◆連載

私のこの一枚 (105)

慢性型ATLの自然寛解後にHTLV-1関連脊髄症（HAM）を発症した症例

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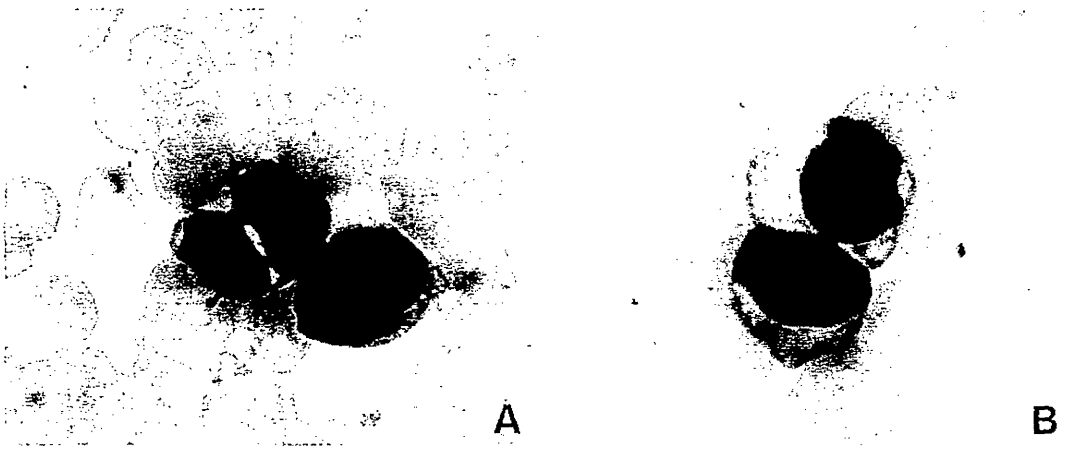

写真　末梢血塗抹標本（メイギムザ染色，×1,000）
A：核に切れ込みを有する異常リンパ球（左）とクロマチンの凝集を認める異常リンパ球（右）。B：胞体の広い正常大リンパ球。
（筆者提供）

はじめに

成人T細胞白血病-リンパ腫（adult T-cell leukemia-lymphoma：ATL）は，ヒトTリンパ球向性ウイルスI型（human T-lymphotropic virus type I：HTLV-1）が原因で発症する末梢性T細胞腫瘍である[1~3]。HTLV-1が原因で発症する疾患は，ATL以外のHTLV-1関連疾患も存在し，免疫機序による慢性炎症性疾患であるHTLV-1関連脊髄症（HTLV-1 associated myelopathy：HAM）が代表的なものである[4, 5]。興味深いことに，ATLとHAMにおける宿主の免疫応答は対照的であり，例えばHTLV-1感染細胞に対する細胞傷害性T細胞（cytotoxic T-cell：CTL）の免疫応答は一般的にATLで低く，HAMでは高い[6]。これまでこのような対照的な両疾患が合併することは稀であるとされてきたが，HAM患者において経過中にATLを合併する症例報告は存在する[7, 8]。今回，古い症例であるが，慢性型ATLが自然寛解した後，HAMを発症したと思われる非常に貴重な症例を経験したので報告する。

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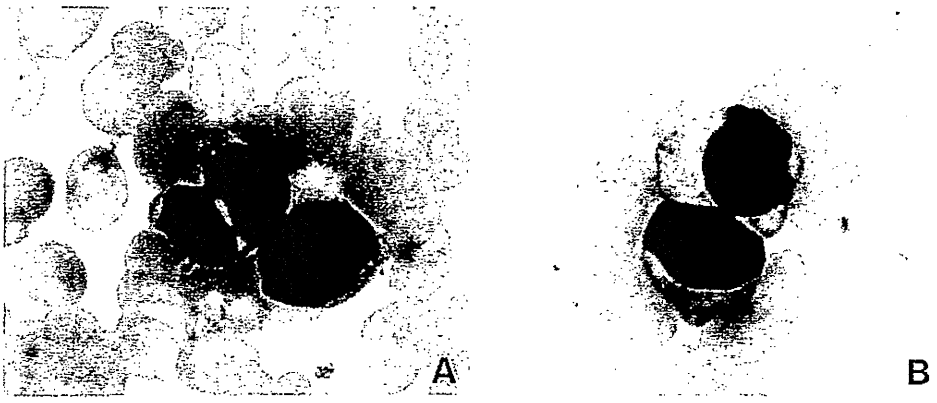

図1　末梢血塗抹標本 (メイギムザ染色, ×1,000, 再掲)
A：核に切れ込みを有する異常リンパ球 (左) とクロマチンの凝集を認める異常リンパ球 (右)。B：胞体の広い正常大リンパ球。 (筆者提供)

表1　入院時検査成績

【検尿】		【末梢血検査】		【血液生化学】	
Protein	(±)	WBC (/μL)	9,100	GOT	24 ka.U
Glucose	(−)	RBC (×10⁴/μL)	346	GPT	15 ka.U
Occult blood	(−)	Hb (g/dL)	11.2	LDH	306 W-U
Urobilinogen	(±)	PLT (×10⁴/μL)	25.3	(基準値	50～450 W-U)
【血沈】		【白血球分類】		ALP	15.9 K.A.U
12 mm/hr		Bas. (%)	0	CHE	0.80 Δ PH
【血清蛋白】		Eos. (%)	0	T.Bil.	0.48 mg/dL
T-P	6.2 g/dL	Neutr. (%)	32	T.Cho.	170 mg/dL
Alb.	63.7%	Mono. (%)	16	TTT	1.4 U
α1-G.	2.9%	Lymph. (%)	27	ZTT	5.1 U
α2-G.	8.0%	Ab.Lymph. (%)	25	BUN	22.3 mg/dL
β-G.	11.8%	【骨髄検査】		Cr	0.9 mg/dL
γ-G.	13.6%	NCC (×10⁴/μL)	7.9	UA	2.8 mg/dL
【CRP】		Mega (/μL)	15	Na	146 mEq/L
(−)		Erythr. (%)	22.8	K	4.0 mEq/L
【PPD】		Bas. (%)	0.4	Cl	108 mEq/L
(−)		Eos. (%)	1.2	Ca	4.6 mEq/L
		Neutr. (%)	52.4	【血清抗 HTLV-1 抗体】	
		Mono. (%)	4.0	＞256 倍 (PA 法)	
		Lymph. (%)	14.0	160 倍 (FA 法)	
		Ab.Lymph. (%)	4.4		

末梢血中の異常リンパ球 (Ab. Lymph.) の増加により白血球数の軽度増加がみられた。また，異常リンパ球の骨髄浸潤も認められた。 (筆者作成)

症　例

症例：50歳，女性，鹿児島県出身。

現病歴：1989年5月，突然両下肢の脱力，歩行障害，排尿障害出現。整形外科にて腰部脊柱管狭窄症の診断を受け，6月28日部分椎弓切除術を受けた。排尿障害は軽快したが，術後筋力低下による歩行障害はしばらく持続した。1989年8月下旬の末梢血検査にて異常リンパ球の出現を指摘され，9月18日当科入院となった。

入院時現症：身長140 cm，体重42 kg，体温36.3℃，脈拍60/分，血圧90/58 mmHg，胸部聴診にて呼吸音・心音異常なし，腹部は圧痛なく，肝臓・脾臓の腫大なし。表在リンパ節は触知せず，皮疹もみられなかった。神経学的所見では上肢・下肢の筋力低下がみられたが，痙性所見はなく，膀胱直腸障害も認められなかった。

入院時検査成績：白血球数9,100/μL，異常リンパ球25%（図1A），リンパ球27%，骨髄検査で異常リンパ球を4.4%認めた（表1）。末梢血中の異常リンパ球は，CD2, 3, 4, 25, HLA-DR陽性で，CD8, 20陰性であった（表2）。血清抗HTLV-1抗体は陽性で，末梢血単核球のサザンブロット検査で，HTLV-1プロウイルスDNAのモノクローナルな組み込みを認めた。以上より，慢性型ATLと診断した。

入院後経過：慢性型ATLと診断したが，ATLによる症状はなく，血清LDH値も正常範囲であったため外来経過観察を行った。患者の末梢血異常リンパ球は自然に減少し，5%未満となった（表3，図1B）。末梢血リンパ球のマーカー検査ではCD4陽性細胞は減少し，CD8陽性細胞の比率の増加によりCD4/CD8比は著明に低下した（表2）。ATLは自然寛解したと判断し，外来経過観察を行っていたが，1990年8月頃より腰痛，歩行障害が出現し，10月頃より全身倦怠感も加わり，11月1日に入院した。再入院時，神経学的には，痙性歩行，上肢・下肢の筋力低下（下肢優位），両下肢深部腱反射の亢進，両下肢の表在知覚低下，右下肢の異常感覚，膀胱直腸障害が認められた（図2）。Babinski反射はみられなかった。髄液検査では，細胞数1/μL，髄液中の抗HTLV-1抗体は陽性であった（表4）。MRI検査では，頸椎のspur formation，胸椎ではTh10-12に黄靭帯骨化症が認められたが，いずれも脊髄への圧迫所見はなかった。ATLはすでに自然寛解し，再燃は認められず，臨床所見と検査所見により慢性型ATLの自然寛解後に発症したHAMと診断した。

