厚生労働科学研究費補助金
(がん対策推進総合研究事業（革新的がん医療実用化研究事業）)
分担研究報告書

フローサイトメーターによるATL細胞、免疫細胞、および HLA-A発現の同時解析に関する研究

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研究要旨
我々は成人T細胞白血病（ATL）に対する樹状細胞療法の臨床試験（ATL-DC1-L1）において、フローサイトメトリー（FCM）を使用したATL細胞と制御性T細胞（Treg）の解析を行ない、治療前後のATL細胞とTreg動態のモニタリングに成功した。これら先行研究の結果を踏まえ、今年度は新しい樹状細胞療法（ATL-DC1-101）におけるATL細胞、Treg、末梢血リンパ球、およびHLA-A発現をより詳細に解析するマルチカラーの染色組合せを考案した。健常人血を用いて一部の染色方法で予備実験を行ない、Tregにおける表面マーカー、およびFoxp3やCTLA-4など細胞内機能分子の同時解析が可能であることが示された。FCMを使用した本解析システムは、ATL-DC1-101における病態のモニタリングとメカニズムの解明の手段として、有用なツールとなることが期待される。

A.研究目的

ATL-DC1-101においてATL細胞、Treg、末梢血リンパ球およびHLA-A発現レベルをより詳細に解析するマルチカラーFACS解析システムを確立し、樹状細胞療法における病態のモニタリングとメカニズムの解明を目指す。

B.研究方法

先行研究の結果を踏まえ、今年度は新しい樹状細胞療法（ATL-DC1-101）におけるマルチカラーの染色組合せを考案した。

また、一部の染色方法については健常人末梢血を使用し、目的とする解析が実施可能であるのか検証した。

（倫理面への配慮）

平成26年12月までの時点で、ATL-DC1-101の患者登録は行なわれなかった。したがって、今回の予備実験では、健常人ボランティアからの末梢血検体を使用した。解析結果は内部コントロールとして、学会や論文での発表は行なわない。

C.研究結果

副次評価項目として「CD4陽性CD25陽性Foxp3陽性細胞数」と「末梢血リンパ球表面マーカー」の解析を、付随研究として「HLA-A発現」を解析するための蛍光標識抗体の組合せを考案した。

1. 副次評価項目

1) 目的・意義

① CD4陽性CD25陽性Foxp3陽性細胞数

ATL細胞は、免疫抑制機能を持つTregのフェノタイプや機能を持つことが報告されている。また、一般にがん患者ではTreg活性が亢進し、がんに対する免疫反応を抑制することが知られている。したがって、本臨床治験においては、治療効果判定のためのATL細胞の検出に加え、ATL細胞以外のTregフェノタイプを判別して解析する。また、Tregフェノタイプが真のTregであるのか確認する目的で、細胞内Foxp3とCTLA-4も同時に解析する。これらの解析により、免疫療法の評価判定とTreg動態を明らかにする。

② 末梢血リンパ球表面マーカー

ポテリジオ投与やDC療法前後の末梢血免疫細胞を測定し、これらの治療に伴う影響をモニタリングする。

2) 染色方法

① CD4陽性CD25陽性Foxp3陽性細胞数

末梢血単核細胞をCD3、CD4、CD7、CD14、CD25、CD45RA、CD127、CCR4、TSLC-1、Foxp3、およびCTLA-4に対する蛍光標識抗体の組合せで染色する（表1、Stain 1）。

② 末梢血リンパ球表面マーカー

T細胞およびNK細胞関連マーカーとして、末梢血単核細胞をCD2、CD3、CD4、CD5、CD7、CD8、CD14、CD16、CD25、CD56、CD235a、お

よびTSLC-1に対する蛍光標識抗体の組合せで染色する（表1、Stain 2）。

また、B細胞および造血幹・前駆細胞関連マーカーとして、末梢血単核細胞をCD3、CD10、CD11c、CD14、CD19、CD20、CD34、CD45、およびCD235aに対する蛍光標識抗体の組合せで染色する（表1、Stain 3）。

2. 付随研究

1)目的・意義

樹状細胞（DC）療法では、DC表面上のHLA-A*02:01、HLA-A*11:01、あるいはHLA-A*24:02にTax由来ペプチドを結合させて患者に投与するが、ATL細胞表面のこれらのHLAがダウンレギュレーションして免疫機構からエスケープする可能性もある。よって、ATL細胞表面の当該HLAの発現レベルも解析する。

2) 染色方法

末梢血単核細胞をCD3、CD4、CD7、CD14、CD235a、HLA-2、HLA-A9、HLA-A11、およびTSLC-1に対する蛍光標識抗体の組合せで染色する（表1、Stain 4）。

3. 検体採取ポイント

スクリーニング（Day -28 ～ Day -14）、Day -7、治療期間（ATL-DC-101投与0週目、2週目、4週目）、後観察期間（6週目、8週目）、追跡調査（24週目までは4週間毎に来院、24週目以降は8週間毎に来院）の各ポイントで採血する（図1）。スクリーニング期間からDC療法後2年間までの間でトータル21回、147 mlの採血量となる。Day -7では「末梢血リンパ球表面マーカー」解析のみ行ない、「Treg解析」は行なわない。中止となった場合は、中止時の採血を行なう。

4. 検体採取法

EDTA添加、静脈血採血。

5. 検体量

7 ml。

6. 搬送法 (温度、搬送時間制限)

常温の宅急便で送付する。採血後24時間以内に解析を開始する。

7. 検体管理・保存条件

必要と認められた場合はATL細胞フェノタイプをソーティングして、医科研の保存施設において液体窒素に凍結保存する。

8. 報告所用日数

検体受け取り後3日以内に、末廣陽子医師にメールでご報告する。

9. 責任者および検査機関

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10. 予備実験

健常人の末梢血を使用し、表1のStain 1の染色組合せで細胞を染色した。健常人血であるため、ATL細胞マーカーであるCD7とTSLC-1による染色は行なわなかった。

一般に活性のあるTreg分画と見なされているのは、$CD3^+CD4^+$細胞の$Foxp3^+CD45RRA^-$分画（青のドット）である。これらの活性型Treg分画はCD25、CCR4、およびCTLA-4が陽性に染まり、活性型のTregとして一致する所見が得られた。

D.考察

平成26年12月の時点で、本治験はまだ患者登録が行なわれていないことから、本年度はフローサイトメトリー解析における蛍光標識抗体の組み合わせを考案するに留まった。

Tregの解析方法に関しては、今だに多くの相反する意見があり、フローサイトメトリー解析のみで真のTregを同定することは困難が伴う。しかしながら、ATL細胞と正常$CD4^+$T細胞を判別した上で、Treg活性の本質につながるFoxp3とCTLA-4を解析する我々の方法は、現時点で可能な最善の解析方法と思われる。

