cells for lysis by HTLV-1 specific CTLs (Anderson et al., 2018). HTLV-1 Tax in exosomes may therefore serve as a source of antigen that could be taken up by CNS resident cells to become targets for lysis by HTLV-1 Tax-specific CTLs. Exosomes may contribute to the continuous activation and inflammation observed in HAM/TSP and may be a potential biomarker that to be therapeutically targeted.

#### **CLINICAL FEATURES**

HAM/TSP is clinically characterized by chronic progressive spastic paraparesis, urinary incontinence and sensory disturbance. The mean age at onset is 43.8 years, and like many other autoimmune diseases, the frequency of cases of HAM/TSP is

greater in women than in men (Nakagawa et al., 1995). Weakness of the lower limbs progresses to an abnormal spastic gait, and some patients may become wheelchair dependent or even bedridden. Hyperreflexia of the lower limbs is commonly seen, often accompanied by clonus and Babinski's sign. Hyperreflexia of upper limbs is occasionally observed in some patients. The disease usually progresses slowly without remission; however, there is a subgroup of patients with rapid progression. The clinical course and rate of progression may vary greatly among patients. Older age at onset was associated with faster disease progression (Olindo et al., 2006; Nozuma et al., 2014). The median time from onset to require a unilateral walking aid was about 7 years, and wheel chair dependency was 18 years (Olindo et al., 2006; Coler-Reilly et al., 2016). Neurogenic bladder symptoms such as nocturia, frequency, and urgency are very common as well as constipation. It is also important to recognize that HAM/TSP patients have a risk to develop ATLL (Tamiya et al., 1995; Coler-Reilly et al., 2016).

#### **DIAGNOSIS**

The diagnosis of HAM/TSP is based upon a combination of characteristic clinical features and confirmation of HTLV-1 infection, along with exclusion of other disorders presenting spastic paraparesis (Yamano and Sato, 2012). The diagnostic criteria for HAM/TSP were initially proposed in 1989 (WHO, 1989). A modified diagnostic criterion is updated as levels of ascertainment as definite, probable, and possible with serological findings and/or detection of proviral DNA and exclusion of other disorders (De Castro-Costa et al., 2006). Recently, a new classification criterion based on disease activity has been proposed to help in providing adequate treatment for HAM/TSP (Sato et al., 2018b). The presence of HTLV-1 antibodies confirmed by western blot and/or a positive PCR should be demonstrated for confirmation of HTLV-1 infection. Recently digital droplet PCR (ddPCR) is used to quantify more precise PVL by determining absolute copy numbers instead of a standard curve in real-time PCR (Brunetto et al., 2014). Detection of anti-HTLV-1 antibodies in cerebrospinal fluid is necessary for the diagnosis of HAM/TSP. CSF examination revealed mild lymphocyte pleocytosis with mildly elevated protein and increased concentration of inflammatory markers such as neopterin, CXCL10, and CXCL9 (Sato et al., 2013). It is also necessary to exclude other disorders resembling HAM/TSP, such as multiple sclerosis (MS), neuromyelitis optica, and spinal cord compression. Neuroimaging is essential to exclude other diseases, but are not specific for HAM/TSP. Typical MRI showed spinal cord atrophy especially in the chronic phase without intensity changes. However, some patients with severe paraparesis and rapid progression show swelling of the spinal cord with T2 hyperintensity (Umehara et al., 2007). Recent advance of quantitative MRI techniques revealed that spinal cord cross-sectional areas correlated with clinical disability score, CD8+ T-cell frequency, and CSF PVL in HAM/TSP patients (Azodi et al., 2017). Reliable non-invasive biomarkers that correlate with the disease activity are clearly needed to inform appropriate treatment(s) for HAM/TSP.

#### **TREATMENT**

Most therapeutic trials in HAM/TSP have aimed to suppress or modulate the immune response, or to reduce the HTLV-1 proviral load in an attempt to decrease the risk or modify the course of the disease. Corticosteroids are most commonly used to reduce the inflammation in the spinal cord, particularly in the early stage. Motor disability might be improved with steroids (Nakagawa et al., 1996; Croda et al., 2008), but improvement is usually not sustained and is difficult to tolerate due to various side effects of steroids. IFN-α is a therapeutic agent whose efficacy was demonstrated in randomized placebocontrolled trials (Izumo et al., 1996; Arimura et al., 2007). However, the therapeutic benefit is small. Reverse transcriptase inhibitors, which are used against HIV-1, were not effective against HTLV-1 in clinical trials (Taylor et al., 2006; Macchi et al., 2011). More recently, a humanized anti-CCR4 monoclonal antibody, mogamulizumab, effectively reduced both the PVL and inflammatory activity in cells obtained from patients with HAM/TSP (Yamauchi et al., 2015). Phase 1/2a clinical trial has demonstrated the safety and short-term effectiveness of mogamulizumab in patients with HAM/TSP (Sato et al., 2018a). Monoclonal antibody against IL-2 Ra resulted in reduction in the PVL and spontaneous proliferation (Lehky et al., 1998), and a clinical trial of humanized Mik-β-1, monoclonal antibody against IL-2/IL-15Rβ, is being evaluated in patients with HAM/TSP (Waldmann, 2015).

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#### CONCLUSION

Recent advances in research on HTLV-1 provide better understanding of the molecular pathogenesis and mechanisms of HAM/TSP, and several clinical trials of novel therapies for patients with HAM/TSP have been initiated. However, long-term improvement of motor disability and quality of life still have not been achieved in HAM/TSP patients, and the clinical management remains challenging. Given that HAM/TSP is characterized by activated T-cells in both the periphery and CNS, studies in HAM/TSP will be highly informative for clarifying the pathogenesis of other neuroinflammatory disorders such as multiple sclerosis. Novel approaches will be required to better define host-virus interactions and host immune response underlying the pathogenesis of HAM/TSP.

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SN wrote the manuscript, and SJ supervised and contributed to the discussion and writing.

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### HTLV-1 HBZ Protein Resides Exclusively in the Cytoplasm of Infected Cells in Asymptomatic Carriers and HAM/TSP Patients

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Forlani G, Baratella M, Tedeschi A, Pique C, Jacobson S and Accolla RS (2019) HTLV-1 HBZ Protein Resides Exclusively in the Cytoplasm of Infected Cells in Asymptomatic Carriers and HAM/TSP Patients. Front. Microbiol. 10:819. doi: 10.3389/fmicb.2019.00819 Human T cell lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) in a subset of infected subjects. Two viral proteins, Tax-1 and HTLV-1 basic leucine zipper factor (HBZ), play important roles in the pathogenesis of both diseases. We recently demonstrated that HBZ, previously considered a nuclear protein, is exclusively localized in the cytoplasm of peripheral blood mononuclear cells (PBMCs) of HAM/TSP patients. Here, the analysis of a larger panel of HAM/TSP cases confirmed that HBZ is a cytoplasmic protein, while Tax-1 preferentially localized in the cytoplasm with fewer speckle-like dots in the nucleus. More importantly, here we report for the first time that HBZ, when expressed in asymptomatic carriers (AC), is also confined in the cytoplasm. Similarly, Tax-1 was preferentially expressed in the cytoplasm in a significant proportion of AC. Interestingly, in both HAM/TSP and AC patients, the expression of HBZ and Tax-1 was rarely found in the same cell. We observed only few cases coexpressing the two oncoprotein in a very limited number of cells. In representative AC and HAM/TSP patients, cells expressing cytoplasmic HBZ were almost exclusively found in the CD4+ T cell compartment and very rarely in CD8+ T cells. Interestingly, at least in the cases analyzed, the expression of thymociteexpressed molecule involved in selection (THEMIS) is dispensable for the cytoplasmic localization of HBZ in both AC and HAM/TSP. The study of an HTLV-1-immortalized cell line established from an HAM/TSP patient confirmed HBZ as a resident cytoplasmic protein not shuttling between the cytoplasm and nucleus. These results extend our previous observation on the dichotomy of HBZ localization between HAM/TSP and ATL, pointing to the exclusive either cytoplasmic or nuclear localization in the two diseased states, respectively. Moreover, they show a rather selective expression in distinct cells of either HBZ or Tax-1. The unprecedented observation that HBZ is expressed only in

the cytoplasm in AC strongly suggests a progressive modification of HBZ localization during the disease states associated to HTLV-1 infection. Future studies will clarify whether the distinct HBZ intracellular localization is a marker or a causative event of disease evolution.

Keywords: HTLV-1, HBZ, Tax-1, HAM/TSP, asymptomatic carriers

#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is the first discovered human oncogenic retrovirus (Poiesz et al., 1980) and is thought to infect at least 10-15 million people worldwide. Several HTLV-1 endemic areas exist in the southern part of Japan, the Caribbean, North and South America, Central and West Africa, and foci in Middle East, Australia, and Melanesia (Gessain and Cassar, 2012). HTLV-1 induces clonal proliferation of infected cells to enhance its propagation, since it is transmitted primarily by cell-to-cell contact (Igakura et al., 2003; Pais-Correia et al., 2009; Pique and Jones, 2012; Bangham et al., 2014). It has been reported that an increased number of infected cells is correlated with a higher rate of transmission by breast-feeding (Li et al., 2004). HTLV-1 is the cause of a severe form of leukemia affecting adult CD4+ T cells (adult T-cell leukemia/lymphoma or ATL) and of a progressive neurological pathology designated HTLV-1associated myelopathy/tropical spastic paraparesis (HAM/TSP), characterized by spastic progressive limb paralysis, sensory dysfunction, and sphincter function defects (Gessain et al., 1985; Osame et al., 1986). Similar to other retroviruses, the HTLV-1 proviral genome has structural genes, gag, pol, and env, coded by the plus strand of the viral genome. HTLV-1 encodes two regulatory genes, tax and rex, and three accessory genes (p12, p13, and p30) in the plus strand of the provirus (Matsuoka and Jeang, 2007). Another regulatory gene, the HTLV-1 bZIP factor (HBZ), is transcribed from the minus strand of the proviral genome (Larocca et al., 1989; Gaudray et al., 2002). It has been demonstrated that the viral proteins Tax-1 and HBZ play important roles in HTLV-1 infectivity as well as growth and survival of leukemic cells (Matsuoka and Jeang, 2011). Tax-1 promotes proviral transcription and tumorigenesis (Grassmann et al., 2005; Tosi et al., 2011; Forlani et al., 2013). However, Tax-1 expression is generally lost in ATL cells while HBZ transcripts are ubiquitously expressed in HTLV-1-infected cells, ATL cells, and peripheral blood mononuclear cells (PBMCs) of HTLV-1-infected individuals (Takeda et al., 2004; Taniguchi et al., 2005; Satou et al., 2006). Moreover, the amount of HBZ transcripts positively correlates with HTLV-1 proviral load (PVL) in asymptomatic carriers (AC), HAM/TSP, and ATL patients (Ma et al., 2016). Therefore, it is likely that other mechanisms are involved in the establishment and persistence of the infection, perhaps involving HBZ. Indeed, HBZ has been reported to promote growth and proliferation of leukemic cells in vivo and in vitro (Satou et al., 2006; Mitobe et al., 2015). There are three different transcriptional isoforms of HBZ: the unspliced (usHBZ) variant and two alternative spliced forms, SP1 and SP2 (Cavanagh et al., 2006; Murata et al., 2006). The SP1 form occurs

more frequently than SP2 (Cavanagh et al., 2006). The sequences of SP1 and usHBZ forms are identical with the exception of the first 7 amino acids and contain 206 amino acids and 209 amino acids, respectively. Although the two protein variants exhibit similar functions (Ma et al., 2016), the spliced form is more abundant than the unspliced form and is found in almost all ATL patients (Usui et al., 2008). All the HBZ protein variants are composed by conserved functional domains: an N-terminal activation domain (AD), a central domain (CD), and a C-terminal basic ZIP domain (bZIP; Gaudray et al., 2002). HBZ displays three nuclear localization signals (NLS) responsible for its nuclear localization (Hivin et al., 2005; Zhao and Matsuoka, 2012) and two functional nuclear export signals (NES) within its N-terminal region (Mukai and Ohshima, 2011), which led us to suppose that HBZ may reside in both cytoplasm and nucleus. Most of the reported subcellular localizations, biochemical interactions, and functional aspects related to HBZ have been assessed in cells overexpressing tagged HBZ. Recently, the availability of the first reported monoclonal antibody (mAb), 4D4-F3, isolated in our laboratory, allowed us to study the expression, localization, and interaction in vivo of endogenous HBZ in HTLV-1-infected ACs, ATL and HAM/TSP patients (Raval et al., 2015; Baratella et al., 2017b). It was found that in chronically infected cell lines and ATL cells, endogenous HBZ interacts and colocalizes with p300 and JunD. Partial colocalization was also observed for CBP and CREB2 (Raval et al., 2015). The amount of HBZ expression in the above cells was 20- to 50-fold less than that found in HBZ-transfected cells (Raval et al., 2015; Shiohama et al., 2016). Subsequent studies have shown that HBZ localizes in different subcellular compartments in ATL and HAM/TSP. While HBZ was found in the nucleus in leukemic cells, with a speckle-like distribution (Raval et al., 2015; Baratella et al., 2017a,b), in HAM/TSP patients, we found for the first time that HBZ localized in the cytoplasm (Baratella et al., 2017b). More recently, a cytoplasmic localization of HBZ in HBZ-transfected T cells was reported (Kinosada et al., 2017), depending on the expression of THEMIS (thymociteexpressed molecule involved in selection), a T-lineage-restricted protein (Brockmeyer et al., 2011; Fu et al., 2013). Interestingly, cytoplasmic HBZ protein was almost selectively found in CD4+ T cells without relationships with CD25 expression, suggesting that CD4+ T cells were either not in rapid proliferation or not included in the classical resting regulatory T cell compartment (Baratella et al., 2017b). Consistently, it has been previously reported that HBZ-specific humoral immune response correlated with reduced CD4+ T cell activation in HAM/TSP patients (Enose-Akahata et al., 2013; Enose-Akahata et al., 2017). The distinct expression patterns of HBZ and Tax-1 in PBMCs of infected AC, ATL, and HAM/TSP patients suggest that Tax-1 and

HBZ have different roles in the course of HTLV-1 pathogenesis. In the present study, the analysis of a larger panel of patients has added new relevant information on this point. PBMCs from 10 HTLV-1 AC and 10 HAM/TSP patients were analyzed. Expression and subcellular distribution of endogenous HBZ and Tax-1 proteins were assessed by confocal microscopy with the 4D4-F3 and A51-2 mAbs, respectively. Consistent with our previous findings, in HAM/TSP, HBZ protein is expressed in a discrete percentage of cells (2-10% of total PBMC), and its expression is confined in the cytoplasm. Of particular interest, we now report the unprecedented finding that HBZ is found in 6 out of 10 AC in a limited number of cells (1-4% of the total PBMC), again with an exclusive cytoplasmic localization. The number of HBZ-positive cells was higher in HAM/TSP compared to AC patients. The coexpression of Tax-1 and HBZ at the singlecell level was a very rare event both in HAM/TSP and in AC. At least for the patients analyzed, the expression of THEMIS was found not strictly required for HBZ cytoplasmic localization. Confirming our previous results, in HAM/TSP, HBZ-positive cells mainly resided in the CD4+ T cell compartment. Similar results were found in AC. These findings might shed light on a new molecular basis for a role of HBZ in the progression of HTLV-1 infection. The unprecedented observation that HBZ is expressed only in the cytoplasm in AC strongly suggests a progressive modification of HBZ localization during the disease states associated to HTLV-1 infection.

#### MATERIALS AND METHODS

#### **Ethics Statement**

We obtained PBMCs from HTLV-1 asymptomatic donors and HAM/TSP patients as part of the NIH natural history protocol # 98-N-0047. All individuals gave written informed consent. The patients' data were analyzed anonymously.

#### Cells

HTLV-1-immortalized CB T cells (CB-CD4/HTLV-1; Ozden et al., 2004) were cultured in RPMI supplemented with 10% fetal calf serum (FCS) and 50 U/ml IL-2 (Sigma). PBMCs from healthy donors, HTLV-1-infected AC, or HAM/TSP patients were purified by Ficoll-Paque TM PLUS (GE-Healthcare Bio-Science, Milan, Italy) from heparinated blood. PBMCs from healthy donors were obtained from the Blood Transfusion Center, Ospedale di Circolo, Fondazione Macchi, Varese, whereas PBMCs of AC and HAM/TSP patients were isolated by Ficoll-Hypaque (Lonza) centrifugation and cryopreserved in liquid nitrogen until use.

#### **HTLV-1 Proviral Load Measurement**

HTLV-1 PVL was measured using ddPCR (Bio-Rad) as previously described (Brunetto et al., 2014). DNA was extracted from the PBMCs and cerebro-spinal fluid (CSF) cell pellets using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. DNA was digested with the restriction enzyme BamH1 (New England Biolabs) for 30 min at 37°C and diluted 1:5 with PCR-certified water. The digested, diluted DNA was mixed with both HTLV-1 tax and

human ribonuclease P protein subunit 30 (RPP30) primers and probes and Bio-Rad 2× Supermix and then emulsified with droplet generator oil using a QX-100 droplet generator according to the manufacturer's instructions (Bio-Rad). The following primers and probe were used to amplify and detect a 154-base-pair region of HTLV-1 tax: ddPCR HTLV-1 tax F: 5'-CTTATTTGGACATTTACCGATG-3'; ddPCR HTLV-1 tax R: 5'-TGAGGCCGTGTGAGAGTAGA-3'; ddPCR HTLV-1 tax probe: 6FAM-TGATTTCCGGGCCCTGC-MGBNFQ. The droplets were then transferred to a 96-well reaction plate (Eppendorf) and heat-sealed with pierceable sealing foil sheets (Thermo Fisher Scientific). The duplex PCR amplification was performed in this sealed 96-well plate using a GeneAmp 9700 thermocycler (Applied Biosystems). Following PCR amplification, the 96-well plate was transferred to a QX100 droplet reader (Bio-Rad). For PVL calculation, QuantaSoft software version 1.3.2.0 (Bio-Rad) was used to quantify the copies per microliter of each queried target per well. All samples were tested in duplicate, unless otherwise specified, and PVL is reported as the average of the two measurements. The PVL was calculated using the following formula: PVL = [quantity of HTLV-1  $tax/(quantity of RPP30/2)] \times 100\%$ .

#### **Treatments**

CB-CD4/HTLV1 cells cultured on glass coverslips precoated with poly-L-lysine were incubated with 20 nM leptomycin B (LMB; Sigma) or the vehicle methanol for 3 h at 37°C, 5% CO<sub>2</sub>. Cells were then washed and processed for confocal microscopy analysis by using the following antibodies: anti-HBZ 4D4-F3 mAb, anti-Tax-1 mAb (clone 168 A51-2 from the NIH AIDS Research and Reference Reagent Program), and anti-RelA rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, United States), followed by the secondary antibodies as specified in the figure legends.

# Immunofluorescence, Flow Cytometry, and Confocal Microscopy

Peripheral blood mononuclear cells from AC, HAM/TSP patients, or normal control individuals, after rapid thawing, were washed with warm RPMI medium and immediately processed for immunofluorescence and flow cytometry analysis or for confocal microscopy, as described (Forlani et al., 2016b). For flow cytometry, the following reagents were used: mouse anti-human HLA class I (clone B9.12); mouse anti-human HLA class II DR (clone D1.12), both revealed by FITClabeled rabbit anti-mouse IgG F(ab')2 antiserum (Sigma, Milan, Italy); FITC mouse anti-human CD3 (clone (UCHT1, BD Pharmingen); FITC mouse anti-human CD4 (clone RPA-T4, BD Pharmingen); PE-Cy5 mouse anti-human CD8a (clone RPA-T8; eBioscience, Milan, Italy); PE mouse anti-human CD16 (clone B73.1, eBioscience, Milan, Italy); FITC mouse anti-human CD19 (clone HIB19, BD Pharmingen); and phycoerythrin (PE) mouse anti-human CD25 (clone M-A251, BD Pharmingen). For confocal microscopy, cells were cultured on glass coverslips precoated with poly-L-lysine (0.2 g/ml, Sigma) for 5 h. The cells were then washed with 1× Pipes-Hepes-EGTA-MgSO4 (PHEM)

buffer, pH 6.9 (60 mM PIPES, 25 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), 10 mM ethylene glycolbis(beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), and 2 mM MgCl<sub>2</sub>), three times, fixed in methanol 7 min at −20°C, and blocked with 1% bovine serum albumin (BSA) in 1× PHEM for 1 h at room temperature (RT). Cells were then stained overnight with anti-HBZ 4D4-F3 mAb, anti-Tax-1 mAb (clone 168 A51-2 from the NIH AIDS Research and Reference Reagent Program), anti-vimentin rabbit polyclonal antibody (Santa Cruz Biotechnology, CA, United States), rabbit anti-CD4 mAb (clone EPR6855, ABCAM), and rabbit anti-THEMIS mAb (clone EPR7353, ABCAM), diluted in PHEM buffer containing 0.5% BSA. The slides were then washed five times with cold 1× PHEM and incubated in the dark for 2 h at RT with the following secondary antibodies from Life Technology (Waltham, MA, United States): goat anti-mouse IgG1 coupled to Alexa Fluor 546 to detect HBZ, goat antimouse IgG2a conjugated to Alexa Fluor 488 to detect Tax-1, and goat anti-rabbit IgG conjugated to Alexa Fluor 488 or to Alexa Fluor 546 to detect vimentin, CD4, or THEMIS. For costaining with directly labeled antibodies, after extensive washing with 1× PHEM, anti-CD8 rabbit mAb directly conjugated to Alexa Fluor 647 (clone EP1150Y, ABCAM) and mouse anti-human CD25 mAb directly conjugated to Alexa Fluor 488 (clone BC96, BioLegend) were added after the indirect immunofluorescence for 2 h at RT. Similarly, after indirect immunofluorescence, the nuclei were stained by incubating the cells with DRAQ5 Fluorescent Probe (Thermo Fisher Scientific, Waltham, MA, United States) for 30 min at RT. CIB-CD4/HTLV1 cells were cultured on glass coverslips precoated with poly-Llysine for 5 h and processed for confocal microscopy analysis as described above.

After washing, the slides were mounted on coverslips with the Fluor Save reagent [Calbiochem, Vimodrone (MI), Italy] and examined by a confocal laser scanning microscope (Leica TCS SP5; HCX PL APO objective lenses, 63× original magnification, numerical aperture 1.25). Images were acquired and analyzed by LAS AF Lite Image (Leica Microsystems, Milan, Italy) and/or Fiji (ImageJ) software.

#### Preparation of Nuclear and Cytoplasmic Extracts and Immunoprecipitation Procedures

Endogenous HBZ protein was precipitated from both the nuclear and cytoplasmic protein fractions prepared from 8  $\times$   $10^6$  CD-CD4/HTLV-1 cells, using an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Thermo Fisher Scientific) according to the manufacturer's instruction. Briefly, cells were washed twice with cold phosphate buffer saline (PBS) and centrifuged at 500g for 3 min. The cell pellet was suspended in 200  $\mu l$  of cytoplasmic extraction reagent I (CERI), supplemented with 0.1% protease inhibitor cocktail (Sigma), by vortexing. The suspension was incubated on ice for 10 min followed by the addition of 11  $\mu l$  of a second cytoplasmic extraction reagent II (CERII), vortexed for 5 s, incubated on ice for 1 min, and centrifuged for 5 min at 16,000g. The supernatant fraction (cytoplasmic

extract) was transferred to a pre-chilled tube. The insoluble pellet fraction, which contains crude nuclei, was resuspended in 100  $\mu l$  of nuclear extraction reagent (NER), supplemented with 0.1% protease inhibitor cocktail (Sigma), by vortexing for 15 s, incubated on ice for 40 min, and then centrifuged for 10 min at 16,000g. Both the nuclear and cytoplasmic protein extracts were used for the subsequent immunoprecipitation experiments.

After preclearing with protein A/G-Sepharose beads, the nuclear and cytoplasmic extracts were incubated with anti-HBZ 4D4-F3 mAb for 1 h on ice and then immunoprecipitated with protein A/G-Sepharose beads overnight at  $4^{\circ}C$ , under rotation. The precipitated proteins were resolved on 9% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting with the anti-HBZ 4D4-F3 mAb, followed by Mouse True Blot ULTRA-anti mouse Ig HRP (e Bioscience). This specific secondary antibody was used to avoid the detection of both heavy and light chains in the precipitated proteins. Ten percent of the nuclear and cytoplasmic protein extracts were analyzed by Western blotting with anti Nup98 (Cell Signaling Technology) and anti- $\beta$ -tubulin (Sigma) mAbs to assess the purity of nuclear and cytoplasmic protein extracts, respectively.

#### **RESULTS**

#### PBMCs of HTLV-1-Positive Asymptomatic Carriers Express HBZ Protein in the Cytoplasm

By analyzing the subcellular distribution of endogenous HBZ in PBMCs of four HAM/TSP patients, we previously demonstrated for the first time that HBZ is a cytoplasmic protein expressed in a discrete percentage, up to 11%, of the cells. Unlike HAM/TSP, we were unable to detect HBZ-positive cells in PBMCs of four AC (Baratella et al., 2017a,b). To better understand the role of HTLV-1 HBZ protein in the progression of HTLV-1-associated diseases, we extended our previous study to include more HAM/TSP and AC patients. We first analyzed by immunofluorescence and confocal microscopy the subcellular localization of both endogenous HBZ and Tax-1 oncoproteins in PBMCs isolated from 10 HTLV-1-positive AC (Table 1). Interestingly, although PBMC from four ACs were negative for HBZ expression, six ACs clearly expressed HBZ protein in 1-4% of their PBMCs (Table 1). More importantly, HBZ was found localized exclusively in the cytoplasm (Figure 1, HBZ). Parallel staining with vimentin, a cytoplasmic marker, and DRAQ5, a nuclear marker, confirmed HBZ cytoplasmic localization without detectable nuclear colocalization (Figure 1, upper panels). Like HAM/TSP patients, in PBMCs from AC, cytoplasmic HBZ appeared distributed in dots, sometimes dispersed all over the cytoplasm or concentrated in restricted areas. The expression and the subcellular distribution of Tax-1 were then analyzed. Tax-1 was expressed in 70% of ACs in a discrete proportion of cells (between 2 and 9% of total PBMCs). Tax-1 was preferentially localized in the cytoplasm, with fewer speckle-like dots in the nucleus (Figure 1, Tax-1). Co-staining with vimentin and

**TABLE 1** | Percent distribution of HBZ+ and Tax-1+ PBMCs in HTLV-associated pathologies.

Patient	Proviral load	Pathology	Total HBZ+ cells (%)	Total Tax-1+ cells (%)	HBZ+ Tax-1+ cells (%)
PH70	3.26	AC*	1	0	0
PH928	1.02	AC	0	3	0
PH1152	6.43	AC	0	2	0
PH1186	0	AC	0	4	0
PH1307	8	AC	3	4	0
PH1320	0	AC	2	9	0.2
PH1339	1.1	AC	4	7	1
PH1443	2.81	AC	1	2	0
PH2113	0.58	AC	0	0	0
PH2116	19.16	AC	2	0	0
PH667	8.84	HAM/TSP	0	11	0
PH1026	27.21	HAM/TSP	6	3	0
PH1205	9.99	HAM/TSP	7	2	0
PH1216	0.74	HAM/TSP	8	15	1
PH1419	6.69	HAM/TSP	4	7	0
PH1509	5.09	HAM/TSP	10	2	0
PH1510	16.59	HAM/TSP	2	4	0
PH2163	20.13	HAM/TSP	1	12	0
PH2176	27.44	HAM/TSP	1	5	0
PH2262	20.44	HAM/TSP	2	3	0

<sup>\*</sup>AC, asymptomatic carriers.

DRAQ5 showed the Tax-1 prominent cytoplasmic localization (**Figure 1**, bottom panels).

Interestingly, expression of both HBZ and Tax-1 was found in PBMCs of only four ACs, but in two ACs coexpression of HBZ and Tax-1 within the same cell was detected in a small proportion

of cells (0.2 or 1% of total PBMCs; **Table 1**, PH1320 and PH1339, respectively), indicating that, in AC, the coexpression of the two viral proteins in the same cell is a rare event. A clear example was the PH1339 AC, whose PBMCs were 4 and 7% positive for HBZ and Tax-1, respectively (**Figures 2a,b**, respectively and **Figure 2d**) while only 1% coexpressed the two viral proteins (**Figures 2f,g**, respectively). In coexpressing cells, HBZ did not colocalize with Tax-1 (**Figure 2i**). Moreover, HBZ was confined in a specific region of the cytoplasm (**Figure 2f**), while Tax-1 was observed in the nucleus and in the cytoplasm surrounding the HBZ-positive staining (**Figure 2g**). DRAQ5 was used to stain the nuclei (**Figures 2c,h**). Differential interference contrast (DIC) images of the cells are shown (**Figures 2e,j**).

In addition, in AC PH70 and PH2116, HBZ was expressed in 1 and 2% of total PBMC, respectively, while Tax-1 was not detected. On the contrary, in AC PH928, PH1152, and PH1186, HBZ was undetectable, while Tax-1 was found in 3, 2, and 4% of total PBMCs, respectively. We were unable to detect both HBZ and Tax-1 in patient PH2113 (**Table 1**). Collectively, these findings demonstrate for the first time that HBZ protein has a cytoplasmic localization in AC and this localization is maintained in HAM/TSP patients.

#### HAM/TSP Pathology Is Characterized by a Relevant Increase in Number of HBZ-Positive Cells With an Exclusive Cytoplasmic Localization

To expand our previous studies in HAM/TSP patients, PBMCs of 10 additional patients were investigated by immunofluorescence and confocal microscopy. Consistent with our previous findings, nine HAM/TSP patients expressed HBZ with a percentage of HBZ-positive cells ranging between 1 and 10% (**Table 1**). Interestingly, the number of HBZ-positive cells was higher in

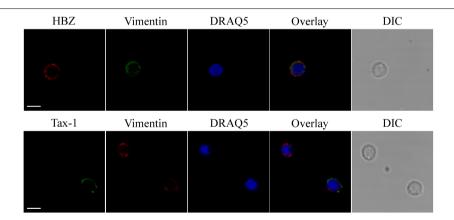


FIGURE 1 | Endogenous HBZ localized in the cytoplasm whereas Tax-1 was distributed both in the nucleus and in the cytoplasm in peripheral blood mononuclear cells (PBMCs) of representative AC PH1307. PBMCs of representative AC PH1307 patient were stained with the 4D4-F3 anti-HBZ monoclonal antibody (mAb) followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red, upper panels) and with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green, lower panels) and analyzed by confocal microscopy. Specific counterstaining of nucleus or cytoplasmic compartments was performed by using DRAQ5 fluorescence probe to detect the nucleus (blue) and anti-vimentin rabbit polyclonal antibody followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green, upper panels) or to Alexa Fluor 546 (red, lower panels) to stain the cytoplasmic compartment. DIC represents the differential interference contrast image. At least 300 cells were analyzed. One representative image of single confocal section for HBZ or Tax-1 staining is shown. All scale bars are 5 μm.

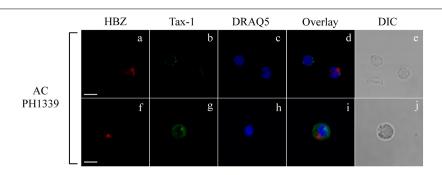


FIGURE 2 | Coexpression of HBZ and Tax-1 in the same cell is a rare event in asymptomatic carriers. PBMCs of representative AC PH1339 patient were co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red, a,f,d,i) and with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green, b,g,d,i) and analyzed by confocal microscopy. DRAQ5 fluorescence probe was used to detect the nucleus (c,h,d,i). DIC represents the differential interference contrast image (e,j). At least 300 cells were analyzed. In the top panel, one representative image of single confocal section is shown; in the bottom panel, one representative image derived from the same sample and obtained from the sum of all z-stacks is shown. All scale bars are 5 μm.

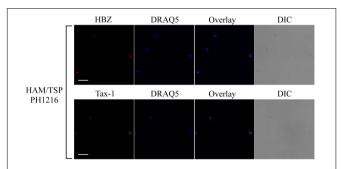


FIGURE 3 | Subcellular localization of endogenous HBZ and Tax-1 in PBMCs of representative HAM/TSP PH1216 patient. (Top panels) PBMCs of representative HAM/TSP patient PH1216 were stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and (Bottom panels) with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green) and analyzed by confocal microscopy. Nucleus was stained with DRAQ5. DIC represents the differential interference contrast image. At least 300 cells were analyzed; two representative fields for HBZ or Tax-1 staining are shown. All scale bars are 5  $\mu$ m.

HAM/TSP patients as compared to AC. As clearly shown for a representative patient PH1216 having 8% of HBZ-positive cells, the viral protein localized in the cytoplasm with a speckled-like distribution dispersed along the cytoplasm or accumulated in a discrete and limited region of it (Figure 3, HBZ). Parallel staining with DRAQ5 confirmed the HBZ cytoplasmic distribution (Figure 3, top panels, overlay). Tax-1 was expressed in all HAM/TSP patients with a percentage of Tax-1-positive cells ranging between 2 and 15% of total PBMCs depending on the patient analyzed (Table 1). Patient PH667 did not show any positivity for HBZ, although it expressed Tax-1 in a considerable number of cells (11%; Table 1).

Tax-1 was expressed mostly as discrete dots localized preferentially in the cytoplasm with fewer dots in the nucleus (representative patient PH1216; **Figure 3**, Tax-1). In this patient, expressing the highest number of HBZ and Tax-1 positive cells, 8 and 15%, respectively (see **Table 1**), most cells expressed either

HBZ or Tax-1 (**Figure 4**, upper panels), with only 1% of the cells coexpressing the two viral proteins. In some cells, HBZ partially colocalized with Tax-1 in the cytoplasm (**Figure 4**, bottom panels, overlay).

Thus, as we observed for AC, in HAM/TSP patients, coexpression of HBZ and Tax-1 oncoproteins is a rare event. Importantly, the cumulatively higher number of HBZ- and Tax-1-expressing cells in HAM/TSP patients compared to HTLV-1-infected AC strongly suggests that evolution toward the neuroinflammatory disease modifies only the number of HBZ- and Tax-1-positive cells but not the HBZ- or the Tax-1-specific intracellular localization. Moreover, the higher number of HBZ- and Tax-1-expressing cells in HAM/TSP compared to AC correlated generally, but not always, with higher PVL (**Table 1**).

#### CD4+ T Cell Subpopulation Represents the Majority of HBZ-Positive Cells in Asymptomatic Carriers and in HAM/TSP Patients

We previously demonstrated that in HAM/TSP patients, cytoplasmic HBZ is almost exclusively found in CD4+ T cells not coexpressing the CD25 marker (Baratella et al., 2017b). In order to identify the cell subpopulations expressing the cytoplasmic HBZ protein in AC patients and extend the analysis to the new HAM/TSP patients, we first analyzed by immunofluorescence and flow cytometry the cell surface phenotype of PBMCs of all AC and HAM/TSP patients.

Peripheral blood mononuclear cells of AC and HAM/TSP patients displayed a phenotype similar to that of PBMCs from healthy donors (**Figure 5**, representative AC PH1339 and representative HAM/TSP PH1509). PH1339 expressed CD3, CD4, and CD8 markers in 61, 44, and 18% of PBMCs, respectively. CD3 and CD4 T cell markers were expressed in a comparable number of cells in patient HAM/TSP PH1509 (70 and 33%, respectively) while cells expressing the CD8 marker were increased to 40%. In the sample analyzed, the CD25 marker was undetectable. The CD19-positive B cells were

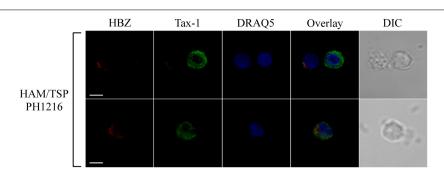


FIGURE 4 | HBZ and Tax-1 were coexpressed at the single-cell level in 1% of total PBMCs of HAM/TSP representative patient PH1216. PBMCs of representative HAM/TSP PH1216 patient were co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green) and analyzed by confocal microscopy. DRAQ5 fluorescence probe was used to detect the nucleus. DIC represents the differential interference contrast image. At least 300 cells were analyzed; representative images for HBZ and Tax-1 co-staining are shown. All scale bars are 5 μm.

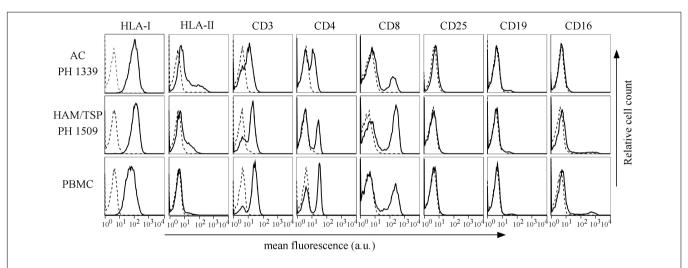


FIGURE 5 | Expression of cells surface markers in PBMCs of AC PH1339, HAM/TSP patient PH1509, and healthy control. The expression of HLA class I, HLA class II DR, CD3, CD4, CD8, CD25, CD19, and CD16 surface molecules on PBMCs from AC PH1339, HAM/TSP patient PH1509, and a healthy control was assessed by immunofluorescence and flow cytometry with antibodies specific for the various markers. Results are expressed as relative number of cells (ordinate) versus the mean intensity of fluorescence in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an appropriate isotype-matched antibody, are depicted as a dashed line.

present in almost equal proportions of PH1307 (4.5%), PH1419 (4.7%), and normal (3.4%) PBMCs. NK cells, as assessed by the CD16 marker, were not detected in PH1307, whereas they represented 11.4 and 10% of PH1419 and normal PBMCs, respectively. HLA-I was expressed in 100% of PBMCs of the patients and the normal donor, and HLA class II was expressed in 17, 12, and 9% of PH1339, PH1509, and normal PBMCs, respectively. Subsequently, we co-stained PBMCs of AC and HAM/TSP patients for either CD4 or CD8 and HBZ, and we analyzed them by confocal microscopy. Representative data obtained from PBMCs of PH1339 AC and PH1509 HAM/TSP patient revealed a higher number of HBZ-positive cells (7 and 10%, respectively).

Almost all HBZ-positive cells expressed CD4 (**Figure 6A**, upper panels). Conversely, HBZ-expressing cells were very rarely detected in the CD8+ T cell compartment (**Figure 6A**, lower panels).

Similarly to AC patients and confirming our previous findings (Baratella et al., 2017b) in HAM/TSP PH1509 patient, cells expressing cytoplasmic HBZ were almost exclusively found in the CD4+ T cells compartment (**Figure 6B**, top panels) and very rarely, less than 1%, in CD8+ T cells (**Figure 6B**, lower panels). From the above data, we conclude that in AC and HAM/TSP patients, cytoplasmic HBZ is almost exclusively expressed in CD4+/CD25- T cells.

# The Expression of THEMIS Is Dispensable for the Cytoplasmic Localization of HBZ in Asymptomatic Carriers and HAM/TSP Patients

Recently, it has been reported that the HBZ-transfected Jurkat T cell line may partially segregate HBZ in the cytoplasm as a result of interaction with THEMIS (Kinosada et al., 2017), a

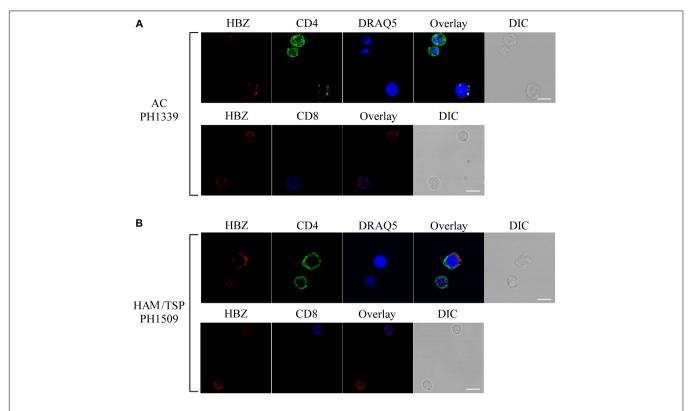


FIGURE 6 | HBZ is preferentially expressed in CD4+ T cells of AC PH1339 and HAM/TSP patient PH1509. Confocal microscopy analysis of PBMCs from AC patient PH1339 and HAM/TSP patient PH1509. (A) Upper panels: PBMC of patient AC PH1339 were co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with the anti-CD4 mAb followed by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody (green). Lower panels: co-staining with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with the anti-CD8 rabbit mAb directly conjugated to Alexa Fluor 647 (blue). (B) PBMCs of patient HAM/TSP PH1509 were co-stained with 4D4-F3 anti-HBZ mAb and either anti-CD4 mAb (upper panels) or anti-CD8 mAb (lower panels) followed by specific secondary antibodies staining as described in panel (A). DIC represents the differential interference contrast image. At least 200 cells were analyzed; representative images derived from each sample are shown. All scale bars are 5 μm.

molecule involved in thymocyte selection and T cell receptor (TCR) signaling.

To assess whether HBZ colocalizes with THEMIS in PBMCs, we analyzed by immunofluorescence and confocal microscopy the expression and subcellular localization of both HBZ and THEMIS in PBMCs of AC and HAM/TSP patients. Figure 7 shows the results obtained in PBMCs of PH1307 and PH1419, representative of an AC and a HAM/TSP patient, respectively. As control, PBMCs from non-infected individuals were also analyzed. As expected, in PBMCs of a healthy donor, THEMIS was expressed in the cytoplasm of a discrete percentage of cells (Figure 7, PBMC, Themis). The co-staining with DRAQ5 nuclear marker confirmed THEMIS as a cytoplasmic resident protein (Figure 7, PBMC, overlay).

Interestingly, in PBMCs of PH1307 AC, coexpression of HBZ and THEMIS could be found in cells expressing both proteins at low level (**Figure 7**, AC PH1307, bottom panels). On the other hand, highly expressing THEMIS-positive cells (**Figure 7**, AC PH1307, top panels, Themis) usually did not show coexpression of HBZ (**Figure 7**, AC PH1307, top panels, THEMIS and overlay). Similarly, highly expressing HBZ cells can be found that do not express THEMIS (**Figure 7**, AC PH1307, top panels, HBZ and overlay).

Unlike AC, in PBMCs of HAM/TSP patient PH1419, the majority of HBZ-positive cells (**Figure 7**, HAM/TSP PH1419, top and middle panels, HBZ) were included in the highly expressing THEMIS (**Figure 7**, HAM/TSP PH1419, top and middle panels, Themis) and the two proteins partially colocalized in the cytoplasm (**Figure 7**, HAM/TSP PH1419, top and middle panels, overlay). Nevertheless, we found cells expressing either THEMIS or HBZ alone (**Figure 7**, HAM/TSP PH1419, bottom panels). Collectively, these findings suggest that colocalization of HBZ and THEMIS is observed, but the cytoplasmic expression of THEMIS is not required for the cytoplasmic expression of HBZ, particularly in HAM/TSP patients.

# An IL-2-Dependent Cell Line From a HAM/TSP Patient Further Demonstrates HBZ as a Cytoplasmic Protein Unable to Shuttle Between Cytoplasm and Nucleus

As shown above, HBZ expression in HTLV-1-infected asymptomatic patients shows a cytoplasmic localization that is further increased both in intensity and in number of positive cells in HAM/TSP patients. To further investigate the biological basis of this peculiar subcellular distribution of HBZ compared

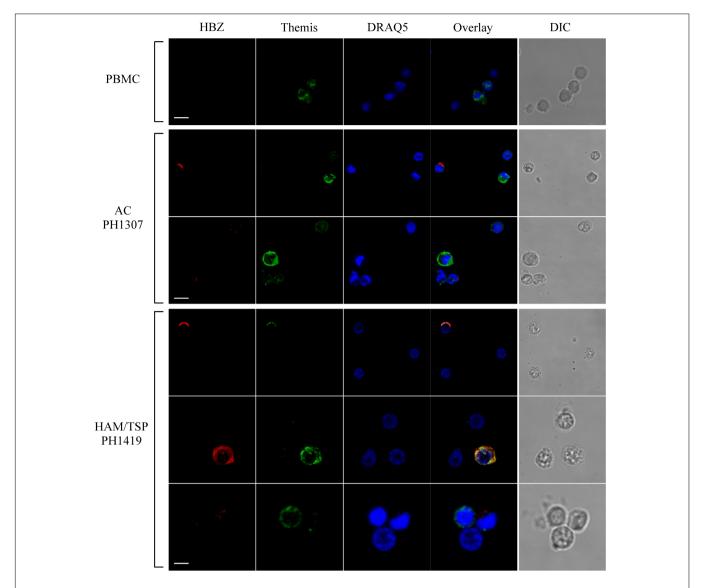


FIGURE 7 | Subcellular localization of endogenous HBZ and THEMIS in PBMC of AC, HAM/TSP patients, and healthy donor. Co-staining of PBMCs of representative AC PH1307 and HAM/TSP 1419 and healthy donor (PBMC) was performed using antibodies against HBZ (red) and THEMIS (green) followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody and by Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibody, respectively. DIC represents the differential interference contrast image. At least 200 cells were analyzed; two representative images derived from each sample are shown. All scale bars are 5 μm.

with the prominent nuclear localization found in ATL cells, we took advantage of an IL-2-dependent HTLV-1-immortalized T cell line, designated CB-CD4/HTLV-1 established from a HAM/TSP patient (Ozden et al., 2004). Interestingly, the cell surface phenotype of CB-CD4/HTLV-1 showed that these cells express CD4, CD25, and HLA-II markers (Figure 8, upper panels) but lack the cell surface expression of CD3. Absence of CD3 expression in HTLV-1-infected cells is not unprecedented since it has been observed also in other cell lines (Raval et al., 2015). As observed in fresh PBMCs of HAM/TSP patients, HBZ was found to be localized in the cytoplasm of CB-CD4/HTLV-1 cells (Figure 9A, HBZ). Parallel staining with DRAQ5 or with vimentin confirmed the exclusive HBZ cytoplasmic localization with a similar speckle-like distribution as the one observed

in PBMCs of HAM/TSP patients (**Figure 9A**, overlay). While virtually all cells expressed HBZ, only 20% of them expressed Tax-1. Interestingly, Tax-1 was mainly localized in the cytoplasm with a dotted-like distribution (**Figure 9B**, Tax-1). Parallel staining with DRAQ5 and vimentin revealed that Tax-1 is concentrated in a specific cytoplasmic area (**Figure 9B**, overlay). Furthermore, we observed that Tax-1 distribution did not overlap with that of HBZ, suggesting that the two proteins localized in different regions of the cytoplasmic compartment (**Figure 9C**, overlay). Altogether these localization studies suggested that HBZ resided exclusively in the cytoplasm of CB-CD4/HTLV-1 cells. To further confirm the cytoplasmic localization of HBZ, nuclear and cytoplasmic fractions of CB-CD4/HTLV-1 cells were immunoprecipitated with the 4D4-F3 anti-HBZ mAb,

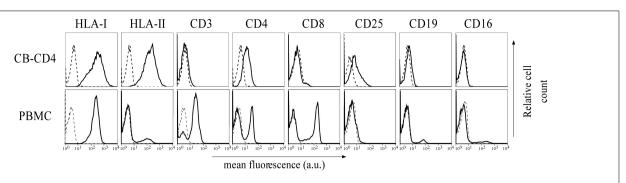


FIGURE 8 | Expression of cell surface markers in the CB-CD4/HTLV-1 cell line. The expression of HLA class I, HLA class II DR, CD3, CD4, CD8, CD25, CD19, and CD16 surface molecules on CB-CD4 HAM/TSP cells and healthy control was assessed by immunofluorescence and flow cytometry with antibodies specific for the markers indicated on the top of the panels. Results are expressed as relative number of cells (ordinate) versus the mean intensity of fluorescence in arbitrary units (abscissa). In each histogram, negative controls, obtained by staining the cells with an appropriate isotype-matched antibody, are depicted as a dashed line.

with the resulting immunoprecipitate run in SDS-PAGE and analyzed by Western blotting with the same antibody. As shown in Figure 10, HBZ protein was found only in the cytoplasmic fraction (C) of CB-CD4/HTLV-1 cells (Figure 10, IP, HBZ). Nucleoporin 98 (Nup98) and Tubulin protein expression was also analyzed (Figure 10, input) to verify the purity of nuclear (N) and cytoplasmic (C) protein extracts. To assess whether, in CB-CD4/HTLV-1 cells, HBZ stably resides in the cytoplasm or shuttles between cytoplasm and nucleus, the cells were treated with Leptomycin B (LMB), an inhibitor of CRM-1-dependent nuclear export, and analyzed by immunofluorescence and confocal microscopy. Results clearly showed that LMB treatment did not affect HBZ cytoplasmic localization in CB-CD4/HTLV-1 T cells (Figure 11A, HBZ, top panel versus bottom panel). Interestingly, cytoplasmic localization of endogenous Tax-1 in CB-CD4/HTLV-1 cells was also not modified by the presence of LMB (Figure 11A, Tax-1, upper panel versus lower panel). This finding was at variance with the results observed in the Tax-1-transfected 293T cell line in which Tax-1 was shuttling to the nucleus as assessed by a similar LMB treatment (Forlani et al., 2016a). The efficacy of LMB treatment was shown by the nuclear accumulation of p65/RelA that, in the absence of LMB treatment, was mostly localized in the cytoplasm (Figure 11B, +LMB). From these results, we conclude that in CB-CD4/HTLV-1 cells, as in HAM/TSP patients, HBZ is specifically confined to the cytoplasm and does not translocate to the nucleus.

#### **DISCUSSION**

Although many advances have been obtained on the molecular and cellular mechanisms underlining the pathogenesis of infection by HTLV-1, the first described human oncogenic retrovirus (Poiesz et al., 1980), the intimate mechanisms associated to the onset of the two major diseases associated to HTLV-1 infection, namely, acute T cell leukemia-lymphoma (ATL) and tropical spastic paraparesis (HAM/TSP), are still not fully understood. In particular, the reason why some HTLV-1-infected patients develop HAM/TSP and others progress toward the neoplastic state is largely elusive.

Two viral products, Tax-1 and HBZ, are certainly involved in the pathogenesis of ATL but most likely with distinct mechanism. Tax-1 is thought to be more important in the triggering than in the maintenance of oncogenic transformation for various actions on the homeostasis of the cell initially infected by HTLV-1, particularly in the constitutive activation of the NF-kB pathway (Grassmann et al., 2005). Indeed, Tax-1 expression is not present in 40% of leukemic patients (Takeda et al., 2004). On the other hand, HBZ is persistently present in ATL and thus it is believed to play a major role in the maintenance of a neoplastic process (Matsuoka and Jeang, 2011). This is further complicated by the observation that Tax-1 and HBZ seem to have several opposing functions on specific cellular pathways involved in the control of cellular activation and proliferation (Matsuoka and Yasunaga, 2013). Mechanisms associated with the neuroinflammatory pathology of HAM/TSP depend on chronic activation of cellular immunity and damage induced by the immune response to the nervous system (Matsuura et al., 2010; Yamano and Sato, 2012; Enose-Akahata et al., 2017). The role of Tax-1 and HBZ seems to be less understood, at least in terms of recognition by immune cells and consequent outcome of protection versus immunopathology (Suemori et al., 2009; Macnamara et al., 2010; Hilburn et al., 2011).

All these events require a precise and more defined analysis of important parameters related to these viral proteins: their temporal expression, their relative amount of expression during the various phases of infection and related pathology, and, importantly, their relative subcellular distribution during the life history of infection and evolution toward distinct pathologies.

To address these issues, we have recently undertaken studies of expression, quantification, and subcellular distribution of HBZ that have been hampered in the past by the lack of suitable reagents to detect and quantify the endogenous viral protein. We have recently demonstrated for the first time that HBZ protein, previously believed as an exclusive nuclear protein, is indeed expressed in the cytoplasm of PBMCs of HAM/TSP patients (Baratella et al., 2017b), in contrast with the predominant nuclear expression in ATL cells (Raval et al., 2015). No careful studies have been reported on the expression of HBZ protein in AC.

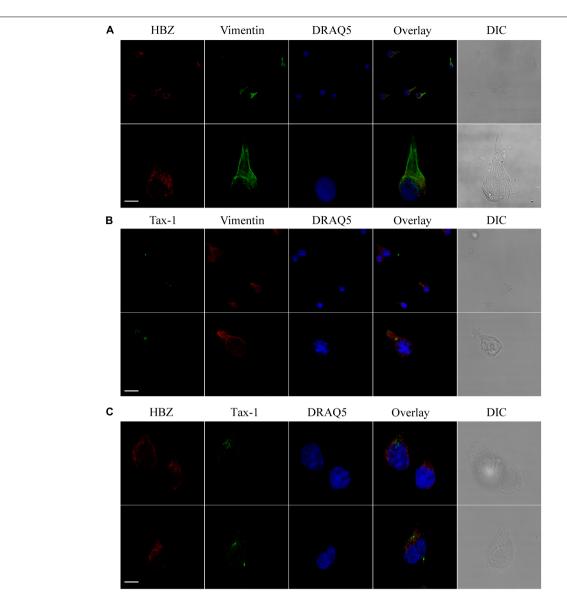


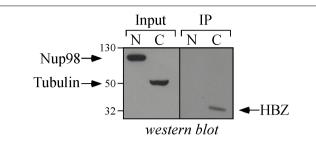
FIGURE 9 | In CB-CD4/HTLV-1 cells, HBZ and Tax-1 reside in different compartments of the cytoplasm. (A) CB-CD4/HTLV-1 cells were stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red), with DRAQ5 to detect the nucleus (blue) and with anti-vimentin rabbit polyclonal antibody followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) to detect the cytoplasmic compartment. DIC represents the differential interference contrast image. Three representative images for HBZ staining are shown. (B) CB-CD4/HTLV-1 cells were stained with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green), with DRAQ5 to detect the nucleus (blue) and with anti-vimentin rabbit polyclonal antibody followed by goat anti-rabbit IgG conjugated to Alexa Fluor 546 (red) to detect the cytoplasmic compartment. DIC represents the differential interference contrast image. Two representative images for Tax-1 staining are shown. (C) CB-CD4/HTLV-1 T cells were co-stained with the 4D4-F3 anti-HBZ mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1 antibody (red) and with the A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green) and analyzed by confocal microscopy. DRAQ5 was used to detect the nucleus. DIC represents the differential interference contrast image. Two representative images are shown. All scale bars are 5 μm.

Here, we have analyzed in detail the expression and subcellular distribution of HBZ and Tax in a cohort of AC and HAM/TSP patients. Several important findings were observed.

The confocal microscopy analysis performed on PBMCs of AC showed that HBZ protein expression could be detected in these cells, although not in all infected individuals and at a reduced number as compared to HAM/TSP. Most importantly,

we show for the first time that in AC, HBZ is localized exclusively in the cytoplasm.

Cytoplasmic HBZ appears to be distributed in dots resembling the nuclear dots found in ATL cells. Thus, in the natural history HTLV-1 infection, expression of HBZ protein is primarily confined to the cytoplasm and, depending on the differential progression toward HTLV-1-associated diseases, HBZ remains cytoplasmic in HAM/TSP patients. Importantly, the increase in



**FIGURE 10** | Biochemical assessment of the cytoplasmic localization of HBZ in CB-CD4/HTLV-1 cells. Nuclear (N) and cytoplasmic (C) protein extracts obtained from CB-CD4/HTLV-1 cells (8  $\times$   $10^6$  cells) were immunoprecipitated with an anti-HBZ 4D4-F3 mAb (IP) and analyzed for the presence of HBZ by Western blotting using the same antibody (HBZ). Ten percent of the nuclear and cytoplasmic protein extracts were analyzed for the expression of Nucleoporin 98 (NUP98) and β-tubulin (Tubulin) by Western blotting (input).

HTLV-1 PVL in these patients is paralleled by the increase in the number of HBZ-positive cells. By contrast, HBZ expression is more associated with the nucleus of ATL patients (Raval et al., 2015), strongly suggesting that the evolution of HTLV-1 infection toward the leukemic state is marked by the migration of HBZ to the nucleus. Within this context, recent studies have

reported that HBZ-transfected cell lines, including the T cell line Jurkat, may partially segregate HBZ in the cytoplasm as a result of interaction with THEMIS (Kinosada et al., 2017), a protein expressed exclusively in T cells (Brockmeyer et al., 2011; Fu et al., 2013). However, confocal microscopy analysis of PBMCs of AC and HAM/TSP patients described in this paper shows that the expression of THEMIS is not necessary for the expression and cytoplasmic localization of endogenous HBZ. Thus, whether the distinct subcellular localization of HBZ observed in ATL as compared to AC and HAM/TSP is due to an active migration of HBZ to the nucleus or to a passive translocation due to the loss or altered expression of THEMIS remains to be elucidated. This does not exclude the idea that different cytoplasmic anchoring molecules or molecular complexes may also be involved in the cytoplasmic localization of HBZ in AC and in HAM/TSP patients (see also discussion below).

In PBMCs of AC, the Tax-1 protein was expressed in a discrete number of cells and preferentially localized in the cytoplasm. Interestingly, the expression of Tax-1 was found to be largely uncoupled to that of HBZ, and in this respect, these findings were similar to those previously reported for HAM/TSP patients (Baratella et al., 2017b). The biological basis of the mutually exclusive expression of either HBZ or Tax-1 in the same cell in

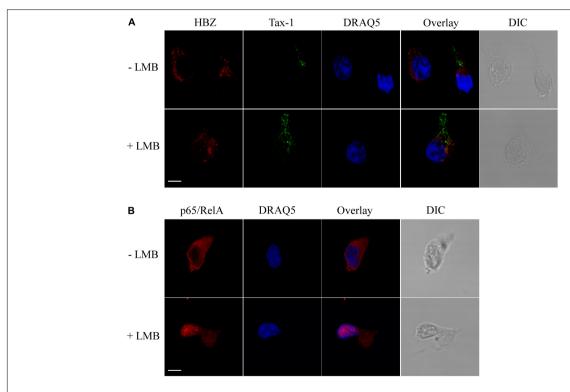


FIGURE 11 | HBZ is a resident cytoplasmic protein and does not shuttle to the nucleus in CB-CD4/HTLV-1 cells. (A) Cells were either treated (+LMB) or not treated (-LMB) with LMB, an inhibitor of nuclear export, before fixing and co-staining with the anti-HBZ 4D4-F3 mAb followed by Alexa Fluor 546-conjugated goat anti-mouse IgG1antibody (red) and A51-2 anti-Tax-1 mAb followed by Alexa Fluor 488-conjugated goat-anti-mouse IgG2a antibody (green). DRAQ5 was used to detect the nucleus (blue). One representative image is shown for each sample. (B) As control of inhibition of nuclear export by LMB, treated (+LMB) and untreated (-LMB) cells were fixed and stained with antibodies against p65/RelA followed by Alexa Fluor 546-conjugated goat-anti-rabbit secondary antibody (red) and analyzed by confocal microscopy. Nucleus was stained with DRAQ5 (blue). DIC represents the differential interference contrast image. One representative image is shown for each sample. All scale bars are 5 μm.

AC and HAM/TSP patients is still unknown and certainly will be the focus of our future investigation.

In this study, we have significantly expanded the number of HAM/TSP patients. We confirmed that HBZ is a cytoplasmic protein, and we showed for the first time that it is expressed in a higher number of PBMCs as compared to AC. This was paralleled by a similar higher number of PBMCs expressing Tax-1, and, as mentioned above, the increase in number of cells expressing HBZ and Tax-1 correlated often, but not always, with the higher PVL found in these patients. A noticeable exception was the PH2116 AC displaying a very high PVL (19.16) and only 2 and 0% of HBZ and Tax-1 positive cells, respectively. Of interest is the observation that this AC with an uncommonly high PVL was the spouse of a HAM/TSP patient and is clinically being followed for any signs and symptoms of neurological disease.

In line with our previous findings (Baratella et al., 2017a,b), additional confocal microscopy analysis of PBMCs of AC and HAM/TSP patients clearly showed that the major, if not the exclusive, HBZ+ cell subpopulation was represented by CD4+ cells. Conversely, only a very limited number of HBZ-positive cells were found in the CD8+ T cell compartment, reinforcing the idea that in HAM/TSP as well as in AC, CD8+ T cell subpopulation is not a primary target of the HTLV-1 virus.

Although CD4+/CD25+ Tregs can be infected by HTLV-1 and HAM/TSP patients have been shown to have a high number of CD4+/CD25+ Tregs with impaired function and higher HBZ mRNA levels (Araya et al., 2011; Enose-Akahata et al., 2018), the results presented here indicate that in HAM/TSP patients, HBZ protein can be detected in CD4+ T cells not displaying the classical phenotype of Treg cells. Thus, the possible uncoupling of HBZ mRNA and protein expression in CD4+CD25+ Tregs as well as in other CD4+ T cell subpopulations in both AC and HAM/TSP patients (Yamano et al., 2009; Leal et al., 2013; Araya et al., 2014; Billman et al., 2017) certainly requires further investigation.

Further studies by confocal and biochemical analysis conducted on CB-CD4/HTLV-1, a T cell line established from an HAM/TSP patient (Ozden et al., 2004), confirmed that HBZ

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protein resides exclusively in the cytoplasm. Tax-1, expressed in 20% of CB-CD4/HTLV-1 cells, also localized mostly in the cytoplasm. The CB-CD4/HTLV-1 cell line was instrumental to unambiguously show that HBZ does not shuttle between the cytoplasm and nucleus at least in a CRM1-dependent manner as assessed by LMB treatment. These data, along with previous data from our group (Baratella et al., 2017b), give support to the idea that in AC and in HAM/TSP patients, HBZ may be actively retained in the cytoplasm (Baratella et al., 2017a).

In conclusion, we believe that the data presented in this research add new relevant information for a better definition of the pathological states associated with HTLV-1 infection, particularly in relation to the distinct subcellular expression of HBZ in the different pathological contests and related pathologies. Whether HBZ cytoplasmic and nuclear localization in the natural history of HTLV-1 infection represents a marker of infection or is part of the mechanism governing the evolution toward HAM/TSP or ATL is the challenge for future investigation.

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GF, MB, and RA conceived the work. GF, MB, and AT performed the experiments. All authors wrote, revised, and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Mother-to-Child HTLV-1 Transmission: Unmet Research Needs

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Rosadas C and Taylor GP (2019) Mother-to-Child HTLV-1 Transmission: Unmet Research Needs. Front. Microbiol. 10:999. doi: 10.3389/fmicb.2019.00999 Mother-to-child transmission (MTCT) of Human T-cell lymphotropic virus type 1 (HTLV-1) causes lifelong infection. At least 5-10 million individuals worldwide are currently living with HTLV-1. Studies of regional variation are required to better understand the contribution of MTCT to the global burden of infection. Although most infected individuals remain asymptomatic ~10% develop high morbidity, high mortality disease. Infection early in life is associated with a higher risk of disease development. Adult T-cell leukemia (ATL), which is caused by HTLV-1 and has a median survival of 8 months is linked to MTCT, indeed evidence of ATL following infection as an adult is sparse. Infective dermatitis also only occurs following neonatal infection. Whilst HTLV-1-associated myelopathy (HAM) follows sexual and iatrogenic infection approximately 30% of patients presenting with HAM/TSP acquired the infection through their mothers. HAM/TSP is a disabling neurodegenerative disease that greatly impact patient's quality of life. To date there is no cure for HTLV-1 infection other than bone marrow transplantation for ATL nor any measure to prevent HTLV-1 associated diseases in an infected individual. In this context, prevention of MTCT is expected to contribute disproportionately to reducing both the incidence of HTLV-1 and the burden of HTLV-1 associated diseases. In order to successfully avoid HTLV-1 MTCT, it is important to understand all the variables involved in this route of infection. Questions remain regarding frequency and risk factors for in utero peri-partum transmission whilst little is known about the efficacy of prelabor cesarean section to reduce these infections. Understanding the contribution of peripartum infection to the burden of disease will be important to gauge the riskbenefit of interventions in this area. Few studies have examined the impact of HTLV-1 infection on fertility or pregnancy outcomes nor the susceptibility of the mother to infection during pregnancy and lactation. Whilst breast-feeding is strongly associated with transmission and avoidance of breast-feeding a proven intervention little is known about the mechanism of transmission from the breast milk to the infant and there have been no clinical trials of antiretroviral therapy (ARV) to prevent this route of transmission.

Keywords: HTLV-1, mother-to-child transmission, pregnancy, outcomes, risk, disease

#### INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) is a human retrovirus that is mostly transmitted through sexual intercourse and from mother-to-child. Early after the first identification of HTLV-1 the possibility of mother-to-child transmission (MTCT) was supported by several pieces of evidence including: (1) high incidence of HTLV-1 infection in children born to HTLV infected mothers; (2) high prevalence of infection in mothers of HTLV-1 seropositive children; (3) presence of approximately 10<sup>3</sup> cells infected with HTLV/mL in milk from carrier mothers; (4) experimental infection in animals using fresh milk cells from HTLV-1 infected women (Hino, 2011); (5) low transmission risk among bottle-fed children when comparing to breast fed; and more recently reduced incidence of infection after implementation of antenatal screening and avoidance of breastfeeding observed in Japan. Thus, this route of infection is well stablished and is responsible for the maintenance of HTLV-1 in several generations of the same family (The T and B-cell malignancy study group, 1988; Plancoulaine et al., 1998; Catalan-Soares et al., 2005; da Costa et al., 2013; Mendes et al., 2016). Moreover, transmission is known to be associated with the risk of adult T-cell leukemia (ATL), a highly aggressive and usually fatal malignancy caused by HTLV-1. Despite almost 40 years passing since the discovery of HTLV-1 many gaps in our understanding of MTCT remain. This review aims to summarize what is known, the current limitations of this knowledge and to identify the most important areas for further research.

### HTLV-1 INFECTION IN PREGNANT WOMEN

What We Need to Know About HTLV-1 Infection in Pregnant Women

- What is the prevalence of HTLV-1 infection in pregnant women in different countries/regions?
- What is the susceptibility to HTLV-1 infection during pregnancy and lactation?
- What is the impact of pregnancy on HTLV-1 infection is viral control affected?
- What is the impact of pregnancy in HTLV-1 associated diseases and *vice-versa*?
- Does HTLV-1 infection impact fertility, miscarriage rates or 3rd trimester pregnancy outcomes including birth weight and gestational age at delivery?
- What is the impact of HTLV-1 infection and/or its diagnosis on psychological health of pregnant women and parturient?

### Prevalence of HTLV-1 Infection in Pregnant Women

HTLV-1 prevalence is considered more representative of the general population than prevalence studies among blood donors due to the impact of pre-screening on donor self-selection. However, many cross-sectional studies also show a significant increase in prevalence with age, particularly in women and

most notably after age 40 (Plancoulaine et al., 1998). This is considered to result from a birth cohort effect as well as horizontal transmission (Satake et al., 2015). Therefore younger women of reproductive age are not fully representative and prevalence is likely to be underestimated in the overall population (Proietti et al., 2005). However, HTLV-1 prevalence among pregnant women is useful to indicate the regions where implementation of measures to prevent MTCT is urgent.

The published prevalence of HTLV-1 infection among pregnant women is represented in the map (Figure 1) and presented in Supplementary Table. Although some studies are not comparable, due to differences in methodologies, the overall picture shows wide variation not only between but within countries. Importantly, many regions lack any data about HTLV-1 prevalence in this population whilst, even where the prevalence has been reported, many studies are out of date and were conducted with less specific assays. To better understand the public health importance of HTLV-1 infection new, adequately powered studies examining HTLV-1 seroprevalence in most regions of the world are a priority.

### Susceptibility to HTLV-1 Infection During Pregnancy and Lactation

The various risk factors for HTLV-1 infection that have been reported in pregnant women or in women of reproductive age are listed in Figure 2 (Sanchez-Palacios et al., 2003; Alarcón et al., 2006; Moxoto et al., 2007; Dal Fabbro et al., 2008; Etenna et al., 2008; Lima and Viana, 2009; Ydy et al., 2009; Ribeiro et al., 2010). Most studies did not perform multivariate analysis to confirm the risk factors. Increasing age and age at first sexual intercourse were strongly associated with HTLV-1 infection, while history of abortion and history of transfusion presented borderline statistical significance in a multivariate analysis (Alarcón et al., 2006). These risk factors were observed in pregnant women assuming that infection occurred previously. An increase in susceptibility to HIV infection during pregnancy and lactation is reported (Thomson et al., 2018), but there are no similar studies in HTLV-1. Therefore, we do not know if pregnancy and lactation impact the susceptibility to HTLV-1 infection.

### The Impact of Pregnancy on HTLV-1 Infection Control

Few studies have examined the impact of pregnancy on HTLV-1 infection. HTLV-1 proviral load (PVL), which is until now, the main predictor of disease outcome, seems to be stable during pregnancy, but increase after delivery in a recent Japanese study of 36 women (Fuchi et al., 2018). However, HTLV-1 PVL before conception was not known. It is assumed that these women had chronic infection. There are limited data on the kinetics of HTLV-1 infection but in one detailed study in a transplant setting, PVL increased rapidly during the first few weeks and had reached steady state by 38–45 days after exposure (Cook et al., 2016). Since pregnancy represents a state of relative immunosupression it would be important to know whether the rate of viral replication and the steady state PVL differ when infection is first acquired during pregnancy, as this would

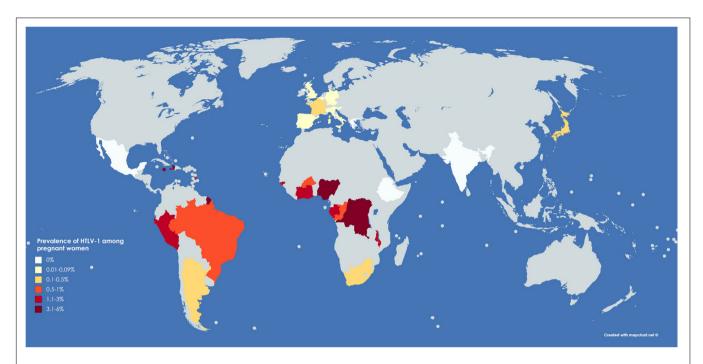


FIGURE 1 | Representative map of world prevalence of HTLV-1 in pregnant women. Original figure created with mapchart.net. Areas in pale gray are those with unknown HTLV-1 prevalence in pregnant women.



FIGURE 2 | Risk factors for HTLV-1 infection in pregnant women or women at reproductive age. Original figure created with mindthegraph.

likely impact on HTLV-1 MTCT. Whether a similar pattern of change in PVL with pregnancy is observed in different regions, in populations with different genetic background and in different environments, where for example, there is a high prevalence of infectious agents or different socio-economic conditions needs to be determined. Similarly, if PVL rises after pregnancy is this transient or sustained and do serial pregnancies matter?

### The Impact of Pregnancy on HTLV-1 Associated Diseases and *vice-versa*

There are few data on the impact of pregnancy on the progression of HTLV-1 associated diseases or vice-versa. ATL in pregnancy has been reported. The majority were acute ATL and most patients died soon after delivery (by pre labor cesarean section [PLCS]), despite treatment (Utsumi et al., 1996; Safdar et al., 2002; Amor et al., 2013). Interestingly, there is one report of chronic ATL in a pregnant woman, previous diagnosed as asymptomatic carrier (AC). In this case, chronic ATL with unfavorable prognosis (aggressive ATL) was diagnosed at 12 weeks of pregnancy. The pregnancy was terminated at 15 weeks following which the unfavorable factors (high lactate dehydrogenase (LDH) and low serum albumin) improved, with re-classification to favorable (indolent ATL) and a watchful waiting strategy was adopted (Fuchi et al., 2016). Interestingly, Fuchi et al. (2018) also identified higher frequency of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup> cells and increased sIL-2R in HTLV-1 infected women compared with un-infected women 1 month after delivery in their recent study. The optimal management of HTLV-1 infection and ATL in this setting is not known. Three cases from the United Kingdom were presented in 2017. MTCT despite PLCS occurred in the first case whilst zidovudine and raltegravir were added to maternal treatment along with neonatal zidovudine and PLCS for HTLV-1 MTCT in the subsequent cases - with no evidence of transmission at last follow-up (Cook et al., 2017).

Although no studies regarding HAM/TSP in pregnant women have been published there is clear potential for pregnancy to impact HAM, directly or indirectly. HAM/TSP

is an inflammatory disease and the severity of many diseases, particularly autoimmune, is reduced during pregnancy due to the change in Th1/Th2 balance (Mor and Cardenas, 2010). Conversely, bladder and bowels symptoms such as frequency and constipation are common in both HAM and pregnancy whilst gait can be expected to worsen due to the physiological changes in pregnancy. Neurogenic bladder which is commonly observed in HAM/TSP (Araujo, 2015), could impact urinary infection rate (Nomata et al., 1992), which, in turn, can induce preterm birth (Dautt-Leyva et al., 2018).

#### Impact of HTLV-1 Infection in Miscarriage Rates and 3rd Trimester Pregnancy Outcomes

A number of studies have explored the impact of HTLV-1 infection on pregnancy. In a study from Mato Grosso do Sul (Brazil) (Dal Fabbro et al., 2008) 6.7% (11/164) of HTLV-1 associated pregnancies resulted in first trimester miscarriage. Moreover, HTLV-1 infected pregnant women reported, more frequently [26.8% (41/153)], history of previous miscarriage than non-infected women (16%). Similar results were observed in Peru, where women with a history of abortion were more likely to be infected with HTLV-1 [prevalence of HTLV-1 infection 3.2% vs. 1.1%, Crude OR (95% CI): 3 (1.8-5.0)] (Alarcón et al., 2006). In the same study, the prevalence of HTLV-1 infection was higher among those women who presented at the hospital for abortion/miscarriage (3.5%) than for prenatal (1.4%) or postnatal care (1.7%) (Alarcón et al., 2006). As induced abortion is illegal in Peru, it is not possible to elicit any conclusion about those cases of abortions/miscarriage. Moreover, multivariate analysis was not performed to check for confounding factors such as the presence of co-infections. It is known that approximately half of all preterm deliveries are associated with histologic evidence of placental inflammation (acute chorioamnionitis (ACA) or chronic chorioamnionitis), regardless of fetus infection (Mor and Cardenas, 2010). Underweight babies, pre-term and higher numbers of hospitalization was observed among HTLV-1 infected children (n = 58) when comparing to noninfected children born from seropositive mothers (n = 42)and may raise the question if underweight babies or preterm births are risk factors for increased HTLV-1 susceptibility (Kendall et al., 2009). In a case-control study in Gabon of 45 HTLV-1 infected mothers each matched to two controls higher rates of preterm delivery (11% vs. 2.7%), complicated pregnancy (36.5% vs. 22%) and cesarean sections (8% vs. 3%) were observed in HTLV-1 infected women compared to the control group, but none of these differences were statistically significant (Ville et al., 1991). Moreover, other studies (of up to 45 HTLV-1 infected women) did not observe any impact of HTLV-1 infection on miscarriage or preterm delivery rates (Saito et al., 1990; Bittencourt et al., 2001; Montano et al., 2004). Clearly the small sample size of these studies precludes definitive conclusions to be drawn and more research regarding this topic is needed. It is also important to understand which mechanisms might be involved in the impact of this virus in pregnancy. HTLV-1 infection is known

to be associated with an immune imbalance, characterized mainly by the production of pro-inflammatory cytokines, more evident in persons with HAM/TSP (Futsch et al., 2018; Rosa et al., 2018) As pro-inflammatory mediators have been already associated with unfavorable pregnancy outcomes in HIV infection (Pfeifer and Bunders, 2016), it is plausible to assume that HTLV-1 infection may also be associated with negative pregnancy outcomes.

#### **HTLV-1** Infection and Fertility

There are limited data on the impact of HTLV-1 on fertility and on assisted reproduction. While HTLV-1 infection can be associated with erectile dysfunction in up to 55.2% men (Caskey et al., 2007; Oliveira et al., 2010; de Oliveira et al., 2017) and with sexual dysfunction in women (Lopes Martins et al., 2018), no differences in the prevalence of HTLV-1 infection according to the fertility status was observed in women from Gabon (Schrijvers et al., 1991). One study about assisted reproduction from Iran, evaluated 32 cycles from HTLV-1 positive women and when comparing to an age matched control group no differences were found among fertilization, implantations, pregnancy rate, number of transferred and cryopreserved embryos, multiple pregnancies nor in abortion rates (Torshizi et al., 2014). However, a question that is raised is: should assisted reproduction be indicated for HTLV discordant couples? Moreover, testing couples that seek assisted reproduction is also important (Epelboin, 2011). Indeed, in the United Kingdom, in accordance with European Regulations, the donors of all tissue used in therapy, including assisted reproduction are tested for HTLV-1 infection (ECDC, 2012). In Brazil, HTLV screening in germinative cells donors and patients seeking for assisted reproduction is also implemented (ANVISA, 2011).

# Impact of HTLV-1 on Psychological Health of Pregnant Women and Parturient

Another point that needs more attention is about the impact of HTLV-1 infection in the psychological health of pregnant women and parturient (Carneiro-Proietti et al., 2014). Pregnancy and birth are unique moments, when women already must face many changes. Little is known about the impact of HTLV-1 infection and/or the diagnosis on the emotional condition of mothers to be (anxiety, depression), parturient nor on the relationship among mothers/babies or with their family members. Clinical experience suggests that various factors impact on this: knowledge and perception of HTLV-1 infection as well as timing of diagnosis. Some have cited anxiety as a reason not to screen for HTLV-1 infection ante-natally referring to a single paper from Japan (UK National Screening Committee, 2017). It should, however, be noted that a national antenatal screening programme for HTLV-1 has been successfully introduced in Japan. There, pregnant women were tested during the third trimester in order to minimize their anxiety and prevent artificial abortions (Hino, 2011).

### HTLV-1 MOTHER TO CHILD TRANSMISSION (MTCT)

What we need to know about HTLV-1 mother-to-child transmission

- Does intra-uterine transmission occur? If so, why it is so rare?
- Which mechanisms are involved in the protection of intrauterine transmission?
- What factors are important in HTLV-1 MTCT?
- What is the impact, if any, of co-infections in the risk of MTCT?
- Which cells are involved with HTLV-1 transmission via oral route?
- What is the mechanism of HTLV-1 transmission across the digestive tract?
- Is the cellular component in breast milk from HTLV-1 infected women similar to healthy uninfected women over time?
- What is the role of different breast milk constituents in the efficiency of infection?
- There is a need to identify maternal neutralizing antibodies and their role in protection.

### What Is Known About Intra-uterine Transmission?

The overall MTCT rates observed in different areas are presented in **Table 1**. There is substantial evidence that ante-natal and perinatal infection are less frequent than transmission through breastfeeding, as summarized by Hino (2011). The low level of infection among babies that are formula fed is one of the most compelling evidence (**Table 2**; Ando et al., 1987, 2003, 2004; Biggar et al., 2006; Bittencourt et al., 2002; Del Mistro et al., 1994; Gotuzzo et al., 2007; Hamedi et al., 2012; Hino et al., 1985; Hisada et al., 2002; Kashiwagi et al., 2004; Kusuhara et al., 1987; Li et al., 2004; Maloney et al., 2003; Monplaisir et al., 1993; Montano et al., 2004; Nyambi et al., 1996; Oki et al., 1992; Paiva et al., 2018; Ribeiro et al., 2012; Takahashi et al., 1991; Tsuji et al., 1990; Ureta-Vidal et al., 1999; Van Tienen et al., 2012; Wiktor et al., 1997; Wiktor et al., 1993).

HTLV-1 proviral DNA was detected in 2/9 placentas from HTLV-1 positive mothers by nested PCR. HTLV-1 antigen (p19) was also identified in placental villous cells cultured from these women (Fujino et al., 1992). Others, using culture and PCR were unable to detect HTLV-1 infected cells or provirus DNA in cord blood (Hino et al., 1985; Saji et al., 1989, 1990; Tsuji et al., 1990; Nyambi et al., 1996) nor in amniotic fluid (Nyambi et al., 1996). Where identification of HTLV-1 proviral DNA in cord blood was reported (Saito et al., 1990), there was no clear association with new-born infection. In a study from Japan, none of seven children with positive HTLV-1 DNA in cord blood seroconverted while nine that seroconverted did not have HTLV-1 DNA in cord blood (Kawase et al., 1992; Hino et al., 1997). In the other hand, at least two cases of bottle-fed infants, born by PLCS, infected by MTCT were already reported (Saito et al., 1990; Cook et al., 2017). While one baby was born from a mother with ATL (Cook et al., 2017), the other one was Rh positive, born from Rh negative mother with indirect Coombs positive test after 24th weeks of pregnancy (Saito et al., 1990). These observations may indicate transplacental infection. Due to these mixed findings, the risk of intra-uterine transmission remains uncertain (Percher et al., 2016). Are transmissions in exclusively formula-fed infants due to infection at the time of delivery rather than earlier and, if so, what is the route of infection and how can this be prevented?

### Protection Against Intra-Uterine Infection

Little is known about the possible mechanisms associated with protection against intra-uterine infection. Apoptosis of infected cells may contribute to control of HTLV-1 infection. Placentas from HTLV-1 infected women presented a higher number of apoptosis-positive cells than those from non-infected women (Fujino et al., 1999). Trophoblasts were infected by HTLV-1 when co-cultured with MT-2 cells resulting in trophoblast apoptosis (Fujino et al., 1999). In another in vitro study, malignantly transformed trophoblast cells (choriocarcinoma) were infected by HTLV-1, however, the infection was essentially non-productive, as viral protein expression could not be detected (Liu et al., 1995). Interestingly, syncytiotrophoblast coinfection with HTLV-1 and human cytomegalovirus, resulted in a productive infection of both virus (Tóth et al., 1995). Similar results were observed with Epstein-Barrar virus coculture (Tóth et al., 1997), raising the question about the role of co-infections in the risk of HTLV-1 MTCT. Women co-infected

TABLE 1 | Rate of HTLV-1 mother-to-child transmission.

References	Country	HTLV-1 mother-to-child transmission rate (%)		
Paiva et al., 2018	Brazil	14.2		
Ureta-Vidal et al., 1999	French Guyana	9.7		
Nyambi et al., 1996	Gabon	17.5		
Del Mistro et al., 1994	Gambia	22		
Van Tienen et al., 2012	Guinea-Bissau	25		
Hamedi et al., 2012	Iran	16.6		
Biggar et al., 2006	Jamaica	17		
Barcellos et al., 2005	Jamaica	22		
Li et al., 2004	Jamaica	22		
Maloney et al., 2003	Jamaica	15.2		
Hisada et al., 2002	Jamaica	18		
Wiktor et al., 1997	Jamaica	18		
Wiktor et al., 1993	Jamaica	23		
Kashiwagi et al., 2004	Japan	16		
Kashiwagi et al., 2004	Japan	3.9		
Takahashi et al., 1991	Japan	8.5		
Tsuji et al., 1990	Japan	21		
Kusuhara et al., 1987	Japan	15.4		
Ando et al., 1987	Japan	46		
Hino et al., 1985	Japan	17		
Monplaisir et al., 1993	Martinique	27		
Gotuzzo et al., 2007	Peru	19		
Montano et al., 2004	Peru	18		

TABLE 2 | Rate of HTLV-1 mother-to-child transmission according to the duration of breastfeeding.