表2　末梢血リンパ球の表面形質

	1989.9.18	1990.2.14	1990.8.1	1990.11.6	1991.7.11
リンパ球数（/μL）	6,188	1,634	1,395	1,360	936
CD2（%）	96.9	89.3	87.3	83.8	93.6
CD3（%）	93.7	83.2	79.9	76.7	85.7
CD4（%）	86.4	61.1	51.5	55.5	53.5
CD8（%）	8.9	23.1	29.5	22.7	34.1
CD20（%）	1.2	4.4	4.8	7.8	7.5
CD25（%）	38.3	17.3	8.2	16.0	9.4
HLA-DR（%）	49.5	26.6	23.3	23.5	17.3
CD4/8	9.71	2.65	1.75	2.44	1.57

末梢血リンパ球のマーカー検査では，ATL細胞を主体としたCD4陽性リンパ球数の減少によってCD8陽性細胞の比率は増加し，CD4/8比は正常化した。

（筆者作成）

表3　血液学的検査成績

		1989.9.18	1990.11.29	1991.7.11
[末梢血検査]				
WBC (/μL)		9,100	3,300	5,200
RBC (×10⁴/μL)		346	351	386
Hb (g/dL)		11.2	11.1	10.1
PLT (×10⁴/μL)		25.3	17.5	31.1
[白血球分類]				
Bas.	(%)	0	0	3
Eos.	(%)	0	3	15
Neutr.	(%)	32	47	45
Mono.	(%)	16	12	19
Lymph.	(%)	27	37	17
Ab.Lymph.	(%)	25	1	1
[骨髄検査]				
NCC (×10⁴/μL)		7.9	10.2	−
Mega (/μL)		15	60	−
Erythr.	(%)	22.8	19.6	−
Bas.	(%)	0.4	0	−
Eos.	(%)	1.2	2.4	−
Neutr.	(%)	52.4	58.4	−
Mono.	(%)	4.0	6.4	−
Lymph.	(%)	14.0	12.0	−
Ab.Lymph.	(%)	4.4	0	−

ATL の自然寛解により末梢血・骨髄中の異常リンパ球 (ATL 細胞) は，ほぼ消失した。　(筆者作成)

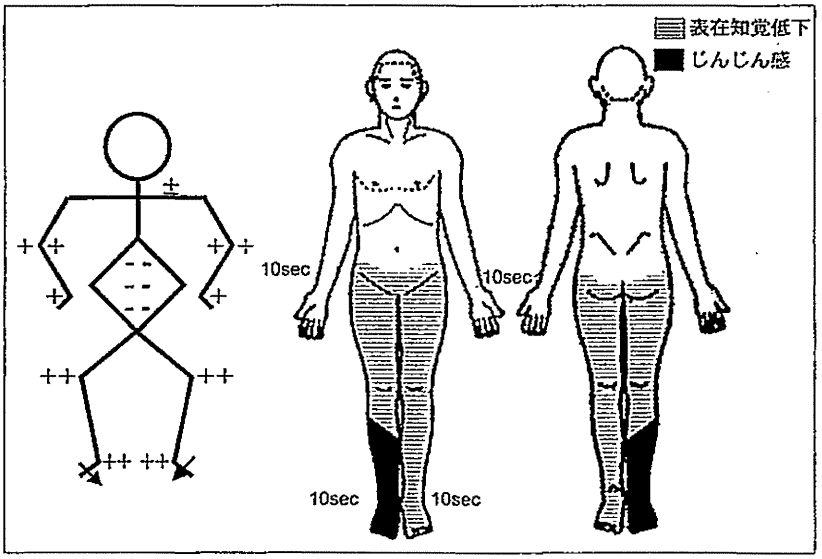

図2　再入院時神経学的所見

両下肢深部腱反射の亢進，両下肢の表在知覚低下、右下肢の異常感覚が認められた。　(筆者作成)

表4　再入院時髄液検査成績

髄液検査
初圧 120 mmH$_2$0 →終圧 80 mmH$_2$0
↓
6 mL 採取
Queckenstedt 試験：圧上昇迅速
性状：無色透明
細胞数　1 μL/dL
蛋白　48 mg/dL
糖　68 mg/dL
Cl　116 mEq/L
ノンネ・アペルト反応（−）
パンディ反応（−）
抗HTLV-1 抗体：32 倍（PA 法）

髄液細胞数の増多は認められず，髄液中の抗HTLV-1 抗体価は陽性であった。

（筆者作成）

Prednisolone（PDN）40 mg/day の投与を開始し，一時的に下肢の疼痛・しびれ感，痙性歩行，膀胱直腸障害などの神経症状の改善が得られたが，PDN の減量とともに神経症状は再び悪化した。その後，PDN を再び 20 mg/day と増量し，症状は残存したまま安定したのでPDN を漸減し，1991 年 12 月 28 日に PDN は中止となった。以後，外来にて経過観察を行っていたが，ATL の再発はみられなかった。1998 年 4 月 21 日以降受診がなくなり，その後の経過は不明である。

考　察

ATL と HAM はともに HTLV-1 が発症の要因となっている疾患である。両疾患の合併に関する報告は，HAM 患者が ATL を発症した症例報告が存在していたが[7, 8]，これまでまとまった報告はない。しかし最近，多数例の HAM 患者の長期追跡調査で ATL の合併が約 2.6%に認められたと報告され，決して稀ではない可能性が示唆されている[9]。一方，ATL 患者が HAM を発症した報告に関しては我々が検索した範囲では認められず，本報告が初めてのケースと思われる。ATL の発症機序は完全に解明されてはいないが，HTLV-1 感染 T 細胞に対する CTL の量的機能的な低下や，感染細胞における遺伝子変化の蓄積が主であると考えられている[6, 10]。一方，HAM では HTLV-1 感染 T 細胞に対する CTL は一般に高応答であり，ウイルスに起因する過剰な免疫応答が脊髄障害を引き起こすと考えられている[11]。このように免疫学的に対照的な両疾患の合併は不思議であり，合併例の特徴を理解することは，両疾患の病態理解に役立つと考えられる。

ATL は稀に自然寛解を起こすことが報告されているが，感染症の治療後やリンパ節生検後にみられることが多い[12, 13]。本例での ATL の自然寛解のメカニズムは不明であるが，ATL の診断の直前に腰椎椎弓切除術が施行されており，この外科的侵襲が自然寛解をもたらした可能性は否定できない。しかし，外科的侵襲や感染症がなぜ自然寛解の誘因になるのか，そのメカニズムについては明らかではない。本症例では CD4 陽性 ATL 細胞の減少に伴い CD8 陽性細胞の比率が増加し，CD4/CD8 比は低下した。古い症例であるため，HTLV-1 に対する免疫応答の詳細は解析できていないが，ATL 細胞や HTLV-1 感染非 ATL 細胞に対する CD8 陽性細胞の免疫応答が回復した可能性が示唆され，その後に HAM を発症したことは，HTLV-1 感染細胞に対する高い免疫応答が HAM 発症に促進的に作用した可能性を示唆する。現状のデータでは，CD8 陽性細胞が，ATL 細胞に対して反応したのか，HTLV-1 感染非 ATL 細胞に反応したのかは明らかではなく，自然寛解と HAM 発症が同じメカニズムで起こったのか，自然寛解の結果，HAM 発症の誘因になったのかは定かではない。いずれにしても，両疾患の発症は宿主免疫と密接に関連があり，本例の ATL から HAM に移行した際の免疫学的な変化は，両疾患の病態を理解する上で非常に興味深い。

本例では，ATLの発症前に突然の下肢脱力，歩行障害，排尿障害がみられている。その原因として，腰部脊柱管狭窄症，ATLの中枢神経浸潤，HAMなどが鑑別として考えられる。HAMに関しては，ある日突然に症状が出現することはなく，またその後改善したという経過からして考えにくい。ATLによる神経症状に関しては完全に否定することは困難であるが，椎弓切除術により症状は軽快しており，腰部脊柱管狭窄症の関与が大きかったものと推測される。しかしながら，前述したようにHAMとATLが合併し得ることが判明してきており，本例のように両疾患の鑑別が重要となる症例も存在することから，今後，HAMとATLによる神経症状を明確に鑑別できる方法や基準を確立することは極めて重要であると考える。

まとめ

慢性型ATLで自然寛解の後，HAMを発症したと思われる稀な症例を経験した。本例はATL細胞の末梢血からの減少とともに正常なCD8陽性細胞の比率が増加しており，HTLV-I感染細胞に対する免疫応答の高まりに伴ってHAMを発症したと推測され，免疫学的に興味深い。このように，ATLとHAMという異なる病態が同一宿主内で変遷し得る可能性が示され，ATLとHAMの相違点や共通点，連続性などについて明らかにすることは，臨床的な重要性のみならず，両疾患の発症メカニズムの解明とその制御に役立つものと思われる。

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A novel and simple method for generation of human dendritic cells from unfractionated peripheral blood mononuclear cells within 2 days: its application for induction of HIV-1-reactive CD4$^+$ T cells in the hu-PBL SCID mice

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Because dendritic cells (DCs) play a critical role in the regulation of adaptive immune responses, they have been ideal candidates for cell-based immunotherapy of cancers and infections in humans. Generally, monocyte-derived DCs (MDDCs) were generated from purified monocytes by multiple steps of time-consuming physical manipulations for an extended period cultivation. In this study, we developed a novel, simple and rapid method for the generation of type-1 helper T cell (Th1)-stimulating human DCs directly from bulk peripheral blood mononuclear cells (PBMCs). PBMCs were cultivated in the presence of 20 ng/ml of granulocyte-macrophage colony-stimulating factor, 20 ng/ml of interleukin-4 (IL-4) and 1,000 U/ml of interferon-β for 24 h followed by 24 h maturation with a cytokine cocktail containing 10 ng/ml of tumor necrosis factor-α (TNF-α), 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2. The phenotype and biological activity of these new DCs for induction of allogeneic T cell proliferation and cytokine production were comparable to those of the MDDCs. Importantly, these new DCs pulsed with inactivated HIV-1 could generated HIV-1-reactive CD4$^+$ T cell responses in humanized mice reconstituted with autologous PBMCs from HIV-1-negative donors. This simple and quick method for generation of functional DCs will be useful for future studies on DC-mediated immunotherapies.