E.結論

FCMを使用した本解析システムは、ATL-DC1-101における病態のモニタリングとメカニズムの解明の手段として、有用なツールとなることが期待される。

G.研究発表

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H.知的財産権の出願・登録状況

特になし

表1　ATL-DC-101染色組合せ

[Fresh PBMCs]

Stain 1: Treg/Foxp3/CTLA-4　　◇SA-PE-Cy7(x20 dilution, 25 μl)②

FITC	PE	PE-Cy5	PerCP-Cy5.5	PE-Cy7	PE-TR	APC	AF700	APC-Cy7	V450	BV510	BV605
CCR4①	(Foxp3)②		CD45RA①	Biotin-TSLC-1①	CD4①	(CD152)②	CD14①	CD3①	CD7	CD127①	CD25①
LDG0411031	E11466-1633		B145933	Lab	910872A	29768	B131136	09317	2153618	B173326	B158071
10 μl	5 μl		5 μl	1 μl	2 μl	5 μl	7 μl	5 μl	5 μl	5 μl	5 μl
e-Bioscience						BD Pharmingen					

Stain 2: Lymphocyte markers (T cells and NK cells)

FITC	PE	PE-Cy5	PerCP-Cy5.5	PE-Cy7	PE-TR	AF647	AF700	APC-Cy7	V450	V500	BV605
CD2	CD5	CD235a/PI	CD8	CD25	CD16	TSLC-1	CD56	CD3	CD7	CD4	CD14
B166331	27053	B126613/-	B129722	51531	910872A	Lab	B131136	09317	2153618	B173326	B158071
5 μl	12 μl	1 μl / 1 μl	5 μl	5 μl	2 μl	1 μl	7 μl	5 μl	5 μl	5 μl	5 μl

Stain 3: Lymphocyte markers (B cells , Stem cells and others)

FITC	PE	PE-Cy5	PerCP-Cy5.5	PE-Cy7	PE-TR	APC	AF700	APC-Cy7	AF405	V500	BV605
CD19	CD34	CD235a/PI	CD20	CD10		CD11c		CD3	CD45		CD14
68976	------	B126613/-	B171352	62232		3079922		09317	773984A		B158071
12 μl	12 μl	1 μl / 1 μl	5 μl	5 μl		20 μl		5 μl	5 μl		5 μl

Stain 4: HLA　　◇SA-PE-Cy7(x20 dilution, 25 μl)②

FITC	PE	PE-Cy5	PerCP-Cy5.5	PE-Cy7	PE-TR	AF647	AF700	APC-Cy7	V450	V500	BV605
HLA-A9①	HLA-A2①	CD235a①/PI		Biotin-HLA-A11①		TSLC-1①		CD3①	CD7①	CD4①	CD14①
017	79836	B126613/-		51531		Lab		09317	2153618	B173326	B158071
10 μl	12 μl	1 μl / 1 μl		5 μl		1 μl		5 μl	5 μl	5 μl	5 μl

Stain 5: Isotype for Stain 4　　◇SA-PE-Cy7(x20 dilution, 25 μl)②

FITC	PE	PE-Cy5	PerCP-Cy5.5	PE-Cy7	PE-TR	AF647	AF700	APC-Cy7	V450	V500	BV605
IgG2b①	IgG2b①	CD235a①/PI		Biotin-IgM①		TSLC-1①		CD3①	CD7①	CD4①	CD14①
B159582	--------	B126613/-		3168672		Lab		09317	2153618	B173326	B158071
12 μl	12 μl	1 μl / 1 μl		5 μl		1 μl		5 μl	5 μl	5 μl	5 μl

図1　ATL-DC-101における採血のタイミング

副次評価項目の採血ポイント(スクリーニングから投与後2年までの間に21回)：
Treg解析 → Stain 1: CD3, CD4, CD7, CD14, CD25, CD45RA, CD127, CCR4, TSLC-1, Foxp3, CTLA-4
末梢血リンパ球解析 → Stain 2: CD2, CD3, CD4, CD5, CD7, CD8, CD14, CD16, CD25, CD56, CD235a, TSLC-1
Stain 3: CD3, CD10, CD11c, CD14, CD19, CD20, CD34, CD45, CD235a
付随研究の採血ポイント（スクリーニングから投与後2年までの間に5回）：
HLA解析 → Stain 4: CD3, CD4, CD7, CD14, CD235a, HLA-2, HLA-A9, HLA-A11, TSLC-1
Stain 5はStain 4のアイソタイプコントロール

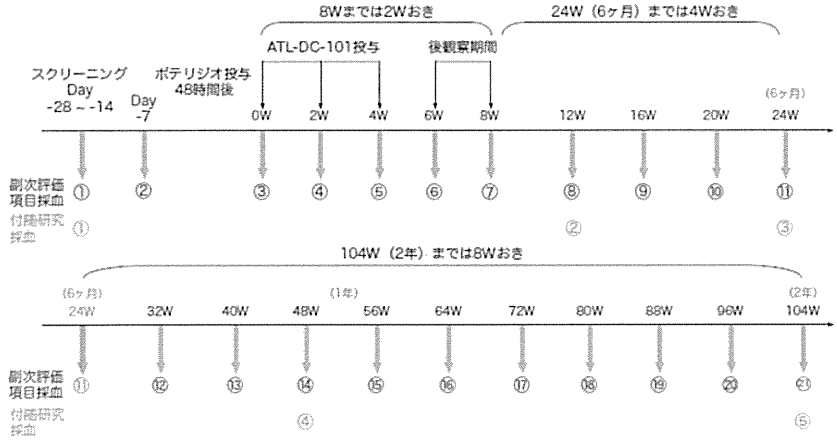
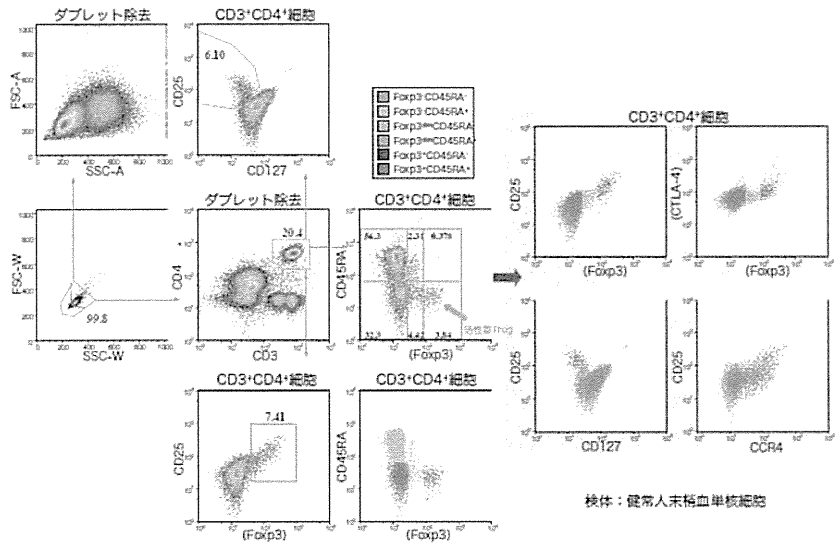