References	Country	HTLV-1 MTCT rate							
		No Breastfeeding	Short-term breastfeeding	Long-term breastfeeding	Freeze-thawed milk				
Paiva et al., 2018	Brazil		4.8%( < 12 m)	23.8%( > 12 m)					
Ureta-Vidal et al., 1999	French Guyana	2.5% (0-3 m)	5.9%(4-6m)	7%(7 – 9 <i>m</i> )					
				10.3%(10 – 12 <i>m</i> )					
				12.9%( > 12 <i>m</i> )					
Wiktor et al., 1997	Jamaica		9%( < 6 m)	32%( > 6 m)					
Wiktor et al., 1993	Jamaica		7%(<6m)	21%( > 6 m)					
Hino, 2011	Japan	2.5%	7.4%(<6m)	20.3%( > 6 m)					
Ando et al., 2004	Japan				0%				
Ando et al., 2003	Japan				4.60%				
Hino et al., 1996	Japan		6%(<6m)	13.7%(>6m)					
				15.7%( > 12 <i>m</i> )					
Oki et al., 1992	Japan		4.5%(<7 m)	14%( > 7 m)					
Oki et al., 1992	Japan	5.6%	3.8%(<7 m)	25%(>7 m)					
Takahashi et al., 1991	Japan	5.7%	4.4%(<6m)	14.4%(>7 m)					

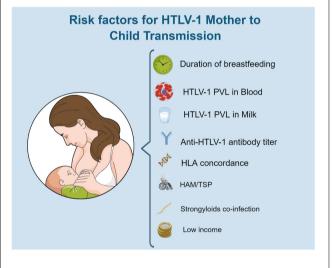
m, months.

with Strongyloides showed higher HTLV-1 transmission rates in breast-fed children when compared to uninfected women (Gotuzzo et al., 2007).

Maternal antibodies may also play a role in protection of infection. Several studies identified anti-HTLV-1 antibodies in cord blood (CB) cells from HTLV-1 carriers. Notably none of them identified the presence of anti-HTLV IgM in cord blood, which is often considered a marker of intrauterine infection (Tsuji et al., 1990; Hino, 2011). The presence of specific IgG, that crosses the placenta, was detect in virtually all tested CB samples (Saji et al., 1989; Tsuji et al., 1990). The strong correlation of CB anti-HTLV-1 IgG with maternal antibodies (coefficient of correlation = 0.85) and the invariable decrease at a rate of 1/10 every 2 months, indicates that they were passively transferred (Tsuji et al., 1990). Importantly, HTLV-1 seropositive cord blood plasma inhibits in vitro infection of neonatal lymphocyte co-cultured with breast milk cells of HTLV-1 positive mothers, pointing to the protective role of passively transferred maternal antibody (Takahashi et al., 1991).

#### Risk Factors for HTLV-1 MTCT

Several factors are reported to influence the HTLV-1 MTCT rate (Figure 3; Nyambi et al., 1996; Ureta-Vidal et al., 1999; Hisada et al., 2002; Carles et al., 2004; Li et al., 2004; Gotuzzo et al., 2007; Kendall et al., 2009; Paiva et al., 2018). Maternal risk factors include HTLV-1 PVL in PBMCs and in breast milk (Yoshinaga et al., 1995). As patients with HTLV-1 associated diseases usually have high PVL, it seems plausible that they are at a high risk of transmitting HTLV to their offspring. In a study conducted in Peru, while 6% of the offspring of ACs were infected, the transmission rates were 19 and 31% among HAM/TSP women and those co-infected with *Strongyloides stercoralis*, respectively, even with similar duration of breastfeeding (Gotuzzo et al., 2007). This raised the hypothesis that women who are predisposed to



**FIGURE 3** | Risk factors for HTLV-1 mother to child transmission. Original figure created with mindthegraph.

HAM/TSP or strongyloidiasis are the same women who transmit the infection to their children (Gotuzzo et al., 2007).

#### **Breast Milk Cells and Transmission**

The cellular mechanism of HTLV transmission through breastfeeding is not well stablished. Breast luminal epithelial cells are permissive to HTLV-1 infection, but their role and importance as a HTLV-1 reservoir *in vivo* is not clear. *In vitro*, these infected cells have altered morphology and increased ability to overcome senescence compared to non-infected cells. They can also infect epithelial cells of milk and intestinal origin as well as leukocytes (from blood and milk). Moreover, infected breast luminal epithelial cells can activate primary T cells, which is important to enhance HTLV-1 infection (Southern and Southern,

1998). Breast milk macrophages (BrMM $\phi$ ) can also be infected with HTLV-1 *in vitro*. Infected cells had provirus integration in their genome, expressed high levels of intracellular HTLV-1 p24 and produced more extracellular HTLV-1 p19 than MT-2 cells. Moreover, HTLV-infected BrMM $\phi$  maintained phagocytic activity to uptake and process foreign antigen and the ability to produce co-stimulatory molecules (CD80, CD86, CD40). In the other hand, they did not express a complete DC phenotype (lack of expression of CD14 and CD11b). The transmissibility of HTLV-1 to T cells by infected BrMM $\phi$  was more efficient than from MT-2 cells. Acid medium did not alter the capacity of HTLV-1 transmission by these cells, indicating that they can transmit HTLV-1 to other cells even after exposure to acid (Takeuchi et al., 2010).

### **Mechanism of Transmission Across the Gastro-Intestinal Tract**

It has been proposed that a baby can ingest a total of 10<sup>8</sup> HTLV-1 infected cells before weaning (Yamanouchi et al., 1985). Postnatal HTLV-1 infection must involve the migration of HTLV-1 infected cells and/or cell-free virions across the epithelium of the oro-pharynx or gastro-intestinal tract. Different mechanisms have been proposed. The infected cells could: (1) cross a disrupted epithelium; (2) cross intact epithelium (as seen with HIV infected cells); (3) attach to epithelial cells and transmit HTLV by cellto-cell contact to epithelial cells; (4) produce cell-free HTLV-1 virions that cross the epithelium by transcytosis (Pique and Jones, 2012). In vitro studies have demonstrated that infected lymphocytes do not alter epithelial barrier integrity, in contrast to what is observed in the blood brain barrier. Moreover, enterocytic cell lines are not permissive for HTLV-1. The same study demonstrated that infected lymphocytes have a lower passage rate through this barrier than non-infected lymphocytes but that HTLV-1 virions can cross the tight epithelial barrier by transcytosis. After transcytosis, the virions were able to infect underlying dendritic cells (Martin-Latil et al., 2012). The importance of a viral synapse, demonstrated ex vivo, has been postulated for lymphocyte - lymphocyte transmission (Igakura et al., 2003). This allows targeted production of HTLV-1 virions at the point of cell-cell contact and explains the lack of cell-free virions in plasma (Demontis et al., 2015). Whether this occurs in MTCT is unknown.

### Maternal Proviral Load and the Risk of MTCT

Maternal HTLV-1 PVL is also a risk factor for HTLV-1 transmission (Ureta-Vidal et al., 1999; Hisada et al., 2002; Paiva et al., 2018). Hisada et al. considered the risk of transmission negligible when maternal PVL is lower than 0.1%. The probability of transmission increases significantly when PVL is higher than 3%. PVL in milk, which correlates with PVL in maternal PBMC (Li et al., 2004), is another risk factor for HTLV-1 transmission. PVL in breast milk was significantly higher among those women who transmitted HTLV to their children (1.3% vs. 0.18%) irrespective of duration of breastfeeding (Li et al., 2004). It has been reported that the number of HTLV-1 infected cells in

breast milk is lower than in peripheral blood (Li et al., 2004), however, while the PVL in blood is usually evaluated in PBMCs, lymphocytes may not be the main cellular population observed in breast milk. In fact, when evaluating the lymphocyte population the amount of HTLV-1 infected cells in blood and breast milk is similar (Yoshinaga et al., 1995). Therefore, an alteration in the cellular composition of breast milk may influence the transmission of HTLV. In this context, conditions like mastitis may affect the risk of HTLV-1 transmission (Barcellos et al., 2005; Hisada et al., 2005), as observed in HIV infection (Semba et al., 1999). Unfortunately, there are no data on this nor on the dynamics of HTLV PVL in milk over time.

#### Duration of Breastfeeding as an Important Risk Factor for MTCT

Duration of breastfeeding is one of the most important risk factors associated with HTLV-1 transmission. While short-term breastfeeding (<6 months) is associated with low transmission rates, breastfeeding for longer periods increase the risk of HTLV-1 transmission (**Table 2**; Takahashi et al., 1991; Oki et al., 1992; Wiktor et al., 1993, 1997; Ureta-Vidal et al., 1999; Ando et al., 2003, 2004; Paiva et al., 2018). This can be associated with different factors, such as: an increased cumulative risk of exposure to HTLV-1 present in breast milk and decrease of maternal neutralizing antibody in infants over time.

Virtually all babies born from HTLV-1 infected mothers have anti-HTLV-1 antibody at birth. These antibodies decrease exponentially in the first 3-6 months and most babies are seronegative at 6-9 months. By 12 months anti-HTLV-1 maternal antibodies are no longer detected in almost all (98.4%) of children (including those who later become infected through late breastfeeding). Among those who were infected, seroconversion occurred 6-24 months after birth with only IgG and not IgM detectable (Takahashi et al., 1991). Therefore, the risk of HTLV-1 infection increases as the duration of breastfeeding increases and the quantity of transplacental maternal antibodies decreases. These antibodies may act in conjunction with the innate immune system. Antibody dependent cellular cytotoxicity (ADCC) can control HTLV-1 infection in vitro in a mechanism that seems to involve NK cells (Biggar et al., 2006). Anti-HTLV-1 antibodies were not detected in 60 breast milk samples, diluted 1:10, from infected HTLV-1 mothers (Kinoshita et al., 1984, 1985, 1987).

### Breast Milk Constituents and It Influence in HTLV Transmission

Milk constituents may also interfere in HTLV-1 transmission. Lactoferrin, for example, is able to facilitate HTLV-1 replication in lymphocytes derived from ACs. It also improves HTLV transmission to CB lymphocytes. This protein can transactivate the long terminal region (LTR) promoter and, interestingly, this activation is more prominent in CB mononuclear cells than in PBMCs. In the other hand, the same protein suppressed viral entry, although to a lesser degree than was observed for HIV (Moriuchi and Moriuchi, 2001). Moriuchi and colleagues also demonstrated that prostaglandin E 2 (PGE2) and transforming growth factor  $\beta$  (TGF- $\beta$ ), that are abundant in breast milk, can

transactivate LTR of HTLV-1, facilitating HTLV-1 replication of infected lymphocytes derived from ACs. Moreover, *in vitro*, both TGF- $\beta$  and PGE2 were able to enhance HTLV-1 transmission to CB lymphocytes (Moriuchi et al., 2001; Moriuchi and Moriuchi, 2002). HTLV-1 tax protein was able to stimulate PGE2 expression in PBMC and CB mononuclear cells (Moriuchi et al., 2001), while TGF- $\beta$  induced biding of HTLV-1 virions and expression of GLUT1 (HTLV-1 receptor) in CD4+ T lymphocyte (Jones et al., 2005).

### Other Maternal Factors That Can Influence HTLV-1 MTCT Risk

Maternal anti-HTLV-1 antibody titer has also been associated with MTCT risk. However, the association between antibody titer and risk of HTLV-1 transmission may be related to the strong correlation observed with antibody titer and PVL (Li et al., 2004). This correlation is also observed in HTLV-1 carriers, despite pregnancy (Mueller et al., 1996).

When PBMC and BMMC of HTLV-1 infected mothers are cultured they produce HTLV-1 antigen positive cells. Interestingly, 27.9% can be considered high HTLV-1 antigen producing mothers, while 72.1% are considered low antigen producing mothers. The transmission rate of HTLV-1 was 37.5% among high and 3.2% for low antigen producing mothers, independent of duration of breastfeeding. While the production observed was parallel in PBMC and BMMC from the same patient, it did not correlate with maternal anti-HTLV-1 antibody titers, indicating that the cytotoxic T cell response could be more important than humoral response to control HTLV-1 load and, consequently, vertical transmission (Yoshinaga et al., 1995).

Human Leukocyte Antigen (HLA) concordance between mothers and child is another recognized risk factor for HTLV-1 transmission. In Jamaica, HTLV-1 transmission risk increased (1.75-fold) with each increase in concordance in HLA class I types (Biggar et al., 2006). Maternal infected cells present in breastmilk seem to play an important role in vertical transmission. If these cells are HLA concordant, they are not recognized as foreign by the infant immune system, and therefore they could persist. Self HLA can also activate NK-cell inhibitor receptors and suppress the immune response. Moreover, HLA concordance can enhance cell-to-cell interaction improving HTLV-1 transmission between cells (Biggar et al., 2006).

Understanding the persistence of HTLV-1 infected maternal lymphocytes in the neonates, whether through the *in utero* transplacental or the post-partum gastro-intestinal transepithelial route will be important for the design of interventions, particularly post-exposure antiretroviral prophylaxis.

### NEONATAL INFECTION AND THE RISK OF DISEASE DEVELOPMENT

What we need to know about neonatal infection

 What are the consequences of neonatal infection in early life as well as in adult life?

- Are these consequences different if infection occurred in utero?
- What is the relative contribution of HTLV-1 MTCT to the global burden of HTLV-1 associated disease?
- How and when should HTLV-1 infection be diagnosed in children?

### Consequences of Neonatal Infection in Early Life as Well as in Adult Life

Orally infected young rats present higher PVL in spleen cells when comparing to intraperitoneal infection. In this animal model, oral infection was associated with impaired HTLV-1 specific cellular immune response (evaluated by IFN-y production and HTLV-1 specific cell proliferation response) and no production of HTLV-1 specific antibodies (Hasegawa et al., 2003). Therefore, oral infection, as occurs mainly in MTCT, may, through unknown mechanism, be associated with low immune response and high PVL which are both associated with risk of disease development (Kannagi et al., 2004). Ueno et al. (2012) reported that adult ACs infected by MTCT had higher PVL (2.41 copies of HTLV-1 pX gene/100 PBMC) than those infected by sexual intercourse (0.34 copies of HTLV-1 pX gene/100 PBMC), however the time from infection was not evaluated in this study (Ueno et al., 2012). This supports the hypothesis that vertical transmission can be associated with high risk of HTLV-1 associated disease onset, which could contribute to explain some high prevalence of disease among certain family members (Pombo-De-Oliveira et al., 2001; da Silva et al., 2013; Alvarez et al., 2016; Mendes et al., 2016). Exposure to HTLV-1 at the beginning of life has been associated with a higher risk of ATL (Hisada et al., 1998, 2001; Kannagi et al., 2004). HAM/TSP development can also occur following vertical transmission (Yoshida et al., 1993; Araújo et al., 2002; Kendall et al., 2009; Oliveira et al., 2018; Varandas et al., 2018).

Data on the impact of HTLV-1 on child development are scarce. HTLV-1 neonatal infection does not seem to impact the early children development, as no difference in reaching the development milestones was detected in a total of 68 HTLV-1 infected compared with 80 uninfected Peruvian children born to HTLV-1 infected mothers (Montano et al., 2004; Kendall et al., 2009). As the risk of HTLV-1 infection during short term breastfeeding is very low, it is possible that most children infected by vertical transmission are not infected at the time of these milestone evaluations.

### What Is the Impact of HTLV-1 on Routine Blood Parameters in Children?

Maloney et al. (2003) evaluated laboratorial parameters of 28 HTLV-1 infected and 280 uninfected children from Jamaica. Although the mean hemoglobin level was similar between these two groups, children with HTLV-1 seemed to have a higher rate of severe anemia (RR = 2.5/CI = 0.8–7.9) (Maloney et al., 2003). HTLV-1 seropositive children with severe anemia had higher HTLV PVL (9.6 copies/100 PBMC) compared to those without anemia (3.4 copies/100 PBMC). Social economic status may partially account for this association, since following

adjustment for maternal income, this association did not achieve statistical significance (p=0.06). The anemia was microcytic hypochromic, indicative of iron deficiency, which can also result from parasitic diseases and malnutrition. Both of which can be linked with poor socioeconomic status (Maloney et al., 2004). The plasma concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-1a, IL-4, IL-6, and IL-10 were not associated with the development of severe anemia in those children, but this could be due to the small sample size (Maloney et al., 2004). Interestingly, HTLV-1 infected children with infective dermatitis were anemic when compared to an age matched control group with atopic eczema (La Grenade et al., 1998).

HTLV-1 infected children in Jamaica also present higher rates of HLA-DR – positive CD4 T cells when compared to non-infected children (1.9% vs. 1.12%, p < 0.001), which strongly correlated with time since seroconversion (r = 0.74, p = 0.009) and the percentage of CD25CD4 T cells (r = 0.7, p = 0.02) (Maloney et al., 1995). Children with infective dermatitis associated with HTLV-1 (IDH) also presented higher T cell activation markers expression (HLA-DR) when compared to children with atopic eczema. They also have high percentage of CD4 and CD8 cells (La Grenade et al., 1998).

### Infective Dermatitis, ATL and HAM/TSP in Children

Infective dermatitis associated with HTLV-1 (IDH) is also observed in children infected early in infancy, and is associated with a high PVL and increased risk for the development of other diseases such as ATL and HAM/TSP (LaGrenade et al., 1990; Bittencourt et al., 2006; Hlela et al., 2013). Varandas et al. (2018) reported that 54% of patients with IDH developed HAM/TSP (Varandas et al., 2018).

Children infected with HTLV-1 report more frequently leg weakness, lumbar pain, paraesthesia/dysesthesia, urinary incontinence and constipation than those uninfected children. Clonus and lower extremity hyperreflexia were associated with HTLV-1 infection, Therefore, it is important to perform neurological evaluation of HTLV-1 infected children, once it can detect otherwise-unrecognized neurological disease (Kendall et al., 2009). HTLV-1 infected children also presented higher rates of eczema, seborrheic dermatitis, and persistent hyperreflexia of lower limbs when comparing to non-infected children in Jamaica (Maloney et al., 2003).

There are many reports of early onset of HTLV-1 associated diseases, such as HAM/TSP and ATL (Araújo et al., 2002; Bittencourt et al., 2006; Varandas et al., 2018). A review about this topic was published recently, and the authors found 27 published cases of juvenile HAM/TSP and 31 cases of ATL in infancy (Oliveira et al., 2018). Rapid progression of HAM/TSP in children has been reported (Oliveira et al., 2018; Varandas et al., 2018) but whether the rate of progression in younger patients is faster than in adults is uncertain. Among adult patients, early age at onset of clinical signs may be associated with rapid progression of HAM/TSP (Kuroda et al., 1991).

Juvenile HAM/TSP occurs predominantly in females and is frequently associated with IDH (history or co-incidence in up

to 55.6%) (Oliveira et al., 2018). In Japan, in 10% of HAM/TSP patients, disease onset was before 15 years of age (Yoshida et al., 1993). Short stature and association with pseudoparathyroidism have been reported in HAM/TSP children (Yoshida et al., 1993; Araújo et al., 2002).

A descriptive study of 5 patients with juvenile HAM/TSP showed normal development among all patients, who reached the development milestone at normal age (Araújo et al., 2002). However, in a Japanese report, slight delay in psychomotor development was observed in juvenile HAM/TSP cases (Yoshida et al., 1993). The most common complaint at the beginning of clinical presentation of the five cases reported in Brazil was the inability to run as fast as children of the same age and/or pain in the legs (Araújo et al., 2002).

ATL usually occurs in adults (Iwanaga et al., 2012; Satake et al., 2015; Nosaka et al., 2017) and the onset of ATL in infants is rarely reported. Genetic mutations in protein-coding genes associated with cell cycle regulation have already been implicated as a possible predisposing factor for ATL outcome in children (Pombo-de-Oliveira et al., 2002). The small number of available samples preclude a definitive conclusion. Among those published cases compiled by Oliveira et al. (2018), 16.1% of pediatric ATL were associate with IDH, while 6.4% also had HAM/TSP. As seen in adults, the clinical presentations in children vary, with distinct prognosis. Acute, chronic and smoldering ATL have all been reported in infants. Interestingly, most cases were reported in Latin-American countries (Oliveira et al., 2018). Despite the severity of the disease, there is a paucity of information on this subject and HTLV-1 infection should be also investigated in oncologic pediatric patients.

# Are the Consequences of HTLV-1 Infection Different If the Transmission Occurred *in utero*?

There are no data on whether *in utero* infection alters HTLV-1 infection outcomes in the short or long term.

## Relative Contribution of HTLV-1 MTCT to the Global Burden of HTLV-1 Associated Disease

Until now, there is no cure to the lifelong HTLV-1 infection nor any preventive measure to avoid the onset of HTLV-1 associated diseases in patients living with HTLV-1. Thus, infection prevention is extremely important. While almost all ATL and IDH cases are associated with maternal transmission (The T and B-cell malignancy study group, 1988), MTCT can account for around 30% of HAM/TSP cases (Bartholomew et al., 1998). As early age at first sexual intercourse can increase the risk of HAM/TSP (Krämer et al., 1995), we can postulate that the infection early in life may also be linked to a higher risk of HAM/TSP. Indeed, Kaplan et al. (1990) estimated a higher cumulative lifetime risk of HAM/TSP among those individuals infected at birth (0.0023) when compared with those infected later in life (0.0018, 0.0014, and 0.008 if infected at ages 20, 30, and 50 years, respectively). These are considered conservative estimates applicable to Japan and may differ from other regions

(Kaplan et al., 1990). The lifetime risk of HAM/TSP is higher in other regions, for example, 1.8% in Jamaica and Trinidad (Maloney et al., 1998) and that is a report of lifetime risk of up to 9% in Brazil (Tanajura et al., 2015). Moreover, in a mathematical model simulating the dynamic changes of HTLV-1 carries rates, the prevention of MTCT was shown to be much more effective to reduce the rates of HTLV-1 infected carriers than the prevention of horizontal transmission (Oguma, 1990). Thus, as almost all ATL and infective dermatitis cases and up to 30% of HAM/TSP cases are associated with MTCT we can conclude that MTCT contributes disproportionately more to the global burden of HTLV-1 associated diseases when comparing to others transmission routes. Therefore, antenatal screening and avoidance of breastfeeding would not only result in a decrease in HTLV-1 incidence, but most importantly a sharp decrease in the onset of HTLV-1 associated diseases. Thus, it can be considered the single best approach to reduce disease.

### How and When Should HTLV-1 Infection Be Diagnosed in Children?

The diagnosis of HTLV-1 neonatal infection can be challenging. Passively transmitted antibodies can be detected in new-borns from HTLV-1 positive mothers. These antibodies decrease gradually and at 12 months the great majority of children do not have anti-HTLV-1 maternal antibodies anymore. In the other hand, seroconversion of infected children can occur from months to more than 2 years after birth. No seroconversion after 3 years of age was reported (Kusuhara et al., 1987; Nyambi et al., 1996). Molecular methods such as PCR may be useful for the diagnosis of neonatal infection (Bittencourt et al., 2006), however, the diagnosis of HTLV-infection before 3 years of age usually does not impact the management of HTLV-1 infection in children, although this may vary by region. Moreover, there is no commercially available PCR for the diagnosis of HTLV-1 infection and the sensitivity of PCR in children should be assessed in further studies. Two distinct approaches are possible: serological - testing children born to non-breasting HTLV-1 infected mothers once at 15 months or if breastfeeding 3 months after completed weaning; molecular - which will allow early diagnosis but will necessitate multiple sampling. At present there is no consensus and research into parental attitudes and their willingness (to be screened, to use formula feeding, short-term breastfeeding, how often the children should be tested, at each age, for example) will be important to inform any guidelines.

### PROPHYLACTIC MEASURES TO AVOID HTLV-1 VERTICAL TRANSMISSION

What we need to know about prophylactic measures

- Does pregnancy impair the detection of HTLV-1 infection?
- Should we recommend avoidance of breastfeeding to all HTLV-1 seropositive women?
- Cost-effectiveness analysis of HTLV-1 antenatal screening in different scenarios

- Should short-term breast feeding, and freeze and thawed milk be recommended as a prophylactic measure to MTCT?
- Safety and efficacy of antiretroviral therapy (ARV) as a prophylactic method for MTCT
- What is the role of neutralizing antibodies in the prevention of MTCT?
- What is the impact, if any, of pre-labor elective cesarean section in the prevention of MTCT?
- Approaches to a successful antenatal screening program: education of general population, doctors, pregnant women

#### Detection of HTLV-1 Infection in Pregnancy

In order to consider any measure to avoid MTCT, it is essential to acknowledge HTLV infection in pregnant women. However, HTLV-1 antenatal screening is only mandatory in Japan and part of clinical practice in other areas, such as French Guiana and some Brazilian States, as Bahia.

In general population serological tests, such as ELISA, are widely recommended as a screening test, followed by confirmatory assay (usually western blot or PCR). More recently, chemiluminescent microparticle immunoassay (CMIA), electrochemiluminescent assays (ECLIA), and line immunoassays (LIA) became available for HTLV-1 diagnosis. Such tests have proven robust for the detection of other blood borne infections in the ante-natal population without loss of sensitivity. However, the National Screening Committee in the United Kingdom have cited lack of evidence that current HTLV assays detect this infection in pregnant women as one reason not to recommend the addition of HTLV-1 testing to the existing national antenatal screening programme (UK National Screening Committee, 2017).

Therefore, the questions which arise are how we should test pregnant women and when? The use of pooled samples to reduce the costs of antenatal screening can be considered (Ramos et al., 2011), but this may affect the sensitivity of the screening test (Andersson et al., 2001). Dried blood spots may also present advantages when compared to venous blood. Dried blood spots on filter paper showed 100% sensitivity, specificity and diagnostic accuracy when compared to venous blood sample (Boa-Sorte et al., 2014). In a study of almost 120,000 pregnant women donating cord blood Glasser et al. (2013) detected a total of 545 reactive samples but some degree of discordance with two screening assays.

Several studies have used different techniques in order to diagnosis HTLV-1 infection during pregnancy (Supplementary Table). Tsuji et al. (1990) did not observed significant difference in the prevalence among pregnant women and age matched blood donors (PA and IF), although a little higher in the first group (3.9% vs. 1.6%) (Tsuji et al., 1990). Beside this, the higher prevalence among pregnant women, when compared to blood donors (Taylor and The Htlv European Research Network (HERN), 1999; Taylor et al., 2001) argues against any lack of sensitivity. On the other hand, the necessity of confirmatory tests for HTLV-1 diagnosis is evident, regardless of which population is being evaluated.

In a scenario of limited resources and many demands in public health, cost-effectiveness analysis is required to guide policy makers. In Nagasaki (Japan), in the late 80's, with 4% prevalence, the cost for detecting a HTLV-1 infected person, for preventing an infection and preventing a case of ATL was 50,000, 250,000, and 5,000,000 (JYE), respectively. At the time of the national screening program implementation in Japan (2010), considering the prevalence of 0.1% the estimated cost was 40-fold higher, according to Hino (2011). A recent study demonstrated that antenatal screening can be cost-effective in United Kingdom (Malik and Taylor, 2018). It is important to evaluate the cost-effectiveness in different scenarios.

Even if antenatal screening program is implemented in a country, success will depend on many factors including education of obstetrics, pediatrics, and general community (Hino, 2011). The majority of HTLV-1 seropositive women affirmed that they had not heard about HTLV-1, even when 72.5% were aware that they had a blood virus and only 10% of pregnant women were aware of other modes of transmission (Oni et al., 2006). The lack of information may influence the mothers to keep breastfeeding, in spite of medical recommendation, in order to maintain confidential their carrier status from family members and community (Hino, 2011). Even in high prevalence countries, such as Brazil, many doctors are not aware of HTLV-1 infection (Zihlmann et al., 2012). Many do not have any experience of ATL and believe that it is treatable and rare, which influences in their commitment to recommend against breastfeeding for HTLV-1 seropositive mothers (Hino, 2011).

#### **Avoidance of Breastfeeding**

The avoidance of breastfeeding has conclusively been shown to be an efficient measure to prevent post-partum HTLV-1 MTCT (Ribeiro et al., 2012; Leal et al., 2015) and is the key component of the Japanese HTLV antenatal screening programme (Nishijima et al., 2019). However, the risks and benefits of formula-feeding need to be evaluated in each setting. In resource-poor settings, where alternatives to breast milk are limited and the prevalence of infectious diseases are high, poor infant outcomes related to gastroenteritis, chest infections etc. may outweigh the benefit of avoiding HTLV-1 infection. Therefore, the recommendation of avoidance of breastfeeding should take in account the conditions of the parturient and their access to methods other than breastfeeding which need to be safe, affordable, accessible, acceptable, and sustainable (Hisada et al., 2005; van Tienen et al., 2012).

In this context, it is also important to consider if the presence of risk factors should be taken into account to establish who should avoid breastfeeding. For example, the PVL in mother's blood or in breast milk, could be considered to guide this recommendation? The availability of adequate substitutes of breastmilk should also be considered (Hisada et al., 2005). Finally, if breast-feeding is considered the best option, can this be made safer for the infant?

Therefore, it is necessary to discuss whether the benefits of breastfeeding will outweigh or not the risk of HTLV-1 infection, and if this is applicable to all scenarios. This remains one of the main questions regarding HTLV-1 MTCT.

#### **Short-Term Breastfeeding**

Early weaning is associated with low risk of HTLV-1 transmission (**Table 2**) and therefore, can be recommended to reduce the transmission, mainly in those situation when there is no or limited access to alternatives for feeding the infants (Hisada et al., 2005).

In fact, Takahashi et al. (1991) showed that the risk of infection by short-term breastfeeding is lower (4.4%) than long term breastfeeding (14.4%) (Takahashi et al., 1991). Similar results were reported by Hino et al. (1996), however, they were reluctant to affirm that short-term breastfeeding is as safe as formula-feeding. Indeed, in a large study from Japan, a higher incidence of HTLV-1 infection among short-term breastfeeding group (7.5%) than in bottle-fed infants was reported (2.5%) (Hino, 2011). Since 2016, Japan recommends only formula feed for all HTLV-1 seropositive mothers. Short-term breastfeeding and freeze-thawing breast milk are not recommended anymore (Nishijima et al., 2019). If short-term breastfeeding is adopted, how long should be the breastfeeding? The advantages and risks of an early weaning at, for example, 3 months and 6 months should be evaluated. Moreover, some mothers may have difficulties to respect early weaning and may breastfeed for longer periods than recommended, which can increase the transmission risk. Thus, this should also be considered when recommending early weaning.

#### **Using Freeze-Thawed Breastmilk**

The process of freezing and subsequently thawing the breast milk results in morphological changes and loss of function of cells present in milk (Ando et al., 1989). Babies fed with breastmilk from infected mothers submitted to frozen and thawed process were not infected (Ando et al., 1989). However, in this study, the duration of breastfeeding was not specified. As this process can be laborious for new mothers, it could result in shortterm breastfeeding, which could also contribute to the low transmission rate. In fact, in the study of Ando et al. (2004) none of 54 babies who were fed with freeze-thawed breastmilk for a short period were infected even after 12 years follow-up. However, the mean time of breastfeeding duration was 2 months (varying between 2 weeks - 6 months) (Ando et al., 2004). As discussed before, short-term breastfeeding results in a low transmission rate. In this study, the process consisted in freezing milk -12°C overnight then thawing it at 37°C in a water bath (Ando et al., 2004).

### ARV Therapy During Pregnancy and in Neonate

Breast milk and breastfeeding present numerous benefits including emotional, nutritional, cognitive and immunological benefits (Lawrence and Lawrence, 2004). So, in a context, where we must balance the benefits of breastfeeding and the risk of HTLV-1 MTCT, alternatives to avoidance of breastfeeding should be considered. In this scenario, the implementation of antiretroviral therapy (ART) could be an alternative. ART is well known as an effective measure to prevent HIV MTCT (Leal et al., 2015). Although antiretroviral drugs do not seem

to be effective in controlling a stablished HTLV infection and in reducing PVL (Taylor et al., 2006), *in vitro* studies demonstrated that drugs, such as Zidovudine, Adefovir, and the prodrugs Adefovir dipivoxil and Tenofovir disoproxil can inhibit HTLV-1 infection of lymphocytes *in vitro* (Macchi et al., 1997; Hill et al., 2003) and prevent *de novo* cellular infection (Bertazzoni et al., 2018). As demonstrated *in vitro*, free infectious HTLV-1 virions can cross the human epithelial barrier via transcytosis and infect subjacent target cells such as dendritic cells (Martin-Latil et al., 2012), so, ARV could be beneficial to avoid or reduce the risk of vertical transmission. However, clinical trials should be implemented in order to assess benefits and risks of ART to prevent HTLV-1 MTCT. Moreover, analysis regarding costs and medical access to therapeutic drugs should be considered.

#### **Neutralizing Antibodies**

The potential of passive immunization to prevent HTLV-1 infection was been demonstrated in distinct animal models (rabbit, mice, monkey) (Sawada et al., 1991; Miyoshi et al., 1992; Murata et al., 1996; Saito et al., 2014). As HTLV-1 has a conserved genome, monoclonal antibodies can be considered instead of polyclonal antibodies obtained from ACs. Human monoclonal antibodies capable of neutralizing HTLV-1 have been developed (Kuroki et al., 1992).

Weekly intraperitoneal inoculation of anti-HTLV antibodies in new-borns rabbits (from 1 week of age and up to weaning) reduced HTLV-1 transmission to rabbit offspring (42.9% vs. 8.3%) (Sawada et al., 1991). HTLV-1 neutralizing monoclonal antibody of rat origin (LAT-27) can be passively transferred from pregnant rats to their offspring. The transferred antibodies were

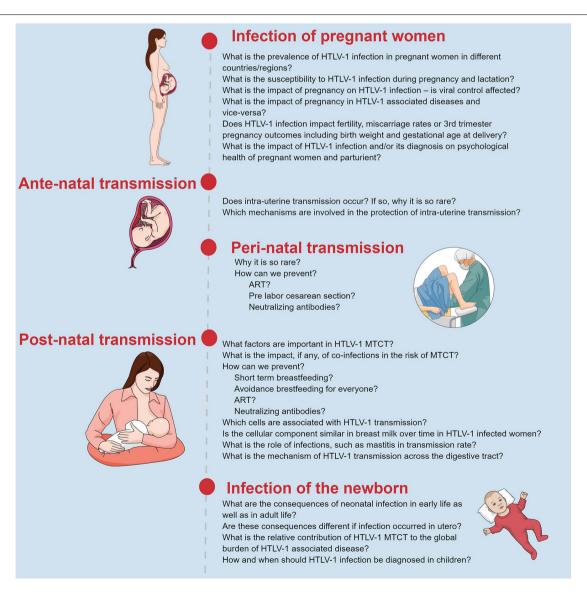


FIGURE 4 | Summary of unmet questions regarding HTLV-1 mother to child transmission. Original figure created with mindthegraph.

able to neutralize HTLV-1 *in vitro* and protected the infants against intra-peritoneum HTLV-1 infection. Moreover, similar results were observed when using humanized LAT-27 antibodies in humanized immunodeficient mice (NOG mice). Both *in vitro* neutralization of HTLV-1 and *in vivo* resistance for HTLV-1 infection were acquired (Fujii et al., 2016). Unfortunately, this antibody was not able to inhibit oral HTLV-1 infection in rats, despite the protection of intraperitoneal infection (Murakami et al., 2017). This reinforces the importance of understanding the cellular mechanism of HTLV-1 transmission in order to develop preventive measures to avoid HTLV-1 vertical transmission.

#### **Pre-labor Elective Cesarean Section**

A study conducted in 1995 evaluated the presence of proviral DNA in maternal blood at delivery, cord blood and oral aspirates from babies delivered by vaginal route and cesarean section. They found that all maternal bloods were positive while all cord bloods were negative. Interestingly, 4/10 new-borns from vaginal route had HTLV-1 proviral DNA in oral aspirates while only one out of seven born by cesarean section had, indicating HTLV exposure during passage through the birth canal (Sawada et al., 1995).

In a cohort of 41 HTLV-1 infected pregnant women in Brazil, 81% delivered by elective cesarean section and all babies were bottle-feed. None of those children were HTLV-1 positive when tested by PCR from 3 to 39 months. The authors hypothesized that the delivery method, together with the avoidance of breastfeeding, contributed to the prevention of vertical transmission (Bittencourt et al., 2002).

In Okinawa (Japan) a decrease in the transmission rate among non-breastfeed children was observed between 1986–1991 (12.8%) and 1995–1999 (3.2%). Although the difference was not statically significant, the authors highlighted that since 1991, doctors have been advised to protect new-borns against HTLV-1 nosocomial infection at delivery. Therefore, obstetricians

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and gynecologists have taken precautions to avoid new-borns contamination at the time of delivery, ensuring that they do not swallow blood during birth. They hypothesize that this action contributed to the observed decrease (Kashiwagi et al., 2004). More studies are clearly needed in order to evaluate the impact of pre-labor elective cesarean section in the prevention of vertical transmission of HTLV-1.

#### FINAL CONSIDERATIONS

Soon after the discovery of HTLV-1, MTCT has been implicated as one of the most important modes for HTLV transmission and for maintaining HTLV-1 within a population. However, little has been done in the world to avoid HTLV transmission from mother to child. Despite the success observed, Japan remains the only country to include HTLV-1 antenatal screening in their national programme. Therefore, although many research questions remain (**Figure 4**), the most important question is why has so little been done to prevent HTLV-1 MTCT thus far?

#### **AUTHOR CONTRIBUTIONS**

CR and GT contributed to conception and design of the study. CR wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.00999/full#supplementary-material

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# Evidence of New Endemic Clusters of Human T-Cell Leukemia Virus (HTLV) Infection in Bahia, Brazil

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**Background:** Salvador, Bahia (northeastern Brazil), has been identified as the epicenter of Human T-cell leukemia virus Human T-cell leukemia virus (HTLV) type 1 infection in the country. This study aims to estimate the rate of HTLV infection and the geographical distribution of this virus in this state.

**Methods:** All HTLV tests (chemiluminescence/ELISA assays/Western Blotting) performed in the Central Laboratory of Public Health of Bahia (LACEN) from 2004 to 2013 were included. Data was extracted from LACEN's database using high volume extract, transformation and load throughput. Infection rate was expressed as the number of infected individuals per 100,000 inhabitants considering municipalities grouped in microregions and/or mesoregions as the unit of analysis.

**Results:** A total of 233,876 individuals were evaluated. Individuals were from 394 out of 417 municipalities of Bahia (94.5%). HTLV chemiluminescence/ELISA assay was found to be reactive for 3,138 individuals from whom 2,323 had WB results (1,978 positives, 62 negative and 282 indeterminate). Out of 1978 reactive samples, 1,813 (91.7%) were positive for HTLV-1, 58 (2.9%) for HTLV-2 and 107 (5.4%) were for both HTLV-1 and HTLV-2. The cumulative mean rate of HTLV-positive cases in Bahia was 14.4 per 100,000 inhabitants. Three microregions presented rates >20 HTLV-positive cases/100,000 inhabitants: Barreiras (24.83 cases per 100,000 inhabitants), Salvador (22.90 cases per 100,000 inhabitants), and Ilhéus-Itabuna (22.60 cases per 100,000 inhabitants).

**Conclusion:** HTLV infection is disseminated in the state of Bahia, with an overall moderate rate of infection. Further studies should be conducted to characterize the epidemiological and clinical profile of HTLV-infected individuals better and to propose effective prevention measures.

Keywords: HTLV, clusters, epidemiology, Bahia, spatial distribution

#### INTRODUCTION

Human T-cell leukemia virus (HTLV) type 1 was the first human oncogenic retrovirus to be identified in 1979 (Poiesz et al., 1980). Subsequently, in 1982, HTLV-2 was identified (Kalyanaraman et al., 1982). HTLV types 3 and 4 were identified only in 2005 in samples of patients from Cameroon (Calattini et al., 2005; Wolfe et al., 2005). To date, only HTLV-1 has been associated with the development of severe diseases, such as adult T-cell leukemia/lymphoma (Hinuma et al., 1981) and tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) (Gessain et al., 1985; Osame et al., 1986), uveitis (Mochizuki et al., 1992) and infective dermatitis (LaGrenade et al., 1990). HTLV-1 infection has also been associated with inflammatory diseases, such as bronchiectasis (Einsiedel et al., 2014; Honarbakhsh and Taylor, 2015), Keratoconjunctivitis sicca (Castro-Lima Vargens et al., 2011) and arthritis (Yakova et al., 2005). In addition, HTLV-1-infected individuals are known to be more susceptible to infectious diseases, such as tuberculosis (Marinho et al., 2005; Verdonck et al., 2007a; Grassi et al., 2016), disseminated Strongyloides stercoralis (Nera et al., 1989) and Norwegian scabies (Brites et al., 2002). The transmission of HTLV-1 occurs through the transfusion of contaminated blood or tissue, including needle sharing among drug users, from mother-to-child, predominantly through breastfeeding (Bittencourt et al., 2001), as well as through sexual contact (Gessain and Cassar, 2012). Recently, the sexual transmission of HTLV-1 was reported as the main route in Salvador, Bahia (Nunes et al., 2017).

It is estimated that at least 5–10 million people are infected with HTLV-1 worldwide, with the highest prevalence occurring in Japan, on the African continent, in the Caribbean, the Melanesian Islands and South America (Gessain and Cassar, 2012). HTLV-2 is less prevalent and is predominantly found in the indigenous populations of North, Central, and South America, as well as in injection drug users in the United States and Europe (Ishak et al., 1995; Hall et al., 1996).

Brazil is home to around 800,000 HTLV-1-infected people and represents one of the largest endemic areas for the diseases associated with HTLV-1 (Gessain and Cassar, 2012). The virus is present throughout the country, with variable prevalence in accordance with geographical region, being highest in the Northeast and North regions. Salvador, the capital of the state of Bahia, has been identified as the epicenter of HTLV-1 infection in Brazil (Galvao-Castro et al., 1997; Catalan-Soares et al., 2005). A population-based study conducted in this city found that 1.76% of the population was infected with HTLV-1. A higher prevalence was found among women, reaching 10% of those aged 50 years or older (Dourado et al., 2003).

Bahia is the largest state in the Brazilian Northeast, with a total of 417 municipalities. Sparse studies have indicated that HTLV-1 infection may be present in some regions of the state. However, these studies have investigated specific population groups, such as pregnant women, blood donors or drug users (Dourado et al., 1998; Magalhaes et al., 2008; Boa-Sorte et al., 2014; Mello et al., 2014). Large-scale population-based assessments are considered the gold standard to determine the prevalence of a given infection; however, the high cost associated with these

make them difficult to perform (Hlela et al., 2009). The present study aimed to estimate the seroprevalence and geographical distribution of HTLV infection in the state of Bahia over a 10-year period (2004–2013). All available serological tests for HTLV-1/2 were evaluated using the database of the Bahia state public health reference laboratory, which tests blood samples from all municipalities in the state.

#### MATERIALS AND METHODS

#### **Ethics Statement**

The Institutional Review Board (IRB) for Human Research at the Gonçalo Moniz Institute of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) provided ethical approval to conduct this study (CAAE number 22478813.7.0000.0040).

#### Study Area

This study was performed in the state of Bahia, Brazil, the fourth largest state in terms of population size, and the fifth largest in terms of area: 565,733 km². The state is comprised of 417 municipalities, which have been grouped into 32 microregions and seven mesoregions by the Brazilian National Institute of Geography and Statistics (IBGE) in accordance with the economic and social similarities among them (**Figure 1**). According to the 2015 Brazilian national census, the state's total population size was 15,203,934 inhabitants, with an overall density of 27 inhabitants per km² (http://www.ibge.gov.br).

#### Study Design

A retrospective ecological study was conducted using data obtained from the Central Laboratory of Public Health of Bahia (LACEN-BA), which is responsible for infectious disease surveillance throughout the state via laboratory analysis. The target population was comprised mainly of blood donors, pregnant women and individuals exhibiting symptoms of infectious disease, referred by blood banks, prenatal physicians or clinicians in the public health system.

All serological tests for HTLV were included among the 32 Bahia microregions from 2004 to 2013. From 2004 to 2008, testing was carried out by ELISA using the Murex HTLV-1/2 immunoassay (DiaSorin S.p.A., Dartford, United Kingdom), from 2009 to 2010 using the anti-HTLV-1/2 Sym Solution kit (Symbiosis Diagnostica LTDA, Leme, Brazil) and, from 2011, the CLIA chemiluminescence assay (Architect rHTLV-1/2, Abbott Diagnostics Division, Wiesbaden, Germany) was used. When HTLV seropositivity was detected, Western blotting (HTLV Blot 2.4, Genelabs Diagnostics®, Singapore) was used throughout the study period for confirmation. HTLV-negative samples were defined as lack of reactivity to HTLV-specific proteins; HTLV-1-positive samples were defined as reactive to GAG (p19 with or without p24) and two ENV (GD21 and rgp46-I) proteins; HTLV-2-positive samples were those reactive to GAG (p24 with or without p19) and two ENV (GD21 and rgp46-II) proteins; HTLV seropositive samples were defined as samples reactive to GAG (p19 and p24) and ENV (GD21). The samples that indicated co-infection of HTLV -1/2 demonstrated reactivity for

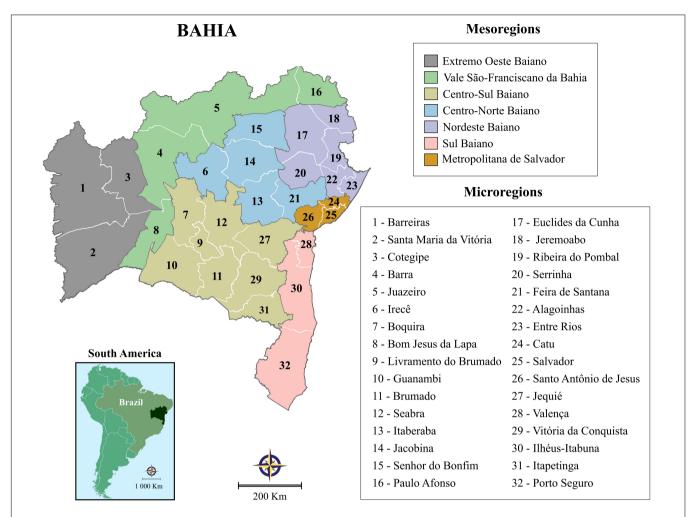


FIGURE 1 | Illustration of the state of Bahia's seven mesoregions and 32 microregions, grouped by IBGE to more accurately reflect economic and social similarities among this state's municipalities.

specific markers of both HTLV-I and HTLV-II: GD21, p19, p24, rgp46-II and rgp46-I. Samples were considered indeterminate when no specific bands for HTLV-I, HTLV-II, or HTLV were detected. All serological tests for HTLV that lacked confirmatory results were excluded.

#### **Data Analysis**

All serological tests performed in each year of the study period were extracted from the SMART LAB laboratory management system using high volume extract, transformation and load throughput. Each individual's unique registration number was considered as the key variable. In order to avoid multiplicity, each individual's most recent serological result was considered. The database was validated using the R software package and analyzed with STATA v13.0. Median and interquartile range (IQR) intervals were calculated for the age variable, and individuals were grouped accordingly. Absolute and relative frequencies were calculated for all categorical variables (age groups: <15/15-30/31-50/>50; sex: male/female, and serological test results: reactive/non-reactive). Geographic information system

techniques and spatial analysis tools were employed to determine the geographical distribution of HTLV throughout the state of Bahia. Population data were acquired from IBGE, supported by national census data from 2010 and official estimates for all other years<sup>1</sup>.

Infection rate was expressed as the number of infected individuals per 100,000 inhabitants. Microregions and/or mesoregions were adopted as the unit of analysis in order to obtain improved accuracy concerning differences among regions. Digital maps were obtained from the IBGE cartographic database in shapefile (.shp), which were subsequently reformatted and analyzed using TerraView version 4.2, open source software freely available from the National Institute for Space Research<sup>2</sup>. Spatial distribution maps based on moving averages were constructed by applying 3-year intervals to data from 2004 to 2013. This method was also used to minimize the effects of random fluctuation for a time-series of infection rates calculated

<sup>1</sup>http://www.ibge.gov.br

<sup>&</sup>lt;sup>2</sup>www.dpi.inpe.br/terraview

for rare events. Annual incidence rates were calculated for each of the 32 micro-regions and the entire state using standardized methods. To assess the relative risk of HTLV in each microregion, maps were constructed using the moving average for the first 3-year rate in Bahia as the denominator assuming no changes in time and space.

#### **RESULTS**

A total of 249,869 serologies for HTLV were performed during the studied period. Due to multiplicity (i.e., the presence of multiple serological test results in the database for a given individual), 15,993 samples were excluded (**Figure 2**). The final sample comprised 233,876 serological tests from unique individuals. Out of 3,138 HTLV-positive samples, the 2,323 samples submitted to Western blotting produced 1,978 positive (85.2%) 63 negative (2.7%) and 282 (12.1%) indeterminate results, yielding an overall prevalence of 0.84%. However, 815 samples were not submitted to Western Blot due to a lack of reagents at some points during the study period, and were excluded from the analysis. Assuming that 85.2% of these 815 samples Blot would have been HTLV positive, it follows that the overall prevalence of HTLV would have been 1.18%.

Of the positive samples, 1,813 (91.7%) were positive for HTLV-1 (prevalence of 0.78%), 58 (2.9%) for HTLV-2 (prevalence of 0.025%) and 107 (5.4%) were positive for both HTLV-1 and HTLV-2 (prevalence of 0.05%). A total of 394 (94.5%) of the 417 municipalities in the state reported at least one positive result during the study period. Information regarding the municipality of sample origin was missing for 1.4% of the HTLV-positive samples. **Figure 3** illustrates the spatiotemporal distribution of HTLV positivity at eight distinct time points. An overall increase in the number of positive cases was observed in all microregions, most notably after 2008, as evidenced by the first 5 years of study (2004–2008) in which 4.3 cases of HTLV per 100,000 inhabitants was found, compared to 10.5 cases per 100,000 inhabitants during the final period (2009–2013).

The cumulative mean rate of HTLV-positive cases in Bahia was 14.4 per 100,000 inhabitants. Considering the grouping of HTLV-positive samples into microregions (**Figure 4**), three demonstrated rates above 20 HTLV-positive cases per 100,000 inhabitants: Barreiras (24.83 cases per 100,000 inhabitants), Salvador (22.90 cases per 100,000 inhabitants), and Ilhéus-Itabuna (22.60 cases per 100,000 inhabitants). Elevated rates of HTLV infection ranging from 8.96 to 19.30 cases per 100,000 inhabitants were also seen in nine other microregions. In the remaining microregions, a homogenous distribution of HTLV positivity was observed. No information was retrieved from two microregions (Santa Maria da Vitória and Jeremoabo).

The microregions of Barreiras, Salvador and Porto Seguro were all notable for high rates of HTLV-1 and HTLV-2 infection, as well as HTLV-1/2 coinfection. HTLV-1 was also predominant in Ihéus-Itabuna and Itapetinga microregions. Higher rates of HTLV-2 infection were also found in the Livramento do Brumado and Juazeiro microregions. HTLV-1/2

coinfection was most prevalent in the Entre Rios and Valença microregions (Figure 4).

The median age of the studied population was 31 years (interquartile range: 25–39 years) and the female:male ratio was 8:1. The median age of HTLV-infected persons was 46 years (IQR: 33–59 years), and 75% were women. Overall, our analysis of HTLV-positivity according to age and sex showed much higher frequencies in individuals aged 31 years or older for both males (85.7%) and females (79.3%) (**Figure 5**). Our stratified age analysis identified 42 positive individuals aged 15 years or younger from all of the mesoregions. The highest frequency of this age group was found in Nordeste Baiano. Three children, two male and one female, younger than 48 months of age, tested positive for HTLV.

In two mesoregions, Extremo Oeste Baiano and Nordeste Baiano, higher frequencies of positive cases were observed among females aged 15–30 years as compared to males. Conversely, a higher frequency of positive cases among males aged >30 years was found in the Vale do São Francisco da Bahia mesoregion.

#### DISCUSSION

Salvador, the capital of the Brazilian state of Bahia, has been considered the epicenter of HTLV-1 infection in Brazil. A population-based study conducted in 2003 estimated a total of 40,000 infected individuals in this city (Dourado et al., 2003). Scattered studies have evidenced the presence of the virus in specific population groups throughout the state, such as pregnant women (Bittencourt et al., 2001; Magalhaes et al., 2008; Mello et al., 2014). To date, no information on the number of HTLVinfected individuals in the State of Bahia (the fourth-most populous in Brazil), nor the geographical distribution of these individuals, has been available. The present results demonstrate that HTLV infection is indeed widespread throughout the state, with an overall rate of 14.4 per 100,000 inhabitants. HTLV-1 can be considered predominant, as it corresponds to 91.2% of all cases, followed by HTLV-1/HTLV-2 coinfection (5.4%) and HTLV-2 (2.9%).

Notably, our results identified one important cluster of HTLV infection comprising four microregions surrounding the Salvador microregion (Entre Rios, Santo Antonio de Jesus and Catu). This cluster consists of 43 cities and concentrates almost a third of the state's population. Geographically, the municipalities in this cluster are mainly located in the area surrounding the Baía de Todos os Santos, which is considered to have a subtropical climate characterized by Atlantic Forest. The population is largely of similar ethnic origin (mainly of African descent) with uniform socialdemographic characteristics. As expected, the Salvador microregion which includes 10 municipalities, presented a high overall rate of HTLV-infection (22.90/100,000 inhabitants). This finding confirms previous reports indicating that Salvador is highly affected by this virus (Dourado et al., 2003). Cruz das Almas, a city located in the Santo Antônio de Jesus microregion was also previously identified as having a 1% prevalence in pregnant women (Magalhaes et al., 2008).

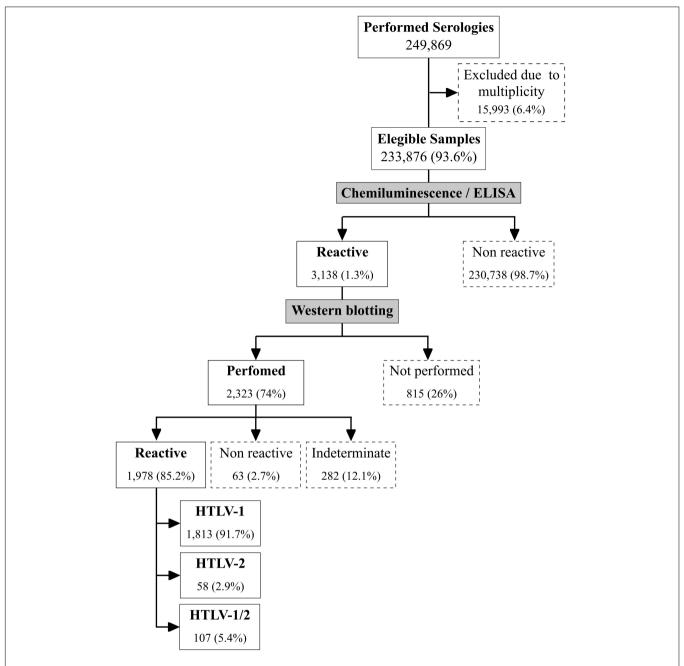


FIGURE 2 | Standards for reporting of diagnostic accuracy studies (STARD) flowchart illustration of study design. Boxes with dotted lines indicate samples excluded from the study.

Unexpectedly, the present results also identified three other endemic clusters: one located in the southernmost (comprising four microregions, 86 municipalities,  $\sim 2.7$  million inhabitants), one in the central region (two microregions, 33 municipalities,  $\sim 750.000$  inhabitants) and another in the westernmost region of the state (comprising one microregion, 7 municipalities,  $\sim 280.000$  inhabitants). Scarce studies identified the presence of HTLV-1 infection in some cities located in these regions: a recent study evaluating pregnant women in Ilhéus and Itabuna, cities in the southern region of Bahia, found 1.05% were infected with

HTLV-1 (Mello et al., 2014), while another study found HTLV-1 infection in people from the Jacobina microregion (Britto et al., 1998; Rego et al., 2008). The Ilhéus-Itabuna microregion is located around the southern coastal region of Bahia, having a humid tropical climate with areas of Atlantic Forest. This regional center is home to important commercial, service, and industrial activities. The economic importance of this region grew during the golden era of cocoa production. Nowadays, the economy is based on tourism and other activities related to the seaport. By contrast, the Jacobina microregion has a

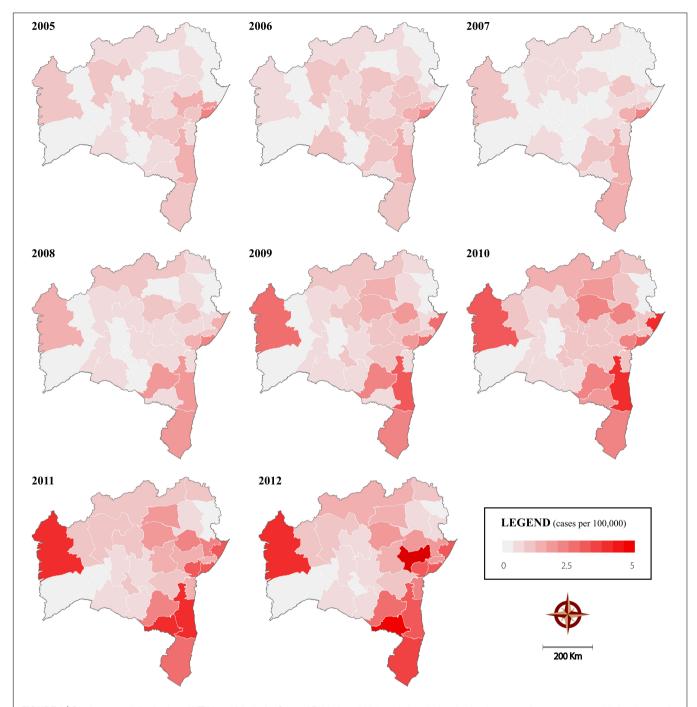


FIGURE 3 | Spatiotemporal distribution of HTLV positivity in the State of Bahia from 2004 to 2013, calculated using 3-year moving averages considering the state's microregions as units of analysis.

tropical climate with a dry season. Surrounding lakes, mountains, rivers and waterfalls favor ecological tourism. Regarding the Barreiras microregion, located in the westernmost part of Bahia, to the best of our knowledge no previous studies reported this area being endemic to HTLV infection. We cannot exclude the possibility that these microregions received samples from surrounding areas. Barreiras is the most important municipality located in the microregion, and the city borders Tocantins state.

This microregion has a tropical climate and the predominant vegetation is the arboreal cerrado. The regional economy is mainly based on agribusiness, and migrants from all over Brazil moved here in the 1970s and 1980s.

While higher rates of HTLV-1/2 co-infection were mainly present in some microregions identified as HTLV-1 clusters, including Salvador, Barreiras and Porto Seguro, the highest rates of HTLV-2 were present in Livramento do Brumado microregion,

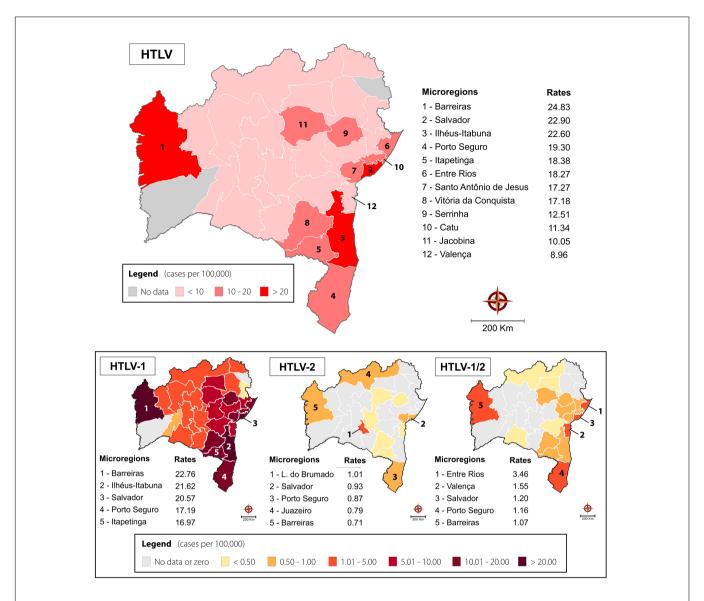


FIGURE 4 | Spatial distribution of overall HTLV, HTLV-1, HTLV-2, and HTLV-1/2 positivity in the State of Bahia from 2004 to 2013, considering microregions as the unit of analysis. The 12 microregions with the highest rates of HTLV positivity per 100,000 inhabitants are highlighted, in addition to the top five according to each respective HTLV type.

located in the center of the state, and Juazeiro, located in the north. In Brazil, few studies have reported on the prevalence of HTLV-1/2 coinfection (Gabbai et al., 1993). HTLV-2 infection has been predominantly reported in indigenous populations residing in northern Brazil (Ishak et al., 1995) and among injectable drug users in urban areas (Gabbai et al., 1993). It is possible that HTLV-1/2 coinfection found in Bahia may result from the introduction of drug users infected with HTLV-2 into areas in which HTLV-1 is already prevalent.

Of note, a progressive increase in the incidence of HTLV infection was observed throughout the study period. This is likely due to an expansion in the number of municipal primary health clinics provided by the federal Family Health Program, as well as increases in testing and counseling centers (aimed primarily

at sexually transmitted diseases) in the countryside of the state. In addition, awareness surrounding HTLV infection increased during the study period and HTLV was included on the list of compulsory disease notifications for the state of Bahia in 2011.

With respect to HTLV infected individuals profile, the mean age was 46 years with a predominance of female (75%). HTLV infection is known to be more prevalent in women, and its prevalence increases with age (Dourado et al., 2003; Mota et al., 2006). Herein, a higher proportion of HTLV-1-infected women was detected in the 31–50 age range among most mesoregions. This could be due to the non-random nature of the sample, which was young (median age of 31 years) and consisted mostly of females. Increased sexual activity is more common in this age range, and virus transmission is known to be more efficient from



men to women (Murphy et al., 1989). It is also possible that compulsory serological HTLV screening for pregnant women, in effect in Bahia, contributed to the higher proportion of women detected in this age range.

By contrast, in the Vale do São Francisco and Centro Norte Baiano mesoregions, men were found to be predominantly infected by HTLV in the age range of 31–50 years. Both mesoregions are highly productive agricultural zones containing municipalities of high population density. In addition, the Centro Norte Baiano mesoregion attracts young men to work in the mining industry. Accordingly, it is theoretically possible that the higher prevalence observed in men of this age range could be due to increased exposure to illicit drugs, as well as to an increased incidence of STDs, such as HIV, gonorrhea and syphilis. In

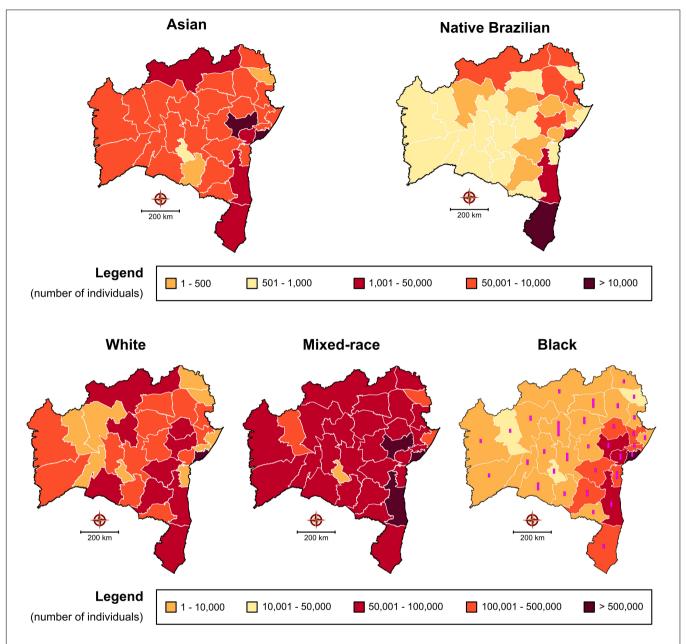


FIGURE 6 | Self-reported ethnicity/color of regional populations in the state of Bahia according to the Brazilian Institute of Statistics and Geography 2010 census. With respect to the representative map of individual identifying as "black," bars represent the number of quilombola communities located in each mesoregion of the state (total number = 656).

addition, lower levels of formal education and income have been attributed to a higher risk of acquiring HTLV infection (Dourado et al., 2003). Moreover, despite the greater efficiency of malefemale transmission, evidence of female-male transmission has also been reported (Brodine et al., 1992). Concerning the possible route of the HTLV transmission, it has been suggested that in Salvador the sexual route predominates, since almost no children was found to be infected (Dourado et al., 2003; Nunes et al., 2017). In the present study the HTLV infection was observed in 42 individuals under 15 years, indicating that in addition to sexual transmission, the vertical route may be an important route.

Brazil is considered an endemic area for HTLV (Gessain and Cassar, 2012). However, the prevalence of this infection in the country varies according to the geographic region, being higher in the North and Northeast (Galvao-Castro et al., 1997). The origin of HTLV in Brazil is linked to the introduction of the virus in the post-Colombian era, by the slave trade from Africa mainly to the northeastern cities of the country (Alcantara et al., 2003; Magalhaes et al., 2008; Rego et al., 2008). The population in Bahia is highly mixed, consisting of mostly black and mixed Western African and Portuguese descendants (Azevedo et al., 1982). **Figure 6** illustrates the distribution in each

microregion of self-reported ethnicity/color (white, mixed-race, black, Asian, or native Brazilian) according to the data reported by the Brazilian Institute for Geography and Statistics (IBGE) in its 2010 census<sup>3</sup>. A diffuse distribution is observed throughout the microregions, especially with respect to white/mixed-race and black, which corresponds to the distribution of HTLV-1 cases, while native Brazilians are more concentrated in the Porto Seguro microregion, which also had a notable rate of HTLV-2 infection. However, the information available prevents us from making direct correlations between ethnic profile and the distribution of HTLV types. Interestingly, the data herein indicate that the microregions with the highest rates of infection in the state of Bahia were areas where communities recognized as quilombos predominated. Originally, quilombos were places of refuge for the African and Afro-descendant slaves who fled slavery. Their descendants often remained in these areas and were then termed quilombolas. Bahia is one of the states with the highest number of quilombolas recognized4, Figure 6 (Black).

One limitation of the present study was the non-random sample, which was predominantly female. However, our analysis considered samples from 94% (394/417) of the municipalities in Bahia, a state with an estimated population of 15,126,371 inhabitants; i.e., just 5.5% of the municipalities (comprising a total of 260,125 inhabitants) did not send any samples to LACEN during the study period. This was especially relevant in two microregions (Santa Maria da Vitória and Jeremoabo), from which no information regarding HTLV testing was obtained. To explain this, we can only speculate that perhaps local health authorities chose to outsource testing to private laboratory services instead of sending samples to LACEN, or that authorities in these municipalities did not properly implement actions designed to increase HTLV detection. In fact, the municipality of Jeremoabo has, to date, not reported a single case of HTLV to the Information System on Diseases of Compulsory Declaration (Sistema de Informação de Agravos de Notificação-Sinan), while Santa Maria da Vitória reported a single case in 2013 and another in 2018 (data not shown). Accordingly, we surmise that health professionals in these areas faced challenges in identifying patients suspected of HTLV infection, and/or did not recognize HTLV infection as a sexually transmitted infection, and therefore did not request appropriate serological testing. While population-based studies are considered the gold standard for estimating infection prevalence, high cost and methodological difficulties can present significant challenges (Hlela et al., 2009). Accordingly, estimates of HTLV prevalence are often based on data obtained from blood banks, pregnant women and other population groups (Verdonck et al., 2007b). In addition, the present study is unable to offer any insight into virus phylogeny, as the retrospective nature of this investigation precluded the performance of molecular analysis to accurately assess results considered indeterminate by Western Blot.

#### CONCLUSION

In conclusion, the present study serves to highlight previously unreported data regarding clusters of HTLV infection throughout the state of Bahia, which is considered to be the Brazilian state with the highest HTLV infection rate. HTLV infection cannot be considered as restricted to the area of Salvador, the capital of Bahia, as the data reported herein identified new endemic areas. Considering the overall prevalence of HTLV in Bahia to be 0.84% (1,978/233,876), it follows that ~130,000 individuals would be infected with HTLV. However, it is important to note that this number could be underestimated, since 26% of the HTLV reactive samples were not submitted to Western blotting, and were thusly excluded. Had 85.2% of these samples been confirmed, and subsequently included in our analysis, the overall prevalence would increase to 1.18%. Further studies are needed to better describe the epidemiological profile of the infected population and to reinforce public policies designed to prevent HTLV transmission, especially in pregnant women.

#### **ETHICS STATEMENT**

As the present study has a retrospective design, evaluating HTLV serologies performed in a 10 years-period in the Public Health Laboratory of Bahia, the Institutional Research Board of Fiocruz approved a study, dispensing the signing of written informed consent.

#### **AUTHOR CONTRIBUTIONS**

All authors read and approved the final version of the manuscript, analyzed and interpreted the data, and drafted the manuscript. FP, MC, MG, and BC conceived and designed the study. FP, MC, CR, RC, and MG acquired the data. FP, MG, FS, and BC critically revised the manuscript.

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<sup>&</sup>lt;sup>3</sup>http://www.ibge.gov.br

<sup>4</sup>http://www.palmares.gov.br/?page\_id=37551

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### **Association Between HTLV-1** Genotypes and Risk of HAM/TSP

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Human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a neurological disorder presenting with spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities, which develops in a small minority of HTLV-1-infected individuals. HTLV-1-specific T cells are efficiently activated through dedicated human leukocyte antigen-mediated mechanisms, a process considered deeply involved with its pathogenesis. It has been reported that the lifetime risk of developing HAM/TSP differs between ethnic groups, and there is an association between HTLV-1 tax gene subgroups (i.e., tax subgroup-A or -B), which correspond to HTLV-1 "cosmopolitan subtype 1a subgroup A (i.e., transcontinental subgroup)" and "cosmopolitan subtype 1a subgroup B (i.e., Japanese subgroup)," respectively, and the risk of HAM/TSP in the Japanese population. These findings suggest that a given host's susceptibility to HAM/TSP is deeply connected with both differences in genetically determined components of the host immune response and HTLV-1 subgroup. Therefore, it is crucial for ongoing work to focus on developing novel treatments and preventative approaches for HAM/TSP. In this review, based on an overview of the topic and our latest research findings, the role of the HTLV-1 subgroup on the effects of virus-host interactions in the pathogenesis of HAM/TSP is discussed.

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#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1), the first human retrovirus to be discovered, is etiologically associated with adult T-cell leukemia (ATL) (Poiesz et al., 1980; Hinuma et al., 1981; Yoshida et al., 1984) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). In HTLV-1 infection, only a minority of infected individuals develop the disease after prolonged viral persistence (2-5% for ATL and 0.25-3.8% for HAM/TSP), whereas the majority remain asymptomatic carriers (ACs) throughout their lives (Uchiyama, 1997; Bangham and Matsuoka, 2017). However, a definitive answer to the fundamental question of why some HTLV-1-infected individuals develop diseases, whereas the vast majority remain healthy in a lifetime has not yet been answered. Since both ATL and HAM/TSP

have unidentifiable causes and/or a lack of curable treatments, evaluation of the individual risk of developing diseases in ACs would certainly be of considerable importance, especially in HTLV-1 endemic areas. This review summarizes past and recent studies of HTLV-1, especially focused on the viral and host genetic risk factors for developing HAM/TSP, in an attempt to answer this fundamental question.

### HOST GENETIC FACTORS GOVERNING HAM/TSP SUSCEPTIBILITY

HTLV-1-associated myelopathy/tropical spastic paraparesis is a rare chronic progressive neuroinflammatory disease of the central nervous system mainly involve the thoracic spinal cord, therefore characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa et al., 1995). Based on the number of infected individuals, the number of HAM/TSP cases in the world has been estimated to be between 60,000 and 100,000 in HTLV-1 endemic areas. Sporadic cases of HAM/TSP have also been reported from non-endemic areas such as the United States and Europe, among immigrants from endemic areas (Araujo and Silva, 2006). In Japan, by the end of 2018, 551 patients of an estimated 3,000 cases were registered in "HAM-net," a recently established registration system that collect personal and clinical data from patients with HAM/TSP (Coler-Reilly et al., 2016).

Shortly after the discovery of HAM/TSP, scientists found that in vitro culture of peripheral blood mononuclear cells (PBMCs) from HTLV-1-infected individuals exhibit "spontaneous lymphocyte proliferation" (SLP) in the absence of exogenous stimuli such as antigens or mitogens. In patients with HAM/TSP, the levels of SLP reflect the severity of the disease (Itoyama et al., 1988; Ijichi et al., 1989), and the levels of SLP are higher in patients with HAM/TSP than those in patients with ATL or in ACs (Itoyama et al., 1988; Eiraku et al., 1992). Meanwhile, patients with HAM/TSP have significantly higher anti-HTLV-1 titers than those in patients with ATL or in ACs (Nakagawa et al., 1995). These observations raised the possibility that the immune hypersensitivity to HTLV-1 observed in patients with HAM/TSP is governed by host genetic factors. Indeed, just 2 years after the discovery of HAM/TSP, Usuku et al. (1988) identified human leukocyte antigen (HLA)-DR1 and -DR4 as two HLA-DR serotypes highly represented in HAM/TSP, hypothesizing that HAM/TSP and ATL correspond to two populations of HTLV-1 carriers with distinct immune responses to HTLV-1. Later on, they also discovered that large quantities of HTLV-1-specific antibodies are present in the cerebrospinal fluid of patients with certain HAM/TSP-related HLA haplotypes (Kitze et al., 1996) and identified an HLA-DRB1\*0101-restricted dominant epitope on HTLV-1 envelope (Env) glycoprotein gp21 (Kitze et al., 1998). They further corroborated the association between host genetic factors and the susceptibility to HAM/TSP by genomic DNA analysis of 224 patients with HAM/TSP and 202 ACs living in Kagoshima Prefecture, located in HTLV-1 endemic southwest Japan (Nagai et al., 1998). Namely, the HTLV-1 proviral load (PVL), which represents the number of HTLV-1-infected cells in vivo because HTLV-1-infected cells in vivo harbor one copy of the integrated HTLV-1 provirus per cell (Cook et al., 2012), is 7- to 16-fold higher in patients with HAM/TSP than in ACs, and the PVL is significantly higher in ACs with genetic relatives with HAM/TSP than in ACs with no family history (Nagai et al., 1998). Interestingly, they also reported that the risk of HAM/TSP increases exponentially once the HTLV-1 PVL exceeds 1% of PBMCs, providing more evidence to support increased PVL (i.e., number of HTLV-1 infected cells in vivo) as a risk factor for HAM/TSP (Nagai et al., 1998). At around the same time, a collaborative study by this Kagoshima group and London group showed that possession of the HLA-class I genes HLA-A\*02 and Cw\*08 is associated with lower risk of HAM/TSP through a statistically significant reduction in the HTLV-1 PVL, whereas HLA-class I HLA-B\*5401 and class II HLA-DRB1\*0101 was associated with a higher risk of HAM/TSP in the same population (Jeffery et al., 1999, 2000). Since the protective effect of HLA-A\*02 has also been reported in the Brazilian population (Borducchi et al., 2003; Catalan-Soares et al., 2009), HLA-A\*02 may play an important role in regulating HTLV-1 infection. Indeed, consistent with these findings, it has been reported that HLA class I-related epitopes targeted by cytotoxic T lymphocytes (CTLs) are most commonly found on HTLV-1 regulatory factor Tax; HLA-A\*02-restricted epitope on Tax, spanning amino acids 11-19, is known to provoke a particularly intense immune response (Kannagi et al., 1991, 1992; Parker et al., 1992); HTLV-1 Env gp21 immunodominant epitope is restricted by HLA-DRB1\*0101 (Yamano et al., 1997; Kitze et al., 1998); and HLA-DRB1\*0101 is associated with the susceptibility to HAM/TSP in independent HTLV-1-infected populations in southwest Japan (Jeffery et al., 1999, 2000) and northeastern Iran (Sabouri et al., 2005). These findings convincingly demonstrated that immune response to HTLV-1 regulated by HLA is an important determinant of the number of HTLV-1 infected cells in vivo and the risk of HAM/TSP.

# ETHNIC AND GEOGRAPHICAL DIFFERENCES IN HLA ASSOCIATIONS WITH THE RISK OF HAM/TSP

As observed in other infections, the reported association between HLA genes and the outcome of HTLV-1 infection shows ethnic and geographical differences. In Iranian HTLV-1-infected individuals, although HLA-DRB1\*0101 is associated with susceptibility to HAM/TSP in the absence of HLA-A\*02, as observed in Kagoshima, southwest Japan, both HLA-A\*02 and HLA-Cw\*08 have no effect on either the risk of developing HAM/TSP or HTLV-1 PVL (Sabouri et al., 2005), suggesting that the underlying mechanism involving HLA-DRB1\*0101 may not be the same between the Iranian and Japanese populations. Furthermore, although the sample size was small, the lack of association between HLA-A\*02 and the risk of HAM/TSP has been reported in other ethnic groups, such as Afro-Caribbean individuals from Martinique (Deschamps et al., 2010),

Jamaica (Goedert et al., 2007), and Spain (Trevino et al., 2013). There are several possible explanations for these observed differences as follows: (1) the immunodominant target epitopes recognized by class I major histocompatibility complex (MHC)restricted CTLs are different among various HTLV-1 strains in different racial and ethnic groups; (2) unknown environmental factors such as co-infection with other pathogens affect HTLV-1 PVL and HAM/TSP risks in different racial and ethnic groups; and (3) different A\*02 subgroups in different racial and ethnic groups affect PVL and HAM/TSP risk because HLA-A\*02 CTL responders to Tax frequently recognize more than one A\*02-restricted Tax epitope (Parker et al., 1994), and A\*02 subgroups differ significantly in their peptidebinding characteristics (Barouch et al., 1995; Sudo et al., 1995). Therefore, further studies of ethnic and geographical differences in associations between HLA and outcomes of HTLV-1 infection are needed using sufficient numbers of samples because most HLA alleles occur with relative infrequency and in strong linkage disequilibrium with other alleles. Furthermore, it is also necessary to consider the differences in other genetic factors, including non-HLA genes, and the different types and subtypes of HTLV-1 distributed in diverse racial/ethnic groups.

### **CLASSIFICATION OF HTLV-1 SUBGROUPS**

HTLV-1-infected individuals are widely distributed across different geographical regions, such as sub-Saharan Africa, South Africa, north-eastern Iran, Melanesia (e.g., Solomon Islands, Papua New Guinea), southwest Japan, Australia (i.e., Aboriginals), the Caribbean, and South America (Verdonck et al., 2007; Gessain and Cassar, 2012). In these endemic areas, seven subtypes of HTLV-1 exist (subtypes 1a-1g), defined based on a phylogenetic analysis of viral long terminal repeats (LTRs): 5 African subtypes (subtypes 1b, 1d, 1e, 1f, and 1g), a Melanesian/Australian subtype (subtype 1c), and a cosmopolitan subtype (subtype 1a). Among them, cosmopolitan subtype A has the broadest worldwide distribution of all of them and is further divided into 5 "subgroups" (subgroups A-E of subtype 1a) (Proietti et al., 2005; Wolfe et al., 2005; van Tienen et al., 2012): transcontinental (A), Japanese (B), West African (C), North African (D), and Afro-Peruvian (E) (Gessain and Cassar, 2012). In the Japanese population, the transcontinental (A) and Japanese (B) subgroups predominate, and sequence variation of HTLV-1 tax gene determines the "HTLV-1 tax subgroups," i.e., tax subgroup-A and subgroup-B correspond to LTR-based "cosmopolitan subtype 1a subgroup A" and "cosmopolitan subtype 1a subgroup B," respectively (Furukawa et al., 2000). As shown in Table 1, subgroup-specific nucleotide substitutions are observed in viral regulatory genes Tax (on the sense-strand) and HTLV-1 bZIP factor (HBZ; on its anti-sense-strand). Namely, tax subgroup-A sequence differed at 4 nucleotides in Tax-coding and 1 nucleotide in HBZ-coding sequences compared with Japanese prototypic ATK-1 strains (Seiki et al., 1983), which result in two amino acid substitutions in Tax and single amino acid substitution in HBZ.

TABLE 1 | Nucleotide variations specific to HTLV-1 tax subgroup.

	Nucleotide variation and amino acid change in <i>tax</i> subgroup <sup>a</sup>						
	HBZ -7247 S→P	Tax 7900 NC	Tax 7962 A→V	Tax 8211 S→N	Tax 8347 NC		
ATK-1	А	С	С	G	А		
tax subgroup-B	Α	С	С	G	Α		
tax subgroup-A	G	Т	Т	Α	С		

<sup>a</sup>Nucleotide position is provided as aligned with the prototype ATK-1 sequence (GenBank accession number: J02029) (Seiki et al., 1983). Amino acid change results from nucleotide substitutions are shown as single-letter amino acid codes (i.e., tax subgroup-B  $\rightarrow$  tax subgroup-A). NC, no change.

### INCIDENCE OF HAM/TSP IN DIFFERENT GEOGRAPHICAL AREAS

Although the incidence of HAM/TSP has a two- to three-fold higher risk in women both among Jamaican and Japanese HTLV-1-infected individuals, the annual incidence of HAM/TSP is much higher among Jamaican individuals than among Japanese individuals (20 and 3 cases, respectively, per 100,000). Thus, it is noteworthy that Jamaican individuals have a higher incidence of "cosmopolitan subtype 1a subgroup A" infection (i.e., Jamaican individuals infected with HTLV-1 harboring subgroup-A nucleotide substitutions) compared with Japanese individuals with a higher incidence of the "cosmopolitan subtype 1a subgroup B" infection (Osame et al., 1990; Tajima, 1990; Kramer et al., 1995; Nakagawa et al., 1995; Hisada et al., 2004). Namely, the lifetime risk of HAM/TSP is different among different ethnic groups. The lifetime incidence of HAM/TSP, i.e., the probability that a neonatal carrier of HTLV-1 will develop HAM by 75 years of age, was reported as 0.25% in the Kagoshima prefecture, which is located in HTLV-1-endemic southwest Japan (Kaplan et al., 1990), and this was significantly lower than the lifetime incidence reported in Jamaica and other Caribbean islands (1.9%) (Maloney et al., 1998) and Brazil (9%) (Rosadas et al., 2018). Interestingly, HLA-A\*02 provides protection against tax subgroup-B, but not against tax subgroup-A in both the Japanese (Furukawa et al., 2000) and Iranian (Sabouri et al., 2005) populations. These findings increase the possibility that regional differences in HAM/TSP prevalence can be explained by HLA haplotypes and corresponding geographical variations in HTLV-1 subtypes and subgroups.