Keywords: dendritic cell, short-term culture, Th1-inducing DCs, anti-HIV-1 T cell response, hu-PBL-SCID

INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) which play a critical role in the regulation of the adaptive immune response through activation and polarization of naive T cells (Banchereau et al., 2000). Since small numbers of activated DCs are highly efficient in generating immune responses against infections and cancers (Moll and Berberich, 2001; Steinman and Banchereau, 2007), the DC therapy represents a new and promising immunotherapeutic approach for treatment of advanced cancers as well as for prevention of infectious diseases. Indeed, the current clinical trials with *ex vivo*-generated DCs (so-called DC vaccine) will yield precious information regarding their potentials as vectors for immunotherapy (Gilboa, 2007; Connolly et al., 2008; Ezzelarab and Thomson, 2011). However, the general protocols to generate DCs are complicated and time consuming. Moreover, since different *ex vivo* DC generation methods affect the DC phenotype and function (Kalantari et al., 2011), it is critical to choose appropriate method for generating functional DCs. In general, the DC precursor monocytes are purified from PBMCs by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). These enriched monocytes are then induced to differentiate into DCs by 5 days-*in vitro* cultivation in medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 followed by a 2-days-maturation procedure (Sallusto and Lanzavecchia, 1994; Gilboa, 2007; Dauer et al., 2008). However, a lines of evidence are increasing that mature monocyte-derived DCs can be generated even after short-term cell culture for 2–3 days (Dauer et al., 2003a,b; Jarnjak-Jankovic et al., 2007; Zhang et al., 2008; Tawab et al., 2009).

In this study, in an attempt to simplify the methods currently being used for optimal DC generation and to develop a standardized method of preparing effective myeloid DC vaccine for immunotherapies, we explored the efficacy of using unfractionated PBMCs as a source of DC precursors and short-term *in vitro* cell culture just for 2 days.

MATERIALS AND METHODS

REAGENTS

The media used were RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (hereafter called RPMI medium) and Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island,

NY, USA) supplemented with 10% FCS with the same antibiotics (hereafter called Iscove's medium). Aldrithiol-2 (AT-2) and low-endotoxin bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). The recombinant human cytokines used included IL-4, GM-CSF, TNF-α and IL-1β (PeproTech, London, UK). Enzyme-linked immunosorbent assay (ELISA) kits for the quantitation of human IFN-γ, human IL-4, human IL-10 and human IL-12 (detecting IL-12 p75 heterodimer) were purchased from Biolegend. The human monocyte negative isolation kits and the human T cell isolation kits were purchased from Invitrogen (Carlsbad, CA, USA). The human naive $CD4^+$ T cell isolation kit was purchased from Miltenyi Biotec (Gladbach, Germany). The Vybrant CFDA SE Cell Tracer Kit was purchased from Invitrogen.

GENERATION OF DCs

Human PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by standard density gradient centrifugation. Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na_2EDTA. For select experiments, monocytes were purified from PBMCs using the $CD14^+$ monocyte negative isolation kit (Invitrogen, Carlsbad, CA, USA). An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% $CD14^+$ cells. To obtain immature MDDCs (iMDDCs), PBMCs (2.5×10^6 cells/ml) or the purified monocytes (5×10^5 cells/ml) were cultured in RPMI medium containing 20 ng/ml of human GM-CSF and 20 ng/ml of human IL-4 at 37°C in 24-well plates in a 5% CO_2 humidified incubator for 5 days. In other experiments, iDCs were generated from either purified monocytes or whole PBMCs by cultivation in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day. These iDCs were matured by incubation in the presence of either 10 ng/ml of LPS (Sigma) or a cocktail containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of prostaglandin E2 (PGE2; TIP cocktail) for 1–2 days.

FLOW CYTOMETRY

Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% sodium azide (FACS buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a predetermined optimal concentration of the appropriate fluorescent dye-labeled mAbs against human cell surface markers on ice for 30 min. The fluorescent dye-labeled monoclonal antibodies (mAbs) against human cell surface molecules used included anti-CD3, anti-CD4, anti-CD8, anti-CD14, CD20, anti-CD80, anti-HLA-DR, and isotype-matched control mAbs (Beckman Coulter, Fullerton, CA, USA), and anti-CD11c, anti-CD86, and anti-CD83 (BioLegend, San Diego, CA, USA). After washing with FACS buffer, cells were fixed in 1% paraformaldehyde (PFA) containing FACS buffer. The cells were then analyzed on FACS-Calibur flow cytometer with CellQuest software (BD Pharmingen, San Diego, CA, USA). Isotype-matched mAbs were utilized as controls to stain an aliquot of the cells to be analyzed for purposes of establishing gates and for determination of the frequency of positively stained cells.

HIV-1 PREPARATION AND INACTIVATION

$HIV\text{-}1_{IIIB}$ (virus that only use CXCR4 as chemokine co-receptor, termed X4) was harvested from Molt-4/IIIB cell cultures. Batches of each HIV-1 preparation were inactivated with Aldrithiol-2 (AT-2; Sigma) as described previously (Yoshida et al., 2003). AT-2 was removed by three successive ultrafiltration in PBS using 100-kDa-cut-off centrifugal filtration devices (Centriprep 100; Amicon, Beverly, MA, USA). Then AT-2–inactivated HIV-1 (iHIV) was purified by pelleting down the virus at 20,000 × *g* for 2 h three times in 0.1% BSA-PBS. The virus pellet was resuspended in 0.1% BSA-PBS, aliquoted, and stored at −80°C until use. The concentration of HIV-1 was estimated by measuring levels of HIV-1 p24 antigen with our in-house p24 ELISA kit (Tanaka et al., 2010). As previously described (Yoshida et al., 2003), activated human PBMCs incubated with an aliquot of 1 μg/ml of the AT-2-treated HIV-1 preparation failed to demonstrate the presence of any detectable infectious virions (data not shown).

STIMULATION OF T CELLS

Enriched populations of naive $CD4^+$ T cells and bulk T cells with >90% purity were isolated from normal human PBMCs by using appropriate negative cell isolation kits. These T cells (4×10^4 cells/well) were first labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), then co-cultured with allogeneic DCs at a T cells: DCs ratio of 50:1 in 100 μl of RPMI medium supplemented with 20 U/ml human IL-2 in 96-well, U-bottomed plates. Cell proliferation and cytokine production were determined on day 4.

hu-PBL-SCID MICE

The BALB/c-$rag2^{-/-}\gamma c^{-/-}$ mice lacking T cells, B cells and natural killer (NK) cells ($Rag2^{-/-}$ mice; Traggiai et al., 2004) were used in this study. The mice were kept in the specific-pathogen-free and P3 animal facilities of the Laboratory Animal Center, University of the Ryukyus. The protocols for the care and use of mice engrafted with human PBMCs and autologous DCs sensitized with inactivated HIV-1 or ovalbumin (OVA) were approved by the committee on animal research of the University of the Ryukyus prior to initiation of the study. Matured DCs (5×10^5 cells) pulsed with either AT-2-inactivated HIV-1 (40 ng of p24) or 100 μg of OVA in 100 μl of RPMI medium for 2 h at 37°C were mixed with autologous fresh PBMCs (3×10^6 cells) in a final volume of 100 μl in serum-free RPMI medium, and the were directly injected into the spleen of Rag2 mice as previously described (Yoshida et al., 2003). One week later, the same number of DCs pulsed with the same antigens were inoculated again into the spleen. One week later, mice were sacrificed, blood was collected by cardiocentesis, and human $CD4^+$ T cells were enriched from splenocytes using a human $CD4^+$ T cell isolation kit according to the manufacturer's instructions. For the measurement of antigen-specific human cellular immune responses, human $CD4^+$ T cell (2×10^5 cells) collected from the spleens of immunized $Rag2^{-/-}$ mice were cultured for 2 days with autologous monocytes (2×10^5 cells) in the presence or absence of inactivated HIV containing 40 ng/ml of p24 in 500 μl of RPMI medium supplemented with 20 U/ml of IL-2 in individual wells of a 48-well plate at 37°C. The concentration

of human IFN-γ or IL-4 produced in the culture supernatants was determined with ELISA kits.