図2　「CD4陽性CD25陽性Foxp3陽性細胞数」の解析

Stain 1: Treg/Foxp3/CTLA-4



厚生労働科学研究費補助金
がん対策推進総合研究事業（革新的がん医療実用化研究事業）

分担研究報告書

ATLに対する樹状細胞ワクチン療法臨床試験の実施

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研究要旨
成人T細胞白血病に対する樹状細胞療法について、治験プロトコール作製に従事した。また、免疫療法のより有効性を高めるべく、リンパ系腫瘍における、腫瘍増殖と免疫応答につき、基礎的研究を行った。その結果、免疫調節因子がリンパ系腫瘍の生存に関与することが明らかとなった。

A．研究目的

極めて難治性である成人T細胞白血病に対して、新たな免疫学的療法の開発を行う。

B．研究方法

移植適応の無い成人T細胞白血病患者を対象に、抗CCR4抗体を併用した樹状細胞療法を行う。細胞株、患者から得られた臨床検体を用いて、より有効な治療開発について検討する。

（倫理面への配慮）

ヘルシンキ宣言、臨床研究に関する倫理指針を遵守して実施する。

C．研究結果

臨床試験実施に向け、プロトコール作成を行った。

リンパ系腫瘍における免疫応答について、臨床検体や、細胞株を用いて検討した所、免疫調整因子であるIDO及びトリプトファン代謝産物のトラニラストがリンパ系腫瘍細胞の増殖抑制効果がある事を明らかとした。

D．考察

成人T細胞白血病は極めて難治性の疾患であり、化学療法のみで長期予後の期待は出来ない。同種造血幹細胞移植での成功例から、免疫学的治療の有効性が期待出来る。本試験の遂行により、この疾患の免疫学的治療の実用化を図るとともに、より有効性を高める為の検討が重要であると考えられる。

E．結論

成人T細胞白血病に対する新規免疫学的治療の実用化に向けて、早期の臨床試験実施が望まれる。

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H. 知的財産権の出願・登録状況

特になし

厚生労働科学研究費補助金
がん対策推進総合研究事業（革新的がん医療実用化研究事業）

分担研究報告書

樹状細胞における膜孔形成性細胞傷害蛋白 Perforin-2 に関する研究

研究分担者：白土　基明　九州大学大学院病態制御内科学　助教

研究要旨
樹状細胞は強力な抗原提示能を有しており、悪性腫瘍に対する免疫療法への応用が試みられている。しかし、その貪食から抗原提示までのメカニズムは未知の部分が多い。我々はマクロファージに発現が報告されている膜孔形成性細胞傷害蛋白 Perforin-2 が樹状細胞においても、特に貪食の盛んな未熟な段階に高発現していることを見いだした。

A．研究目的

樹状細胞は感染免疫・腫瘍免疫においてT細胞に抗原を提示する重要な役割を持っている。われわれは膜孔形成性細胞傷害蛋白 Perforin-2 の樹状細胞での発現と機能につき解明する。

B．研究方法

健常人末梢血より CD14 陽性細胞を磁気ビーズにて単離し、GM-CSF、IL-4 を添加して培養し未熟樹状細胞を誘導した。その後、TNF-α を加えてさらに培養し、成熟樹状細胞を誘導した。それぞれの Perforin-2 蛋白、mRNA をそれぞれ Western blot、RT-PCR にて検討した。

また、Perforin-2 に対する miRNA を組来んだベクターを作成し樹状細胞に導入し Perforin-2 の発現を低下させて抗原提示への影響を検討した。

（倫理面への配慮）
健常人からの採血の際は文書で説明し同意を得た。

C．研究結果

健常人末梢血より CD14 陽性単球分画を単離し、GM-CSF、IL-4 による刺激下に 7 日間培養し得られた細胞は形態的、表面マーカー上、未熟樹状細胞と考えられた。さらに TNF-α を加えて 2 日間培養を続けると、成熟樹状細胞となった。Perforin-2 蛋白の発現は未熟樹状細胞で高まり、成熟するとやや低下した。しかし mRNA の発現は単球分画が最も高く、次第に低下した。

Perforin-2 に対する siRNA ベクター（GFP 遺伝子を含む）を作成し、Nucleofector にて未熟樹状細胞に導入を試みた。GFP 陽性率による導入効率に 10%～40%とばらつきが見られた。

D．考察

健常人末梢血単球分画より誘導した樹状細胞において、Perforin-2が発現していることを確認できた。蛋白レベルでは未熟樹状細胞において高発現であり、貪食過程において重要な役割を果たしている可能性が示唆された。蛋白発現とmRNAレベルが相関しておらず、転写後調節の関与が考えられた。

樹状細胞への遺伝子導入は可能であったが、効率にばらつきが見られ、改良の余地がある。また、今後mRNAレベルで抑制されていることを確認する予定である。

E.結論

樹状細胞に膜孔形成性細胞傷害蛋白 Perforin-2 が発現していることを確認した。未熟樹状細胞に高発現することより貪食と抗原プロセッシングに関与している可能性がある。

G．研究発表

1．論文発表
なし

2．学会発表
なし

H．知的財産権の出願・登録状況

特になし

研究成果の刊行に関する一覧表

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bjh research paper

Clinical outcomes of a novel therapeutic vaccine with Tax peptide-pulsed dendritic cells for adult T cell leukaemia/lymphoma in a pilot study

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Summary

Adult T cell leukaemia/lymphoma (ATL) is a human T cell leukaemia virus type-I (HTLV-I)-infected T cell malignancy with poor prognosis. We herein developed a novel therapeutic vaccine designed to augment an HTLV-I Tax-specific cytotoxic T lymphocyte (CTL) response that has been implicated in anti-ATL effects, and conducted a pilot study to investigate its safety and efficacy. Three previously treated ATL patients, classified as intermediate- to high-risk, were subcutaneously administered with the vaccine, consisting of autologous dendritic cells (DCs) pulsed with Tax peptides corresponding to the CTL epitopes. In all patients, the performance status improved after vaccination without severe adverse events, and Tax-specific CTL responses were observed with peaks at 16–20 weeks. Two patients achieved partial remission in the first 8 weeks, one of whom later achieved complete remission, maintaining their remission status without any additional chemotherapy 24 and 19 months after vaccination, respectively. The third patient, whose tumour cells lacked the ability to express Tax at biopsy, obtained stable disease in the first 8 weeks and later developed slowly progressive disease although additional therapy was not required for 14 months. The clinical outcomes of this pilot study indicate that the Tax peptide-pulsed DC vaccine is a safe and promising immunotherapy for ATL.