Interestingly, HTLV-1 in Iranian individuals, which showed no association between HLA-A\*02 and the risk of HAM/TSP, possessed 10 different nucleotides in the *tax* region compared with Japanese prototypic strain ATK-1 (= *tax* subgroup B) (Seiki et al., 1983). Among these, nucleotides 7900, 7962, 8211, and 8347 were identical with "*tax* subgroup A," whereas there are six more nucleotide substitutions that induced four additional different amino acids in the Japanese *tax* subgroup A (**Table 2**). Because Iranian *tax* induces HAM/TSP with lower PVL than Japanese strains (Sabouri et al., 2005), it is plausible that the Iranian HTLV-1 strain, which belongs to "cosmopolitan subtype 1a subgroup A,"

TABLE 2 | Nucleotide variations specific to Iranian HTLV-1 Tax.

	Nucleotide variation, by position and amino acid change <sup>a</sup>									
Subgroup (n)	7625 M→V	7814 I→V	7858 NC	7900 NC	7962 A→V	7994 N→H	8211 S→N	8316 G→E	8317 NC	8347 NC
ATK-1 ( = tax subgroup-B)	А	А	Т	С	С	А	G	G	С	А
tax subgroup-A	Α	Α	Т	Т	Т	Α	Α	G	С	С
Iranian tax	G	G	С	Т	Т	С	Α	Α	G	С

<sup>&</sup>lt;sup>a</sup>Nucleotide position corresponds to that of prototypic strain ATK-1. Amino acid change results from nucleotide substitution. NC, no change.

has a much higher risk for HAM/TSP than the Japanese strains of both tax subgroup A and B. Meanwhile, using 211 Japanese samples, Nozuma et al. (2017) recently confirmed previous findings by Furukawa et al. (2000), i.e., transcontinental subgroup of HTLV-1 ( = tax subgroup A) is susceptible to HAM/TSP compared with the Japanese subgroup ( = tax subgroup B). These findings suggested that a high-risk subgroup of HTLV-1 explains the observed differences in HTLV-1 PVL and higher risk for HAM/TSP.

### NUCLEOTIDE SEQUENCE VARIATION OF HTLV-1 IN PATIENTS WITH HAM/TSP

Since HTLV-1 infection precedes both HAM/TSP and ATL, the possibility that certain HTLV-1 strains cause one condition and others cause the other has been investigated. However, the resounding conclusion was that no such disease-specific strains existed (Daenke et al., 1990; Kinoshita et al., 1991). Nonetheless, later work identified mutations in the region of the viral genome coding for tax associated with HAM/TSP morbidity (Kira et al., 1994; Renjifo et al., 1995). In one of these studies, mutant proviral tax sequences were frequently isolated from lesioned tissue sampled from the spines of patients with HAM/TSP (Kira et al., 1994). Subsequently, proviral sequences with similar mutations were found in PBMCs from ACs, proving both that tax mutants are not unique to patients with HAM/TSP and that infected cells do not exclusively accumulate around spinal cord lesions (Saito et al., 1995). Another study found a single point mutation, located at nucleotide 7959 of the tax region, to be associated with HAM/TSP status (Renjifo et al., 1995). However, later work showed it to be specific to virus strains of a specific geographical area, rather than a disease-specific mutation (Mahieux et al., 1995). These findings led many to conclude that there were simply no HAM/TSP-specific mutations in the HTLV-1 genome.

Then, in Furukawa et al. (2000) reported the existence of two tax subgroups, i.e., "tax subgroup-A" and "tax subgroup-B," with distinct sequences based on an analysis of a large number of specimens from patients with HAM/TSP and ATL and from ACs in Kagoshima Prefecture, located in HTLV-1 endemic southwest Japan. Most interestingly, they found tax subgroup-A carriers to be at about 2.5 times the risk of developing HAM/TSP compared to tax subgroup-B carriers, independent of HLA haplotype (Furukawa et al., 2000). As already mentioned, they explicitly showed that tax subgroup-A and -B correspond to the transcontinental (A) and Japanese (B) subgroups within cosmopolitan subtype 1a, a taxonomy defined based on LTR sequence similarity (Furukawa et al., 2000). Table 3 and Figure 1 show the results of our analysis of the tax subgroups of patients with HAM/TSP and ACs living in Kagoshima and Okinawa Prefectures. Okinawa prefecture consists of 160 islands, of which 49 islands are inhabited, and is located in the subtropical southern-most point of Japan. In Okinawa prefecture, tax subgroup-A and -B, respectively, comprised 63.6 and 36.4% of patients with HAM/TSP: moreover, tax subgroup-A was significantly more common among ACs here than in Kagoshima. Kagoshima had significantly more tax subgroup-B carriers, but the HAM risk was higher among tax subgroup-A carriers; curiously, however, the tax subgroup was not associated with any differences in HAM/TSP susceptibility in Okinawa, despite the higher frequency of tax subgroup-A (Table 3). In future studies, it is important to examine the HAM/TSP prevalence rates in these two prefectures to clarify whether or not the pathogenesis of HAM/TSP is influenced at all by the HTLV-1 subgroup.

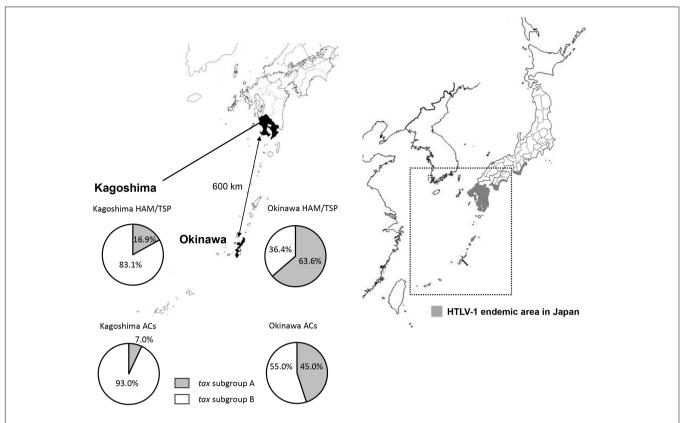
### TAX SUBTYPE-SPECIFIC FUNCTIONAL DIFFERENCES

We have previously reported that although no clear differences were noted in clinical and laboratory characteristics, clinical

TABLE 3 | HTLV-1 tax subgroup-A is associated with the risk of HAM/TSP in Kagoshima but not in Okinawa, Japan.

Population	HAM/TSP		ACs					
	tax subgroup-A	tax subgroup-B	tax subgroup-A	tax subgroup-B	$\chi^{2a}$	Р	OR	CI, 95%
Kagoshima	41 (16.9%)	201 (83.1%)	14 (7.0%)	186 (93.0%)	9.04	0.0264	2.71	1.43–5.13
Okinawa	21 (63.6%)	12 (36.4%)	9 (45.0%)	11 (55.0%)	1.08	0.298	1.76	0.69-6.63

<sup>&</sup>lt;sup>a</sup>Reported as one-tailed with Yates correction. Odds ratio used the approximation of Woolf. The significant value is indicated in bold. OR, Odds ratio; Cl, confidence interval; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; ACs, asymptomatic carriers.



**FIGURE 1** Geographical distribution of HTLV-1 *tax* subgroups in Kagoshima and Okinawa Prefecture, Japan. The distribution of HTLV-1 *tax* subgroups varied by geographic region in southern Japan. A significantly higher prevalence of *tax* subgroup-A was found in Okinawa prefecture than Kagoshima prefecture both in HAM/TSP patients and asymptomatic carriers (ACs) (Okinawa vs. Kagoshima, HAM/TSP: 63.6 vs. 16.9%, p < 0.001, ACs: 45.0 vs. 7.0%, p < 0.001, p-value assessed by  $\chi^2$  test).

course, and treatment response between HAM/TSP patients with subgroup-A and subgroup-B, different HTLV-1 subgroups are characterized by different patterns of viral and host gene expression in patients with HAM/TSP (Yasuma et al., 2016). Namely, HBZ mRNA expression was significantly higher in patients with HAM/TSP with tax subgroup-B than in those with tax subgroup-A in HTLV-1-infected cells, and there is a positive correlation between the expression of HBZ mRNA and its target Foxp3 mRNA in patients with HAM/TSP with tax subgroup-B, but not in patients with tax subgroup-A. It has previously been reported that Tax suppresses (Arnulf et al., 2002; Lee et al., 2002) and HBZ enhances (Zhao et al., 2011) transforming growth factor (TGF)-β-mediated signaling activity and HBZ induces Foxp3 expression in naïve T cells via Smad3-dependent TGF-β signaling (Zhao et al., 2011). We therefore examined whether there were any differences in the ability of subgroup-specific Tax or HBZ to activate the Foxp3 promoter. However, no functional differences were observed between the Tax and HBZ proteins of each subgroup (i.e., HBZ-A and HBZ-B or Tax-A and Tax-B) based on reporter gene assays using three different reporters, i.e., the HTLV-1-3'-LTRpromoter luciferase reporter, human Foxp3 promoter luciferase reporter, and human TGF-β-responsive concatemer-containing

luciferase reporter (Yasuma et al., 2016). Meanwhile, coexpression of Tax did not significantly influence the function of HBZ to activate the Foxp3-promoter in transfected cells (Yasuma et al., 2016). These results indicate that different patterns of viral and host gene expression in patients with HAM/TSP observed in different HTLV-1 *tax* subgroups are characterized via independent mechanisms of direct transcriptional regulation.

More recently, we further studied the function of each subgroup-specific Tax using microarray analysis, reporter gene assays, and evaluation of viral-host proteinprotein interactions (Naito et al., 2018). In this study, we transformed human acute T-cell leukemia cell line Jurkat Tet-On cells with a construct containing either full-length tax subgroup-A or -B gene downstream of a tetracyclineresponsive promoter, and then used comprehensive microarray analysis to identify genes of which expression levels changed following Tax protein induction. Interestingly, induced Tax protein (i.e., Tax-A or Tax-B) potently activated the expression of their target genes, and Tax-A and -B elicited the expression of different combinations of both alreadyreported and unreported target genes (Naito et al., 2018). Furthermore, although the chemokine CXCL10, which has been proposed as a prognostic biomarker for HAM/TSP (Sato et al., 2013), was more efficiently induced by Tax-A than by Tax-B through the nuclear factor (NF)- $\kappa$ B pathway, there was no difference in the ability of each subgroup of Tax (i.e., Tax-A or Tax-B) to activate the CXCL10 gene promoter evaluated using a reporter gene assay with HTLV-1-negative human T-cell line Jurkat (Naito et al., 2018). Meanwhile, chromatin immunoprecipitation assays revealed that the ternary complex containing Tax-A is more efficiently recruited onto the CXCL10 promoter, which contains two NF- $\kappa$ B binding sites, than that containing Tax-B (Naito et al., 2018).

#### **CONCLUDING REMARKS**

As discussed above, each subgroup-specific Tax or HBZ have a unique expression signature of both viral and host

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genes, suggesting that differential expression of pathogenesis-related genes by subgroup-specific viral regulatory proteins may be associated with the onset of HAM/TSP. However, the associations between HAM/TSP susceptibility and *tax* subgroups still remain to be fully clarified. Moreover, to date, no research has observed subgroup-based differences in the ability of Tax to regulate transcription via independent mechanisms of direct transcriptional regulation nor compared HTLV-1 immunogenicity between subgroups A and B. Future research in these directions is eagerly anticipated to identify attractive targets for novel therapeutics for HAM/TSP.

#### **AUTHOR CONTRIBUTIONS**

MS wrote this review manuscript.

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# TRAF3 Is Required for NF-kB Pathway Activation Mediated by HTLV Tax Proteins

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Fochi S, Bergamo E, Serena M, Mutascio S, Journo C, Mahieux R, Ciminale V, Bertazzoni U, Zipeto D and Romanelli MG (2019) TRAF3 Is Required for NF-kB Pathway Activation Mediated by HTLV Tax Proteins. Front. Microbiol. 10:1302. doi: 10.3389/fmicb.2019.01302 Human T-cell leukemia viruses type 1 (HTLV-1) and type 2 (HTLV-2) share a common genome organization and expression strategy but have distinct pathological properties. HTLV-1 is the etiological agent of Adult T-cell Leukemia (ATL) and of HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP), whereas HTLV-2 does not cause hematological disorders and is only sporadically associated with cases of subacute myelopathy. Both HTLV genomes encode two regulatory proteins that play a pivotal role in pathogenesis: the transactivating Tax-1 and Tax-2 proteins and the antisense proteins HBZ and APH-2, respectively. We recently reported that Tax-1 and Tax-2 form complexes with the TNF-receptor associated factor 3, TRAF3, a negative regulator of the non-canonical NF-κB pathway. The NF-κB pathway is constitutively activated by the Tax proteins, whereas it is inhibited by HBZ and APH-2. The antagonistic effects of Tax and antisense proteins on NF-kB activation have not yet been fully clarified. Here, we investigated the effect of TRAF3 interaction with HTLV regulatory proteins and in particular its consequence on the subcellular distribution of the effector p65/RelA protein. We demonstrated that Tax-1 and Tax-2 efficiency on NF-κB activation is impaired in TRAF3 deficient cells obtained by CRISPR/Cas9 editing. We also found that APH-2 is more effective than HBZ in preventing Tax-dependent NF-kB activation. We further observed that TRAF3 co-localizes with Tax-2 and APH-2 in cytoplasmic complexes together with NF-kB essential modulator NEMO and TAB2, differently from HBZ and TRAF3. These results contribute to untangle the mechanism of NF-κB inhibition by HBZ and APH-2, highlighting the different role of the HTLV-1 and HTLV-2 regulatory proteins in the NF-κB activation.

Keywords: HTLV, NF-κB, Tax, HBZ, APH-2, TRAF3

#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus discovered. It is estimated that 10–20 million people are infected worldwide by this oncogenic virus. The majority of infected carriers remain asymptomatic, but 5–10% of infected subjects are at risk of developing either Adult T-cell Leukemia (ATL), a highly aggressive peripheral T-cell malignancy, or a chronic

neurodegenerative disorder called HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP) (Kannian and Green, 2010; Ishitsuka and Tamura, 2014; Bangham et al., 2015). HTLV-1 is genetically related to HTLV type 2 (HTLV-2), which is associated with lymphocyte proliferation and rare cases of subacute myelopathy but does not cause hematological disorders (Araujo and Hall, 2004; Bartman et al., 2008). The difference in the pathobiology of HTLV-1 and HTLV-2 might provide important clues to further understand the mechanisms leading to their distinct clinical outcomes. HTLV-1 and HTLV-2 express the transactivating proteins Tax-1 and Tax-2, respectively, which can immortalize human CD4+ T-cell (Cheng et al., 2012; Ciminale et al., 2014; Ma et al., 2016). Both Tax proteins activate viral RNA transcription from the LTR promoter (Journo et al., 2009), interact and modulate the expression of a wide range of cellular proteins and deregulate multiple cellular pathways (Romanelli et al., 2013). Among them, Tax proteins induce the hyper-activation of NF-κB transcription factors, thus altering the control mechanisms of cell proliferation and survival (Harhaj et al., 2007; Kfoury et al., 2012; Fochi et al., 2018). The mechanism of Tax-mediated NF-κB activation has been intensively studied in the past years showing that Tax-1 interacts with the regulatory IKK-γ subunit of IκB kinase complex, also known as NF-kB essential modulator (NEMO). This interaction results in the constitutive activation of IKKa and IKKβ, that leads to degradation of the inhibitor IκB and activation of the canonical NF-κB pathway (Xiao et al., 2006; Sun, 2017). Several other factors involved in the persistent activation of the NF-kB pathway have been found to interact with Tax-1, including TAB2 (TAK1 binding protein 2), TAX1BP (Tax1 binding protein 1), NRP/Optineurin, TRAF6 and CADM1 (cell adhesion molecule 1) (Avesani et al., 2010; Journo et al., 2013; Pujari et al., 2015). It has been demonstrated that persistent NF-κB activation by Tax-1 induces cell senescence (Kuo and Giam, 2006; Zhi et al., 2011) and this effect may be counteracted by the expression of the HTLV-1 antisense protein HTLV-1 bZIP factor (HBZ). HBZ is an inhibitor of the activation of 5'LTR promoter and of Tax-1-mediated transcription. HBZ is constitutively expressed in most ATL cases and enhances the proliferation of T-cells in vitro and in vivo (Satou et al., 2006). The combined action of Tax-1 and HBZ is considered relevant for the proliferation of HTLV-1 infected cells and persistent infection (Barbeau et al., 2013; Zhao, 2016). Several studies have demonstrated that HBZ and Tax-1 exert opposite functions in the deregulation of cellular signaling pathways that may help the virus to escape from immune surveillance (Gaudray et al., 2002; Zhao, 2016; Bangham and Matsuoka, 2017; Baratella et al., 2017; Karimi et al., 2017). HBZ selectively inhibits the canonical NF-κB pathway activated by Tax-1 together with the host transcription factor p65, by repressing the p65 ability to bind DNA (Zhao et al., 2009). Furthermore, HBZ reduces p65 acetylation and enhances its degradation through the PDLIM2 E3 ubiquitin ligase, resulting in the reduction of the expression of several NFκB target genes (Zhao et al., 2009; Wurm et al., 2012). Recently, Ma et al. (2017) have demonstrated that HBZ-mediated NF-κB inhibition contributes to the suppression of cyclin D1 gene expression, favoring the G1/S phase transition of the cell cycle.

HTLV-2 also expresses an antisense transcript, encoding APH-2 (antisense protein of HTLV-2) (Halin et al., 2009), which is widely expressed in vivo (Douceron et al., 2012). Unlike Tax-1 and Tax-2, which show a high degree of conservation, APH-2 shows less than 30% similarity to HBZ and does not contain a conventional basic leucine zipper domain. APH-2 is likewise able to inhibit Tax-2-mediated viral transcription by interacting with CREB (Halin et al., 2009; Yin et al., 2012), but its repressive activity is weaker compared to HBZ. It was recently reported that APH-2, like HBZ, represses p65 transactivation. However, APH-2 does not reduce the level of p65 expression nor induces its ubiquitination (Panfil et al., 2016). It is not yet established whether APH-2 inhibits Tax-2-mediated NF-κB activation. Of note, while both Tax-1 and Tax-2 activate the canonical NF-κB pathway, only Tax-1 activates the non-canonical one by recruiting NEMO and IKKα to p100 and promoting the release of p52/RelB active heterodimers into the nucleus (Shoji et al., 2009; Motai et al., 2016). These different mechanisms still need to be adequately addressed. We have recently demonstrated that both Tax-1 and Tax-2 interact with the TNF-receptor associated factor 3 (TRAF3), an adaptor protein that participates in the crosstalk between the type I interferon (IFN-I), the mitogen-activated protein kinase (MAPK) and the NF-κB pathways (Diani et al., 2015). TRAF3 positively regulates IFN-I production, while it inhibits the MAPK pathway and the non-canonical NF-κB pathway (Häcker et al., 2011). TRAF3 is a component of a multiprotein complex containing TRAF2 and the cellular inhibitor of apoptosis proteins cIAP1 and cIAP2, which restrict the activation of the non-canonical NF-κB pathway. TRAF3 also participates in the degradation of the alternative NF-κB inducing kinase NIK (Hauer et al., 2005; Vallabhapurapu et al., 2008; Zarnegar et al., 2008; Hildebrand et al., 2011), acting as a negative regulator of the non-canonical NF-κB pathway (Yang and Sun, 2015). The accumulation of NIK leads to IKKα activation and p100 processing to yield p52 (Sun, 2017). We have also demonstrated that the IFN-β promoter activation is increased when Tax-1 and TRAF3 are co-expressed with IKKE or TBK1 (Diani et al., 2015). The impact of TRAF3 on HTLV-mediated NF-κB activation has not yet been understood. In the present study, we demonstrate that TRAF3 plays a critical role in Tax-mediated NF-κB activation. We further show that APH-2, unlike HBZ, may form complexes with Tax-2 and key factors of the NF-κB signaling pathway, decreasing p65 nuclear translocation. These results can contribute to highlight a novel regulatory mechanism of NF-кВ activation mediated by HTLV proteins.

#### **MATERIALS AND METHODS**

#### **Cell Lines and Transfection**

HeLa, HEK293T, TRAF3 knock-out (TRAF3-KO) and U2OS cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), and Penicillin G (100 U/L)/Streptomycin (100 mcg/L). Jurkat T-cells (clone E6-1) were grown to a density of  $5 \times 10^5$  cells/mL in RPMI-1640 medium supplemented

with 10% FCS, L-glutamine (2 mM) and Penicillin G (100 U/L)/Streptomycin (100 mcg/L). All cell lines were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. For immunoprecipitation and confocal analysis,  $4\times10^5$  HEK293T and HeLa cells were seeded in 6-well plates. For transactivation studies,  $2\times10^5$  HEK293T cells were seeded in 12-well plates and transfected using TransIT®-LT1 transfection reagent (MIR2300, Mirus Bio), following the manufacturer's protocol. For confocal and transactivation analysis of Jurkat cells,  $2\times10^6$  cells were transfected by electroporation using the Neon Transfection System (Thermo Fisher Scientific), applying three pulsations of 10 ms at 1,325 V.

#### CRISPR/Cas9 Knockout of TRAF3

To induce the TRAF3 knockout, two guide RNA (gRNA) sequences 5'-AGCCCGAAGCAGACCGAGTG-3' 5'-TCTTGACACGCTGTACATTT-3' were designed to target exon 1 and exon 2 of the TRAF3 gene, respectively. gRNAs were selected using online tools1 (Ran et al., 2013). A third gRNA sequence, 5'-CCAGTTTTTGTCCCTGAACA-3' was selected to target exon 1 of TRAF3 gene (Chen et al., 2015). Potential off-target sites were predicted using the online tool "CHOPCHOP"<sup>2</sup> (Labun et al., 2016). Each selected gRNA was cloned independently, using the T4 DNA ligase (Promega) into the BbsI restriction sites of the pSpCas9(BB)-2A-Puro (PX459) V2.0 vector (#62988, Addgene) and transfected in HEK293T cells using the TransIT®-LT1 transfection reagent (MIR2300, Mirus Bio), following the manufacturer's protocol. Cells were selected using 0.5 µg/ml puromycin for 3 days after transfection. Clonal cell lines were isolated by limiting dilution and the absence of the TRAF3 protein was tested by western blot using an anti-TRAF3 specific antibody.

#### **Plasmids**

pJFE-Tax-1, pJFE-Tax-2B full length expression vectors and the plasmid constructs expressing Tax-M22 and Tax-1 K1-10R mutants have been previously described (Turci et al., 2006, 2012). pFlag-APH-2, pGFP-APH-2, pFlag-HBZ, expression plasmids were kindly provided by Dr. Sheehy (Marban et al., 2012). pRSV-RelA/p65, pCMVF-TAB2 and Flag-TRAF3, HA-TRAF3 expression vectors have been previously described (Avesani et al., 2010; Diani et al., 2015). pEF-p52 was kindly provided by Dr. Matsuoka. pcDNA3-VSV-APH-2, pSG5M-Tax2-His, His-HBZ have been previously described (Journo et al., 2013; Dubuisson et al., 2018). pSpCas9(BB)-2A-Puro (PX459) V2.0 vector was purchased from Addgene. NF-κB-Luc and phRG-TK plasmids have been previously described (Bergamo et al., 2017).

#### **Antibodies**

The following primary antibodies were used: mouse monoclonal anti-Flag M2 (F3165, Sigma-Aldrich), rabbit polyclonal anti-Flag (F7425, Sigma-Aldrich), mouse monoclonal anti-VSV (V5507, Sigma-Aldrich), rabbit polyclonal anti-VSV (V4888, Sigma-Aldrich), goat polyclonal anti-His (ab9136, Abcam), rabbit

polyclonal anti-HA (H6908, Sigma-Aldrich), mouse monoclonal anti-HA (16B12, Covance), rabbit polyclonal anti IκB-α (#9242, Cell Signaling), rabbit polyclonal anti-TRAF3 (18099-1-AP, ProteinTech), mouse monoclonal anti-p65 (C-20) (sc-372, Santa Cruz), rabbit polyclonal anti-p65 (MAB3026, Merck Millipore), mouse monoclonal anti-IKKy (611306, BD Bioscience), rabbit monoclonal anti-NF-κB2 p100/p52 (#3017, Cell Signaling Technology), mouse monoclonal anti-β-tubulin (bsm-33034M, Bioss Antibodies), mouse monoclonal anti-Tax-1 derives from hybridoma 168-A51 (AIDS research and Reagent Program, National Institutes of Health), rabbit polyclonal anti-Tax-2 has been previously described (Turci et al., 2012). Horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG (31430, Thermo Fisher Scientific) and anti-rabbit IgG (31460, Thermo Fisher Scientific) were used in western blotting. The secondary antibodies anti-rabbit488 (ab98488, Abcam), anti-goat488 (ab150129, Abcam), anti-mouse549 (DI-2549, Vector Lab), anti-goat TexasRed (705-075-147, Jackson Imm. Res.), (ab150107, Abcam), anti-rabbit649 (STAR36D649, AbD serotec) were used in immunofluorescence.

#### **Co-immunoprecipitations**

HEK293T cells were harvested 24 h after transfection with 1  $\mu g$  of each expression vector. Cells were lysed in non-denaturing buffer (10 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% TritonX-100) supplemented with protease inhibitors Complete Protease Inhibitor Cocktail EDTA-free (Roche). Lysates were sonicated twice for 5 s, frozen at  $-80^{\circ}\mathrm{C}$  for 1 h and then centrifuged for 30 min at 14000 rpm at 4°C. Proteins were subjected to co-immunoprecipitation with the appropriate primary antibodies overnight at 4°C. Immunocomplexes were linked to magnetic beads of Dynabeads Protein G or A (LifeTechnologies) for 30 min at 4°C. The beads were then washed 3 times and resuspended in elution buffer containing NuPAGE loading buffer (LifeTechnologies) and 0.25 mM DTT.

#### **Western Blotting**

Total protein concentration in cell lysates was determined by Bradford Coomassie brilliant blue assay (Sigma-Aldrich). Equal amounts of cellular proteins were resolved in SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (GE Healthcare). Membranes were first saturated in TBS solution containing 5% non-fat milk and 0.1% Tween20, and then incubated with specific primary and secondary antibodies. Anti- $\beta$ -tubulin was used as loading control. Bound antibodies were revealed using ECL prime western blotting detection reagent (GE Healthcare), according to the manufacturer's instructions. Densitometry analysis of western blot protein bands was performed using the GelQuant. NET software provided by http://biochemlabsolutions.com.

#### **Luciferase Reporter Assay**

Luciferase functional quantitative assay was performed as previously described (Bergamo et al., 2017). Briefly, HEK293T, TRAF3-KO and Jurkat cells were transfected with 500 ng of the NF-κB-Luc reporter plasmid together with the appropriate expression vectors or an empty vector. Transfection efficiency

<sup>1</sup> http://crispr.mit.edu

<sup>&</sup>lt;sup>2</sup>http://chopchop.cbu.uib.no/

was normalized using 100 ng of phRG-TK plasmid (Renilla luciferase vector). TNF- $\alpha$ -induced NF- $\kappa$ B-activation was analyzed following transient expression of a NF- $\kappa$ B-luciferase reporter vector and cell stimulation with 25 ng/mL TNF- $\alpha$  for 18 h. Luciferase activity was assayed 24 h post-transfection using the Dual-Luciferase reporter assay system (Promega). Levels of Firefly and Renilla luciferase were measured with a 20/20n Single Tube Luminometer (Promega). Experiments were repeated at least three times and luciferase activity was measured after deduction of the activity levels with the promoter alone.

#### Fluorescence Microscopy

HeLa, HEK293T, TRAF3-KO, U2OS and Jurkat cells were transfected with 1 or 2 µg of each appropriate expression vector; p65 protein nuclear translocation was analyzed following 25 ng/mL TNF-α cell stimulation for 20 min. Cells were seeded on cover glasses and 24 h post-transfection HeLa, HEK293T, TRAF3-KO, and U2OS cells were fixed with 4% paraformaldehyde/PBS, whereas Jurkat cells were fixed with formalin (HT5011, Sigma-Aldrich), for 20 min. Cells were then permeabilized with 0.5% Triton X-100/PBS for 20 min, blocked with a 5% milk/PBS solution and incubated with appropriate primary and conjugated secondary antibodies. The cover glasses were then mounted in DAPI-containing Fluoromount-G (Southern Biotech). For confocal analyses, slides were examined under a LSM800 (Carl Zeiss MicroImaging) confocal microscope equipped with 63× 1.4 plan apochromat oil-immersion objective using the ZEN software. For epifluorescence imaging, slides were examined under an AxioImager.Z1 microscope (Zeiss) using the Methamorph software. To quantify p65 nuclear translocation, the ratios of the nuclear/total p65 mean brightness staining were calculated using ImageJ software. RGB profiles were calculated using the RGB profiler plugin.

## RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from HEK293T and TRAF3-KO cells using TRIzol  $^{\rm TM}$  (Invitrogen), according to the manufacturers' recommendations. RNA was quantified by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and absorbance ratio at 260/280 and 260/230 were measured. Total RNA (1  $\mu g$ ) was reverse transcribed at 50°C for 45 min using oligo

dT primers and the SuperScript<sup>TM</sup> III First-Strand Synthesis System (Invitrogen).

PCR reactions were performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) and ran on CFX Connect<sup>TM</sup> Real-Time Detection System (Bio-Rad). Primer pairs for TNF Alpha Induced Protein 3 (TNFAIP3/A20), Apoptosis Regulator Bcl-2 (BCL2), Interleukin 6 (IL-6), and Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) gene expression analysis are listed in **Table 1**. PCR cycles included an initial step of 10 min at 95°C required for enzyme activation, followed by 40 cycles of amplification: denaturation for 15 s at 95°C, annealing and extension for 60 s at 60°C. The relative fold change values were calculated using the  $2^{-\Delta \Delta Ct}$  method (Livak and Schmittgen, 2001). RPLP0 was used as internal reference gene.

#### **Statistical Analyses**

All data are presented as the means  $\pm$  standard deviation from at least three independent experiments. Statistical significance was assessed by the Student's t-test. Differences were considered to be significant when P < 0.05 (\*), and strongly significant when P < 0.01 (\*\*), and P < 0.001 (\*\*\*).

#### **RESULTS**

## TRAF3 Is Required for an Efficient Activation of NF-κB Mediated by Tax

It has been shown that ATL cells exhibit high expression levels of NIK, a positive regulator of the NF-κB pathway and that NIK turnover is regulated by protein complexes that contain the E3 ubiquitin ligase TRAF3 (Chan and Greene, 2012). Based on our previous data showing that TRAF3 interacts with both Tax-1 and Tax-2 (Diani et al., 2015), we investigated the contribution of TRAF3 in the modulation of NF-κB mediated by the viral regulatory proteins. We produced TRAF3-KO HEK293T cell lines introducing insertion/deletion (indel) mutations by transfection of TRAF3 RNA-guided Cas9 nuclease expressing plasmid. TRAF3-KO clones were first characterized for their ability to activate an NF-kB promoter. Using the luciferase reporter assay, we first found that in the absence of any stimulus, the basal NF-kB promoter activity was increased 50 folds in TRAF3-KO cell lines as compared to wild type cells (WT) (Figure 1A). The luciferase activity was restored by the rescue of TRAF3 expression in transfected TRAF3-KO

TABLE 1 | qRT-PCR primer sequences.

Gene	Accession no.	Primer sequence (5' -≥ 3')	PCR product (pb)
TNFAIP3 (A20)	NM_001270507.1	F: CTGGGACCATGGCACAACTC R: CGGAAGGTTCCATGGGATTC	182
BCL2	NM_000633.2	F: GATGTGATGCCTCTGCGAAG R: CATGCTGATGTCTCTGGAATCT	92
IL6	NM_000600.4	F: TACATCCTCGACGGCATCTC R: TGGCTTGTTCCTCACTACTCT	247
RPLP0	NM_001002.4	F: ACATGTTGCTGGCCAATAAGGT R: CCTAAAGCCTGGAAAAAGGAGG	127

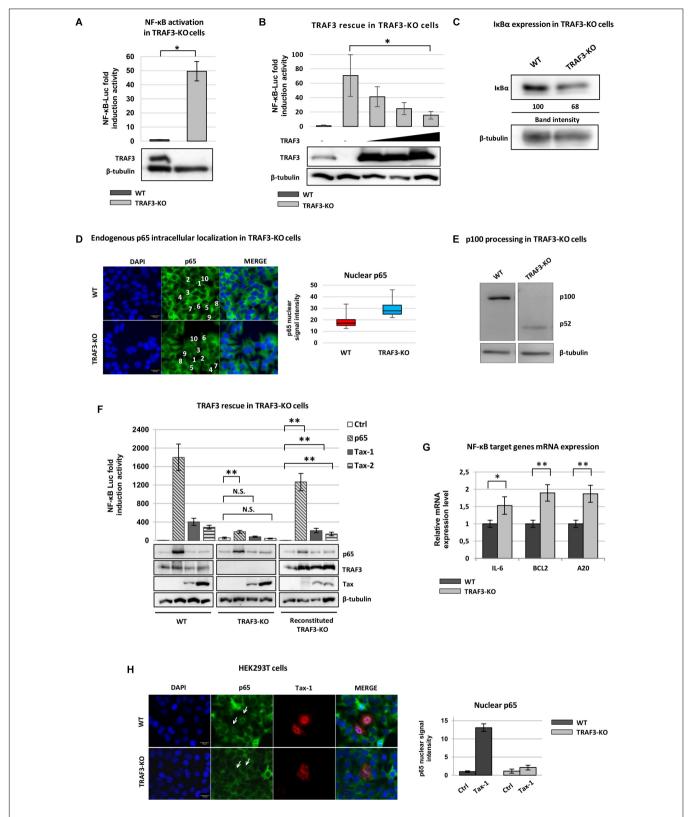


FIGURE 1 | TRAF3 is required for an efficient activation of NF-κB mediated by Tax. (A) Luciferase levels were measured from HEK293T (WT) and TRAF3-KO cells transfected with phRG-TK renilla vector (control for transfection efficiency) and NF-κB-luciferase reporter expressing vector. (B) Luciferase levels were measured from HEK293T (WT) and TRAF3-KO cells transfected with phRG-TK renilla vector, NF-κB-luciferase reporter and increasing amount of Flag-TRAF3 expressing vector. (Continued)

#### FIGURE 1 | Continued

(C) HEK293T (WT) and TRAF3-KO cells were lysed and immunoblot analysis was performed to compare the levels of endogenous  $I_KB$ . The amount of  $I_KB$  was measured relative to the amount of β-tubulin. (D) HEK293T (WT) and TRAF3-KO cells were stained with anti-p65 (green signal) primary antibody. Nuclei were stained with DAPI (blue signal). Scalebar, 10 µm. Total cells and nuclei of cells (n = 10) indicated by a number from 1 to 10 in white were delineated using ImageJ software, and the mean brightness ratio of the nuclear ReIA/p65 staining was calculated and presented as box-plot. (E) Immunoblot analysis of p100/p52 proteins expression levels in HEK293T (WT) and TRAF3-KO cells. (F) HEK293T (WT) and TRAF3-KO cells were transfected with phRG-TK renilla vector, NF- $I_K$ B-luciferase reporter and p65, Tax-1, Tax-2, or TRAF3 expression plasmids. Cell lysates were collected and luciferase levels were measured. Immunoblot analysis was performed to detect of TRAF3, p65, Tax-1 and Tax-2 (N.S. = not significant). (G) RNA from HEK293T (WT) and TRAF3-KO cells were analyzed by qRT-PCR for NF- $I_K$ B-related transcripts. The values were normalized to RPLP0 and the results were expressed as relative fold change of expression levels. (H) HEK293T (WT) and TRAF3-KO cells were transfected with Tax-1 encoding vector and stained with anti-p65 (green signal) and anti-Tax-1 (red signal) primary antibody. Nuclei were stained with DAPI (blue signal). Total cells and nuclei were delineated using ImageJ software, and the mean brightness ratio of the nuclear p65 staining was calculated (right panel). Scalebar, 10  $I_I$ M. P < 0.05 (\*) and P < 0.01 (\*\*).

cells (Figure 1B). The basal activation of NF-κB in TRAF3-KO cells was further confirmed by analyzing IkB protein level. As expected, we measured a reduced amount of IkB (30% decrease) in TRAF3-KO cells as compared to WT cells (Figure 1C). When the basal endogenous p65 distribution was analyzed in TRAF3-KO cells, the presence of p65 in the nucleus, even if limited to a weak signal, was observed (Figure 1D). The mean brightness ratio of the nuclear p65 staining was increased 1.5 fold in the TRAF3-KO cells compared to the WT. This indicates that the canonical NF-kB pathway is constitutively although partially activated in the absence of TRAF3. To define if the alternative NF-κB pathway is also activated in the absence of TRAF3, the processing of the p100 protein was analyzed. As shown in Figure 1E, accumulation of p52 was detectable in the TRAF3-KO cell line, but not in the WT cells. These results showing that the depletion of TRAF3 enables detectable translocation of p65 into the nucleus, as well as p100 processing, suggest that both the canonical and non-canonical NF-κB may be partially activated.

When we analyzed the NF-kB activation induced by the expression of p65 in TRAF3-KO cells, we unexpectedly measured a limited induction compared to WT cells (about 9-fold decrease). A similar dramatic reduction of NF-kB induction by Tax-1 or Tax-2 was observed in TRAF3-KO cells compared to WT cells (about 5-fold and 6-fold decrease, respectively) (Figure 1F). Reconstituted TRAF3 expression restored the efficiency of p65- and Tax-mediated activation. In order to explore the mechanism that impairs the full activation of NF-κB mediated by p65 in the absence of TRAF3, we analyzed TNF- $\alpha$ -mediated induction of the NF-κB promoter in TRAF3-KO cells. After TNF-α stimulation, we measured a significant NF-κB activation in TRAF3-KO cells, although lower when compared to WT cells (Supplementary Figure 1A). When we analyzed p65 nuclear translocation induced by TNF-α, we observed p65 nuclear translocation in WT and TRAF3-KO cells (Supplementary **Figure 1B**), indicating that the NF-κB pathway was activated, although the resulting transcriptional activity of p65 was reduced.

To explore the possibility that in TRAF3-KO cells the basal activation of NF-κB may lead to the expression of NF-κB target genes that control p65 activity by negative feedback loops, we analyzed the expression of A20 and BCL2 genes, which are well known negative regulators of NF-κB (Grimm et al., 1996; Pujari et al., 2013). We found that both genes were significantly more expressed in TRAF3-KO cells compared to WT suggesting

that limited p65-mediated induction of NF-κB in TRAF3-KO cells can be due to negative feedback mechanisms (**Figure 1G**). Furthermore, the results showed an increased expression of IL-6, an NF-κB target gene (Libermann and Baltimore, 1990) (**Figure 1G**) compared to the WT cells.

In agreement with the results showing that Tax-1 mediated NF-κB activation is impaired in TRAF3-KO cells, we observed that Tax-1 failed to induce endogenous p65 nuclear translocation in transfected cells (**Figure 1H**). Taken together, these results strongly suggest that NF-κB hyper-activation by Tax is strictly dependent upon TRAF3 expression.

## HBZ and APH-2 Interact With p65 and Suppress the NF-κB Pathway

Since our results indicate that TRAF3 is involved in Tax-induced NF- $\kappa$ B hyper-activation, and it is known that HBZ and APH-2 interfere with NF- $\kappa$ B signaling, we aimed to analyze whether the antisense proteins might interfere with TRAF3 functions. Before analyzing the specific interplay of HBZ and APH-2 with TRAF3, we first aimed at precisely comparing their ability of antagonize Tax-induced NF- $\kappa$ B activation.

HEK293T cells transfected with increasing amounts of Tax proteins in the presence or absence of HBZ or APH-2 were analyzed by luciferase assay (Figures 2A,B). As expected, when HBZ was present together with low levels of Tax-1, the NF-κB activation was significantly reduced (Figure 2A, lanes 2 vs. 5), whereas no HBZ inhibitory effect was evident when Tax-1 was expressed at high levels in transfected cells (Figure 2A, lane 3 vs. 6). APH-2 effect on Tax-mediated NF-кВ activation was then analyzed (Figure 2B). Similar to HBZ, APH-2 inhibited Tax-2-induced NF-κB activity, but unexpectedly and unlike Tax-1, high expression of Tax-2 did not restore the activation of NF-κB (Figure 2B). Of note, this inhibitory effect of APH-2 was evident despite the fact that APH-2 was less expressed than HBZ in transfected cells, as previously reported (Panfil et al., 2016; Dubuisson et al., 2018). Because APH-2 appeared to be more potent than HBZ in the inhibition of Tax-mediated NF-κB activation, the effect of HBZ and APH-2 expression on the NF-κB promoter activation mediated by p65 over-expression was then compared in HEK293T cells. The inhibition was statistically significant for both proteins and reached similar levels (Figures 2C,D). The results were also reproduced in Jurkat cells in the presence of APH-2 (**Supplementary Figure 2A**).

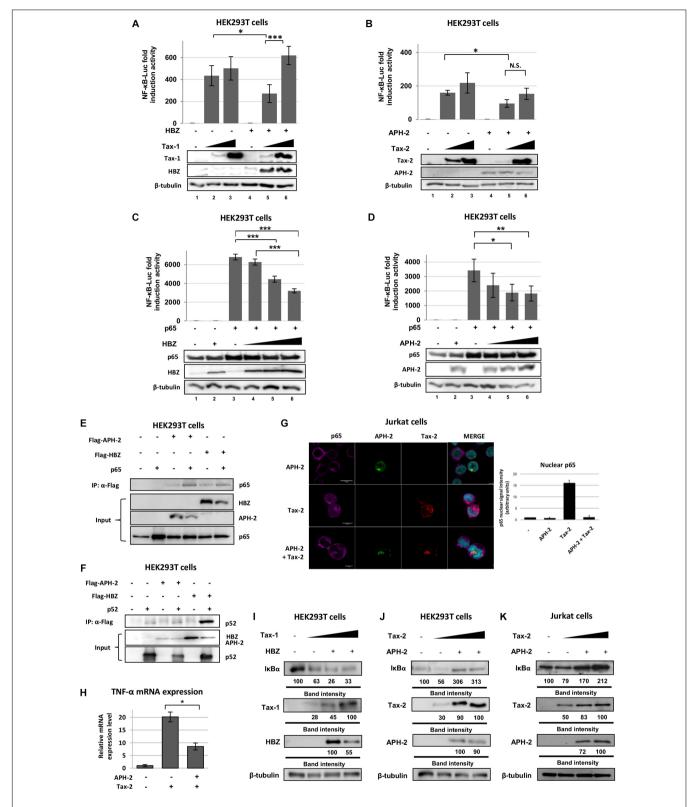


FIGURE 2 | HBZ and APH-2 interact with p65 and suppress the NF- $\kappa$ B pathway. HEK293T cells were transfected with phRG-TK renilla vector, NF- $\kappa$ B-luciferase reporter, Flag-HBZ and increasing amounts of Tax-1 (A) or Flag-APH-2 and increasing amounts of Tax-2 expression plasmids (B) (N.S. = not significant). HEK293T cells were transfected with phRG-TK renilla vector, NF- $\kappa$ B-luciferase reporter, p65 expression plasmid, and increasing amounts of Flag-HBZ (C) or Flag-APH-2 (D) expression vectors. Cell lysates were collected and luciferase levels were measured (Top). Immunoblot analysis was performed to detect the expression levels of the (Continued)

#### FIGURE 2 | Continued

transfected proteins (Bottom). **(E,F)** HEK293T cells were transfected with p65 or p52, Flag-APH-2, or Flag-HBZ expression vectors. Tagged proteins were immunoprecipitated with an anti-Flag antibody and the presence of the p65 or p52 proteins was examined by western blot. **(G)** Jurkat cells were transfected with VSV-APH-2 and Tax-2 expression vectors. Samples were stained with anti-VSV, anti-Tax-2 and anti-p65 primary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and endogenous p65 (magenta signal), respectively. Total cells and nuclei were delineated using ImageJ software, and the mean brightness ratio of the nuclear p65 staining was calculated (right panel). Values obtained in non-transfected cells were then subtracted from those for APH-2, Tax or APH-2+Tax-expressing cells. Scalebar, 10  $\mu$ m. **(H)** TNF- $\alpha$  expression was analyzed by qRT-PCR from RNA of HEK293T cells transfected with VSV-APH-2 and Tax-2. The values were normalized to RPLP0 and the results were expressed as relative fold change of expression levels. **(I–K)** HEK293T and Jurkat cells were co-transfected with Flag-APH-2 and increasing amount of Tax-1 or with Flag-APH-2 and increasing amount of Tax-2. Immunoblot analysis was performed to compare the levels of endogenous lkB in the presence of the viral regulatory proteins. The amount of lkB was measured relative to the amount of  $\beta$ -tubulin. P < 0.05 (\*), P < 0.01 (\*\*), and P < 0.001 (\*\*\*).

To further compare the mechanism of NF-κB inhibition by both antisense proteins, we then analyzed the interaction of HBZ and APH-2 with the transcription factors p65 and p52 by immunoprecipitation. Figure 2E shows that p65 was detected in complexes with both proteins, indicating that both antisense proteins interact with the classical transcription factor p65. When HBZ and APH-2 interaction with p52 was analyzed, it revealed that HBZ was present in complex with p52, unlike APH-2 (Figure 2F). These results suggested that HBZ and APH-2 share the ability to form complexes with p65, but they differ in their interaction with p52, the final effector of the alternative NF-kB signaling. Given that p65 translocates into the nucleus when Tax is expressed, the cellular localization of p65 in the presence of APH-2 and/or Tax-2 was analyzed. The results showed that endogenous p65 localized in the cytoplasm in the presence of APH-2 (Figure 2G). Interestingly, it was found that the co-expression of APH-2 and Tax-2 resulted in their cytoplasmic co-localization and in the impairment of p65 nuclear translocation (5-fold decrease) (Figure 2G). The same results were observed in U2OS cells (Supplementary Figure 2B). We performed qRT-PCR analysis in the presence of Tax-2 and APH-2, analyzing the expression of TNF- $\alpha$  as an example of an NF-κB target gene. In the presence of APH-2 and Tax-2, TNF-α expression was reduced more than 10-fold (Figure 2H). We then analyzed the effect of HBZ and APH-2 on IkB degradation, which allows p65 nuclear translocation and which is known to be induced by Tax (Harhaj and Harhaj, 2005). As expected, the expression of IkB was reduced to about 40% in the presence of Tax-1 and 80% in the presence of both Tax-1 and HBZ proteins (Figure 2I), whereas in the presence of both APH-2 and Tax-2 proteins, an increase in IkB expression was observed (Figure 2J). A similar result was obtained in Jurkat cells (Figure 2K). Taken together, these results suggest that while HBZ and APH-2 both inhibit Tax- and p65-mediated NF-κB activation, they may differ in their inhibition mechanism. APH-2 may inhibit p65 translocation by affecting IkB degradation, thus limiting Tax-2-mediated NF-κB activation.

## APH-2 Is Recruited to Cytoplasmic Structures in the Presence of Tax-2

To investigate how APH-2 might inhibit NF-κB activity upstream of IκB degradation, we analyzed APH-2 subcellular localization. Indeed, it has previously been reported that while Tax-1 does not interact with HBZ, Tax-2 does interact with APH-2 (Zhao et al., 2009; Marban et al., 2012). Given the different

cellular distribution of Tax-1 and Tax-2 (Meertens et al., 2004; Bertazzoni et al., 2011), it is possible that HBZ and APH-2 differ in the mechanism of NF-κB inhibition due to their differential recruitment by host factors in subcellular compartments. To test this hypothesis, HEK293T cells were transfected with Tax-1 or Tax-2 and Flag-HBZ or Flag-APH-2 expressing vectors, and the presence of Tax in the immunocomplexes precipitated with an anti-Flag antibody was analyzed (Figure 3A). The results, as expected, confirmed that Tax-2 formed complexes with APH-2, but no interaction of Tax-1 with HBZ was observed. Confocal microscopy analyses in transfected HeLa cells showed that HBZ alone localized in the nucleus and that when Tax-1 was co-expressed with HBZ, no co-localization was observed (Figure 3B). Interestingly, the co-expression of APH-2 and Tax-2 resulted in APH-2 redistribution and its co-localization with Tax-2 in cytoplasmic structures (Figure 3C). This result was also reproduced in Jurkat cells (Figure 3D). To ensure that recruitment of APH-2 in cytoplasmic structures is a property of Tax-2 but not of Tax-1, HEK293T cells were transfected with both APH-2 and Tax-1, or with both HBZ and Tax-2 vectors and their intracellular localization was analyzed by fluorescent microscopy. We observed that Tax-1 and APH-2 did not co-localize in the cytoplasm (Supplementary Figure 3A). In the presence of HBZ, Tax-2 was mainly distributed in the cytoplasm and HBZ in the nucleus (Supplementary Figure 3B). Taken together, these results suggest that HBZ and APH-2 differ in their subcellular distribution when Tax proteins are expressed and that the cytoplasmic co-localization occurs only in the presence of Tax-2 and APH-2. This might explain how HBZ and APH-2 differ in the mechanism of NF-kB inhibition.

## APH-2 Is Present in Cytoplasmic Complexes With TAB2, NEMO, and Tax-2

We have previously shown that Tax-2 co-localizes in the cytoplasm with the NF-κB pathway factors TAB2, NEMO and p65 (Avesani et al., 2010). Given that APH-2 co-localizes with Tax-2 in the cytoplasm, we speculated that APH-2 may be recruited in complexes containing these NF-κB factors. To test this hypothesis, the co-localization of APH-2 with TAB2 and NEMO in Jurkat cells was analyzed by confocal microscopy (**Figure 4**). The results showed a redistribution of TAB2 in cytoplasmic structures in the presence of Tax-2 and APH-2. TAB2 partially co-localized with APH-2 in the absence of Tax-2 (**Figure 4A**). APH-2 co-localized with NEMO only in the presence of Tax-2 (**Figure 4B**). The co-localization of

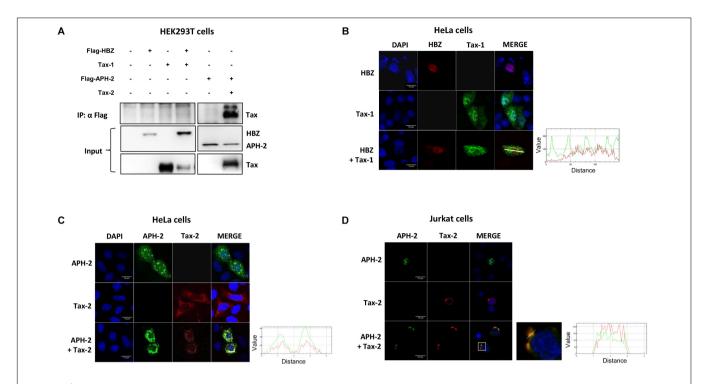


FIGURE 3 | APH-2 is recruited to cytoplasmic structures in the presence of Tax-2. (A) HEK293T cells were transfected with Tax-1 and Flag-HBZ or Tax-2 and Flag-APH-2 expression vectors. Tagged proteins were immunoprecipitated with an anti-Flag antibody and the presence of Tax protein was examined by western blot. (B,C) HeLa cells were transfected with Tax-1 and Flag-HBZ or Tax-2 and GFP-APH-2 expression plasmids. Cells were stained with anti-Flag and anti-Tax-1 primary antibodies to detect HBZ (red signal) and Tax-1 (green signal), respectively; anti-Tax-2 primary antibody was used to detect Tax-2 (red signal) whereas APH-2 is fused to a GFP protein (green signal). (D) Jurkat cells were transfected with VSV-APH-2 and Tax-2 vectors. Cells were stained with anti-VSV, anti-Tax-2 primary antibodies to detect APH-2 (green signal) and Tax-2 (red signal), respectively. Co-localization of Tax-2 signal with APH-2 signal was calculated using Mander's coefficient (M<sub>1</sub> = 0.91; M<sub>2</sub> = 0.76). Nuclei were stained with DAPI (blue signal). Enlargements are shown next to the "Merge" panel. The intensity of fluorescence along the white line drawn on the merged images is plotted in the diagrams. Scalebar, 10  $\mu$ m.

Tax-2 and APH-2 with NEMO was also reproduced in U2OS cells (**Supplementary Figure 4A**). Taken together, these results suggest that in the presence of Tax-2, APH-2 localized in the cytoplasm with TAB2 and NEMO.

## HBZ and APH-2 Interfere With TRAF3 Functions

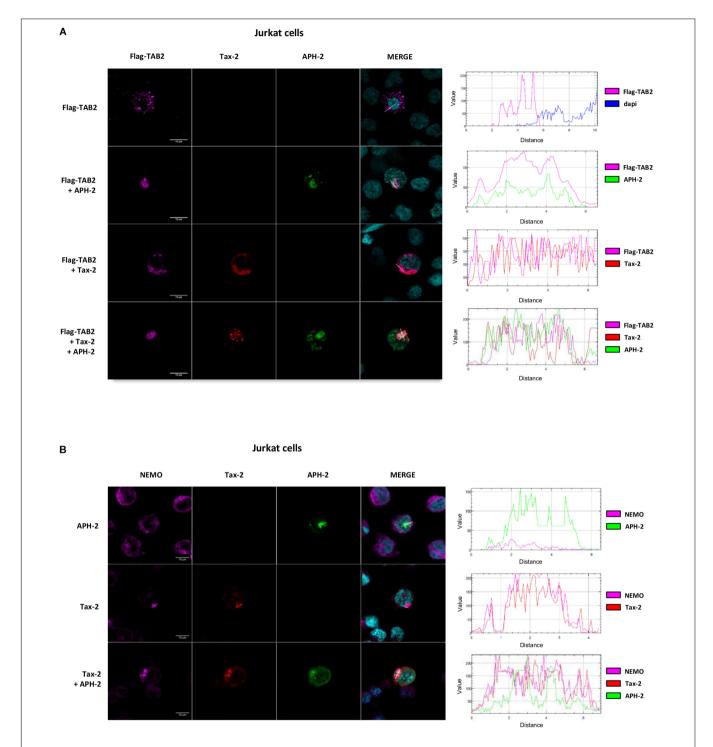
Since the antisense proteins HBZ and APH-2 both repress NF-κB activity, we investigated the possible recruitment of HBZ and APH-2 in cytoplasmic complexes containing TRAF3. We analyzed TRAF3 distribution in the presence of viral antisense proteins by confocal microscopy in Jurkat cells. We found that HBZ (green signal) and TRAF3 (magenta signal) did not co-localize, whereas TRAF3 partially co-localized with APH-2 in the cytoplasm (Figure 5A). We confirmed that TRAF3 is present in immunocomplexes containing APH-2 by co-immunoprecipitation (Figure 5B). Furthermore, we detected TRAF3 in complexes containing both APH-2 and Tax-2 (Figure 5B). We analyzed by confocal microscopy TRAF3 distribution in the presence of APH-2 and Tax-2, and we found that APH-2 (green signal), TRAF3 (magenta signal) and Tax-2 (red signal) co-localized in the cytoplasm (Figure 5C). Co-localization of Tax-2 and APH-2 with TRAF3 was also observed in U2OS cells (Supplementary Figure 4B). This

indicates that APH-2, but not HBZ, could modulate Tax-2 induced NF- $\kappa$ B activation by associating with Tax-2 and TRAF3 complexes.

To assess whether HBZ may deregulate the NF-κB pathway by affecting the expression of TRAF3, as it was previously shown with p65, HEK293T cells were transfected with increasing amounts of HBZ and the expression levels of TRAF3 was evaluated. The results demonstrated a reduction of the expression of TRAF3 protein in the presence of HBZ and APH-2 (**Figure 5D**). A reduced expression of endogenous TRAF3 was also found in the presence of increasing amounts of HBZ and APH-2 (**Supplementary Figures 5A,B**). These results suggest that TRAF3 may be involved in the mechanisms of NF-κB deregulation mediated by HTLV proteins and that its expression may be altered by both HBZ and APH-2 proteins.

#### **DISCUSSION**

Dysregulation of the NF- $\kappa$ B pathway can induce alterations of physiological cellular responses and can lead to oncogenesis (Park and Hong, 2016). It has been demonstrated that persistent NF- $\kappa$ B activation derived from Tax expression induces cell senescence, and the inhibitory effect of the antisense protein HBZ on NF- $\kappa$ B may represent an adaptation of HTLV-1 that contributes to



**FIGURE 4** | APH-2 is present in cytoplasmic complexes with TAB2, NEMO, and Tax-2. Jurkat cells were transfected with VSV-APH-2, Tax-2 and Flag-TAB2 expression vectors. Samples were stained with anti-VSV, anti-Tax-2, anti-Flag and anti-NEMO primary antibodies to detect APH-2 (green signal), Tax-2 (red signal), TAB2 (magenta signal), and NEMO (magenta signal), respectively **(A,B)**. Nuclei were stained with DAPI (cyan signal). The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams. Scalebar, 10  $\mu$ m.

viral persistence and, in the long term, to ATL development (Giam and Semmes, 2016). It has also been reported that the inhibition of the NF- $\kappa$ B pathway results in the induction of apoptosis of ATL cells (Watanabe et al., 2005). The HTLV

regulatory proteins interaction with canonical NF- $\kappa$ B factors leading to NF- $\kappa$ B dysregulation has been intensively investigated, but limited information is available about the interaction with factors that play a critical role in the non-canonical pathway

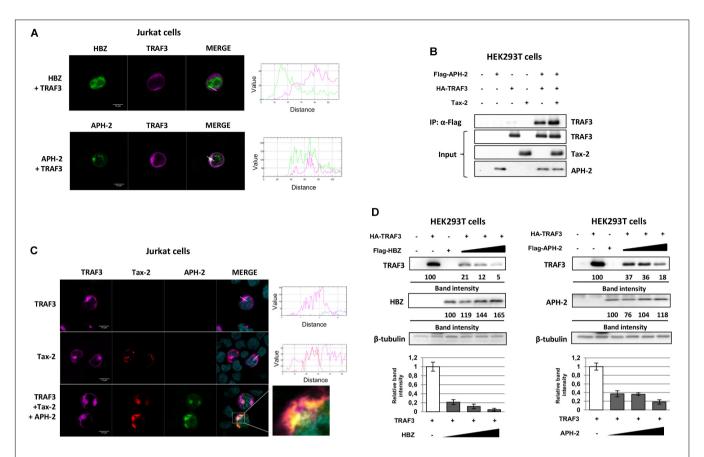


FIGURE 5 | HBZ and APH-2 interfere with TRAF3 functions. (A) Jurkat cells were transfected with Flag-HBZ or VSV-APH-2 expression vectors. Samples were stained with an anti-TRAF3 and anti-Flag or anti-VSV primary antibodies to detect TRAF3 (magenta signal) and HBZ or APH-2 (green signal), respectively. (B) HEK293T cells were transfected with HA-TRAF3, Tax-2 and Flag-APH-2 expression vectors. Tagged proteins were immunoprecipitated with an anti-Flag antibody and the presence of the TRAF3 protein was examined with an anti-HA primary antibody, by western blot. (C) Jurkat cells were transfected with VSV-APH-2 and Tax-2 expression vectors. Samples were stained with anti-VSV, anti-Tax-2 and anti-HA primary antibodies to detect APH-2 (green signal), Tax-2 (red signal), and TRAF3 (magenta signal), respectively. Nuclei were stained with DAPI (cyan signal). The intensity of fluorescence overlapping the white line drawn on the merged images is plotted in the diagrams. Enlargements are shown bottom the "Merge" panel. Scale bar, 10 μm. (D) HEK293T were co-transfected with HA-TRAF3 and increasing amount of Flag-HBZ or Flag-APH-2. Immunoblot analysis (top) was performed and the amount of TRAF3, HBZ and APH-2 was measured relative to the amount of β-tubulin (bottom).

(Schmitz et al., 2014; Fochi et al., 2018). We focused our attention on TRAF3, a ubiquitin ligase that controls the expression levels of the NIK kinase and that has been demonstrated to be abnormally expressed in freshly isolated ATL cells (Watanabe, 2017). TRAF3-deficient mice display increased non-canonical NF-κB activation as well as increased B cell survival, suggesting that TRAF3 may act as a negative regulator of the NF-κB signaling in vivo (Gardam et al., 2008). It has been demonstrated that TRAF3 knockout results in NIK stabilization, inducing p100 processing (Liao et al., 2004; He et al., 2006). In TRAF3-deficient cell lines, we found that the NF-kB pathway is activated and that both a partial nuclear distribution of the transcriptional factor p65 and p100 processing are taking place. These results confirm that TRAF3 is a negative regulator of the NF-κB pathway, but it may also participate to the functional regulation between the two NF-kB pathways. We also found that p65 and TNF-α partially induced NF-κB activation in TRAF3-KO cell lines. This effect may be explained by negative feedback loop mechanisms derived by the expression of NF- $\kappa B$  target genes acting as negative regulators. This hypothesis is supported by the qRT-PCR analyses of A20 and BCL-2 genes in TRAF3-KO cells, showing that they are over-expressed as compared to WT cells. It is known that A20 mediates a negative regulation of the canonical NF-κB pathway (Pujari et al., 2013) and BCL-2 overexpression specifically represses NF-κB-dependent transactivation by attenuating the transactivation potential of p65 (Grimm et al., 1996). These results are consistent with previous data showing that TRAF3 depletion causes an accumulation of distinct subsets of NF-κB inhibitors, including A20 (Bista et al., 2010).

We have previously shown that Tax interacts with TRAF3 and here we demonstrate for the first time that the TRAF3 factor is required for an efficient NF- $\kappa$ B activation by both Tax-1 and Tax-2.

Unlike Tax-1, Tax-2 cannot induce p100 processing to p52, but can activate the canonical NF- $\kappa$ B pathway as well as Tax-1 (Xiao et al., 2001; Higuchi et al., 2007). We found that Tax-mediated activation of NF- $\kappa$ B is impaired in TRAF3-deficient cells and

that it is restored only after rescuing TRAF3 expression. A similar essential role of TRAF3 has been demonstrated for the Epstein-Barr virus-encoded oncoprotein latent membrane protein 1 (LMP1). In fact, LMP1 alone can transform rodent fibroblasts and activate NF-κB (Cahir McFarland et al., 1999), but in the absence of TRAF3, LMP1-induced activation of JNK, p38, and NF-κB are impaired (Xie et al., 2004). It has been also demonstrated that the vFlip protein expressed by Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) activates NF-κB interacting with the IKK complex and the TRAF2/3 complex (Field et al., 2003). Interestingly, we found that TRAF3 is required not only for Tax-1, but also for Tax-2 NF-κB activation, supporting a mechanism in which TRAF3 may be involved in the association with factors that cooperate in the canonical and non-canonical NF-κB pathway.

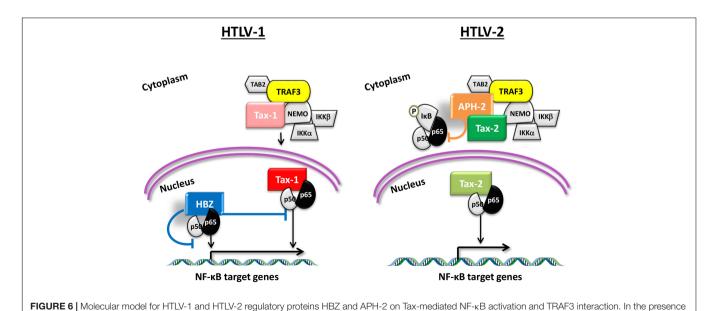
We report here a reduction of TRAF3 expression in the presence of the antisense proteins. We would have expected an activation of the non-canonical NF- $\kappa$ B pathway, but we have not observed an accumulation of p52 in the presence of HBZ (data not shown). This result is in agreement with data demonstrating a lowered expression of p100 in the presence of HBZ that leads to lowered amounts of the non-canonical transcription factor p52 (Zhi et al., 2011). The HBZ effect in the non-canonical NF- $\kappa$ B activation still remains controversial since Zhao et al. (2009) suggest that HBZ inhibits selectively the canonical NF- $\kappa$ B pathway in the presence of Tax-1.

Zhao et al. (2009) and Panfil et al. (2016) demonstrated that both HBZ and APH-2 interact with p65 and reduce p65-mediated NF-κB promoter activity. We show here that HBZ and APH-2 may exert a different molecular mechanism in the down-modulation of the NF-κB pathway. We found that HBZ is less efficient in dampening Tax-mediated NF-κB activity compared to APH-2 and that co-expression of HBZ and high levels of Tax-1 results in restoring NF-κB activation,

whereas APH-2 maintains its inhibitory effect despite high expression levels of Tax-2. We also observed that APH-2 inhibits p65-induced NF-κB activation in both HEK293T and Jurkat cell models. This is in contrast to the previous report showing that APH-2 inhibits the p65-induced NF-κB activation only in HEK293T cells (Panfil et al., 2016). This discrepancy may be due to differences in the efficiency of cell transfection, as declared by the authors.

By analyzing the subcellular distribution of the protein complexes formed by Tax and the host interacting proteins, we observed that APH-2, but not HBZ, is recruited in the cytoplasmic structures containing NEMO and TAB2. We have also demonstrated that the co-expression of Tax-2 and APH-2 results in the accumulation of the NF-kB inhibitor IkB and in a partial impairment of p65 nuclear translocation, whereas IkB expression is reduced when Tax-1 and HBZ are co-expressed. From the results obtained, we propose a model in which the recruitment of HBZ and APH-2 within the protein complexes formed by Tax follows different patterns. While APH-2 is partially retained in the cytoplasmic compartments, HBZ translocates more efficiently into the nucleus, thus inhibiting the transcription factor activity of p65 (Figure 6). This model is also supported by the results of Marban et al. (2012) that demonstrate a redistribution of APH-2 in the cytoplasm in the presence of Tax-2, preventing APH-2 from activating AP-1 transcription.

Our results clearly demonstrate that TRAF3 is required for NF- $\kappa$ B dysregulation mediated by Tax, thus representing a novel factor recruited by the viral regulatory proteins to alter cell pathways. In fact, TRAF3 not only acts on NF- $\kappa$ B pathway, but also plays significant roles in the immunity-related signal transduction (Häcker et al., 2011). Cells lacking TRAF3 are defective in IFN-I responses activated by different TLRs (Oganesyan et al., 2006). The induction of IFN-I elicits the



nuclear translocation

of Tax-1, HBZ inhibits p65 action in the nucleus competing with Tax-1. The recruitment of APH-2 in Tax-2-cytoplasmic complexes containing TRAF3 limits the p65

activation of the antiviral cellular gene expression that coincides with the inhibition of viral replication. The key role of TRAF3 in promoting antiviral signaling is supported by a recent study demonstrating that the deletion of TRAF3 in adult mice attenuated their host defense against vesicular stomatitis virus (VSV) infection (Xie et al., 2019). In the non-canonical NF-κB pathway, TRAF3, in concert with TRAF2, cIAP1 and cIAP2, promotes the constitutive degradation of NIK that in turn serves as negative regulator of IFN (Jin et al., 2014; Parvatiyar et al., 2018). A feedback inhibition mechanism has been also proposed to induce TRAF3 degradation, affecting IFN production and NIK accumulation (Nakhaei et al., 2009). Recent evidence demonstrates that the HTLV antisense proteins interact with IRF-1, a transcriptional regulator of IFN-I pathway. In particular, APH-2 enhances IRF-1 DNA binding and steady-state expression levels, whereas HBZ interacts with IRF-1 and causes its degradation (Panfil et al., 2016). The loss of IRF-1 expression has been observed in several cases of leukemia (Alsamman and El-Masry, 2018). It is reasonable to think that the recruitment of APH-2 and Tax-2 in complexes with TRAF3 may positively affect IFN response. This hypothesis could be linked to the demonstration that in rabbits, the lack of APH-2 increases the infection rate of HTLV-2 mutants in comparison to HTLV-2 WT (Yin et al., 2012). Further studies will be required to analyze the interplay of TRAF3 and the HTLV antisense proteins in deregulating the type I IFN induction.

#### CONCLUSION

In summary, we demonstrated that TRAF3 cell factor is required for Tax-mediated NF-κB activation. Our findings allow to further understand the roles of host factors that may be targeted by HTLV regulatory proteins and participate in the crosstalk between IFN and NF-κB pathway. We also found that APH-2 is more effective than HBZ in the inhibition of Tax-mediated NF-κB activation. We suggest that the recruitment of APH-2 in Tax-2-cytoplasmic structures with NF-κB factors that affect

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the p65 nuclear translocation may explain the different effects of APH-2 and HBZ. This work allows to shed light on the role of HTLV-induced NF-kB pathway activation and ultimately in the identification of potential therapeutic targets.

#### **DATA AVAILABILITY**

The datasets generated for this study are available on request to the corresponding author.

#### **AUTHOR CONTRIBUTIONS**

SF, MS, SM, and EB performed the study and contributed to manuscript discussion. SF contributed to manuscript drafting and writing. RM, CJ, VC, DZ, and UB provided intellectual input and contributed to manuscript drafting. MR conceived and designed the experiments, coordinated the study, and contributed to manuscript drafting and writing.

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SF conducted this study as fulfillment of her Ph.D. degree.

#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.01302/full#supplementary-material

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# Inhibition of HTLV-1 Infection by HIV-1 First- and Second-Generation Integrase Strand Transfer Inhibitors

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More than 10 million people worldwide are infected with the retrovirus human T-cell lymphotropic virus type 1 (HTLV-1). Infection phenotypes can range from asymptomatic to severe adult T-cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy. HTLV-1, like human immunodeficiency virus type 1 (HIV-1), is a blood-borne pathogen and viral infection happens in a similar fashion, with the major mode of transmission through breastfeeding. There is a strong correlation between time of infection and disease development, with a higher incidence of ATLL in patients infected during childhood. There is no successful therapeutic or preventative regimen for HTLV-1. It is therefore essential to develop therapies to inhibit transmission or block the onset/development of HTLV-1 associated diseases. Recently, we have seen the overwhelming success of integrase strand transfer inhibitors (INSTIs) in the treatment of HIV-1. Previously, raltegravir was shown to inhibit HTLV-1 infection. Here, we tested FDA-approved and two Phase II HIV-1 INSTIs in vitro and in a cell-to-cell infection model and show that they are highly active in blocking HTLV-1 infection, with bictegravir  $(EC_{50} = 0.30 \pm 0.17 \text{ nM})$  performing best overall. INSTIs, in particular bictegravir, are more potent in blocking HTLV-1 transmission than tenofovir disproxil fumarate (TDF), an RT inhibitor. Our data suggest that HIV-1 INSTIs could present a good clinical strategy in HTLV-1 management and justifies the inclusion of INSTIs in clinical trials.

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#### INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) belongs to the delta-retrovirus genus and is the second most clinically relevant retrovirus after human immunodeficiency virus type 1 (HIV-1). Like HIV-1, HTLV-1 is a blood-borne pathogen and is transmitted horizontally by sexual intercourse, contact with infected blood, and vertically from mother to child during breastfeeding and labor. HTLV-1 is a highly potent oncogenic virus, despite the fact that pathology develops only in about 10% of carriers. The two main pathologies associated with infection are an aggressive form of blood cancer – adult T-cell leukaemia/lymphoma (ATLL) (Mahieux and Gessain, 2007) and a spectrum of neuromuscular disorders called HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Matsuura et al., 2016). There is no therapeutic or preventative treatment for HTLV-1 and disease prognosis is very poor – particularly for acute ATLL, where the median survival rate is about 2 months (Beltran et al., 2011).

Human T-cell lymphotropic virus type 1 infection is of significant epidemiological importance. It is estimated that between 10 and 20 million people are infected worldwide (Gessain and Cassar, 2012). This figure is most certainly underestimated, since infection can be asymptomatic and diagnosis and lack of understanding and care in public health (Zihlmann et al., 2012) in many countries is still poor. In some endemic areas, the prevalence of HTLV-1 infection is staggeringly high [above 35% in regions of central Australia (Einsiedel et al., 2014, 2018) and above 5% in the Kyushu island of Japan (Gessain and Cassar, 2012)]. A recent open letter, instigated by the co-discoverer of the retrovirus family – Robert Gallo (Martin et al., 2018), appeals to the scientific and medical community to take urgent measures in order to prevent the further spread of HTLV-1.

Central to the life cycle of HTLV-1 is the integrase (IN) enzyme - a nucleotidyl transferase responsible for insertion of the DNA copy of the viral RNA genome into host cell DNA. Following reverse transcription, IN engages with the viral long terminal repeat (LTR) ends of the DNA thereby forming a synaptic complex also called the intasome. The integration reaction takes place in two steps: (i) in the 3' processing reaction, IN removes two (or, depending on the genus, three) nucleotides following the invariant CA-dinucleotide thereby generating 3'-OH groups necessary for the next step in the integration reaction; (ii) following nuclear entry, the intasome engages with host chromatin and the reactive 3'-OH groups attack the scissile phosphodiester backbone on opposing strands of the host DNA stably inserting the vDNA into the host genome (Li et al., 2006; Maertens et al., 2010; Cherepanov et al., 2011). The retroviral IN is composed of three domains, with the central catalytic core domain (CCD) being the most conserved. The CCD encompasses the integrase catalytic triad (DDE motif), which coordinates two catalytically critical divalent metal ions (usually Mg<sup>2+</sup>). The irreversible insertion of both vDNA ends into host DNA, termed "concerted integration," results in a stably integrated provirus which is copied with every cell division. The proviral expression products further stimulate cell proliferation and lead to the smoldering, mitotic spread of HTLV-1 (Azran et al., 2004; Bindhu et al., 2004). Due to the imperative role of IN in successful retroviral infection, several IN strand transfer inhibitors (INSTIs) have been developed and released for use in HIV-1 therapy, and have since revolutionized the treatment of this virus (Summa et al., 2008; Serrao et al., 2009; Gillette et al., 2014). These diketo acid derivatives utilize the active site pocket in the intasome through simultaneous chelation of the catalytic pair of Mg<sup>2+</sup> ions by the diketo group oxygens, and  $\pi$ - $\pi$  stacking of their halobenzyl moiety to the terminal vDNA adenine. INSTIs compete for binding with the target host DNA and displace the reactive 3' termini of vDNA away from the active site (Hare et al., 2010a,b). Successive iterations of INSTIs developed have addressed the issues of drug resistance, safety and dissociation rate in HIV-1 treatment.

In Rabaaoui et al. (2008), first reported on the inhibition of HTLV-1 integration by styrylquinolines and diketo acids, the latter being precursors of the currently used HIV-1 INSTIs. More recently, raltegravir and MK-2048 were shown to efficiently

block HTLV-1 infection (Seegulam and Ratner, 2011). Here, we present data on a comprehensive panel of INSTIs that are FDA-approved or awaiting approval for treatment of HIV-1 infection that were tested for their efficacy to block HTLV-1 integration *in vitro* and in cell-to-cell infection. We describe an optimized *in vitro* assay that allows for easy screening of INSTI efficacy and IC<sub>50</sub> determination. *Ex vivo* experiments in Jurkat cells infected with HTLV-1 by cell-to-cell transmission indicate that the INSTI inhibition profiles are very similar to those of HIV-1. Taken together, we present data that support the use of INSTIs as prophylactic treatment, and may justify their use in clinical trials.

#### MATERIALS AND METHODS

## **Expression and Purification of Recombinant HTLV-1 Integrase**

Human T-cell lymphotropic virus type 1 IN (GenBank ID: AHX00119.1) was expressed and purified from pET28a-SUMO-HTLV-1 IN as described previously (Maertens, 2016) with minor modifications. Briefly, following elution from the cation exchange column, positive fractions containing HTLV-1 IN were pooled and dialyzed against 25 mM piperazine-N,N'bis(2-ethanesulfonic acid) (PIPES) pH 6, 300 mM NaCl at 4°C overnight. The resulting solution was injected onto an S200 16/60 Superdex size-exclusion column (GE Healthcare, United Kingdom) pre-equilibrated in dialysis buffer. The peak fractions corresponding to IN were pooled and concentrated in a 10 kDa molecular weight cut-off centrifugal ultrafiltration device to a concentration of about 10 mg/mL and supplemented with 2 mM DTT and 10% glycerol. Protein was aliquoted, flash frozen in liquid nitrogen and stored at  $-80^{\circ}$ C until further use. The B' $\gamma$  regulator subunit of PP2A (further referred to as B' $\gamma$ ) was purified as described previously (Maertens, 2016).

#### **Integrase Strand Transfer Assays**

Donor DNA mimicking the U5 3'-processed LTR ends of the viral DNA copy was prepared by annealing oligonucleotides (Supplementary Table S1), in 100 mM Tris pH 7.4, 400 mM NaCl. The optimized conditions for the strand transfer reactions are as follows: a solution was prepared containing 73 mM PIPES pH 6.0, 175 mM NaCl, 16.7 mM MgCl<sub>2</sub>, 5.8 μM ZnCl<sub>2</sub>, 12.8 mM DTT, 0.53 μM donor DNA, and 0.8 μM HTLV-1 integrase. Following a 30 min room temperature incubation in the presence or absence of INSTIs in the final concentration of 5% DMSO, the reaction was initiated by addition of 300 ng of pGEM-9Zf(-) supercoiled plasmid DNA. The total volume of each reaction was 150 µL. Reactions were carried out at 37°C for 30 min and were processed as described previously (Maertens et al., 2010). All reactions were performed in these conditions unless otherwise stated in the Figure legend. Reactions that included B'y were done with equimolar ratio of IN to  $B'\gamma$ . The precipitated DNA was resuspended in agarose loading dye and analyzed on a 1.5% agarose gel run in 1× TAE buffer and stained with ethidium bromide. Bands corresponding to products of concerted integration were quantified by densitometry in ImageLab 4.1 (Bio-Rad). All experiments were done in triplicate.

Quantified data points were fitted to a dose-response curve in Prism 7. Cumulative standard deviation for each drug was calculated as an average of the upper limit and lower limit values:

$$\begin{aligned} \text{Lower limit} &= \frac{EC_{50}}{10^{\log SE(EC_{50})}} \\ \text{Upper limit} &= EC_{50} \times 10^{\log SE(EC_{50})} \end{aligned}$$

Where SE denotes the standard error computed by Prism.

#### **Cell Lines and Media**

The MT-2 and Jurkat (E6.1) T cell lines (ATCC) were maintained in RPMI supplemented with 10% foetal bovine serum (FBS), 100 U penicillin 100  $\mu g/mL$  streptomycin and 0.25  $\mu g/mL$  fungizone. Cells were cultured at 37°C with 5% CO $_2$  in a humidified atmosphere.

## HTLV-1 Infection by Cell-to-Cell Transmission

Jurkat cells were pre-treated with increasing amounts of INSTI  $(0.2 \text{ pM}-2 \mu\text{M} \text{ in DMSO})$ , or the carrier (DMSO) for 24 h. MT-2 cells were resuspended to a concentration of  $2 \times 10^6$  cells/mL and exposed to a sub-lethal dose of gamma-irradiation (40,000 Rad). Cells were resuspended in serum-free RPMI to a concentration of  $2 \times 10^6$  cells/mL in the presence of drug, and  $0.5 \times 10^6$  of each cell type were co-cultured for 18 h. Following co-culture, cells were washed in PBS and resuspended in 1 mL of depletion buffer (0.1% FBS, 2 mM EGTA, PBS) before gentle tumbling (1 h, 4°C) with 25  $\mu$ L of anti-CD25 + magnetic beads (DynaBeads, Thermo Fisher Scientific) to deplete MT-2 cells. Following depletion, the unbound fraction, i.e., Jurkat cells were maintained in RPMI with the drug present for 16 days before genomic DNA harvest (DNeasy kit, Qiagen). All experiments were done in triplicate. Depletion of MT-2 cells was verified by FACS. For cellular toxicity measurements, Jurkat cells were grown in the presence of the INSTIs as described above. Viability was measured by live dead stain (Hoechst 33258) and analyzed by FACS.

## FACS Protocol for MT-2 and Jurkat Identification

Samples of Jurkat and MT-2 cultures pre- and post-depletion were immunostained for CD3 and CD25 to determine the efficiency of anti-CD25 magnetic depletion.  $1\times10^6$  cells were washed extensively in PBS + 3% FBS before incubation with 0.05 µg Alexa 647-conjugated anti-CD3 + (Biolegend, clone UCHT1) and 0.1 µg Alexa488 conjugated anti-CD25 + (Biolegend, clone BC96) antibodies in the dark for 30 min. Isotype control antibodies were used to determine specificity of the antibodies and staining protocol. Following extensive washing, samples were fixed in 2% formaldehyde for FACS analysis. Single cells were gated, CD3 and CD25 were identified by emission/excitation at 640/670 nm and 488/530 nm, respectively. Data were analyzed on FlowJo software version 10.7.

## Determination of HTLV-1 Proviral Load and Alu-PCR

Proviral load (PVL) was estimated as previously described (Manivannan et al., 2016; Rowan et al., 2016). Genomic DNA concentrations were measured by nanospectroscopy (DeNovix), and samples were diluted to 5 ng/µL for real-time PCR (qPCR) analysis. qPCR reactions were performed for both the HTLV-1 tax gene and the human GAPDH gene (Supplementary **Table S2**). Gene copy numbers were determined by comparison to standard curves generated from a clone with a known single integration site (clone 11.50, courtesy of Prof. Charles Bangham). PVL was calculated by comparison of tax and GAPDH copy numbers as a fraction, multiplied by 100 to generate a percentile value assuming a single copy of tax and two copies of GAPDH per infected cell (Lairmore et al., 1989). Alu-PCR was performed as described previously (Alais et al., 2015), with the modification that the nested PCR was also run as a qPCR using SYBR-green (primers used are shown in **Supplementary Table S2**). Integrated provirus copy numbers were normalized to GAPDH, and DMSO treated HTLV-1 infected Jurkat cells were arbitrarily set to 100%. Averages and standard deviations of three independent experiments are shown. P-values were calculated using the student's t-test.