STATISTICAL ANALYSIS

Data were analyzed by Student's *t* test with the with Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

GENERATION OF MYELOID MATURE DCs DIRECTLY FROM PBMCs WITHIN 2 DAYS

In order to reduce the cost, labor and any loss of potential precursors from PBMCs, we have previously established a novel culture method for generating functional human DCs from unfractionated PBMC in which whole PBMCs were cultured in the presence of IL-4 and GM-CSF for 5 days followed by a 2-day maturation in media containing poly I:C and IL-1β (Kodama et al., 2010). However, there were considerable lot variations in commercial poly:IC in the DC-maturation activity (data not shown). Therefore, we tested a previously reported maturation cytokine cocktail containing TNF-α, IL-1β, IL-6 and PGE2 (Jonuleit et al., 1997). In a preliminary study, we found that IL-6 was not necessary to mature DCs from purified monocytes in the present cell culture conditions, probably due to the use of serum-containing media. Thus, we used a cytokine cocktail

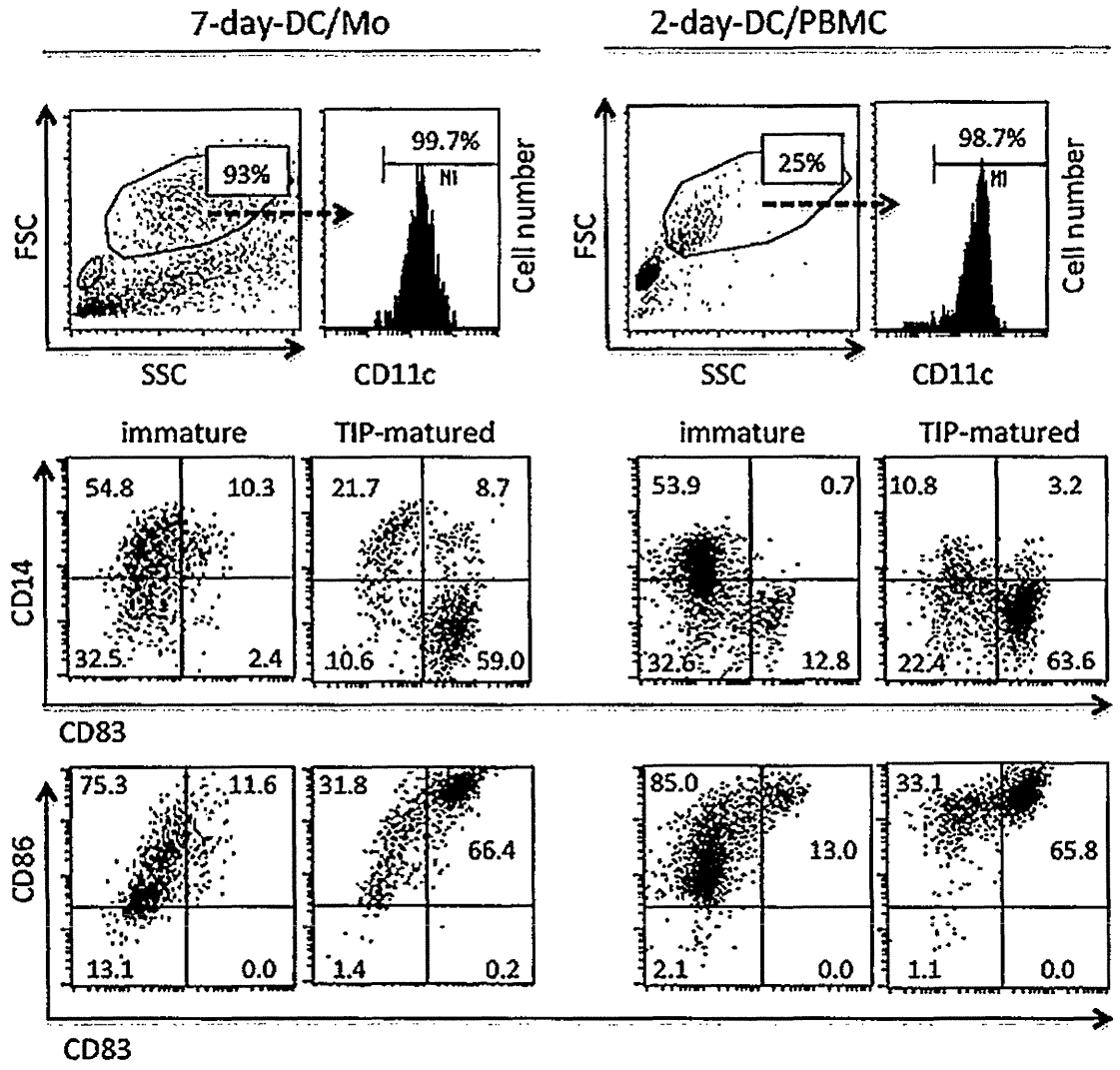

FIGURE 1 | Generation of functional human myeloid DCs directly from PBMCs *in vitro* within 2 days. MDDCs generated purified monocytes for 7 days (7-day-DC/Mo) or whole PBMCs cultured in medium containing GM-CSF, IL-4 and IFN-β (2-day-DC/PBMC), either non-treated or treated with the maturation cocktail containing TNF-α, IL-1β and PGE2 (TIP) were examined for the expression of mature DC markers by a flow cytometry. Cells were stained with antibodies to CD11c, CD14, CD83 and CD86, and analyzed on a subpopulation gated for FSC^{high}, SSC^{high} and $CD11c^{+}$. The percentages of the positive cells in the gated population were shown in the dot plots. Data shown are representative of three independent experiments using blood from three different donors.

containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β and 1 μg/ml of PGE2 (hereafter called TIP cocktail) throughout the present study.

Based on our previous report that monocytes can be differentiated into mature DCs within 2 days (Zhang et al., 2008), we tested whether Th1-inducing DCs could be generated from unfractionated PBMCs. PBMCs (2.5×10^6 cells/ml) were cultured in RPMI medium containing GM-CSF (20 ng/ml), IL-4 (20 ng/ml) and IFN-β (1,000 U/ml) for 1 day followed by additional 1 day cultivation in the presence or absence of the TIP cocktail. The phenotypes of $CD11c^+$ large cells in these 2-day PBMC cultures were compared with those of MDDCs derived from purified monocyte for 7 days (7-day-DC/Mo; **Figure 1**). The proportion of FSC^{high} and SSC^{high} cells in the 2-day-DC/PBMC culture was 20~25% of total viable cells depending on donors and these cells expressed CD11c (data not shown). After maturation with the TIP cocktail, similar to the 7-day-DC/Mo, the large $CD11c^+$ cells in the 2-day PBMC cultures became $CD14^{low}$, $CD86^{high}$ and $CD83^{high}$, a typical marker of matured myeloid DCs (Ohshima et al., 1997). The other viable cell populations in the 2-day PBMC cultures were $CD3^+$ T cells (54.0~59.2%), $CD56^+$ NK cells (8.4~9.3%) and $CD19^+$ B cells (6.5~8.6%; $n = 3$). These data showed that the present culture method was applicable to generate myeloid mature DCs from bulk PBMCs within 2 days (2-day-DC/PBMC).

Then we tested cytokine production by these 2-day-DC/PBMC. Interestingly, in contrast to the DCs matured in the presence of LPS, the production of IL-12 and IL-10 by the TIP matured 2-day-DC/P was minimum (**Figure 2**). To investigate whether the 2-day-DC/PBMC were immunologically functional, we examined their ability to stimulate allogeneic T cell proliferation. Like the MDDCs (7-day-DC/Mo), the 2-day-DC/PBMC could stimulate allogeneic T cell proliferation (**Figure 3A**). Then we quantitated the levels of IFN-γ and IL-4 in the culture supernatants from allogeneic $CD4^+$ T cells co-cultured with various DCs. As shown in **Figure 3B**, among the four DC preparations including the 7-day-DC/Mo, 7-day-DCs from PBMCs (7-day-DC/PBMC), 2-day-DCs from monocytes (2-day-DC/Mo) and 2-day-DC/PBMC, the 2-day-DC/PBMC were most potent in induction of IFN-γ production. The bulk 2-day-DC/PBMC alone did not produce detectable IFN-γ (<20 pg/ml) in the present culture conditions (data not shown). The levels of IL-4 and IL-10 were below detection (<5 pg/ml) in all the samples tested (data not shown). These results indicated that the 2-day-DC/PBMC had a potential to induce Th1 response.

INDUCTION OF HIV-1-REACTIVE HUMAN $CD4^+$ T CELL RESPONSES IN hu-PBL-SCID MICE

Finally, we examined whether the short-term generated 2-day-DC/PBMC could induce HIV-1-reactive immune responses *in vivo* in comparison to MDDCs (7-day-DC/Mo) using our hu-PBL-SCID mice model (Yoshida et al., 2003). SCID mice were *intra-splenically* transplanted with DCs loaded with AT-2-inactivated HIV-1 together with autologous fresh PBMCs. On day 7, these mice were received an *intra-splenic* booster injection with similarly prepared antigen-pulsed DCs. Seven days after the booster injection, mice were sacrificed and examined for antigen-specific human immune responses. **Figure 4** showed that after *in vitro* re-stimulation with autologous APCs pulsed with inactivated HIV-1, enriched human $CD4^+$ T cells from two out of three mice immunized with MDDCs (7-day-DC/Mo) pulsed with HIV-1 and those from three out of four mice immunized with 2-day-DC/PBMC pulsed with HIV-1 produced IFN-γ in antigen-dependent way, indicating that the 2-day-DC/PBMC could induce HIV-1 antigen-reactive human T responses *in vivo* as potent as MDDCs. In the re-stimulated culture supernatants, no IL-4 or IL-10 was detected (<5 pg/ml) using ELISA (data not shown). In addition, no detectable antibodies against HIV-1 were detected