Keywords: adult T cell leukaemia/lymphoma, tumour vaccine, dendritic cell, human T cell leukaemia virus type-I, cytotoxic T lymphocyte.

Adult T cell leukaemia/lymphoma (ATL) is an aggressive lymphoproliferative disease caused by human T cell leukaemia virus type-I (HTLV-I) infection (Uchiyama *et al*, 1977; Poiesz *et al*, 1980; Hinuma *et al*, 1981). In particular, the acute and lymphoma types of ATL are characterized by a poor prognosis. Although the chronic and smouldering types

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of ATL exhibit milder disease progression, these diseases also result in poor clinical outcome once they have converted to the acute or lymphoma types.

One reason for the poor clinical outcome associated with ATL is rapid progression of the disease at onset, which requires a prompt diagnosis and effective first-line therapy. Currently available first-line therapies for ATL include intensive multi-agent chemotherapy (Tsukasaki *et al*, 2012), interferon-α combined with zidovudine (Gill *et al*, 1995; Hermine *et al*, 1995) and an anti-CCR4 antibody (mogamulizumab) (Ishida *et al*, 2012).

Frequent relapse is another reason for the poor prognosis of ATL, requiring subsequent administration of second-line therapy that can produce a long-lasting anti-ATL effect. Haematopoietic stem cell transplantation (HSCT) has been reported to achieve a long-lasting remission in 30–40% of ATL patients, although it occasionally induces treatment-related mortality in a similar percentage of recipients (Utsunomiya *et al*, 2001; Okamura *et al*, 2005; Hishizawa *et al*, 2010; Ishida *et al*, 2013). In addition to the graft-*versus*-host response (Tanosaki *et al*, 2008), the actions of Tax-specific cytotoxic T lymphocytes (CTLs) have been implicated in the graft-*versus*-ATL effects of HSCT. This is based on our previous finding that ATL patients who obtained complete remission following HSCT often exhibit activation of $CD8^+$ CTLs specific for HTLV-I Tax (Harashima *et al*, 2004).

In untreated ATL patients, Tax-specific CTLs are either undetectable or dysfunctional, if present (Takamori *et al*, 2011). Although ATL patients are in a severe immune suppressive state, the impaired CTL response is not merely a result of general immune suppression in the advanced disease, but also observed in the patients with earlier stages of the disease in a selective manner for HTLV-I-specific responses (Takamori *et al*, 2011). The anti-tumour effects of Tax-specific T cells have been well characterized in animal models, where Tax-coding DNA and Tax-peptide vaccines have been shown to induce T cell immunity, thus eradicating HTLV-I-infected lymphomas in rats (Ohashi *et al*, 2000; Hanabuchi *et al*, 2001).

The efficacy of the vaccine targeting Tax in human ATL patients remains unclear, and no such treatment has ever been attempted as an actual therapy. This is partly because the HTLV-I gene expression levels are believed to be very low *in vivo* (Kurihara *et al*, 2005; Rende *et al*, 2011), and ATL cells occasionally lack the ability to express Tax (Takeda *et al*, 2004). However, our previous finding of the Tax-specific CTL activation in ATL patients following HSCT from uninfected donors indicated the presence of a sufficient level of Tax expression for the CTL response *in vivo* (Harashima *et al*, 2004).

These findings prompted us to attempt to develop a therapeutic anti-ATL vaccine designed to augment a Tax-specific CTL response that may partly reproduce the long-lasting anti-tumour effects of HSCT as second-line therapy for ATL. For the vaccine antigen, we used synthetic oligopeptides corresponding to the major epitopes recognized by Tax-specific CTL identified in our previous studies of post-HSCT ATL patients (Harashima *et al*, 2004, 2005). These epitopes are restricted to HLA-A2, A24 or A11, all of which are common in the Japanese population. For the vaccine adjuvant, we used autologous dendritic cells (DCs) induced from the peripheral monocytes. Although previous reports suggested dysfunctions of DCs in ATL patients (Makino *et al*, 2000; Hishizawa *et al*, 2004), the monocyte-derived DCs obtained from ATL patients retained the ability of antigen presentation in our preliminary experiments. The use of autologous DCs loaded with tumour antigens have been reported in various tumour vaccine trials of different tumours (Nagayama *et al*, 2003; Ueda *et al*, 2004; Linette *et al*, 2005; Fuessel *et al*, 2006; Thomas-Kaskel *et al*, 2006; Wierecky *et al*, 2006).

The present pilot study investigated the safety and efficacy of the Tax peptide-pulsed dendritic cell (Tax-DC) vaccine when administered to augment Tax-specific CTL responses in ATL patients.

Materials and methods

Study design

This clinical study was approved by the institutional ethics committee and registered as UMIN000011423. Three ATL patients possessing HLA-A*02:01, A*24:02 and/or A*11:01, in stable condition at least 4 weeks after the administration of previous therapy, provided their written informed consent and were enrolled in this study, which investigated the safety and efficacy of the Tax peptide-pulsed DC (Tax-DC) vaccine between September 2012 and February 2013.

HTLV-I proviruses in the peripheral blood mononuclear cells (PBMCs) were examined for the potential Tax expression and conservation of targeted CTL epitopes by analysing their nucleotide sequences beforehand. All patients were subcutaneously administered with Tax peptide-pulsed autologous DCs (5×10^6) three times at 2-week intervals (Fig 1A) at Kyushu University Hospital.

Patients

Patient 1 was a 69-year-old male who was diagnosed with acute ATL in August 2011. After receiving four courses of multi-agent chemotherapy, he achieved stable disease (SD). Although additional treatment with lenalidomide was administered for a few weeks, it was discontinued due to the development of thrombocytopenia. The patient was registered to the study in September 2012.