## Construction of Integrase Structural Models

Human T-cell lymphotropic virus type 1 amino acid sequence (residues 53-216, corresponding to the catalytic domain) was submitted for automatic homology model building to the online software Phyre2 (Kelley et al., 2015). The search yielded a high-quality model with confidence score of 100% and 99% coverage. For structural comparisons, a previously reported structural model of HIV-1 intasome was used, which was constructed on the basis of the PFV intasome crystal structure in complex with raltegravir and elvitegravir (Krishnan et al., 2010). HTLV-1 and HIV-1 models were aligned and annotated in PyMol (Schrodinger, 2015). Multiple sequence alignments were conducted in Jalview (Waterhouse et al., 2009) with the MAFFT algorithm (Katoh and Standley, 2013). ESPript was used for graphical representation of the alignments (Gouet et al., 1999). The sequence conservation scores annotation on the structural models was conducted with the Alebrijes script1.

#### **RESULTS**

## *In vitro* Catalytic Strand Transfer Activity of HTLV-1 IN

Full-length wild type HTLV-1 IN was recombinantly expressed, purified, and characterized. HTLV-1 IN *in vitro* activity on radiolabeled vDNA donor mimics (Balakrishnan and Jonsson, 1997; Muller and Krausslich, 1999), as well as IN strand transfer activity dependence on the human host factor  $B'\gamma$  have been reported previously (Maertens, 2016). Here, we have attempted to

¹https://github.com/mbarski/Alebrijes

further characterize the strand transfer activity of HTLV-1 IN and optimized the assay for efficient testing of integrase inhibitors.

Recombinant IN enzyme was incubated with short (16–20 nt) double-stranded oligonucleotides mimicking the 3′ processed viral LTR end (derived either from the U5 or the U3, vDNA) and a supercoiled plasmid functioning as the target (tDNA) for integration. The biologically relevant concerted integration where two vDNA mimics are inserted in the tDNA, results in linearization of the plasmid, whilst products of aberrant half-site integration co-migrate with nicked plasmid DNA (**Figure 1A**). These products can be easily separated on an agarose gel (**Figure 1B**).

Human T-cell lymphotropic virus type 1 IN was active with all donor lengths tested (16-20 nt) but was clearly discriminative against the U3-derived donors (Figure 1C and Supplementary Figure S1). Certain buffering species significantly improved IN in vitro strand transfer activity (Figure 1D). PIPES provided such optimal conditions for IN. Indeed, in the PIPES buffer, no  $B'\gamma$ was needed to observe efficient concerted integration activity by HTLV-1 IN. In fact, in this buffer,  $B'\gamma$  only slightly stimulated strand transfer activity of HTLV-1 IN (Figure 1D, compare lanes 4 and 5). Conversely, using the closely related HEPES buffer at the same pH neccesitated the presence of B' $\gamma$  for any discernable activity (Figure 1D, compare lanes 6 and 7). Finally, in contrast to previous findings where HTLV-1 IN activity was only observed in NaCl concentrations below 100 mM (Muller and Krausslich, 1999), under our optimized conditions, concentrations of up to 200 mM had no adverse effect on activity (**Figure 1E**).

## Inhibition Profiles of INSTIs in HTLV-1 IN Strand Transfer

A panel of six INSTIs was selected (**Figure 2A**), to encompass the inhibitors currently approved for use (raltegravir, elvitegravir, dolutegravir, and bictegravir) as well as those under development (MK-2048, BMS-707035). Strand transfer assays were performed with recombinantly produced IN in presence of each of the respective inhibitors. Although raltegravir and MK-2048 were previously tested to inhibit HTLV-1 (Seegulam and Ratner, 2011), we included them in our study to allow comparison of inhibition profiles under the same assay conditions.

All tested compounds caused significant inhibition of *in vitro* strand transfer at nanomolar concentrations (**Figures 2B,C** and **Table 1**). Elvitegravir and the second-generation inhibitors dolutegravir and bictegravir showed activity superior to raltegravir, which is consistent with the observations of these compounds' activity against HIV-1 IN (Pandey et al., 2007; Marinello et al., 2008; **Figure 2C**). The biggest improvement was shown by elvitegravir; about 5.3-fold less drug was needed than raltegravir to reach 50% inhibition (**Table 1**). The Hill slope of the HTLV-1-tested INSTIs oscillates quite consistently around 1.0 – a low Hill slope characteristic of this group of inhibitors (**Table 1**; Sampah et al., 2011).

#### Inhibition of HTLV-1 Infection by INSTIs

In order to convincingly establish the usefulness of the selected INSTIs for the purpose of limiting HTLV-1 infection and

for potential prophylactic use, we tested a selected panel of INSTIs for their efficacy to block HTLV-1 infection. HTLV-1 is most efficiently spread by cell-to-cell transmission, hence we infected Jurkat cells by co-culture with the persistently HTLV-1 infected MT-2 cell line. To remove any potential carry-over of proviral DNA originating from the MT-2 cells, the MT-2 cells were gamma-irradiated before co-culture with Jurkat cells and depleted using anti-CD25 antibodies conjugated to magnetic beads. Depletion of the MT-2 cells was verified by FACS (Supplementary Figure S2). As an additional control, a condition where only the irradiated MT-2 cells were plated was taken alongside the other conditions. Thus, these cells were used for CD25 depletion and the flow-through was kept in culture and diluted in parallel to the infected Jurkat cells. Following 16 days of expansion, no MT-2 cells were found in these "MT-2 only" conditions further strengthening our observation that the signal observed in our PVL and Alu-PCR assays is specifically derived from newly infected cells.

Infection of Jurkat cells was determined by quantifying the PVL (Demontis et al., 2013). On average 13.4  $\pm$  2.2% (absolute value for the PVL, n = 9) of Jurkat cells are infected under these experimental conditions. Infections were done in the presence of DMSO (vehicle of the drugs) or a range of drug concentrations (0.2 pM to 2 µM). The resulting dose-response curves show a strong inhibition profile of tested INSTIs, active even at picomolar concentrations (Figure 2D). This is inline with numerous observations of INSTIs activity for HIV-1<sup>2,3,4</sup>, the INSTI EC<sub>50</sub> values for both viruses approaching the very low nanomolar range (Table 1). Interestingly, within the context of HTLV-1 replication, bictegravir showed about 20-30-fold higher activity than raltegravir or elvitegravir, respectively. No cellular toxicity was observed at the drug concentrations used (Table 1). Alu-qPCR further confirmed the strong inhibition of integration by these INSTIs (Figures 2E,F). Using this assay, we also investigated the efficacy of tenofovir disproxil fumarate (TDF) to inhibit HTLV-1 transmission. Whilst not as powerful as bictegravir, TDF is still quite potent  $(EC_{50} = 17.78 \pm 7.16 \text{ nM})$ , inhibiting HTLV-1 transmission only two- to threefold less efficient than raltegravir or elvitegravir, respectively (Supplementary Figure S3).

#### **DISCUSSION**

Despite the urgent need to curtail HTLV-1 infection and spread, there has been no structural information available on the delta-retroviral IN, and the data on its inhibition by the HIV-1 INSTIs is outdated. By successful recombinant expression, purification and further characterization of activity of full-length HTLV-1 IN, we have laid the groundwork for accurate assessment of its inhibition by a panel of selected INSTIs. In contrast to a

<sup>&</sup>lt;sup>2</sup>https://www.accessdata.fda.gov/drugsatfda\_docs/label/2013/022145s029lbl.pdf (accessed December 20, 2018)

<sup>&</sup>lt;sup>3</sup>https://www.accessdata.fda.gov/drugsatfda\_docs/label/2014/203093s000lbl.pdf (accessed December 20, 2018)

<sup>&</sup>lt;sup>4</sup>https://www.accessdata.fda.gov/drugsatfda\_docs/label/2013/204790lbl.pdf (accessed December 20, 2018)

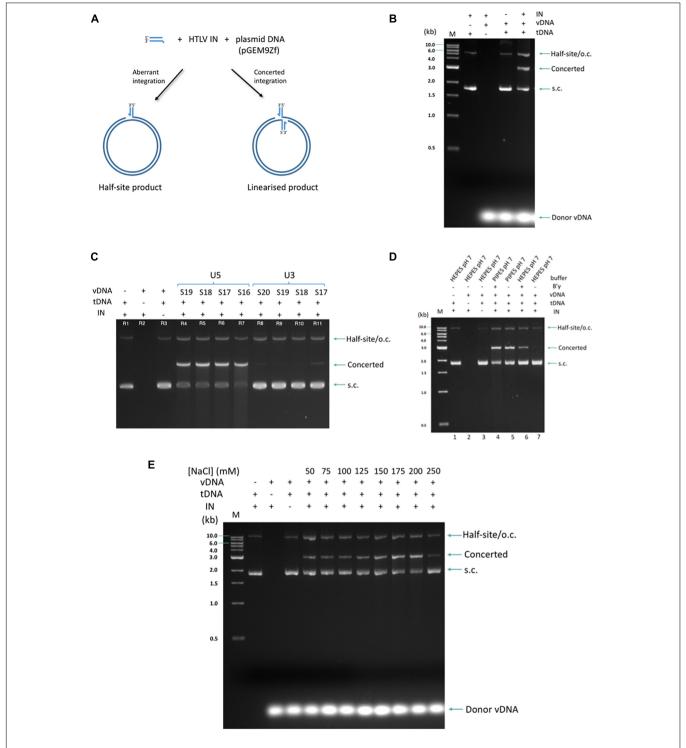


FIGURE 1 | Strand transfer activity of recombinant HTLV-1. (A) The formation of two types of product, concerted and half-site, is shown in the schematic. (B) The products are resolved on a 1.5% agarose gel and visualized with standard ethidum bromide treatment. M denotes the marker ladder (NEB, 1 kB), vDNA (U5\_S18 3′-pre-processed, 18 nucleotide-long, double-stranded oligonucleotide donor mimicking the viral U5 LTR region), tDNA (supercoiled plasmid pGEM9Zf). (C) Activity of IN for U5- and U3-derived donors of different lengths is assayed. Sx: substrate of x nt in length. (D) HTLV-1 IN is sensitive to the use of different buffering species. When performing the strand transfer reaction in Hepes pH 7, B′γ strongly stimulates the concerted integration activity of HTLV-1 IN. In contrast, under optimal conditions (Pipes pH 7), no B′γ is required for high strand transfer activity. (E) Under conditions of optimal activity, IN remains active in the presence of 200 mM NaCl. Migration of DNA species in the gel is indicated on the right of the gel. S.c., supercoiled; o.c., open circular. The 1 kb DNA ladder (NEB, indicated on the left of the gel) was used as a reference. Presence of IN, B′γ, vDNA, tDNA, and buffer conditions are indicated above each gel.

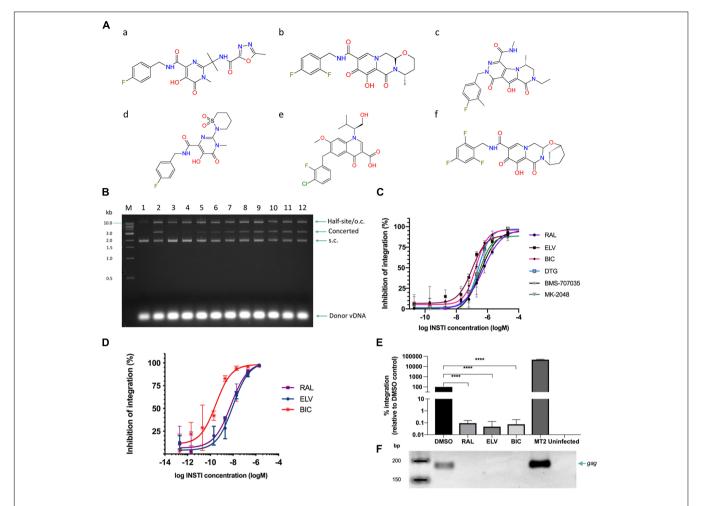


FIGURE 2 | INSTIs efficiently block HTLV-1 integration *in vitro* and HTLV-1 infection by cell-to-cell transmission. (A) Chemical structures of the INSTIs used in this study. (a) raltegravir, (b) dolutegravir, (c) MK-2048, (d) BMS-707035, (e) elvitegravir, and (f) bictegravir. Strand transfer is significantly inhibited by the addition of raltegravir (B) or other tested INSTIs (C). (B) Lane 1, negative control: no IN; lane 2: positive control, IN in the absence of drug. Lanes 3–12: in the presence of raltegravir; lane 3: 200 μM, lane 4: 20 μM, lane 5: 2 μM, lane 6: 634 nM, lane 7: 200 nM, lane 8: 63.4 nM, lane 9: 20 nM, lane 10: 2 nM, lane 11: 200 pM, and lane 12: 20 pM. Migration of DNA species in the gel is indicated on the right of the gel. S.c., supercolled; o.c., open circular. The 1 kb DNA ladder (NEB, indicated on the left of the gel) was used as a reference. For each of the six compounds tested, the concerted integration bands were quantified by densitometry and values plotted as dose-response curves (C). (D–F) INSTIs efficiently block HTLV-1 infection in Jurkat cells. Jurkat cells were pre-treated with INSTIs (2 μM down to 0.2 pM serially diluted 1/10) and infected with HTLV-1 by co-culture with gamma-irrradiated MT-2 cells. Following depletion of MT-2 cells and expansion of the infected Jurkat cells, genomic DNA was isolated. (D) Infection was measured by determining the relative PVL of INSTI treated cells compared to DMSO treated control cells. The vertical axis shows the percentage of inhibition of HTLV-1 IN defined as the percentage of (1 – relative PVL). (E) Integrated provirus was quantified by Alu-qPCR and normalized to *GAPDH* numbers. DMSO treated Jurkat cells infected with HTLV-1 were arbitrarily set to 100%. Averages and standard deviations from three independent experiments are shown. RAL, raltegravir; ELV, elvitegravir; BIC, bictegravir; Uninfected stands for uninfected Jurkat cells (negative control). Data shown here is for the samples treated with 2 μM of the indicated drug. \*\*\*\*: ρ-valu

previous study which first described the *in vitro* 3'-processing, strand transfer and disintegration activity of HTLV-1 IN using radiolabeled probes (Muller and Krausslich, 1999), the advantage of the integration assay employed here is that the biologically relevant concerted integration activity can be discerned from the aberrant half-site integration events and quantified separately. Stabilization of IN at high concentrations also allows to avoid the use of radioactivity. By optimizing conditions for HTLV-1 IN activity, the need for the presence of detergent and the host factor  $B'\gamma$  for efficient strand transfer activity has been removed. Furthermore, the observation of equal stimulation by

either B' $\gamma$  or optimized buffering conditions suggests that the said *in vitro* stimulation of HTLV-1 IN activity by B' $\gamma$  occurs by its entropic stabilization upon binding of the enzyme, rather than by a specific mechanism improving substrate recognition or catalysis. It has also been shown that HTLV-1 IN is active on a range of vDNA LTR donor mimicks with lengths as short as 16 bp – although, as reported previously (Muller and Krausslich, 1999), it strongly discriminates against the U3-derived donors. The discrimination between U5 and U3 ends is not unique for HTLV-1 IN, indeed HIV-1 and SIV IN also display a similar preference

TABLE 1 | Calculated parameters of in vitro HTLV-1 IN strand-transfer and HTLV-1 infection inhibition by INSTIs.

				HIV-1 ————————————————————————————————————			
	Inhibition of enzymatic activity					Antiviral activity	
INSTI	IC <sub>50</sub> (μM)	IC <sub>95</sub> (μΜ)	Hill slope	EC <sub>50</sub> (nM)	EC <sub>95</sub> (μM)	CC <sub>50</sub> (μM)	EC <sub>50</sub> (nM)
Raltegravir	$0.53 \pm 0.102$	29.92 ± 5.75	0.73	6.42 ± 4.24	1.02 ± 0.675	>20	9.4 ± 1.4 <sup>a</sup>
Elvitegravir	$0.10 \pm 0.016$	$1.9 \pm 0.304$	1.0	$9.57 \pm 5.54$	$1.27 \pm 0.735$	>20	$2.4 \pm 0.9^{a}$
Dolutegravir	$0.26 \pm 0.059$	$3.02 \pm 0.485$	1.2	ND	ND	ND	$1.4 \pm 0.3^{a}$
MK-2048	$0.29 \pm 0.069$	$4.22 \pm 0.965$	1.1	ND	ND	ND	1.9 <sup>b</sup>
BMS-707035	$0.35 \pm 0.074$	$12.2 \pm 2.90$	0.83	ND	ND	ND	2.5 <sup>c</sup>
Bictegravir	$0.18 \pm 0.029$	$2.09 \pm 0.437$	1.2	$0.302 \pm 0.173$	$0.0318 \pm 0.0182$	>20	$1.6 \pm 0.4^{a}$

<sup>&</sup>lt;sup>a</sup>Tsiang et al. (2016), <sup>b</sup>Bar-Magen et al. (2010), <sup>c</sup>Naidu et al. (2018).

for U5 LTR ends (Cherepanov et al., 1999; Goodarzi et al., 1999). Although the presence of half-site product is apparent, under optimized conditions the concerted integration product predominates (**Figure 1**).

The similarity of HTLV-1 IN INSTI sensitivity to that of HIV-1 is striking (Table 1; Tsiang et al., 2016). The apparent disparity between the *in vitro*- and *in cellulo*-derived IC<sub>50</sub> values is caused by the stark difference in IN concentration to be inhibited in these assays. Since IN is known not to form a steady-state or turn over (Dicker et al., 2007), its concentration is positively correlated with the resulting INSTI IC50. Furthermore, in our cell-to-cell infection model, bictegravir performed about 20- to 30-fold better than either raltegravir or elvitegravir. In in vitro assays, bictegravir is only three times as active as raltegravir, and  $\sim$ 55% as active as elvitegravir. Why bictegravir is so much more efficient in blocking HTLV-1 infection than in vitro integration remains enigmatic. We speculate that Jurkat cells used in our experiments might be more efficient in taking up bictegravir than raltegravir or elvitegravir, compared to the MT-2 or primary T cells used by Tsiang et al. (2016).

Recently, Pasquier et al. (2018) reported a thorough study investigating the efficacy of HIV-1 reverse transcriptase (RT) inhibitors in blocking HTLV-1 infection. Whilst zidovudine was able to block HTLV-1 infection somewhat (<10-20% inhibition at 50 µM concentration), lamivudine and stavudine were not (Pasquier et al., 2018). Tenofovir disproxil fumarate were shown to inhibit HTLV-1 transmission; at concentrations of 10  $\mu M$  about 50% of HTLV-1 infection was blocked (Pasquier et al., 2018). In our hands, we measured an EC<sub>50</sub> of  $17.78 \pm 7.16$  nM for TDF. The discrepancy is likely due to the different assays used. Whilst Pasquier et al. (2018), reads out luciferase activity from an HTLV-1 LTR promoter 24 h post co-culture with live C91PL cells, we deplete the gammairradiated MT-2 cells and measure (integrated) proviral DNA 16 days post-infection. Nevertheless, this is very promising data. Indeed, whilst zidovudine in combination with IFN-alpha or valproic acid was shown to reduce PVL in some cases (Afonso et al., 2010; Trevino et al., 2012; Cook et al., 2018), there is hope for improvement. The fact that INSTIs are as potent as TDF, and in the case of bictegravir  $\sim$ 60 more potent than TDF in blocking HTLV-1 infection (EC50 values between 0.3 and

10 nM, Figures 2D-F), may justify the inclusion of INSTIs in clinical trials.

The occurrence of escape mutants is a real challenge in HIV-1 patients and drives the research toward the design of alternative anti-retroviral therapy (Harada and Yoshimura, 2017). The mutation rate of HTLV-1 RT is about one fourth of HIV-1 RT (Mansky, 2000). This alone cannot explain the apparent lack of HTLV-1 quasi species, which is in part due to its different mode of replication. Although limited clinical data is available, it is promising that so far, no resistance mutations have been observed in HTLV patients experiencing a strong reduction in PVL upon treatment with RT inhibitors for more than 1 year (Machuca et al., 2001).

Taken together, our results strongly suggest that HTLV-1 IN is as sensitive to the tested panel of INSTIs as HIV-1. Although there are significant differences in the overall sequence similarity between the two proteins as well as the infection dynamics of these viruses, the residues within the active site are highly conserved (Figure 3). Whilst it is tempting to speculate why elvitegravir is fivefold better in inhibiting in vitro HTLV-1 IN activity, we note that the loop important for drug interaction (including the homologous residues to HIV-1 Y143, N144, and Q146) is not well structured in our HTLV-1 homology model (Figure 3A). It is therefore difficult, at this stage, to explain the differences in inhibition efficiency between raltegravir and elvitegravir. A structure of the HTLV-1 (or related deltaretroviral) intasome bound to INSTIs is needed. In addition, structural information available on bictegravir bound to a retroor lentiviral intasome is currently lacking.

Early on in infection, HTLV-1 proliferates mostly by viral replication. Unlike HIV-1, these dynamics change dramatically a few weeks later, where the PVL is most significantly increased by the expansion of infected clones and viral replication is turned down. The time window to use anti-retrovirals in recently HTLV-1 exposed patients to block infection is very narrow (Cook et al., 2016). It is well known that a high PVL increases the odds of developing HAM/TSP (Nagai et al., 1998; Matsuzaki et al., 2001; Takenouchi et al., 2003; Olindo et al., 2005, 2006) and ATLL (Iwanaga et al., 2010; Yonekura et al., 2015). Therefore, reducing the PVL by use of antiretrovirals might prevent an asymptomatic carrier from developing HTLV-1 associated

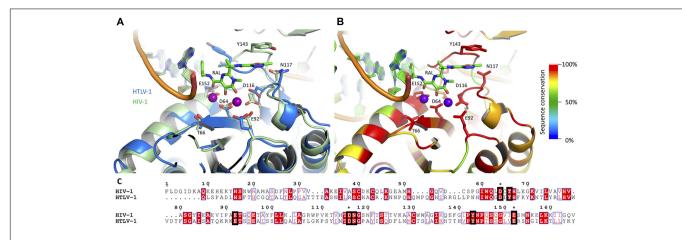


FIGURE 3 | The active sites of HIV-1 and HTLV-1 IN are highly conserved. A homology model of HTLV-1 CCD is presented (A), overlaid on a previously reported model of HIV-1 intasome, based on the crystal structure of PFV intasome with raltegravir present in the active site (Krishnan et al., 2010). (C) A pairwise sequence similarity between the HIV-1 and HTLV-1 INs was conducted with MAFFT (Katoh and Standley, 2013), and the resulting similarity score for each residue was overlaid on the structure, represented in a color scale (red for highest conservation through to blue for lowest/no conservation), using the Alebrije script (B). The catalytic DDE triad as well as residues known to be involved in INSTI resistance are shown in stick representation (B) and indicated in the alignment with black squares (C). The residues of the DDE motif are also marked with asterisks. Catalytically important magnesium atoms are represented in purple (B). The predominance of residues highly conserved between the two INs is apparent within the active site. Pairwise alignment represented using ESPRIPT (Gouet et al., 1999), the model in panel (B) was produced using PyMOL (Schrodinger, 2015).

disease. HTLV-1 screening of organ donors is not standard procedure. Recent analysis of living organ donor recipients that were HTLV-1 negative before transplantation show a high incidence of seroconversion and development of HAM (40%) within 4 years of transplantation (Taylor, 2018; Yamauchi et al., 2019). Whether the fast development of HAM is correlated with immunosuppressive therapy is unclear but use of potent INSTIs (either given to the donor to reduce PVL in the organ at the time of explant or administered peri-transplantation) could possibly reduce transmission rate. A neat study in which naturally STLV-1 infected baboons were treated with a combination of valproic acid and zidovudine showed a decline in PVL (Afonso et al., 2010); Cook et al. (2018) reported the long-term clinical remission after cessation of zidovudine in combination with interferonalpha in one patient diagnosed with chronic ATL; and Soriano and colleagues on the other hand reported a decrease in PVL in raltegravir treated HAM patients but not in asymptomatic carriers (Trevino et al., 2012).

The presented data should encourage the use of INSTIs and bictegravir in particular, especially in the preventative treatment for HTLV-1 transmission among drug users, serodiscordant couples, organ recipients and pregnant/breastfeeding mothers. Moreover, more clinical trials are needed to investigate the efficacy of using anti-retrovirals (combining RT and INSTI inhibitors with or without IFN-alpha or valproic acid) in reducing PVL thereby reducing chances of transmission between partners, but also in cases of organ donor transplantation, and possibly preventing the evolution of asymptomatic carrier to HTLV-1 associated disease. Treating pregnant women with INSTIs might warrant some caution; given the reduced exposure of elvitegravir during pregnancy it increases the risk of virological failure and mother-to-child transmission (Momper et al., 2018; van der Galien et al., 2019). The WHO issued a drug safety alert

for dolutegravir<sup>5</sup> following the observation that children born to HIV-1 positive mothers treated with dolutegravir at the time of conception were more likely to have neural tube defects (Zash et al., 2018). Data obtained from a recent study in France did not support a pharmacovigilance signal on neural tube defects in women exposed to dolutegravir, raltegravir or elvitegravir (Chouchana et al., 2019). The limitation of all these studies are the small number of patients involved. Nevertheless, caution is warranted and careful monitoring and sharing of information in regard to anti-retroviral therapy during pregnancy to investigate the risks involved, is paramount. Bictegravir has only recently been FDA approved6, thus investigations are needed to see whether this INSTI, a close relative of dolutegravir, is safe to use during pregnancy. For now, raltegravir is the recommended INSTI to suppress viral load in HIV-1 pregnant women and prevent mother-to-child transmission, and no adverse effects have been found (Chouchana et al., 2019; Rasi et al., 2019; van der Galien et al., 2019).

Currently, the most devastating pathology caused by HTLV-1 is ATLL. Prognosis is very poor – most patients die within several months after presentation (Beltran et al., 2011). At the same time, it is known that the likelihood of an HTLV-1 carrier developing ATLL is correlated with exposure early in life – most commonly during breastfeeding or child-birth (Carneiro-Proietti et al., 2014). In conclusion, using INSTIs as a prophylactic could have a dramatic impact on limiting the spread of the virus within many high-risk populations and preventing the development of ATLL among them.

<sup>&</sup>lt;sup>5</sup>https://www.who.int/medicines/publications/drugalerts/Statement\_on\_DTG\_ 18May\_2018final.pdf?ua=1 (accessed May 07, 2019)

<sup>&</sup>lt;sup>6</sup>https://www.accessdata.fda.gov/drugsatfda\_docs/label/2018/210251s000lbl.pdf (accessed December 20, 2018)

#### **DATA AVAILABILITY**

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

#### **AUTHOR CONTRIBUTIONS**

MB designed, executed, and analyzed the experiments, and wrote the manuscript. JM designed, executed, and analyzed the experiments, and edited the manuscript. GM designed and analyzed the experiments, and wrote the manuscript.

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#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.01877/full#supplementary-material

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# Post-transcriptional Regulation of HTLV Gene Expression: Rex to the Rescue

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Human T-lymphotropic virus type 1 (HTLV-1) and other members of the Deltaretrovirus genus code for a regulatory protein named Rex that binds to the Rex-responsive element present on viral mRNAs. Rex rescues viral mRNAs from complete splicing or degradation and guides them to the cytoplasm for translation. The activity of Rex is essential for expression of viral transcripts coding for the virion components and thus represents a potential target for virus eradication. We present an overview of the functional properties of the HTLV-1 and HTLV-2 Rex proteins (Rex-1 and Rex-2), outline mechanisms controlling Rex function, and discuss similarities and differences in the sequences of Rex coded by HTLV-1, -2, -3, and -4 that may influence their molecular anatomy and functional properties.

Keywords: HTLV-1, HTLV-2, splicing, RNA export, Rex

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#### INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) infects approximately 10 million persons worldwide (Willems et al., 2017). HTLV-1 is the causative agent of adult T-cell leukemia/lymphoma (ATLL), tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) and several inflammatory diseases (Futsch et al., 2017). HTLV-1 is classified in seven molecular subtypes, named a, b, c, d, e, f, and g, with characteristic geographic distributions in several endemic regions (Gessain and Cassar, 2012). The closely related virus HTLV-2 circulates as two major subtypes, named a and b, mainly in indigenous populations of South America and western and central Africa, and in injection drug users (Roucoux and Murphy, 2004). Although the pathogenic spectrum of HTLV-2 is not clearly defined (Ciminale et al., 2014), infection with this virus may be associated with neurological disease (Araujo and Hall, 2004) and appears to significantly increase all-cause and cancer-related mortality (Biswas et al., 2010). Two other HTLVs, named HTLV-3 (Calattini et al., 2005; Wolfe et al., 2005) and HTLV-4 (Switzer et al., 2009), were identified in individuals living in the rainforests of Cameroon and are of unknown pathogenicity (Mahieux and Gessain, 2011). The HTLVs are classified in the Deltaretrovirus genus, which also includes the closely related simian T-lymphotropic viruses and bovine leukemia virus. Deltaretroviruses are considered to be "complex" retroviruses, as they produce regulatory and accessory proteins and exhibit 2-phase expression of alternatively spliced mRNAs (Cullen, 1991; Cavallari et al., 2011).

The replication cycle of the HTLVs (and all Deltaretroviruses) is controlled by the viral regulatory proteins Tax and Rex, which are coded in open reading frames (ORFs) named x-IV and x-III, respectively, located on the plus-strand of the proviral genome. Tax increases transcription from the 5'LTR promoter as well as the promoters of many cellular genes, and plays a key role in

viral replication and cell transformation (Romanelli et al., 2013). As illustrated in **Figure 1**, Rex regulates viral mRNA expression at the post-transcriptional level by interacting with a complex stem-loop RNA structure termed the Rex-responsive element (RXRE), present at the 3' portion of all plus-strand viral transcripts. This interaction relieves the inhibitory effects of the RXRE and of cisacting repressive sequences (CRS) present in incompletely spliced mRNAs, rescues these mRNAs from splicing or degradation, and allows their exit from the nucleus through a pathway mediated by the cellular export factor CRM1 (also referred to as exportin 1/XPO1) (Younis and Green, 2005).

The RXRE is located in the 3'LTR, with RXRE-1 mapping to a short portion of U3 and most of R (Toyoshima et al., 1990) and RXRE-2 including the entire R segment and a small portion of U5 (Kim et al., 1991). Its stem-loop structure brings the polyadenylation signal close to the polyadenylation site (Bar-Shira et al., 1991), ensuring efficient polyadenylation of viral mRNAs. This position suggests that all transcripts, including multiply spliced species, may have some degree of Rex-responsiveness; this was confirmed for constructs expressing HTLV-1 spliced mRNAs as intronless cDNAs (D'Agostino et al., 1999; Bai et al., 2012). An incomplete RXRE (R for RXRE-1 and R-U5 for RXRE-2) is also present at the 5' end of the unspliced transcript.

In addition to CRS, introns and the RXRE, HTLV transcripts contain multiple stop codons, two ribosomal frameshifting signals and multiple splice acceptors that could be recognized by the non-sense-mediated decay (NMD) machinery. A study of HTLV-1-infected cells provided evidence that Rex contributes to suppress NMD of viral and cellular transcripts through a mechanism that does not involve interaction with the RXRE (Nakano et al., 2013). Results of *in vitro* experiments suggest that Rex-1 may also interfere with the activity of Dicer (Abe et al., 2010), a key component of the siRNA- and microRNA processing machinery. Its interactions with NMD and Dicer-dependent pathways suggest that Rex may have broad effects on cellular RNA processing and expression.

Studies of Rex and its HIV homolog Rev have contributed substantially to our knowledge of RNA processing as well as nucleo-cytoplasmic shuttling of proteins and RNA (Shida, 2012; Nakano and Watanabe, 2016; Rekosh and Hammarskjold, 2018). The following sections provide a brief description of Rex coded by HTLV-1 and HTLV-2, referred to as Rex-1 and Rex-2, respectively, and point out mechanisms that control Rex's activity. We also comment on similarities and differences in the sequences of Rex-1, Rex-2, and Rex proteins coded by HTLV-3 and HTLV-4, whose activities have not been studied to date, and highlight aspects of Rex function that remain to be understood.

## FUNCTIONAL DOMAINS IN REX-1 AND REX-2

Almost all of the information on Rex was gathered from studies of Rex-1 coded by subtype-a isolate ATK-1 and Rex-2 coded by subtype-a isolate Mo. Rex-1 (ATK-1) and Rex-2 (Mo) contain 189- and 170 amino acids, respectively, and four main functional

domains (**Figure 2A**; Younis and Green, 2005; Shida, 2012). An amino-terminal, arginine-rich nuclear/nucleolar localization signal (NLS) targets Rex to the nucleus through binding to importin  $\beta$ . This sequence also functions as an RNA binding domain (RBD) that mediates binding to the RXRE. A centrally positioned nuclear export sequence (NES) mediates binding of Rex to CRM1. The NES is flanked by two regions required for the formation of Rex multimers, a process that is facilitated by interaction with CRM1. A phosphorylation-regulated carboxy-terminal domain enhances Rex's stability and function (Kesic et al., 2009a,b; Xie et al., 2009).

**Figure 2B** shows a CLUSTAL alignment of Rex-1 (ATK-1), Rex-2 (Mo) and the Rex-3 and Rex-4 proteins coded by 2 full-length HTLV-3 and HTLV-4 isolates (GenBank nos. listed in **Supplementary Table 1**). Overall, the NLS/RBD and NES show higher sequence identity compared to the multimerizationand stability domains. However, 9 of the 21 residues comprising Rex-2's stability domain show perfect identity among the Rex proteins, suggesting a conserved functional role for this region. Percent-identity calculations showed that Rex-1 (ATK-1) is more similar to Rex-2 (Mo) than to Rex-3 or Rex-4, while Rex-2 (Mo) is most similar to Rex-4 (see **Supplementary Table 2**).

Rex-1 proteins coded by isolates of subtypes a, b, and c also showed some variation, especially between subtype-a and subtype-c ORFs (86.77–88.36% identity, see **Supplementary Table 3**). One subtype-a isolate, from an ATL patient in Iran, codes for Rex with 20 additional carboxy-terminal amino acids, a feature that might influence its stability domain (see **Supplementary Figure 1**). Alignments of 11 Rex-2 ORFs (5 subtype-a and 6 subtype-b) revealed 93.53–95.88% identity between the two subtypes (**Supplementary Table 4**), with distinct "signatures" of amino acids at positions 104, 105, 123, 126, and 136 (see arrows in **Supplementary Figure 1**). Interestingly, Rex-2 (Mo) was not the most common subtype-a sequence.

## CONTROL OF REX FUNCTION BY PHOSPHORYLATION

Early studies of Rex-1 showed that it migrates as a 27-kDa band in SDS-PAGE and is phosphorylated on multiple serines and threonines (Adachi et al., 1990, 1992). Mass spectrometry analysis of Rex-1 (ATK-1) (Kesic et al., 2009a) identified phosphorylation of Thr-22, Ser-36, Thr-37, Ser-70, Ser-97, Ser-106, and Thr-174 (see **Figure 2A**). Assays with a Gag-RXRE reporter plasmid and Rex-1 (ATK-1) mutants carrying phosphoablative (alanine) and phosphomimetic (aspartic acid) substitutions of these residues indicated that phospho-Ser-97, located in the NES, and phosho-Thr-174, located in the stability domain, contribute substantially to Rex activity (Kesic et al., 2009a).

Five of the seven phosphoacceptor sites identified in Rex-1 (ATK-1) (i.e., Thr-22, Ser-36, Thr-37, Ser-97, and Thr-174; **Figure 2C**) are conserved across the 15 HTLV-1 isolates aligned in **Supplementary Figure 1**. It is noteworthy that Ser-36 and Ser-97 are also conserved in Rex-2, Rex-3, and Rex-4 (see **Supplementary Figure 1**).

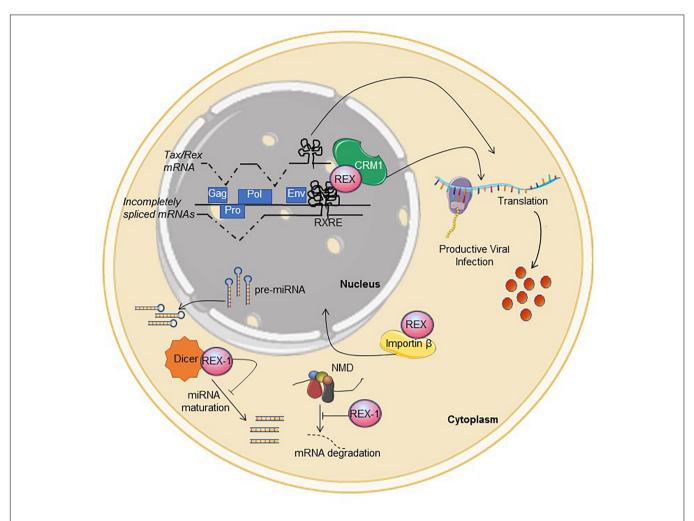


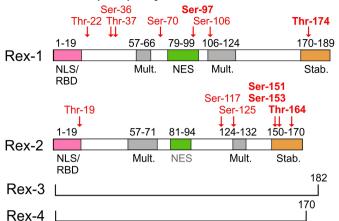
FIGURE 1 | Functional activities described for Rex. See text for description. Interference with NMD and Dicer were described for Rex-1. The figure was made using SMART images (https://smart.servier.com).

The Rex-2 (Mo) ORF produces two isoforms of 24- and 26 kDa that differ in phosphorylation (Green et al., 1991). Mass spectrometry analysis (Kesic et al., 2009b) showed that p26-Rex-2 is phosphorylated on Thr-19, Ser-117, Ser-125, Ser-151, Ser-153, and Thr-164 (see Figure 2A), while p24Rex-2 is phosphorylated on Ser-117 and Thr-164. Functional assays on point mutants revealed an important role for phosphorylation on Ser-151, Ser-153, and Thr-164, with phospho-Ser-151 particularly important for Rex-2's ability to accumulate in the nucleus, bind to the RXRE and exert full functional activity (Narayan et al., 2001, 2003; Kesic et al., 2009b). These studies also suggested that sequential phosphorylation of Thr-164 followed by Ser-151 and Ser-153 converts the protein from a closed to an open conformation that exposes the NLS/RBD (Kesic et al., 2009b). Residues equivalent to Thr-19, Ser-125, and Thr-164 are present in all of the Rex proteins aligned in Supplementary Figure 1 (Figure 2C). The position of Thr-164 in a conserved region known to be important for stability and activation of Rex-2 suggests that it might be functionally relevant in all Rex proteins.

The protein kinases responsible for Rex phosphorylation have not been experimentally identified. Analysis of the Rex-1 (ATK-1) and Rex-2 (Mo) ORFs with the NetPhos and ScanSite prediction tools yielded one or more candidate kinases for most of the phosphoacceptor sites identified by mass spectrometry (Kesic et al., 2009a,b) as well as many other potential phosphoacceptor sites and kinases (Supplementary Table 5). Although the NetPhos and ScanSite predictions in general showed little agreement, both algorithms identified kinases potentially targeting Ser-97 in Rex-1 and the equivalent serine in Rex-2, Rex-3, and Rex-4 (Supplementary Table 5). Its conservation and position within the NES suggests that Ser-97 phosphorylation might influence Rex's interaction with CRM1 and thereby modulate the nuclear export of cargo RNA.

Most of the studies of Rex phosphorylation have been carried out in cell lines of non-lymphoid derivation (e.g., 293T, Cos, HeLa) transfected with Rex expression plasmids. As the expression of protein kinases can be highly cell-type specific, it will be important to study the protein in cells that are natural targets of HTLV infection *in vivo* – predominantly





#### **B** Alignments

```
MPKTRR-RPRRSQRKRPPTPWPTSQGLDRVFFSDTQSTCLETVYKATGAPSLGDYVRPAY 59
Rex-1 (ATK-1)
Rex-2 (Mo)
              MPKTRORTRRARRNRPPTPWPISQDLDRASYMDTPSTCLAIVYRPIGVPSQVVYVPPAY 60
Rex-3 (Py143)
              MPKTRKQRSRRPRNQRPSTPWPISQVSDRAFSTGTLSTFSATVYRPIGAPFLGGFVPLGY
                                                                           60
Rex-4 (1863LE) MPKTRPPTTRARRNRPPTPWPTSQDSGRASSMDTPSMCLAIVFKPIGAPSPVDYAPPAY 60
               *****: * ** :.:** **** ** .*.
                                                        *:: *:*
              IVTPYWPPVQSIRSPGTPSMDALSAQLYSSLSLDSPPSPPREPLRPSRSLPROSLIOPPT 119
Rex-1 (ATK-1)
Rex-2 (Mo)
               IDMPSWPPVQSTNSPGTPSMDALSALLSNTLSLASPPSPPREPQGPSRSLPLPPLLSPPR 120
Rex-3 (Pv143)
              TAMPCWPRAPNTRI.PGTPSMDAT.SAOI.YNTI.SI.GSPPSPPKET.PAPSRFSPPOPT.T.RPPR 120
Rex-4 (1863LE)
              IATPSWPPAPSTRSPGTPSMDELSARLSNTLSLASPPSPPNEPPRPSKSLPHQPLLSPPR 120
                  * ** . . . ****** *** * .:*** *****
Rex-1 (ATK-1)
              FHPPSSRPCANTPPSEMDTWNPPLGSTSQPCLFQTPDSGPKTCTPSGEAPLSACTSTSFP 179
Rex-2 (Mo)
              FHLPSFNOCESTPPTEMDAWNOPSGISSPPSPSPNLASVPKTSTPPGEKP----- 170
              FLHPSSTPLKNTPPSETIASSSPWESSCQPCPSPTLGSGPKTSTPYGAAPSCVSTSISSP 180
Rex-3 (Py143)
Rex-4 (1863LE) FHPPSFSPCGGTAPTATDVLKQPLESSSPPLHFLSQASGPKTSTPSGERP-----
                        .* *: . . *
                                      :. * . . * ***. * * . . . * * *
Rex-1 (ATK-1)
              PPSPGPSCPT
                          189
Rex-2 (Mo)
                          170
                          182
Rex-3 (Py143)
              PP----
Rex-4 (1863LE)
                          170
```

#### C Conservation of phospho-acceptor residues

#### Rex-1

Conservation	Thr-22	Ser-36	Thr-37	Ser-70	Ser-97	Ser-106	Thr-174
Rex-1 (15 isolates)	15/15	15/15	15/15	13/15	15/15	14/15	15/15
Rex-2 (11 isolates)	0/11	11/11	11/11	11/11	11/11	11/11	-
Rex-3 (1 isolate)	0/1	1/1	1/1	0/1	1/1	1/1	1/1
Rex-4 (1 isolate)	1/1	1/1	0/1	1/1	1/1	1/1	-

#### Rex-2

Conservation	Thr-19	Ser-117	Ser-125	Ser-151	Ser-153	Thr-164
Rex-1 (15 isolates)	15/15	0/15	15/15	0/15	2/15	15/15
Rex-2 (11 isolates)	11/11	11/11	11/11	11/11	11/11	11/11
Rex-3 (1 isolate)	1/1	0/1	1/1	0/1	1/1	1/1
Rex-4 (1 isolate)	1/1	1/1	1/1	0/1	0/1	1/1

FIGURE 2 | Domain structure and sequence comparison of HTLV Rex proteins. (A) shows the functional domain structure of Rex-1 and Rex-2. Positions of phosphoacceptor sites identified in Rex-1 (ATK-1) (Kesic et al., 2009a) and Rex-2 (Mo) (Kesic et al., 2009b) are indicated in red type. Diagrams are modified from Kesic et al. (2009a,b). (B) shows a multiple sequence alignment of Rex proteins obtained with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Amino acids are labeled in different colors according to their biochemical properties (red, small/hydrophobic; blue, acidic; magenta, basic; green, hydroxyl/sulfhydryl/amine/Glycine). Asterisks indicate single conserved residues; and periods indicate residues with similar properties. GenBank Accession IDs: HTLV-1 (ATK-1): J02029.1; HTLV-2 (Mo): M10060.1; HTLV-3 (Pyl 43): DQ462191.1; HTLV-4 (1863LE): EF488483.1 (see also Supplementary Figure 1). Tables in (C) show conservation of the indicated serines and threonines in Rex-1 and Rex-2 proteins coded by the panel of 28 viral isolates listed in Supplementary Table 1.

CD4 <sup>+</sup> T-cells for HTLV-1 (Richardson et al., 1990; Melamed et al., 2015) and CD8 <sup>+</sup> T-cells for HTLV-2 (Ijichi et al., 1992; Melamed et al., 2014). The identification of Rex's functionally relevant phospho-acceptor sites in these cells may have clinical implications, as inhibitors for some of the kinases predicted by NetPhos and ScanSite are already used in cancer therapy (Roskoski, 2019).

#### TRUNCATED REX ISOFORMS

As depicted in **Figure 3**, Tax and Rex are expressed from a doubly spliced, bicistronic mRNA containing exons 1, 2, and 3. In addition to mRNA 1-2-3, HTLV-1, and HTLV-2 also produce transcripts that code for truncated forms of Rex. In HTLV-1, an mRNA that contains exon 1 linked to exon 3 (Orita et al., 1991) produces p21Rex, whose initiator methionine corresponds

to methionine 79 of full-length Rex-1. p21Rex contains the NES, second multimerization domain segment and C-terminal stability domain of Rex-1, but lacks the N-terminal RBD/NLS (**Figure 3A**). While two studies indicated that p21Rex does not alter the function of full-length Rex-1 (Ciminale et al., 1997; Bai et al., 2012), another indicated an inhibitory effect (Heger et al., 1999), and a fourth study indicated that p21Rex, similar to full-length Rex, stabilizes the unspliced transcript through NMD inhibition (Nakano et al., 2013). Met-79 is present in all of the Rex ORFs examined in **Supplementary Figure 1**, so all of these viruses should be able to express p21Rex.

In HTLV-2, spliced mRNAs 1-3 and 1-B code for truncated Rex-2 proteins named p22/p20Rex and p17Rex (Ciminale et al., 1997; **Figure 3B**). These mRNAs also contain the x-II ORF, which codes for the regulatory protein p28 (Ciminale et al., 1995). The p22/20Rex isoforms initiate at Met-33 of full-length Rex-2 and contain both segments of the multimerization domain,

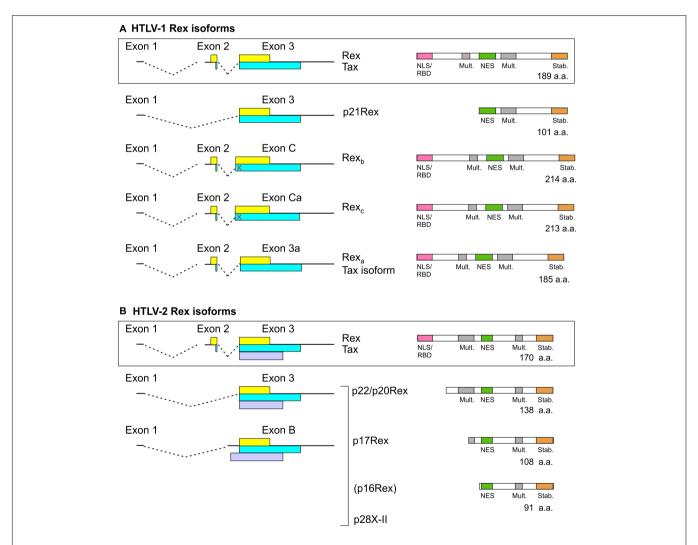


FIGURE 3 | Rex isoforms coded by HTLV-1 (A) and HTLV-2 (B). mRNAs coding for Tax/Rex and Rex isoforms are indicated on the left. The Tax and Rex ORFs are indicated by yellow and blue boxes, respectively, and the HTLV-2 p28 (x-II) ORF is indicated by a light purple box. The domain structures of Rex isoforms are indicated on the right. The red X indicates a stop codon upstream of the exon 3 splice acceptor that is in frame with the Tax ORF. p16Rex is a very low-abundance truncated Rex-2 isoform (Ciminale et al., 1995).

the NES and the stability domain, while p17Rex starts at Met-63. Functional analyses of p22/p20 showed that they sequester full-length Rex-2 in the nucleus and interfere with its ability to activate expression of a RXRE-dependent RNA reporter. This effect was attributed to changes in Rex-2 phosphorylation induced by the truncated proteins rather than through the formation of inactive heteromultimers (Ciminale et al., 1997).

While Met-33 is not present in any of the 15 Rex-1 proteins or in Rex-3 shown in **Supplementary Figure 1**, it is present in 10 out of 11 Rex-2 proteins and in Rex-4. It is noteworthy that the Rex-1 ORFs coded by 2 Australian isolates examined in **Supplementary Figure 1** contain a methionine seven codons downstream the position aligning with Met-33. These isolates, as well as HTLV-4, thus have the potential to produce proteins similar to p22/p20Rex and p21Rex.

#### **FUNCTIONAL REX ISOFORMS**

A search for novel monocistronic HTLV-1 transcripts coding for only Tax or Rex revealed the production of mRNAs that contain exons 1 and 2 linked to splice acceptors (SA) located upstream (C, Ca) or downstream (3a) of the canonical exon 3 SA; the use of these SA results in insertion or deletion of amino acids in the Rex ORF just after the NLS/RBD (Figure 3A; Rende et al., 2015). Transcripts 1-2-C and 1-2-Ca code for longer Rex-1 isoforms named Rex<sub>b</sub> (214 a.a.) and Rex<sub>c</sub> (213 a.a.), respectively, but do not produce Tax, and mRNA 1-2-3a produces a 185-residue Rex-1 isoform named Rex<sub>a</sub> and a Tax isoform lacking four amino-proximal amino acids. Results of functional assays showed that all three Rex isoforms are functionally active. However, Rexb and Rexc are mainly cytoplasmic, suggesting that their extra amino acids affect the NLS and/or NES (Rende et al., 2015). It will be interesting to compare the expression of the alternatively spliced Tax/Rex and Rex isoform mRNAs in the context of asymptomatic infection and HTLV-1-associated pathologies.

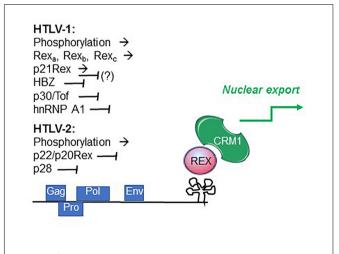
The possibility that the other HTLVs produce analogous Rex isoforms remains to be investigated. SAs C, Ca and 3a are present in all 15 HTLV-1 isolates listed in **Supplementary Table 1**. However, six of the isolates, including the prototype ATK-1, contain a stop codon between SAs C and 3, and are thus predicted to produce Rex<sub>a</sub> but not Rex<sub>b</sub> or Rex<sub>c</sub>. Among the HTLV-2, HTLV-3, and HTLV-4 isolates in **Supplementary Table 1**, all but one are likewise predicted to produce a Rex<sub>a</sub>-like protein but not Rex<sub>b</sub> or Rex<sub>c</sub> due to the absence of the corresponding SAs and presence of one or more stop codons. HTLV-2b isolate Gu lacks all three alternative SAs and contains stop codons, and thus should not produce any of the extra Rex isoforms.

## THE TWO-PHASE MODEL OF HTLV EXPRESSION AND VIRAL LATENCY

The first investigation of HTLV-1 mRNA expression kinetics, performed by transfecting a full-length HTLV-1 provirus

and northern blotting, revealed early (Rex-independent) expression of multiply spliced mRNA and late (Rex-dependent) accumulation of singly spliced and unspliced mRNA (Hidaka et al., 1988). This two-phase model was supported and refined by time-course studies that employed quantitative RT-PCR with splice-site specific primers to detect individual alternatively spliced transcripts. Experiments performed in 293T cells transfected with an HTLV-1 molecular clone confirmed early accumulation of the Tax/Rex mRNA followed by a steady increase in the unspliced transcript and singly spliced env mRNA, but did not indicate Rex-dependence of other alternatively spliced species (Li et al., 2009, 2012). In partial contrast with these results, analyses of transfected and infected cell lines and cells from infected patients revealed that some of the alternatively spliced transcripts coding for accessory/regulatory proteins accumulate during the late phase together with the unspliced Gag-Pro-Pol and singly spliced Env mRNAs, indicating their Rex-dependence (Rende et al., 2011; Cavallari et al., 2016). All of the late mRNAs contain a 75-nucleotide 3' CRS located between the splice acceptors for exons C and 3 that is absent from the Rex-independent mRNAs (Cavallari et al., 2016). These experiments also showed that mitosis partially overcomes the Rex-dependence of some transcripts, suggesting that Rex function is critical in the context of resting or slowly dividing cells (Cavallari et al., 2016). The 2-phase timecourse of mRNA expression was also evident in studies demonstrating the ability of Rex and p21Rex to block NMD (Nakano et al., 2013).

Mathematical modeling of HTLV-1 expression indicated the requirement for a delay in Rex function compared with Tax in order to support the 2-phase kinetics observed experimentally (Corradin et al., 2010). However, it is difficult to explain how Rex function might be delayed compared to Tax, given their co-expression by mRNA 1-2-3 (an early transcript). Possible contributing factors include (i) the shorter half-life of Tax and progressive accumulation of Rex, which is relatively stable (Rende et al., 2011); (ii) the temporal pattern of expression of potential



**FIGURE 4** | Factors controlling Rex function. Summarized are factors that promote  $(\rightarrow)$  and interfere with  $(\neg)$  Rex function, as described in the text.

D'Agostino et al.

HTLV Rex

inhibitors of Rex (e.g., p21Rex mRNA 1-3, expressed early); and (iii) late-phase accumulation of the monocistronic mRNAs coding for functional Rex isoforms  $\text{Rex}_b$  and  $\text{Rex}_c$  but not Tax.

It is also possible that changes in the availability of cellular factors influence the relative activity of the 2 proteins during the expression cycle. An example is heterogenous nuclear ribonucleoprotein A1 (hnRNP A1), an important regulator of RNA processing that was shown to interfere with Rex function by competitively binding to the RXRE (Duc Dodon et al., 2002) (**Figure 4**).

Recent studies indicated that individual HTLV-1-infected cells undergo alternating bursts of viral gene expression and latency (Billman et al., 2017; Mahgoub et al., 2018). Shutdown of viral gene expression following the burst phase may involve the activity of the late-phase protein p30Tof, a nuclear/nucleolar product of the x-II ORF (Ciminale et al., 1992; Koralnik et al., 1993). In addition to important transcriptional effects, p30Tof blocks the Tax/Rex mRNA in the nucleus (Nicot et al., 2004), a property shared by the HTLV-2 x-II ORF protein p28 (Anupam et al., 2013). p30Tof also binds directly to Rex-1, resulting in interference with RXRE binding (Sinha-Datta et al., 2007) and failure to export RXRE-containing RNA cargo (Baydoun et al., 2007). Analyses of the intranuclear trafficking of Rex-1 showed that it normally encounters CRM1 within the nucleoli, and then travels as Rex-CRM1 complexes to nucleoplasmic foci, where it binds to RXRE-containing mRNA that will eventually be exported through the nuclear pore complex. p30/Tof blocks Rex-CRM1 complexes in the nucleoli, thus precluding their interaction with RNA cargo and blocking the RNA export leg (Baydoun et al., 2007). HBZ, a multifunctional regulatory protein coded on the minus strand of HTLV-1 (Ma et al., 2016), was also shown to interfere with Rex function (Philip et al., 2014) (Figure 4).

Studies of HTLV-2 expression kinetics indicated a similar 2-phase pattern of mRNA production (Bender et al., 2012). The observed late-phase expression of abundant levels of mRNA 1-3, coding for p22/20Rex and p28, suggests that these proteins may engage a negative feedback loop in the late phase through p22/p20Rex-mediated interference with Rex function and through p28-mediated retention of the Tax/Rex mRNA in the nucleus (Younis et al., 2004), thus favoring a shut-down of productive infection (Bender et al., 2012). The influence of the HTLV-2 minus-strand protein APH-2 on Rex function remains to be investigated.

#### **CONCLUSION AND PERSPECTIVES**

Comparisons of the biological properties of wild-type and Rex-defective HTLV-1 molecular clones indicated that while Rex is not essential for *in vitro* immortalization of cultured T-cells (a hallmark of HTLV-1 and HTLV-2), it is required for establishment of persistent infection in a rabbit model (Ye et al., 2003). This is an important finding, as persistent

viral replication is considered to be a key factor that drives the inflammatory response to HTLV-1, with risk for developing TSP/HAM, and ensures the generation of a vast population of infected cells at risk for neoplastic transformation (Bangham, 2018).

The discovery of Rex-1 as an essential factor for expression of Gag/Pro/Pol RNA (Inoue et al., 1986) opened up a research field that yielded a wealth of information on the mechanisms regulating retroviral gene expression as well as cellular mRNA processing pathways. Further studies of Rex are needed in order to understand how its activity may be fine-tuned through phosphorylation and interactions with alternative Rex isoforms, other HTLV regulatory proteins, and cellular factors involved in mRNA processing, export and translation (Figure 4). These control points could be of key importance to turn on and off Rex function during the early/late phases the kinetics of expression of viral genes as well as in the bursts of expression revealed by the more recent single-cell analysis. It will be critical to focus future investigations of Rex-controlled HTLV gene expression on the natural cell targets of the virus, and to determine whether Rex phosphorylation, function and the pattern of splicing of viral transcripts change over time in infected individuals and, in the case of HTLV-1, are associated with development of disease. Answers to these questions could also pave the way to the development of novel therapeutic strategies to eradicate HTLV infection.

#### **AUTHOR CONTRIBUTIONS**

All authors worked together to prepare the manuscript. DD performed the sequence alignments and prepared the **Supplementary Material**.

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#### **SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.01958/full#supplementary-material

D'Agostino et al. HTLV Rex

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D'Agostino et al.

HTLV Rex

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# Cerebrospinal Fluid CXCL10 as a Candidate Surrogate Marker for HTLV-1-Associated Myelopathy/ Tropical Spastic Paraparesis

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Human T-cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a debilitating, progressive disease without effective treatment; therefore, development of disease modifying therapy that improves long-term functional outcomes is an unmet need for patients. However, it is virtually impossible to consider this as a primary endpoint in clinical trials owing to the prolonged disease course. Therefore, development of surrogate markers that help predict the effectiveness of new interventions is essential. Currently, several candidate surrogate markers have been identified for HAM/TSP. Cerebrospinal fluid (CSF) C-X-C motif chemokine 10 (CXCL10) is involved in the pathogenesis of HAM/TSP and was shown to correlate with disease progression. However, it remains unclear whether changes in CSF CXCL10 levels are observed in response to treatment and whether these correlate with prognosis. Here we investigated several markers, including CSF CXCL10, in this respect. Data pertaining to patient characteristics and results of motor function evaluation and CSF examination of 13 HAM/TSP patients who received steroid treatment were retrospectively analyzed. Osame motor disability scores (OMDS), 10 m walking time, and CSF levels of CXCL10, neopterin, total protein, cell counts, and anti-HTLV-1 antibody titer were compared before and after steroid therapy. Levels of all CSF markers, with the exception of cell count, were significantly decreased after treatment. Nine of the 13 patients (69.2%) showed improvement in OMDS and were considered responders. Pre-treatment CSF levels of CXCL10 and anti-HTLV-1 antibody titer in responders were higher than those in non-responders (p = 0.020 and p = 0.045, respectively). Patients who continued lowdose oral prednisolone maintenance therapy after methylprednisolone pulse therapy showed sustained improvement in OMDS and CSF CXCL10 and neopterin levels lasting for 2 years. In contrast, OMDS and the CSF marker levels in patients who

discontinued treatment returned to pre-treatment levels. This rebound phenomenon was also observed in patients who discontinued oral prednisolone therapy independently of pulse therapy. Our findings suggest that CSF CXCL10 may serve as a therapy-response and therapy-predictive marker for HAM/TSP. In addition, since decrease in CSF CXCL10 level was associated with good functional prognosis, CSF CXCL10 is a potential surrogate marker for treatment of HAM/TSP.

Keywords: HTLV-1, HAM/TSP, cerebrospinal fluid, neopterin, CXCL10, biomarker

#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a human pathogenic retrovirus (Poiesz et al., 1980). A proportion of HTLV-1 carriers develop adult T-cell leukemia/lymphoma (Uchiyama et al., 1977; Hinuma et al., 1981) and/or HTLV-1associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). HAM/TSP is a neuroinflammatory disease characterized by infiltration of HTLV-1-infected T-cells into the spinal cord; the resultant chronic inflammation is believed to lead to spinal cord damage (Yamano and Sato, 2012; Bangham et al., 2015). The disease is characterized by spastic paraparesis, bladder and rectal disturbance, and sensory abnormality. The symptoms typically last throughout the life of the afflicted individual. Therefore, the true endpoint of treatment for HAM/TSP is the improvement of long-term functional prognosis. Gait disturbance, which is the main symptom of HAM/TSP, generally worsens over several years (Olindo et al., 2006; Martin et al., 2010; Coler-Reilly et al., 2016); therefore, a clinical trial with substantially long followup is required to prove the efficacy of treatment for the true endpoint. However, such trials are difficult to conduct and cannot immediately deliver new drugs to patients suffering from illness. Therefore, instead of this true endpoint, it is necessary to identify a surrogate endpoint that can facilitate quicker evaluation of the therapeutic efficacy in patients with HAM/TSP. This is a significant unmet need for patients with HAM/TSP.

An ideal surrogate marker should qualify the following criteria (Buyse et al., 2010; Zhao et al., 2015):

- 1. good correlation with disease progression;
- 2. biological plausibility that shows association with the true endpoint;
- correlation with the true endpoint independent of treatment; and
- 4. the effect of treatment on the surrogate marker correlates with its effect on the true endpoint.

Several candidate surrogate markers in the context of HAM/TSP have already been identified; these include Cerebrospinal fluid (CSF) markers (neopterin, C-X-C motif chemokine 10 (CXCL10), anti-HTLV-1 antibody titer, cell count, and total protein) and HTLV-1 proviral load (Matsuzaki et al., 2001; Olindo et al., 2005; Sato et al., 2013; Matsuura et al., 2016). Among these, CXCL10 (a chemokine) and neopterin (a metabolite of guanosine triphosphate) are abundantly present in the CSF of HAM/TSP patients, and their concentrations

were shown to correlate with the degree of disease progression (Nomoto et al., 1991; Narikawa et al., 2005; Sato et al., 2013). In particular, CXCL10 has been implicated in the causative mechanism of chronic inflammation in HAM/TSP (Ando et al., 2013; Araya et al., 2014). CXCL10 produced by astrocytes in the spinal cord of HAM/TSP patients recruits CXCR3-positive cells (including HTLV-1-infected cells and inflammatory cells) into the spinal cord; in addition, interferon y produced by the recruited cells further induces CXCL10 production from astrocytes in the spinal cord lesions (Ando et al., 2013). Therefore, CSF CXCL10 is one of the key players in the pathogenesis of HAM/TSP. Furthermore, patients with high levels of CSF CXCL10 were shown to have greater disease activity and poorer long-term prognosis as compared with their counterparts with low CSF CXCL10 levels (Sato et al., 2018b). Thus, CSF CXCL10 qualifies criteria 1-3 for surrogate markers. However, whether it qualifies criterion 4 is not clear. In other words, whether decrease in CSF CXCL10 concentration induced by treatment is associated with improved long-term functional prognosis is not clear. Therefore, in the present study, we first examined whether candidate markers including CSF CXCL10 change in response to treatment. Second, we aimed to verify whether the change in marker level is related to the clinical course for at least 2 years.

Corticosteroids are commonly used to treat HAM/TSP in endemic areas (Bangham et al., 2015). These are effective in suppressing inflammation, especially in the spinal cord. There are two main steroid therapies for patients with HAM/TSP: oral prednisolone therapy and intravenous highdose methylprednisolone pulse therapy. The effectiveness of oral prednisolone therapy was shown in a retrospective study that included a control group (Coler-Reilly et al., 2017). However, there is no clear consensus on the effectiveness of methylprednisolone pulse therapy (Duncan and Rudge, 1990; Araujo et al., 1993; Nakagawa et al., 1996). Even in studies that demonstrated its effectiveness, the beneficial effects were only transient (Duncan and Rudge, 1990; Croda et al., 2008). There have been no reports about the need for maintenance therapy so far. In addition, it is not clear as to which category of patient is suitable for this steroid treatment. We hypothesized that pulse therapy is more effective for patients with high disease activity, and oral prednisolone treatment is necessary as an additional treatment in order to sustain its effect. In this study, we sought to examine the effectiveness of these steroid therapies and to identify markers that predict therapeutic response; in addition, we sought to clarify the group of patients suitable for this treatment.

In this study, we retrospectively investigated the clinical course and the time course of CSF marker levels (including CXCL10) in 22 HAM/TSP patients (13 patients who received methylprednisolone pulse therapy, five patients who did not receive any steroid therapy, and four patients who discontinued low-dose oral prednisolone therapy). Next, we sought to identify patients who are more likely to benefit from steroid therapy; for this purpose, we compared the pre-treatment marker levels between patients who showed improvement in Osame motor disability scores (OMDS) post-treatment and those who did not show improvement post-treatment. Furthermore, in order to examine the linkage between the clinical course and the chronological changes in CSF marker levels, we investigated three groups: patients who received prednisolone maintenance therapy after methylprednisolone pulse therapy, patients who did not receive prednisolone maintenance therapy, and independently of pulse therapy, patients who discontinued oral prednisolone therapy.

#### **MATERIALS AND METHODS**

#### **Ethical Considerations**

The study was approved by the Institutional Review Board of St. Marianna University School of Medicine (#1646) and Fukuoka University Hospital (#14-2-08). Prior to the collection of blood or CSF samples, all participants provided written informed consent for analysis of their samples for research purposes as part of their clinical care.

#### Study Design and Subjects

This was a retrospective observational study. All subjects were diagnosed with HAM/TSP based on the World Health Organization criteria (Osame, 1990). The clinical characteristics and treatment details of all subjects are summarized in Table 1. The treatment group composed of 13 HAM/TSP patients; this included seven patients who received methylprednisolone pulse therapy at the Fukuoka University Hospital between April 2012 and August 2014 (patient nos. 1-7) and six patients who received methylprednisolone pulse therapy at the St. Marianna University Hospital between February 2012 and May 2014 (patient nos. 8-13). The untreated group included five HAM/TSP patients who underwent CSF examination at two time points and who did not receive steroids or interferon-α between the two time points (patient nos. 14-18). As a separate analysis group, we used the data from 4 HAM/TSP patients who had received and subsequently discontinued oral prednisolone therapy (patient nos. 19–22).

#### **Treatment Details**

At the Fukuoka University Hospital, methylprednisolone 1000 mg per day was instilled intravenously for three consecutive days; after a gap of 4 days, methylprednisolone 1000 mg per day was infused intravenously again for three consecutive days. Directly after the pulse therapy, two patients received oral prednisolone therapy whereas five patients did not receive oral prednisolone therapy (Table 1). At the St. Marianna University

Hospital, methylprednisolone (250, 500, or 1000 mg per day) was instilled intravenously for three consecutive days; subsequently, all patients (n = 6) received oral prednisolone therapy (**Table 1**). Since the dose of oral prednisolone was gradually tapered, **Table 1** shows both the starting dose and the 2-year dose. In this paper, a series of treatments implemented at the two hospitals are collectively described as "steroid therapy." In four patients (nos. 19–22), 3–5 mg of oral prednisolone was administered daily for at least 6 months.

#### **Disease Evaluation**

Data pertaining to OMDS, (Table 2) and 10 m timed walk were collected as clinical outcome measures. The OMDS was evaluated before treatment and 1 month after treatment at both the university hospitals. Subjects whose OMDS improved 1 month after treatment compared with that at baseline were defined as responders, and those who did not show improvement were defined as non-responders. Subsequently, OMDS was measured every month for at least 6 months. Only patients who were able to walk for 10 m with or without walking aids underwent the 10 m timed walk. We could collect the data on 10 m timed walk before and about 2 weeks after treatment was performed in both hospitals. Since the 10 m timed walk was not performed regularly at the Fukuoka University Hospital, there are many missing data in this respect.

#### **Measurement of Biomarkers**

Data pertaining to the following biomarkers were collected: CSF markers (CXCL10, neopterin, total protein, anti-HTLV-1 antibody titer, and cell counts) and HTLV-1-proviral load in peripheral blood mononuclear cells (PBMCs). In both hospitals, lumbar puncture for CSF examination is performed before and about 2 weeks after the start of treatment (mean  $\pm$  standard deviation (SD): 2.5  $\pm$  0.9 weeks from the first day of pulse therapy). CSF samples and PBMCs were prepared as described previously (Sato et al., 2013). Briefly, CSF obtained by lumbar puncture was used for routine laboratory tests and further analysis. The anti-HTLV-1 antibody titer in CSF was determined using the gelatin particle agglutination test (Serodia-HTLV-1; Fujirebio, Tokyo, Japan). Neopterin level in CSF was measured using high-performance liquid chromatography at a commercial laboratory (SRL Inc., Tokyo, Japan). CXCL10 in CSF was measured using a cytometric bead array (BD Biosciences, Franklin Lakes, NJ, United States). PBMCs were isolated with standard procedures using Pancoll density gradient centrifugation (density 1.077 g/mL; PAN-Biotech GmbH, Aidenbach, Germany). HTLV-1 proviral load was measured using real-time PCR, following DNA extraction from PBMCs, as previously described (Yamano et al., 2002).

#### Statistical Analysis

The Wilcoxon signed rank test was used to compare the preand post-treatment biomarker levels in the same patient. The Mann–Whitney U test was used to compare baseline values of biomarkers between two patient groups (responders and non-responders). Fisher's exact test was used to examine the relationship between additional oral prednisolone therapy and

TABLE 1 | Characteristics of patients with HAM/TSP enrolled in this study.

Hospital name	Patient no.	Age (years)	Sex	Disease duration	OMDS at baseline	mPSL dose (mg/day)	PSL dose from after pulse therapy until year 2 (mg/day)
Fukuoka University Hospital (FUH)	1	61	F	3 months	6	1000	0.0
	2	71	F	8 months	9	1000	$50.0 \to 10.0$
	3	71	F	6 months	4	1000	0.0
	4	72	F	2 years	5	1000	0.0*
	5	48	F	16 years	4	1000	0.0
	6	67	F	4 years	4	1000	$20.0 \to 5.0$
	7	68	F	9 years	4	1000	0.0
St. Marianna University Hospital (SMUH)	8	58	F	1 year	6	1000	20.0 → 10.0
	9	53	M	2 years	6	250	$40.0 \rightarrow 7.0$
	10	67	F	4 years	3	500	$15.0 \to 2.0$
	11	33	F	17 years	6	500	$20.0 \rightarrow 5.0$
	12	67	F	19 years	8	500	$30.0 \to 8.0$
	13	62	F	10 years	6	1000	$25.0 \rightarrow 7.0$
SMUH	14	71	F	18 years	5	0	N/A
	15	71	F	13 years	5	0	N/A
	16	59	F	1 year	1	0	N/A
	17	64	F	4 years	4	0	N/A
	18	45	F	10 years	4	0	N/A
Hospital name	Patient	Age	Sex	Disease	OMDS at	PSL dose	
	no.	(years)		duration	baseline	(mg/day)	
FUH	19	58	F	3 years	3	5	
SMUH	20	59	F	12 years	5	3	
	21	64	F	18 years	5	3	
	22	67	F	4 years	4	5	

OMDS, osame motor disability score; mPSL, methylprednisolone; PSL, prednisolone; F, female; M, male; N/A, not applicable. \*Patient no. 4 received oral prednisolone therapy from 3 months after treatment because of high levels of CXCL10 and neopterin (Figure 3).

the change in OMDS during the observation period. Statistical analyses and graph composition were performed using R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria) or GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, United States). All *p*-values are two tailed, and the threshold of significance was set at 0.05.

#### **RESULTS**

#### **Subject Characteristics**

The median age (range) of 13 HAM/TSP patients (patient nos. 1–13; 1 male and 12 females) who received methylprednisolone pulse therapy in the treatment group was 67 (33–72) years; the median (range) baseline OMDS was 6 (3–9) (**Table 1**). We defined rapid progressors as those who developed OMDS grade 4 or above within 1 year or developed OMDS grade 5 or above within 2 years from the onset of motor symptoms. The percentage of rapid progressors was 46.2% (6 out of 13 patients); these included four of seven patients at the Fukuoka University Hospital (patient nos. 1–4) and two of six patients at the St. Marianna University Hospital (patient nos. 8 and 9).

The characteristics of five HAM/TSP patients (patient nos. 14–18; all females) in the untreated group were median age (range), 64 (45–71) years; median (range) baseline OMDS, 4 (1–5). There were no rapid progressors in this group. In a separate analysis group (4 HAM/TSP patients; nos. 19–22; all females) who received low-dose oral prednisolone therapy, the median age (range) was 61.5 (58–67) years and the median (range) baseline OMDS was 4.5 (3–5). There were also no rapid progressors in this group.

## Effect of Steroid Therapy on Lower Limb Motor Function

The percentage of patients whose OMDS improved by  $\geq 1$  after 1 month from the start of steroid therapy was 57.1% (four of seven patients) at the Fukuoka University Hospital and 83.3% (five of six patients) at the St. Marianna University Hospital; the overall percentage was 69.2% (**Table 3**). Among these, five patients (38.5%) showed improvement of OMDS by  $\geq 2$ . One patient could not walk 10 m; therefore, the 10 m walking time was evaluated in 12 patients. The percentage of patients whose 10 m walking time improved by 10% after about 2 weeks from the start of pulse therapy was 100% (6 of 6) at the Fukuoka University

TABLE 2 | Osame motor disability score.

Grade	Motor disability			
0	No walking or running abnormalities			
1	Normal gait but runs slowly			
2	Abnormal gait (stumbling, stiffness)			
3	Unable to run			
4	Needs handrail to climb stairs			
5	Needs a cane (unilateral support) to walk			
6	Needs bilateral support to walk			
7	Can walk 5-10 m with bilateral support			
8	Can walk 1-5 m with bilateral support			
9	Cannot walk, but able to crawl			
10	Cannot crawl, but able to move using arms			
11	Cannot move around, but able to turn over in bed			
12	Cannot turn over in bed			
13	Cannot even move toes			

TABLE 3 | Effects of the steroid therapy on lower limb motor function.

	Changes in OMDS in response to steroid therapy					
Hospital	≥2-grade	1-grade	No	Deterioration	Total	
name	improvement	improvement	change			
FUH	3 (42.9%)	1 (14.3%)	3 (42.9%)	0 (0.0%)	7	
SMUH	2 (33.3%)	3 (50.0%)	1 (16.7%)	0 (0.0%)	6	
Total	5 (38.5%)	4 (30.8%)	4 (30.8%)	0 (0.0%)	13	

#### Changes in 10 m WT in response to steroid therapy

Hospital name	≥30% improvement	30%> ≥10% improvement	changes within 10%	≥10% deterioration	Total
FUH	1 (16.7%)	5 (83.3%)	0 (0.0%)	0 (0.0%)	6*
SMUH	2 (33.3%)	3 (50.0%)	1 (16.7%)	0 (0.0%)	6
Total	3 (25.0%)	8 (66.7%)	1 (8.3%)	0 (0.0%)	12

<sup>\*</sup>One non-ambulatory patient was excluded. OMDS, osame motor disability score; FUH, Fukuoka University Hospital; SMUH, St. Marianna University Hospital; 10 m WT, 10-meter walking time.

Hospital and 83.3% (5 of 6) at the St. Marianna University Hospital; the overall percentage was 91.7% (11 of 12) (**Table 3**). Among these, three patients (25.0%) showed improvement in 10 m walking time by 30%. None of the patients showed deterioration in OMDS or 10 m walking time at the time of the above evaluation.

#### Markers of Response to Steroid Therapy

We compared the pre- and post-treatment levels of five CSF markers that are believed to reflect the level of spinal cord inflammation in HAM/TSP patients (n=11 or 12). As shown in **Figure 1** (left), the levels of CXCL10, neopterin, total protein, and anti-HTLV-1 antibody in CSF of HAM/TSP patients who received steroid therapy were significantly reduced 2 weeks after treatment, compared with the pre-treatment levels (p=0.0005, p=0.0005, p=0.0005, and p=0.0078, respectively). CSF cell

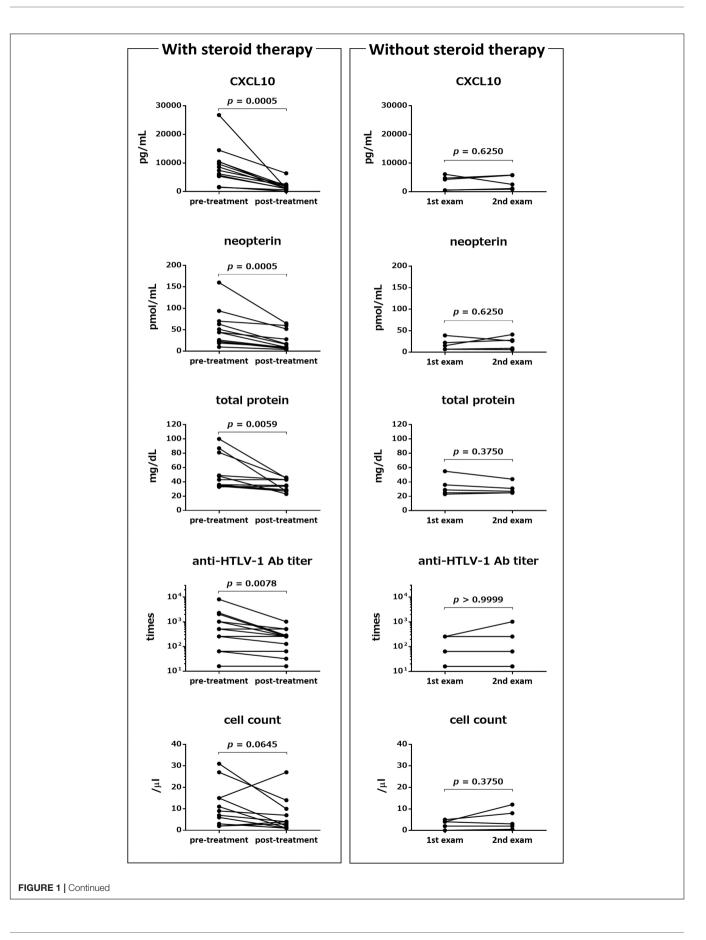
counts also tended to decrease; however, the difference was not significant (p = 0.0645). When comparing the pre- and post-treatment levels for each hospital, significant reduction was observed only for two out of the five CSF markers (CXCL10 and neopterin) (data not shown). In contrast, none of the five markers showed a significant reduction in HAM/TSP patients (n = 5) who were not treated with steroids or interferon- $\alpha$  (**Figure 1**, right).

## Predictors of Response to Steroid Therapy

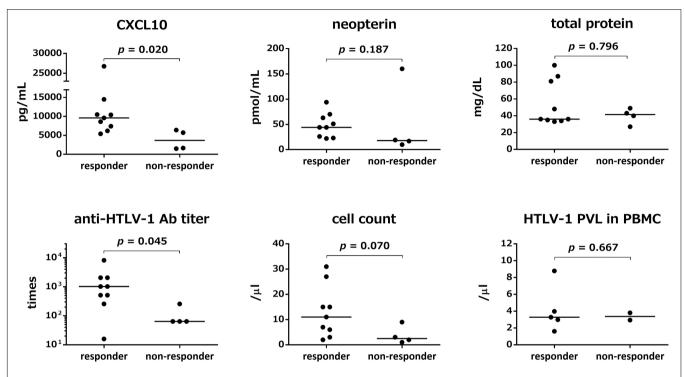
HTLV-1-associated myelopathy/tropical spastic paraparesis patients who showed improvement in OMDS were defined as responders (9 out of 13 patients). In order to identify predictors of therapeutic response, we compared the pre-treatment marker levels between responders and non-responders (Figure 2). In addition to the five CSF markers described above, we also assessed the HTLV-1 proviral load in PBMCs. Pre-treatment CSF levels of CXCL10 and anti-HTLV-1 antibody titer in responders were significantly higher than those in non-responders (p = 0.020and p = 0.045, respectively). CSF neopterin concentration was not significantly different (p = 0.187); however, after exclusion of one non-responder (outlier), CSF neopterin was significantly higher in responders (p = 0.009). CSF cell count showed a higher tendency in responders (p = 0.070). There was no significant difference between the two groups with respect to total protein level (p = 0.796); nevertheless, the total protein level was high in some of the responders. Although the number of patients who we investigated was limited, the HTLV-1 proviral load in PBMCs did not differ significantly between responders and non-responders (p = 0.667).

## Relationship Between Clinical Course and CSF Marker Levels

Next, the clinical course and the time course of CSF marker levels for approximately 2 years after initiation of treatment were compared between the group that received maintenance therapy with oral prednisolone after methylprednisolone pulse therapy (n = 8) and the group that did not receive oral prednisolone (n = 5). The OMDS in seven of eight HAM/TSP patients who received maintenance therapy improved compared with that at baseline; the improvement was maintained afterward in all but one patient (no. 6) who showed no change throughout 2 years (Figure 3A, upper left). Six of seven patients who were able to walk 10 m with or without walking aids showed sustained improvement in the 10 m walking time (Figure 3A, lower left). In contrast, three out of five patients who had not received maintenance therapy showed improvement in OMDS 1 month after pulse therapy; however, the OMDS subsequently deteriorated and returned to the original level in 2 or 3 months (Figure 3A, upper right). Data pertaining to 10 m walking time from 2 weeks after treatment were only available for two patients. The results showed initial improvement followed by deterioration in the 10 m walking time (Figure 3A, lower right). As shown in Table 4, there was a significant association between maintenance therapy with oral prednisolone and change in OMDS during the observation period (p = 0.035). We were



**FIGURE 1** Effects of steroid therapy on Cerebrospinal fluid (CSF) markers. Left: Comparison of pre-treatment levels of the following five CSF markers with those approximately 2 weeks after steroid therapy (mean  $\pm$  standard deviation (SD):  $2.5 \pm 0.9$  weeks from the first day of pulse therapy): C-X-C motif chemokine 10 (CXCL10), neopterin, total protein, anti-HTLV-1 antibody (Ab) titer, and cell count. Post-treatment CSF markers were not available for one or two patients among the 13 patients who received methylprednisolone pulse therapy (n = 12: CXCL10, neopterin, and anti-HTLV-1 antibody titer; n = 11: total protein and cell count). Right: Comparison of the same five CSF markers between two time points (mean  $\pm$  SD:  $16.4 \pm 5.7$  months) in five patients who did not receive any steroid treatment and interferon alpha treatment. Statistical analysis was performed using a Wilcoxon signed rank test. Ab, antibody.