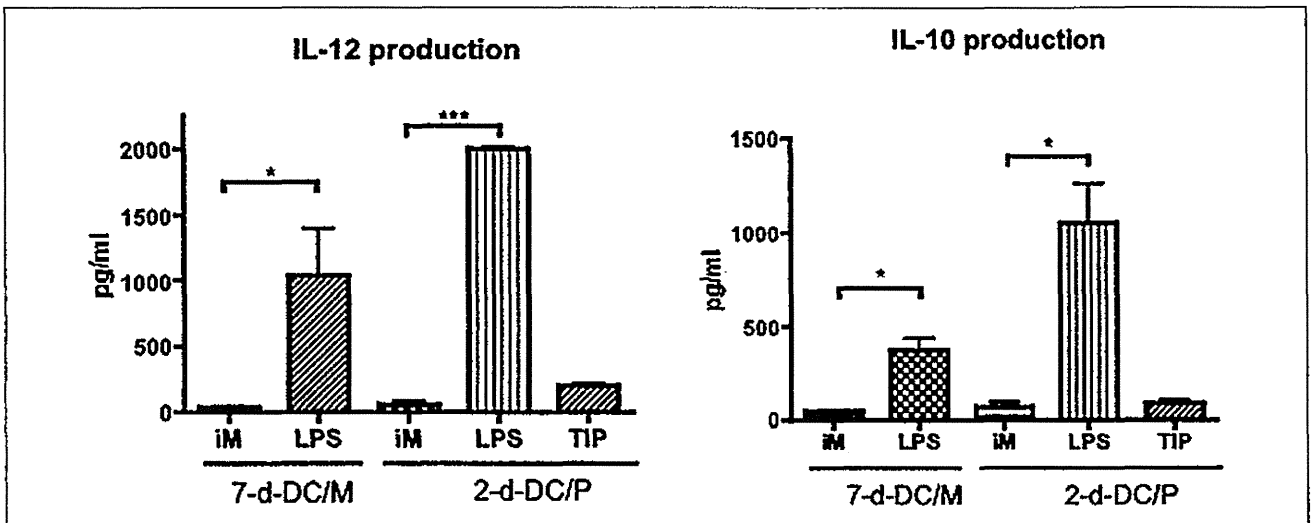

FIGURE 2 | Production of cytokines by the 2-day-DC/PBMC. The levels of IL-12 (bioactive p70 heterodimer) and IL-10 in the supernatants from MDDCs (7-d-DC/M) or 2-day-DC/PBMC (2-d-DC/P) either non-matured (iM) or matured with LPS or the TIP cocktail were determined by ELISA. Data are mean ± SD of triplicate cultures. $^*P < 0.05$, $^{***}P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

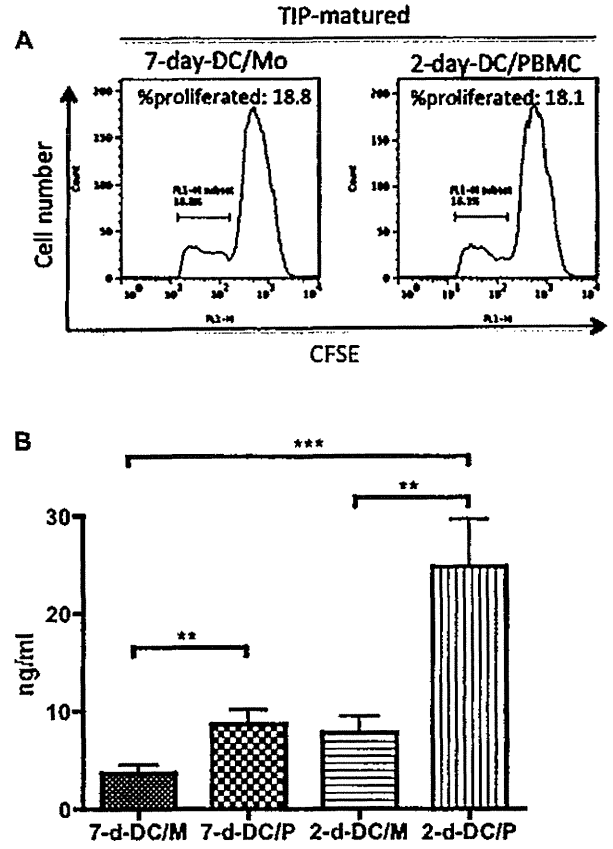

FIGURE 3 | High levels of production of IFN-γ by allogeneicT cells stimulated with 2-day-DCs generated from PBMCs. Matured MDDCs (7-day-DC/Mo) and 2-day-DCs generated from PBMCs (2-day-DC/PBMC) were co-cultured with CFSE-labeled allogeneic $CD4^+$ T cells at DC toT cell ratio of 1:50 for 4 days. The cell number of DCs in 2-day-DC/PBMC was manually counted using a Burker-Turk hemo-cytometer in which only large cells were counted. **(A)** Percentages of proliferated cells were examined by flow cytometry. **(B)** The levels of IFN-γ produced in the supernatants were quantitated by ELISA. In addition to the DCs shown in **(A)**, MDDCs generated from PBMCs for 7 days (7-d-DC/P) and 2-day-DCs generated from purified monocytes (2-d-DC/M) were also tested. Data shown are representative of three independent experiments using blood from two different donors.

as determined by using a commercial Western blot assay kit in plasma samples from all the DCs-HIV-1-immunized mice (data not shown).

Altogether, these results demonstrated that human myeloid DCs directly generated from PBMCs by the present short-term cultivation method were potent in induction of functional Th1 responses both *in vitro* and *in vivo*.

DISCUSSION

In the present study, we have developed a novel, simple and rapid protocol for generating Th1-stimulating human myeloid DCs directly from unfractionated PBMCs. These 2-day-DC/PBMC were potent in both stimulating allogeneic T cells *in vitro* and inducing HIV-1-reactive Th1 responses in hu-PBL-SCID mice. The use of whole PBMCs as DC precursors might reduce any loss of monocytes in the step of purification by adherence (Jonuleit et al., 2001), elutriation (Berger et al., 2005) or positive or negative selection using immunomagnetic beads (Babatz et al., 2003). One possible concern on using whole PBMCs was that the non-monocyte cells, such as T, B or NK cells, in the PBMCs might interfere with differentiation and function of DCs. However, in the present study there was no obvious difference in DC maturation and function between in PBMC and purified monocyte cultures.

For the final maturation, we used a cytokine cocktail containing TNF-α and IL-1β and PGE2 (TIP coctail). Simultaneous use of these three reagents in TIP was essential for maturation of DCs since use of the reagents either in single or in two combinations failed to mature DCs (data not shown). In general, IL-6 that is included in the maturation cytokine cocktail TNF-α and IL-1β and PGE2 to mature DCs was not necessary in the present culture conditions. The reason remains to be studied, but it is possible that IL-6 is required in serum-free culture conditions. The present 2-day-DC/PBMC matured by TIP produced lower IL-12 than those matured by LPS. Low levels production of IL-12 might be ascribed to the use of PGE2 that inhibits bioactive IL-12 heterodimer production (Kalinski et al., 2001; Kalim and Groettrup, 2013). Despite of the low level production of IL-12, the TIP-matured 2-day-DC/PBMC were potent in stimulating IFN-γ, but not IL-4 or IL-10, production by allogeneic T cells. The reason for higher potentials of 2-day-DC/PBMC to induce Th1 cells than MDDCs remains to be clarified. It is speculated that natural DCs contained in the 2-day-PBMC-derived DCs might enhance the activation. Indeed, 2-day-DC/PBMC culture generated from $CD14^+$ cell-depleted PBMCs were able to stimulate allogeneic $CD4^+$ T cells to a lesser extent (data not shown). However, we cannot clearly determine if the stimulation was mediated by remaining monocytes. Further study is required to solve this issue. Importantly, as the previous study (Yoshida et al., 2003), the present study showed the induction of primary HIV-1-specific human $CD4^+$ T cell immune responses in hu-PBL-SCID mice by DC-based immunization, demonstrating that the present 2-day-PBMC-derived DCs might have a potential for clinical use in DC-based immunization in humans against HIV-1. It was of interest that the levels of IFN-γ production were higher in $CD4^+$ T cells immunized with 2-day-DC/PBMC than those immunized with 7-day-DC/Mo. It is possible that 2-day-DC/PBMC could live longer than 7-day-DC/Mo *in vivo* to stimulate antigen-specific $CD4^+$ T cells. In addition, because myeloid DCs are susceptible to HIV-1 infection (Knight et al., 1990), the use of these IFN-β-treated DCs will be beneficial for HIV-1-infected individuals.

In conclusion, the present study provided a new method to generate functional human myeloid DCs directly from PBMCs in a short-term culture period. These DCs will be useful for studies exploring potentials of DC-based immunization for not only infectious diseases but also cancers *in vitro* and *in vivo*.