Patient 2 was a 67-year-old female who was diagnosed with acute ATL in December 2011. She presented with remarkable systemic lymphadenopathy and splenomegaly, in addition to an extremely high level of soluble interleukin-2 receptor (sIL2R; 57 815 u/ml). She received four courses of

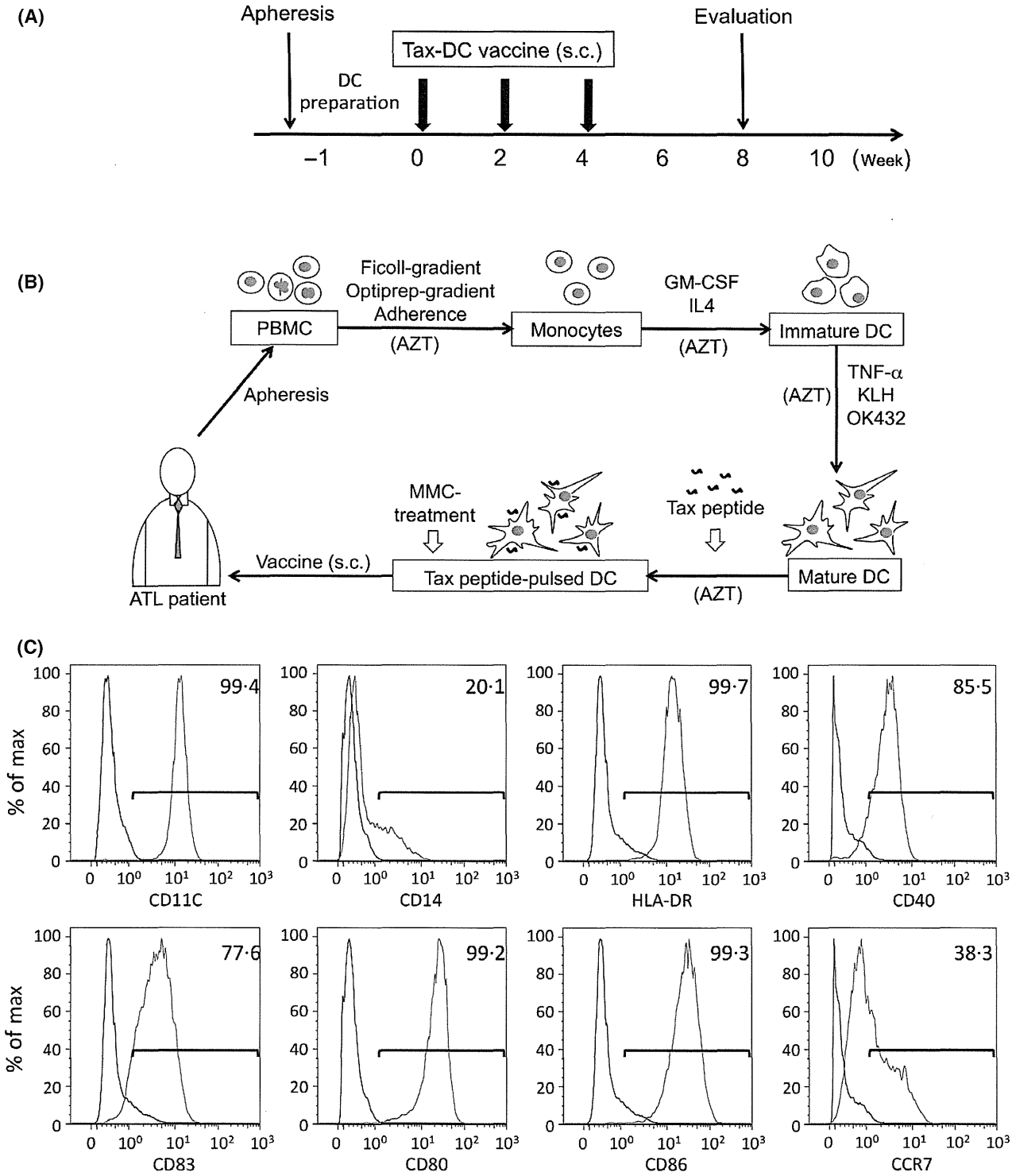


Fig 1. Outline of the Tax-DC vaccine therapy. (A) Schedule for the Tax-DC vaccine therapy. (B) Preparation of the monocyte-derived dendritic cells (DCs). Monocytes were enriched via serial density gradient centrifugation, and the adherent cells were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4) for 5 d, followed by 48 h of culture with TNF-α, keyhole limpet haemocyanin (KLH), and OK432. A total of 10 μmol/l of zidovudine (AZT) was added whole throughout the culture. The matured DCs were pulsed with synthetic Tax peptides, treated with Mitomycin C (MMC), and then cryopreserved prior to subcutaneous injection. (C) Representative phenotype of mature dendritic cells prepared from Patient 1 prior to administration, as evaluated using flow cytometry. The red histograms indicate the results of staining with monoclonal antibodies for the indicated molecules, while the black histograms indicate the results of staining with control antibodies. ATL, Adult T cell leukaemia/lymphoma; PBMC, peripheral blood mononuclear cells.

multi-agent chemotherapy and achieved a partial remission (PR). Due to the development of disease recurrence with rapid progression after 2 months, treatment with mogamulizumab and low-dose chemotherapy (sobuzoxane + etoposide) was added. After obtaining a second PR, the patient was registered to the study in November 2012.

Patient 3 was a 56-year-old female diagnosed with acute ATL who presented with severe pneumocystis pneumonia in August 2012. After receiving two courses of multi-agent chemotherapy followed by two courses of mogamulizumab combined with chemotherapy, she achieved a PR. Further intensive treatment was not planned due to the development of severe respiratory dysfunction. The patient was registered to the study in February 2013.

The clinical information of the patients at enrollment is summarized in Table I.

Preparation of Tax peptide-pulsed DCs

Monocyte-derived DCs were generated from apheresis samples collected from the peripheral blood (6 l) of ATL patients at institutional cell processing facilities according to the good manufacturing practice (GMP) standard using a previously reported method, with some modifications (Nagayama *et al*, 2003) (Fig 1B). Briefly, monocytes enriched via serial density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and density-adjusted Optiprep (1·073 g/ml; Axis-Shield PoC, Oslo, Norway) were cultured at 37°C for 2 h, after which the adherent cells were cultured in CellGro DC medium (CellGenix GmbH, Freiburg, Germany) with 1000 iu/ml of granulocyte-macrophage colony-stimulating factor (Leukine; Bayer HealthCare Pharmaceuticals, Seattle, WA, USA) and 100 iu/ml of IL4 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 d. The resulting monocyte-derived DCs were matured in the presence of 10 ng/ml of TNF-α (Miltenyi Biotec) and 12·5 μg/ml of keyhole limpet haemocyanin (KLH; Calbiochem, La Jolla, CA, USA) for 48 h, with 0·1 Clinical unit (Klinische Einheit; KE)/ml of OK432 (Picibanil; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) for the last 24 h. The matured DCs were pulsed with 2 μg/ml of synthetic peptides (NeoMPS; PolyPeptide Laboratories Group, San Diego, CA, USA), including Tax11-19 (LLFGYPVYV) (Kannagi *et al*, 1992) or Tax301-309 (SFHSLHLLY) (Harashima *et al*, 2004) restricted to HLA-A*02:01 or -A*24:02 respectively, and treated with Mitomycin C (MMC; Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) (50 μg/ml) in order to inactivate the ATL cells potentially contained in the preparation. As DCs are reported to be susceptible for HTLV-I infection (Jones *et al*, 2008), 10 μmol/l of zidovudine (Retrovir, AZT; GlaxoSmithKline, Research Triangle Park, NC, USA) was added whole throughout the culture to avoid *de novo* infection. The peptide-pulsed DCs were then washed and examined for safety by checking for contamination with bacteria, fungi, mycoplasma and/or endotoxins, then cryopreserved until use. The cells (5×10^6) were subsequently thawed and washed prior to administration.