**FIGURE 2** Comparison of pre-treatment marker values between responders and non-responders to steroid therapy. Responders (n = 9) refer to patients who showed improved Osame motor disability scores (OMDS) by one or more grade 1 month after the start of steroid therapy. Non-responders (n = 4) refer to patients who did not show any change in OMDS. The pre-treatment values of five CSF markers (CXCL10, neopterin, total protein, anti-HTLV-1 antibody titer, and cell count) and HTLV-1 proviral load in Peripheral blood mononuclear cells (PBMCs) were compared between responders and non-responders. Data were analyzed by Mann–Whitney U test. Ab, antibody; PVL, proviral load; PBMC, peripheral blood mononuclear cells.

able to obtain longitudinal data pertaining to CXCL10 and neopterin levels in CSF from seven of eight patients who received maintenance therapy and three of five patients who did not receive maintenance therapy. As shown in Figure 3B, both marker levels decreased after the pulse therapy; subsequently, there was a rapid increase in the levels in patients who did not receive maintenance therapy (Figure 3B, right) compared with those who received maintenance therapy (Figure 3B, left). The rapid increase in marker levels and the deterioration of OMDS and 10 m walking time occurred in the same time frame. Furthermore, we investigated the clinical course and the time course of CSF marker levels in four HAM/TSP patients who received and subsequently discontinued low-dose oral prednisolone therapy (Figure 4). The OMDS and 10 m walking time showed the best values at the point of treatment discontinuation and gradually deteriorated after discontinuation (Figure 4A). The CXCL10 and neopterin levels in all four patients decreased with the treatment and increased after discontinuation (Figure 4B).

#### DISCUSSION

The present study demonstrated that CSF CXCL10 is a marker of therapeutic response in HAM/TSP patients. Steroidinduced reduction in CSF CXCL10 occurred simultaneously with the amelioration of clinical symptoms (Figures 1, 3, left and Table 3); conversely, withdrawal of steroid treatment increased CSF CXCL10 alongside the deterioration in clinical symptoms (Figures 3, right, 4). In addition, maintenance of reduced CSF CXCL10 by oral prednisolone therapy after pulse therapy was associated with maintenance of motor function improvement for 2 years (Figure 3 and Table 4). These data suggested that CSF CXCL10 qualifies the following criteria for a surrogate marker: "the effect of treatment on the surrogate marker correlates with its effect on the true endpoint." In other words, treatment-induced decrease in CSF CXCL10 concentration was associated with improvement in functional prognosis. Unfortunately, the relatively short observation period (2 years) in this study is a limitation.

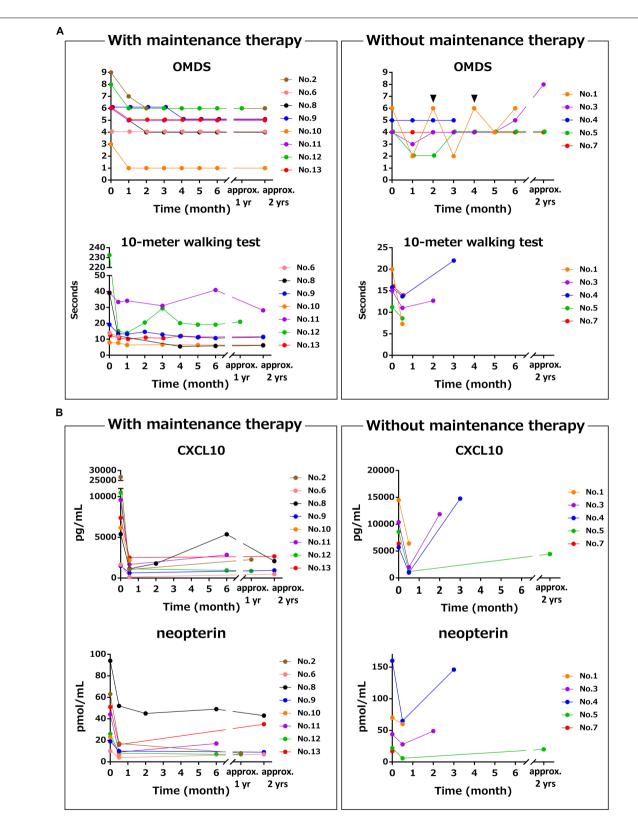


FIGURE 3 | Differences in time course of motor function and CSF markers with and without maintenance therapy using oral prednisolone after methylprednisolone pulse therapy. These graphs demonstrate the time course of (A) OMDS and 10 m walking time and (B) CSF CXCL10 concentration and CSF neopterin concentration in eight HAM/TSP patients who received maintenance therapy (left) and five HAM/TSP patients who did not receive maintenance therapy (right). The data of (Continued)

#### FIGURE 3 | Continued

the 10 m timed walk were obtained from seven patients, as one of eight patients who received maintenance therapy was unable to walk 10 m. Among the five patients who did not receive maintenance therapy, patient no. 1 repeatedly received methylprednisolone pulse therapy every 2 months. The arrowhead indicates the time of administration. Patient no. 4 received oral prednisolone therapy after the third CSF test because of the clinical deterioration with high levels of CXCL10 and neopterin. OMDS, Osame motor disability score.

However, our previous retrospective study has already revealed that patients with high disease activity have high CSF CXCL10 levels and poor functional prognosis after decades; conversely, patients with low disease activity have low CSF CXCL10 levels and good functional long-term prognosis (Sato et al., 2018b). Furthermore, in HAM/TSP patients who received anti-CCR4 antibody therapy, low CSF CXCL10 levels were maintained for about 10 months; during this period, clinical improvement was maintained (Sato et al., 2018a). Therefore, treatmentinduced decrease in CSF CXCL10 level is likely to improve the long-term functional prognosis of HAM/TSP patients. CSF CXCL10 has been shown to be involved in the pathogenesis of HAM/TSP (Ando et al., 2013); in addition, it demonstrated a correlation with the degree of progression and long-term functional prognosis (Sato et al., 2013, 2018b). Thus, it is likely that CSF CXCL10 is a biomarker that meets all the conditions as a surrogate marker for treatment of HAM/TSP. If correct, maintenance of CSF CXCL10 at low levels should be an important therapeutic goal in order to improve long-term functional prognosis.

In addition to CSF CXCL10, CSF neopterin is a promising candidate surrogate marker. Among the CSF markers examined in this study, CSF levels of neopterin, total protein, and anti-HTLV-1 antibody titer as well as CXCL10 were also significantly reduced by steroid therapy (Figure 1). However, among these, only CSF CXCL10 and CSF neopterin were significantly reduced by steroid therapy at both university hospitals (data not shown). Steroid therapy has also been shown to reduce CSF neopterin level (Nakagawa et al., 1996; Nagai et al., 2013). Furthermore, in the present study, the chronological changes in CSF neopterin concentration with steroid treatment were similar to those in CSF CXCL10, both of which were also associated with the clinical course (Figures 3, 4). Therefore, CSF neopterin is also a potential surrogate marker for the treatment of HAM/TSP. However, since the association between CSF neopterin and HAM/TSP pathogenesis is still unclear, CSF CXCL10 is currently considered to be the most suitable surrogate marker.

**TABLE 4** | Association between maintenance therapy and change in OMDS during the observation period.

	observation period			
	Deterioration	No deterioration	Total	
Patients with maintenance therapy	0	8	8	
Patients without maintenance therapy	3	2	5	

Change in OMDS during the

p = 0.035 by Fisher's exact test.

There is no clear consensus on the effectiveness of methylprednisolone pulse therapy in patients with HAM/TSP. In the current study, although methylprednisolone pulse therapy improved clinical symptoms in many patients with disease activity, its effectiveness was transient in patients without steroid maintenance therapy. In addition, this study suggested that maintenance therapy with oral prednisolone is necessary to sustain this improved condition (Figure 3). Indeed, about 90% of patients who received methylprednisolone pulse therapy improved 10 m walking time by 10% or more in about 2 weeks after treatment, and about 70% of them showed improved OMDS 1 month after treatment (Table 3). However, absence of maintenance therapy led to the worsening of motor ability of their lower limbs along with an increase in CSF marker levels; the CSF marker levels returned to their baseline level within a few months. On the other hand, patients who received maintenance therapy showed persistently low CSF marker levels and sustained improvement of lower limb motor ability (Figure 3). There was a significant association between administration of maintenance therapy and the change in OMDS during the observation period (Table 4). Additionally, continuous use of low-dose prednisolone was earlier shown to improve long-term motor functional prognosis (Coler-Reilly et al., 2017). These findings suggest the benefits of low-dose oral prednisolone maintenance therapy after pulse therapy. However, the optimal duration of maintenance therapy and its safety aspects are not clear. Future prospective studies should evaluate the efficacy and safety of low-dose oral prednisolone maintenance therapy after adjusting for patient characteristics.

The present study found that CSF CXCL10 concentration and CSF anti-HTLV-1 antibody titer may help predict the response to steroid therapy. It is likely that patients with high levels of these markers represent a subgroup of patients in whom steroid therapy is likely to be particularly effective. Indeed, responders who showed a high degree of clinical improvement by steroid therapy showed significantly higher levels of CSF CXCL10 concentration and CSF anti-HTLV-1 antibody titer before the start of treatment compared with non-responders (Figure 2). As for CSF neopterin, there was no significant difference between responders and non-responders, prior to exclusion of outlier data. However, CSF neopterin is also likely to be one of the predictors of therapeutic response, as suggested by a previous study (Nagai et al., 2013). The above results suggest that steroid therapy is particularly useful for patients who exhibit high concentrations of CXCL10 and neopterin in CSF; in contrast, steroid treatment offers limited benefit for patients with low or normal levels of these CSF markers. We have already shown that HAM/TSP patients can be classified into three groups according to disease activity, which is assessed on the basis of CSF concentrations of CXCL10 and neopterin (Sato et al., 2018b)

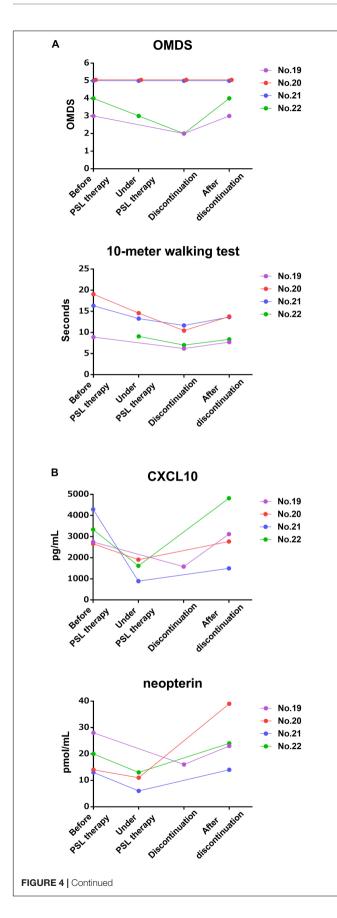


FIGURE 4 | Time course of motor function and CSF markers in patients received and subsequently discontinued oral prednisolone therapy. These graphs demonstrate the time course of (A) OMDS and 10 m walking time and (B) CSF CXCL10 concentration and CSF neopterin concentration in four HAM/TSP patients treated with low-dose prednisolone from before treatment to after treatment discontinuation. The pre-treatment data of the 10 m walking time in patient no. 22 could not be obtained. OMDS, Osame motor disability score: PSL. prednisolone.

and clinical progression rate. In this context, our study underlines the importance of so-called stratified medicine, wherein the treatment strategy is decided according to the pre-treatment disease activity.

In some studies, methylprednisolone pulse therapy was not found to be very effective in HAM/TSP patients. This may be attributable to the inclusion of non-responders with low levels of inflammation in the spinal cord. Indeed, these studies did not involve assessment of pre-treatment disease activity (inflammatory markers) in the spinal cord (Duncan and Rudge, 1990; Araujo et al., 1993). Therefore, it is critical to take into account the extent of disease activity in order to predict therapeutic efficacy.

This study has mainly two limitations. The observation period of 2 years was relatively short considering the prolonged clinical course of HAM/TSP. Second, there were many missing data due to retrospective data collection. Future prospective study involving data collection over a longer period of time may provide more definitive evidence. However, a prospective study may pose a challenge as denial of maintenance therapy to patients with high disease activity is ethically difficult after the importance of maintenance therapy becomes apparent.

#### CONCLUSION

In conclusion, the present study suggests that CSF CXCL10 may serve both as a marker and predictor of therapeutic response in patients with HAM/TSP. In addition, since it is likely that the therapeutic effect on CSF CXCL10 is associated with the effect on long-term functional prognosis of HAM/TSP patients, CSF CXCL10 has a potential to serve as a surrogate marker for treatment of HAM/TSP.

#### **DATA AVAILABILITY**

The datasets generated for this study are available on request to the corresponding author.

#### **ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by the Institutional Review Board of St. Marianna University School of Medicine (#1646) and Fukuoka University Hospital (#14-2-08). The patients/participants provided their written informed consent to participate in this study.

#### **AUTHOR CONTRIBUTIONS**

KT, YY, and YT conceived and designed the study. KT, TS, JT, SF, JY, and YY collected the clinical data. KT and TS analyzed the results. KT, TS, NY, NA, JY, AC-R, MN, YH, and YY drafted and corrected the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Safety of Infliximab for the Eye Under Human T-Cell Leukemia Virus Type 1 Infectious Conditions *in vitro*

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Uchida M, Kamoi K, Ando N, Wei C, Karube H and Ohno-Matsui K (2019) Safety of Infliximab for the Eye Under Human T-Cell Leukemia Virus Type 1 Infectious Conditions in vitro. Front. Microbiol. 10:2148. doi: 10.3389/fmicb.2019.02148 Use of biologics has been widely advocated for inflammatory diseases recently. Anti-tumor necrosis factor (TNF)- $\alpha$  antibody therapy is reportedly effective against ocular inflammation. However, side effects of TNF- $\alpha$  inhibition have been reported, particularly in the form of exacerbation of infections such as tuberculosis. Paradoxical reactions such as exacerbated inflammation are also well known. Around 20 million humans are infected with human T-cell leukemia virus type 1 (HTLV-1) globally, and this virus can cause adult T-cell leukemia, HTLV-1-associated myelopathy and HTLV-1 uveitis. As for ophthalmic concerns, it has not been identified whether anti-TNF-α antibody stimulates HTLV-1-infected cells and ocular cells to induce HTLV-1 uveitis in HTLV-1 carriers. Here we investigated the effects of anti-TNF- $\alpha$  antibody on ocular status under HTLV-1 infectious conditions using ocular cells and HTLV-1-infected cells in vitro. We used the ARPE-19 human retinal pigment epithelial cell line as ocular cells considered to play an important role in the blood-ocular barrier, and the MT2 HTLV-1-infected cell line. Jurkat cells were used as controls. Infliximab (IFX) was used as an anti-TNF- $\alpha$  antibody to achieve TNF- $\alpha$  inhibition. We evaluated the production of inflammatory cytokines and intercellular adhesion molecule (ICAM)-1, proliferation of ARPE-19, expression of TNF-α receptor (TNF-R) and HTLV-1 proviral DNA, and the percentage of apoptotic ARPE-19. Inflammatory cytokines such as interleukin (IL)-6, IL-8, TNF, and ICAM-1 were significantly elevated through contact between ARPE-19 and MT2. Treatment with IFX tented to inhibit TNF production, although the level of production was low, but changes in IL-6, IL-8, and ICAM-1 remained unaffected. Expression of TNFR was unaltered by IFX treatment. HTLV-1 proviral DNA was not significantly changed with treatment. No change in cell growth rate or apoptotic rate of ARPE-19 was seen with the addition of IFX. In conclusion, IFX did not exacerbate production of inflammatory cytokines, and did not affect expression of TNFR, proliferation of ARPE-19, HTLV-1 proviral load, or apoptosis of ARPE-19. These results suggest that IFX does not exacerbate HTLV-1-related inflammation in the eye and represents an acceptable treatment option under HTLV-1 infectious conditions.

Keywords: human T-cell leukemia virus type 1, anti-TNF-alpha antibody, infliximab, ocular inflammation, uveitis

Uchida et al.

Anti-TNF for Eye With HTLV-1

#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that infects an estimated 20 million people globally (Watanabe, 2011). Various HTLV-1 endemic areas exist around the world, and about 1 million HTLV-1-infected individuals live in Japan, mainly in southwestern regions such as Kyushu and the Okinawa islands (Satake et al., 2012). HTLV-1 causes adult T-cell lymphoma (ATL) (Uchiyama et al., 1977), HTLV-1-associated myelopathy (HAM) (Osame et al., 1986), and HTLV-1 uveitis (HU) (Mochizuki et al., 1992). HU is considered by ophthalmologists as one of the most common ocular inflammatory diseases in HTLV-1 endemic areas (Terada et al., 2017a). This pathological entity was established after a series of seroepidemiological, clinical, molecular biological, and virological studies (Kamoi and Mochizuki, 2012a,b). HU occurs with a sudden onset of floaters and foggy vision, and is classified as an intermediate uveitis (Takahashi et al., 2000). HU is considered to be caused by inflammatory cytokines produced by HTLV-1-infected T-cells accumulating in the eyes (Kamoi and Mochizuki, 2012b).

Biologics have been widely advocated for use against inflammatory diseases recently. In particular, anti-tumor necrosis factor (TNF)-α antibody therapy has seen wide use for rheumatoid arthritis (RA) (Bathon et al., 2000), psoriasis (Leonardi et al., 2003), ankylosing spondylitis (Gorman et al., 2002), and inflammatory bowel disease (Rutgeerts et al., 2005). In addition, anti-TNF-α antibody therapy has been reported as effective for ocular inflammation (Joseph et al., 2003). In the ophthalmological field in Japan, infliximab (IFX) has been approved for use in the treatment of Behcet disease since 2008, and adalimumab has been approved for use against non-infectious uveitis since 2016 (Diaz-Llopis et al., 2008). Anti-TNF-α antibody acts to neutralize activities against soluble TNF-α, block TNF-α binding to TNF receptors on cells, and induce apoptosis and arrest of the cell cycle in G0/G1 in TNF-α-producing cells (Lim et al., 2018).

Although significant efficacy of anti-TNF has been reported, side effects of TNF-α inhibition such as infection (Lim et al., 2018), malignant tumor (Wang et al., 2016), autoimmune disease (Perez-De-Lis et al., 2017), and demyelinating disease (Gill et al., 2017) have been reported. In particular, exacerbation of infection was a notable side effect, and a meta-analysis indicated that caution is needed for patients infected with tuberculosis, syphilis, toxoplasma, herpes, cytomegalovirus, bacteria, or fungi (Minozzi et al., 2016). In addition, paradoxical reactions such as exacerbation of inflammation are well known. Such paradoxical effects have mainly been reported as skin reactions, granulomatous diseases, and ocular inflammation (uveitis) (Wendling and Prati, 2014; Mylonas and Conrad, 2018).

However, problematically, the risks of administering TNF- $\alpha$  inhibitors to patients infected with HTLV-1 have yet to be clarified, because few basic and clinical studies have been conducted for this retrovirus. Clinically, cases of rheumatoid arthritis complicated with HTLV-1 infection reportedly did not show any sign of exacerbated infection, such as increased proviral load (PVL), after providing anti-TNF- $\alpha$  antibody therapy (Umekita et al., 2015). Basically, TNF- $\alpha$  inhibition showed no

effect on a HTLV-1-infected cell line from patients with HAM in vitro (Fukui et al., 2017).

As mentioned above, several reports have examined the safety of anti-TNF- $\alpha$  antibody therapy in internal medicine fields, but no report from the ophthalmological field has clarified whether TNF- $\alpha$  inhibition can affect ocular conditions in HTLV-1 carriers. HU is one of the most important clinical entities among HTLV-1-infected individuals. As a result, the risk of induction of HTLV-1-related ocular inflammation (i.e., HU) following administration of anti-TNF- $\alpha$  antibody into HTLV-1 carriers is a concern warranting investigation. In addition, guidelines for the use of anti-TNF- $\alpha$  antibody have not mentioned screening for HTLV-1 infection prior to initiation of treatment.

The present study investigated the effects of anti-TNF- $\alpha$  antibody on ocular status among HTLV-1 carriers using an ocular cell line and an HTLV-1-infected cell line *in vitro*. The present investigation chose a retinal pigment epithelium (RPE) cell line as a representative ocular cell line, as the RPE plays a major role in the blood-ocular barrier and the maintenance of immunological homeostasis in the eye (Holtkamp et al., 2001).

#### MATERIALS AND METHODS

## Cell Lines, Cell Culture, and Human T-Cell Leukemia Virus Type 1 Infection *in vitro*

We used the ARPE-19 human retinal pigment epithelial cell line (American Type Culture Collection, Manassas, VA) as ocular cells, and the MT-2 cell line as an HTLV-1-infected T-cell line. We used Jurkat cells as a control T-cell line. MT2 and Jurkat cells were cultured in RPMI 1640 (Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Japan, Tokyo, Japan) and 1% penicillin/streptomycin. ARPE-19 cells were cultured in minimum essential medium (Wako Pure Chemical Corporation) with the same supplements. All cell lines were incubated in a humidified incubator at 37°C under an atmosphere of 5% CO2. In vitro infection by HTLV-1 was performed using the standard co-culture method (Akagi et al., 1986; Graziano et al., 1987; Liu et al., 2006). Briefly, ARPE-19 cells were plated and co-cultured with three times the number of MT2 or Jurkat cells at 48 h using cell culture inserts (Thermo Fisher Scientific, Waltham, MA).  $1.5 \times 10^5$  ARPE-19 cells were used in cytometric bead assay (CBA) and annexin V assay. 2 × 10<sup>4</sup> ARPE-19 cells were used in cell counts, TNF receptor analysis, and the measurement of HTLV-1 proviral load.

#### **Anti-Tumor Necrosis Factor-α Inhibitor**

IFX (Mitsubishi Tanabe Pharma, Osaka, Japan) was used as an anti-TNF- $\alpha$  antibody, with 10 µg/ml/well, in line with a previously established *in vitro* method for HTLV-1-associated myelopathy experiments (Fukui et al., 2017).

#### **Cytometric Bead Assay**

Cultured supernatants were examined using CBA human inflammation cytokine kits (BD Biosciences, San Jose, CA).

Uchida et al.

Anti-TNF for Eye With HTLV-1

Results were analyzed with FCAP Array version 3.0 software (BD Biosciences) according to the instructions from the manufacturer. Cytokines measured by the kits included interleukin (IL)-6, IL-8, IL-1 $\beta$ , IL-12p70, IL-10, and TNF.

#### Cytokine Enzyme-Linked Immunosorbent Assays

To measure levels of soluble intercellular adhesion molecule (ICAM)-1 in supernatants, ELISA kits (R&D Systems, Minneapolis, MN) were used in accordance with the instructions from the manufacturer.

#### **Cell Counts**

ARPE-19 cells  $(2 \times 10^4)$  were co-cultured with three times the number of MT2 or Jurkat cells with or without IFX. After 0, 24, 48, or 72 h of co-culture, we removed the supernatants, trypsinized ARPE-19, and counted the number of ARPE-19 cells under light microscopy.

### Anti-Tumor Necrosis Factor-α Receptor Analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to examine the cell surface expression of TNF-R1 and TNF-R2, using fluorescein isothiocyanate (FITC)-conjugated anti-CD120a (TNF-R1) and anti-CD120b (TNF-R2) human monoclonal antibodies (MBL International, Woburn, MA). ARPE-19 cells were washed and trypsinized, then incubated with TNF-R1 and TNF-R2 antibodies according to the instructions from the manufacturer. Analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

#### **Immunohistochemistry**

ARPE-19 cells were cultured on glass bottom plates (AGC Techno Glass, Shizuoka, Japan) for 24 h, then co-cultured with MT2 and Jurkat cells using cell culture inserts (Thermo Fisher Scientific) for 48 h. After three washes with phosphatebuffered saline (PBS) (Wako Pure Chemical Corporation), ARPE-19 cells were fixed by cold fixing buffer (methanol/ acetone, 1:1) at -20°C for 20 min and blocked with 10% FBS in PBS for 15 min. Cells were then incubated in the diluted primary antibodies for 1 h at room temperature, followed by incubation with Alexa fluor488-labeled anti-rabbit secondary antibody (Abcam, Tokyo, Japan) along with 4',6-diamidino-2-phenylindole dihydrochloride (Cosmo Bio, Tokyo, Japan) incubation for 1 h at room temperature. The following antibodies were used as primary antibodies: TNF receptor 1 polyclonal antibody (Bioss Antibodies, Woburn, MA) and TNF receptor 2 polyclonal antibody (Proteintech, Chicago, IL). We scanned using a TCS-SP8 microscope (Leica Micro Systems, Wetzlar, Germany).

## Measurement of Human T-Cell Leukemia Virus Type 1 Proviral Load

DNA was prepared from each sample using EZ1 Virus Mini Kits v2.0 (Qiagen, Hilden, Germany) according to the

instructions from the manufacturer. Quantitative real-time polymerase chain reaction (PCR) assay was used to measure the PVL of HTLV-1 in cells, as described previously (Matsuda et al., 2005; Fukui et al., 2017). PVL was quantified using the HTLV-1 Tax primer (forward, 5'-CCCACTTCC CAGGGTTTGGA-3'; reverse, 5'-GGCCAGTAGGGCG TGA-3') and probe (5'-FAM-CCAGTCTACGTGTTTTGGA GACTGTG TACA-TAMRA-3'). Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control.

#### **Annexin V Staining**

FACS analysis to evaluate of apoptosis and cell death using Annexin V-FITC was performed using assay kits (MBL, Nagoya, Japan) according to the instructions from the manufacturer. Apoptotic cells were defined by detecting propidium iodidenegative, annexin V-positive cells. The rate of apoptotic cells among all cells was calculated by FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

#### **Statistical Analysis**

Student's unpaired t-test or Welch's unpaired t-test after Bonferroni correction for multiple testing, which adjusts the values of p by multiplying them by the number of tests (Krzywinsk and Altman, 2014), was used to determine significant differences in the levels of cytokines, number of ARPE-19 cells, and percentage of apoptotic cells. Values of p < 0.05 were considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). More precisely, EZR is a modified version of R Commander (version 1.6-3) designed to add statistical functions used frequently in biostatistics.

#### **RESULTS**

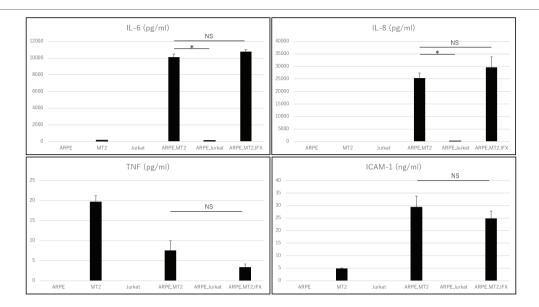
### Inflammatory Cytokines and Intercellular Adhesion Molecule-1

Levels of IL-6, IL-8, IL-1 $\beta$ , IL-12p70, IL-10, TNF, and ICAM-1 secreted by each of ARPE-19, MT2, Jurkat, ARPE-19 co-cultured with MT2, and ARPE-19 co-cultured with Jurkat were measured (**Figure 1**). MT2 spontaneously secreted IL-6, TNF, and ICAM-1. Levels of IL-6, IL-8, TNF, and ICAM-1 in the ARPE-19 co-cultured with MT2 were increased significantly more than those co-cultured with Jurkat. Compared to MT2 alone, levels of IL-6, IL-8, and ICAM-1 were increased. On the other hand, levels of TNF decreased in the ARPE-19 co-cultured with MT2. Levels of IL-12p70, IL-1 $\beta$ , and IL-10 were below the limits of detection under all conditions.

## Changes in Inflammatory Cytokines and Intercellular Adhesion Molecule-1 on ARPE-19 Treated With Infliximab

Changes in the levels of inflammatory cytokines and ICAM-1 secreted by ARPE-19 co-cultured with MT2 under treatment with IFX were examined. IL-6, IL-8, and ICAM-1 were unaffected

Uchida et al. Anti-TNF for Eye With HTLV-1



**FIGURE 1** Inflammatory cytokines and ICAM-1 secreted by ARPE, MT2, Jurkat, and ARPE-19 co-cultured with MT2 or Jurkat, and ARPE-19 co-cultured with MT2 with addition of IFX at 48 h. Although TNF tends to be inhibited by IFX, no significant difference is detected (p = 0.14). Data were obtained from three independent biological replicates performed in triplicate. Error bars represent standard deviations (\*p < 0.05; NS, not significant).

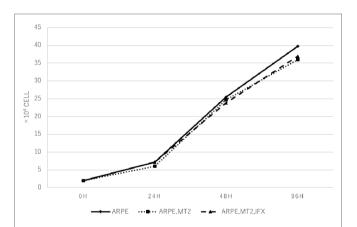
by addition of IFX. TNF tended to be inhibited, but did not reach the level of statistical significance for suppression (p = 0.14). Levels of IL-12p70, IL-1 $\beta$ , and IL-10 were below measurable limits under all conditions (**Figure 1**).

#### Changes in Cell Growth Rate in ARPE-19 Treated With Infliximab

Changes in the cell growth rate of ARPE-19 co-cultured with MT2 treated with IFX were examined. Numbers of ARPE-19 cells after co-culture for 0, 24, 48, and 96 h increased in a time-dependent manner, and counts of ARPE-19 co-cultured with MT2 also increased in a similar manner. ARPE-19 cells co-cultured with MT2 tended to be slightly less numerous than ARPE-19 cells alone after co-culture for 96 h, but no significant differences were found. Furthermore, no significant differences were apparent between the numbers of ARPE-19 cells co-cultured with MT2 treated with and without IFX (Figure 2).

## Expression of TNF-R1 and TNF-R2 on ARPE-19 Treated With Infliximab

To identify changes in the expression of TNF-R1 and TNF-R2 caused by IFX, we performed FACS analysis and immunohistochemical staining on ARPE-19 co-cultured with MT2 with addition of IFX. In FACS analysis, the number of TNF-R1 and TNF-R2 on ARPE-19 cells was unchanged by co-culture with MT2 or Jurkat cells (data not shown). Likewise, there were no differences in the histograms of TNF-R1 and TNF-R2 on ARPE-19 by treatment with IFX (**Figure 3A**). Furthermore, in immunohistochemical staining, expression of TNF-R was unaltered by IFX treatment (**Figure 3B**).



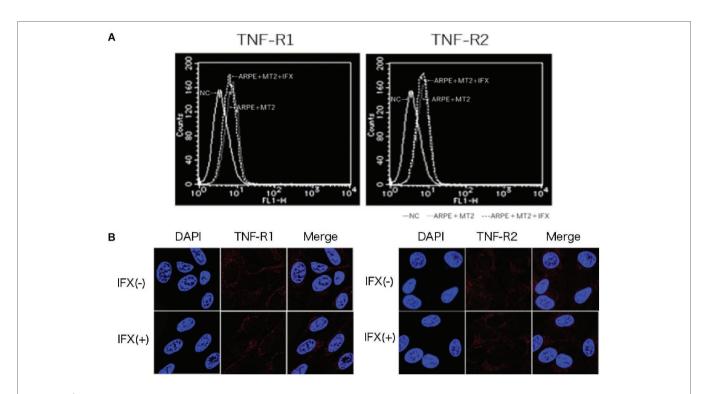
**FIGURE 2** | The number of ARPE-19 cells per well at 0, 24, 48, and 96 h. ARPE-19 cells increase in a time-dependent manner and no significant differences among the number of ARPE-19 cells co-cultured with MT2 cells with or without IFX are seen at each point. We conducted independent experiments and plotted the average values.

#### Detection of Human T-Cell Leukemia Virus Type 1 Proviral DNA in Human T-Cell Leukemia Virus Type 1-Infected ARPE-19 Cells Treated With Infliximab

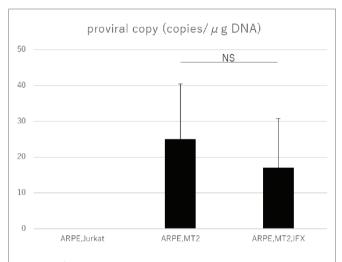
PVL was measured by real-time PCR to assess whether IFX affected HTLV-1 related disease progression in ARPE-19 cells. PVL in ARPE-19 cells co-cultured with MT2 cells was significantly increased compared to that in co-culture with Jurkat cells. As for controls, undetectable PVL was confirmed in ARPE-19 cells co-cultured with Jurkat cells. In HTLV-1-infected ARPE-19, PVL did not increase with IFX treatment (Figure 4).

Uchida et al.

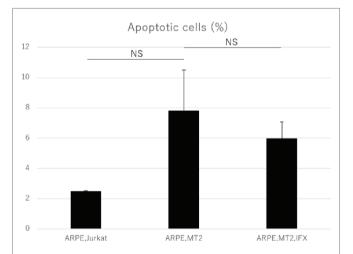
Anti-TNF for Eye With HTLV-1



**FIGURE 3** | (A) Histogram of TNF-R1 and TNF-R2 on ARPE-19 in FACS analysis. Histograms of TNF-R on ARPE-19 and ARPE-19 after co-culture with MT2 with or without IFX. Results are representative of four experiments. (B) Expression of TNF-R1 and TNF-R2 on ARPE-19 in immunohistochemical staining. No significant changes in expression of TNF-R1 or TNF-R2 are seen for ARPE-19 treated with or without IFX.



**FIGURE 4** PVL in ARPE-19 cells co-cultured with Jurkat, MT2, or MT2 treated with IFX. The PVL of HTLV-1-infected ARPE-19 is not increased, but is slightly decreased by IFX. Error bars represent standard deviations (NS, not significant).



**FIGURE 5** | Rate of apoptotic ARPE-19 cells in co-culture with Jurkat, MT2, and MT2 treated with IFX. No significant change in apoptotic rate of ARPE-19 is seen with IFX; rather, there is a slight decrease with IFX. Error bars represent standard deviations (NS, not significant).

#### Assessment of Apoptosis in Human T-Cell Leukemia Virus Type 1-Infected ARPE-19 Cells Treated With Infliximab

Annexin V assay was performed to assess the apoptosis in ARPE-19 co-cultured with MT2 and the effect of IFX on

ARPE-19 was examined. The apoptotic rate of ARPE-19 co-cultured with MT2 tended to be increased compared to that in co-culture with Jurkat, but the difference was not significant (p = 0.10). No change in the apoptotic rate of ARPE-19 co-cultured with MT2 was seen with the addition of IFX (**Figure 5**).

Uchida et al. Anti-TNF for Eye With HTLV-1

#### DISCUSSION

Biologics are reportedly effective for ocular inflammation and have been used worldwide. Although HTLV-1 carriers are common all over the world, no basic studies have been conducted to clarify the safety of biologics for HTLV-1-infected patients in the ophthalmological field. In Japan, several decades have passed since the first biologic was applied to inflammatory diseases, and Japan is now one of the countries with the greatest use of biologics by physicians. A case has already been reported in which an RA patient with HTLV-1 infection experienced exacerbation of HTLV-1-associated conditions such as myopathy and uveitis after using a biologic agent (Terada et al., 2017b). Clarification of the safety of biologics on HTLV-1-infected patients is thus considered an urgent issue. This study focused on IFX, as the first biologic agent applied to inflammatory diseases, and clarified the effect of IFX on ocular conditions under an HTLV-1-infected status.

The intraocular concentrations of cytokines and chemokines greatly influence the pathophysiology of ocular inflammation (Mochizuki et al., 2013). The RPE plays an important role in the ocular-blood barrier (Holtkamp et al., 2001) and in the intraocular immune response by producing various inflammatory cytokines and expressing adhesion molecules such as ICAM-1 (Elner et al., 1992). Immunosuppressive and immunomodulatory therapies such as biologics have been reported to provide beneficial effects in patients with this disorder. IFX is an effective agent for various inflammatory conditions and many reports have indicated that IFX has potential for decreasing various cytokines in patients with inflammatory diseases. However, whether TNF-α inhibition can affect the eye of HTLV-1 carriers has not been clarified. In fact, paradoxical reactions, i.e., exacerbated inflammation, are also well known with anti-TNF-α treatment. In particular, ocular inflammation (uveitis) has been reported as a major paradoxical effect from treatment with anti-TNF-α antibody (Wendling and Prati, 2014). We therefore first checked the effect of IFX on inflammatory cytokines such as IL-6, IL-8, IL-1β, IL-12p70, IL-10, and TNF. Inflammatory cytokine investigations showed that levels of IL-6 and IL-8 increased when HTLV-1-infected T-cells came into contact with RPE (Figure 1). Those inflammatory cytokines resulted in inflammatory conditions in the eye, suggesting that HTLV-1 carriers potentially face a credible threat of intraocular inflammation. In such a state, however, IFX treatments did not affect levels of IL-6 or IL-8 (Figure 1). This means that IFX did not encourage acceleration of the inflammatory conditions that result in HU in the eyes of HTLV-1-infected patients.

As for TNF- $\alpha$ , our experiments showed that levels of this cytokine were low, but decreased further when HTLV-1-infected T-cells came into contact with RPE, compared to HTLV-1-infected T-cells alone (**Figure 1**). This phenomenon is related to the nature of RPE, which plays a role in local immunosuppressive ability to protect the blood-ocular barrier (Mochizuki et al., 2013). The result showed that suppression of TNF- $\alpha$  seems to be one of the characteristic features of the RPE response to HTLV-1 infection in a local immunosuppressive role. Although no significant reduction was identified, IFX treatment led to a relative reduction in TNF- $\alpha$  under this

condition (**Figure 1**), providing positive evidence for IFX-induced suppression of ocular inflammation. However, this experiment was not specifically aimed at identifying the effectiveness of anti-TNF- $\alpha$  antibody for HTLV-1 uveitis, but rather at confirming the safety of anti-TNF- $\alpha$  antibody in terms of the ocular circumstances of HTLV-1 carriers.

We next focused on another important molecule, ICAM-1. ICAM-1 acts to mediate cell-to-cell interactions, (Lebedeva et al., 2005) and ICAM-1 has been reported as necessary for HTLV-1 infection. ICAM-1 has also been reported to be upregulated by HTLV-1 infection (Liu et al., 2006) and contributes to this efficient transmission (Barnard et al., 2005). From the viewpoint of ocular circumstance, it has been well known that increase of soluble ICAM is detected in the ocular fluid samples from patients with various types of uveitis, including Behçet disease-related uveitis which is a major targeted disease of infliximab. Our result showed that soluble ICAM-1 secreted by ARPE-19 was increased by contact with HTLV-1-infected cells, as expected (Figure 1). However, IFX treatment did not influence the level of soluble ICAM-1 under this condition (Figure 1), and was therefore estimated to play no role in further accelerating HTLV-1 infection or upregulation, which is consistent with results from an in vitro investigation of HTLV-1-associated myelopathy (Fukui et al., 2017).

Inhibition of IFX is mediated *via* interference of TNF binding to two known receptors, namely TNF-R1, which binds to soluble TNF, and TNF-R2, which binds to membrane-bound TNF. As shown in previous studies, TNF-R1 and TNF-R2 are expressed on the RPE (Kociok et al., 1998; Lee et al., 2015), and changes in these receptors are therefore thought to influence the function of the blood-ocular barrier through TNF-mediated inflammation. In the present study, FACS analysis and immunohistochemical staining identified no change in TNF-R1 or TNF-R2 expression on ARPE-19 by IFX treatment (**Figures 3A,B**). This suggests that IFX did not accelerate TNF-mediated alterations in the eye.

The PVL in peripheral blood is related to the development of HTLV-1-associated diseases (Demontis et al., 2013), and PVL is used as a marker for HTLV-1 disease progression (Moens et al., 2009). Many investigations have looked into PVL in peripheral blood, but information on PVL in human residential tissue and cells is scant. The present study therefore tried to check whether changes to PVL in RPE would occur under IFX administration. A previous investigation identified RPE as a potential reservoir for HTLV-1, as HTLV-1 proviral DNA was detected in ARPE-19 cells co-cultured with MT2 cells (Liu et al., 2006). Our study confirmed that proviral DNA in RPE was detectable in the co-culture experiment, as previously reported. More importantly, we were able to demonstrate that PVL in RPE was not increased by IFX treatment (Figure 4), indicating that IFX did not affect PVL in RPE. This result suggested that IFX was unrelated to HTLV-1 provirus-related disturbance, potentially leading to a breakdown of the bloodocular barrier, in RPE.

An increase in apoptotic ARPE cells represents critical damage to the maintenance of immunological homeostasis in the eye (Mochizuki et al., 2013). In the present study, the apoptotic rate of ARPE-19 cells co-cultured with MT2 cells tended to

Uchida et al.

Anti-TNF for Eye With HTLV-1

be increased compared to that in co-culture with Jurkat cells, but no significant difference was identified (**Figure 5**). From one perspective, apoptosis contributes to suppression of cell carcinogenesis and control of infection spread (Tabakin-Fix et al., 2006), so this apoptotic rate in the case of ARPE-19 co-cultured with MT2 might be considered as an infection-control reaction of ARPE. This result also may be related to the slightly reduced proliferation of ARPE-19 co-cultured with MT2 at 96 h (**Figure 2**). In addition, the apoptotic rate of HTLV-1-infected ARPE-19 cells was not increased by the addition of IFX (**Figure 5**). This result indicated that further damage did not occur in HTLV-1-infected ARPE-19 cells with IFX treatment.

Some limitations to this study merit attention when considering the potential effects of IFX therapy in patients with ocular inflammation complicated by HTLV-1 infection. For instance, in our experiments involving inflammatory cytokines, we did not observe the increases in IL-1 and IL-10 in co-cultures of ARPE-19 and MT2 that were described in previous clinical reports (Sagawa et al., 1995). This in vitro investigation using cell lines might not accurately reflect all aspects of clinical events. As we placed particular focus on changes to ocular tissue in this investigation, we did not examine the risk of ATL induction using IFX. Although previous basic and clinical papers have not indicated any increased PVL in peripheral blood mononuclear cells with IFX treatment (Umekita et al., 2015; Fukui et al., 2017), we would next focus on the alteration of HTLV-1-infected T cells by IFX treatment through Tax analysis, DNA damage, and cell-cycle progression. In conjunction with basic research, clinical long-term tracking investigations of IFX-treated patients might also be needed.

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#### CONCLUSION

IFX did not exacerbate the production of inflammatory cytokines, and did not affect the expression of TNFR, cell proliferation, HTLV-1 PVL, or apoptosis on RPE. These results suggest that IFX did not exacerbate HTLV-1-related inflammation in the eye and represents an acceptable treatment option, at least according to HTLV-1 infectious conditions *in vitro*.

#### **AUTHOR CONTRIBUTIONS**

MU performed the experiments and wrote the draft of the manuscript. KK designed the experiments, analyzed the data, and wrote the manuscript. NA, CW, and HK performed the experiments. 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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Uchida et al.

Anti-TNF for Eye With HTLV-1

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# HTLV-1 Extracellular Vesicles Promote Cell-to-Cell Contact

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Pinto DO, DeMarino C, Pleet ML, Cowen M, Branscome H, Al Sharif S, Jones J, Dutartre H, Lepene B, Liotta LA, Mahieux R and Kashanchi F (2019) HTLV-1 Extracellular Vesicles Promote Cell-to-Cell Contact. Front. Microbiol. 10:2147. doi: 10.3389/fmicb.2019.02147 Human T-cell leukemia virus-1 (HTLV-1) is a neglected and incurable retrovirus estimated to infect 5 to 10 million worldwide. Specific indigenous Australian populations report infection rates of more than 40%, suggesting a potential evolution of the virus with global implications. HTLV-1 causes adult T-cell leukemia/lymphoma (ATLL), and a neurological disease named HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). Even though HTLV-1 transmission primarily occurs from cell-to-cell, there is still a gap of knowledge regarding the mechanisms of viral spread and disease progression. We have recently shown that Extracellular Vesicles (EVs) ubiquitously produced by cells may be used by HTLV-1 to transport viral proteins and RNA, and elicit adverse effects on recipient uninfected cells. The viral proteins Tax and HBZ are involved in disease progression and impairment of autophagy in infected cells. Here, we show that activation of HTLV-1 via ionizing radiation (IR) causes a significant increase of intracellular Tax, but not EV-associated Tax. Also, lower density EVs from HTLV-1-infected cells, separated by an Iodixanol density gradient, are positive for gp61+++/Tax+++/HBZ+ proteins (HTLV-1 EVs). We found that HTLV-1 EVs are not infectious when tested in multiple cell lines. However, these EVs promote cell-to-cell contact of uninfected cells, a phenotype which was enhanced with IR, potentially promoting viral spread. We treated humanized NOG mice with HTLV-1 EVs prior to infection and observed an increase in viral RNA synthesis in mice compared to control (EVs from uninfected cells). Proviral DNA levels were also quantified in blood, lung, spleen, liver, and brain post-treatment with HTLV-1 EVs, and we observed a consistent increase in viral DNA levels across all tissues, especially the brain. Finally, we show direct implications of EVs in viral spread and disease progression and suggest a two-step model of infection including the release of EVs from donor cells and recruitment of recipient cells as well as an increase in recipient cell-to-cell contact promoting viral spread.

Keywords: HTLV-1, extracellular vesicle, viral spread, tax, cell-to-cell contact, infection, RNA, DNA

#### INTRODUCTION

Human T-cell leukemia virus-1 (HTLV-1) is a neglected bloodborne pathogen that indiscriminately infects people of all ages around the world (Gallo, 2011; Gallo et al., 2016; Tagaya and Gallo, 2017; Martin et al., 2018). Approximately 5-10 million people worldwide carry HTLV-1, although this estimate does not include epidemiological data from highly populated regions, such as China, India, Maghreb, and East Africa, and very limited epidemiological data from Africa where the virus is endemic. Therefore actual numbers may be closer to 20 million (Kondo et al., 1989; Koga et al., 2010; Gessain and Cassar, 2012; Einsiedel et al., 2016). More recently, reports of high HTLV-1 infection rates, of 40% and above, in remote Australian populations are cause of global concern (Einsiedel et al., 2016, 2018). HTLV-1infected individuals remain asymptomatic and possibly unaware of their infection status (Hinuma et al., 1981; Gonçalves et al., 2010; Gessain and Cassar, 2012), which is especially concerning since a significant route of transmission occurs from mother to child via breastfeeding (Fujino and Nagata, 2000; Einsiedel et al., 2016). Moreover, it is likely that, due to a lack of universal screening recommendations, more people globally are at risk of infection via sexual contact, blood transfusions and organ transplants (Gallo et al., 2016; de Morais et al., 2017; Caswell et al., 2019).

Human T-cell leukemia virus-1 is associated with Adult T-cell leukemia/lymphoma [ATLL; 3-5% (Hinuma et al., 1981; Poiesz et al., 1981; Aoki et al., 1984; Kondo et al., 1989; Hirai et al., 1992; Iwanaga et al., 2012; Coffin, 2015; Tagaya and Gallo, 2017)] and HTLV-1 associated myelopathy/tropical spastic paraparesis [HAM/TSP; 0.25-3.8% (Gessain et al., 1985; Kaplan et al., 1990; Bangham et al., 2015; Naito et al., 2018)]. Recently, another HTLV-1-associated disease, bronchiectasis, has been linked to high mortality rates (28%) in HTLV-1 seropositive patients (Einsiedel et al., 2018). Depending on the country, current ATLL treatments may include a combination of interferon (IFN) and Zidovudine (AZT), allogeneic stem cell transplantation, or mogamulizumab, a humanized anti-CCR4 monoclonal antibody (Gill et al., 1995; Hermine et al., 2002; Tsukasaki et al., 2009; Subramaniam et al., 2012), however, these treatments are ineffective against some ATLL forms, and in cases of relapse (Phillips et al., 2016). Due to chronic stage of HTLV-1 infection, disease progression may be stalled for several years in an asymptomatic state (latent), with increasing risk over time of developing ATLL (Iwanaga et al., 2010) or HAM/TSP, and of transmitting HTLV-1.

In contrast to human immunodeficiency virus type-1 (HIV-1), HTLV-1 transmission occurs primarily via cell-to-cell contact and formation of a virological synapse, viral biofilm, cellular conduit, or tunneling nanotube (Igakura et al., 2003; Majorovits et al., 2008; Nejmeddine et al., 2009; Pique and Jones, 2012; Alais et al., 2015; Wang et al., 2017; Omsland et al., 2018). Characterization of the proteins involved in viral transmission suggests participation of adhesion proteins (i.e., ICAM-1 and LFA-1), viral proteins [i.e., p12, p8, gp61 (precursor of gp46 envelope), and Tax], and cellular proteins (i.e., Hsc70, CD43, and CD45) (Igakura et al., 2003; Nejmeddine et al., 2009; Van

Prooyen et al., 2010b; Mazurov et al., 2012; Pique and Jones, 2012; Pise-Masison et al., 2014; Dutartre et al., 2016; Gross and Thoma-Kress, 2016; Gross et al., 2016; Futsch et al., 2017; Omsland et al., 2018). Although cell surface and viral proteins have been identified as players in cell-to-cell adhesion, there is still a gap of knowledge regarding the mechanisms by which HTLV-1 may increase viral spread. We propose that the small membrane-bound structures, known as Extracellular Vesicles (EVs), contribute to recipient cell recruitment and increased HTLV-1 viral spread.

Extracellular Vesicles have gained considerable attention due to their increasingly apparent role in cell-to-cell communication and in mediating disease (Gould et al., 2003; Keller et al., 2006; Fleming et al., 2014; Jaworski et al., 2014a; Sampey et al., 2014; Vader et al., 2014; Ahsan et al., 2016; Anderson et al., 2016; Pleet et al., 2017, 2018a,b; Barclay et al., 2017b; Anderson M. et al., 2018; DeMarino et al., 2018). EVs encompass a wide range of sizes based on their biogenesis pathway, such as exosomes (50-100 nm) and microvesicles (50-10,000 nm) (Keller et al., 2006; Raposo and Stoorvogel, 2013; Fleming et al., 2014; Kastelowitz and Yin, 2014; Sampey et al., 2014; Vader et al., 2014; van Dongen et al., 2016; van Niel et al., 2018). The detection of viral proteins in EVs secreted from infected cells denotes their potential to mediate disease. For instance, we have previously shown that the HTLV-1 protein Tax, implicated in driving oncogenesis and subsequent development of ATLL (Baratella et al., 2017; Billman et al., 2017; Tagaya and Gallo, 2017; Mahgoub et al., 2018), may be found in EVs from infected cells (Jaworski et al., 2014a). Also, we recently found that HAM/TSP patient cerebral spinal fluid (CSF) and peripheral blood mononuclear cells (PBMCs) consistently express Tax associated with EVs that may trigger inflammatory responses known to cause neurodegeneration (Anderson M.R. et al., 2018). However, it is still unclear which type of EV and associated cargo may participate in HTLV-1 pathogenesis, as well as the extent of the cellular targets (i.e., neuronal, myeloid, and T-cells) that may be involved in this pathogen-EV-

Our lab has previously shown that low levels of ionizing radiation (IR) can be used to activate latent viral reservoirs in vitro and in vivo across multiple tissues (blood, liver, lung, brain, and spleen) (Iordanskiy et al., 2015; Iordanskiy and Kashanchi, 2016). IR is also used as a tool to block cell cycle progression of HTLV-1-infected cells prior to administration in animal models of HTLV-1 infection (Tezuka et al., 2014, 2018). In this manuscript, we initially used IR as a probe to study HTLV-1 in a transcriptionally active setting, as to better resemble patients expressing higher levels of viral transcripts. We further explored the potential uses of IR in modulating EV release, as well as viral activation. Specific EV types derived from infected cells in distinct transcriptional states may potentially elicit varied effects on neighboring cells, such as activating uninfected T-cells or promoting viral spread. Understanding the mechanistic differences between latent and transcriptionally active HTLV-1 may allow for the development of clinical tools in the early detection of disease (i.e., EV/viral biomarkers) important for ATLL or HAM/TSP.

Here, we have attempted to address whether treatments such as IR affect EV release and cargo packaging (i.e., gp61<sup>+++</sup>/Tax<sup>+++</sup>/HBZ<sup>+</sup>; referred to as HTLV-1 EVs). We characterized the cargo of HTLV-1 EVs separated by a novel technique to isolate virus away from EVs. Additionally, we tested the functional role of EVs in promoting cell-to-cell contact and subsequent viral spread and identified CD45 and ICAM-1 as possible players in EV-mediated cell-to-cell contact. Finally, we examined the *in vivo* functional roles of HTLV-1 EVs in promoting spread and proviral integration. Collectively, we propose a novel two-step model of HTLV-1 infection, which involves EV-mediated priming of uninfected recipient cells and increased cell-to-cell contact resulting in an enhanced viral spread.

#### **RESULTS**

#### Viral Activation via IR Increases Intracellular Tax and EV Release

Our previous studies have shown that Tax protein may be encapsulated in EVs isolated from HTLV-1-infected cells (Jaworski et al., 2014a). Additionally, our more recent data have shown that EV-associated Tax can be isolated from HAM/TSP patient PBMCs and CSF samples (Anderson M.R. et al., 2018). These data demonstrate the potential clinical relevance and functional roles of EVs in HTLV-1 infection. We sought to elucidate the potential functional roles of EVs in HTLV-1 infection, particularly concerning viral spread. We wanted to understand the fundamental differences in Tax expression and EV release between latent and activated viral settings using ionizing radiation (IR), which can be used to activate virus (Iordanskiy et al., 2015). HTLV-1-infected HUT102 cells were treated with IR (10 Gy) and then incubated for 5 days to allow for maximal EV release, as described previously for HTLV-1 and other viruses (Narayanan et al., 2013; Jaworski et al., 2014a,b; Sampey et al., 2016; Barclay et al., 2017b; Anderson M.R. et al., 2018).

Western blot (WB) analysis was used to assess intracellular and EV-associated Tax protein expression levels related to viral activation. Intracellular Tax levels increased after treatment with IR (Figure 1A; lanes 1-3 vs. 4-6), while no increase in EVassociated Tax levels were observed (Figure 1A; lanes 7, 8 vs. 10-12). The p53 protein is typically used as a marker for double-stranded DNA damage induced by irradiation. HTLV-1 infected cells express high levels of inactive p53 (Yamato et al., 1993; Mori et al., 1997; Mulloy et al., 1998; Pise-Masison et al., 1998, 2000; de la Fuente et al., 2000; Mahieux et al., 2000; Grassmann et al., 2005; Pise-Masison and Brady, 2005). As expected, intracellular p53 levels were unchanged post-IR treatment (Figure 1A; compare lanes 1-6) however, p53 levels in EVs were increased after IR (compare lanes 7–12). Cytochrome c protein levels were also evaluated as a negative control for EVs (Figure 1A; lanes 7–12; as previously described in Jaworski et al., 2014a). Signal quantification was performed and confirmed a significant increase only in intracellular Tax (Figure 1B). These data potentially suggest that the mechanisms driving Tax protein packaging into EVs are either not altered by viral activation or are close to their maximal capacity. Alternatively, IR and an excess of Tax may also activate autophagy that degrades excess Tax that may have otherwise been secreted in EVs.

We next asked whether IR had any effects on EVs release from HTLV-1-infected cells. Nanotracking analysis (NTA; ZetaView) was used to quantitate EVs isolated from HTLV-1-infected cells (**Figure 1C**). This revealed a significant increase (\*\*\* $p \le 0.001$ ) for HUT102 cells treated with IR. The overall diameter of EVs did not change between control and IR lanes (Figure 1D). Finally, to ensure that culture settings and IR treatment were not deleterious to HUT102 cells, cell viability was measured at day 0 and day 5 for control and IR treated cells, revealing no significant change (Figure 1E). Uninfected T-cells (CEM) were also assayed under the same culture conditions revealing no significant change in viability (data not shown; Supplementary Figure S1). Altogether, these data suggest that IR may cause an increase in EV release from HTLV-1-infected cells without significantly altering EV biogenesis pathways, as per the unchanged EV diameter measured before and after treatment.

## IR Activates HTLV-1 Transcription Without Increasing Viral RNA Levels in EVs

The functional roles of EVs in transport of viral proteins and RNA is a topic of significant importance to the understanding of overall viral pathogenesis, especially since EVs have been shown to modulate immune responses (Muller et al., 2016; Pleet et al., 2016; Sampey et al., 2016; Anderson M.R. et al., 2018) and package viral cargo that may regulate gene expression (Ung et al., 2014; Barclay et al., 2017b). For instance, we have recently observed that in chronic HIV-1 infection, EVs from uninfected cells may activate viral transcription (Barclay et al., 2017b). In the case of HTLV-1, we have previously shown that viral RNA may be packaged into EVs released from infected cells (Jaworski et al., 2014a). These observations highlight the importance of understanding the cargo associated with EVs and its potential effects on recipient cells. We hypothesized that IR would increase HTLV-1 RNA transcripts intracellularly and that this increase would also result in increased incorporation of HTLV-1 RNA into EVs. Intracellular HTLV-1 env, tax, and hbz transcripts were quantified by RT-qPCR in HUT102 cells treated with IR (Figure 2A). Viral activation via IR specifically increased intracellular levels of tax (2.346  $\times$  10<sup>8</sup> to 3.63  $\times$  10<sup>8</sup> copies/mL; *p*-value  $\leq 0.05$ ), env (1.42  $\times 10^8$  to 2.16  $\times 10^8$  copies/mL; p-value  $\leq 0.01$ ), and hbz RNA (4.78  $\times 10^2$  to 2.80  $\times 10^5$ copies/mL; p-value < 0.001) (Figure 2A). However, gapdh cellular housekeeping gene remained unaffected by IR treatment. Overall, these data suggest that IR selectively promotes HTLV-1 transcription at both the 5' and 3' long terminal repeat (LTR).

To validate observations from **Figure 2A**, an experiment was performed using MT-2 and MT-4 HTLV-1-infected cell lines, with and without IR treatment (**Figure 2B**). RT-qPCR was performed specifically for *env* RNA since it generates the important precursor envelope protein gp61, and proteolytic byproducts gp46 and gp21. HTLV-1 envelope protein may have

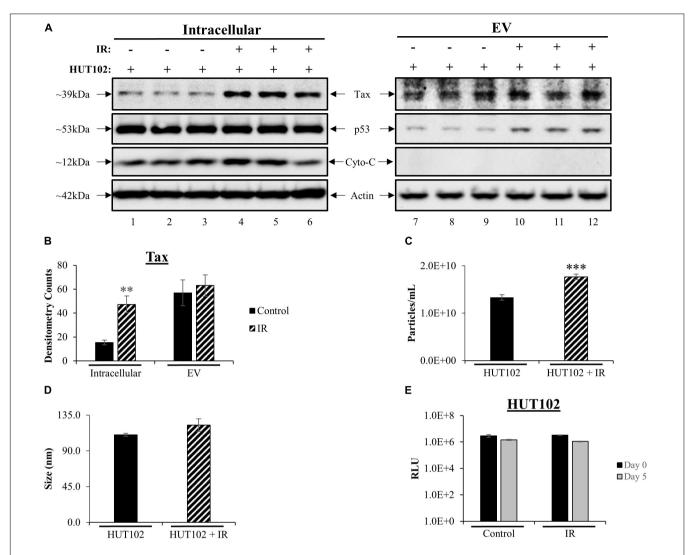


FIGURE 1 | Viral Activation with IR Increases Intracellular Tax and EV Release. HUT102 cells  $(1 \times 10^6 \text{ cells/mL})$  from three distinct cultures were treated with IR (10 Gy) and cultured for 5 days in EV-depleted media. (A) From 1 mL of culture, cell pellets were lysed, and EVs captured and enriched using Nanotrap (NT) particles NT080/NT082. WB analysis was performed on cell lysates (lanes 1–6) and EVs (lanes 7–12) for detection of Tax, p53 (IR control), Cytochrome c (negative control for EV) and Actin protein expression. (B) Densitometry analysis was used for each Tax band and normalized to its corresponding Actin band. Biological triplicates were averaged together (lanes 1–3, 4–6, 7–9, and 10–12) and a two-tailed student t-test used to evaluate statistical significance between control (no treatment) and IR treatment. (C,D) ZetaView analysis of supernatant material derived from 1  $\times$  10<sup>6</sup> HUT102 cells/mL ( $\pm$ IR; 10 Gy) was analyzed to determine EV size and concentration from three technical replicates. ZetaView analysis of EVs from HUT102 cells was evaluated in multiple independent experiments (n > 3) and the trends were replicated consistently. (E) Cell viability was evaluated for HUT102 cells at 1  $\times$  10<sup>6</sup> cells/mL for control and IR conditions after 5 days. A two-tailed student t-test was performed to evaluate statistical significance with \*\*\*p-value  $\leq$  0.001 indicating the highest statistical significance, followed by \*\*p-value  $\leq$  0.01.

crucial roles in promoting cell-to-cell contact; therefore, it is important to study the effects of viral activation (i.e., IR) on *env* RNA transcription and packaging into EVs. RT-qPCR data revealed a statistically significant increase (p-value  $\leq 0.001$ ) in intracellular *env* in all three IR treated cell lines, HUT102 (4.67  $\times$  10<sup>7</sup> to 5.82  $\times$  10<sup>7</sup> copies/mL), MT-2 (7.28  $\times$  10<sup>5</sup> to 1.08  $\times$  10<sup>6</sup> copies/mL), and MT-4 (4.96  $\times$  10<sup>6</sup> to 9.88  $\times$  10<sup>6</sup> copies/mL; **Figure 2B**). However, we found a decrease in *env* RNA in EVs from HUT102 (4.09  $\times$  10<sup>6</sup> to 1.98  $\times$  10<sup>6</sup> copies/mL; p-value  $\leq$  0.01) and MT-2 (1.53  $\times$  10<sup>6</sup> to 5.18  $\times$  10<sup>5</sup> copies/mL; p-value  $\leq$  0.001), but not from MT-4 (4.42  $\times$  10<sup>5</sup> to 4.70  $\times$  10<sup>5</sup> copies/mL) EVs (**Figure 2B**). These data suggest that while

IR causes an increase in overall intracellular *env* RNA across all three HTLV-1-infected cell lines, it does not increase its packaging into EVs.

#### HTLV-1-Infected Cells Secrete EVs Containing Differentially Packaged Viral Proteins and RNA

We previously developed a novel EV isolation and separation method using density gradients, which allow isolation of EVs away from viruses and into distinct EV groups, large protein complexes, and free protein fraction (DeMarino et al., 2018,

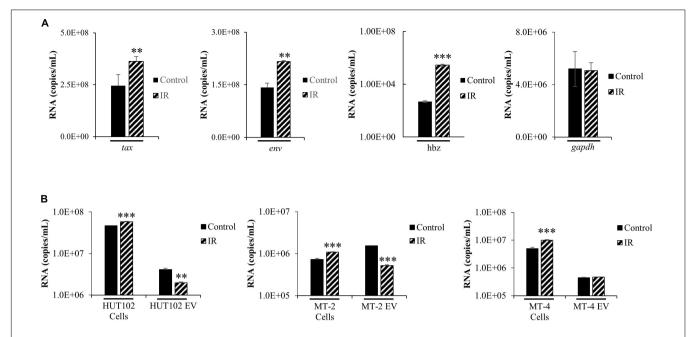


FIGURE 2 | HTLV-1 Activation Increases Intracellular Viral RNA Transcription, but not RNA packaging into EVs. HUT102 cells (1  $\times$  10<sup>6</sup> cells/mL) were treated ( $\pm$ IR; 10 Gy) and cultured as described previously. (A) RNA was isolated from a 1 mL culture of HUT102 cells for analysis by RT-qPCR of tax, env, hbz, and gapdh. At least three independent experiments, each in technical triplicate, were performed and observed consistent reproducibility. (B) Similarly, 1 mL cultures of HUT102, MT-2, and MT-4 cells were cultured and EVs nanotrapped (NT080/082) and used for RT-qPCR analysis of env RNA. RT-qPCRs were performed in technical triplicate. A two-tailed student t-test was used to evaluate statistical significance with \*\*\*p-values  $\leq$  0.001, \*\*p-values  $\leq$  0.01, and \*p-values  $\leq$  0.05 indicating the level of significance.

2019). We reasoned that the differences in EV density are potentially due to a combination of EV cargo/packaging and size. The isolation method consists of the addition of ExoMax reagent for the precipitation of EVs, followed by the use of an Iodixanol gradient for the separation of distinct EV populations away from the potential virus. EVs from HUT102 cells ( $\pm IR$ ) were separated into 11 fractions and then enriched with Nanotrap particles (NT080/082/086) for downstream detection of HTLV-1 matrix (p19), envelope (gp61 and gp46), Tax, HBZ, and Actin (control) proteins. WB analysis showed the presence of p19 in fractions 13.2 to 18 (Figure 3A; lanes 7-11), while cells treated with IR yielded EVs that primarily displayed p19 in fraction 18 (Figure 3A; lane 22). This finding is in line with previous observations in the context of HIV-1 infection which suggest that fraction 18 may contain a mixture of EVs and potentially virus, or at least an abundance of viral components in autophagosomes (DeMarino et al., 2018). Interestingly, mature envelope protein, gp46, was almost exclusively present in fraction 18, unaffected by IR (Figure 3A; lanes 11 and 22). Low levels of gp46 were also detected in the left side of the gradient mainly at fraction 6. Not surprisingly, colocalization of gp46 with p19 (on the right side of the gradient; fraction 18) suggests the presence of a potential viral particle, whereas the presence of gp46 in fraction 6 may suggest free gp46. On the other hand, a strong signal for the unprocessed form of HTLV-1 envelope protein (gp61) was detected in lower density fractions 6 to 13.2 (lanes 1–7) and in IR treated fractions 6 to 15.6 (lanes 12-20), while absent in high-density fractions 16.8 and 18 (lanes 10, 11 and 21, 22). This data provides further

confirmation that processed proteins and virus may colocalize in the high-density fraction 18 (lanes 11–22), and unprocessed proteins in low-density EV fractions. Next, Tax was detected in lower density fractions 6 to 10.8 (lanes 1–5). However, upon IR treatment, a shift in Tax packaging was observed into the higher density fractions (12 to 15.6; lanes 17–20). The signal for Tax was strong in fraction 18 (lane 11) and more so in IR treated fraction 18 (lane 22). These findings suggest that upon IR treatment Tax is potentially packaged into higher density EVs, potentially due to the increased transcription of *tax* mRNA as previously observed in **Figure 2A**. Finally, we observed low levels of antisense protein, HBZ, in fractions 6 and 18 without IR treatment, while IR treated fractions contained almost no HBZ protein.

To more closely inspect the overall levels of Tax in EVs after viral activation, we performed densitometry analysis of gradient separated Tax bands from three distinct biological replicates and found that IR caused a non-significant increase of the total Tax protein levels (**Figure 3B**). Next, RT-qPCR analysis of each fraction was performed to characterize HTLV-1 *env*, *tax*, *hbz* RNA levels in EVs. In **Figure 3C**, fractions 6 to 12 contained very similar quantities of *env* RNA, with fraction 6 quantifying an average of  $2.11 \times 10^3$  copies (control) and  $9.00 \times 10$  copies (IR) of total *env* RNA. Higher *env* RNA levels were detected as the density of the fractions increased, with the most abundant quantities of RNA in fraction  $18 [3.4 \times 10^5 \text{ (control)}]$  and  $6.7 \times 10^4 \text{ copies}$  (IR)] (**Figure 3C**). IR treatment resulted in a decrease in *env* RNA levels in every fraction (**Figure 3C**, fractions 6 to 18), potentially due to alterations in packaging.

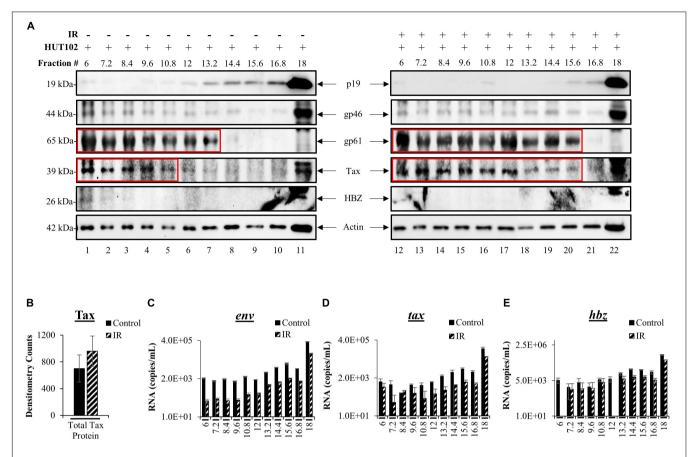


FIGURE 3 | HTLV-1 proteins are differentially packaged after Viral Activation. HUT102 cells were treated with IR (10 Gy) and cultured in Exo-Free media for 5 days. Supernatants were collected from control and IR treated cells for precipitation by ExoMAX and separation into 11 fractions by OptiPrep/ultracentrifugation. Each resulting fraction was concentrated with NT080/082/086 particle mixture overnight. (A) WB analysis was conducted for each fraction from control (lanes 1–11) and IR treated cells (lanes 12–22), and probed for p19, gp61, gp46, HBZ, Actin. Each figure represents two blots with identical exposure combined into one for control and one for IR. (B) Densitometry analysis was performed on Tax protein bands from three independent lodixanol gradients, averaged, and normalized to Actin. EV RNA was analyzed by RT-qPCR for (C) env RNA, (D) tax RNA, (E) and hbz RNA. Error bars represent one standard deviation above and below the averaged RNA copies/mL of technical triplicates.

However, it is important to note that *env* RNA levels could be the result of amplification of both *env* mRNA and genomic RNA. Similar trends were observed for both *tax* (**Figure 3D**) and *hbz* (**Figure 3E**). RNA copy numbers for *tax* were the highest in fraction 18 at  $1.2 \times 10^5$  (control) and  $3.9 \times 10^4$  (IR) RNA copies (**Figure 3D**). Similarly, *hbz* RNA levels were highest in fraction 18, quantifying at  $4.4 \times 10^5$  (control) and  $1.8 \times 10^5$  (IR), which is interesting as an increase in *hbz* RNA did not result in HBZ protein synthesis. Overall, these data indicate that viral proteins and RNA are present in different density EVs and that processed viral components are potentially more abundant in the highest density EVs, while unprocessed proteins in lower density EVs.

#### Characterization of the Effects of EVs From HTLV-1-Infected Cells on Recipient Cells

Whether EVs derived from HTLV-1-infected cells are capable of establishing a new infection is not known. However, the presence of viral proteins and RNAs in EVs from infected cells

implies that they may be able to cause disease in recipient cells. We observed previously that fraction 18 contained a higher abundance of processed viral proteins (p19 and gp46) and RNA (env, tax, and hbz), which led us to hypothesize that this fraction may potentially be infectious. Therefore, we investigated the effects of all HTLV-1 EV fractions (±IR) on uninfected recipient T-cells (CEM and Jurkat). Recipient cells were harvested after 5 days and analyzed for the presence of viral RNA or proteins. Both p19 and Tax were not detected in CEM cells treated with EV fractions (lanes 1-22), except for the presence of p19 in fraction 18 (Figure 4A; lane 11). The results in Figure 4B, in which EV fractions from HTLV-1-infected cells were added to Jurkat recipient cells, demonstrated a trend similar to that observed in CEM recipient cells for all measured proteins. Interestingly, in both CEM and Jurkat cells, fraction 18 from IR treated HUT102 cells did not elicit the expression of p19 in recipients (Figures 4A,B; lane 22). Altogether, these data suggest that while fractions isolated from HTLV-1-infected cells contain viral proteins and RNA, only fraction 18 may have the potential

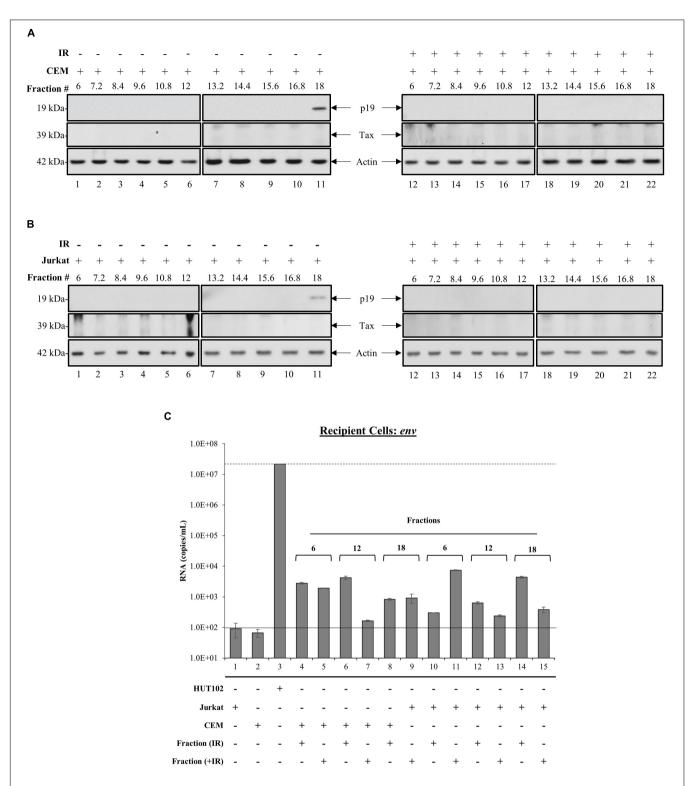


FIGURE 4 | Treatment with HTLV-1 EVs does not generate HTLV-1 viral proteins and RNA in Recipient cells. CEM and Jurkat cells were treated with density fractions (6 to 18) of HUT102 EVs for 5 days. WB analysis was conducted on CEM (A) and Jurkat (B) cells treated with HUT102 control EV fractions (lanes 1–11) and HUT102 IR-treated EV fractions (lanes 12–22) for p19, Tax, and Actin. Selected lanes were taken from the same blot with identical exposure settings presented in the figure. (C) RT-qPCR was conducted on WCE from Jurkat, CEM, and HUT102 cells (controls; lanes 1, 2, and 3, respectively). Jurkat cells (±IR) treated with fractions 6 (lanes 4 and 5), 12 (lanes 6 and 7), and 18 (lanes 8 and 9), and CEM cells (±IR) treated with fractions 6 (lanes 10 and 11), 12 (lanes 12 and 13), and 18 (lanes 14 and 15) for the presence of env RNA. Negative controls levels were denoted by a solid line (-) and positive control levels for infection by a dashed line (—). We used fraction 6 as negative control for presence of virus; fraction 18 as a potential source of virus; and fraction 12 as a control for fraction 18, which contained EVs with Tax/gp61.

to establish a new infection, and possibly includes both EVs and mature virions.

To further elucidate the functionality of fraction 18, we asked whether the observed p19 was due to carryover or instead novel viral replication and protein translation. The effects of each EV fraction (±IR) on uninfected recipient cells was assessed by RT-qPCR of env RNA levels in negative controls (Figure 4C; solid line) Jurkat (lane 1) and CEM cells (lane 2), positive control (Figure 4C; dashed line) HUT102 (lane 3); and recipient CEM/Jurkat cells treated with ±IR fractions 6, 12, and 18. Jurkat and CEM cells showed a background of 92 and 67 RNA copies, respectively (Figure 4C). Positive control HUT102 cells showed a baseline env RNA copy number of  $2.2 \times 10^7$ , which served as a control for infection. Quantitation of Jurkat or CEM cells treated with EV fractions from HTLV-1-infected cells (±IR) resulted in RNA levels above negative controls (Figure 4C; lanes 1 and 2), but below positive control (Figure 4C; lane 3), regardless of IR treatment (Figure 4C; lanes 4-15). This data further validates that EVs from HTLV-1-infected cells do not cause a new round of infection, yet the EVs may be successful at delivering their cargo into recipient cells, as evident by baseline env RNA levels detected in all cells receiving EVs.

We next sought to investigate whether a heterogeneous EV population, rather than those separated explicitly by a density gradient, may establish infection in uninfected T-cells, as measured by an increase in RNA levels above and beyond negative controls (Figures 5A-C; solid line) to a positive control (Figures 5A-C; dashed line). EVs from HTLV-1-infected HUT102, MT-2, and MT-4 donor cells ( $\pm$ IR) were enriched using NT080/082 and used for subsequent co-culture with recipient cells (CEM and Jurkat). Following incubation, recipient cells were harvested and analyzed by RT-qPCR (Figure 5). Not surprisingly, EVs from HUT102 cells did not result in an increase in env RNA copy numbers in recipient cells (lanes 4 and 5) compared to the amount of env RNA present in the EV input (Figure 5A; lane 3). Similar data was also observed for MT-2 (Figure 5B) and MT-4 (Figure 5C) EVs. Overall this data suggests that HTLV-1 EVs may not be infectious, however, EVs are capable of carrying viral RNA and/or proteins into the recipient/target cell.

## **EVs From HTLV-1-Infected Cells Promote Cell-to-Cell Contact**

We have previously shown that EVs have functional effects on recipients cells (Narayanan et al., 2013; Sampey et al., 2016; Anderson M.R. et al., 2018). Although our data suggest that HTLV-1 EVs may not be infectious in T-cells, *env* RNA was still detected in recipient cells, therefore we asked whether EVs may have functional effects on neighboring recipient cells (i.e., cargo delivery). During prior treatments of recipient cells with HTLV-1 EVs, increased cell aggregation was noted. We hypothesized that HTLV-1 EVs might promote increased cell-to-cell contact of recipient uninfected cells. It was first necessary to track HTLV-1 EVs to determine if they colocalized with recipient cells.

EVs derived from donor CEM cells (Control EVs), HUT102 cells (HTLV-1 EVs), or HUT102 IR-activated cells (HTLV-1/IR EVs) were fluorescently labeled with BODIPY, and added to recipient CEM cells at EV concentrations normalized to volume to better resemble physiological conditions (Figure 6A). Twentyfour hours post-treatment, Control EVs localized sparsely in the extracellular medium, and minimally with recipient cells. Only 9% of all CEM cells were found agglutinated together. Aggregates did not seem to be EV-mediated and may represent background levels (Figure 6A; upper panel). Interestingly, HTLV-1 EVs (**Figure 6A**; middle panel) were found to colocalize with cell membranes of recipient cells agglutinated together resulting in a total of 59% of cell agglutination (only EVmediated aggregates counted), and similarly for HTLV-1/IR EVs (Figure 6A; lower panel) at 54% agglutination. However, it was noted that HTLV-1/IR EVs caused a 2-fold increase in the maximum number of cells agglutinated (from 24  $\pm$  2 to  $40 \pm 2$  cells/clump). Additionally, cell viability of recipient cells was assessed up to 5 days of post-EV treatment and analysis revealed no detrimental effects on viability (Figure 6B). On the contrary, HTLV-1 EV treated recipient cells (lanes 3 and 4) showed significantly improved viability (p-value  $\leq 0.05$ ) when compared to untreated cells (Figure 6B; lane 1). These data suggest that HTLV-1 EVs promote cell-to-cell contact without decreasing cell viability, a finding that may be important for viral spread.

## Increased Cell-to-Cell Contact Promotes Potential Increase in Viral Spread

We consistently observed increased agglutination of recipient CEM cells treated with HTLV-1 EVs normalized to volume. We next validated our observations by testing the effects of EVs on recipient cells by normalizing treatment to EVs concentration (1:10,000; Cell:EV ratio) at day 5 post-EV treatment, and by the use of multiple different uninfected recipient cells (i.e., CEM and Jurkat). Additionally, we asked whether the observed increased agglutination could also correlate to increased viral transcription on recipient cells. To test this, IR treated HUT102 donor cells were added at day 5 and cultured for 4 additional days prior to performing RT-qPCR analysis (Figure 7). In this scenario, IR treatment of HTLV-1-infected donor cells (which inhibits cellular replication) is the source of the viral spread.

Initial microscopic analysis revealed the highest agglutination levels in recipient cells when treated with HTLV-1/IR EVs, suggestive of high levels of cell-to-cell contact (**Figure 7A**; black arrows in the upper panel; larger clumps). HTLV-1 EVs (**Figure 7A**; middle panel) elicited a moderate amount of cell-to-cell contact between uninfected cells, while minimal contact was observed in the control EV treatment (**Figure 7A**; lower panel). Quantitation of HTLV-1 RNA by RT-qPCR indicated that *tax* RNA levels increased above background when pretreated with HTLV-1/IR EVs (**Figure 7A**; compare *tax* lanes 1–4) and to a lesser extent when treated with HTLV-1 EVs (compare *tax* lanes 1–3). Similar results were obtained for *env* and *hbz*. Overall, these data suggest that HTLV-1 EVs (±IR)

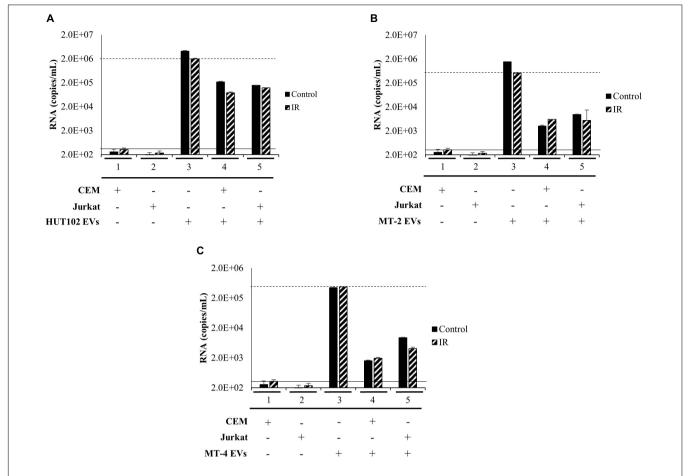


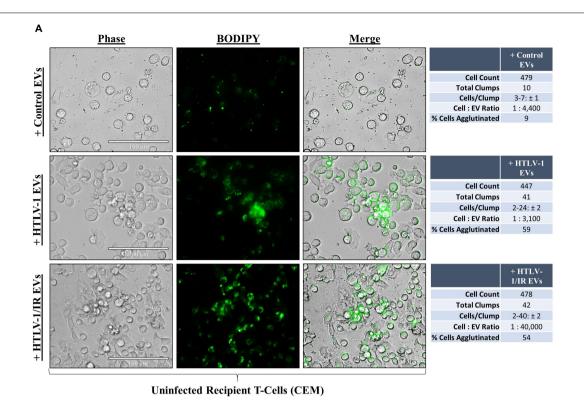
FIGURE 5 | EVs isolated from HTLV-1-Infected Cells are not Infectious. T-cell lines (CEM and Jurkat) were cultured in EV-depleted media and incubated with EVs from HTLV-1-infected cells (HUT102 (A), MT-2 (B), or MT-4 (C); ±IR) for 5 days. RT-qPCR for env RNA was performed for control CEM cells (lane 1), Jurkat cells (lane 2). Additionally, HTLV-1 EVs (HUT102, MT-2, or MT-4; lane 3) was also analyzed to determine relative RNA levels of the starting material. RT-qPCR quantitation of recipient CEM and Jurkat cells were analyzed to assess infectivity (lanes 4 and 5, respectively). Negative controls levels were denoted by a solid line (-) and positive control levels for infection by a dashed line (—).

promote increased levels of viral transcription in recipient CEM T-cells.