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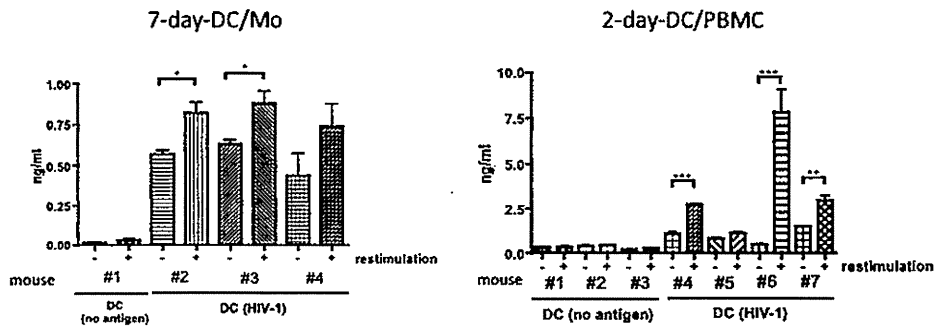

FIGURE 4 | Functional activity of the 2-days-DCs in hu-PBL-SCID mice. Fresh autologous PBMCs from normal human donors were transferred into the Rag2$^{-/-}$ mouse spleen together with autologous mature MDDCs (7-day-DC/Mo) or 2-days-PBMC-derived DCs (2-day-D/PBMC) pulsed with no antigen (no antigen) or AT-2-inactivated HIV-1 (40 ng of p24). On day 7 after the first transplantation, these mice were received an *intra splenic* booster injection with similarly prepared DCs. Seven days after the booster injection, mice were sacrificed and human CD4$^+$ T cells were purified from splenocytes. These CD4$^+$ T cells were co-cultured with autologous APCs (adherent PBMCs) in the presence or absence of antigens (restimulation) for 2 days at 37°C. IFN-γ levels produced in the culture supernatants were measured by ELISA. Data show mean ± SD of triplicate cultures. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data shown are representative of three independent experiments using blood from two different donors.

AUTHOR CONTRIBUTIONS

Akira Kodama designed and performed the experiments, analyzed the data and wrote the paper. Reiko Tanaka and Mineki Saito performed the experiments, analyzed the data and wrote the paper. Aftab A. Ansari participated in the design of the study and helped to draft the manuscript. Yuetsu Tanaka designed and supervised the research, performed experiments and wrote the paper. All authors checked the final version of this manuscript.

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REVIEW

Neuroimmunological aspects of human T cell leukemia virus type 1-associated myelopathy/tropical spastic paraparesis

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Abstract Human T cell leukemia virus type 1 (HTLV-1) is a human retrovirus etiologically associated with adult T cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Only approximately 0.25–4 % of infected individuals develop HAM/TSP; the majority of infected individuals remain lifelong asymptomatic carriers. Recent data suggest that immunological aspects of host–virus interactions might play an important role in the development and pathogenesis of HAM/TSP. This review outlines and discusses the current understanding, ongoing developments, and future perspectives of HAM/TSP research.

Keywords HTLV-1 · HAM/TSP · Host immune response

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease: a malignancy of mature $CD4^+$ T cells called adult T cell leukemia/lymphoma (ATL) (Hinuma et al. 1981; Poiesz et al. 1980; Yoshida et al. 1984) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al. 1985; Osame et al. 1986). Like human immunodeficiency virus (HIV), HTLV-1 is never eliminated from the host despite vigorous cellular and humoral immune responses. However, in contrast to HIV infection, few with HTLV-1 develop disease; only approximately 2–3 % of infected persons develop ATL (Tajima 1990), another 0.25–4 % develop HAM/TSP (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990), and the majority of infected individuals remain lifelong asymptomatic carriers (ACs). Therefore, evaluation of the individual risk of developing disease in ACs would certainly be of considerable importance, especially in HTLV-1 endemic areas.

The viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection appear to regulate the development of HTLV-1-associated diseases (Bangham and Osame 2005). In particular, a strong immune response, especially the cytotoxic T lymphocyte (CTL) response, to HTLV-1 is seen in patients with HAM/TSP and suggested to be strongly associated with the pathogenesis of HTLV-1-associated diseases (Matsuura et al. 2010; Saito et al. 2012). For more than two decades, the investigation of HTLV-1-mediated immunopathogenesis has focused on Tax, an HTLV-1-encoded viral oncoprotein, because Tax activates many cellular genes by binding to groups of transcription factors and co-activators and is necessary and sufficient for cellular transformation. However, recent reports have identified that another regulatory protein, HTLV-1 basic leucine zipper factor (HBZ), also has a critical role in the development of ATL and HAM/TSP (Matsuoka and Jeang 2011). This review summarizes past and recent studies of HAM/TSP, attempting to answer the following fundamental questions: Why do some HTLV-1-infected people develop disease whereas the vast majority remain healthy? How does HTLV-1 persist in the individual host despite a strong host immune response? How is the inflammatory lesion in HAM/TSP initiated and maintained?

History and epidemiology of HTLV-1

HTLV-1 belongs to the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily and infects 10–20 million people worldwide (de The and Bomford 1993; Proietti et al. 2005; Uchiyama 1997). HTLV-1 can be transmitted through sexual contact (Roucoux et al. 2005), intravenous drug use (Proietti et al. 2005), and breastfeeding from mother to child (Hino

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et al. 1985; Kinoshita et al. 1987). At present, the infection is endemic in southwest Japan, the Caribbean, Sub-Saharan Africa, and South America, with smaller foci in Southeast Asia, South Africa, and northeast Iran (Verdonck et al. 2007). HTLV-1 was initially isolated in 1980 from two T cell lymphoblastoid cell lines and the blood of a patient originally thought to have a cutaneous T cell lymphoma (Poiesz et al. 1980). It was the first retrovirus ever associated with cancer in a human. Three years before the isolation of HTLV-1, Takatsuki et al. reported ATL, a rare form of leukemia endemic to southwest Japan, as a distinct clinical entity (Uchiyama et al. 1977). In 1981, Hinuma et al. clearly demonstrated that ATL was caused by a new human retrovirus, originally termed ATLV (Hinuma et al. 1981; Miyoshi et al. 1981). Since then, ATLV and HTLV have been shown to be identical, and a single name, HTLV-1, has been adopted. In the mid-1980s, epidemiological data linked HTLV-1 infection to a chronic progressive neurological disease, which was termed tropical spastic paraparesis in the Caribbean (Gessain et al. 1985) and HTLV-1-associated myelopathy in Japan (Osame et al. 1986). HTLV-1-positive TSP and HAM were subsequently found to be clinically and pathologically identical, and the disease was given a single designation as HAM/TSP (Hollsberg and Hafler 1993). To date, more than 3,000 cases of HAM/TSP have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in non-endemic areas such as the USA and Europe, mainly in immigrants from an HTLV-1 endemic area (Araujo and Silva 2006). HTLV-1 can cause other chronic inflammatory diseases such as uveitis (Mochizuki et al. 1992), arthropathy (Nishioka et al. 1989), pulmonary lymphocytic alveolitis (Maruyama et al. 1988; Sugimoto et al. 1989; Sugimoto et al. 1987), polymyositis (Higuchi et al. 1992; Morgan et al. 1989), Sjögren syndrome (Terada et al. 1994), and infective dermatitis (LaGrenade et al. 1990), although there is no clear evidence for an etiological role of HTLV-1 in these diseases.

Clinical and pathological features of HAM/TSP

HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities (Nakagawa et al. 1996). The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than that for the onset of ATL (Nakagawa et al. 1995; Olindo et al. 2006). HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life, that is, through sexual contact almost exclusively from male to female, intravenous drug use, contaminated blood transfusions, etc. The mean age at onset is 43.8 years, and like autoimmune diseases, the frequency of cases of HAM/TSP is greater in women than in men (the male-to-female ratio of occurrence is 1:2.3) (Nakagawa et al. 1995). In addition to HTLV-1 antibody (Ab) positivity both in serum and cerebrospinal fluid (CSF), the presence of atypical lymphocytes (the so-called "flower cells") in peripheral blood and CSF, a moderate pleocytosis, and raised protein content in CSF is observed in patients with HAM/TSP (Araujo and Silva 2006). Oligoclonal immunoglobulin bands in the CSF; raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interferon (IFN)-γ; and increased intrathecal Ab synthesis specific for HTLV-1 antigens have also been described (Jacobson 2002). Clinical progression of HAM/TSP is associated with an increase in the proviral load (PVL) in individual patients, and a high ratio of PVL in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease (Takenouchi et al. 2003). Thus, a pro-inflammatory environment associated with increased numbers of HTLV-1-infected cells is a characteristic immunological profile of HAM/TSP.

Pathological analysis of HAM/TSP autopsy materials showed the loss of myelin and axons in the lateral, anterior, and posterior columns of the spinal cord. These lesions are associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, reactive astrocytosis, and fibrillary gliosis, predominantly at the thoracic level (Iwasaki 1990; Izumo et al. 2000; Yoshioka et al. 1993), suggesting that the immune response against HTLV-1 causes the inflammatory spinal cord damage seen in patients with HAM/TSP (Bangham 2000). In patients with active chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain (Aye et al. 2000). The peripheral nerve pathology of patients with HAM/TSP with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineural fibrosis (Bhigjee et al. 1993; Kiwaki et al. 2003).