Evaluation of adverse events and the clinical response

Toxic effects were graded according to the Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf). The clinical response was evaluated according to the criteria proposed by the international consensus meetings that led to the modification of the Japan Clinical Oncology Group criteria (Tsukasaki *et al*, 2009). Briefly, complete remission (CR) was defined as the disappearance of all clinical, microscopic and radiographic evidence of disease. PR was defined as a ≥50% reduction in the level of measurable disease without the appearance of new lesions. In addition, the diagnosis of a PR was required to satisfy a 50% or greater reduction in the absolute abnormal lymphocyte count in the peripheral blood. Progressive disease (PD) or relapsed disease was defined as a ≥50% increase from the nadir in the sum of the products of measurable disease or the appearance of new lesions, excluding the skin. Stable disease (SD) was defined as the failure to attain CR/PR nor PD.

The soluble IL2 receptor (sIL2R) level, HTLV-I proviral load and Tax-specific CTL response were monitored in addition to the results of general laboratory tests. Adverse effects

Table I. Patient characteristics at enrollment.

	Patient 1	Patient 2	Patient 3
Age (years)/gender	70/ male	68/ female	57/ female
HLA-A allele	24:02, 31:01	24:02, 26:03	02:01, 11:01
Subtype of ATL	Acute	Acute	Acute
Previous therapy	mEPOCH, lenalidomide	mEPOCH, mogamulizumab + PVP	mEPOCH, mogamulizumab + PVP
Disease status	SD	PR	PR
Interval from previous therapy	2·5 months	1·5 months	2 months
Duration since diagnosis	14 months	11 months	6 months
Complication	Allergic dermatitis	Breast cancer, DM, NASH	Interstitial pneumonia

mEPOCH, modified combination chemotherapy with etoposide + prednisone + vincristine + doxorubicin + carboplatin; PVP, combination chemotherapy with sobuzoxane + etoposide; SD, stable disease; PR, partial remission; DM, diabetes mellitus; NASH, nonalcoholic steatohepatitis.

and the clinical response were monitored and evaluated at 8 weeks after the initiation of the Tax-DC vaccine therapy.

Tax-specific CTL analysis

Phycoerythrin (PE)-conjugated HLA-A*0201/Tax11-19, HLA-A*1101/Tax88-96 and HLA-A*2402/Tax301-309 tetramers were purchased from Medical & Biological Laboratories, Co., Ltd. (Nagoya, Japan). Whole blood samples or PBMCs were stained with PE-conjugated Tax/HLA tetramers, together with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 and PE/cyanin 5 (Cy5)-conjugated anti-human CD8 monoclonal antibodies (mAbs) (BioLegend, San Diego, CA, USA), then fixed in Becton Dickinson (BD) FACS lysing solution (BD Biosciences, San Jose, CA, USA), followed by analysis on the FACS Calibur system using the CELLQUEST software program (BD Biosciences). For staining intracellular IFN-γ production, PBMCs pre-stained with PE-conjugated Tax/HLA tetramers and anti-human CD8-PE/Cy5 mAb were incubated at 37°C for 6 h in the presence of cognate Tax peptides (10 μmol/l), with brefeldin A (10 μg/ml; Sigma Aldrich, St. Louis, MO, USA) for the last 5 h. The cells were then permeablized using BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained with FITC-conjugated anti-human IFN-γ mAb (4S.B3; BioLegend).

Detection of HTLV-I gene expression

To detect intracellular HTLV-I antigens, cells were serially treated with 4% paraformaldehyde for 10 min and 100% methanol for 10 min on ice, and then stained with Alexa Fluor 488-labelled anti-Tax Lt-4 (Lee *et al*, 1989) or isotype control mAbs followed by flow cytometry.

To quantify HTLV-I *pX* mRNA, total RNA extracted by using Isogen (Nippon Gene, Tokyo, Japan) were treated with DNase (Ambion, Austin, TX, USA), and subjected to quantitative reverse transcription polymerase chain reaction (RT-PCR) with the primer sets specific for HTLV-I *pX* (forward, 5′-CGG ATA CCC AGT CTA CGT GTT TGG AGA CT-3′; reverse, 5′-GAG CCG ATA ACG CGT CCA TCG ATG GGG TCC-3′) and *GAPDH* (forward, 5′-TGA TTT TGG AGG GAT CTC GCT CCT GGA AGA-3′; reverse, 5′-GTG AAG GTC GGA GTC AAC GGA TTT GGT CGT-3′) by using LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) after reverse transcription with oligo(dT)20 primers. The *pX* mRNA levels were standardized against *GAPDH* mRNA copy numbers.

Results

Feasibility of the DC preparation in ATL patients

We obtained 4·3–10·6 × 10^7 DCs with 72·2–91·3% purity. The cells exhibited the phenotype of mature DCs ($CD11c^+$, $CD80^+$, $CD86^+$, $CD83^+$, $CD40^+$, $HLA\text{-}DR^+$). The representative results obtained in Patient 1 are shown in Fig 1C. The HTLV-I proviral load of the PBMCs in the input apheresis samples were 114·8, 36·7 and 25·5 copies/1000 cells in the three patients respectively, with final loads in the DCs of 5·9, 5·0 and 10·3 copies/1000 cells, respectively.

Clinical courses after the Tax-DC vaccine therapy in the ATL patients

The clinical outcomes of the Tax-DC vaccine therapy in the three patients are summarized in Table II.

Table II. Clinical responses after the Tax-DC vaccine therapy in the three ATL patients.

Clinical response in 8 weeks after initiation of the vaccine therapy	Patient 1*		Patient 2†		Patient 3‡	
Time at evaluation	Pre-therapy	8 weeks	Pre-therapy	8 weeks	Pre-therapy	8 weeks
KPS (%)	70	90	70	80	70	90
LDH (iu/l)	473	245	250	326	329	268
sIL2R (u/ml)	19 056	1866	806	1462	1739	871
HTLV-I PVL (copies/1000 PBMCs)	114·8	12·4	36·7	14·9	17·7	29·6
Clinical response	–	PR	–	SD	–	PR
Long-term outcomes						
TTNT (months from registration)	25+		15		20+	
Survival (months from diagnosis)	39+		34		26+	

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; HTLV-I PVL, human T cell leukaemia virus type-I proviral load, PBMCs, peripheral blood mononuclear cells; SD, stable disease, PR, partial remission; TTNT, time to next anti-tumour therapy.