Jurkat recipient T-cells were also tested. They showed similar levels of cell agglutination with HTLV-1/IR and HTLV-1 EVs, and minimal levels with Control EVs (Figure 7B). Subsequently, HTLV-1 donor cells (HUT102, IR activated) were added and incubated as described for CEM cells. RTqPCR analysis indicated that tax RNA levels increased above background when pretreated with HTLV-1/IR (Figure 7B; compare tax lanes 1-4) and HTLV-1 EVs (compare tax lanes 1-3), but decreased from the background when treated with Control EVs (compare tax lanes 1 and 2). RNA levels for env increased the most above background when treated with HTLV-1/IR EVs (env lane 4), to a lesser extent when treated with HTLV-1 EVs (env lane 3) and the least with Control EVs (env lane 2). Finally, hbz levels exhibited the highest increase above background when treated with HTLV-1/IR EVs (hbz lane 4), moderately with HTLV-1 EVs (hbz lane 3), and the least with Control EVs (hbz lane 2). These data suggest that HTLV-1 EVs (±IR) may have functional effects in priming distinct recipient T-cells for infection (i.e., Jurkat and CEM). Overall, these data suggest that HTLV-1 EVs promote cell-to-cell contact of uninfected recipient cells, which could potentially facilitate viral spread.

#### Antibodies Against Specific Cellular Surface Receptors Inhibit Cell-to-Cell Contact

HTLV-1/IR EVs consistently promoted increased cell-to-cell contact, and CEM recipient cells were the most receptive to this effect. We, therefore, attempted to elucidate the mechanism by which EVs promote this phenotype. HTLV-1 transmission primarily occurs via cell-to-cell contact facilitated by several potential proteins involved in cell adhesion, such as CD45 (viral biofilm), ICAM-1, VCAM-1, and LFA-1 (cellular conduits and virological synapse), and Tax/Gag proteins (tunneling nanotubes) (Van Prooyen et al., 2010b; Gross and Thoma-Kress, 2016; Gross et al., 2016; Omsland et al., 2018). We hypothesized that HTLV-1/IR EVs could carry



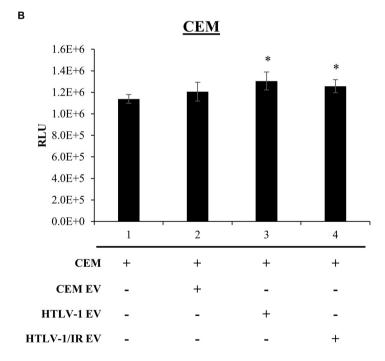
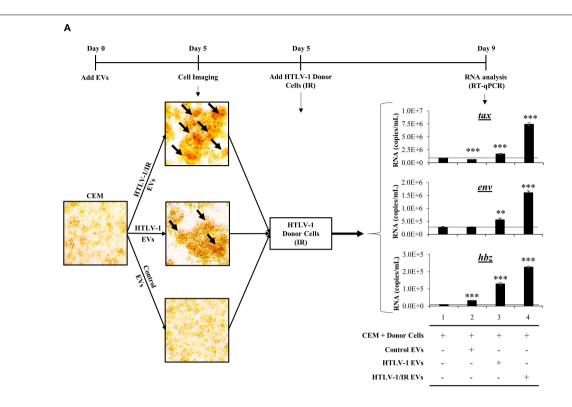


FIGURE 6 | EVs from HTLV-1-Infected Cells Promote Cell-to-Cell Contact. (A) Uninfected recipient cells (CEM) in biological triplicate were cultured at  $1 \times 10^5$  cells/mL in a  $100 \,\mu$ L well and treated with equal volumes of CEM EVs ( $4.43 \times 10^8$  EVs; 1:4,400), HTLV-1 EVs ( $3.05 \times 10^8$  EVs; 1:3,300), and HTLV-1/IR EVs ( $4.02 \times 10^9$  EVs;  $4.02 \times$ 



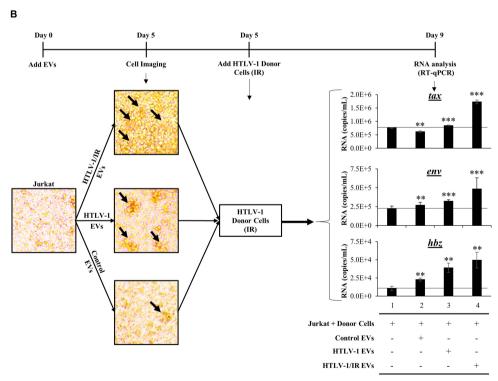


FIGURE 7 | HTLV-1 EVs Promote Cell-to-Cell Contact and Increase Infectivity of HTLV-Infected Cells. Uninfected recipient CEM (A) and Jurkat (B) cells were cultured in biological triplicate in EV-depleted media with EVs from CEM cells (Control EVs), HUT102 cells (HTLV-1 EVs), and irradiated HUT102 cells (HTLV-1/IR EVs) at a ratio of 1 cell to 10,000 EVs for 5 days prior to microscopic analysis. Images are representative of three independent experiments. Following microscopy, irradiated HUT102 cells (HTLV-1 Donor Cells; 10 Gy) and fresh Exo-Free media were added to the culture at a ratio of 1:100 for 4 days. Subsequent RT-qPCR analysis was performed for the presence of tax, env, and hbz. A two-tailed student t-test was used to evaluate statistical significance with \*\*\*p-values  $\leq$  0.001, and \*p-values  $\leq$  0.05 indicating the level of significance.

proteins involved in cell adhesion. Thus, neutralization with antibodies would potentially inhibit HTLV-1 EV-mediated cell-to-cell contact.

Recipient cells (CEM) were cultured in triplicate and treated with HTLV-1/IR EVs as shown in Figure 7. Simultaneously, neutralizing antibodies against CD45, ICAM-1, VCAM-1, Tax, or gp61/46 was added to test for cell-to-cell contact inhibition (data not shown) and one optimal concentration for each was used for blocking experiments. The most noticeable differences were observed using α-CD45 (Figure 8A; Tier I) and α-ICAM-1 (Figure 8A; Tier II) which prevented EVmediated cell agglutination, whereas  $\alpha$ -VCAM-1 (**Figure 8A**; Tier V) had no effect against EV-mediated cell-to-cell contact. Out of the two antibodies that effectively prevented cell-tocell contact, α-CD45 treated cells showed morphology that resembled untreated CEM cells. However, although addition of α-ICAM prevented agglutination, cell morphology was altered (smaller size) and the pH of the media remained unchanged after 5 days, potentially suggesting an either lowering of intracellular ATP levels or a general cellular toxicity. Use of  $\alpha$ -Tax (Tier III) and  $\alpha$ -gp61/46 (Tier IV) antibodies only partially inhibited agglutination and, therefore, were not considered as primary proteins responsible for HTLV-1 EV mediated cellto-cell contact (data not shown). Overall, the data suggest that CD45 is a prime candidate in the mediation of cell-tocell contact in HTLV-1 infection. However, it was still unclear whether CD45 protein was on the surface of the EV or on the recipient cell membrane.

Next, it was important to evaluate the viability of antibody treated cells, especially since antibodies may concomitantly inhibit normal cellular process leading to cell death. Additionally, viral transmission requires viable cells for effective viral spread. Therefore, we validated recipient cell viability using five neutralizing antibodies (and a duplicate  $\alpha$ -CD45 antibody). Cell viability analyses revealed no significant change in cell survival after antibody treatment (**Figure 8B**), as a two-tailed *t*-test confirmed no significance between changes in viability when compared to either untreated CEM cells (lane 1) or HTLV-1/IR EV treatment (lane 3). These data suggest that the inhibition of cell-to-cell contact by  $\alpha$ -CD45 is potentially limited to interactions induced by EVs, and not physiologically necessary interactions in the tested *in vitro* settings.

We next explored whether CD45 and/or ICAM-1 are present in HTLV-1 EVs ( $\pm$ IR), hence contributing to viral spread. Supernatant from 5-day old cultures in biological triplicate were separated and enriched for EVs by NT080/082 (**Figure 8C**). WB analysis revealed trace amounts of CD45 in HTLV-1 EVs (**Figure 8C**; lanes 1 and 3) and no significant difference in HTLV-1/IR EVs (**Figure 8C**; lanes 4–6). ICAM-1 was detected in HTLV-1 EVs and HTLV-1/IR EVs (lanes 1–6). These data show the potential presence of CD45 and ICAM-1 in HTLV-1 EVs ( $\pm$ IR), supporting prior observations about their potential role in mediating increased levels of cell-to-cell contact.

Finally, two distinct donor PBMC cultures were treated with IL-2 and PHA for 3 days prior to treatment with HTLV-1/IR EVs (as described previously; 1:10,000; Cell:EV

ratio) and neutralizing antibodies (α-CD45 or α-ICAM-1) were added in fresh EV-depleted RPMI media for 4 days. PBMCs treated with HTLV-1/IR EVs showed increased cell agglutination (Figure 8D; upper panel for both PBMCs), which was blocked by  $\alpha$ -CD45 (**Figure 8D**; middle panel) and  $\alpha$ -ICAM-1 treatment (Figure 8D; lower panel). Next, at day 4, HTLV-1 donor cells (IR; 10 Gy) were added as the source of virus at a ratio of 1 donor cell to 100 recipient cells (1:100). At day 8, RT-qPCR analysis revealed that env, tax, and hbz RNA levels were present in PBMCs above the background (starting material; lane 1). Addition of HTLV-1/IR EVs did not alter RNA levels dramatically. However, addition of α-CD45 antibody resulted in a decrease in env and tax levels for PBMC 1; and in env and hbz for PBMC 2 (compare lane 3 and 4). Addition of α-ICAM antibody resulted in a decrease in env RNA levels for both PBMC 1 and 2 (compare lane 3 and 5). Collectively, these data suggest that CD45 may be an important molecular target in EV-mediated cell-to-cell contact, followed by ICAM-1, since their inhibition potentially suppresses viral transmission in PBMCs.

#### EVs From HTLV-1-Infected Cells Promote Viral RNA Transcription and DNA Integration Across Multiple Tissues in vivo

Finally, to test the effect of EVs in vivo, we utilized HTLV-1infected NOD/Shi-scid/IL-2Ryc null (NOG) mice, which have shown successful viral spread and ATLL development in vivo (Tezuka et al., 2014, 2018). NOG mice received human CD34<sup>+</sup> cells (8 weeks), followed by HTLV-1 EV injection (HUT102; 6×, 10 μg each for 2 weeks), and subsequent treatment with IR activated HTLV-1 donor cells (50 million cells) for 3 weeks (Figure 9A). As previously described, IR treatment of HTLV-1-infected cells is the source of the viral spread. NOG mice were treated with EVs from HTLV-1-infected cells or EVs from uninfected control cells followed by injection of IR activated HTLV-1 donor cells. All mice exposed to HTLV-1-infected cells (NOG 1-6) showed upregulation of HTLV-1 RNA in blood cells (Figure 9B). Interestingly, NOG mice that received HTLV-1 EVs showed enhanced viral replication compared to mice which received Control EVs (Figure 9B; compare NOG 1-3 vs. NOG 4-6). Uninfected and untreated NOG mice controls (Figure 9B; C1-3) served as a baseline for infection. We then examined the presence of HTLV-1 DNA in various tissues. Data in Figure 9C indicates a 90% increase in HTLV-1 DNA, which was detected in the blood of animals that received HTLV-1 EV treatment compared to control EV treatment. HTLV-1 DNA levels were also increased in other tissues (Figure 9D) after HTLV-1 EV treatment, such as in lung (59% increase), spleen (93% increase), liver (81% increase; p-value  $\leq 0.05$ ), and brain (97% increase). Therefore, animals which received HTLV-1 EVs showed higher viral load in all tissues, including the brain, suggesting the virus was potentially able to expand into the anatomically privileged tissues. Collectively, these data indicate that EVs may contribute to viral spread in vivo, by promoting cell-to-cell contact and in turn potentiating the infection.

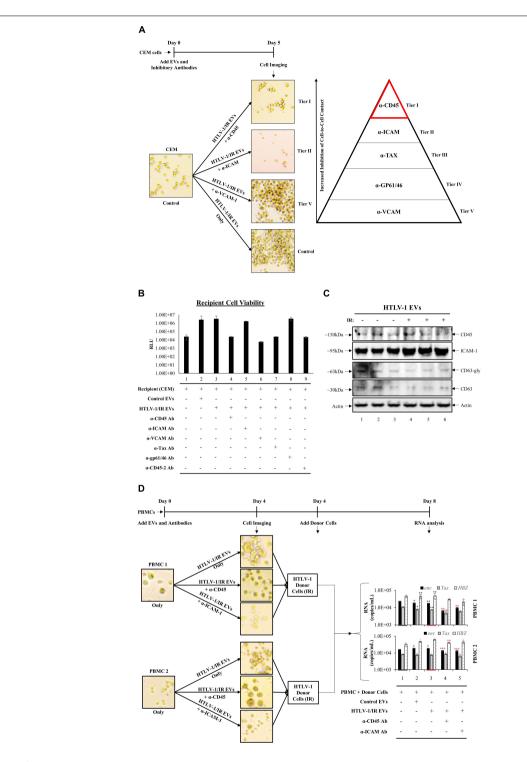


FIGURE 8 | Antibodies against Specific Cellular Surface Receptors Inhibit Cell-to-Cell Contact. (A) CEM recipient cells, in biological triplicate, were treated with HTLV-1 EVs (1 cell: 10,000 EVs) and treated with antibodies at concentrations derived from titration (data not shown) for α-CD45 (0.2 μg/mL), α-ICAM-1 (20 μg/mL), α-VCAM-1 (20 μg/mL), α-Tax (7.5 μg/mL of three Tab antibodies; 1:100 dilution), α-gp61/46 (5 μL of 1:10 dilution; according to Palker et al., 1992; 45 μL/1 ×  $10^6$  cells/mL), and imaged at day 5. (B) Cell viability of CEM cells (5 ×  $10^5$  cells/mL) treated with HTLV-1/IR EVs (5 ×  $10^9$  cells/mL) and neutralizing antibodies at day 5 after treatment. (C) Western blot analysis for CD45, ICAM-1, CD63 and Actin was performed on HTLV-1 EVs (from HUT102 cells) enriched by NT080/082 from supernatants of 5 days old CEM cultures at 1 ×  $10^6$  cells/mL. (D) PBMCs were cultured for 3 days and with IL2 and PHA on day 0 and day 3 prior to treatment with HTLV-1/IR EVs (1 PBMC: 10,000 EVs) and α-CD45 and ICAM-1 for 4 days, and subsequent addition of IR treated donor cells (1 HUT102 cell: 100 PBMCs) and RT-qPCR analysis for env, tax, and hbz at day 8. Only 2 out of 3 PBMC experiments are shown. Black Asterisks (\*) are used to compare lanes 2 and 3 to lane 1, and red asterisks (\*) are used to compare lanes 4 and 5 to lane 3.

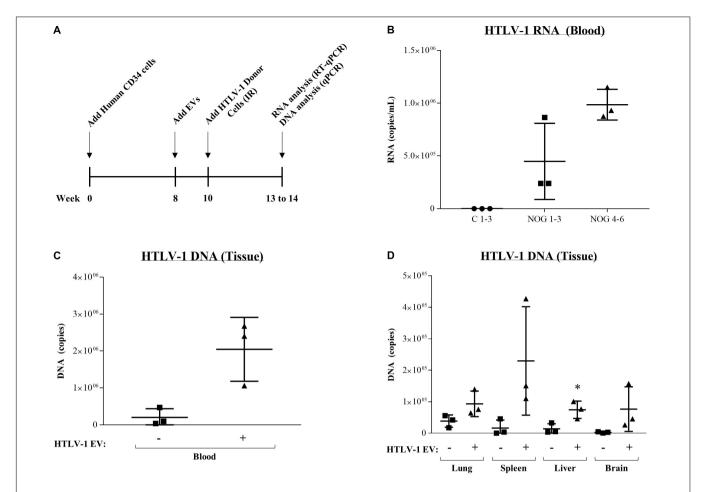


FIGURE 9 | EVs from HTLV-1-infected cells increase viral RNA and DNA in NOG mouse model. (A) NOG mice received human CD34 cells (8 weeks), followed by control EV or HTLV-1 EV injection (HUT102;  $6 \times$ ,  $10 \mu g$  for 2 weeks), and subsequent treatment with IR activated HTLV-1 donor cells (50 million) for 3 weeks. IR treatment of HTLV-1-infected cells (inhibition of cellular replication) is the source of the viral spread. (B) RT-qPCR of viral RNA from blood of NOG mice (n = 9) that received no treatment (C 1–3; denoted by a circle), Control EV treatment (NOG 1–3; denoted by a square, and HTLV-1 EVs (NOG 4–6; denoted by a triangle). (C) Presence of HTLV-1 DNA (using PCR for env region) from the blood of NOG mice. (D) Analysis of HTLV-1 DNA (env) from NOG mice from Lung, Spleen, Liver, and Brain. A two-tailed student t-test was used to evaluate statistical significance with \*\*p-values  $\leq 0.01$  and \*p-values  $\leq 0.05$  indicating the level of significance compared to control.

#### **DISCUSSION**

Our data validate findings that HTLV-1-infected cells secrete EVs that contain Tax protein. The presence of Tax in EVs are in line with previous findings of EV associated Tax in cell lines, PBMCs, and CSF from HAM/TSP patients (Jaworski et al., 2014a; El-Saghir et al., 2016; Barclay et al., 2017a; Anderson M.R. et al., 2018; Otaguiri et al., 2018). Moreover, they show that an IR treated infected cell may potentially contain significantly higher levels of intracellular Tax. Tax levels could contribute to cellular events related to EV biogenesis, such as release, as well as other related machineries including control of autophagy. IR treated infected cells also secreted higher numbers of EVs that may contain more Tax, either as free Tax, or EVs that originate from multiple sources (i.e., Golgi vs. MVBs). Future experiments will better define the nature of these Tax containing entities.

Tax is the main oncoprotein in HTLV-1, therefore its association with EVs may have potential effects in perpetuating

Tax-mediated pathogenesis, including the induction of NF-κB in recipient cells which may promote the development of ATLL (Azimi et al., 1998; de la Fuente et al., 2000; Azran et al., 2004). Additionally, expression of EV-associated Tax has been found consistently in HAM/TSP patients with functional roles such as overexpression of activated CD8+ Cytotoxic T-cells (Anderson M.R. et al., 2018). This overexpression may have direct implications in the development of HAM/TSP. We expand on previous work by showing that IR treatment may have modulatory effects on Tax-containing EVs (Figure 1). We found that IR not only increases the association of Tax with EVs, but it also causes an upregulation in the amount of EVs secreted from HTLV-1-infected cells. It is important to note that IR is a well-characterized stress inducer (Bartkova et al., 2005; Gorgoulis et al., 2005) and we have previously shown that it can activate LTR gene expression of HIV-1 (Iordanskiy et al., 2015). Interestingly, here we observed IR activates both 5'LTR and 3'LTR transcription in multiple

different HTLV-1-infected cell lines as shown by increases in tax, env, and hbz gene expression (**Figure 2**). Interestingly, the increase in transcription was higher for the 3'LTR ( $\sim$ 2.5-log), since there was complete suppression of the 3'LTR prior to IR activation. In contrast, the 5'LTR activation was not as dramatic, since there were originally higher background levels of basal transcription from this promoter. Therefore, the use of IR to potentially reactivate HTLV-1 may likewise stimulate the increased production of EVs and packaging of Tax and other viral products into these EVs. Increased levels of Tax-containing EVs may thereby highly impact recipient cells during pathogenesis.

We next sought to characterize whether specific EV types were responsible for carrying select HTLV-1 cargo and whether such EVs were infectious. For the past several years, our lab has focused on optimizing the isolation of EVs from multiple different viral infection settings, including HTLV-1 (Jaworski et al., 2014a; Barclay et al., 2017a), HIV-1 (Narayanan et al., 2013; Jaworski et al., 2014b; Sampey et al., 2016; Barclay et al., 2017b; DeMarino et al., 2018), Ebola virus (Pleet et al., 2016, 2018b), and Rift Valley Fever virus (Ahsan et al., 2016). Separation of EVs by density through the use of Iodixanol gradients has been described previously as a method to reliably separate EVs away from viruses such as HIV-1 (DeMarino et al., 2018). Here, we found that HTLV-1 processed proteins, such as envelope, settled in the highest density fraction (#18), suggesting potential presence of either virus or EVs that contained virus (Figure 3). Fractions 7.2 to 13 contained mostly unprocessed proteins, such as gp61, and fraction 6 contained trace levels of gp46. Overall, this data allows an initial categorization of HTLV-1 EVs into three groups: large density EV complex (fraction 18), lower density EV complex (fraction 7.2 to 13), and potentially  $\leq$ 35 nm EVs or free protein complexes (fraction 6). However, upon treatment of recipient T-cells with each EV fraction, neither fraction was able to show infection in recipient cells. Only fraction 18 resulted in a band for the Gag p19 HTLV-1, although we cannot formally discard the possibility that the p19 was carried over from NTs used for concentrating the fractions prior to their addition to cells (Figure 4). The use of all EVs types, as opposed to those separated by Iodixanol gradients, on recipient cells also resulted in no infection, validating the lack of infectivity of HTLV-1 EVs (Figure 5). It is important to note that the amount of viral protein or RNA in recipient cells was 1 to 3 logs less than starting NT concentrated material, suggesting that recipient cells might be processing the cargo of the delivered EVs.

Even though HTLV-1 EVs were not infectious, they promoted agglutinated phenotypes through cell-to-cell contact by direct interaction with uninfected recipient cell membranes (**Figure 6**). Here, we observed that HTLV-1 viral spread was most efficient when the recipient cells were primed by HTLV-1 EVs (±IR) followed by the addition of infected donor cells (**Figure 7**). This was true for all recipient cells as HTLV-1 RNA levels on recipient cells were consistently higher after HTLV-1 EV treatment. Interestingly, CEM recipient cells showed significant clustering of cells indicative of cell-to-cell contact mediated by HTLV-1 EVs (±IR). In this scenario, where the uninfected cells are agglutinated and an infected cell is in close proximity, the

chances of viral spread may increase via known mechanisms of viral transmission, such as virological synapse, viral biofilm, cellular conduits, and/or tunneling nanotubes.

We also have performed similar experiments in monocyte U937 cells (Supplementary Figure S2). Microscopic analysis revealed higher numbers of cell aggregates post-, pre-treatment with HTLV-1/IR EVs (upper panel), followed by HTLV-1 EVs (middle panel), and no noticeable clustering with Control EVs (lower panel). To determine if these phenotypes correlated with changes in HTLV-1 RNA levels, RT-qPCR was performed. Hbz RNA levels were increased by 2-fold HTLV-1 EVs and 4-fold with HTLV-1/IR EVs in monocytes. However, tax RNA levels did not increase with either treatment, indicating that there might be an inherent difference between T-cell and monocyte infection of HTLV-1. Along, these lines, it has previously been shown that monocytes do not support in vitro HTLV-1 infection and can undergo SAMHD1-dependent apoptosis through abortive cDNA synthesis (Sze et al., 2013). Although our EV results on monocyte show agglutination, future experiments will determine whether there is low levels of full cDNA synthesis, resulting in low copy number integrations and regulation with the 3'LTR resulting in hbz RNA transcripts.

Our data suggest that CD45 (or its binding partner CD43) in HTLV-1 EVs is a potential mediator between HTLV-1 donor cells and recipient T-cells (Figure 8), allowing the assumption that HTLV-1 EVs promote viral transmission via viral biofilm formation or use of ICAM-1 through virological synapse. Altogether, these data suggest that HTLV-1 EVs may amplify viral spread via upregulated cell-to-cell contact between uninfected cells.

We then sought to further validate the in vitro increase in potential viral spread, through an increase in cell-to-cell contact, in an in vivo humanized mouse model. The NOG mice treated with HTLV-1 EVs showed an increase in blood RNA levels (3 out of 3) compared to mice that received control EV treatment (Figure 9B). Moreover, HTLV-1 EVs consistently caused an increase in HTLV-1 DNA copies in all tissues from animals treated with HTLV-1 EVs (Figures 9C,D). Most interestingly, HTLV-1 DNA levels in anatomically privileged tissues, such as the brain, were drastically elevated in animals treated with HTLV-1 EVs versus below background levels in mice that received only Control EVs. These data suggest that HTLV-1 EVs not only promote viral spread via clustering of uninfected cells, but they potentially allow for viral spread across anatomical barriers, pointing toward evidence of the potential importance of EVs in CNS related HTLV-1 pathogenesis (i.e., HAM/TSP), as well as in the establishment of latent viral reservoirs.

Finally, to better explain the observed EV-mediated phenotype in HTLV-1 infection, we propose a two-step model for viral spread. Our current model indicates that EVs from HTLV-1-infected cells ( $\pm$ IR) may play an important role in priming uninfected recipient cells for infection (**Figure 10**). Following EV release from HTLV-1-infected donor cells, these EVs may reach nearby uninfected recipient cells (especially in tissues), activate (potentially through NF- $\kappa$ B), and/or attract cells toward infected donor cells. Subsequently, following the migration of the uninfected recipient cells, cell-to-cell contact may occur.

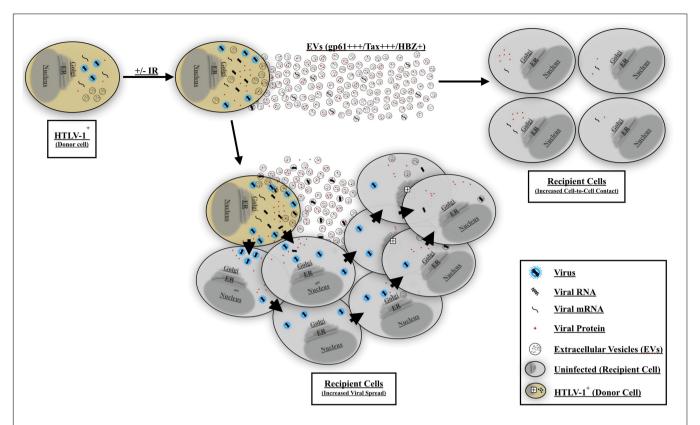


FIGURE 10 | Proposed model for EV-mediated HTLV-1 viral spread and pathogenesis. HTLV-1 EVs carrying viral cargo are released and prime uninfected recipient cells. The recipient cells become activated and/or migrate toward the HTLV-1-infected donor cell thereby promoting increased cell-to-cell contact. HTLV-1 donor cells may then transmit the virus to adjacent recipient cells and facilitate viral spread. Viral spread may occur via viral biofilm, virological synapse, cellular conduits, or TNT formation

Neighboring cells may form a viral biofilm and/or virological synapse by which virus may pass to the adjacent recipient cells (Igakura et al., 2003; Majorovits et al., 2008; Nejmeddine et al., 2009; Gross and Thoma-Kress, 2016; Omsland et al., 2018). Other potential mechanisms of viral spread may also include formation of cellular conduit, and/or tunneling nanotube (TNT) (Pais-Correia et al., 2010; Van Prooyen et al., 2010a,b; Pise-Masison et al., 2014; Omsland et al., 2018) and other viral and cellular proteins (Van Prooyen et al., 2010a; Chevalier et al., 2014; Gross et al., 2016; Omsland et al., 2018). Ongoing and future experiments should aim to further elucidate the mechanisms of EV mediated viral spread and HTLV-1 pathogenesis, specifically studies should focus on the type of EV involved and the associated set of proteins that mediate cell-to-cell contact.

#### **MATERIALS AND METHODS**

#### Cells

Human T-cell leukemia virus-1-infected cell lines HUT102, MT-2, and MT-4, uninfected T-cell (CEM and Jurkat), and promonocytic (U937) cell lines were cultured in RPMI-1640 media (Quality Biological) supplemented with 10% heatinactivated exosome-free fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin

for 5 days (at 37°C and 5% CO<sub>2</sub>) before harvesting for downstream experiments. For IR experiments, all cells were plated at a density of 1  $\times$  10<sup>6</sup> cells/mL, while co-culture experiments were plated at a density of 5  $\times$  10<sup>6</sup> cells/mL at day 0. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from healthy, anonymous donors using Ficoll gradient centrifugation and then expanded in medium containing 1  $\mu$ g/mL PHA-L and 30 or 50 IU/mL rhIL-2. After 2 days of cultivation the cells were washed and then cultured in the medium 30 IU/mL rhIL-2 without PHA-L.

#### X-Ray Irradiation

All ionizing radiation (IR) treatments were performed in a RS-2000 X-Ray Irradiator (Rad Source, Suwanee, GA, United States) for a total radiation dose of 10 Gy per *in vitro* experiment. Samples were irradiated at day 0 and used for NOG mouse studies or allowed to incubate for 5 days prior to harvesting for downstream assays.

# EV Enrichment Using Nanotrap Particles (NTs)

Nanotrap particles (Ceres Nanosciences, Inc.) were used to enrich EVs from low volume, cell-free supernatant samples (1 mL) as previously described (Narayanan et al., 2013; Ahsan et al., 2016;

Pleet et al., 2016, 2018b; Sampey et al., 2016; Barclay et al., 2017b; DeMarino et al., 2018). Briefly, a 30% slurry of NT082 (Ceres #CN2010), NT080 (Ceres #CN1030), and  $1\times$  Phosphate Buffered Saline (PBS) were combined, and 30  $\mu L$  was added to 1 mL of each sample supernatant. The samples were then rotated overnight at  $4^{\circ}C$  to capture EVs. The resulting pellet was washed once with  $1\times$  PBS and used for downstream assays.

#### **Western Blot Analysis**

Cell were harvested, pelleted, washed once in 1× PBS and resuspended in lysis buffer [50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, 0.5% Non-idet P-40, 50 mM NaF, 0.2 mM Na3VO4, 1 mM DTT, and 1 complete protease inhibitor mixture tablet/50 mL (Roche Applied Science)]. Lysate suspensions were incubated on ice for 20–30 min and vortexed at intervals of 5 min, followed by centrifugation at 10,000 × g at 4°C for 10 min to remove cell debris. Protein concentrations from lysates were quantified using Bradford protein assay (BIO-RAD).

For Western blot analysis, cell lysates (10-30 µg) were resuspended in 10 µL Laemmli buffer, heated at 95°C for 3 min, and loaded onto a 4-20% Tris-glycine SDS gel. Nanotrap particle pellets were resuspended in 10 µL Laemmli buffer, heated at 95°C for 3 min and vortexed repeatedly until fully resuspended (3 to 4 cycles). Material eluted from the Nanotraps were then loaded onto a 4-20% Tris-glycine gel. Transfer of proteins was done overnight at 50 mA onto PVDF membranes (Millipore). Membranes were blocked in 5% skim milk in 1× PBS containing 0.1% Tween 20 for 30 min at 4°C, then incubated overnight at 4°C with primary antibody at manufacturer recommended dilutions. Antibodies for protein targets include α-Tax (Tax antibodies 169, 170, and 171, monoclonal mouse; generous gift of Dr. Scott Gitlin, University of Michigan) (Jaworski et al., 2014a), α-p19 (Santa Cruz Biotechnology, sc-1665), α-gp61/46 (NIH AIDS Reagent Program Cat. #1578), 4D4-F3 α-HBZ (monoclonal mouse; generous gift from Dr. Roberto S. Accolla), α-CD45 (Santa Cruz Biotechnology, sc-1123), α-ICAM-1 (Santa Cruz Biotechnology, sc-8439), α-p53 (Abcam; ab32389), α-Cytochrome c (Santa Cruz Biotechnology, sc-13560), α-CD63 (Santa Cruz Biotechnology, sc-365604), and α-Actin (Abcam; ab469900). The next day, membranes were washed and incubated with the appropriate HRP-conjugated secondary antibody for 2 h at 4°C and developed using Clarity or Clarity Max Western ECL Substrate (BIO-RAD). Luminescence was imaged on a ChemiDoc Touch Imaging System (BIO-RAD). Raw densitometry counts were obtained using ImageJ software and by subtracting the background of each membrane. Data was normalized to Actin inputs for each protein of interest.

# ZetaView Nanoparticle Tracking Analysis (NTA)

Nanotracking analysis for EV quantification and sizing was performed using ZetaView® Z-NTA (Particle Metrix) with its corresponding software (ZetaView 8.04.02). Calibration was performed using 100 nm polystyrene nanostandard particles (Applied Microspheres) prior to sample readings at a sensitivity

of 65 and a minimum brightness of 20. Instrument parameters were set as previously described (DeMarino et al., 2018; Pleet et al., 2018b) for each reading. For each sample, 1 mL of sample diluted in DI water was loaded into the cell, followed by measurements of size and concentration of particles at 11 unique positions throughout the cell in three independent reads. After automated analysis and removal of any outliers, the mode diameter size and the concentration of the sample were calculated by the machine software. Measurement data from the ZetaView were analyzed using the corresponding manufacturer software, and raw data were plotted using Microsoft Excel 2016. The mode size of particles detected is presented in our data.

#### **EV Tracking and Fluorescent Microscopy**

Between 50 and 80  $\mu Ls$  ultracentrifuged (at  $100,\!000 \times g)$  HUT102 cell supernatants (from 5-day old culture) at approximately  $1 \times 10^9$  EVs/mL were mixed with  $1.5~\mu L$  of BODIPY  $^{TM}$  493/503 (Cat. # D3922; Invitrogen  $^{TM}$ ) to label EVs for 30 min at 37°C, and run on a Pharmacia G-50 spin column (1 mL bed volume in PBS buffer; 2000 rpm/2 min; Sorval RT6000D) to remove unbound dye. Resulting BODIPY EVs were quantified again by ZetaView and added onto recipient cells normalized to volume in biological triplicate. Treated cells were analyzed with an EVOS-FL-Auto microscope (Life Technologies), under  $20\times$  and  $40\times$  magnification with phase objective and fluorescence.

# RNA Isolation, Generation of cDNA, and Real-Time qPCR

For analysis of HTLV-1 RNA, total RNA was isolated from either cell pellets of Nanotrap pellets. TRIzol Reagent (Invitrogen) was used according to manufacturer's protocol. Total RNA was quantitated by a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and used to generate cDNA with the GoScript Reverse Transcription System (Promega) using Oligo (dT). Next, RTqPCR analysis was performed using 2 μL aliquots of undiluted cDNA, SYBR Green master mix (Bio-Rad), and specific primer sets were used: HTLV-1 env (env-Reverse 5'-CCA TCG TTA GCG CTT CCA GCC CC-3', Tm = 64.4°C; env-Forward 5'-CGG GAT CCT AGC GTG GGA ACA GGT-3',  $Tm = 64.5^{\circ}C$ ), tax (tax-Reverse 5'- AAC ACG TAG ACT GGG TAT CC-3', Tm = 53.6°C; tax-Forward 5'- ATC CCG TGG AGA CTC CTC AA-3', Tm =  $57.6^{\circ}$ C), and hbz (hbz-Reverse 5'-TGA CAC AGG CAA GCA TCG-3', Tm = 55.7°C; hbz-Forward 5'-AGA ACG CGA CTC AAC CGG-3', Tm =  $57.8^{\circ}$ C). Serial dilutions of DNA from HUT102 cells were used as quantitative standards. PCR conditions were as follows: for env primers 50°C for 2 min, 95°C for 2.5 min, then 40 cycles of: 95°C for 15 s, 64°C for 40 s; for tax primers 50°C for 2 min, 95°C for 2 min, Then 40 cycles of: 95°C for 15 s, 51°C for 20 s, and 72°C for 40 s; and hbz: 50°C for 2 min, 95°C for 2 min, Then 40 cycles of: 95°C for 15 s, 65.7°C for 20 s, and 72°C for 40 s. For RTqPCR of gapdh, 2 µL aliquots of undiluted cDNA, using iQ Supermix (Bio-Rad), and specific set of primers sets and probes were used: cellular *gapdh* (*gapdh*-Reverse 5'-GAA GGT GAA GGT CGG AGT CAA C-3', Tm = 57.5°C; *gapdh*-Forward 5'-CAG AGT TAA AAG CAG CCC TGG T-3' Tm = 57.5°C; and *gapdh*-Probe 5'-/56-FAM/TTT GGT CGT ATT GGG CGC CT/36-TAMSP/-3', Tm = 59.8°C). PCR conditions were as follows: for *gapdh* primers and probe 50°C for 2 min, 95°C for 2 min, then 39 cycles of: 95°C for 15 s, 50°C for 30 s. The quantification of samples was determined based on cycle threshold (Ct) values relative to the standard curve for each plate. Reactions were carried out in triplicate using the Bio-Rad CFX96 Real-Time System.

# **ExoMAX EV Enrichment and Density Gradient Separation**

HUT102 cells were seeded at a density of  $1 \times 10^6$  cells/mL in 30 mL of EV-depleted media and subjected to treatment (10 Gy). Following a 5 days incubation, supernatants were harvested using centrifugation ( $10,000 \times g$  for 10 min) and EVs were precipitated by incubation with ExoMAX<sup>TM</sup> reagent (1:1 ratio to sample volume; SBI) at 4°C overnight. Samples were then centrifuged ( $1,500 \times g$  for 30 min) and resuspended in 300  $\mu$ L of  $1 \times$  PBS and loaded onto an Iodixanol gradient (OptiPrep<sup>TM</sup>; Sigma; 11 density fractions ranging from 6 to 18%). Separation of EVs into each fraction was accomplished by ultracentrifugation ( $24,100 \times g$  for 90 min) in a SW41 Ti rotor (Beckman). Each fraction was collected into clean microcentrifuge tubes and EVs were concentrated with an NT080/082/086 particle mixture overnight as described above and used for downstream assays.

#### **Co-culture Treatments**

Recipient cells (CEM, Jurkat, and U937) were incubated with corresponding EVs at a 1:10,000 ratio (5  $\times$   $10^6$  cells: 5  $\times$   $10^{10}$  EVs ratio) for 5 days. At day 5, HTLV-1 donor cells (HUT102) were subjected to IR (10 Gy) and co-cultured with the EV-treated, uninfected recipient cells at a ratio of 1:100 (5  $\times$   $10^4$  HTLV-1 donor cell: 5  $\times$   $10^6$  uninfected recipient cell ratio) and allowed to incubate for an additional 4 days prior to harvesting for downstream assays.

#### **Cell Contact Inhibition Assay**

Antibodies against proteins involved in cell-to-cell contact were used at a final concentration of 0.2  $\mu g/mL$  for  $\alpha\text{-CD45}$  (Santa Cruz Biotechnology, sc-1123), 0.2  $\mu g/mL$  for the second  $\alpha\text{-CD45-2}$  (Santa Cruz Biotechnology, sc-53666), 20  $\mu g/mL$  for  $\alpha\text{-ICAM-1}$  (Santa Cruz Biotechnology, sc-8439), 20  $\mu g/mL$  for  $\alpha\text{-VCAM-1}$  (Santa Cruz Biotechnology, sc-13160), 0.12  $\mu g/mL$  for  $\alpha\text{-Tax}$  (1:100 dilution from a 7.5  $\mu g/mL$  stock of a mix of Tax antibodies 169, 170, and 171, monoclonal mouse), and 1:10 dilution for  $\alpha\text{-gp61/46}$  (NIH AIDS Reagents Program; Cat. #1578).

#### **Cell Viability Assay**

Cells were cultured in technical triplicates (i.e.,  $1\times10^6$  cells/mL or at  $5\times10^5$  cells/mL) in 100  $\mu$ L of fresh EV-depleted

RPMI media in a 96 well assay plate (Corning Inc.; Cat#: 3610) and treated with HTLV-1 EVs as specified for each experiment. Antibodies were used to treat recipient cells to test for recipient cell viability at the concentrations specified above. Cells were allowed to incubate for zero or 5 days and CellTiter-Glo reagent (Promega; Cat#: G7572) was used at a 1:1 ratio to detect cell viability on a GloMax Explorer (Promega). EV-depleted RPMI media alone was cultured and used as background.

#### **Animal Model**

NOD/Shi-scid/IL-2Rgc null (NOG) female pregnant mice were obtained from Jackson labs. NOG pups received human CD34<sup>+</sup> cells (fetal liver embryonic cells; 500,000) 2 days post-initial IR treatment. Humanized animals (8-10 weeks) were treated with total EVs from HUT102 cells, 6 times over 2 weeks (10 µg in 200 µl PBS at intraperitoneal injection each). The animals were then injected with IR treated HTLV-1 donor cells (50 million, intraperitoneally) and kept for 3 weeks. IR treatment stops the replication of infected cells and allows for viral spread into new human cells in the animals. Blood was collected from the animals and white cells were analyzed for the presence of HTLV-1 cell-associated RNA. If positive, then the animals were sacrificed and DNA isolated from tissues, as described previously (Iordanskiy et al., 2015). Briefly, the animals were sacrificed and various tissues were cut with a fresh razor (0.25-0.75 cm<sup>3</sup>) in PBS. The samples were then treated with a mixture of trypsin/EDTA for 30 min/37°C (10× the volume of tissue chunks). They were then spun using Eppendorf (5415 C) for 5 min at 6,000 rpm. The suspended cell mixture, as well as blood samples, were then used to isolate total cellular DNA using Wizard Genomic DNA Purification Kit (Promega) according to manufacturer's protocol. All mouse experiments were approved by the George Mason University Institutional Animal Care and Use Committee (IACUC; 0188).

#### Statistical Analysis

Standard deviation was calculated in all quantitative experiments done in triplicate. All p-values were calculated using 2-tailed Student's t-tests (Microsoft Excel) and were considered statistically significant when p < 0.05.

#### **DATA AVAILABILITY**

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

#### **ETHICS STATEMENT**

This study was carried out in accordance with the recommendations of "IACUC# 0188, The Committee on Animal Research." The protocol was approved by "The Committee on Animal Research" at GMU.

#### **AUTHOR CONTRIBUTIONS**

DP, CD, SAS, MP, and MC performed all the experiments. DP wrote the manuscript. CD, MP, HB, RM, and FK contributed the writing support of the manuscript. DP and CD created the figures. DP, HD, BL, JJ, LL, RM, and FK performed the overall direction of research.

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#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.02147/full#supplementary-material

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# Biomarkers and Preclinical Models for Adult T-Cell Leukemia-Lymphoma Treatment

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Adult T-cell leukemia/lymphoma (ATL) is an aggressive lymphoproliferative malignancy with a very poor prognosis. Despite several recent advances, new therapeutic approaches are critical, and this will require successful preclinical studies, including studies in ATL cell culture systems, and mouse models. Identification of accurate, reproducible biomarkers will be a crucial component of preclinical and clinical studies. This review summarizes the current state-of-the-art in each of these fields, and provides recommendations for future approaches. This problem is an important unmet need in HTLV research.

#### **OPEN ACCESS**

 $\textbf{Keywords: adult T-cell leukemia-lymphoma, preclinical, xenograft, mouse models, tax, \textbf{HBZ}, biomarkers}$ 

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#### INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is a refractory T cell malignancy. The most common clinical variants, acute and lymphoma subtypes, are associated with a median survival of less than 1 year (Katsuya et al., 2015). More indolent variants, smoldering and chronic ATL, are also associated with poor prognoses, with median survivals of 2.6 and 4.6 years, respectively. In the last several years, substantial progress was made in therapy for ATL. These achievements include the following advances. First, established expert guidelines for the diagnosis and treatment of ATL provide evidence-based recommendations for treatment (Cook et al., 2019). Second, aggressive chemotherapy regimens have been shown to be superior to standard CHOP regimens for treatment of the lymphoma subtype (Tsukasaki et al., 2007). Third, zidovudine plus interferon with or without arsenic trioxide has shown strong responses in the acute subtype of ATL (Bazarbachi et al., 2010). Fourth, allogeneic stem cell transplant has demonstrated potential for cure in eligible subjects (Hishizawa et al., 2010). Fifth, mogamulizumab has shown significant activity in multiple ATL subtypes (Ishida et al., 2015). Sixth, lenalidomide has also shown activity in relapsed and refractory subtypes of ATL (Ishida et al., 2016).

Despite, these advances, the prognosis of ATL remains quite poor. Advances in treatment require the establishment of biomarkers for response predictions. Moreover, improved preclinical models are required for identifying potentially active therapies. The focus of this review is to assess the current status of ATL biomarkers and preclinical models for improving ATL therapy.

#### BIOMARKERS FOR ATL

Cancer biomarkers refer to a substance or process that is indicative of the presence of cancer in the body (Goossens et al., 2015). A biomarker may be a molecule secreted by a cancer, or a specific

response of the body to the presence of the tumor. Genetic, epigenetic, proteomic, and imaging biomarkers can be used for cancer diagnosis, prognosis, or treatment predictions. For the purpose of the current review, we are focused on biomarkers that may provide a quantifiable and accurate predictive measure of ATL response or lack of response to therapy. Ideally, the same biomarker can be used in preclinical and clinical studies. If biomarkers can be identified that are highly predictive of changes in tumor load in patients as well as patient survival, they can assist in defining the mechanism of action of a therapy and/or validating that the intended target is inhibited. In addition, the identification of predictive biomarkers can reduce the cost and duration of future preclinical and clinical studies, and improve safety by decreasing the duration of exposure to ineffective and potentially toxic therapies.

Major prognostic indicators for ATL were defined in a study of 854 patients with ATL (Tsukasaki et al., 2009). The parameters identified by multivariate analysis to have prognostic potential were poor performance status, high lactic dehydrogenase (LDH) levels, age greater than 40 years, more than three involved lesions, and hypercalcemia. Using these factors, a risk model was constructed that may have applications for individual patients. Additional factors associated with a poor prognosis include thrombocytopenia, eosinophilia, bone marrow involvement, high interleukin 5 (IL5) levels in the serum, CC-chemokine receptor 4 (CCR4) expression, lung-resistance-related protein, p53 mutation, and p16 deletion (Tsukasaki et al., 2009). Several markers have also been used to define poor risk chronic ATL (Katsuya et al., 2017).

The proviral DNA level (PVL) may provide prognostic and/or predictive information (Iwanaga et al., 2010). Quantitation of proviral levels in peripheral blood mononuclear cells is performed by quantitative PCR methods, by comparison with a cellular gene (Brunetto et al., 2014). The presence of a single provirus per infected cell allows one to use PVL as a measure of the number of infected cells (Cook et al., 2014). Levels of PVL have been used to follow patients after allogeneic hematopoietic stem cell transplants for ATL (Shiratori et al., 2008). However, it must be recognized that PVL measures the number of infected cells rather than number of malignant cells. Most assays have utilized the tax gene for PCR amplification. Moreover, the number of proviruses per peripheral blood mononuclear cell (PBMC) may be higher in acutely infected cells as compared to chronically infected cells. It must also be kept in mind that PBMCs include B lymphocytes, and CD8+ lymphocytes in addition to CD4+ lymphocytes. Although CD4+ lymphocytes are the predominant infected cell in vivo, significant viral loads can be detected in CD8+ lymphocytes, which are rarely transformed by HTLV-1 (Goon et al., 2002). In addition, although the primers for PCR are typically based on highly conserved HTLV-1 sequences, polymorphisms, mutations, or deletions within tax would affect PVL measurements. Lastly use of a cellular gene as a control may be problematic given high levels of aneuploidy associated with ATL, which may affect the copy number of the chosen cellular gene (Kamihira et al., 1994).

Cell-free circulating tumor DNA (cfDNA) has been used to monitor tumor load in a number of types of malignancies

(Corcoran and Chabner, 2018). The precise mechanism of release of cfDNA is unclear. Biological processes suggested to be involved include apoptosis and necrosis from dying cells, or active release from viable tumor cells. The typical size of fragmented cfDNA is 166 base pairs, which corresponds to the length of DNA wrapped around a nucleosome plus a linker (Cole et al., 2016). Various techniques have been proposed for collection, extraction, and analysis of cfDNA (Stewart and Tsui, 2018). A recent abstract presentation described the application of this methodology for monitoring HTLV-1 infection (Haddow et al., 2019). In this study, plasma DNA was isolated as well as genomic DNA from PBMCs from 36 subjects. HTLV-1 DNA was detectable by qPCR in 30 of 36 samples with 0-31 copies of tax/ml plasma (P-cfDNAPVL). A strong correlation was found between P-cfDNAPVL and PBMC-PVL. It should be noted, however, that none of the individuals participating in this study had ATL.

Cell-associated HTLV-1 RNA may also be used to quantify virus gene expression (Yamano et al., 2002). Since helix basic zipper gene (HBZ) is continuously expressed in HTLV-1 infected individuals, it is predicted that levels of hbz mRNA may correlate well with virus load (Mitobe et al., 2015). Since tax is generally not expressed or only transiently expressed in ATL, detection of tax RNA in PBMCs may provide a measure of plus strand expression (Kataoka et al., 2015). Levels of tax RNA in PBMCs may be elevated in acutely infected cells, or in cells manifesting virus re-activation from latency. Another measure of active virus replication is the level of 1- or 2-LTR circles (Sloan and Wainberg, 2011). These are considered by-products of reverse transcription and entry of preintegration complexes in the nucleus. The 1- or 2-LTR circles are thought to form as a result of recombination and/or ligation in the nucleus, but are not likely to be templates for integration (Bukrinsky et al., 1993). In one study of HTLV-1 LTR circles, 1-LTR circles were detected in 14 of 20 patients at a mean of 1.38 copies/100 PBMCs. The 2-LTR circles were detected in 30 of 31 patients (Fox et al., 2016).

HTLV-1 infected cells may increase in an individual through (1) retrovirus replication, virus release, and infection of new target cells, and/or (2) clonal expansion of infected cells carrying an integrated provirus that undergo mitotic cell division (Bangham and Ratner, 2015). Although HTLV-1 integrates at a wide range of different sites in the human genome, with limited preference for specific genetic features, ATL cells within an individual are in 91% of cases, characterized by a predominant single integration site (Cook et al., 2014). In 24% of ATL cases an intermediate abundance clone is present. This was first identified by Southern blot analysis of HTLV-1 provirus integration, and more recently by next-generation sequencing methods of integration sites (Yamaguchi et al., 1984; Gillet et al., 2011). Alternatively, variant allele frequency measurements of specific ATL genetic abnormalities provide an alternative measurement of clonal outgrowth of ATL (Farmanbar et al., 2018). Laydon et al. (2014) and Farmanbar et al. (2019) also reported quantification of HTLV-1 clonality using TCR diversity analysis. However, we have identified subjects in whom two or more TCR gene rearrangements are present within a population of ATL cells with a single proviral integration site (Rauch et al., 2019). Moreover, intratumor heterogeneity has been shown to

be a complex and early event in the development of ATL (Farmanbar et al., 2018).

Cell surface marker analysis may also be used to monitor tumor load. Levels of survivin, a member of the inhibitor of apoptosis (IAP) family was reported to be predictive of clinical response to proteasome inhibitor bortezomib or anti-CD25 antibody, daclizumab (Pise-Masison et al., 2009). More recently, a multicolor flow method has been described as a useful technique to monitor ATL cells in infected patients. For this purpose, Kobayashi et al., showed that CD4 cells expressing cell adhesion molecule-1 (CADM1), but with reduced expression of cell surface co-stimulatory protein, CD7 represent transformed cells. More recently, CD45RA+ T memory stem (T<sub>SCM</sub>) cells were identified as cancer initiating cell capable of reconstituting ATL clones (Nagai et al., 2015).

Several serum markers have also been used as prognostic or predictive markers. The α-chain of the interleukin 2 receptor (IL-2R; CD25) is over-expressed on ATL cells, and sIL-2R have been used to monitor disease, particularly in Japanese patients (Katsuya et al., 2012, 2017). The soluble form of IL-2R is released as a result of proteolytic shedding from activated T cells. Levels of sIL-2R was reported to be a poor prognostic marker in a wide range of B cell lymphomas (Kusano et al., 2017). CD30 is a member of the tumor necrosis factor (TNF) receptor family that is expressed on activated, but not resting T and B cells, and is associated with signal transduction that leads to activation of nuclear factor kappa B (NFkB). Elevated levels of soluble CD30 are also linked with various T-cell neoplasms, including ATL (Nishioka et al., 2005). OX-40 is another member of the TNF receptor superfamily, that co-stimulates activated T cells following interaction with its own ligand. High levels of OX40 have also been reported as a marker of ATL (Tanaka et al., 2019). Lastly, plasma-associated exosomes may also be useful for monitoring HTLV-1 infection (Jeannin et al., 2018).

In summary, numerous potential biomarkers for ATL have been proposed, but systematic comparison and analysis of their utility in monitoring treatment response remains to be performed.

# CELL CULTURE MODELS WITH ATL CELLS

Numerous studies have used HTLV-1 infected cell lines for preclinical evaluation of potential anti-ATL therapies. However, it is unclear whether these cell lines recapitulate the key oncogenic features of ATL. HTLV-1 infected cell lines have been subdivided into Tax-expressing (Tax+) and Tax non-expressing (Tax-) cell lines. It is generally believed that Tax expression is lost in ATL (Kataoka et al., 2015). This has been attributed to escape from immunosurveillance (Hanon et al., 2000). Tax is an important target of CTL responses to ATL. Moreover, Tax expression is associated with expression of other plus strand HTLV genes, which also contribute to ATL immune responses (Bangham and Osame, 2005). Alternatively, Tax has been implicated in the induction of cellular senescence through NFκB hyper-activation (Zhi et al., 2011). Repression

of Tax has been suggested as a means of overcoming this inhibitory activity.

Molecular mechanisms that account for loss of Tax expression in 50% of ATL cases include deletions in the integrated provirus in ATL cells, DNA methylation, and/or chromatin modifications associated with epigenomic shut-off of the HTLV-1 genome (Takeda et al., 2004; Taniguchi et al., 2005). It has been suggested that tumors capable of expressing Tax compared to those unable to express Tax, may represent two biologically distinct subtypes of ATL, perhaps analogous to the different latency programs in EBV-associated malignancies (Mahgoub et al., 2018).

Tax has been suggested to be an ATL initiation factor, but it is unclear whether it is required for ATL maintenance (Baratella et al., 2017). It is possible that HBZ is a tumor maintenance factor. HBZ has weak tumorigenic activity, and is continuously expressed in HTLV-1 infected cells in patients (Mitobe et al., 2015; Esser et al., 2017).

Transient expression of Tax from a minority of cells in culture was shown to occur in certain ATL cell lines and primary T cell cultures from HTLV-1 infected patients (Mahgoub et al., 2018). In the MT1 cell line, Mahagoub et al., showed that 0.05–3.0% of cells with bursts of Tax expression at any time, lasting on average 19 hrs. During Tax expression, antiapoptotic and NFκB-related genes were expressed. Moreover, they showed upregulation of Tax expression by cytotoxic stress, such as oxidative stress. Repression of Tax expression with siRNA resulted in apoptosis of the culture. Billman et al. (2017) showed similar bursts of Tax expression in T cell clones from HTLV-1 infected subjects. Using single molecule FISH, they found that 1-10% of cells expressed Tax. Moreover, cells expressing Tax did not express HBZ. In addition, to cellular stress, they found that hypoxia or glucose metabolism also enhanced Tax expression.

Based on these observations, it is recommended that preclinical studies demonstrate activity in ATL cell lines which are Tax-independent, as well as cell lines that are Tax-dependent with transient bursts of Tax. However, it should be noted that improved characterization of ATL cell lines is required. Do these cell lines fully recapitulate the biological and molecular characteristics of ATL in the patients from which they were derived? What cultures conditions provide the closest representation of ATL? Are these characteristics representative of all ATL disease subtypes? Are there specific characteristics that differentiate ATL cells derived from smoldering or chronic subtypes of ATL from acute or lymphoma subtypes? Can these ATL cell lines recapitulate key features of the disease when inoculated into an animal model?

#### **MOUSE MODELS OF ATL**

Although rats, rabbits, and monkeys have been used in a small number of preclinical models for ATL, the majority of studies have utilized various mouse models (Lairmore et al., 2005). However, HTLV-1 does not infect or replicate in murine cells. Blocks are present at the level of viral entry and Tax expression, and perhaps other steps in virus replication (Trejo and Ratner, 2000). Thus, an ideal animal model for preclinical studies of

HTLV-associated tumors is still lacking. This is particularly important in studies of immune therapies, where distinct features of the microenvironment play a critical role in efficacy.

Genetically engineered mouse (GEM) models provide valuable tools for cancer research (Richmond and Zu, 2008). An advantage of such models is that they allow analysis of many genetic backgrounds using a variety of mouse strains. In addition, the tumor exists in the presence of a competent immune system. These models allow definition of mutations that mimic those identified in human tumors. In addition, they allow one to follow tumor development from early time points. Disadvantages include the fact that a limited number of genes may not reflect the complex heterogeneity of human tumor cells, they are costly and time consuming, and tumor development is often slow and variable.

Several different GEM models have been developed with Tax and/or HBZ, resulting in lymphoproliferative disease (Zimmerman et al., 2011). Ratner et al., expressed Tax with the human granzyme promoter (Grossman et al., 1995). These mice have been engineered with a marker to monitor Tax level non-invasively, which led to observations of dynamic changes in Tax levels over time and induction with various inflammatory stimuli (Rauch et al., 2009a,b). Greene et al., reported on an inducible Tax murine model using EmuSR alpha promoter and a doxycycline-repressible enhancer (Kwon et al., 2005). These mice developed polyclonal lymphocytic inflammation in the skin. Hasegawa et al. (2006) expressed Tax in developing thymocytes using the Lck proximal promoter. After 10 months, the majority of these mice developed hepatomegaly and mesenteric tumors, characterized as diffuse large-cell lymphomas. All of these models demonstrated dependence on NFkB activation (Portis et al., 2001; Kwon et al., 2005; Hasegawa et al., 2006). Injection of the Tax transgenic cells from Hasegawa's transgenic mice into SCID mice resulted in an aggressive leukemia characterized by "flower" cells, as well as extensive lymph node and skin involvement, similar to the transgenic mice. Cancer initiating cells isolated from these tumors reproduced the disease phenotype (Yamazaki et al., 2009). Treatment of transgenic mice with arsenic and interferon, resulted in Tax protein degradation, and depletion of cancer initiating cells (ElHajj et al., 2010). Bellon et al. (2016) used xenografts from these Tax transgenic tumors to study the anti-proliferative activity of Pim1 kinases inhibitors. ElHajj et al. (2014) showed that synthetic retinoids inhibited Tax transgenic tumor cell growth and induced apoptosis in NSG mice. They ascribed this effect to enhanced degradation of Tax. HBZ transgenic mice also develop lymphoid malignancies, but with a longer latency than Tax transgenic animals, and with an incomplete penetrance (Satou et al., 2011; Esser et al., 2017).

Xenografts of human tumor cell lines in mice (CDX models) have provided valuable models for screening and evaluation of candidate anticancer drugs (Richmond and Zu, 2008). Such models can predict the drug response of a tumor in human patients, and are rapid. The major disadvantage is that the mice are immunocompromised, thus, obscuring the effect of the microenvironment. The severe combined immunodeficiency (SCID) mouse was developed in the 1980s, and has been commonly used for xenograft studies of cancer. Although the

non-sense mutation in the protein kinase DNA-activated catalytic polypeptide (Pkrdc) results in loss of B and T lymphocytes in these mice, they retain normal macrophage, dendritic, and natural killer cell functions (Zimmerman et al., 2011). Several different ATL cell lines have been engrafted successfully into SCID mice, including Hut 102, RV-ATL, and SLB-1 cells. Further refinements of the mouse models have incorporated into SCID mice, various defects of innate immune function. These include SCID/beige and NOD/SCID mice, with or without mutations of IL-2Ry. Ishitsuka et al. (2012) used SCID mice inoculated subcutaneously with Hut 102 cells to assess the activity of the pro-apoptosic drug ABT-737. Maeda et al. (2010) used nonobese diabetic (NOD)/SCID mice injected with Hut 102 cells, to assess growth inhibitory activity of anti-CD30 monoclonal antibodies. Ishikawa et al. (2018) used SCID mice inoculated with Hut 102 cells to demonstrate the effects of inhibitors of mTOR and/or PI3K. Tsumuraya et al. (2011) used SCID mice inoculated with Hut 102 cells to demonstrate inhibition of hippuristanol, a polyoxygenated steroid. They ascribed this effect to inhibition of eukaryotic initiation factor eIF4A. Other studies with Hut102 cells in SCID mice have examined NFkB inhibitors curcurmin, dehydroxymethyl epoxyquinomicin (HDMEQ), and synthetic retinoid NIK-333, mevalonate pathway inhibitor incadronate, combined NFkB and AP-1 inhibitors carotenoid fucoxanthinol, modified galectin hG9NC, and retinoic acid receptor inhibitor tamibarotene (Zimmerman et al., 2011). Additional studies have used MT1, MT2, and TL-Om1 cells to study effects of DHMEQ, and MET-1 cells to examine the effects of IL-2R monoclonal antibody, anti-CD52 antibody campath-1H, anti-CD2 antibody MEDI-507, and flavoperidol (Zimmerman et al., 2011). More recently, Zhang et al. (2015) used EC40515(+) cells to study the activity of JAK inhibitors on subcutaneous growth in NOD/SCID, IL2Rynull (NSG) mice.

Patient-derived xenograft (PDX) models provide a powerful tool to investigate tumor biology and preclinical activity of potential therapies (Jung et al., 2018). PDX models have the advantage over CDX models in providing cancer-associated stromal cells with primary tumors which may provide a more realistic heterogeneity of tumor cells (Richmond and Zu, 2008). Their major shortcoming is that they are expensive and technically complicated. Kondo et al. (1993) demonstrated successful xenotransplantation into SCID mice of PBMCs from six of eight ATL subjects. Similar models have been used by other investigators subsequently (Phillips et al., 2000; Kawano et al., 2005). Nakamura et al. (2015) reported on the use of NOD/SCID mice null for Jak3 injected with primary PBMCs from ATL patients. The malignant cells were serially transplanted between animals. They used this model to demonstrate activity of the anti-oxidant, pyrrolidine dithiocarbamate (PDTC). In recent work, Xiang et al. (2019) described the development of ATL in mice injected with purified CD4+ cells from patients with ATL in NSG mice, but not with total PBMCs. The latter finding suggests that non-CD4+ cells antagonize ATL development in this model.

NSG mice have been humanized with hematopoietic stem cells isolated from umbilical cord PBMCs based on their expression of CD34 or CD133 (Villaudy et al., 2011; Ikebe et al., 2013; Tezuka et al., 2014). These mice can then be infected by inoculation

of irradiated HTLV-1 producer cells. After 6–12 weeks, these animals develop an aggressive lymphoproliferative disorder with many of the manifestations of human ATL, including flower cells, visceral infiltration of CD4+ cells, lytic bone lesions, hypercalcemia, and oligoclonal expansion of infected cells (Villaudy et al., 2011; Tezuka et al., 2014; Xiang et al., 2019). These models have the advantage that they can examine effects of drugs on HTLV-1 replication *in vivo*. However, these models have the disadvantage of long duration and variable levels of humanization, incomplete disease penetrance, and variable levels of virus replication.

#### CONCLUSION

Further studies of biomarkers, cell culture and animal models of ATL are very important to develop improve

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therapies for this devastating malignancy. Critical analysis of each assay and model in preclinical vs. clinical studies of new therapeutic approaches is warranted. Strengths and weaknesses of each approach should be analyzed, compared, and validated.

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LR reviewed the literature and wrote the manuscript.

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### Progress in Allogeneic Hematopoietic Cell Transplantation in Adult T-Cell Leukemia-Lymphoma

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Utsunomiya A (2019) Progress in Allogeneic Hematopoietic Cell Transplantation in Adult T-Cell Leukemia-Lymphoma. Front. Microbiol. 10:2235. doi: 10.3389/fmicb.2019.02235 The prognosis of aggressive adult T-cell leukemia-lymphoma (ATL) remains poor because of frequent infections and drug resistance. Dose-intensified chemotherapy followed by autologous stem cell transplantation failed to improve the prognosis of patients with ATL; however, we first revealed that allogeneic hematopoietic cell transplantation (allo-HCT) might improve their prognosis. We showed that reducedintensity stem cell transplantation using peripheral blood was feasible for elderly patients. Further, the prognosis of patients in remission, who receive cord blood transplantation, has been recently improved and is equivalent to that of patients who receive transplants from other stem cell sources. As for the timing of HCT, the patients who underwent transplantation early showed better outcomes than those who underwent transplantation late. Based on the analysis of patients with aggressive ATL, including those who received transplants, we identified five prognostic factors for poor outcomes: acute-type ATL, poor performance status, high soluble interleukin-2 receptor levels, hypercalcemia, and high C-reactive protein level. Next, we developed a new prognostic index: the modified ATL-PI. The overall survival (OS) rates were significantly higher in patients who underwent allo-HCT than those who did not in the intermediate and high-risk groups stratified using the modified ATL-PI. Two new anti-cancer agents, mogamulizumab and lenalidomide, were recently approved for ATL patients in Japan. They are expected to induce longer survival in ATL patients when administered along with transplantation. However, a retrospective analysis that the risk of severe, acute, and corticosteroid-refractory graft-versus-host disease was higher in patients who received mogamulizumab before allo-HCT, and that mogamulizumab might increase the transplant-related mortality (TRM) rates and decrease the OS rates compared to those of patients who did not receive mogamulizumab. However, our recent study showed that administration of mogamulizumab before allo-HCT tended to improve the survival of patients with ATL. In conclusion, allo-HCT procedures for patients with aggressive

ATL have considerably progressed and have helped improve the prognosis of these patients; however, many concerns still remain to be resolved. Further development of allo-HCT by using new molecular targeting agents is required for the improvement of cure rates in patients with ATL.

Keywords: ATL, HTLV-1, allogeneic hematopoietic cell transplantation, graft-versus-ATL effect, HTLV-1 proviral load, molecular targeting agent, mogamulizumab, immunotherapy

#### INTRODUCTION

Adult T-cell leukemia-lymphoma (ATL) is an intractable peripheral T-cell malignancy caused by human T-cell leukemia virus type-I (HTLV-1). The overall survival (OS) rates by conventional chemotherapy are very low because of drug resistance for anti-cancer agents and susceptibility for various kinds of infectious diseases (Ishitsuka and Tamura, 2014; Utsunomiva et al., 2015).

Dose-intensified chemotherapy followed by autologous stem cell transplantation failed to improve the prognosis of patients with ATL because of high ATL relapse rates and frequent infectious complications (Asou et al., 1985; Tsukasaki et al., 1999). Conversely, the successful treatment of a patient with aggressive ATL with allogeneic hematopoietic cell transplantation (allo-HCT) was reported in 1987. However, the patient died from interstitial pneumonitis due to cytomegalovirus (CMV) infection 117 days after transplantation (Sobue et al., 1987). Subsequently, several ATL cases that were successfully treated using allo-HCT were reported (Ljungman et al., 1994; Borg et al., 1996; Obama et al., 1999; Kishi et al., 2001). In 2001, we first reported the possibility of improved outcomes in patients with aggressive ATL by using allo-HCT (Utsunomiya et al., 2001). Although the prognosis of patients with aggressive ATL improved by allo-HCT, transplant-related mortality (TRM) rates in patients who received transplantation with myeloablative conditioning (MAC) regimen were very high (Utsunomiya et al., 2001; Kami et al., 2003; Fukushima et al., 2005). However, the TRM rates of patients who received reduced-intensity stem cell transplantation (RIST) decreased compared to those of patients transplanted using MAC (Tanosaki et al., 2008). Although the prognosis of patients with ATL who underwent cord blood transplantation (CBT) was poorer than that of patients transplanted with other stem cell sources, it has recently improved (Hishizawa et al., 2010; Nakamura et al., 2012; Fukushima et al., 2013; Kato et al., 2014).

The Japan Society for Hematopoietic Cell Transplantation established a hematopoietic cell transplantation registry program named Transplant Registry Unified Management Program (TRUMP; Atsuta et al., 2007). According to this Japanese TRUMP database, approximately 2000 patients with ATL who had undergone allo-HCT were enrolled until 2017 in Japan (The Japanese Data Center for Hematopoietic Cell Transplantation/The Japan Society for Hematopoietic Cell Transplantation, 2017)<sup>1</sup>. Conversely, in countries other Japan, such as Europe, only a small number of

patients with ATL underwent allo-HCT [retrospective study by Bazarbachi et al. (2014)].

At present, allo-HCT is thought to be the only potential curative treatment for patients with aggressive ATL. In this study, I review the progresses in allo-HCT for ATL in Japan.

#### **DEVELOPMENT OF ALLO-HCT FOR ATL**

#### **Stem Cell Sources**

Bone marrow cells, peripheral blood stem cells, and cord blood cells are generally used as stem cell sources for allo-HCT for patients with ATL like in other hematological malignancies. A nationwide survey in Japan revealed that the OS rates of patients with ATL receiving CBT were lower than those of patients receiving bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (Hishizawa et al., 2010; Ishida et al., 2012a). Subsequently, two retrospective studies showed that the prognosis of patients with ATL in remission who underwent CBT had improved and was equivalent to that of patients who were transplanted with other stem cell sources, such as bone marrow cells or peripheral blood stem cells (Table 1; Nakamura et al., 2012; Fukushima et al., 2013). Recently, in Japan, the number of patients with ATL receiving CBT has rapidly increased because available related donors have markedly decreased, and coordination time for allo-HCT in unrelated transplantation is too long, which hinders early transplantation at an appropriate time. The analysis of the Japanese TRUMP database revealed that prognosis of patients with ATL who underwent CBT has gradually improved recently. The TRM rates of patients with ATL in remission who underwent CBT decreased. By decreasing the TRM rates, the OS rates of patients with ATL who underwent CBT improved like those of patients transplanted with other stem cell sources (Table 1). If patients with ATL receiving allo-HCT have suitable related donors, a stem cell source, BM, or PB, would be chosen considering patient age, disease status, and performance status. Early transplantation from alternative donors, such as cord blood or HLA haploidentical donor would be recommended if a patient does not have suitable related donors for allo-HCT. As the Japan Cord Blood Bank Network supplies cord blood units to recipients undergoing CBT, cord blood is the most appropriate stem cell source in Japan.

#### Indication of Allo-HCT for ATL

Before treatment, the clinical subtypes of ATL should be determined after ATL diagnosis (Shimoyama, 1991). The therapeutic strategy for patients with ATL can be decided

<sup>&</sup>lt;sup>1</sup>http://www.jdchct.or.jp/data/report/2017/

TABLE 1 | Cord blood transplantation for adult T-cell leukemia-lymphoma.

References	Pt. No.	Median age (range)	Sex M/F	Subtype	Disease status at CBT	Conditioning regimen	Cause of death	Outcome
Tamura et al., 2006	11	56 (39–61)	Unk	Unk	CR: 2 Non-CR: 9	MAC: 2 RIC: 9	TRM: 5 ATL: 5	Unk
Hishizawa et al., 2010	90	Unk	52/38	Unk	CR: 26 Non-CR: 57 Unk: 7	MAC: 14 RIC: 64 Unk: 12	TRM: 45 ATL: 25 Unk: 4	3Y-OS: 17% (95% CI 9-25)
Nakamura et al., 2012	10	51 (31–64)	6/4	Acute: 9 Lymphoma: 1	CR: 2 PR: 4 SD: 1 PD: 3	MAC: 6 RIC: 4	ATL: 4 Sepsis: 1 GVHD + ATL: 1	2Y-OS: 40% (95% CI 67-12)
Ishida et al., 2013	174	Unk	Unk	Unk	Unk	Unk	Unk	3Y-OS: 21% (95% CI 15-29)
Fukushima et al., 2013	27	52 (41–63)	18/9	Acute: 17 Lymphoma: 10	CR: 5 PR: 11 PIF: 5 REL: 6	MAC: 9 RIC: 18	TRM: 10 ATL: 9	3Y-OS: 27.4%
Kato et al., 2014	175	55 (27–79)	105/70	Unk: 175	CR: 50 Non-CR: 116 Unk: 9	MAC: 63 RIC: 108 Unk: 4	TRM: 77 ATL: 52	2Y-OS: 20.6% (95% CI 14-27)
Choi et al., 2016	15	62 (55–69)	8/7	Acute: 13 Lymphoma: 2	CR: 5 PR: 10	RIC: 15	TRM: 3 ATL: 4	2Y-OS: 53.3%
Tokunaga et al., 2018	150	61 (24–78)	88/62	Acute: 107 Lymphoma: 39 Other: 4	CR: 62 PR: 86 Unk: 2	MAC: 54 RIC: 95 Unk: 1	TRM: 54 ATL 46	1Y-OS: 38.3% (95% CI 30-47)

ATL, adult T-cell leukemia-lymphoma; CBT, cord blood transplantation; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; PIF, primary induction failure; REL, refractory after relapse; OS, overall survival; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; TRM, transplant-related mortality; GVHD, graft-versus-host disease; Unk, unknown.

by further dividing ATL into two categories: aggressive type, including acute, lymphoma and unfavorable chronic type; and indolent type, including favorable chronic and smoldering type (Shimoyama, 1994; Tsukasaki et al., 2009). We proposed the treatment algorithm for patients with ATL based on these clinical subtypes and clinical characteristics (**Figure 1**; Utsunomiya et al., 2015). All young patients with ATL who obtained complete remission (CR), partial remission (PR), and stable disease (SD) by chemotherapy and maintained good performance status (PS) were recommended for allo-HCT. Even in relapsed patients with ATL who underwent chemotherapy, the OS rate in patients who received allo-HCT after relapse was higher than that in patients who did not (Fuji et al., 2018b). This suggests that allo-HCT is one of the promising salvage therapies for even relapsed patients with ATL.

Recently, the Guidelines Committee of the American Society for Blood and Marrow Transplantation proposed clinical practice recommendations in mature T cell and NK-T cell lymphomas, which include indication and timing of HCT for patients with ATL (**Figure 2**; Kharfan-Dabaja et al., 2017). These recommendations of transplantation for patients with ATL are similar to our proposal of indications for allo-HCT (Utsunomiya et al., 2015; Kharfan-Dabaja et al., 2017). Furthermore, the most recent International Consensus Meeting Report strongly recommends up-front allo-HCT for all suitable patients with aggressive ATL (Cook et al., 2019).

Concerning donor eligibility criteria, the use of HTLV-1 carriers as donors in allo-HCT for ATL patients is controversial. Although HTLV-1 carriers are omitted for donors in unrelated transplantation and CBT, they are not excluded for donors in related transplantation. When allo-HCT is planned for patients with ATL by using an HTLV-1 carrier donor, no monoclonal or oligoclonal integration by Southern blot analysis of HTLV-1 proviral DNA in the blood is required for the determination

of donor. Four factors, including high HTLV-1 proviral loads (PVLs) in the blood, higher age, family history of ATL, and HTLV-1 carriers with any symptoms, were reported to be associated with the risk for ATL development in HTLV-1 carriers (Iwanaga et al., 2010). Donor eligibility of HTLV-1 carriers in allo-HCT for patients with ATL should be determined by considering these risk factors. The recent International Consensus Meeting Report recommends the use of HTLV-1 seronegative donors in allo-HCT for patients with ATL in order to reduce the risk of donor cell-derived ATL after transplantation (Tamaki and Matsuoka, 2006; Cook et al., 2019).

#### Preconditioning

Allogeneic hematopoietic cell transplantation for patients with ATL was developed using MAC as for other hematological malignancies. Although the prognosis of patients with aggressive ATL was improved by allo-HCT by using MAC, the TRM rates were very high, ranging from 40 to 56% (Utsunomiya et al., 2001; Kami et al., 2003; Fukushima et al., 2005). Recently, the median age of ATL at diagnosis was reported to be 68 years (Nosaka et al., 2017). Higher age of patients with ATL was probably associated with high TRM rates in patients undergoing allo-HCT by using MAC. In order to reduce the high TRM rates, we conducted prospective clinical trials of non-myeloablative stem cell transplantation (Slavin et al., 1998) for elderly patients with aggressive ATL (≥50 years) by using granulocyte colonystimulating factor-mobilized peripheral blood stem cells from HLA-matched sibling donors. We succeeded in reducing the TRM rates in about 15% of elderly patients with aggressive ATL who received RIST (Okamura et al., 2005; Tanosaki et al., 2008). Further, we showed that RIST performed using unrelated bone marrow donors was feasible and highly effective for elderly patients with aggressive ATL (Choi et al., 2014). We also showed that cumulative incidence of TRM was 6.7% at 100 days

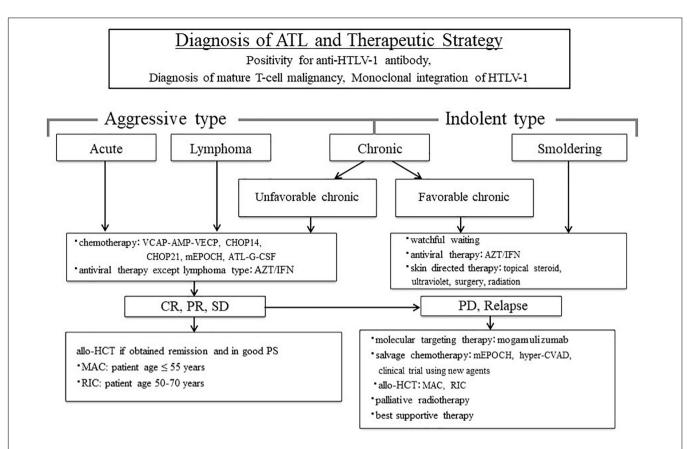


FIGURE 1 | Treatment algorithm for adult T-cell leukemia-lymphoma (ATL) patients. The figure is reproduced modified from Figure 1 in Utsunomiya et al. (2015). ATL diagnosis is based on anti-HTLV-1 antibody positivity in the serum, the presence of mature T-cell malignancy, and the Southern blot detection of monoclonal integration of HTLV-1 proviral DNA in the tumor cells. ATL treatment is usually determined according to the clinical subtypes and prognostic factors. The presence of an aggressive-type ATL (acute, lymphoma, and unfavorable chronic types) or indolent-type ATL (favorable chronic and smoldering types) is critical to make treatment decisions. Patients with an aggressive-type generally receive immediate combination chemotherapy or antiviral therapy with zidovudine and interferon-α (AZT/IFN), except for those with lymphoma-type ATL. The international consensus meeting primarily recommends the VCAP-AMP-VECP regimen. Other therapeutic regimens include CHOP14, CHOP21, mEPOCH, and ATL-G-CSF. Patients undergo further treatment with allogeneic hematopoietic cell transplantation, which is particularly effective in young patients with good performance status, and those who have achieved remission before transplantation. In Japan, patients with an indolent-type ATL without any skin lesions are usually followed-up under a watchful waiting policy until the disease transforms to an aggressive type. Antiviral therapy is frequently used for patients with favorable chronic and smoldering type of ATL in non-Japanese nations, and skin-directed therapy is applied for smoldering ATL with skin manifestations. allo-HCT, allogeneic hematopoietic cell transplantation; ATL-G-CSF, combination chemotherapy consisting of vincristine, vindesine, doxorubicin, mitoxantrone, cyclophosphamide, etoposide, ranimustine, and prednisone with granulocyte-colony stimulating factor support; AZT/IFN, zidovudine and interferon-α; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP14 is performed every 2 weeks, and CHOP21 is performed every 3 weeks); CR, complete remission; hyper-CVAD, cyclophosphamide, vincristine, doxorubicin, and dexamethasone; MAC, myeloablative conditioning; mEPOCH, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin (EPOCH) with modifications; PD, progressive disease; PR, partial remission; PS, performance status; RIC, reduced-intensity conditioning; SD, stable disease; VCAP-AMP-VECP, vincristine, cyclophosphamide, doxorubicin and prednisone (VCAP)-doxorubicin, ranimustine and prednisone (AMP)-vindesine, etoposide, carboplatin, and prednisone (VECP). Reprinted with permission of Cancer Science.

after CBT performed using reduced-intensity conditioning (RIC; Bacigalupo et al., 2009; Giralt et al., 2009; Choi et al., 2016). In the retrospective analysis of the Japanese TRUMP database, the OS rates were not different between patients who were transplanted using MAC, and those who were transplanted using RIC (Ishida et al., 2012a). However, prospective clinical trials considering patient's age, disease status, and PS at the time of transplantation are needed for the clarification of this issue.

#### **Outcomes of Allo-HCT**

Almost all outcomes of allo-HCT for patients with ATL have been reported by Japanese researchers except one from

the European Society for Blood and Marrow Transplantation (EBMT)-Lymphoma Working Party, in which the outcomes of patients receiving transplantation were almost the same as those reported in Japanese patients (Utsunomiya et al., 2001; Hishizawa et al., 2010; Ishida et al., 2012a; Kanda et al., 2012; Bazarbachi et al., 2014; Activities and Outcomes of Hematopoietic Cell Transplantation in Japan, 2017<sup>2</sup>). To our knowledge, in the first report of Japanese nationwide retrospective analysis, we revealed that the 3-year OS rates in patients with ATL receiving allo-HCT from HLA-matched

<sup>&</sup>lt;sup>2</sup>http://www.jdchct.or.jp/en/data/slide/2017/

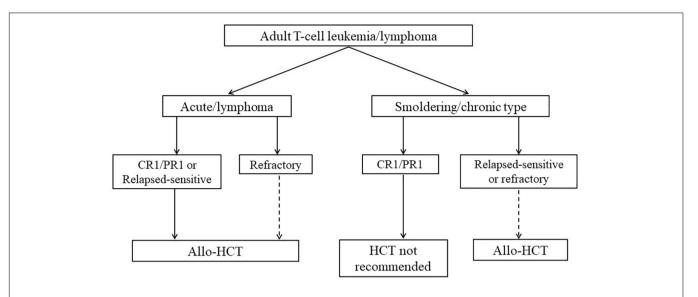


FIGURE 2 | Algorithm summarizing indications for HCT in ATL. The figure is reproduced from Figure 5 in Kharfan-Dabaja et al. (2017). The dashed line denotes a weak recommendation. CR, complete remission; PR, partial remission; allo-HCT, allogeneic hematopoietic cell transplantation. Reprinted with permission of Biology of Blood and Marrow Transplantation.

related donors, HLA-mismatched related donors, unrelated donor bone marrow, and unrelated donor cord blood were 41, 24, 39, and 17%, respectively (Hishizawa et al., 2010). Although the prognosis of patients with ATL who underwent CBT was poor previously, the OS rates of patients receiving CBT has gradually improved recently by the decrease of TRM, especially in patients who underwent transplantation in remission (Table 1).