Treatment of HAM/TSP

To date, no generally agreed standard treatment regimen has been established for HAM/TSP, and no treatment has proven to be consistently effective on a long-term basis. Therefore, clinical practice for treatment of patients with HAM/TSP is based on case series and open, nonrandomized, uncontrolled studies. Mild to moderate beneficial effects have been reported for a number of agents in open-label studies including corticosteroids (Nakagawa et al. 1996), danazol (Harrington et al. 1991), pentoxifylline (Shirabe et al. 1997), immunosuppressants such as ciclosporin A (Martin et al. 2012), high-dose intravenous gamma globulin (Kuroda et al. 1991), plasmapheresis (Matsuo et al. 1988), antibiotics (erythromycin and fosfomycin), and vitamin C (Nakagawa et al. 1996). It should be noteworthy that oral prednisolone was effective in 81.7 %

of 131 patients in a large-scale case series study (Nakagawa et al. 1996). However, the complications of corticosteroids limit their use, particularly in post-menopausal women, who are at higher risk for developing HAM/TSP. Multicenter double-blind randomized placebo-controlled trials for the IFN-α treatment indicate that IFN-α is an effective therapy with an acceptable side effects profile (Izumo et al. 1996), although the benefit of long-term IFN-α therapy has not been well studied. In regard to oral antiviral drugs zidovudine plus lamivudine, no evidence of significant benefit yet exists from randomized placebo-controlled trials (Taylor et al. 2006). Recently, oral administration of histone deacetylase inhibitor valproic acid (VPA) has been conducted as a single-center, open-label trial (Olindo et al. 2011). Although administration of VPA induced a transient increase of HTLV-1 expression to expose virus-positive cells to the host immune response, clinical measures and PVL were stable overall. It has also been reported that the antibiotic minocycline significantly inhibited spontaneous lymphocyte proliferation and degranulation/IFN-γ expression in $CD8^+$ T cells of patients with HAM/TSP, suggesting its potential for treatment (Enose-Akahata et al. 2012). Overall, more clinical trials with adequate power are needed in the future.

Risk factors for developing HAM/TSP

It is well-known that HAM/TSP occurs in only a minority of HTLV-1-infected individuals. A previous population association study in HTLV-1 endemic southwest Japan revealed that one of the major risk factors is the HTLV-1 PVL, because the PVL is significantly higher in patients with HAM/TSP than in ACs (Nagai et al. 1998). A higher PVL in patients with HAM/TSP than in ACs was also observed in other endemic areas such as the Caribbean (Manns et al. 1999), South America (Adaui et al. 2006), and the Middle East (Sabouri et al. 2005). In southwest Japan, it was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in patients with HAM/TSP and genetic relatives. Namely, possession of the HLA class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP in the same population (Jeffery et al. 2000; Jeffery et al. 1999). Because the function of class I HLA proteins is to present antigenic peptides to CTL, these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of PVL and the risk of HAM/TSP. In accordance with this observation, it has been reported that CTL spontaneously kill autologous HTLV-1-infected cells ex vivo (Hanon et al. 2000), granzymes and perforin are more highly expressed in individuals with a low PVL (Vine et al. 2004), and the lytic efficiency of the CD8+ T cell response (i.e., the fraction of autologous HTLV-1-expressing cells eliminated per CD8+ T cell per day) was inversely correlated with both PVL and the rate of spontaneous proviral expression (Kattan et al. 2009). Furthermore, the major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of patients with HAM/TSP showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted CD8+ lymphocytes compared with PBMCs (Nagai et al. 2001b). These findings indicate that an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system might be closely associated with the pathogenesis of HAM/TSP (Hayashi et al. 2008a), and the CTLs against HTLV-1 reduce both PVL and the risk of HAM/TSP. Recently, using a combination of computational and experimental approaches, MacNamara et al. reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, also determines the outcome of HTLV-1 infection (Macnamara et al. 2010).

Meanwhile, analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP. Namely, the TNF-α promoter −863 A allele (Vine et al. 2002) and the longer CA repeat alleles of matrix metalloproteinase 9 promoter (Kodama et al. 2004) predisposed to HAM/TSP, whereas IL-10 −592 A (Sabouri et al. 2004), stromal-derived factor 1 +801A (Vine et al. 2002), and IL-15 +191 C alleles (Vine et al. 2002) conferred protection against HAM/TSP. The polymorphisms in the matrix metalloproteinase 9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene (Kodama et al. 2004; Sabouri et al. 2004). However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in a larger sample size may provide evidence for the association of non-HLA genes with the pathogenesis of HAM/TSP.

It has been reported that the lifetime risk of developing HAM/TSP differs among ethnic groups, ranging between 0.25 and 4 %. The annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times greater risk for women in both populations (Hisada et al. 2004; Kramer et al. 1995; Nakagawa et al. 1995; Osame et al. 1990). Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 Tax gene variation and the risk of HAM/TSP (Furukawa et al. 2000). Tax subgroup A, which belongs to cosmopolitan

subtype A, was more frequently observed in patients with HAM/TSP, and this association was independent of the protective effect of HLA-A*02. Interestingly, HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, Tax subgroup B, which belongs to cosmopolitan subtype B, but not against Tax subgroup A in the Japanese population (Furukawa et al. 2000). Jamaican subjects, who had a higher annual incidence of HAM/TSP, also have cosmopolitan subtype A, whereas approximately 80 % of Japanese subjects, who had a lower annual incidence of HAM/TSP, have cosmopolitan subtype B. Interestingly, HLA-A*02 did not appear to provide protection against HAM/TSP development with cosmopolitan subtype A in a population in Iran (Sabouri et al. 2005).

To test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T cell clone, Gillet et al. recently reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome (Gillet et al. 2011). They mapped >91,000 unique insertion sites (UISs) of the provirus in primary PBMCs from 61 HTLV-1-infected individuals and showed that a typical HTLV-1-infected host carries between 500 and 5,000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index to quantify the clonality of HTLV-1-infected cells in vivo and found that the oligoclonality index did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between the oligoclonality index and HTLV-1 PVL in either ACs or patients with HAM/TSP. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, to a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion in vivo is favored by a proviral integration site near a region of host chromatin undergoing active transcription or same-sense transcriptional orientation of the provirus. In contrast, negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T cell clones with low pathogenic potential. More recent reports indicate that circulating HTLV-1-positive cells each contain a single integrated proviral copy (Cook et al. 2012), and cells expressing HTLV-1 Tax protein (i.e., viral protein expression) were significantly more frequent in clones of low abundance in vivo, whereas certain transcription start sites immediately upstream of the viral integration site were associated with virus latency (i.e., no viral protein expression). In particular, Tax-expressing, more "pathogenic" clones were efficiently controlled by the immune response, especially CTLs, whereas non-Tax-expressing "invisible" infected clones were associated with mitotic clonal expansion in vivo (Melamed et al. 2013).

The innate immune response in HAM/TSP

Type I IFN is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of IFN-stimulated genes, which execute the antiviral effects of IFN (Samuel 2001). The IFN-stimulated genes then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself (Liu et al. 2011). In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 messenger RNA is very low and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture in vitro (Hanon et al. 2000). However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T cells is suppressed by stromal cells (i.e., epithelial cells and fibroblasts) in culture through type I IFNs (Kinpara et al. 2009). Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression in vitro and in vivo, at least through type I IFN. More recently, it has been reported that IFN-stimulated genes were overexpressed in circulating leukocytes and the expression correlated with the clinical severity of HAM/TSP (Tattermusch et al. 2012).

Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of natural killer (NK) cells (especially the CD3+ CD16+ subset) than ACs, although the results were not normalized with respect to the PVL (Yu et al. 1991). Because an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the non-polymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in patients with HAM/TSP (Saito et al. 2003). The results clearly showed a lower frequency of HLA-E tetramer-binding cells in patients with HAM/TSP than in ACs; as in the earlier studies (Yu et al. 1991), this reduction in frequency was particularly notable in the CD3+ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding CD3− cells between patients with HAM/TSP and ACs (Saito et al. 2003). Recent reports also suggest that the frequency of invariant natural killer T (NKT) cells in the peripheral blood of patients with HAM/TSP is significantly decreased when compared with that in healthy subjects and/or ACs (Azakami et al. 2009; Ndhlovu et al. 2009). These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota-containing

fermented milk in patients with HAM/TSP resulted in a significant increase in NK cell activity with improvements in clinical symptoms (Matsuzaki et al. 2005). Thus, circulating NK and NKT cells might also play an important role in the disease progression and pathogenesis of HAM/TSP.

The acquired immune response in HAM/TSP

It has been reported that patients with HAM/TSP generally have higher anti-HTLV-1 Ab titers than ACs with a similar PVL (Ishihara et al. 1994; Kira et al. 1992; Nagasato et al. 1991), suggesting the existence of an augmented humoral immune response to HTLV-1. Interestingly, although Ab responses to the immunodominant epitopes of the HTLV-1 Envelope (Env) proteins were similar in all three clinical groups of HTLV-1 infection (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was higher in patients with HAM/TSP (71–93 %) than in patients with ATL (4–31 %) or ACs (27–37 %) (Lal et al. 1994). A recent report indicates that the Ab response against HBZ was associated with reduced CD4+ T cell activation in patients with HAM/TSP, and HBZ-specific Ab inhibited spontaneous in vitro lymphocyte proliferation in the PBMCs of patients with HAM/TSP (Enose-Akahata et al. 2013). Among these anti–HTLV-1 antibodies, anti-Env Ab is particularly important because some anti-Env Abs have neutralizing activity against HTLV-1. Antisera raised against recombinant HTLV-1 Env polypeptides (Kiyokawa et al. 1984; Nakamura et al. 1987), vaccinia virus containing the HTLV-1 env gene (Hakoda et al. 1995; Shida et al. 1987), immunization with neutralizing epitope peptides (Tanaka et al. 1994), and passive transfer of human immunoglobulin G that has neutralizing activity (Murata et al. 1996; Tanaka et al. 1993) were all shown to neutralize HTLV-1 infectivity. In HTLV-1 infection, the roles of HTLV-1 neutralizing Ab in vivo are still largely unknown. It will be interesting to examine whether HTLV-1 neutralizing Ab titers correlate with disease status and PVL in infected individuals. Because the mutation rate of HTLV-1 provirus is significantly lower than that of HIV-1, passive immunization with human monoclonal Ab may be a beneficial and effective method to prevent HTLV-1 infection.