*The size of the lymph nodes in Patient 1 repeatedly increased and decreased, especially at time points later than 6 months after initiation of vaccine therapy.

†Patient 2 was considered to have developed a progressive disease at 6 months after the initiation of the vaccine therapy.

‡Patient 3 achieved complete remission at 6 months after the initiation of the vaccine therapy.

Patient 1 was positive for HLA-A*24:02 and vaccinated with Tax 301-309 peptide-pulsed DCs. Following the first administration of the Tax-DC vaccine, he developed a fever (grade 2), dermatitis (grade 2) and diarrhoea (grade 1). The white blood cell count, level of ATL cells in the peripheral blood and LDH level in the serum showed remarkable fluctuation during the vaccination, and then stabilized after the third administration of the vaccine (Fig 2A). In Patient 1, the level of sIL2R, which is a sensitive tumour marker for ATL, decreased from 19 056 to 1866 u/ml (normal range: <570 u/ml) by 8 weeks of therapy (Fig 2B). In addition, his surface lymph nodes decreased in size (Fig 2C), and he achieved a partial remission (PR) that persisted for at least 24 weeks. He returned to his normal life, and his Karnofsky performance status (KPS) improved from 70% to 100%. Although the size of the patient's lymph nodes and the level of sIL2R fluctuated at later time points, he has remained in remission for more than 24 months after the completion of the Tax-DC vaccine therapy, without any additional anti-tumour treatment.

Patient 2 had HLA-A*24:02 and was vaccinated with Tax 301–309 peptide-pulsed DCs. She developed a low-grade fever and dermatitis (grade 2) after each vaccine administration. However, no severe adverse events were observed during her clinical course. At 8 weeks of therapy, she was considered to have achieved SD. Although there was no objective response, an improvement in the KPS was noted. She was subsequently considered to have developed PD 6 months after the initiation of the Tax-DC vaccine therapy. Nevertheless, due to slow progression of the disease and her stable general condition, she was followed without any additional anti-tumour therapy until 14 months after the completion of vaccination. The patient died of infection 23 months after the initiation of the vaccine therapy.

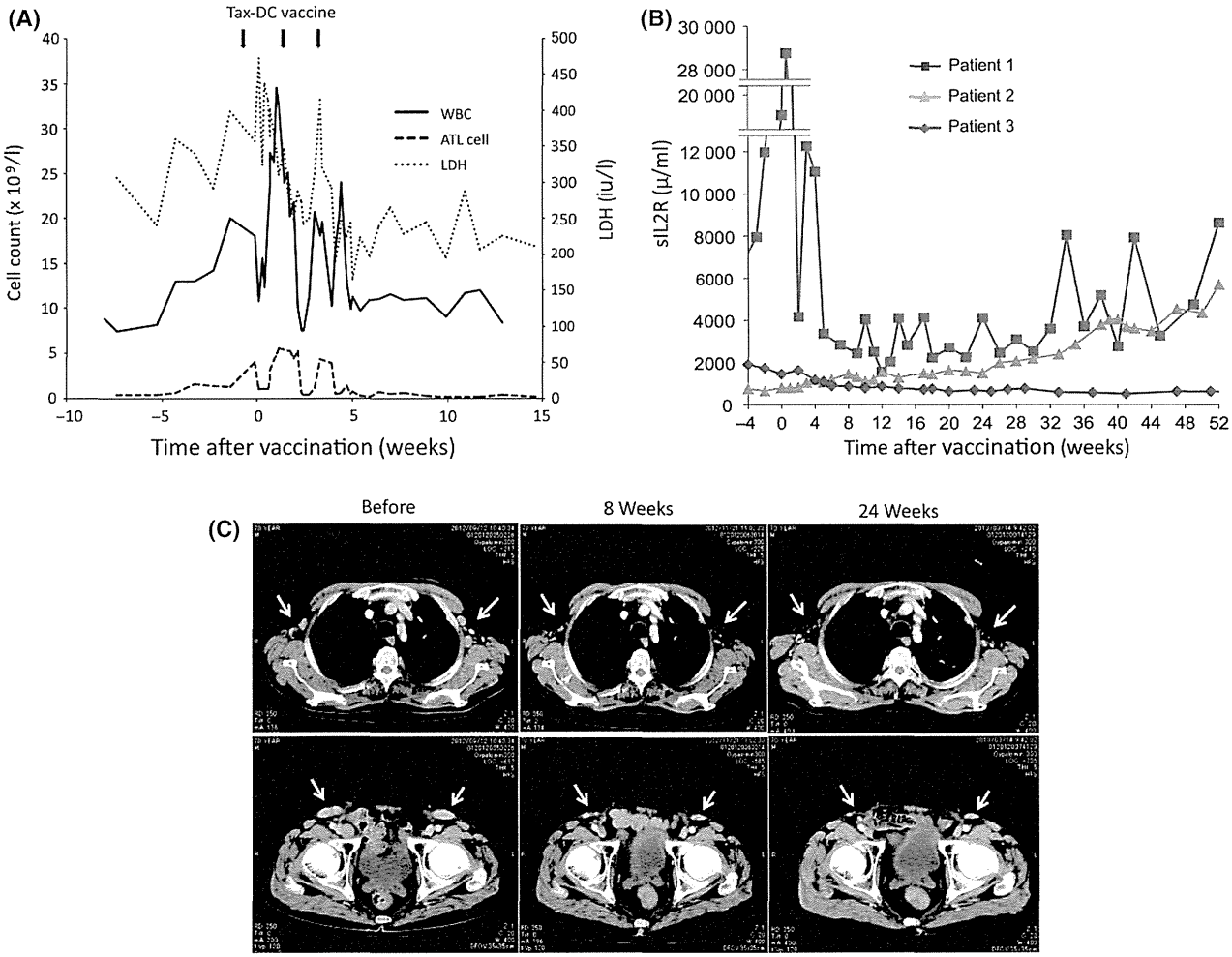


Fig 2. Clinical courses of the patients after the Tax-DC vaccine therapy. (A) Changes in the peripheral white blood cell count (WBC, solid line), ATL cell count (dashed line) and lactate dehydrogenase (LDH) level (fine dotted line) during the initial 15 weeks in Patient 1. The arrows indicate the days of Tax-DC vaccine administration. (B) Kinetics of the sIL2R levels in the sera obtained from Patients 1 (red), 2 (green) and 3 (blue) during the long-term observation period after the initiation of the Tax-DC vaccine therapy. (C) Computerized tomography images of the axillary (top) and inguinal (bottom) lymph nodes (arrows) of Patient 1 before and 8 and 24 weeks after the initiation of the Tax-DC vaccine therapy.