The timing of allo-HCT is very important because obtaining CR is not easy in patients with ATL, and relapse or regrowth of ATL occurs suddenly. The patients who underwent transplantation early (within the first 100 days of diagnosis) showed better outcomes than those who underwent transplantation late (after 100 days of diagnosis). Notably, the survival of patients in non-CR who received early transplantation was significantly better than those who received late transplantation (Fuji et al., 2016a). The rates of ATL-related death were not different in both the groups, but the TRM rates were significantly higher in late transplant patients than in early transplant ones. These results suggest that early transplant is required for better outcomes for patients with ATL in good PS because ATL cells are resistant to anti-cancer agents (Kuwazuru et al., 1990; Ohno et al., 2001a,b); hence, persistent chemotherapy deteriorates patients' conditions without obtaining CR. When patents with ATL who plan allo-HCT achieve CR or PR, immediate allo-HCT would be recommended after stopping residual courses of chemotherapy.

Recent Japanese TRUMP data show that the 5-year and 10-year OS rates of patients with ATL who underwent allo-HCT with various kinds of stem cell sources, including bone marrow, peripheral blood stem cell, and cord blood, and conditioning regimens, including MAC and RIC, were 29.6 and 25.0%, respectively (Activities and Outcomes of Hematopoietic Cell

Transplantation in Japan, 2017; see text footnote 2). Although the prognosis of ATL patients who underwent allo-HCT is improving, their OS rates are lower than those of patients with other hematological malignancies (**Figure 3**; Activities and Outcomes of Hematopoietic Cell Transplantation in Japan, 2017; see text footnote 2).

### Prognostic Factors in ATL Patients Who Underwent Allo-HCT

Prognostic factors for poor outcomes such as advanced stage, poor PS, and three continuous variables [age, serum albumin level, and soluble interleukin-2 receptor (sIL-2R) levels] in patients with aggressive ATL were identified using nationwide retrospective analysis, and then the prognostic index (PI) for aggressive ATL (aggressive ATL-PI) was proposed by Katsuya et al. (2012). By using this aggressive ATL-PI, they divided patients into three risk groups (low, intermediate, and high risk; Katsuya et al., 2012). Although aggressive ATL-PI is very useful for the prediction of the prognosis of patients with aggressive ATL, this index was developed by analyzing only patients with aggressive ATL who did not receive allo-HCT. In another nationwide retrospective study that included patients with aggressive ATL aged 70 years or younger who underwent allo-HCT, we identified five prognostic factors for poor outcomes: acute-type ATL, poor PS, high sIL-2R levels (>5,000 U/mL), hypercalcemia (adjusted calcium level ≥12 mg/dL), and high C-reactive protein level (≥2.5 mg/dL). We then developed a new PI: the modified ATL-PI (Fuji et al., 2017). The prognoses of patients were clearly separated according to the three risk groups (low, intermediate, and high-risk). In particular, the OS rates of patients who underwent allo-HCT were significantly higher than those of patients who did not undergo allo-HCT in the intermediate

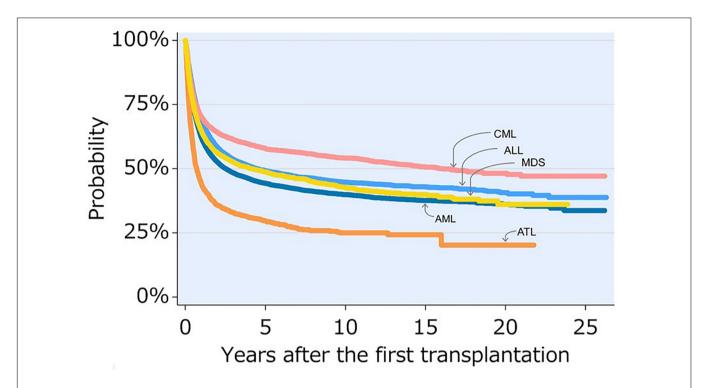


FIGURE 3 | Overall survival after transplant for leukemia 1991–2016. This figure is reproduced from JDCHCT home-page (http://www.jdchct.or.jp/en/data/slide/2017/). Activities and Outcomes of Hematopoietic Cell Transplantation in Japan (2017) provided by the Japanese Data Center for Hematopoietic Cell Transplantation (JDCHCT). CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; ATL, adult T-cell leukemia-lymphoma. Reprinted with permission of the Japanese Data Center for Hematopoietic Cell Transplantation.

and high-risk groups stratified using the modified ATL-PI (Fuji et al., 2017).

Older age, male sex, non-CR, poor PS, and unrelated HCT were reported to be prognostic factors for poor outcomes in patients with ATL who underwent allo-HCT (Hishizawa et al., 2010). Subsequently, high sIL-2R level at the time of transplantation was also reported to be a poor prognostic factor for disease progression and OS after transplantation (Shigematsu et al., 2014). They also revealed that high sIL-2R level at diagnosis was a poor prognostic factor for OS after transplantation (Shigematsu et al., 2014). We also showed that high sIL-2R was a poor prognostic factor in patients with ATL who underwent allo-HCT by performing a retrospective analysis (Tokunaga et al., 2017). Further, we showed that hematopoietic cell transplantation-specific comorbidity index (HCT-CI; Sorror et al., 2005) and EBMT score (Gratwohl et al., 2009) at transplant were reliable prognostic factors in patients with ATL who underwent allo-HCT (Tokunaga et al., 2017). However, the EBMT score was not associated with the risk of non-relapse mortality (NRM) because about 95% of patients with ATL who underwent allo-HCT were over than 40 years of age in the Japanese TRUMP database (Yoshimitsu et al., 2018a). We attempted to develop another new ATL HCT prognostic index (ATL-HCT-PI). We determined three independent risk factors, including optimized HCT-CI, donor-recipient sex combination (female donor-male recipient), and older age (>64 years; Yoshimitsu et al., 2018a). By using these three risk factors, we

divided patients into 3 risk groups, namely low, intermediate, and high risk, and then developed a new ATL-HCT-PI, which could be used to stratify NRM and OS rates in patients with ATL according to the three risk groups (2-year NRM of 22.0, 27.7, and 44.4%, and 2-year OS of 47.3, 39.1, and 15.7% for ATL-HCT-PI low-, intermediate-, and high-risk patients, respectively; Yoshimitsu et al., 2018a).

As for cytogenetic analysis and clinical significance in patients with ATL, chromosomal abnormalities in patients with ATL were associated with lower OS rates (Itoyama et al., 2001). The OS rates in patients who had multiple chromosomal breaks (at least 6) were significantly poorer than those of patients who had less than 6 chromosomal breaks (P < 0.01). The OS rates in patients with abnormal break points in 1p, 1q, 3q, and 17q were significantly poorer than those in patients without these break points (Itoyama et al., 2001). By using the Japanese TRUMP database, we retrospectively analyzed whether chromosomal abnormalities influence the prognosis of patients with ATL, irrespective of whether they underwent allo-HCT, or not. Structural abnormalities such as 2q and 5q were associated with poor prognosis in patients with ATL who underwent allo-HCT (Nakano et al., 2018).

Recently, genetic alterations in ATL cells have been reported in detail (Kataoka et al., 2015). Several genetic alterations are reported to be associated with the prognosis of patients with ATL (Kataoka et al., 2018). In particular, the amplification of PD-L1 and deletion of CDKN2A were detected more frequently

in patients with aggressive ATL than in those with indolent ATL, and PKRCB mutation and PD-L1 amplification were poor prognostic factors in patients with aggressive ATL (Kataoka et al., 2018). Unfortunately, the association between genetic alterations and prognosis of patients with ATL who underwent allo-HCT has not been reported. In the future, elucidating whether allo-HCT can overcome these gene alterations associated with poor prognosis in patients with aggressive ATL, or whether patients with these gene alterations can receive allo-HCT is necessary.

### IMMUNE REACTION AFTER TRANSPLANTATION IN ATL

#### **Graft-Versus-ATL Effect**

Graft-versus-leukemia (GVL) reactions were reported to be observed in patients who received allogeneic BMT. GVL reactions are frequently observed in patients complicated with graft-versus-host disease (GVHD); therefore, they are thought to be one of the effects of GVHD. The relapse rates of transplant in identical twins were reported to be higher than those in related siblings without GVHD; hence, GVL reactions were independent phenomena from GVHD (Horowitz et al., 1990). Subsequently, GVHD was reported to induce GVL effect, and the impact on relapse and disease-free survival after transplantation was strong in patients who were transplanted using RIC regimen (Weisdorf et al., 2012).

Immune reactions for tumor cells were occasionally observed in patients with ATL in whom spontaneous regression of ATL occurred (Shimamoto et al., 1993; Matsushita et al., 1999; Takezako et al., 2000). Maintaining remission or another CR after relapse in patients who underwent allo-HCT was thought to be associated with graft-versus-ATL (GV-ATL) effect (Obama et al., 1999; Utsunomiya et al., 2001). We retrospectively analyzed 10 patients with ATL who relapsed after allo-HCT and survived for more than 100 days at our institution. Nine ATL patients relapsed in the skin and other organs, and 5 relapsed only in the skin. Immunosuppressants (ISs) were stopped immediately after the diagnosis of relapse, and then another CR was obtained in 6 patients without chemotherapy and/or donor lymphocyte infusion (DLI). Surprisingly, 4 out of the five patients who had relapsed only in the skin obtained CR only by the cessation of ISs. DLI was applied in two patients who did not show GVHD after the cessation of ISs, and then one patient obtained CR, but died of severe acute GVHD at 5.3 months after transplant. These effects were considered to suggest GV-ATL effects in relapsed patients with ATL after allo-HCT (Yonekura et al., 2008). Itonaga et al. (2013) similarly analyzed the outcomes of 35 patients with relapsed ATL after allo-HCT. They reported that only cytoreductive chemotherapy failed to obtain remission, whereas cytoreductive therapy followed by donor lymphocyte infusion was effective for relapsed ATL in 4 out of 6 patients (Itonaga et al., 2013). This indicates that the reduction of tumor burden requires obtaining GV-ATL effect in patients with ATL who relapsed after allo-HCT.

In patients with ATL who underwent allo-HCT, the prognoses of patients associated with mild acute GVHD (grade I/II) were superior than those of patients without acute GVHD or associated with severe acute GVHD (grade III/IV; Okamura et al., 2005; Kanda et al., 2012; Ishida et al., 2013). As for CBT in patients with ATL, the prognosis of patients associated with mild acute GVHD was slightly better than that of patients without acute GVHD or with severe acute GVHD (Kato et al., 2014). The prognosis of patients with limited chronic GVHD was slightly better than that of patients without chronic GVHD or with extensive chronic GVHD (Kato et al., 2014). These phenomena strongly suggested the existence of GV-ATL effect after allo-HCT in patients with ATL.

# Tax-Specific Cytotoxic T Lymphocyte Response

We reported that another CR was frequently observed in patients with ATL who relapsed after allo-HCT only by the cessation of ISs (Yonekura et al., 2008). One representative case has been shown in **Figures 4**, **5**. A 62-year-old male patient with acute ATL was transplanted from an HLA-matched sibling with anti-HTLV-1 antibody in his serum. He relapsed in the skin, peripheral blood (PB), and lymph nodes 28 days after RIST. IS therapy was stopped immediately after the diagnosis of relapse by skin biopsy. Another CR was observed accompanied by the disappearance of skin lesions, peripheral ATL cells, and superficial lymph nodes. Subsequently, different skin lesions and generalized skin rash appeared in the patient 14 days after the cessation of ISs, and the patient was diagnosed as having Grade II acute GVHD by skin biopsy (Figures 4, 5). Acute skin GVHD was resolved by the administration of steroid hormones and cyclosporine A. Subsequently, CMV infection was detected using the CMV pp65 antigenemia test, and the patient recovered from CMV infection by intravenous ganciclovir administration. The patient was maintained in CR for more than 10 years.

Tax-specific cytotoxic T-lymphocyte (CTL) responses were recognized in the recipient (case mentioned above) PB at the time of another CR after the cessation of ISs; the Tax-specific CTLs were derived from donor lymphocytes (Harashima et al., 2004). Conversely, Tax-specific CTL response was hardly detectable in donor PB (Harashima et al., 2004). In this patient, the Tax-specific CTL responses were maintained for more than 10 years.

Immunotherapy targeting HTLV-1 Tax was considered for long disease control in patients with ATL at least in whom ATL cells expressed Tax, because partial contribution of Tax-specific CTL responses has been suggested to the CR after cessation of ISs in relapsed patients with ATL after RIST (Harashima et al., 2004). Following many basic researches to augment Tax-specific response, Suehiro et al. (2015) conducted a new immunotherapy by using the Tax peptide-pulsed dendritic cell vaccine (Tax-DC vaccine) for patients with aggressive ATL who were controlled in stable condition for at least 1 month by chemotherapy. Three patients were enrolled in that pilot study; by using this immunotherapy, they obtained good clinical responses, which included PR in 2 patients and SD in 1. Responses were maintained for more than 1 year without any additional chemotherapy in the

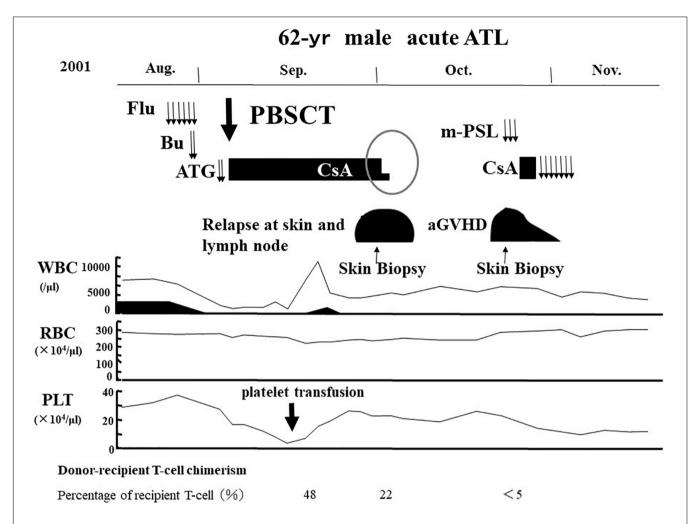


FIGURE 4 | Representative case of graft-versus-ATL effect. ATL, adult T-cell leukemia-lymphoma; Flu, fludarabine; Bu, busulfan; ATG, anti-thymocyte globulin; PBSCT, peripheral blood stem cell transplantation; CsA, cyclosporine A; m-PSL, methylprednisolone; aGVHD, acute graft-versus-host disease; WBC, white blood cell; RBC, red blood cell; PLT, platelet.

former two patients (Suehiro et al., 2015). At present, phase Ia/Ib study of Tax-DC vaccine has been completed; we intend to start phase II study to investigate the efficacy of this vaccine therapy for patients with ATL.

Interestingly, we also recognized HBZ-specific CD4 T cell responses in patients with ATL after allo-HCT, but not in patients with ATL who did not receive transplant (Narita et al., 2014). In some patients with ATL who underwent allo-HCT, HBZ-specific CD4 T cell responses might have been associated with longer remission after transplant.

# HTLV-1-Infected Cells in the PB After Transplant

Four kinds of HTLV-1 infected cells were found in the PB of patients with ATL who received allo-HCT, i.e., two recipient HTLV-1-infected cells, including residual ATL cells and non-ATL recipient cells, and two donor HTLV-1-infected cells, including cells from HTLV-1 carrier donors and cells infected to donor cells from recipient cells after transplant (Yamasaki et al., 2007;

Utsunomiya, 2017). Therefore, HTLV-1-infected cells after allo-HCT do not represent only residual ATL cells but also other HTLV-1-infected cells both from recipient and donor origin. Yamasaki et al. (2007) reported that small number of HTLV-1-infected cells in patients with ATL who underwent allo-HCT were heterogenous and were frequently derived from donors. Considering this fact, we need to evaluate not only disease status but also donor-recipient chimerism; Clonality analysis of HTLV-1 proviral DNA of PB mononuclear cells should be conducted to differentiate ATL relapse from donor-cell derived ATL, when HTLV-1-infected cells increased in the PB after allo-HCT in patients with ATL.

#### Kinetics of HTLV-1 Proviral Loads After Transplant

We reported the kinetics of HTLV-1 PVLs in the PB of patients with ATL who received RIST (Choi et al., 2011). In this study, half of donors were HTLV-1 carriers. In patients who were transplanted from HTLV-1 carrier donors, HTLV-1 PVLs in

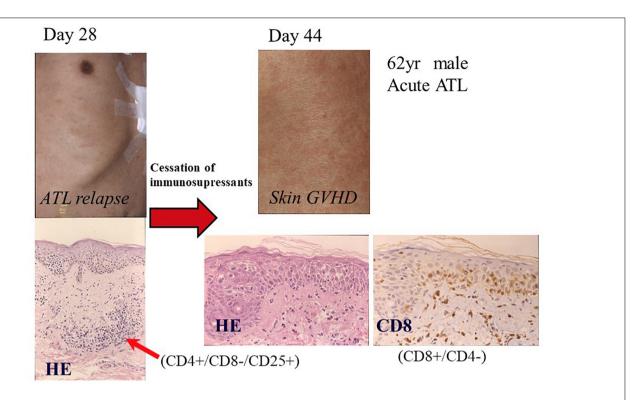


FIGURE 5 | Graft-versus-ATL effect after the cessation of immunosuppressants. Pathological images are reproduced from Figures 3, 4 of Yonekura et al. (2008). Day 28: Skin lesions appeared on day 28 after transplantation (left upper part), which was diagnosed as ATL relapse by skin biopsy. Pathological findings showed ATL cell infiltration in the dermis formed Pautrier's microabscess and dense dermal infiltration by lymphoid cells with nuclear atypia (left lower portion; HE, hematoxylin-eosin stain; original magnification, ×100). The infiltrates were positive for CD4 and CD25, and negative for CD8. Day 44: Generalized erythema reappeared after disappearance of nodules in the skin after the cessation of immunosuppressants on day 44 after transplantation (right upper portion). Pathological findings by skin biopsy revealed spongiosis, hydropic basal cell degeneration, and dermal edema, which were consistent with acute graft-versus-host disease (HE, original magnification, ×400). Infiltrating lymphocytes were predominantly positive for CD8 (original magnification, ×400). Reprinted by courtesy of Bone Marrow Transplantation.

the PB were maintained in relatively high levels consistently because HTLV-1-infected cells were transplanted to recipients together with hematopoietic stem cells. In patients who were transplanted from HTLV-1 negative normal donors, HTLV-1 PVLs markedly decreased in most cases, and two types of HTLV-1 PVL fluctuation patterns were noted: first, HTLV-1 PVLs in PB became detectable, which were equivalent to HTLV-1 carrier levels; second, HTLV-1 PVLs were maintained in undetectable levels for a long time (Choi et al., 2011).

Although HTLV-1 PVLs in the PB do not represent only residual ATL cells after transplant, evaluation of the kinetics of HTLV-1 PVLs in ATL patients who underwent allo-HCT is useful for detecting the early ATL relapse and diagnosis of donor cell-derived ATL in combination with donor-recipient chimerism analysis of peripheral lymphocytes.

The eradication of HTLV-1 by allo-HCT has been reported in a young male patient with congenital pure red-cell anemia (Kawa et al., 1998). HTLV-1 PVLs frequently disappeared from the PB after allo-HCT in patients who were transplanted from HTLV-1 negative donors, but thereafter small number of HTLV-1-infected cells frequently appeared in the PB (Yamasaki et al., 2007; Choi et al., 2011). Prevention of HTLV-1 infection to donor cells from recipients who received transplant from HTLV-1 negative

donors should be considered by administering antiviral drugs after transplant.

### COMPLICATIONS OF TRANSPLANT FOR ATL

# Early Complications After Allo-HCT in ATL: Focusing on Viral Infection

Adult T-cell leukemia-lymphoma patients are severely immunocompromised and have frequent complications of opportunistic infections, including various kinds of viral infection. In patients with ATL, CMV, Epstein–Barr virus, and human herpes virus-6 (HHV-6) infections were frequently observed after cytotoxic chemotherapy (Ogata et al., 2011). High incidence of CMV infection was reported in patients with ATL after allo-HCT (Nakano et al., 2014). CMV infection has been reported to be associated with poor prognosis of patients with ATL who received allo-HCT (Sawayama et al., 2019). HHV-6 infection has also been reported in patients with ATL after CBT or unrelated BMT (Ogata et al., 2008, 2013). Monitoring for CMV antigenemia and HHV-6 in ATL patients

who undergo allo-HCT is necessary for the early detection and empirical therapy for these viral infections. There is no report that empirical therapy for HHV-6 can reduce the incidence of HHV-6 infection and TRM in patients with ATL who underwent allo-HCT. However, patients who developed HHV-6 encephalitis may have aggravation of symptoms within a short period (Ogata et al., 2013). There is a report that prophylaxis for HHV-6 using foscarnet reduced the incidence of HHV-6 infection where it is possible that HHV-6 prophylaxis prevented the progression of HHV-6 encephalitis; nevertheless, the incidence of encephalitis did not decrease (Ogata et al., 2018). Empirical therapy for HHV-6 infection in patients with ATL who received allo-HCT is recommended.

# Late Complications: Focusing on Donor Cell Leukemia and Lymphoma

Donor cell leukemia (DCL) has been reported in patients who underwent allo-HCT for various kinds of hematological malignancies (Wiseman, 2011). Although DCL was thought to be a very rare complication previously, about 5% of patients who underwent allo-HCT were recently reported to develop DCL (Wiseman, 2011). One of the reasons for the increase of DCL is thought to be the increase of donor age because of the relevance of RIC regimen for allo-HCT. The risk of DCL might be high in patients who undergo allo-HCT because of the long survival time.

Two cases of donor cell-derived ATL have been reported (Tamaki and Matsuoka, 2006; Nakamizo et al., 2011). Both patients were transplanted from HTLV-1 carrier donors. One patient developed donor cell-derived ATL 133 days after transplant. DCL was thought to be derived from HTLV-1-infected donor cells because ATL occurred in considerably short time after allo-HCT. In this case, HTLV-1-infected abnormal clone was not detected by Southern blot analysis of PB lymphocytes in the donor (Tamaki and Matsuoka, 2006). The occurrence of donor cell-derived ATL after allo-HCT, especially in the case of transplantation from HTLV-1 carrier donor, should be paid special attention.

Two cases of DCL/lymphoma other than ATL were reported: one was a patient with acute myelogenous leukemia, and the other was Burkitt lymphoma that occurred about 11 years after allo-HCT (Matsunaga et al., 2005; Takeuchi et al., 2015).

### NEW STRATEGY FOR ATL BY USING TRANSPLANT

#### New Agents for Allogeneic Hematopoietic Cell Transplantation in ATL

Recently, new molecular targeting agents such as anti-CC chemokine receptor 4 (CCR4) antibody, mogamulizumab, and lenalidomide have been approved for ATL patients in Japan.

First, we showed that approximately 90% of patients with ATL had CCR4-positive ATL cells, and the survival of CCR4-positive patients was lower than that of patients without CCR4 expression (Ishida et al., 2003, 2004b). Defucosylated

chimeric anti-CCR4 monoclonal antibody, KM2760, which enhanced antibody-dependent cellular cytotoxicity (ADCC), was developed in Japan (Niwa et al., 2004). We showed that KM2760 had strong cytotoxicity for fresh ATL cells derived from patients with ATL in the autologous setting by using patients' effector cells (Ishida et al., 2004a). Subsequently, humanized anti-CCR4 monoclonal antibody, KW-0761/mogamulizumab was further developed in Japan; we showed that mogamulizumab had the same strong cytotoxic effect for fresh ATL cells derived from ATL patients in the same autologous setting as that for KM2760 (Ishii et al., 2010). Based on several preclinical experiments of mogamulizumab for ATL cells, we conducted two prospective clinical studies (phases I and II) for patients with relapsed aggressive ATL in order to investigate the safety and efficacy of mogamulizumab. These two prospective studies revealed acceptable safety and good efficacy of mogamulizumab for patients with relapsed aggressive ATL. Mogamulizumab was thus approved for patients with relapsed or refractory ATL in Japan (Yamamoto et al., 2010; Ishida et al., 2012b). We further conducted a randomized study to evaluate the efficacy of VCAP-AMP-VECP [vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP)-doxorubicin, ranimustine and prednisone (AMP)-vindesine, etoposide, carboplatin and prednisone (VECP)] therapy alone (Tsukasaki et al., 2007) versus that in combination with mogamulizumab for newly diagnosed patients with aggressive ATL. We found that the CR rate in combination therapy was higher than that in VCAP-AMP-VECP therapy alone (52 vs. 33%; Ishida et al., 2015). Thus, mogamulizumab was approved for patients with newly diagnosed aggressive ATL in combination with chemotherapy in Japan in 2014.

The higher CR rates of patients with ATL who were treated with mogamulizumab in combination with chemotherapy were expected to improve the prognosis of patients who received allo-HCT. Unexpectedly, nationwide retrospective analysis revealed that mogamulizumab administration before allo-HCT for patients with ATL was associated with an increased risk of severe, acute, steroid-resistant GVHD, as well as higher NRM at 1 year. The 1-year OS in patients who had used mogamulizumab before allo-HCT was significantly lower than that in patients treated without mogamulizumab (Fuji et al., 2016b). When the OS and TRM rates were analyzed according to the interval between the last mogamulizumab administration and allo-HCT, the TRM rates decreased and the OS rates improved in patients in whom the interval was equal to and more than 50 days and were equivalent to those in patients not receiving mogamulizumab (Fuji et al., 2016b). Sugio et al. (2016) also retrospectively analyzed 25 patients with ATL receiving mogamulizumab before allo-HCT. Severe acute GVHD (Grade III/IV) was observed in 34.7% patients. By analyzing the interval between the last administration of mogamulizumab and allo-HCT, they showed that the frequency of severe acute GVHD (Grade III/IV) was significantly higher in patients receiving mogamulizumab within 80 days before transplant than in those having more than 80-day interval (Sugio et al., 2016).

Although a prospective randomized study revealed that the CR rate of mogamulizumab in combination with VCAP-AMP-VECP therapy was significantly higher than that of VCAP-AMP-VECP therapy alone, the OS in both groups was not significantly different (Ishida et al., 2015). Of the 53 patients enrolled in that clinical trial, 12 underwent allo-HCT after protocol therapy. Subsequent follow-up study revealed that the OS in patients who underwent transplant who did not receive mogamulizumab before allo-HCT was better than that of patients who received mogamulizumab before transplant (Ishida et al., 2019). Mogamulizumab can decrease not only CCR4-positive ATL cells but also normal regulatory T cells expressing CCR4. The half-life of plasma concentration after the eighth administration of mogamulizumab at 1.0 mg/kg is reported to be about 18 days, suggesting that several months are required for the disappearance of mogamulizumab in PB after intravenous administration (Ishida et al., 2012b). Mogamulizumab induced strong cytotoxic effect for CCR4-positive cells in low serum concentration because the ADCC activity of mogamulizumab was markedly enhanced by the defucosylation of IgG Fc portion of antibodies (Ishida et al., 2004a; Niwa et al., 2004). The decrease of normal regulatory T-cells might be associated with an increased risk of severe acute GVHD after transplant and poor prognosis of patients with ATL who underwent allo-HCT.

Conversely, at our institution, the 1-year OS rate of nine patients with ATL who underwent allo-HCT after 1-2 times of mogamulizumab administration was not less than that of 113 patients who did not receive mogamulizumab (55.6 vs. 37.2%, P = 0.843; Fuji et al., 2018a). Recently, hematologists and transplant physicians in Japan discussed the appropriate use of mogamulizumab for patients with aggressive ATL who planned to undergo allo-HCT, regarding the frequency and timing of mogamulizumab administration and the interval between the last mogamulizumab administration and transplantation. Thus, we proposed the appropriate use of mogamulizumab for patients with aggressive ATL before allo-HCT as follows: (1) mogamulizumab should not be administered to patients with aggressive ATL who plan to undergo transplantation if patients can obtain CR or PR without mogamulizumab; (2) mogamulizumab should be administered a few times only when patients cannot obtain remission by ordinary combination chemotherapy; (3) allo-HCT should be recommended with at least 50-day interval, if possible, between last mogamulizumab administration and transplant, and (4) no consensus about GVHD prophylaxis, choice of stem cell source and prophylaxis for infectious diseases (Fuji et al., 2018a).

Anti-CC chemokine receptor 4 mutations were detected in 14/53 ATL samples (26%) and consisted exclusively of nonsense or frameshift mutations that truncated the coding region at C329, Q330, or Y331 in the carboxy terminus (Nakagawa et al., 2014). They showed that the CCR4-Q330 non-sense isoform was a gain-of-function mutation. Another group analyzed CCR4 gene alterations in patients with ATL, and gene mutations were found in 27% of ATL patients who had frameshift and non-sense

mutations. The frameshift mutations in patients with ATL were associated with poor prognosis (Yoshida et al., 2016). We also analyzed CCR4 gene alterations in 116 patients with ATL; CCR4 mutations were found in 33% of the patients. Mogamulizumab therapy was extremely effective for ATL patients with CCR4 mutations. CCR4 mutation status had no significant impact in patients who neither receive mogamulizumab-containing treatment nor allo-HCT. OS in patients with CCR4 mutations who did receive mogamulizumab-containing treatment but no allo-HCT was significantly superior to that in patients without CCR4 mutations (Sakamoto et al., 2018). These results suggest that mogamulizumab therapy can obtain long-term survival in ATL patients with CCR4 mutations. In the near future, CCR4 mutations in patients with ATL need to be detected because they might influence the decision of therapeutic strategies for patients with ATL, including transplantation.

Another new drug, lenalidomide is useful for patients with ATL. Lenalidomide is one of the key drugs for multiple myeloma (MM) therapy. Clinical trials of lenalidomide in phases I and II revealed the efficacy for patients with relapsed ATL, and then lenalidomide was approved for patients with relapsed ATL in Japan in 2017 (Ishida et al., 2016; Ogura et al., 2016). Unfortunately, the efficacy of lenalidomide for patients with ATL who underwent transplant has not been reported in detail, although it is expected to be one of the promising key drugs for salvage therapy for relapsed patients with ATL. In MM patients, lenalidomide for maintenance therapy to prevent relapse or progression after non-myeloablative allo-HCT was not feasible because of the increased acute GVHD (Kneppers et al., 2011). Conversely, another study showed that small amount of lenalidomide was feasible for MM patients after allo-HCT, although the frequency of acute GVHD was high (Alsina et al., 2014). Further, a study showed that half of the MM patients who relapsed after allo-HCT could be controlled by lenalidomide administration (Bensinger et al., 2014). Only one patient with ATL was reported to have been successfully treated by a small dosage of lenalidomide for relapse after allo-HCT (Ando et al., 2018). The appropriate use of lenalidomide could be useful for patients with ATL who relapsed after allo-HCT or who planned to undergo allo-HCT. The therapeutic strategy for using lenalidomide for patients with ATL who received allo-HCT should be established in the near future.

#### New Methods of Allo-HCT for ATL

Haploidentical HCT was performed for highly aggressive hematological malignancies by using non- MAC because of the decrease in HLA-matched related siblings and long period for coordination in unrelated HCT cases (Ogawa et al., 2006). Recently, an approach using post-cyclophosphamide administration in haploidentical HCT has become popular (Luznik et al., 2008; Kasamon et al., 2010; Sugita et al., 2015).

Recently, we evaluated the prognosis of haploidentical HCT for ATL patients without post-cyclophosphamide administration by using Japanese TRUMP database. Although the TRM rate of haploidentical HCT without post-cyclophosphamide

administration was very high, which included 1-year non-ATL-related death of 41.3%, about 20% for the patients survived for more than 5 years (Yoshimitsu et al., 2018b). The safety and efficacy of haploidentical HCT was investigated by two prospective multicenter clinical studies in Japan that are underway and are using both MAC and RIC for patients with ATL followed by post-cyclophosphamide administration. The outcomes of haploidentical HCT for patients with ATL without post-cyclophosphamide administration reported by Yoshimitsu et al. (2018b) are very important compared to those of patients who underwent haploidentical HCT followed by post-cyclophosphamide administration. Because ATL known to occur at higher ages (median age, about 68 years; Nosaka et al., 2017), developing haploidentical HCT by using RIC for patients with ATL followed by post-cyclophosphamide administration is necessary.

#### **FUTURE DIRECTION**

hematopoietic cell transplantation Allogeneic immunotherapy based on allogeneic immune reactions after transplant. Conversely, autologous stem cell transplantation is an intensive chemotherapy that involves numerous anticancer agents and/or radiotherapy. Therefore, the time to obtain immunological effects against tumor cells and reduction of tumor volume before allo-HCT are necessary for obtaining remission. In particular, ATL is an aggressive hematological malignancy that can relapse immediately even in CR. Immunological effects in patients with aggressive ATL can be obtained by determining the disease status and PS at the time of HCT. Strong immune responses after transplant can be induced using allo-HCT in combination with pure immunotherapy without stem cell transplant to produce good prognoses in patients with ATL in the near future. Furthermore, new strategies of allo-HCT for patients with ATL are is needed for the improving the cure rates of patients.

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#### CONCLUSION

The history and progression of allo-HCT for patients with ATL are reviewed. Although allo-HCT procedures for patients with ATL have markedly progressed, which include the development of RIC, haploidentical transplant, and diversity of indications for allo-HCT, the TRM rates after transplant remain considerably high owing to older age of patients with ATL and high frequency of infectious complications. Further development of allo-HCT by using new molecular targeting agents is required for the improvement of the cure rates of patients with ATL.

#### **AUTHOR CONTRIBUTIONS**

AU designed the study and wrote the draft of the manuscript.

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### Collagen IV (COL4A1, COL4A2), a Component of the Viral Biofilm, Is Induced by the HTLV-1 Oncoprotein Tax and Impacts Virus Transmission

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Millen S, Gross C, Donhauser N, Mann MC, Péloponèse J-M Jr and Thoma-Kress AK (2019) Collagen IV (COL4A1, COL4A2), a Component of the Viral Biofilm, Is Induced by the HTLV-1 Oncoprotein Tax and Impacts Virus Transmission. Front. Microbiol. 10:2439. doi: 10.3389/fmicb.2019.02439 Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent for Adult T-Cell Leukemia/Lymphoma (ATLL) and HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). HTLV-1 infects CD4+ T-cells via cell-to-cell transmission requiring reorganization of the cytoskeleton and expression of the viral transactivator and oncoprotein Tax. Viruses spread at the virological synapse (VS), a virus-induced specialized cell-cell contact, by polarized budding into synaptic clefts, and by cell surface transfer of viral biofilms (VBs). Since little is known about Tax's role in formation of the VB, we asked which component of the VB is regulated by Tax and important for HTLV-1 transmission. Collagens are not only structural proteins of the extracellular matrix and basal membrane but also represent an important component of the VB. Here, we report that among the collagens known to be present in VBs, COL4 is specifically upregulated in the presence of HTLV-1 infection. Further, we found that transient expression of Tax is sufficient to induce COL4A1 and COL4A2 transcripts in Jurkat and CCRF-CEM T-cells, while robust induction of COL4 protein requires continuous Tax expression as shown in Tax-transformed T-cell lines. Repression of Tax led to a significant reduction of COL4A1/A2 transcripts and COL4 protein. Mechanistically, luciferase-based promoter studies indicate that Tax activates the COL4A2 and, to a less extent, the COL4A1 promoter. Imaging showing partial co-localization of COL4 with the viral Gag protein in VBs at the VS and transfer of COL4 and Gag to target cells suggests a role of COL4 in VB formation. Strikingly, in chronically infected C91-PL cells, knockout of COL4A2 impaired Gag transfer between infected T-cells and acceptor T-cells, while release of virus-like particles was unaffected. Taken together, we identified COL4 (COL4A1, COL4A2) as a component of the VB and a novel cellular target of Tax with COL4A2 appearing to impact virus transmission. Thus, this study is the first to provide a link between Tax's activity and VB formation by hijacking COL4 protein functions.

Keywords: HTLV-1, Tax-1, collagen 4, collagen IV, COL4A1, COL4A2, virus transmission, viral biofilm

#### INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a highly oncogenic retrovirus causing Adult T-cell Leukemia/Lymphoma (ATLL) or inflammatory diseases in up to 10% of infected individuals (Tagaya and Gallo, 2017). Worldwide, at least 5-10 million people are infected with this yet neglected human retrovirus, however, it is estimated that there is a much higher number of unknown cases since statistics on HTLV-1 prevalence are lacking for several densely populated regions (Gessain and Cassar, 2012). HTLV-1 is highly endemic in Southwestern parts of Japan, Sub-Saharan Africa, South America, the Caribbean, and parts of the Middle East and Australo-Melanesia (Gessain and Cassar, 2012). In central Australia, up to 48% of certain ethnic communities in the socially disadvantaged Indigenous population are HTLV-1-infected (Einsiedel et al., 2016), which currently accounts for global worries (Martin et al., 2018). Since up to 90% of infected patients stay lifelong asymptomatic and blood donors are not screened for HTLV-1 infection in most countries, asymptomatic carriers are mainly unaware of their infection and may pass the infection to other people (Caswell et al., 2019). HTLV-1 is transmitted via cell-containing body fluids such as breast milk, blood products, semen, and via organ transplants (Pique and Jones, 2012; Gross and Thoma-Kress, 2016). Upon binding to its receptor, HTLV-1 infects its target cells, which are mainly CD4<sup>+</sup> T-cells and to a less extent CD8<sup>+</sup> T-cells, dendritic cells (DC), or monocytes (Macatonia et al., 1992; de Castro-Amarante et al., 2015; Melamed et al., 2015). Recent work suggests that infection of hematopoietic stem cells contributes to spread of HTLV-1 in vivo (Furuta et al., 2017).

Upon infection and reverse transcription, HTLV-1 integrates into the host cell genome and persists in vivo mainly in its provirus form (9.1 kb), which is flanked by long terminal repeats (LTR). In addition to structural proteins and enzymes common for retroviruses, HTLV-1 encodes regulatory (Tax, Rex) and accessory (p12/p8, p13, p30, HBZ) proteins (Currer et al., 2012). HTLV-1 replicates either by infecting new cells or by mitotic division and clonal proliferation of infected CD4<sup>+</sup> T-cells. Cell-free transmission of HTLV-1 between T-cells is inefficient, free virions can hardly be detected in infected individuals and are poorly infectious for most cell types (Fan et al., 1992; Derse et al., 2001; Alais et al., 2015; Demontis et al., 2015). Efficient infection of CD4<sup>+</sup> T-cells requires cell-cell contacts, and virus propagation from cell-to-cell depends on specific interactions between cellular and viral proteins. Two types of cell-cell contacts seem to be critical for HTLV-1 transmission: tight cell-cell contacts and cellular conduits (Igakura et al., 2003; Van Prooyen et al., 2010; Gross and Thoma-Kress, 2016). For transmission at tight cell-cell contacts, two non-exclusive mechanisms of virus transmission at the virological synapse (VS), a virus-induced specialized cell-cell contact, have been proposed, polarized budding of HTLV-1 into synaptic clefts (Igakura et al., 2003), and cell surface transfer of so-called viral biofilms (VBs) at the VS (Pais-Correia et al., 2010). In VBs, extracellular concentrated viral particles are embedded in a carbohydraterich structure that is induced and spatially reorganized by viral infection. In detail, viral assemblies are surrounded by cellular

lectins (Galectin-3), heparan sulfate proteoglycans (Agrin), Tetherin (BST-2 or CD317), and components of the extracellular matrix like collagens of unknown composition (Pais-Correia et al., 2010). Further, monoclonal antibody screening revealed that the antigens CD4, CD150, CD70, CD80, and CD25 are concentrated in the VB and the latter three are inducible by Tax (Tarasevich et al., 2015). HTLV-1 transmission via VBs seems to constitute a major route of transmission in vitro since removal of biofilms severely impairs cell-to-cell transmission (Pais-Correia et al., 2010). Further, in vitro studies have shown that DC can be infected cell-free with high concentrations of isolated VBs, which then mediate efficient cell-cell contactdependent infection of CD4+ T-cells (Alais et al., 2015). Moreover, recent work identified isolated viral biofilm-like structures as new viral structures activating innate immunity by triggering type I interferon (IFN) production of plasmacytoid DCs (Assil et al., 2019).

Among the HTLV-1-encoded proteins, the viral regulatory protein Tax is a crucial regulator of virus transmission. Tax is not only essential for HTLV-1 replication by transactivating the HTLV-1 LTR (U3R) promoter, but Tax is also a potent transactivator of cellular transcription, important for initiating oncogenic transformation, and crucial for viral spread (Chevalier et al., 2012; Currer et al., 2012). Tax regulates virus transmission by inducing and cooperating with intercellular adhesion molecule 1 (ICAM-1) and inducing polarization of the microtubule organizing center (MTOC), which leads to formation of the VS (Fukudome et al., 1992; Nejmeddine et al., 2005, 2009). Recent work suggests that next to Tax also HBZ contributes to HTLV-1-infectivity by upregulating ICAM-1 (Fazio et al., 2019). Use of single-cycle replication-dependent HTLV-1 reporter vectors revealed that Tax also enhances actinand tubulin-dependent transmission of HTLV-1 virus-like particles (Mazurov et al., 2010). Cellular factors that are crucial for HTLV-1 infection are rather poorly described in the context of virus transmission. We and others could show that Tax interferes with a variety of cellular genes involved in virus transmission (Kress et al., 2011; Mazurov et al., 2012; Chevalier et al., 2014; Gross et al., 2016). We could show that Tax enhances HTLV-1 cell-to-cell transmission by inducing the actin-bundling protein Fascin (Kress et al., 2011; Gross et al., 2016), potentially by facilitating the recruitment of viral particles to budding sites. Although Tax had been shown to regulate several host cell factors playing a role in virus transmission (Pique and Jones, 2012; Gross and Thoma-Kress, 2016), little is known about Tax's role in formation of the VB (Tarasevich et al., 2015). Here, we propose that Tax regulates a certain collagen which is part of the VB.

The collagen superfamily is composed of 29 different subtypes (COL I- XXIX, or COL1-29) with collagens representing the main structural proteins in various species and tissues. Collagens are important for structure maintenance, tensile stress resistance, cell adhesion, migration, cell–cell interactions, and chemotaxis (Leitinger, 2011). Collagen IV (COL4), a network-building collagen of the basal membrane, is not only a scaffold protein of the extracellular matrix, but it also binds to different integrin and non-integrin receptors to fulfill its various functions. The COL4 family consists of the six family members COL IV

α1 (COL4A1) - COL IV α6 (COL4A6) (Khoshnoodi et al., 2008; Leitinger, 2011). Two COL4A1 (ca. 160 kDa each) and one COL4A2 polypeptide chains (ca. 167 kDa) form a tightly packed triple helix (COL4) by twisting around each other. COL4 consists of an N-terminal collagenous domain and a C-terminal non-collagenous domain (NC1) (Leitinger, 2011). COL4A1 and COL4A2 genes are located head-to-head on opposite strands and transcriptional regulation of COL4A1 and COL4A2 is controlled by a bi-directional promoter region and further regulatory elements located distantly (Poschl et al., 1988; Soininen et al., 1988). CD4<sup>+</sup> T-lymphocytes, the main target cell type of HTLV-1 in vivo, do not express COL4. However, analysis of the VB in chronically HTLV-1-infected T-cell lines and primary T-cells from HTLV-1-infected patients using a pan-collagen antibody recognizing collagens 1-5 revealed that collagens are enriched in VBs (Pais-Correia et al., 2010).

Here, we report that among the collagens known to be present in VBs, COL4 is specifically upregulated in the presence of HTLV-1 infection and Tax is sufficient to induce *COL4A1* and *COL4A2* transcripts, while robust induction of COL4 protein requires continuous Tax expression. Imaging showing partial colocalization of COL4 with the viral Gag protein in VBs at the VS and transfer of COL4 and Gag to target cells suggests a role of COL4 in VB formation. Strikingly, in chronically infected C91-PL cells, repression of COL4 impaired Gag transfer between infected T-cells and acceptor T-cells, while release of virus-like particles was unaffected. Thus, this study is the first to provide a link between Tax's activity, VB formation, and virus transmission by hijacking COL4 protein functions.

#### **MATERIALS AND METHODS**

#### **Cell Lines**

The HTLV-1 in vitro transformed CD4<sup>+</sup> T-cell lines C8166-45 (Salahuddin et al., 1983), MT-2 (Yoshida et al., 1982) and C91-PL (Ho et al., 1984), as well as the ATL-derived CD4<sup>+</sup> T-cell line HuT-102 (Gazdar et al., 1980) and the CD4<sup>+</sup> T-cell lines JPX9/JPX9M (Ohtani et al., 1989) carrying a cassette of Tax wildtype (JPX9) or of a mutated Tax (JPX9M) under control of a metallothioenin-sensitive promoter, were cultured in RPMI 1640 medium (GIBCO, Life Technologies, Darmstadt, Germany) containing 10% fetal calf serum (FCS; Sigma Aldrich, Darmstadt, Germany), L-glutamine (0.35 g/l) and penicillin/streptomycin (Pen/Strep; 0.12 g/l each). Induction of Tax or Tax mutant expression in JPX9/JPX9M cells was triggered by addition of 20  $\mu M$  cadmium chloride (CdCl<sub>2</sub>) to the culture medium. The HTLV-1 in vitro transformed CD4<sup>+</sup> T-cell line MS-9, containing only one single proviral genome (Shuh et al., 1999), as well as the Tax-transformed CD4+ T-cell lines Tesi (Schmitt et al., 1998), Tri (Grassmann et al., 1989) and TAXI-1 (Waldele et al., 2004), were kept in RPMI 1640 (40%) and Panserin 401 medium (40%; PAN-Biotech, Aidenbach, Germany), supplemented with 20% FCS, L-glutamine, Pen/Strep and 100 U/ml (MS-9), 40 U/ml (Tesi, Tri) or 20 U/ml (TAXI-1) interleukin-2 (IL-2; Roche Diagnostics, Mannheim, Germany). For repression of Tax protein in Tesi cells, 1 µg/ml tetracycline (Tet) was added to the

culture medium for 10 days. The CD4+ T-cell lines Jurkat (Schneider et al., 1977), HuT-78 (Gazdar et al., 1980), CCRF-CEM (Foley et al., 1965), Molt-4 (Minowada et al., 1972), and the primary effusion lymphoma derived B-cell line JSC-1 (Cannon et al., 2000) were cultivated in RPMI 1640 (45%) and Panserin 401 medium (45%), supplemented with 10% FCS, Lglutamine and Pen/Strep. The Burkitt lymphoma derived B-cell lines Bjab (Menezes et al., 1975) and Raji (Pulvertaft, 1964), the Hodgkin lymphoma derived B-cell lines KM-H2 (Kamesaki et al., 1986), L-428 (Schaadt et al., 1979) and lymphoid cell line HDLM-2 (Drexler et al., 1986), as well as the primary effusion lymphoma derived B-cell line BC-3 (Arvanitakis et al., 1996) and the recombinant, Tio-expressing Herpesvirus saimiri (HVS) C488 transformed peripheral blood lymphocyte cell lines 1765 and 1766 (Albrecht et al., 2004) were cultured in RPMI 1640 (45%) and Panserin 401 medium (45%), supplemented with 10% FCS, L-glutamine and gentamycin (0.1 g/l). The StpC/Tip-expressing HVS C488 immortalized human cord blood lymphocyte cell line 1851/1 was kindly provided by B. Biesinger and J.C. Albrecht (Institute of Clinical and Molecular Virology, Erlangen, Germany). Cells were kept in RPMI 1640 (45%) and Panserin 401 medium (45%), supplemented with 10% FCS, L-glutamine, gentamycin and 10 U/ml IL-2. HEK-293T were cultured in DMEM (GIBCO, Life Technologies) containing 10% FCS, L-glutamine and Pen/Strep. In the case of C91-PL cells stably transduced with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) constructs, 2 µg/ml puromycin was added to the culture medium.

#### **Primary Cells From Healthy Individuals**

Peripheral blood mononuclear cells (PBMC) from healthy individuals were isolated from leukocyte cones derived from thrombocyte apheresis in the transfusion medicine section of the University Hospital Erlangen, which was approved by the Ethics Committee of the Medical Faculty of Friedrich-Alexander-Universität Erlangen-Nürnberg (Az. 220\_16B; Erlangen, Germany). PBMC were isolated by density gradient centrifugation using Biocoll Separating Solution (Biochrom GmbH, Berlin, Germany). Isolated PBMC were subsequently cultivated with a density of  $2^*10^6$  cells/ml in RPMI 1640 containing 10% FCS, L-glutamine and Pen/Strep. For stimulation of PBMC, cells were incubated in PBMC medium permanently containing 50 U/ml IL-2 and initially 2  $\mu g/ml$  phytohemagglutinin (PHA) overnight.

#### Patient Samples and Controls

Blood samples from HTLV-1 infected patients were obtained from the CHU of Martinique. Clinical collections of samples for research purpose are stored at the Center of Biological Resources of Martinique (CeRBiM). HTLV-1 asymptomatic carrier (AC) patients were recruited according to World Health Organization (WHO) criteria. AC had no neurologic or haematological symptoms. HAM/TSP patients were subgrouped according to their progression rate into slowly (TSP-L) or rapidly progressing patients (TSP-R). Rapid progressors are patients deteriorated more than three grades of a motor disability score within 2 years after initial examination (Olindo et al., 2006). According to

the French Bioethics laws, the collection of samples has been declared to and approved by the ethics committee of the French Ministry of Research. Because the protocol is non-interventional, no informed consent was required, as stated by the French Public Health code and therefore the study was conducted anonymously. Non-infected CD4+ control T-cells were isolated from PBMC derived from buffy coats of healthy blood donors using the MACS negative depletion system (Miltenvi Biotec, Auburn, CA, United States). No contaminating CD8<sup>+</sup> T cells, B-cells, monocytes, or natural killer cells were detected by flow cytometry. Purified CD4+ T cells were then cultured in RPMI 1640 medium (GIBCO, Life Technologies) supplemented with 10% FCS from Sigma Aldrich, 25 mM Hepes buffer, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µM 2β-mercaptoethanol. CD4<sup>+</sup> T cells (1\*10<sup>6</sup>/well) were activated in flat-bottomed 6-well plates pre-coated with the anti-CD3 mAb  $(10 \mu g/ml)$  in the presence of soluble anti-CD28 mAb  $(1 \mu g/ml)$ . Cultures were harvested after 1 week for total RNA isolation.

#### **Microarray Analysis**

Transcriptome analysis was carried out on the Affymetrix HGU133plus 2.0 platform (Affymetrix, Santa Clara, CA, United States) in biological replicates as described before (Pichler et al., 2008; Kress et al., 2010). Briefly, the transcriptome of Tax-expressing Tesi cells was compared to that of Tesi/Tet cells, where Tax expression was repressed. Further, the HTLV-1 *in vitro*-transformed cell line (MT-2) was compared to postmitotic CD4<sup>+</sup> T lymphocytes. The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible at www.ncbi.nlm.nih.gov/geo and GEO Series accession numbers are GSE10508 and GSE17718.

### Quantitative Real-Time RT-PCR (qPCR) and RT-PCR

Total cellular RNA from transfected cells or untransfected cell lines was isolated (NucleoSpin® RNA, Macherey Nagel, Düren, Germany) and reversely transcribed to cDNA by applying random hexamer primers and Superscript<sup>TM</sup> II Reverse Transcriptase (both Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturers' instructions. Quantitative real-time RT-PCR (qPCR) was performed using 200 ng of cDNA and SensiMix<sup>TM</sup> II Probe Kit (Bioline GmbH, Luckenwalde, Germany) or TaqMan® Universal PCR Master Mix in an ABI Prism 7500 Sequence Analyzer (both Applied Biosystems, Foster City, CA, United States) according to the manufacturers' instructions. Primer sequences and FAM (6-carboxyfluorescein)/TAMRA (tetramethylrhodamine)labeled probes for detection of  $\beta$ -actin (ACTB) and Tax transcripts have been described before (Pichler et al., 2008). COL4A1 and COL4A2 transcripts were detected using TagMan Gene Expression Assays Hs00266237\_m1 (COL4A1) and Hs01098858\_m1 (COL4A2; both Applied Biosystems). Transcript expression levels were computed from standard curves generated by pJET1.2/blunt plasmids (Fermentas, St.-Leon Roth, Germany) bearing respective target sequences and the mean of technical triplicates was calculated for all

samples. Every experiment was independently performed at least three times and relative copy numbers (rcn) were calculated by normalization of respective transcript levels on those of β-actin. Total RNA from HTLV-1-infected patients and uninfected controls was prepared from whole cells using Trizol (Invitrogen, Thermo Fisher Scientific) as previously described (Terol et al., 2017). After reverse transcription (RT) using 5x All-In-One RT MasterMix (ABM, Vancouver, BC, Canada), the abundance of transcripts was assessed by qPCR analysis using the SYBR green PCR master mix (Roche Diagnostics) and gene-specific primer sets. Data were analyzed using LightCycler® 480 Software (Roche Diagnostics). Primers for COL4A1 (qHsaCID0010223) and COL4A2 (qHsaCED0044576) and the housekeeping gene HPRT-1 (qHsaCID0016375) were from BioRad (Hercules, CA, United States). For qualitative analysis of mRNA expression, 500 ng of cDNA were subjected to RT-PCR using dNTPs (250 µM each) and DreamTag DNA polymerase (2 U, both Thermo Fisher Scientific). Primer (0.6 μM each) sequences were as follows: Tax-RT-fwd 5'-CAGCCCAC TTCCCAGGGTTTGGAC-3', Tax-RT-rev 5'-GTGTGAGAGT AGAAATGAGGGGT-3', ACTB-RT-fwd 5'-CGGGAAATCGTG CGTGACAT-3', ACTB-RT-rev 5'-GAACTTTGGGGGATGCT CGC-3'.

#### **Western Blot**

Cell lines or transfected cells were resuspended in lysis buffer [150 mM NaCl, 10 mM Tris/HCl (pH 7.0), 10 mM EDTA, 1% Triton<sup>TM</sup> X-100, 2 mM DTT and protease inhibitors leupeptin, aprotinin (20 µg/ml each) and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and subjected to repeated freeze-and-thaw cycles between −196°C (liquid nitrogen) and 30°C. In the case of Tax protein detection, lysates were additionally sonicated three times for 20 s. Equal amounts of proteins (between 30 and 50 µg) were denatured for 5 min at 95°C in sodium dodecyl sulfate (SDS) loading dye (10 mM Tris/HCl (pH 6.8), 10% glycerin, 2% SDS, 0.1% bromophenol blue, 5% β-mercaptoethanol). Samples were subjected to SDS-PAGE and immunoblot using nitrocellulose transfer membranes (Whatmann®, Protran®, Whatmann GmbH, Dassel, Germany) or Immobilon®-FL PVDF transfer membranes (Merck Millipore, Billerica, MA, United States) using standard protocols. Proteins were detected with the following primary antibodies: rabbit polyclonal anti-COL4 (ab6586, Abcam), mouse anti-Tax (derived from the hybridoma cell line 168B17-46-34, provided by B. Langton through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Langton et al., 1988), rabbit polyclonal anti-Tio serum (a kind gift of Brigitte Biesinger and Jens Albrecht, Albrecht et al., 1999), mouse monoclonal anti-HTLV-1 gag p19 (TP-7, ZeptoMetrix Corporation), mouse monoclonal anti-GFP (GSN24, Sigma), mouse monoclonal anti-Hsp90 α/β (F-8, Santa Cruz Biotechnology), mouse monoclonal anti-α-Tubulin (T9026, Sigma), mouse monoclonal anti-ß-actin (AC-15, Sigma) and mouse monoclonal anti-GAPDH (3B1E9, GenScript). For detection of proteins blotted on nitrocellulose membranes, secondary antibodies anti-mouse or anti-rabbit conjugated with horseradish peroxidase (HRP; GE Healthcare, Little Chalfont, United Kingdom) were employed. For detection of PVDF-transferred proteins, secondary antibodies antimouse or anti-rabbit Alexa Fluor® 647 (Life Technologies GmbH) were applied. Peroxidase activity was assessed by enhanced chemiluminescence using a CCD camera (Fujifilm LAS-1000 Intelligent Dark Box; Fujifilm). Fluorescence signals were detected using the Advanced Fluorescence Imager camera (ChemoStar, Intas Science Imaging GmbH, Göttingen, Germany).

### Confocal Laser Scanning Microscopy Detection of COL4 Protein Expression

For detection of COL4 protein expression, 1.8\*10<sup>5</sup> MT-2, HuT-102, C91-PL, C8166-45, or Jurkat T-cells were resuspended in 30 µl PBS and spotted on epoxy-resin coated glass slides (medco Diagnostika GmbH, Hengersberg, Germany) or on poly-L-lysine coated coverslips by gentle desiccation. Cells were fixed in 2% paraformaldehyde (PFA; 1 h, 25°C) and washed five times with PBS/0.1% Tween® 20. Permeabilization was performed with PBS/0.2% Triton<sup>TM</sup> X-100 (20 min, 4°C) where indicated, or cells were left unpermeabilized by incubation in mere PBS (20 min, 4°C). Cells were washed twice and incubated with PBS/5% FCS/1% bovine serum albumin (BSA; 1 h, 25°C). Both primary antibodies, rabbit polyclonal anti-COL4 (ab6586, Abcam) and mouse monoclonal anti-CD98 (ab2528, Abcam), were applied together in blocking solution (45 min, 37°C). Cells were washed three times and incubated with secondary antibodies antimouse Alexa Fluor® 488 and anti-rabbit Alexa Fluor® 647 (both Life Technologies GmbH) in blocking solution (45 min, 37°C) one after the other with three washing steps in between. Finally, samples were covered with ProLong<sup>TM</sup> Gold Antifade Mountant with DAPI (Life Technologies GmbH) according to the manufacturer's instructions. Images were acquired using a Leica TCS SP5 confocal laser scanning microscope equipped with a 63 × 1.4 HCX PL APO CS oil immersion objective lens (Leica Microsystems GmbH, Wetzlar, Germany). Images were analyzed using LAS AF software (Leica) and Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, United States).

### Co-culture Assays Between HTLV-1 Infected MS-9 Cells and Uninfected Jurkat T-cells

HTLV-1 negative Jurkat acceptor cells were prestained with the live cell dye *CellTracker*<sup>TM</sup> *Blue 7-Amino-4-Chlormethylcumarin* (CMAC; Thermo Fisher Scientific; 20 μM, 45 min, 37°C). After five washing steps in serum-free medium, prestained Jurkat T-cells and HTLV-1 positive MS-9 donor cells were co-cultured at a ratio of 1:1 on poly-L-lysine-coated coverslips (20 or 50 min, 37°C) and fixed with 2% PFA (1 h, 25°C). Cells were permeabilized and stained as described in Detection of COL4 Protein Expression. Primary antibodies mouse monoclonal anti-HTLV-1 gag p19 (TP-7, ZeptoMetrix Corporation) and rabbit polyclonal anti-COL4 in blocking solution (45 min, 37°C) were used. Secondary antibody staining was performed as described in "Detection of COL4 Protein Expression." Mounting of cells was performed with *ProLong*<sup>TM</sup> *Gold Antifade Mountant without DAPI* (Life Technologies GmbH). After acquisition

(see Detection of COL4 Protein Expression), images were analyzed and signal intensities were quantified using *LAS AF* software (Leica) and *Adobe Photoshop CS5* (Adobe Systems). For quantitative evaluation, manual counting of cells was performed, analyzing in total 1524 cells of 83 optical fields. Transfer of Gag and/or COL4 protein from MS-9 donor cells to Jurkat acceptor cells was determined and normalized on the number of Gag or COL4 positive MS-9 cells and on the ratio of Jurkat acceptor to MS-9 donor cells.

#### **Plasmids and Transfection**

#### **Plasmids**

The following plasmids were used: the Tax expression vectors pEFneo-Tax1 (Shoji et al., 2009) and pc-Tax (Rimsky et al., 1988); pEFneo and pcDNA3.1 (Life Technologies; controls); the Rex-GFP expression plasmid pCMV-Rex1-GFP (kind gift from Donna M. D'Agostino, University of Padova); the luciferase-reporter control vector pGL3-Basic (Promega, Mannheim, Germany), luciferase-reporter vectors harboring the *U3R* sequence of the HTLV-1 LTR pGL3-U3R-Luc (U3R-Luc; Mann et al., 2014) or sequences of the *COL4A1* or *COL4A2* promoter pGL3-COL4A1-Luc (COL4A1-Luc) and pGL3-COL4A2-Luc (COL4A2-Luc) (Turner et al., 2015); the HTLV-1 packaging vector pCMV-HT1MΔXho (HT1MΔEnv) harboring parts of the HTLV-1 proviral genome lacking the envelope protein (Derse et al., 2001).

#### Transfection

For COL4 induction,  $10^*10^6$  Jurkat or CCRF-CEM T-cells were transfected with 100  $\mu g$  Tax expression plasmid or the empty vector control. Cells were separated before harvest to generate RNA and Western Blot lysates. For luciferase reporter assays,  $5^*10^6$  Jurkat cells were transfected with 30  $\mu g$  Tax expression plasmid and 20  $\mu g$  of the respective reporter vector. Jurkat and CCRF-CEM T-cells were electroporated using the *Gene Pulser X Electroporation System* (BioRad) at 290 V and 1500  $\mu F$ .

### Generation of Stable Knockout C91-PL Cells

For knockout of COL4A1 and COL4A2 by CRISPR technology, the lentiCRISPRv2 vector system was employed [Sanjana et al., 2014; kind gift from Feng Zhang (Addgene plasmid #52961)]. The guide RNA sequence of pCAS-Scramble (OriGene; GCACTACCAGAGCTAACTCA), a non-specific RNA sequence, served as negative control. Guide-RNA sequences targeting COL4A1 and COL4A2 were designed with the CRISPR gRNA Design tool (DNA2.0, Atum, Newark, CA, United States) and were for COL4A1: CCAGGAGGTCCCGGTTCACC (COL4A1\_1) and GTGCTCCTCGTGGAGCAGAA (COL4A1\_2) and for COL4A2: CCTGGAGACGCCGGCTTACC (COL4A2 1) and GAAAGTCGCTTACCGCCGTA (COL4A2\_2). Oligos were inserted into lentiCRISPRv2 vector replacing the 2 kb filler sequence by BsmBI restriction enzyme via standard cloning procedures. The resulting vectors were designated lentiCRISPRv2-scramble-guide (scramble), lentiCRISPRv2-COL4A1-guide1 (COL4A1\_1), lentiCRISPRv2-COL4A1-guide2 (COL4A1\_2), lentiCRISPRv2-COL4A2-guide1 (COL4A2\_1) and lentiCRISPRv2-COL4A2-guide2 (COL4A2 2). For lentiviral production, 5\*106 293T cells were seeded in 10 cm dishes and 24 h later, cells were transfected with GeneJuice® transfection reagent (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol using a total amount of 15 µg DNA: 6 μg CRISPR vector, 6 μg HIV-1 gag-pol expression vector psPAX2 and 3 µg VSV-G expression vector pMD2.G (kind gifts from Didier Trono, Addgene plasmids #12260 and #12259, respectively). At 72 h after transfection, lentivirus-containing supernatants were concentrated (4000 g, 15 min) using Amicon® Ultra®-15 Centrifugal Filters (Ultracel-100K; Merck Millipore). 1\*106 C91-PL cells were spin-infected with 1.5 ml concentrated virus (150 g, 2 h, 32°C) and adjusted to 2\*10<sup>5</sup> C91-PL cells/ml. CRISPR-vectors COL4A1\_1 and COL4A1\_2 or COL4A2\_1 and COL4A2\_2 were transduced as pools to generate a COL4A1 or a COL4A2 knockout. Initial selection was performed 72 h after transduction by addition of 2 µg/ml puromycin to the cell culture medium.

#### Luciferase Reporter Assays

Jurkat T-cells were transfected, as described above, in quadruplicates. One sample was subjected to western blot analysis as described above. The remaining triplicate samples were processed for luciferase reporter assays determining firefly luciferase activity as described before (Mann et al., 2014). Relative light units (RLU) obtained from U3-Luc, COL4A1-Luc or COL4A2-Luc were normalized on protein content and on the respective values derived from co-transfection with the pGL3-Basic negative control vector, which represents background activity.

#### Gag p19 ELISA

 $0.5^*10^6$  stably transduced C91-PL cells were seeded in 24 well plates and incubated for 48 h. Supernatants were subsequently sterile filtrated by passing through a 0.45  $\mu m$  filter and virus release was determined using gag p19 ELISA according to the manufacturer's instruction (ZeptoMetrix Corporation, Buffalo, NY, United States). Values were obtained using Softmax Pro Version 5.3 software (MDS Analytical Technologies, Sunnyvale, CA, United States). Five independent experiments, each performed in duplicate, were performed.

#### Flow Cytometry

 $1^*10^6$  C91-PL-scramble, C91-PL-CRISPR-COL4A1, or C91-PL-CRISPR-COL4A2 cells were co-cultured with  $1^*10^6$  Jurkat T-cells for 1 h at  $37^\circ$ C. The latter have been prestained with CMAC as described earlier (Donhauser et al., 2018). Co-cultures of Jurkat T-cells with C91-PL cells treated with cytochalasin D (5 μM, 24 h) or the solvent control DMSO served as controls. Cells were stained using mouse monoclonal antibodies anti-gag p19 (ZeptoMetrix Corporation) and anti-mouse AlexaFluor® 647-conjugated secondary antibodies (Life Technologies GmbH) as described earlier (Gross et al., 2016). Cells were discriminated by CMAC-staining (Jurkat: CMAC-positive; C91-PL: CMAC-negative) and their different size (FSC/SSC). The percentage of Gag-positive cells within CMAC-positive cells (Jurkat T-cells)

was examined to measure Gag transfer from C91-PL to Jurkat T-cells.

#### **RESULTS**

## COL4 Is Specifically Upregulated in HTLV-1-Infected T-cells

Viral biofilms (VBs) depict, next to formation of the virological synapse (VS), a fundamental role in HTLV-1 transmission via close cell-to-cell contacts (Pais-Correia et al., 2010). Several cellular factors have been identified to account for the formation of VB (Pais-Correia et al., 2010), but only little is known about the involvement of HTLV-1/Tax in regulating expression of these cellular components of the VB (Tarasevich et al., 2015). Thus, we re-evaluated microarray data we had performed earlier (Pichler et al., 2008; Kress et al., 2010) and analyzed the expression of the initially identified components of the VB (Pais-Correia et al., 2010) on their ability to be induced by HTLV-1/Tax. For this purpose, we compared the transcriptome of HTLV-1 positive (MT-2) or Tax-positive (Tesi) T-cells to respective HTLV-1 negative (postmitotic CD4<sup>+</sup>) or Tax-negative (Tesi/Tet) T-cells. Briefly, Tesi cells had been established by transforming human cord blood lymphocytes with a tetracyclinerepressible Tax gene using a rhadinoviral vector (Schmitt et al., 1998). Addition of tetracycline (Tet; 1 µg/ml, 10 days) to the culture medium leads to repression of Tax (Tesi/Tet) (Pichler et al., 2008; Kress et al., 2010). We could show that Agrin and Tetherin (BST2, bone marrow stromal antigen 2; CD317 antigen) are present in all cell culture systems independent of HTLV-1 infection or Tax-transformation (Table 1; see all probe sets in Supplementary Table 1). Fucosyltransferase 4 (FUT4), which is next to Fucosyltransferase 9 (FUT9) able to synthesize the carbohydrate sialyl LewisX (3-fucosyl-N-acetyllactosamine) (Nakayama et al., 2001) enriched in VBs (Pais-Correia et al., 2010), showed 2-3 fold elevated transcript levels in the presence of Tax only (Tesi vs. Tesi/Tet, MT-2 vs. CD4<sup>+</sup>, **Table 1**). Contrary, FUT9 was absent, suggesting that FUT4 is responsible for synthesis of sialyl LewisX in HTLV-1/Tax-positive cells. On the other hand, mRNA levels of Galectin-3 (LGALS3) were present, but only slightly upregulated in one cell culture model (MT-2 vs. CD4<sup>+</sup>, **Table 1**). Interestingly, throughout the group of Collagens type 1 to 5, only the heterotrimeric couple of COL4A1 and COL4A2 seemed to show elevated transcript levels (Table 1). Although also COL1A1 seemed to be upregulated in the presence of HTLV-1/Tax, COL1A2, the matching partner of COL1A1 to form the COL1A1-A1-A2 heterotrimer, could not be detected in any cellular system we tested. Therefore, we further focused on COL4A1 and COL4A2 only. Confirming microarray data by quantitative PCR (qPCR), in the HTLV-1 positive T-cell lines MT-2, C91-PL and HuT-102 detectable amounts of COL4A1 and COL4A2 as well as Tax were present (Figures 1A-C). An exception is the cell line C8166-45 which expresses Tax but not COL4A1 and COL4A2 (Figures 1A-C). This T-cell line is HTLV-1 positive yet Rex-deficient and impaired for expression of the structural proteins Gag and Env and therefore does not produce infectious viral particles (Bhat et al., 1993), which may serve as

TABLE 1 | Transcriptional expression of components of the viral biofilm.

Gene	Average signals <sup>a</sup>								
	Tax-positive		Tax-negative		Fold change <sup>b</sup>				
	MT-2	Tesi	CD4+	Tesi/Tet	MT-2 vs. CD4 <sup>+</sup>	p-value	Tesi vs. Tesi/Tet	p-value	Probe set
AGRIN	68	150	65	122	1	0.79	1	0.13	212285_s_at
AGRIN	(5)	(6)	(6)	(9)	n.a.		n.a.		217410_at
BST2	468	730	516	650	-1	0.29	1	0.51	201641_at
C0L1A1	263	161	(2)	65	272	0.06	2	0.17	1556499_s_at
C0L1A2	(2)	(2)	(2)	(2)	n.a.		n.a.		229218_at
C0L2A1	(5)	(13)	(10)	(11)	n.a.		n.a.		213492_at
C0L3A1	(1)	57	(3)	70	n.a.		-1	0.14	232458_at
C0L4A1	1780	360	(16)	92	109	< 0.01	4	< 0.01	211980_at
COL4A2	1325	624	(4)	233	331	< 0.01	3	0.06	211964_at
COL4A3	(1)	(7)	(1)	(7)	n.a.		n.a.		216896_at
COL4A4	(4)	(9)	(9)	(15)	n.a.		n.a.		241565_at
COL4A5	(2)	13	(1)	(14)	n.a.		-1	0.72	234387_at
COL4A6	(14)	(16)	(2)	21	n.a.		-1	0.26	1564654_at
COL4A6	(7)	(3)	(7)	(6)	n.a.		n.a.		210945_at
C0L5A1	(4)	(2)	(4)	(8)	n.a.		n.a.		212489_at
COL5A2	(8)	(4)	(18)	(8)	n.a.		n.a.		221730_at
COL5A3	(2)	(3)	(4)	(2)	n.a.		n.a.		218975_at
FUT4	148	90	71	31	2	< 0.01	3	0.03	209892_at
FUT9	(2)	(1)	(1)	(1)	n.a.		n.a.		207696_at
LGALS3	2043	4345	1014	4490	2	<0.01	-1	0.54	208949_s_at

<sup>&</sup>lt;sup>a</sup>Average signals were rounded to integers; values in brackets were deemed absent calls. <sup>b</sup>Values depicting the fold change of transcript expression were rounded to integers; p-values were rounded on two decimals; vs., versus; n.a., not applicable; red shaded, fold change  $\geq 2$ .

an explanation for the lack of COL4A1 and COL4A2 transcripts. As expected, all HTLV-1 negative T-cell lines tested (Jurkat, HuT-78, CCRF-CEM, Molt-4) proved absence of COL4A1, COL4A2 and Tax (Figures 1A-C). Tax protein expression in all HTLV-1 positive T-cells could be observed, with the typical additional Tax-Env fusion protein in MT-2 cells (Figure 1D). Corresponding to the presence or absence of COL4A1 and COL4A2 transcripts COL4 protein was detected on Western Blot level (Figure 1D). Since the microarray (Table 1) pointed out that all other members of the Collagen 4 family (COL4A3-COL4A6) showed absence of mRNA, we assumed that the Western Blot signal generated with the antibody targeting COL4 is specific for COL4A1 and COL4A2. Notably, only very little copy numbers of COL4A1 and COL4A2, as low as  $1*10^{-3}$ , are sufficient to induce robust COL4 protein expression, indicating great stability of this extracellular matrix protein. To prove uniqueness of COL4 upregulation by HTLV-1 in cellular transformation and tumor formation, we compared various tumor-derived B-cell lines on the presence of COL4 protein. B-cell lines from Burkitt lymphoma (BL; Bjab, Raji), Primary Effusion lymphoma (PEL; JSC-1, BC-3; Kaposi's sarcoma-associated herpesvirus-positive) as well as Hodgkin lymphoma (HL; KM-H2, L428, HDLM-2) consistently lacked COL4 protein in comparison to the HTLV-1 positive T-cell line MT-2 (Supplementary Figure 1). Hence, upregulation of COL4 is not a general hallmark of cellular transformation per se but is specific for HTLV-1 oncogenesis. Moving from cell culture into human, we analyzed ex vivo samples from HAM/TSP patients

in comparison to non-infected CD4<sup>+</sup> T-cells. No differences of *COL4A1* mRNA could be observed in CD8<sup>+</sup>-depleted PBMC between non-infected donors (CD4<sup>+</sup>-NI), asymptomatic carriers (AC) and slowly (TSP-L) or rapidly progressing patients (TSP-R) (**Figure 1E**). Slowly progressing HAM/TSP patients showed statistically significant elevated transcript levels of *COL4A2* compared to non-infected donors (CD4<sup>+</sup>-NI), asymptomatic carriers and rapidly progressing patients (**Figure 1F**). Although expression of *Tax* did not correlate with *COL4A2* since TSP-R but not TSP-L express high level of *Tax* (**Figure 1G**), we cannot exclude that induction of COL4 by the virus might indeed affect and modulate onset of HTLV-1 associated diseases.

# COL4 Protein Localizes to Extra- and Intracellular Compartments in HTLV-1 Positive T-cells

Collagens are proteins of the extracellular matrix (ECM) that are secreted upon translation and could be shown to contribute to formation of the VB (Schnoor et al., 2008; Pais-Correia et al., 2010). However, since antibodies recognizing collagens 1–5 were used in previous work (Pais-Correia et al., 2010), the exact subcellular localization of COL4 protein remained unclear. Therefore, we performed confocal microscopy comparing permeabilized and non-permeabilized HTLV-1 positive T-cells that had been spotted on epoxy-resin coated glass slides. Specifically, we costained COL4 protein

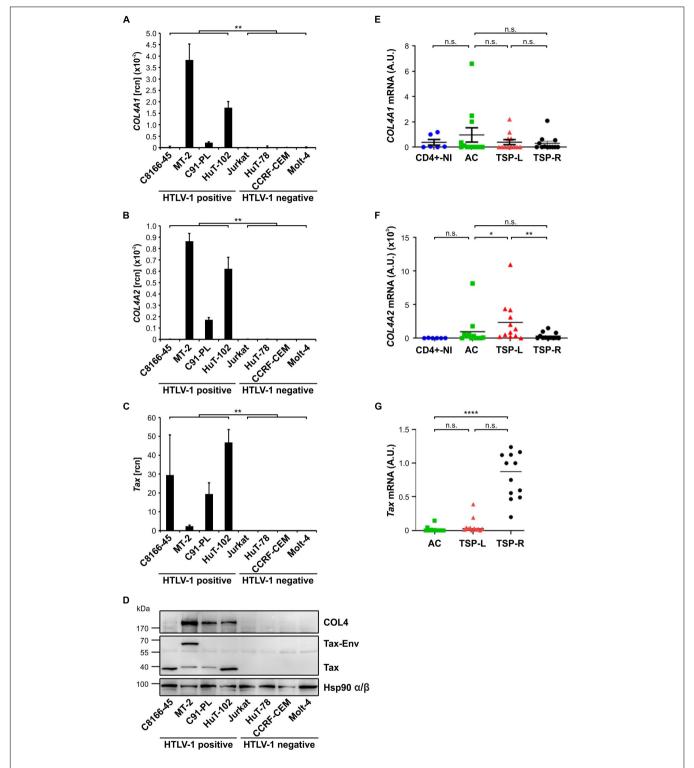


FIGURE 1 | COL4 is specifically upregulated in HTLV-1 positive T-cell lines as well as COL4A2 in ex vivo HAM/TSP patient samples. (A-D) Analysis of COL4 expression in HTLV-1 positive (C8166-45, MT-2, C91-PL, HuT-102) and HTLV-1 negative T-cell lines (Jurkat, HuT-78, CCRF-CEM, Molt-4). (A-C) mRNA of (A) COL4A1, (B) COL4A2, and (C) Tax was quantified by qPCR in the respective HTLV-1 positive and negative T-cell lines. The mean relative copy numbers (rcn), normalized on ACTB, of three individual experiments  $\pm$  SE are depicted. Values were compared using two-tailed Mann–Whitney U test (\*\*p < 0.01). (D) COL4 protein was detected in HTLV-1 positive and negative T-cell lines by means of Western Blot. Staining of Tax and Hsp90  $\alpha/\beta$  served as expression or loading control, respectively. (E) COL4A1, (F) COL4A2, and (G) Tax transcripts were quantified by qPCR comparing mRNA isolated from patient samples of HTLV-1 asymptomatic carriers (AC), slowly progressing HAM/TSP patients (TSP-L), rapidly progressing HAM/TSP patients (TSP-R), and uninfected controls (CD4+-NI). Statistical analysis was performed applying two-tailed Mann–Whitney U test (\*p < 0.05; \*\*p < 0.01; \*\*\*\*\*p < 0.0001; n.s., not significant).

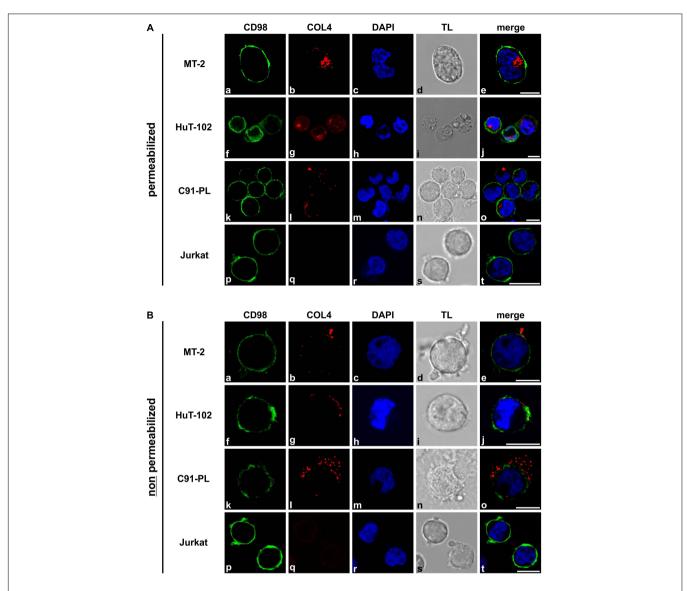


FIGURE 2 | COL4 protein localizes to extra- and intracellular compartments in HTLV-1 positive T-cells. (A,B) Immunofluorescence staining was performed in the HTLV-1 positive T-cell lines MT-2, HuT-102 and C91-PL and in the HTLV-1 negative T-cell line Jurkat. Cells were spotted on epoxy-resin coated glass slides. The plasma membrane was visualized in green by using a mouse primary antibody against CD98, together with the secondary antibody anti-mouse Alexa Fluor<sup>®</sup> 488. COL4 protein was depicted in red, employing a COL4 targeting rabbit primary antibody that was recognized by the secondary antibody anti-rabbit Alexa Fluor<sup>®</sup> 647. DAPI staining of nuclei (blue) and transmitted light (TL) served as controls. Cells in (Aa-t) were permeabilized before antibody treatment, cells in (Ba-t) were left without permeabilization showing extracellular COL4 protein only. The scale bars represent 10 μm.

with the same antibodies as used for western blot analysis (Figure 2, red) together with the plasma membrane marker CD98 (Figure 2, green), and with the DNA-tracer DAPI (Figure 2, blue) to be able to discriminate between extra- and intracellular portions of COL4 protein. Upon permeabilization, the HTLV-1 positive T-cell lines MT-2, HuT-102 and C91-PL proved to exhibit strong intracellular signals for COL4 protein whereas the HTLV-1 negative T-cell line Jurkat showed no COL4 protein (Figures 2Ab,g,l,q). In T-cells that have not been permeabilized, staining of COL4 protein could as well be detected in all HTLV-1 positive but not in Jurkat T-cells (Figures 2Bb,g,l,q). However, taking into account the signal of

the plasma membrane marker CD98 (Figures 2Ba,f,k) and that cells had not been permeabilized, COL4 protein localized only to extracellular compartments of HTLV-1-infected cells in this setting (Figures 2Be,j,o). Of note, permeabilization of the cells may have negative impact on the detection of extracellular COL4 by destruction of collagenous structures since extracellular COL4 was nearly undetectable upon permeabilization (Figures 2Ab,g,l) in this setting. Similar results were obtained repeating the experiment staining HTLV-1 positive T-cells for Collagens 1 to 5, confirming findings obtained from Pais-Correia and colleagues (Supplementary Figure 2; Pais-Correia et al., 2010). Manual counting of cells confirmed that the number of COL4 or

COL1-5 positive T-cells comparing permeabilized and non-permeabilized cells only slightly differed throughout the different cell types. However, upon permeabilization, the proportion of strong COL4 and COL1-5 signals vastly increased amongst all HTLV-1 positive T-cells (data not shown). In summary, COL4 protein localizes to both, intra- and extracellular, compartments in HTLV-1 positive cells.