Antiviral CD4+ T cell responses are of central importance in driving B cell and CD8+ T cell responses in vivo. The most common HTLV-1 antigen recognized by CD4+ T cells is the Env protein (Goon et al. 2004b; Kitze et al. 1998), in contrast to the immunodominance of Tax in the CD8+ T cell response (Goon et al. 2004a; Jacobson et al. 1990; Kannagi et al. 1991). At a similar PVL, patients with HAM/TSP had a significantly increased frequency of virus-specific CD4+ T cells compared with ACs (Goon et al. 2004b; Nose et al. 2007). The antiviral T-helper (Th) 1 phenotype is also dominant among HTLV-1-specific CD4+ T cells in both ACs and patients with HAM/TSP (Goon et al. 2002), and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4+ T cells in patients with HAM/TSP compared with ACs of a similar PVL (Goon et al. 2002; Goon et al. 2003). A role for CD4+ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan (Jeffery et al. 1999, 2000) and northeast Iran (Sabouri et al. 2005). Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4+ T cells directly ex vivo (Nose et al. 2007). The results showed that the frequency of tetramer+ CD4+ T cells was significantly higher in patients with HAM/TSP than in ACs with a similar PVL. Furthermore, direct ex vivo analysis of tetramer+ CD4+ T cells from two unrelated DRB1*0101-positive patients with HAM/TSP indicated that certain T cell receptor Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region 3 from both patients. These results suggest that the observed increase in virus-specific CD4+ T cells in patients with HAM/TSP, which may contribute to CD4+ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected CD4+ T cells but was the result of in vivo selection by specific MHC–peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 tetramer+ CD8+ T cells (Saito et al. 2001) and muscle-infiltrating cells from patients with HAM/TSP and HTLV-1-infected patients with polymyositis (Saito et al. 2002).

Previous reports indicated that HTLV-1-specific CD8+ CTLs are typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax (Bangham 2000). Further, as already mentioned, the median PVL in PBMCs of patients with HAM/TSP was more than 10 times higher than that in ACs, and a high PVL was also associated with an increased risk of progression to disease (Nagai et al. 1998). Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower PVL and a lower risk of HAM/TSP (Jeffery et al. 2000; Jeffery et al. 1999), and CD8+ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals (Hanon et al. 2000). These data have raised the hypothesis that the class I-restricted CD8+ CTL response plays a critical part in limiting HTLV-1 replication in vivo and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk of developing HAM/TSP. The analysis of gene expression profiles using microarrays in circulating CD4+ and CD8+ lymphocytes indicated that granzymes and perforin are more highly expressed in individuals with a low PVL (Vine et al. 2004), suggesting that a strong CTL response is associated

with a low PVL and a low risk of HAM/TSP. In accordance with this observation, the lytic capacity of HTLV-1-specific CTLs in patients with HAM/TSP and ACs quantified by a CD107a mobilization assay showed significantly lower CD107a staining in HTLV-1-specific CTLs in patients with HAM/TSP than in ACs (Sabouri et al. 2008); this suggests that patients with HAM/TSP have a high frequency of HTLV-1-specific CD8+ T cells with poor lytic capacity, whereas ACs have a lower frequency of cells with high lytic capacity. Moreover, it has been reported that the high CTL avidity, which is closely associated with the lytic efficiency of CTLs, correlates with low PVL and proviral gene expression (Kattan et al. 2009), indicating that the efficient control of HTLV-1 in vivo depends on the quality of CTLs, which determines the position of virus–host equilibrium and also the outcome of persistent HTLV-1 infection. More recently, MacNamara et al. (Macnamara et al. 2010) showed that HLA class 1 alleles, which strongly bind oligopeptides from the HBZ protein, enable the host to have a more effective immune response against HTLV-1; therefore, such individuals have a lower PVL and are more likely to be asymptomatic. Another recent report showed the presence of HBZ-specific CD4+ and CD8+ cells in vivo in patients with HAM/TSP and in ACs and a significant association between the HBZ-specific CD8+ cell response and asymptomatic HTLV-1 infection (Hilburn et al. 2011). These findings provide strong evidence to support the hypothesis of the crucial role of CTLs and confirm the importance of HBZ for persistent infection. However, because the frequency of HTLV-1-specific CD8+ T cells was significantly elevated in patients with HAM/TSP compared with ACs (Greten et al. 1998; Nagai et al. 2001a), and these cells have the potential to produce proinflammatory cytokines (Kubota et al. 1998), there is debate on the role of HTLV-1-specific CD8+ T cells, namely, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP or whether the dominant effect of such cells in vivo is protective against disease, although a protective role and a pathogenic role of CTLs are not mutually exclusive. Indeed, there are other examples of viral infections in which the virus-specific CTLs exert both beneficial (antiviral) and detrimental (inflammatory) effects, such as lymphocytic choriomeningitis virus infection in the mouse (Klenerman and Zinkernagel 1997). It is difficult to separate cause and effect in analyzing the association between T cell attributes and the efficiency of viral control in a persistent infection at equilibrium.

Regulatory T cells (Tregs) are important mediators of peripheral immune tolerance and play an important role in chronic viral infections. HTLV-1 preferentially and persistently infects CD4+ CD25+ lymphocytes in vivo (Yamano et al. 2005), which contain the majority of the Foxp3+ Tregs (Sakaguchi et al. 2006). In patients with HAM/TSP, the percentage of Foxp3+ Tregs in CD4+ CD25+ cells is lower than that in ACs and uninfected healthy controls (Oh et al. 2006; Yamano et al. 2005), whereas the percentage of Foxp3+ cells in the CD4+ population tends to be higher in patients with HAM/TSP than in ACs (Best et al. 2009; Hayashi et al. 2008b; Toulza et al. 2008). Because CD25 is induced by HTLV-1 Tax oncoprotein (Inoue et al. 1986), the proportion of Foxp3+ cells falls in the CD4+ CD25+ population, which contains both Tregs and activated non-Tregs, in HTLV-1-infected individuals, especially patients with HAM/TSP. Therefore, it is inappropriate to use CD25 as a marker of Tregs in HTLV-1 infection, and the best current working definition of Treg phenotype is CD4+ Foxp3+. The high frequency of CD4+ Foxp3+ T cells in HTLV-1-infected individuals is maintained by CCL22 produced by HTLV-1-infected PBMCs (Toulza et al. 2010). The frequency of HTLV-1-negative Foxp3+ CD4+ cells positively correlated with the HTLV-1 PVL (Hayashi et al. 2008a; Toulza et al. 2008), and the CTL activity negatively correlated with the frequency of HTLV-1-negative Foxp3+ CD4+ cells (Toulza et al. 2008). These results suggest that an increase in HTLV-1-negative FoxP3+ CD4+ Tregs is one of the chief determinants of the efficiency of T cell-mediated immune control of HTLV-1. If such Tregs reduce CTL activity, which in turn increases the HTLV-1 PVL, this activity increases the risk of developing HAM/TSP.

Dendritic cells and the other reservoirs of HTLV-1

Dendritic cells (DCs) are antigen-presenting cells that play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from patients with HAM/TSP were infected with HTLV-1 (Macatonia et al. 1992), and the development of HAM/TSP is associated with rapid maturation of DCs (Ali et al. 1993). In vitro culture of lymphocytes from HTLV-1-infected individuals results in "spontaneous lymphocyte proliferation" (SLP), which is the in vitro proliferation of PBMCs without any exogenous stimuli such as antigen or mitogen. In patients with HAM/TSP, the levels of SLP reflect the severity of the disease (Ijichi et al. 1989; Itoyama et al. 1988). Interestingly, depletion of DCs from the PBMCs of patients with HAM/TSP abolished SLP, whereas supplementing DCs restores proliferation (Macatonia et al. 1992); supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC class II, CD86, and CD58 can block SLP (Makino et al. 1999). Recently, Jones et al. demonstrated that human-derived myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1 and that HTLV-1-infected DCs can rapidly transfer virus to autologous primary CD4+ T cells (Jones et al. 2008). Furthermore, it was recently demonstrated that transmission of HTLV-1 from DCs to T cells was mediated primarily by DC-SIGN (Jain et al. 2009), and the DCs are the major cell type responsible for the generation and maintenance of Tax-specific CD8+ T cells both in vitro and in vivo (Manuel et al. 2009).

These findings suggest that the interaction of DCs with HTLV-1 is also crucial for the pathogenesis of HAM/TSP. Moreover, using transgenic mouse models that permit conditional transient depletion of CD11c+ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection (Rahman et al. 2011, 2010). Because HTLV-1 can impair the differentiation of monocytes into DCs (Nascimento et al. 2011), the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

Conclusions

During the three decades since the discovery of HTLV-1, advances in research have successfully helped us to understand the clinical features of HTLV-1-associated diseases and the virological properties of HTLV-1, although the precise mechanism of disease pathophysiology is still incompletely understood and treatment is still unsatisfactory. Accumulating evidence suggests that the virus–host immunological interactions play a pivotal role in the pathogenesis of HAM/TSP. A genetically determined, less efficient CTL response against HTLV-1 may cause higher PVL and antigen expression in infected individuals, which lead to the activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of the development of HAM/TSP. Future studies should be conducted to identify the precise mechanism of disease development to allow effective treatment and prevention of disease. This will require the development of a humanized small animal model that could be exploited as a tool for screening and evaluation of HTLV-1-associated diseases.

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