# Transient Expression of Tax Is Sufficient to Induce *COL4A1* and *COL4A2* Transcripts but Not COL4 Protein

The HTLV-1 oncoprotein Tax interferes with several cellular genes in the context of viral transmission (Nejmeddine et al., 2005; Chevalier et al., 2014; Gross et al., 2016; Gross and Thoma-Kress, 2016). Therefore, we asked whether Tax protein is responsible for induction of COL4. We transiently expressed Tax protein in the two T-cell lines Jurkat and CCRF-CEM by electroporation and performed qPCR and western blot 48 h after transfection. In the presence of Tax, COL4A1 as well as COL4A2 transcripts are indeed significantly elevated (Figures 3A,B). However, in spite of robust Tax protein expression, no COL4 protein could be detected next to the MT-2 control stain on Western Blot level (Figure 3C). Although we found that Tax alone is sufficient to upregulate COL4A1 and COL4A2 transcripts, we asked whether the extent of this upregulation could be different in the context of the HTLV-1 genome. However, comparable transcript levels of COL4A1 and COL4A2 were induced by either the HTLV-1 packaging plasmid pCMV-HT1-ΔEnv carrying parts of the HTLV-1 genome except Env and parts of the 5'LTR, or by Tax expression plasmids in Jurkat T-cells (**Figure 3D**). Yet, it cannot be excluded that another viral protein may affect COL4 transcript and protein expression. This is supported by the notion that the Rex-deficient cell line C8166-45 showed expression of Tax, but not of COL4 (Figures 1A-D). Our current findings argue against a role of Rex in regulating COL4 expression since co-expression of Rex-GFP and Tax did not further enhance COL4A1 and COL4A2 transcript levels (Supplementary Figure 3A), nor did co-expression of Rex-GFP induce COL4 protein (Supplementary Figure 3B). To confirm results obtained from transient transfection systems, we moved to JPX9/JPX9M, Tax-inducible T-cells which carry a Tax transgene controlled by a cadmium chloride (CdCl<sub>2</sub>)-inducible promoter (Ohtani et al., 1989). As corresponding control, JPX9M cells were employed, a T-cell line being able to induce a functionally inactive Tax mutant protein with a premature stop codon. At 0, 24, and 48 h after induction of Tax expression JPX9 and JPX9M cells were analyzed for expression of COL4 (Figures 3E-H). Confirming results obtained from Jurkat and CCRF-CEM T-cells, expression of Tax (Figure 3G) led to a time-dependent induction of COL4A1 and COL4A2 in JPX9 cells, reaching similar copy numbers as after transient transfection 48 h after addition of CdCl<sub>2</sub> (**Figures 3E,F**). This effect was likely due to presence of Tax protein as expression of a Tax mutant variant in JPX9M T-cells was not sufficient to induce COL4A1 or COL4A2 (Figures 3E,F). However, in spite of COL4A1 and COL4A2 transcript induction by Tax, no COL4 protein could be detected in Western Blot compared to

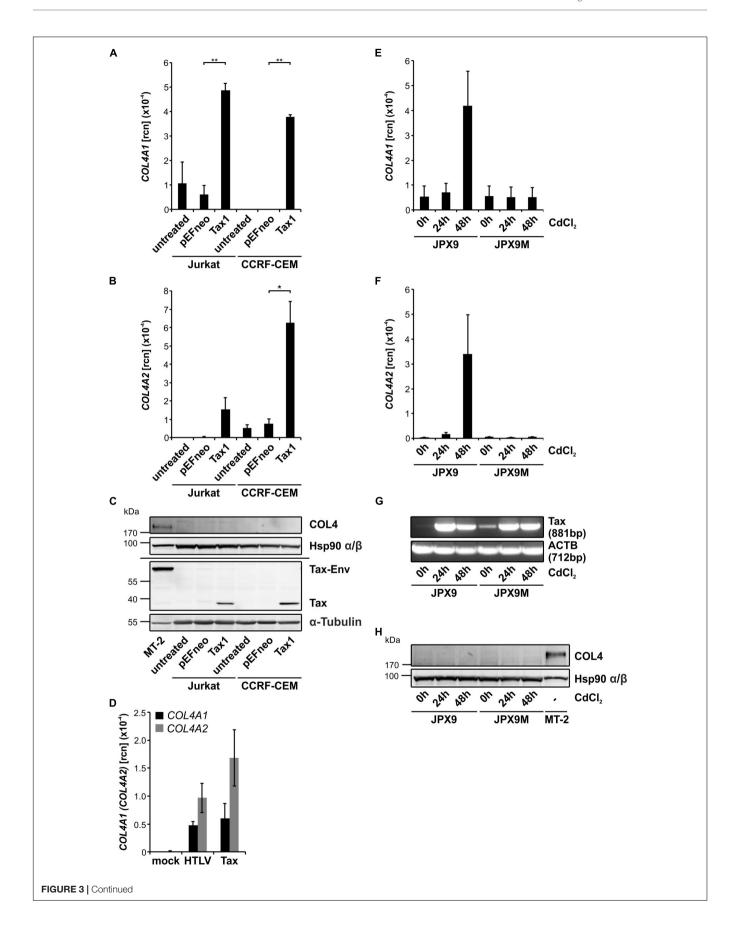
MT-2 cells (**Figure 3H**). Therefore, transient expression of Tax is only sufficient to induce *COL4A1* and *COL4A2* transcripts, but additional mechanisms are required to promote presence of COL4 protein.

# Tax Slightly Transactivates the Bi-Directional *COL4A1-/COL4A2-* Promoter

Expression of COL4 protein is not only tissue-dependent and typically absent in T-cells but is also highly regulated at several levels, including RNA stability, alternative splicing and posttranslational modifications (Khoshnoodi et al., 2008). Since COL4 expression is upregulated in HTLV-1-infected T-cells (Figures 1A-D) and Tax is sufficient to induce COL4A1 and COL4A2 transcripts (Figures 3A,B,D,E) we were interested in the question how Tax interferes with COL4A1 or COL4A2 regulation on genomic level. Therefore, we analyzed the short and bidirectional promoter of COL4A1 and COL4A2 for the presence of transcription factor binding sites by bioinformatics (Cartharius et al., 2005; Turner et al., 2015). We could not identify DNA sequences specific for promoter activation by Tax via the CREB pathway as were for example Tax responsive elements (TRE) (Shimotohno et al., 1986; Beimling and Moelling, 1992; Suzuki et al., 1993). However, we detected common and well described binding sites for transcription factors as Specificity Protein 1 (SP1) or Nuclear Factor 1 (NF1) (Figure 4A). Hence, we asked whether induction of COL4A1 and COL4A2 was due to indirect promoter activation by Tax. As a control, we made use of a luciferase-based reporter construct under the control of the U3R-fragment of the HTLV-1 promoter that is known to be transactivated by Tax via the CREB pathway (Xu et al., 1990; Mann et al., 2014). Upon co-transfection in Jurkat T-cells, Tax was able to significantly increase luciferase reporter activity by U3R promoter activation compared to an empty vector control as expected (Figure 4B). Performing the same experiment with luciferase-based reporter constructs specific for the COL4A1 or the COL4A2 promoter, co-expression of Tax in Jurkat T-cells in comparison to the respective empty vector control revealed that Tax augmented luciferase activity of the COL4A1 and COL4A2 reporter about 1.5-fold, even reaching significance for COL4A2 (**Figure 4C**). This slight transactivation of the *COL4A1/COL4A2* promoter by Tax is supportive of a more complex regulation of type IV collagen gene expression. Tax protein expression during the reporter assays remained detectably constant and was checked by Western Blot analysis (Figure 4D). Summarizing, in spite of lacking Tax-specific binding sites, Tax is able to transactivate the COL4A2 and, to a less extent, the COL4A1 promoter potentially providing a mechanistic explanation for induction of COL4A1 and COL4A2 transcripts.

#### Continuous Expression of Tax, but Not of the Herpesvirus Ateles Oncoprotein Tio, Is Necessary to Induce and Maintain COL4 Protein Expression

Although Tax is able to induce COL4A1 and COL4A2 transcripts, transient expression of Tax was not sufficient to induce COL4



185

FIGURE 3 | Transient expression of Tax is sufficient to induce COL4A1 and COL4A2 transcripts, but not COL4 protein. (A) COL4A1 and (B) COL4A2 transcript levels were quantified by qPCR upon transient expression of 100 μg Tax expression plasmid (pEFneo-Tax1) or empty vector (pEFneo) in  $10*10^6$  Jurkat and CCRF-CEM T-cells. The mean relative copy numbers (rcn), normalized on ACTB, of three independent experiments  $\pm$  SE are depicted. Student's t-test was conducted for statistical analysis (\*p < 0.05; \*\*p < 0.01). (C) Immunoblotting for COL4 protein was performed in mock- (pEFneo) and Tax-transfected T-cell lines Jurkat and CCRF-CEM (100 μg DNA,  $10*10^6$  cells) as well as for MT-2 cells as positive control. Tax and GAPDH or  $\alpha$ -Tubulin staining were carried out as controls. (D) COL4A1 and COL4A2 transcript levels were quantified by qPCR upon transient expression of 50 μg Tax expression plasmid (pCTax), 17.5 μg of the HTLV-1 packaging plasmid pCMV-HT1- $\Delta$ Env (HTLV) supplemented with 32.5 μg empty vector (pcDNA), or 50 μg empty vector (pcDNA) alone in  $5*10^6$  Jurkat cells. The mean relative copy numbers (rcn)  $\pm$  SE, normalized on ACTB, of one representative experiment is depicted. (E,F) mRNA of (E) COL4A1 and (F) COL4A2 transcripts was measured by qPCR in the Tax-inducible T-cell lines JPX9 and JPX9M at the indicated time points after induction of Tax wildtype protein (JPX9M) by addition of 20 μM  $CdCl_2$ . The mean relative copy numbers (rcn), normalized on ACTB, of three independent experiments  $\pm$  SE are depicted. (G) For qualitative analysis, Tax and ACTB transcripts were amplified by RT-PCR from JPX9 and JPX9M cell lysates at the indicated time points after induction of Tax wildtype (JPX9M). (JPX9M) or Tax mutant protein (JPX9M). (JPX9M) or Tax mutant protein (JPX9M). (JPX9M) or Tax mutant (JPX9M) protein expression. MT-2 cells served as positive control for COL4 protein expression.

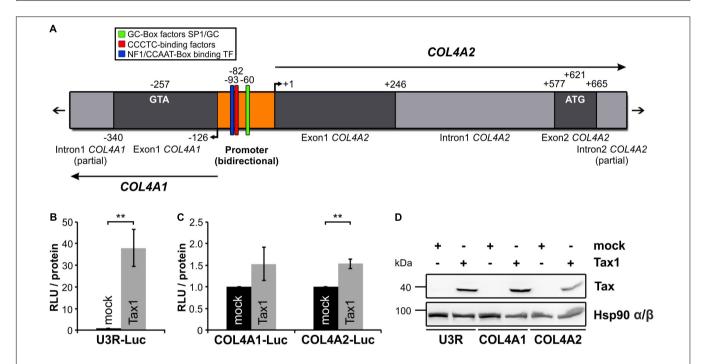


FIGURE 4 | Tax transactivates the COL4A1 and COL4A2 promoter. (A) Schematic representation of the COL4A1/2 genomic organization. In the shared and bidirectional COL4A1/2 promoter region, putative genetic sites of GC-Box factors Specificity Protein 1 (SP1)/GC, CCCTC-binding factors and Nuclear Factor 1 (NF1)/CCAAT-Box binding transcription factors are indicated. Bioinformatic analysis was performed using the MatInspector software tool. (B–D) Conduction of luciferase-based reporter assays. (B,C)  $5*10^6$  Jurkat T-cells were co-transfected with a mock (pEFneo) or Tax expression vector (pEFneo-Tax1; 30 μg DNA) together with (B) a luciferase reporter vector for the U3R promoter from the HTLV-1 5′LTR region (U3R-Luc; 20 μg DNA) as a positive control, or (C) luciferase reporter vectors specific for the COL4A1 or COL4A2 promoter region (COL4A1-Luc, COL4A2-Luc; 20 μg DNA). Values were normalized on protein content and the luciferase background activity measured by co-expression of a promoter-less luciferase vector (pGL3-Basic). The mean of five independent experiments ± SE is depicted and Student's t-test was performed (\*\*p < 0.01) comparing the different reporter vectors with their respective mock control. (D) Immunoblot staining of Tax and Hsp90 α/β protein served as expression control.

protein (**Figures 3C,F**). Thus, we asked whether an endured expression time of Tax protein leads to induction of COL4. As prolonged transient Tax expression in T-cells tends to lead to apoptosis after a certain period of time (Nicot and Harrod, 2000), we made use of the established Tax-transformed T-cell lines Tri, Tesi and TAXI-1 (Grassmann et al., 1992; Schmitt et al., 1998). These cell lines are derived from primary human T-cells immortalized by an expression cassette for Tax, which was transduced with a rhadinoviral vector (recombinant Herpesvirus saimiri). Compared to the control T-cell lines Jurkat (HTLV-1/Tax neg., COL4 neg.) and MT-2 (HTLV-1/Tax pos., COL4 pos.),

robust expression of COL4 protein was a unique phenotype of the Tax-transformed T-cell lines Tri, Tesi and TAXI-1, suggesting that continuous expression of Tax can induce COL4 protein (**Figure 5A**). Due to different Tax protein expression levels in Tri, TAXI-1, and Tesi, varying from very low levels near the detection limit to strong, we also measured *Tax* transcripts confirming that all Tax-transformed cell lines express Tax although at different levels (**Figure 5B**). To exclude that T-cell transformation by any viral protein or the rhadinoviral vectors used to generate Tri, Tesi and TAXI-1 cells induces COL4 protein, we additionally analyzed CD4<sup>+</sup> T-cell lines that were transformed by the Herpesvirus

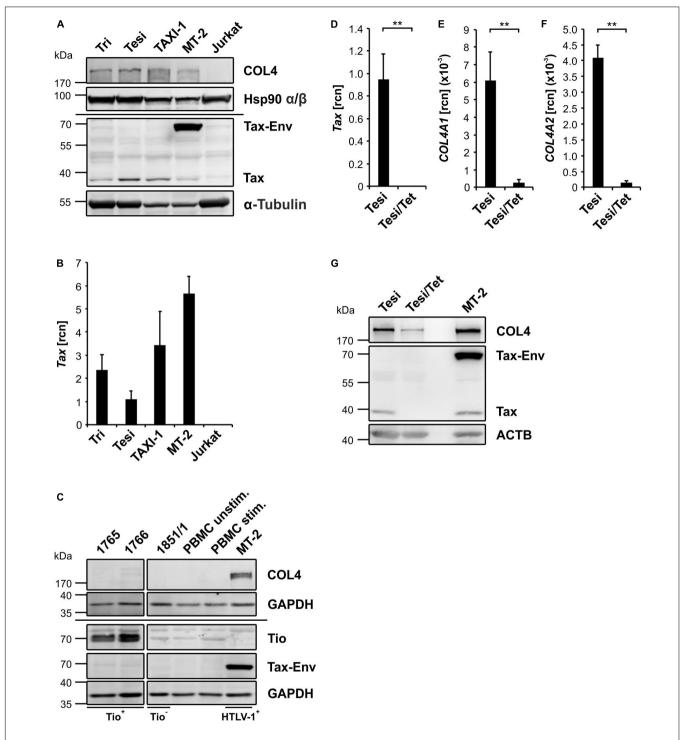


FIGURE 5 | Continuous expression of Tax is necessary to induce and maintain COL4 protein expression. (A,C) Immunoblot of COL4 protein was carried out in (A) Tax-transformed T-cell lines (Tri, Tesi, TAXI-1) and (C) T-cell lines transformed by the oncoprotein Tio, encoded by Herpesvirus ateles (1765, 1766). The HTLV-1 positive T-cell line MT-2 served as positive control. In contrast, negative controls were the Tax and Tio negative T-cell lines Jurkat and 1851/1, as well as peripheral blood mononuclear cells (PBMC), that were either left unstimulated (unstim.) or stimulated (stim.) with 50 U/ml IL-2 together with 2 μg/ml PHA overnight followed by prolonged incubation with 50 U/ml IL-2 only. Staining of Tax, Tio and GAPDH were performed as control. (B) qPCR was performed to analyze abundance of Tax transcripts in Tri, Tesi, TAXI-1 and MT-2 T-cell lines. The mean relative copy numbers (rcn), normalized on ACTB, of one representative experiment ± SE are depicted. (D-F) Transcript levels of (D) Tax, (E) COL4A1, and (F) COL4A2 were detected in the Tax-transformed T-cell line Tesi in the presence of Tax (Tesi) or after repression of Tax for 10 days by addition of 1 μg/ml tetracycline (Tesi/Tet). The mean relative copy numbers (rcn), normalized on ACTB, of three independent experiments ± SD are shown. Student's t-test was conducted for statistical analysis (\*\*p < 0.01). (G) COL4 and Tax protein were detected in Tax-transformed T-cell lines expressing (Tesi) or repressing (Tesi/Tet) Tax protein and, as a control, in the HTLV-1 positive T-cell line MT-2. β-actin (ACTB) served as loading control.

ateles oncoprotein Tio, which is - similar to Tax - a potent inducer of NF-κB signaling (Heinemann et al., 2006; de Jong et al., 2010). For this purpose, we analyzed peripheral blood lymphocytes that were transduced by a Tio-expressing chimeric Herpesvirus saimiri resulting in the cell lines 1765 and 1766 (Albrecht et al., 2004) and the Tio-negative control cell line 1851/1 (Figure 5C). Compared to the Tio-, Tax- and COL4-negative cell line 1851/1 as well as primary stimulated and unstimulated PBMC, the HTLV-1 positive T-cell line MT-2 exhibited strong Tax-Env fusion and COL4 protein signals (Figure 5C). In contrast, albeit revealing clear Tio oncoprotein expression, none of the Tio-transformed cell lines 1765 and 1766 showed enhanced COL4 protein expression as MT-2 (Figure 5C). This finding further proved the uniqueness of Tax oncoprotein to induce COL4 protein in transformed T-cells and excluded that rhadinoviral Herpesvirus saimiri vectors induce COL4. To check whether Tax is important for maintenance of COL4 protein, we employed the Tesi/Tet cellular system, a Tax-transformed T-cell line with Tax expression (Tesi) being able to be repressed in presence of tetracycline (Tesi/Tet) (Schmitt et al., 1998). Tesi cells presented robust Tax transcript levels, which immediately dropped to almost zero after incubation with tetracycline indicating that the Tax repression system profoundly worked (Figure 5D). In the presence of Tax, COL4A1 and COL4A2 transcripts could readily be detected in Tesi cells. Intriguingly, repression of Tax in Tesi/Tet cells consecutively led to a nearly complete decline not only of COL4A1 but also COL4A2 transcripts (Figures 5E,F). In Western Blots, Tesi T-cells exhibited strong Tax as well as COL4 protein expression (Figure 5G). However, in Tesi/Tet, Tax protein almost vanished and COL4 protein decreased dramatically. Prolonged presence of Tax protein is therefore not only sufficient for induction of COL4 protein, but also necessary for maintenance in T-cells.

#### COL4 and Gag p19 Partially Co-localize and Accumulate at the Virological Synapse and Can Be Transferred to HTLV-1 Negative T-cells in Co-culture

Since COL4 is upregulated in productively HTLV-1-infected T-cells, we next checked on implications of COL4 induction on the VB. We performed confocal laser scanning microscopy analyzing distribution of COL4 and Gag protein in the HTLV-1 positive T-cell lines MT-2 and C91-PL (Figure 6A), which had been spotted on poly-L-lysine coated coverslips. C8166-45 cells, which do not produce viral particles (Bhat et al., 1993), served as negative control for Gag and COL4 (Figures 1A-D). We found that COL4 (red) is predominantly expressed near or at the plasma membrane (Figures 6Ac,h), and dotlike spread all over the cell (Figure 6Ac). Moreover, Gag (green), as detected by Gag p19-specific antibodies, is strongly expressed in MT-2 and C91-PL cells and localizes to the plasma membrane (Figures 6Ac,h), while it is absent in C8166-45 cells (Figure 6Am). A partial overlap between Gag and COL4 fluorescence signals (yellow) could be observed as indicated by white arrows (Figures 6Ae,j, inset). For further analysis in more detail, MT-2 cells expressing huge amounts of Gag protein were not suitable. Therefore, we switched the cellular system to MS-9 cells, which is an HTLV-1 immortalized T-cell line containing only one proviral genome, thus expressing reasonable amounts of Gag protein (Shuh et al., 1999). To analyze involvement of COL4 in VB formation during formation of the VS and virus transfer, we performed confocal laser scanning microscopy of MS-9 cells in co-culture with Jurkat T-cells (Figure 6B). The latter cell line was pre-stained with CellTracker<sup>TM</sup> Blue CMAC (7-Amino-4-Chlormethylcumarin; CMAC) as described previously (Donhauser et al., 2018) to discriminate between uninfected Jurkat T-cells (blue) and infected (non-blue) MS-9 cells. Briefly, CMAC is a dye that turns membrane-impermeable having crossed the plasma membrane. Cells were co-cultured (ratio 1:1) for 30 min at 37°C, spotted on poly-L-lysine coated coverslips, fixed and stained. Interestingly, examination of cellcell contacts between infected MS-9 donor cells and Jurkat acceptor cells, stained in blue, revealed that the virological synapse was formed concentrating both COL4 (red) as well as Gag protein (green; detected with Gag p19-specific antibodies) toward the uninfected Jurkat T-cell (Figure 6B) in 50% of all VS detected. Further, Gag accumulated in clusters at the VS which are reminiscent of VBs (Pais-Correia et al., 2010), and even a partial overlap between Gag and COL4 protein could be observed as a yellow fluorescence signal at the virological synapse as indicated by a white arrow (Figure 6Be, inset). The partial co-localization was better determined by defining a region of interest (ROI) covering overlapping fluorescence signals which proved to have similar distributions (Supplementary Figure 4). Thus, COL4 as a component of the extracellular matrix may provide the scaffold for the attachment and concentration of budding viral particles into the VB. Going further, we were not only able to detect COL4 protein on HTLV-1 positive T-cells oriented toward target cells but we could also see, in some cases, patches of COL4 being transferred to HTLV-1 negative Jurkat acceptor cells (Figure 6C). In two examples, co-cultured MS-9 donor and Jurkat target cells are depicted showing parts of COL4 protein located on pre-stained COL4 negative Jurkat T-cells as indicated by black arrows (Figure 6Ce, inset j). Moreover, not only transferred COL4 but also Gag protein could be visualized presumably indicating transfer of VBs (Figure 6Cj, white arrow). Quantitative evaluation of imaging data revealed that after 20 min of co-culture, transfer of Gag could be observed in 14% of co-cultured Jurkat T-cells, suggesting that these cells are HTLV-1-infected (Figure 6D, black bars). A similar proportion of Jurkat T-cells (12%) received both Gag and COL4 (**Figure 6D**, hatched bars). Over time (t = 50 min), the proportion of cells receiving only Gag did not increase (13%), however, the frequency of Gag-positive cells receiving both Gag and COL4 slightly increased to 19% (p > 0.05), suggesting that receiving COL4 may be advantageous for getting HTLV-1-infected after pro-longed co-culture. Since we also observed patches of COL4 in Jurkat T-cells following co-culture with HTLV-1-infected MS-9 cells (Figure 6C), we next quantitated the frequency of COL4 transfer (Figure 6D). After 20 min of coculture, 8% of co-cultured Jurkat T-cells received COL4 protein only (Figure 6D, gray bars). Interestingly, with a prolonged co-culture of 50 min, the frequency of COL4-positive Jurkat

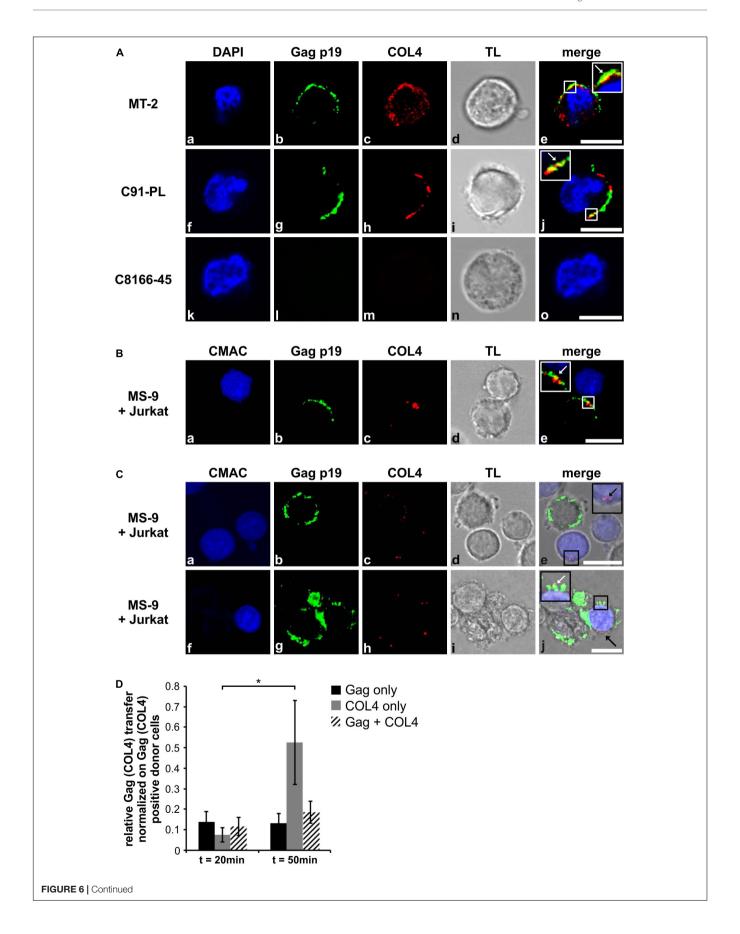


FIGURE 6 | COL4 and Gag p19 partially co-localize and accumulate at the virological synapse and can be transferred to HTLV-1 negative T-cells in co-culture. (Aa-o) Immunofluorescence analysis was conducted in the HTLV-1 positive T-cell lines MT-2, C91-PL and C8166-45 spotted on poly-L-lysine coated coverslips. Permeabilized cells were stained with primary antibodies mouse anti-Gag p19 and the secondary antibody anti-mouse Alexa Fluor<sup>®</sup> 488 (green), as well as rabbit anti-COL4 and the secondary antibody anti-rabbit Alexa Fluor<sup>®</sup> 555 (red). DAPI staining of nuclei and transmitted light (TL) served as control. Insets show enlarged areas. A white arrow indicates partial co-localization of Gag and COL4 protein (yellow). The scale bar represents 10 μm. (Ba-e,Ca-j) Jurkat T-cells that had been prestained with CellTracker<sup>TM</sup> Blue 7-Amino-4-Chlormethylcumarin (CMAC; 45 min, 20 μM, 37°C) were co-cultured with HTLV-1-infected MS-9 T-cells at a ratio of 1:1 for 20 or 50 min on poly-L-lysine coated coverslips. Following permeabilization, cells were stained with primary antibodies mouse anti-Gag p19 and the secondary antibody anti-mouse Alexa Fluor<sup>®</sup> 488 (green), as well as rabbit anti-COL4 and the secondary antibody anti-rabbit Alexa Fluor<sup>®</sup> 555 (red). Transmitted light served as control. Insets show enlarged areas. (Ba-e) A white arrow indicates partial co-localization of Gag and COL4 protein (yellow). (Ca-i) Black arrows indicate transferred COL4 protein from HTLV-1 positive MS-9 to Jurkat T-cells, a white arrow indicates partial co-localization of transferred Gag and COL4 protein (yellow). The scale bars represent 10 μm. (D) Manual counting was performed to quantify Gag and COL4 transfer from MS-9 donor cells to Jurkat acceptor cells. Gag only (black bars), COL4 only (gray bars) or Gag and COL4 (hatched bars) double positive Jurkat cells after 20 or 50 min of co-culture were determined and normalized on the number of Gag and/or COL4 positive MS-9 donor cells and on the ratio of Jurkat acceptor to MS-9 donor cells.

T-cells significantly increased to 53% (**Figure 6D**, gray bars) suggesting that transfer of COL4 between HTLV-1-infected cells to uninfected cells also occurs independent of virus transfer and dominates over the transfer of Gag at late time points post co-culture. Gag transfer appears to take place and be saturated very fast upon formation of cell-cell contacts whereas transfer of COL4 seems to be critical at later time points, indicating that Gag and COL4 exert mutual but also separate functions. Thus, we propose that COL4 protein plays a role not only in formation of the VS by being concentrated toward the cell-cell contact but that COL4 is also involved in formation of the VB and transfer to HTLV-1 negative target cells.

#### Repression of COL4 Protein in Chronically Infected C91-PL Cells Impairs HTLV-1 Transfer to Co-cultured T-cells

To study the impact of COL4 on HTLV-1 cell-to-cell transmission, we generated COL4 knockouts in the chronically HTLV-1-infected T-cell line C91-PL (Figure 7). This cell line expresses high amounts of COL4 (Figure 1D), partially co-localizing with HTLV-1 Gag (Figure 6Aj), and produces infectious viruses and VB (Alais et al., 2015). Briefly, we stably transduced C91-PL cells with lentiviral CRISPR vectors carrying either guide RNAs targeting COL4A1, COL4A2 or a scrambled guide RNA (scramble). First, we verified the knockout of COL4 in C91-PL cells by western blot and qPCR. We could show that targeting COL4A2 resulted in a sharp decrease of COL4 protein and COL4A2 mRNA compared to control cells (scramble), while targeting COL4A1 had only little impact on COL4 expression (Figures 7A,B). Interestingly, repression of COL4 did neither impair expression of Gag p55, processing to Gag p19 (Figure 7A), nor release of Gag p19 (Figure 7C) in chronically infected C91-PL cells. Since measuring of virus release by ELISA only quantitates virus-like particles and not necessarily infectious particles, we decided to measure HTLV-1 cell-to-cell transmission also directly by a flow cytometry based assay that allows monitoring of Gag transfer to target cells (Chevalier et al., 2014; Gross et al., 2016). For this purpose, we co-cultured C91-PL cells with uninfected Jurkat T-cells that had been pre-stained with CellTracker<sup>TM</sup> Blue CMAC, which is retained in living cells for several generations and transferred

to daughter cells, but not to neighboring cells (King et al., 2004; Donhauser et al., 2018). After 1 h of co-culture, cells were stained for Gag using anti-Gag p19 antibodies and flow cytometry was performed to discriminate cells according to the CMAC-staining into CMAC-negative donor cells (C91-PL) and CMAC-positive acceptor cells (Jurkat) and to determine the number of newly infected, Gag p19-positive Jurkat T-cells (Figure 7D). Flow cytometry revealed that repression of COL4A2 in C91-PL cells led to a significant reduction of HTLV-1 transfer to target cells as Gag p19-positive Jurkat T-cells were only 71% compared to the scramble control (Figure 7E). C91-PL cells pre-treated with cytochalasin D, which impairs actinpolymerization and thus, virus transmission, served as assay control (Figure 7E). Overall, our data show that COL4 affects Gag transfer between chronically infected T-cells and acceptor T-cells, while release of virus-like particles is unaffected by COL4 in this cell system, indicating an important role of COL4 in HTLV-1 cell-to-cell transmission.

#### DISCUSSION

HTLV-1 spreads at the virological synapse (VS), a virus-induced specialized cell-cell contact, by polarized budding into synaptic clefts (Igakura et al., 2003), and by cell surface transfer of viral biofilms (VBs) (Pais-Correia et al., 2010). In this study we shed further light on the role of the regulatory protein Tax in formation of the VB. We report that collagen IV (COL4), especially the collagen chains COL4A1 and COL4A2, are not only structural components of the basement membrane, but are also part of the VB and might thus be important for anchoring of HTLV-1 virions to the infected cell and for HTLV-1 transmission. Our study extends earlier work by Pais-Correia et al. (2010), who found collagens enriched in the VB in HTLV-1-infected cells, but randomly distributed in uninfected cells using pan-collagen antibodies, which recognize collagens 1-5. We specified in this study that COL4 is part of the VB and regulated by Tax. However, in our study using the same antibodies as Pais-Correia et al. (2010) (Supplementary Figure 2), we could not detect any COL1-5-specific signal in uninfected Jurkat T-cells, but only in HTLV-1-infected T-cells. Using COL4-specific antibodies we could neither detect COL4 in uninfected Jurkat T-cells nor in PBMCs, but only

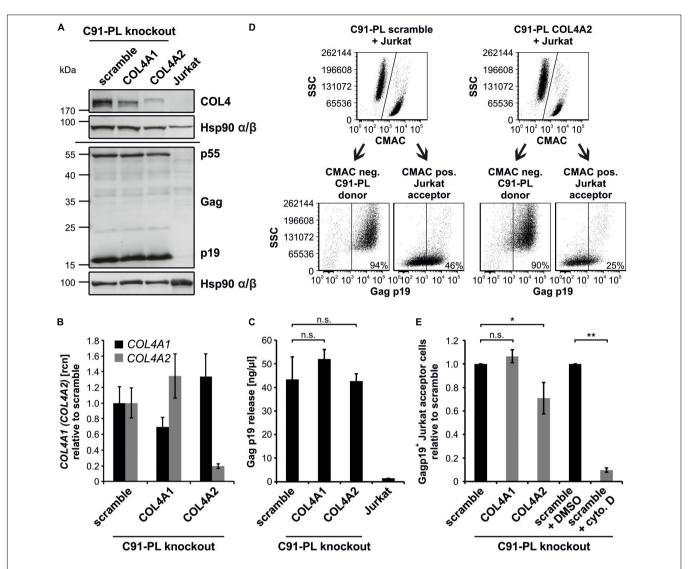


FIGURE 7 | Repression of COL4 protein impairs virus transmission to uninfected T-cells, but not Gag processing and virus release in chronically infected C91-PL cells. (A) Western Blot analysis to detect COL4 as well as Gag p55 precursor and processed Gag p19 matrix protein was carried out in C91-PL cells stably transduced either by a CRISPR control vector (scramble) or vectors to generate a knockout for *COL4A1* or *COL4A2*. Hsp90 α/β served as loading control, lysates of Jurkat T-cells as negative staining control. (B) qPCR was performed to determine expression levels of *COL4A1* and *COL4A2* mRNA in transduced C91-PL cell lines from (A). The mean relative copy numbers (rcn), normalized on *ACTB* and the C91-PL scramble control cell line, of one representative experiment ± SD are shown. (C) The amount of Gag p19 protein in the supernatant of transduced C91-PL cell lines from (A) was assessed by Gag p19 ELISA. The mean of five independent experiments ± SD is depicted and was compared using Student's *t*-test (n.s.; not significant). Jurkat T-cells were employed as negative control. (D,E) Jurkat T-cells, that had been prestained with CellTracker<sup>TM</sup> Blue 7-Amino-4-Chlormethylcumarin (CMAC), were co-cultured with stably transduced C91-PL cell lines from (A) at a ratio of 1:1 for 1 h. The transfer of Gag p19 to Jurkat acceptor cells was measured by flow cytometry using the primary antibody mouse anti-gag p19 and the secondary antibody anti-mouse Alexa Fluor® 647. (D) The representative gating strategy for co-cultures of scramble and COL4A2 knockout C91-PL with Jurkat cells reveals discrimination between CMAC-positive Jurkat acceptor T-cells and CMAC-negative C91-PL donor cells, further detecting Gag p19 in the distinct cell populations. (E) The amount of Gag p19 positive Jurkat acceptor cells normalized on the scramble C91-PL control cells of three independent experiments ± SD is depicted and was compared using Student's *t*-test (n.s., not significant; \* $^{*}$   $^{*}$   $^{*}$   $^{*}$   $^{*}$   $^{*}$   $^{*}$   $^{*}$   $^$ 

in the presence of HTLV-1 infection. Thus, it cannot be excluded that Pais-Correia detected additional collagens, like Collagen 1 in their study (Pais-Correia et al., 2010), which is detected by the pan -collagen antibody and whose alpha1 chain had been described to be transcriptionally induced by Tax in earlier work (Munoz et al., 1995) and in our microarray (**Table 1**).

Having found that COL4 upregulation is a unique feature of HTLV-1-infected cells with exception of the Rex-deficient cell line C8166-45 (Bhat et al., 1993), we searched for the mechanism of COL4 upregulation. In contrast to a variety of transformed B- and T-cell lines, COL4 protein was only upregulated in presence of continuous Tax-expression in either HTLV-1-infected or Tax-transformed T-cell lines suggesting that Tax is sufficient to induce

COL4 expression, with the exception of C8166-45 cells. Thus, it cannot be excluded that another viral protein or a host factor that is absent in C8166-45 cells co-regulates COL4 expression together with Tax. Our study argues against a role of the viral Rex protein in regulating COL4 expression since co-expression of Rex-GFP and Tax did not further enhance COL4A1 and COL4A2 transcripts nor did it induce COL4 protein. Indeed, we found that Tax alone is sufficient to induce COL4A1 and COL4A2 transcripts, while robust induction of COL4 protein required continuous Tax expression. Tax's moderate capacity to trans-activate the bi-directional COL4A1/COL4A2 promoter is reminiscent of earlier work, which has shown that activity of this promoter is controlled by additional regulatory elements present on distant portions of both COL4A1 and COL4A2 genes as well as the presence of an enhancer within the first intron of COL4A2 (Poschl et al., 1988; Pollner et al., 1990). Further, it is conceivable that Tax may affect COL4 expression at a posttranscriptional level since copy numbers of COL4A1 and COL4A2 are very low despite high levels of COL4 protein expression in HTLV-1-transformed cells and since transient expression of Tax alone is not sufficient to induce COL4 protein. However, our data using rhadinoviral-transformed T-cell lines expressing Tax clearly shows that COL4 protein is upregulated following continuous expression of Tax. Upregulation of COL4 does not seem to be a general feature of rhadinoviral-transformed T-cells like the Taxexpressing cell lines Tri, Tesi or TAXI-1 (Grassmann et al., 1992; Schmitt et al., 1998), as T-cells transformed with the tumorigenic rhadinoviruses Herpesvirus saimiri encoding the oncoproteins StpC/Tip, or encoding the Herpesvirus ateles oncoprotein Tio did not express COL4 similar to uninfected PBMC.

COL4 is the most important scaffold for the basement membrane and plays an important role in regulating tumor invasion and metastasis (Leitinger, 2011). It may also be advantageous for Tax-transformed T-cells, and potentially for ATLL-cells, too, to upregulate COL4. HTLV-1 may have evolved a mechanism to protect the infected and transformed cell by a collagen shield from adverse effects associated with the disease. Further, COL4 might be important for cell-cell-communication and for interaction with the tumor microenvironment by interactions between COL4 and its various receptors (Leitinger, 2011). Despite the upregulation of certain matrix metalloproteinases (MMP), MMP2 and MMP9 (Mori et al., 2002), which are unique type of proteinases that hydrolyze type IV collagen (Salo et al., 1983), we found upregulation of COL4 in HTLV-1-infected cells. Since COL4 plays an important role in regulating metastasis, the interplay between MMP activity and COL4 expression might be tightly regulated. Finally, HTLV-1/Tax may have evolved mechanisms to protect the transformed cells from apoptosis by upregulation of COL4. Earlier work has shown that collagens have a pro-survival and anti-apoptotic function in virus-transformed tumor cells by binding to their receptor DDR1 (Cader et al., 2013), which results in JNK-, ERKand p38 MAPK-signaling (Leitinger, 2011).

Our data obtained in cell culture have also suggested a role of upregulated COL4 protein levels for VB formation *in vitro* and this may also be true for infected patients. Tax is necessary for COL4 maintenance *in vitro*. In primary cells derived from

HAM/TSP patients, Tax expression is very low. Thus, detection of COL4 in patients would require higher numbers of Tax-expressing cells than usually observed. Since Tax is expressed in bursts and in individual cells (Billman et al., 2017; Mahgoub et al., 2018), it would be interesting to see, whether this transient expression of Tax *in vivo* is sufficient to upregulate COL4. Further, it remains to be determined whether COL4 is upregulated in ATLL patients where it could protect the infected tumor cell or may be important for interaction with the tumor microenvironment. Importantly, we found that *COL4A2* is upregulated in slowly progressing TSP/HAM patients suggesting that COL4A2 might be necessary for virus transmission *in vivo*.

COL4 is not only a structural component of the basement membrane, but also serves additional important functions like binding to other matrix proteins and to cells, anchoring to basement membrane (Tryggvason et al., 1990), and, as our work suggests, potentially also anchoring of HTLV-1. Having found that COL4 and the viral Gag p19 accumulate and co-localize in clusters at the VS and that COL4 and Gag are transferred to uninfected target cells, we hypothesize that COL4 is part of the VB and may be important for tethering of HTLV-1, and thus, HTLV-1 cell-to-cell transmission. This led us to the assumption that repression of COL4, and thus, interference with the VB, leads to an increase of HTLV-1 release similar to earlier work using heparin to disrupt the VB (Pais-Correia et al., 2010). However, against expectation, knockout of COL4 did not alter release of virus-like particles and HTLV-1 Gag processing in chronically infected C91-PL cells. Therefore, the interplay between COL4, its receptors on T-cells and potential virus-anchoring factors like BST-2 has to be further analyzed. Interestingly, HTLV-1 cell-tocell transmission to co-cultured target T-cells was significantly impaired upon knockout of COL4A2 in C91-PL cells. Since C91-PL cells form an infectious VB (Alais et al., 2015), our data suggest that COL4 may be important for cell-to-cell transmission of the VB. Of note, targeting COL4A2 by gene editing strategies was more efficient than targeting COL4A1 to repress COL4 protein expression in C91-PL cells, which may be due to the regulatory role of the COL4A2 chain in directing chain composition in the assembly of COL4A1-COL4A1-COL4A2 triple-helical molecules (Khoshnoodi et al., 2006). Thus, future studies should aim to repress COL4A1 protein as well to further dissect the distinct roles of COL4A1 and COL4A2 in COL4 protein formation and downstream functions.

Our observations showing that virus transmission is reduced upon repression of COL4 in C91-PL cells is indirectly supported by imaging of COL4 and Gag transfer between chronically infected MS-9 cells and Jurkat T-cells, indicating that COL4 seems to be important for tethering and cell-to-cell transfer of HTLV-1. Further, our data suggesting that COL4 is indeed part of the VB, and is transferred to uninfected target cells together with Gag are in line with earlier work, which has shown that extracellular matrix components are transferred together with viral components to target cells (Pais-Correia et al., 2010). However, our data specify that COL4 is transferred to target cells and that this may also happen independent of HTLV-1 Gag transfer, which is reminiscent of a mechanism termed trogocytosis. During trogocytosis, two cells form a transient

intimate interaction during which the membranes appear to fuse and plasma membrane proteins are exchanged between the two cells. This mechanism is e.g., exploited for bacterial transfer when host cells exchange plasma membrane proteins and cytosol via a trogocytosis-related process, which leaves both donor and recipient cells intact and viable (Steele et al., 2016). Thus, further work is needed to clarify the role of collagen transfer between cells and the mechanism how COL4 affects HTLV-1cell-to-cell transmission.

#### CONCLUSION

In conclusion, our study identifies the extracellular matrix protein COL4 as a novel target of Tax and a component of the viral biofilm (VB), and might thus contribute to a better understanding of cellular processes regulating not only VB formation, but also virus transmission.

#### **DATA AVAILABILITY STATEMENT**

The microarray data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible at http://www.ncbi.nlm.nih.gov/geo and GEO Series accession numbers are GSE10508 and GSE17718 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10508; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17718).

#### **ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Ethics Committee of the French Ministry of Research and Ethics Committee of the Medical Faculty of Friedrich-Alexander-Universität Erlangen-Nürnberg. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

#### **AUTHOR CONTRIBUTIONS**

SM performed and designed the experiments, analyzed the data, and wrote the manuscript. CG performed and designed the experiments, and analyzed the data. ND performed the

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experiments and analyzed the data. MM performed the experiments. J-MP performed the experiments and analyzed the data. AT-K conceived of the study, designed the experiments, analyzed the data, and wrote the manuscript.

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#### SUPPLEMENTARY MATERIAL

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# In vitro Evaluation of the Safety of Adalimumab for the Eye Under HTLV-1 Infection Status: A Preliminary Study

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<sup>1</sup>Department of Ophthalmology & Visual Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan, <sup>2</sup>Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan

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Kurozumi-Karube H, Kamoi K, Ando N, Uchida M, Hamaguchi I and Ohno-Matsui K (2020) In vitro Evaluation of the Safety of Adalimumab for the Eye Under HTLV-1 Infection Status: A Preliminary Study. Front. Microbiol. 11:522579. doi: 10.3389/fmicb.2020.522579 Adalimumab (ADA), a fully human monoclonal tumor necrosis factor (TNF)- $\alpha$  antibody, is one of the most widely used biologics in the treatment of inflammatory diseases. However, ADA can exacerbate infectious conditions, induce paradoxical reactions such as inflammation, and cause neoplasia. Human T-cell leukemia virus type 1 (HTLV-1) is an infectious agent that induces inflammation and neoplastic infiltration in the eye. To date, numerous HTLV-1 carriers have been treated with adalimumab to suppress inflammation out of necessity, when standard anti-inflammatory drugs such as steroids and immunosuppressive agents have proven inadequate to control the inflammation. Here, we clarify the safety of adalimumab for the eye under HTLV-1 infectious conditions in vitro. We used the adult retinal pigment epithelial cell line (ARPE)-19 cell line as ocular resident cells, and used MT2 and TL-Om1 as HTLV-1-infected cells. ARPE-19 and MT2/TL-Om1 were co-cultured, and then adalimumab was administered. Production of cytokines and chemokines, TNF-α receptor (TNF-R), HTLV-1 proviral load (PVL), and apoptosis were measured to assess the effects of adalimumab. Contact between ARPE-19 and MT2/ TL-Om1 produced inflammatory cytokines such as TNF, interleukin (IL)-6, IL-8 and IL-10, and transduced chemokines such as interferon-inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monokine induced by interferon-γ (MIG), and regulated on activation, normal T cell expressed and secreted (RANTES). No inflammatory cytokines and chemokines were exacerbated by adalimumab. Expression of TNF-R on ARPE-19 and MT2/TL-Om1 cells, HTLV-1 PVLs of MT2/TL-Om1 cells, and cell growth rate and apoptotic rate of ARPE-19 were unaffected by adalimumab. In conclusion, adalimumab does not appear to exacerbate HTLV-1-associated inflammatory conditions in the eye or increase PVL in HTLV-1-infected T cells. These data suggest that adalimumab could be used safely for the eye under HTLV-1 infectious conditions from the perspective of in vitro assessment.

Keywords: human T-cell leukemia virus type 1, anti-TNF- $\alpha$  antibody, adalimumab, safety assessment, side effect, HTLV-1 uveitis, ocular inflammation, ocular infiltration

#### INTRODUCTION

Tumor necrosis factor (TNF)- $\alpha$  antibody was introduced in the late 1990s as a molecularly targeted agent and has since seen wide use against a variety of inflammatory diseases, including rheumatoid arthritis (RA; Bathon et al., 2000), psoriasis (Leonardi et al., 2003), ankylosing spondylitis (Braun et al., 2005), ulcerative colitis (Rutgeerts et al., 2005), and inflammatory bowel disease (Rutgeerts et al., 2004).

Adalimumab (ADA), a fully human monoclonal TNF- $\alpha$  antibody, is most widely used as a biologic therapy for a variety of inflammatory diseases (Mullard, 2018). However, multiple side effects have been reported from adalimumab, including induction of infections, malignancies, and paradoxical reactions such as exacerbation of inflammation (Scheinfeld, 2005).

Human T-cell leukemia virus type 1 (HTLV-1) is known to be prevalent in the southwestern part of Japan, sub-Saharan Africa, South America, the Caribbean area, and foci in the Middle East and Australo-Melanesia (Gessain and Cassar, 2012). A recent investigation revealed the infection was not a local problem but spread all over the world. An estimated 20 million people carry the virus worldwide (Watanabe, 2011). Infection by HTLV-1 causes various human diseases, representing hematological neoplasms such as adult T-cell leukemia/lymphoma (ATL; Uchiyama et al., 1977), and inflammatory diseases such as HTLV-1-associated myelopathy (HAM; Osame et al., 1986) and HTLV-1 uveitis (HU; Mochizuki et al., 1992; Kamoi, 2020).

In the eye, HTLV-1 shows pathogenicity for inflammation and neoplastic infiltration (Kamoi and Mochizuki, 2012a; Kamoi, 2020). HU is a defined clinical entity caused by HTLV-1 and represents the most common sight-threatening ocular inflammatory disease in HTLV-1 endemic countries (Terada et al., 2017a; Kamoi et al., 2020). Significant amounts of cytokines and chemokines can be detected in intraocular fluid from HU patients. These cytokines and chemokines cause intraocular inflammation and result in irreversible ocular tissue damage (Kamoi and Mochizuki, 2012a,b). Proviral load (PVL) is also reported to be closely related to the development of HTLV-1-associated diseases (Iwanaga et al., 2010) and is associated with HU activity (Ono et al., 1998).

Another important HTLV-1-related disorder in the eye is ATL-related ocular manifestations (Liu et al., 2010). Our recent nationwide survey identified ocular infiltration as the most frequent manifestation among ATL patients (Kamoi et al., 2018). ATL might also sometimes originate in the eye (Maruyama et al., 2013). A number of studies have identified neoplastic transformation from HTLV-1-infected T cells to ATL cells as clearly related to increased PVL (Okayama et al., 2004; Iwanaga et al., 2010).

To date, numerous HTLV-1 carriers have been treated with adalimumab to suppress inflammation out of necessity, when standard anti-inflammatory drugs such as steroids and immunosuppressive agents have proven inadequate to control the inflammation (Umekita et al., 2015; Umekita and Okayama, 2020). However, information is lacking about the safety of adalimumab for the eye under conditions of HTLV-1 infection. If the use of adalimumab in HTLV-1 carriers affects immunity, HTLV-1-associated disease in the eye might arise. In other

words, there is a possibility that adalimumab could instead induce HTLV-1-associated intraocular inflammation (i.e., HU) and transformation of HTLV-1-infected T cells to neoplastic ATL cells in the eye. Looking back to clinical practice, the guidelines for the use of adalimumab make no mention of screening for HTLV-1 infection before starting treatment.

Recently, we reported an assessment of infliximab for the eve under HTLV-1 infection status in vitro (Uchida et al., 2019). In Japan, infliximab was the first biologic introduced into the field of ophthalmology, but has only been approved for use against a single pathology, Behçet disease (Ohno et al., 2019). On the other hand, adalimumab can be used for non-infectious uveitis such as sarcoidosis, Vogt-Koyanagi Harada disease, and many other diseases (Jaffe et al., 2016). In other words, the number of patients indicated for the application of adalimumab is much greater than the number of patients indicated for the application of infliximab. Information on adalimumab is thus much more valuable for ophthalmologists, who previously did not need to check for HTLV-1 infection before administering adalimumab. In addition, adalimumab is a fully human monoclonal TNF- $\alpha$  antibody, whereas infliximab is a chimeric monoclonal TNF-α antibody. These structural differences might result in different effects on the eye under conditions of HTLV-1 infection.

The present *in vitro* study therefore investigated the possible effects of adalimumab on the eye under HTLV-1 infection status using an ocular cell line and HTLV-1-infected T-cell lines. A retinal pigment epithelium (RPE) cell line was chosen as the ocular cell line, because the RPE plays a major role in the blood-ocular barrier through which HTLV-1-infected cells invade into the eye (Kamoi and Ohno-Matsui, 2019), as well as a role in the maintenance of immunological homeostasis in the eye (Holtkamp et al., 2001; Mochizuki et al., 2013).

#### MATERIALS AND METHODS

#### **Cell Culture**

As ocular cells, the adult retinal pigment epithelial cell line (ARPE)-19 human retinal pigment epithelial cell line (American Type Culture Collection, Manassas, VA), a spontaneously immortalized cell line, was cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare Japan, Tokyo, Japan) and 1% penicillin and streptomycin (P/S) antibiotic solution. The MT2 cell line and TL-Om1 were used as HTLV-1-infected T-cell lines, and Jurkat cells were used as a control T-cell line. MT2, TL-Om1 and Jurkat cells were cultured in RPMI 1640 medium (Wako Pure Chemical Corporation) with the same supplements. All cell lines were incubated at 37°C, under 5% CO<sub>2</sub>. Cell culture inserts with 0.4-µm pores (Greiner Bio-One, Kremsmünster, Austria) were used to prevent the contamination of ARPE cells by other cells.

#### TNF- $\alpha$ Inhibitor

Adalimumab (Humira®; AbbVie, Chicago, IL) was used as an anti-TNF- $\alpha$  antibody. In line with the previous established method (Fukui et al., 2017), adalimumab concentrations of 0.1, 1.0, and 10  $\mu$ g/ml were used.

#### **Cytometric Bead Array**

We seeded  $1.5 \times 10^5$  ARPE cells in 6-well cell culture plates and incubated them for 24 h. Subsequently, 5 × 105 MT2, TL-Om1 or Jurkat cells were co-cultured following 48 h with 0, 0.1, 1.0, or 10 μg/ml of adalimumab. We performed cytometric bead array (CBA) using culture supernatants and CBA human inflammation cytokine kits (BD Bioscience, San Jose, CA). FCAP Array version 3.0 software (BD Bioscience) was used for analyses in accordance with the instructions from the manufacturer. Cytokines measured by the kits included interleukin (IL)-6, IL-1β, IL-12p70, IL-8, IL-10, and TNF, and chemokines included regulated on activation, normal T cell expressed and secreted [RANTES; also known as C-C motif chemokine ligand (CCL)5], monokine induced by interferon-γ [MIG; also known as C-X-C motif chemokine ligand (CXCL)9], monocyte chemotactic protein-1 (MCP-1; also known as CCL2), and interferon-inducible protein-10 (IP-10; also known as CXCL10). Minimum limits of detection for cytokines/chemokines were as follows: IL-6, 2.5 pg/ml; IL-1β, 7.2 pg/ml; IL-12p70, 1.9 pg/ml; IL-8, 0.2 pg/ml; IL-10, 3.3 pg/ml; TNF, 3.7 pg/ml; RANTES, 1.0 pg/ml; MIG, 2.5 pg/ml; MCP-1, 2.7 pg/ml; and IP-10, 2.8 pg/ml.

#### **Cell Growth Analysis**

ARPE-19 cells ( $2 \times 10^4$ ) were co-cultured with three times the number of MT2, TL-Om1 or Jurkat cells using 0.4-µm pore insert, with 0 or 10 µg/ml of adalimumab in 24-well cell culture plates. After 0, 24, 48, or 72 h of co-culture, we removed the supernatants, trypsinized ARPE-19, and counted the number of ARPE-19 cells under light microscopy.

#### **TNF-\alpha** Receptor Analysis

ARPE-19 cells ( $1.5 \times 10^5$ ) were co-cultured with three times the number of MT2, TL-Om1, or Jurkat cells using a 0.4-µm pore insert, with 0 or 10 µg/ml of adalimumab. MT2 or TL-Om1 cells were cultured in RPMI1640 containing 10% FBS and 1% P/S with 0 or 10 µg/ml of adalimumab. Analysis of surface expression of TNF-R1 and TNF-R2 on ARPE-19, MT2 and TL-Om1 were performed using human TNF RI/TNFRSF1A PE-conjugated antibody (R&D Systems, Minneapolis, MN) and human TNF RII/TNFRSF1B fluorescein-conjugated antibody (R&D Systems) compared to an isotype control antibody. Samples were measured using FACSCalibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

#### Immunofluorescence Microscopy

ARPE-19 cells were cultured on glass plates in a cell culture plate (AGC Techno Glass, Shizuoka, Japan) for 24 h, then co-cultured with MT2, TL-Om1 cells using cell culture inserts (Thermo Fisher Scientific, San Jose, CA) for 48 h. ARPE-19 cells were permeabilized and fixed by cold fixing buffer (methanol/acetone, 1:1) at -20°C for 20 min. After blocking with 5% normal goat serum in phosphate-buffered saline for 15 min, cells were incubated in the diluted primary antibodies for 1 h at room temperature, followed by incubation with Alexa Fluor 488-labeled anti-rabbit secondary antibody (Abcam, Cambridge, MA) for 1 h at room temperature. After nuclear staining with

4',6-diamidino-2-phenylindole, cells were scanned using a TCS-SP8 microscope (Leica Micro Systems, Wetzlar, Germany). TNF-R1 polyclonal antibody (Bioss Antibodies, Woburn, MA) and TNF-R2 polyclonal antibody (Proteintech, Chicago, IL), or rabbit IgG control (Abcam) were used as primary antibodies.

#### Measurement of HTLV-1 Proviral Load

MT2 or TL-Om1 cells were cultured in RPMI1640 containing 10% FBS and 1% P/S with 0 or 10  $\mu$ g/ml of adalimumab. EZ1 Virus Mini Kits v2.0 (Qiagen, Hilden, Germany) was used to prepare the DNA from each sample. PVL of HTLV-1 in cells was measured using quantitative real-time PCR assay as described previously (Matsuda et al., 2005; Fukui et al., 2017; Uchida et al., 2019). PVL was quantified using the HTLV-1 Tax primer (forward, 5'-CCCACTTCC CAGGGTTTGGA-3'; reverse, 5'-GGCCAGTAGGGCG TGA-3') and probe (5'-FAM-CCAGTCTACGTGTTTGGA GACTGTGTACA-TAMRA-3'). Glyceraldehyde-3-phosphate dehydrogenase was used as the internal control.

#### **Annexin V Staining**

Evaluations of apoptosis and cell death were performed using Annexin V-FITC/PI double-staining assay kits (MBL, Nagoya, Japan) in line with the instructions from the manufacturer. Briefly, cells were stained using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V for 15 min in room air. Fluorescence-activated cell sorting (FACS) analysis was performed immediately using a FACSCalibur flow cytometer and CellQuest software. PI-negative, annexin V-positive cells were defined as apoptotic. ARPE-19 cells were used at  $2\times10^4$  cells, and MT2, TL-Om1, or Jurkat cells were used at  $6\times10^4$  cells, respectively, with cell culture inserts.

#### Statistical Analysis

After one-way analysis of variance, Student's unpaired t-test or Welch's unpaired-t-test after Bonferroni correction for multiple testing was used to determine significant differences in levels of cytokines, numbers of ARPE-19 cells and percentages of apoptotic cells. Values of p < 0.05 were considered significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR is a modified version of R Commander designed to add statistical functions frequently used in biostatistics.

#### **RESULTS**

### Production of Inflammatory Cytokine and Chemokines

Levels of cytokines (IL-6, IL-8, IL-1 $\beta$ , IL-12p70, IL-10, and TNF) and chemokines (RANTES, MIG, MCP-1, and IP-10) secreted by each of ARPE-19, MT2, Jurkat, ARPE-19 co-cultured with MT2/TL-Om1, and ARPE-19 co-cultured with Jurkat were measured (**Figure 1**).

In terms of cytokines, MT2 spontaneously secreted IL-6, IL-10, and TNF. IL-8 was not secreted from MT2, whereas TL-Om1 did not spontaneously secrete any cytokines. ARPE-19 did not secrete any cytokines measured in this experiment.

IL-6, IL-10, and TNF were produced in ARPE-19 co-cultured with MT2, but not in ARPE-19 alone or in co-culture with TL-Om1 and Jurkat. IL-8 was induced only through contact between ARPE-19 and MT2 cells. Compared to MT2 alone, levels of IL-6 and IL-8 were increased. On the other hand, levels of TNF and IL-10 appeared similar in ARPE-19 co-cultured with MT2. No cytokine release was detected by contact between ARPE-19 and Tl-Om1. Levels of IL-12p70 and IL-1 $\beta$  were below the limits of detection under all conditions upon co-culture with ARPE-19 cells (data not shown).

As for chemokines, MT2 spontaneously secreted MIG and RANTES. IP-10 and MCP-1 were not secreted from MT2. TL-Om1 spontaneously secreted RANTES and IP-10, but did not secrete MIG or MCP-1. ARPE-19 did not secrete any chemokines measured in this experiment (**Figure 2**).

All measured chemokines (RANTES, MIG, MCP-1, and IP-10) were produced in significantly greater quantities by ARPE-19 co-cultured with MT2 than by ARPE-19 co-cultured with Jurkat. IP-10 and MCP-1 were induced through the contact. Compared to MT2 alone, levels of all chemokines

were also increased. As for production of chemokines by ARPE-19 co-cultured with TL-Om1, RANTES and IP-10 were decreased in comparison with production by TL-Om1 alone.

## Changes in Cytokine and Chemokine of ARPE-19 Co-cultured With MT2 or TL-Om1 Cells Treated With Adalimumab

Cytokine and chemokine levels in culture supernatant of ARPE-19 co-cultured with MT2, TL-Om1, and Jurkat cells as well as ARPE-19 alone were measured at 48 h after addition of adalimumab.

Among the inflammatory cytokines, production levels of IL-6, IL-8, and IL-10 were unaltered by addition of adalimumab at each concentration in all co-culture combinations. In ARPE-19 co-cultured with MT2, TNF tended to be inhibited in a concentration-dependent manner, but did not show any significant difference at 0.1  $\mu$ g/ml of adalimumab. TNF was completely inhibited at concentrations of adalimumab  $\geq$ 1.0  $\mu$ g/ml (**Figure 1**). A similar effect was observed with MT2 alone.

Of chemokines, production levels of RANTES, MIG, MCP-1, and IP-10 were unaltered by addition of adalimumab at each concentration in ARPE-19 co-cultured with MT2 or TL-Om1 (**Figure 2**).

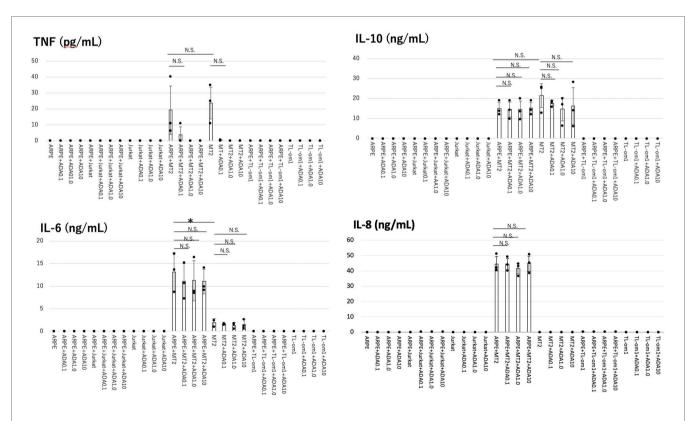


FIGURE 1 | Levels of inflammatory cytokines measured in the culture supernatants of adult retinal pigment epithelial cell line (ARPE)-19, MT2, TL-Om1, or Jurkat, and ARPE-19 co-cultured with MT2, TL-Om1, or Jurkat, with addition of adalimumab (ADA). ARPE cells (1.5 × 10 $^{\circ}$  cells) were co-cultured with 5 × 10 $^{\circ}$  MT2, TL-Om1 or Jurkat cells following 48 h with 0, 0.1, 1.0, or 10 μg/ml of ADA. Production levels of IL-6, IL-10, and IL-8 did not change with adalimumab addition. Adalimumab decreased tumor necrosis factor (TNF) secretion in a concentration-dependent manner. TNF was not detected with ≥1.0 μg/ml of adalimumab. Data were obtained from three independent experiments. Error bars represent SD (\*p < 0.05; NS, not significant). ADA, adalimumab.

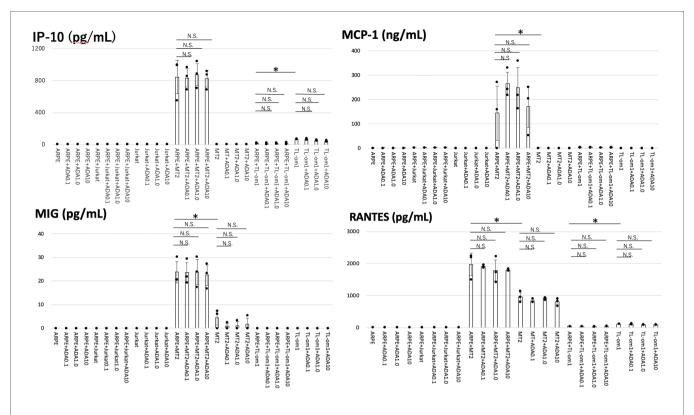
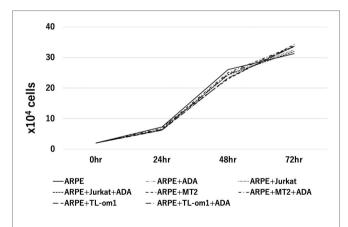


FIGURE 2 | Levels of chemokines were measured in the culture supernatants of ARPE-19, MT2, TL-Om1, or Jurkat, and ARPE-19 co-cultured with MT2, TL-Om1 or Jurkat with addition of adalimumab. Coculture conditions were the same as in Figure 1. Adalimumab concentrations were 0.1, 1.0, or 10 μg/ml. Production levels of interferon-inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), monokine induced by interferon-γ (MIG), and regulated on activation, normal T cell expressed and secreted (RANTES) in co-culture of ARPE-19 cells and MT2 or TL-Om1 cells did not change with addition of adalimumab. Error bars represent SD (\*p < 0.05; NS, not significant). ADA, adalimumab.



**FIGURE 3** | Cell growth curve of ARPE-19, showing number of ARPE-19 cells per well at 0, 24, 48, and 96 h. The number of ARPE cells was  $2.0 \times 10^4$  at the beginning of culture. No significant differences among numbers of ARPE-19 cells co-cultured with MT2 or TL-Om1 cells with or without  $10~\mu\text{g/ml}$  of adalimumab were seen at each point. The number of cells was counted three times independently. ADA, adalimumab.

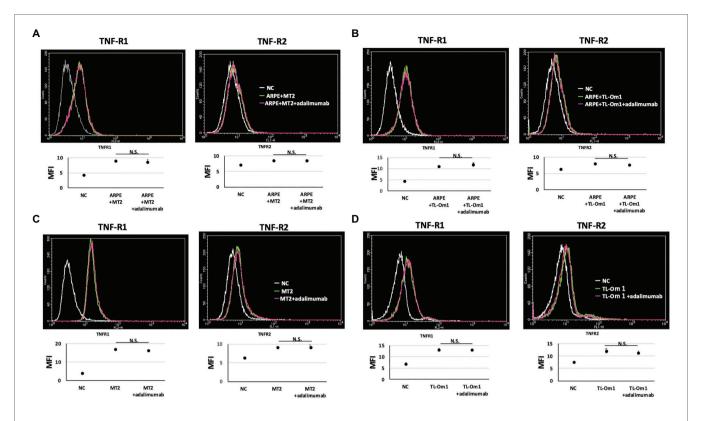
## Changes in Cell Growth in ARPE-19 Treated With Adalimumab

Cell growth rates of ARPE-19 co-cultured with MT2 or TL-Om1 and treated with adalimumab were examined (Figure 3).

As controls, cell growth rates were measured for ARPE-19 alone, ARPE-19 treated with adalimumab, ARPE-19 co-cultured with Jurkat, and ARPE-19 co-cultured with Jurkat treated with adalimumab. Numbers of ARPE-19 cells were counted after 0, 24, 48, and 72 h of co-culturing. Growth curves showed a time-dependent form and were similar among ARPE-19, ARPE-19 co-cultured with MT2, TL-Om1, or Jurkat, and ARPE-19 co-cultured with MT2, TL-Om1, or Jurkat treated with adalimumab. Addition of adalimumab under this co-cultured condition did not affect the growth rate of ARPE-19 cells (Figure 3).

## Expressions of TNF-R1 and TNF-R2 on ARPE-19 Cells Co-cultured With MT2 or TL-Om1 Cells Treated With Adalimumab

To identify changes in expressions of TNF-R1 and TNF-R2 caused by adalimumab, we assessed expressions of TNF-R1 and -R2 on ARPE-19 and MT2 or TL-Om1 at 48 h after addition of adalimumab, by performing FACS analysis. Treatment with 10  $\mu$ g/ml of adalimumab resulted in no significant change in mean fluorescence intensity (MFI) of TNF-R1- or TNF-R2-labeled ARPE-19 cells (**Figures 4A,B**). Addition of adalimumab also did not affect expressions of TNF-R1 or -R2 on MT2 or TL-Om1 (**Figures 4C,D**). We also performed immunofluorescence staining. Expressions of TNF-R1 (**Figure 5A**) and TNF-R2 (**Figure 5B**) on ARPE-19 co-cultured with MT2 cells were unaltered by the presence or



**FIGURE 4** | TNF-R1 and TNF-R2 on ARPE-19 in fluorescence-activated cell sorting (FACS) analysis. Results are representative of three experiments and show that expressions of TNF-R1 and -R2 on ARPE-19 cells ( $1.5 \times 10^5$  cells) co-cultured with MT2 cells (**A**) or TL-Om1 cells ( $5 \times 10^5$  cells; **B**) were unaffected by 10  $\mu$ g/ml of adalimumab. Expressions of TNF-R1 and TNF-R2 on MT2 cells (**C**) and TL-Om1 cells (**D**) were also unaffected by 10  $\mu$ g/ml of adalimumab. Histograms (upper figure) and mean fluorescence intensity (MFI; lower figure) for expression of TNF-R1 and TNF-R2. Error bars represent SD. NS, not significant.

absence of adalimumab. Expressions of TNF-R1 (**Figure 5C**) and TNF-R2 (**Figure 5D**) on ARPE-19 co-cultured with TL-Om1 cells were also unaltered by the presence or absence of adalimumab.

## Detection of HTLV-1 Proviral DNA in HTLV-1-Infected T Cells Treated With Adalimumab

Proviral load in HTLV-1-infected cells is the most frequently used biomarker for prognosis and disease progression (Iwanaga et al., 2010). Therefore, to identify whether adalimumab induces changes in PVL for MT2 or TL-Om1 cells, we measured PVL at 48 h after addition of adalimumab. To assess the potential effects of adalimumab on HTLV-1-infected cells, we checked the PVL of MT2 or TL-Om1 cells at 48 h after addition of adalimumab. Mean PVLs in MT2 and TL-Om1 were  $6.32 \times 10^5$  and  $3.70 \times 10^5$  copies/µg DNA, respectively. On the other hand, mean PVLs in MT2 and TL-Om1 treated with adalimumab were  $6.02 \times 10^5$  and  $3.30 \times 10^5$  copies/µg DNA, respectively. No significant difference was detected according to the presence or absence of adalimumab (**Figure 6**).

## Assessment of Apoptosis in ARPE-19 Cells Treated With Adalimumab

To assess apoptosis in ARPE-19 cells, we performed annexin V staining of ARPE-19 at 48 h after addition of adalimumab.

Apoptosis was seen in 3.74% of ARPE-19 cells co-cultured with MT2 cells, and in 2.63% of ARPE-19 cells co-cultured with TL-Om1 cells. Frequencies of apoptosis for ARPE-19 in each co-culture were 3.34 and 2.55% with addition of adalimumab, respectively. No significant change in apoptotic rate was seen for ARPE-19 cells co-cultured with MT2 or TL-Om1, with or without addition of adalimumab (**Figure** 7).

#### **DISCUSSION**

This study focused on adalimumab, as one of the most widely used anti-TNF-α antibodies in the treatment of inflammatory diseases (Mullard, 2018), and investigated the effects of adalimumab on the eye under HTLV-1 infection status *in vitro*. The study demonstrated that adalimumab did not cause significant changes in the production of inflammatory cytokines IL-6, IL-8, and IL-10 or chemokines MCP-10, MIG, RANTES, and IP-10, proliferation of ARPE-19 cells, expression of TNF-R1 or -R2, or apoptosis, compared to non-treated ARPE-19 cells under HTLV-1 infectious conditions. In addition, HTLV-1-infected T cells were unaffected by adalimumab in terms of cytokine/chemokine production, TNF-R1 or -R2 expression, and PVL increases. This implies that adalimumab did not exacerbate

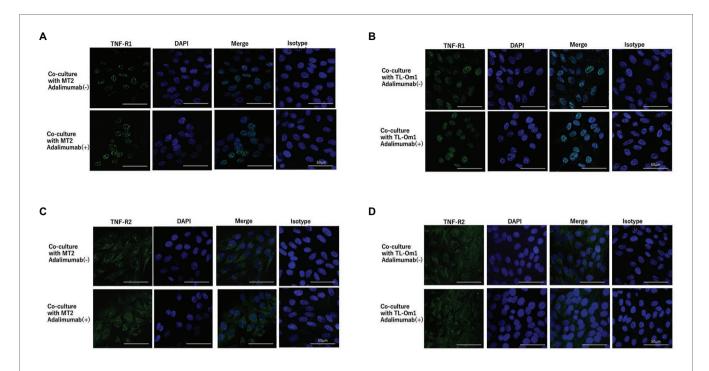
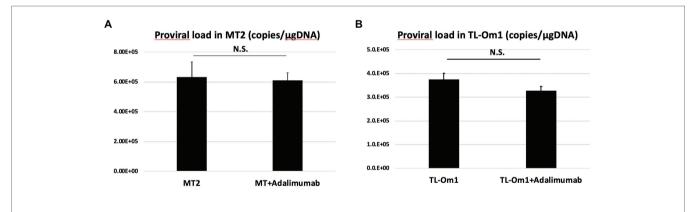


FIGURE 5 | Immunofluorescence staining of TNF-R1 (A,B) and TNF-R2 (C,D) on ARPE-19 co-cultured with MT2 or TL-Om1. ARPE-19 cells were treated with rabbit monoclonal anti-TNF-R1 or anti-TNF-R2 antibodies followed by Alexa Fluor 488-labeled anti-rabbit secondary antibody. DAPI was used to stain the nucleus. No significant changes in expressions of TNF-R1 or TNF-R2 are seen for ARPE-19 treated with or without 10 μg/ml of adalimumab. (A,B) Green color represents TNF-R1 expression and blue represents DAPI. (C,D) Green color represents TNF-R2 and blue represents DAPI. Isotype control indicates staining control with rabbit lqG control as the first antibody.

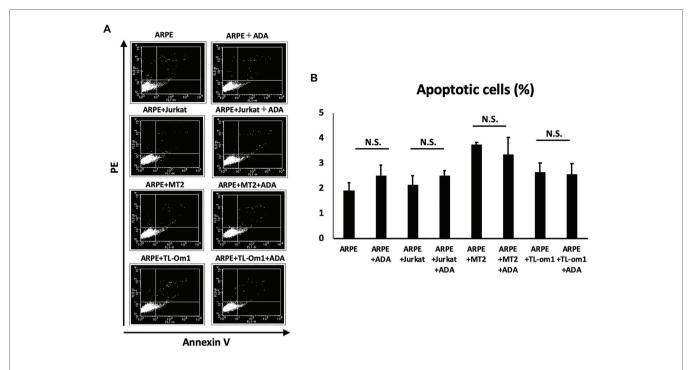


**FIGURE 6** | Proviral load (PVL) in MT2 cells **(A)** or TL-Om1 cells **(B)** treated with 10  $\mu$ g/ml of adalimumab. The number of cells was  $5 \times 10^5$ , respectively. PVL of both cells was unchanged by adalimumab. Error bars represent SD. NS, not significant.

HTLV-1-related inflammation in the eye, at least from the perspective of *in vitro* experiments.

Globally, HTLV-1 infection has gained attention since the high prevalence among Aborigines in central Australia became widely recognized (Martin et al., 2018). Japan is the most endemic country among the developed nations in terms of HTLV-1 infection, with an estimated 1.08 million infected individuals. Japan is one of the countries where patients with inflammatory diseases are treated with biologic agents. Among HTLV-1-infected patients, many patients need biologics because of severe inflammatory diseases such as RA, psoriasis,

or ankylosing spondylitis uncontrollable by standard steroid or immunosuppressive drugs (Umekita et al., 2015). However, little is known about whether biologics can be safely used by infected individuals, particularly in the field of ophthalmology. Biologics may upregulate HTLV-1 activities and change the immunological homeostasis of the eye, which might result in induction of HTLV-1-associated ocular diseases. In fact, we have already reported a case in which an RA patient with HTLV-1 infection received biologics, which resulted in activation of HTLV-1 status and induced HU in the eye (Terada et al., 2017b).



**FIGURE 7** (A) Annexin-V staining assay results for apoptosis of ARPE-19 cells. Extent of apoptosis was determined as the percentage of Annexin V-positive, PI-negative cells. (B) Rate of apoptotic ARPE-19 cells (2 × 10<sup>4</sup> cells) in co-culture with MT2, TL-Om1, or Jurkat cells (6 × 10<sup>4</sup> cells), and MT2, TL-Om1, or Jurkat cells (6 × 10<sup>4</sup> cells), and MT2, TL-Om1, or Jurkat cells (6 × 10<sup>4</sup> cells). The option of adalimumab. No significant change in apoptotic rate of ARPE-19 is seen with adalimumab. Error bars represent SD. NS, not significant; ADA, adalimumab.

In general, adalimumab is an effective drug for various inflammatory diseases, and many reports have suggested that adalimumab can decrease the levels of various cytokines in patients of inflammatory diseases (Scheinfeld, 2004). However, paradoxical response, or exacerbation of inflammation, is also well known during anti-TNF- $\alpha$  therapy. In particular, the occurrence of uveitis (ocular inflammation) has been reported as a major paradoxical effect (Wendling and Prati, 2014). In addition, HU can be seen among HTLV-1 carriers, which means activation of HTLV-1 by adalimumab might disturb the immunological homeostasis of the eye. We therefore checked the effects of adalimumab on inflammatory cytokines (IL-6, IL-8, IL-1 $\beta$ , IL-12p70, IL-10, and TNF) and chemokines (MCP-10, MIG, RANTES, and IP-10) under HTLV-1 infectious conditions, i.e., under conditions of MT2/TL-Om1 cells in contact with ARPE-19 cells.

Inflammatory cytokine investigations showed that levels of IL-6 and IL-8 increased when MT2 cells came into contact with ARPE-19 (**Figure 1**). These inflammatory cytokines can lead to damage to ocular tissues, suggesting that HTLV-1 carriers face a potential threat of inflammation in the eye (Uchida et al., 2019). As for TNF-production, despite low-level detection, no significant change was seen when MT2 cells came into contact with ARPE-19, compared to HTLV-1-infected T cells, but mean TNF levels were slightly decreased with this contact. Previous reports have mentioned that this might be explained by the notion that RPE is thought to play a role in local immunosuppressive ability (Mochizuki et al., 2013; Uchida et al., 2019) and this finding was not thought to rule out an immunosuppressive role of RPE.

As for the effect of adalimumab administration, levels of IL-6, IL-8, and IL-10 production were unaffected by adalimumab treatment, suggesting that adalimumab does not exacerbate inflammation in the eye under HTLV-1 infectious conditions. The level of TNF production after contact between HTLV-1-infected cells and ARPE-19 was low, but decreased in a dose-dependent manner with addition of adalimumab. Complete suppression of TNF was also seen with higher-dose adalimumab administration (**Figure 1**). These results suggest that adalimumab might be somewhat effective for reducing immune reactions through inhibition of TNF in the eye.

In terms of chemokines, production of MCP-1, MIG, IP-10, and RANTES increased when MT2 came into contact with ARPE-19 cells, and production levels were unaffected by adalimumab treatment (**Figure 2**). Decreases in RANTES and IP-10 were seen in the contact between TL-Om1 and ARPE-19, which might relate to the local immunosuppressive ability of RPE as mentioned above (Mochizuki et al., 2013; Uchida et al., 2019). Levels of these chemokines were also unaffected by adalimumab treatment (**Figure 2**). These results also suggest that adalimumab does not accelerate chemokine-mediated attraction of inflammatory cells into the eye among HTLV-1-infected patients (Charo and Ransohoff, 2006).

Adalimumab-mediated inhibition could be due to interference with the binding of TNF to two known receptors. TNF-R1 binds to soluble TNF and TNF-R2 binds to membrane-bound TNF. TNF-R1 and TNF-R2 are both known to be expressed on RPE (Kociok et al., 1998; Shi et al., 2008; Lee et al., 2015), and changes in these receptors are therefore thought to affect

blood-ocular barrier function *via* TNF-mediated inflammation. TNF-R1 and TNF-R2 are also expressed on HTLV-1-infected T cells (Harashima et al., 2001). We therefore checked for changes in TNF-R1 and -R2 expression on ARPE-19 and two types of HTLV-1-infected T cells by FACS analysis and immunofluorescence staining. These experiments identified no changes in TNF-R1 or -R2 expression on ARPE-19 or the two types of HTLV-1-infected T cells through FACS analysis (**Figure 4**) and immunofluorescence (**Figure 5**). This suggests that adalimumab has no effect on TNF-mediated intraocular alterations.

We next investigated the effects on PVL in two types of HTLV-1-infected T cells through adalimumab administration. The increase in PVL among HTLV-1-infected T cells relates to the development of ATL, HAM, and HU (Ono et al., 1998; Matsuzaki et al., 2001; Kulkarni and Bangham, 2018). PVL in peripheral blood is also a well-known marker of HTLV-1 disease progression (Demontis et al., 2013). Checking PVL in infected T cells is thus important to clarify the risks of inducing HTLV-1-associated diseases. Considering local circumstances in the eye, the risk of inducing HTLV-1-associated inflammation and transformation to malignant cells in the eye through adalimumab administration is a concern.

As for the effects of adalimumab for MT2 or TL-Om1, the PVL of each cell was also unchanged by adding adalimumab, consistent with previous reports (Umekita et al., 2015; Fukui et al., 2017). This indicates that competent transformation of infected T cells was not stimulated by adalimumab (Figure 6).

Increases in RPE cells undergoing apoptosis critically damage the maintenance of immunological homeostasis in the eye (Mochizuki et al., 2013). In this study, adalimumab treatment did not affect the frequency of RPE apoptosis in co-culture with MT2 or TL-Om1, compared to that in co-culture with control Jurkat cells (**Figure 7**). In addition, if adalimumab promotes cell growth of HTLV-1-infected RPE, RPE proliferation should be observed. However, adalimumab also did not affect the growth curve of ARPE-19 (**Figure 3**). These results suggest that adalimumab does not promote the growth of ARPE cells under HTLV-1 infectious conditions.

Various limitations to the present study need to be considered when interpreting the results. We selected MT2 and TL-Om1 as HTLV-1-infected T-cell lines in this investigation. Diversity among MT2 cell lines was pointed out in a previous paper (Nomura et al., 2019). We therefore performed additional experiments using another type of HTLV-1-infected T-cell line, TL-Om1. The same results were obtained in terms of the safety of adalimumab under HTLV-1 infectious conditions *in vitro* with these different types of cells. This study did not reflect all aspects of *in vivo* situations and was instead positioned as a preliminary *in vitro* study. In addition, consideration needs to be given to the aspects of the clinical response to adalimumab in the eye and the effects of adalimumab therapy in patients

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Bathon, J. M., Martin, R. W., Fleischmann, R. M., Tesser, J. R., Schiff, M. H., Keystone, E. C., et al. (2000). A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N. Engl. J. Med. 343, 1586–1593. doi: 10.1056/NEJM200011303432201 with inflammatory diseases complicated by HTLV-1 infection. However, this *in vitro* study suggested that the use of adalimumab in HTLV-1-infected patients with inflammatory disease may not aggravate inflammation or induce proliferation in the eye, and this agent may thus be safe to apply in patients with inflammation from an ophthalmological perspective.

#### CONCLUSION

We have shown that adalimumab did not affect production of inflammatory cytokines, expression of TNF-R, cell proliferation, or apoptosis in RPE under HTLV-1 infectious conditions *in vitro*. In addition, adalimumab did not affect increases in PVL in HTLV-1-infected T cells. These results suggest that adalimumab did not exacerbate HTLV-1-related inflammation in the eye. Adalimumab may be safe for use in the eye under HTLV-1 infectious conditions, at least from the perspective of this preliminary *in vitro* assessment.

#### DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

#### **AUTHOR CONTRIBUTIONS**

HK-K performed the experiments and wrote the draft of the manuscript. KK designed the experiments, analyzed the data, and wrote the manuscript. NA and MU performed the experiments. IH and KO-M contributed to the analysis and interpretation of data, and assisted in the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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