Patient 3 had HLA-A*02:01 and A*11:01. Although peptides of CTL epitopes for both HLA alleles were available, we chose the Tax11-19 peptide for HLA-A2 because HLA-A2 has a higher frequency in Japanese individuals. After each vaccination, the patient developed a low-grade fever and dermatitis (grade 2); however, no other severe adverse events were noted. She achieved a PR with an improvement in the KPS 8 weeks after the initiation of the Tax-DC vaccine therapy. Thereafter, the level of sIL2R returned to normal (Fig 2B). The patient subsequently achieved a CR at 6 months and has remained in this status for more than 19 months after the completion of the Tax-DC vaccine therapy.

Immunological responses after the Tax-DC vaccine therapy

In Patient 1, Tax-specific $CD8^+$ CTLs (HLA-A*24:02/Tax301-309 $tetramer^+$) were detectable prior to vaccination, and their frequency in peripheral $CD8^+$ cells transiently decreased during the Tax-DC vaccine administration, then recovered and maintained a constant level with some fluctuation (Fig 3A). The IFN-γ production from Tax-specific CTL also fluctuated. It is noteworthy that a vigorous proliferative response of Tax-specific CTLs was observed *in vitro* in the PBMC sample obtained at 20 weeks after the initiation of the Tax-DC vaccine therapy (Fig 3B), in which the proportion of HLA-A*24:02/Tax301-309 $tetramer^+$ cells in $CD8^+$ cells increased up to 22·5% within 2 weeks of the culture. A mild proliferative response of CTLs was also observed at 12 weeks. Samples obtained from the same patient prior to vaccination lacked such strong responses, implying a functional improvement in CTLs after the Tax-DC vaccine therapy.

Similar to that observed in Patient 1, a markedly increased level of spontaneous *in vitro* proliferative responses of Tax-specific CTLs was observed in the PBMC samples obtained from Patient 2 at 16 weeks after the initiation of the Tax-DC vaccine therapy, although the CTLs of this patient had exhibited a proliferative response prior to vaccination to a lesser degree (Fig 3B). The IFN-γ producing response of the CTL in this patient slightly improved after vaccination and showed some peaks at later time points.

As the size of the lymph nodes in Patient 2 did not improve within the first 8 weeks, a biopsy of the inguinal lymph node was performed at 9 weeks during the study period. The tumour cells isolated from the lymph node were $CD4^+$ $CD8^+$ $CCR4^+$ (Fig 4A) and possessed HTLV-I proviruses (849·5 copies/1000 cells). However, HTLV-I Tax proteins or mRNA expression was not induced in the lymph node cells after a short-term *in vitro* culture, whereas the viral expression was inducible in the PBMC sample of the same patient before vaccination (Fig 4B,C).

Tax-specific CTLs were below detectable levels prior to vaccination in Patient 3. However, 2 weeks after the initiation of the vaccine therapy with Tax 11–19 peptide-pulsed DCs, $CD8^+$ Tax-specific CTLs became detectable with HLA-A*0201/Tax11-19 tetramers, but not HLA-A*1101/Tax88-96 tetramers (Fig 3A). Although the IFN-γ producing response was barely detectable because of the low CTL frequency, an *in vitro* proliferative response of Tax-specific CTLs was observed in the PBMC samples obtained from Patient 3 most clearly at 16 weeks of the Tax-DC vaccine therapy, upon stimulation with Tax11-19 peptides, but not Tax 88–96 peptides (Fig 3B).

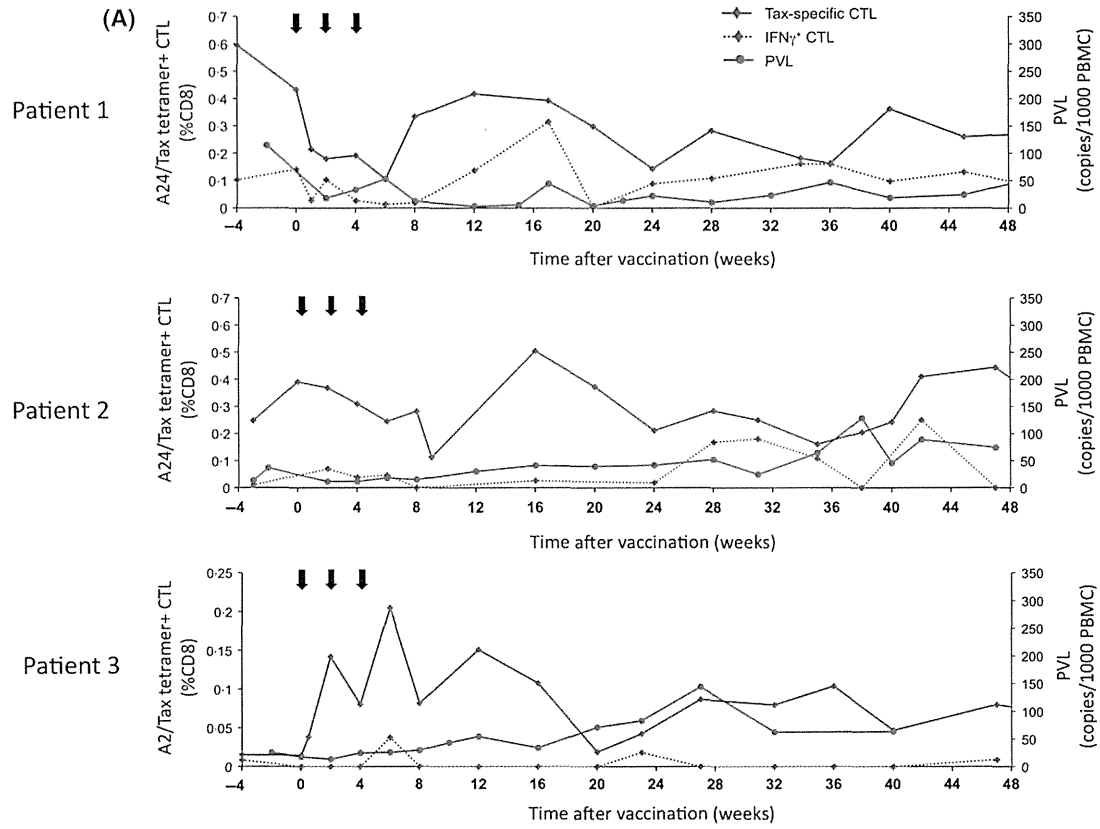
In all three patients, the level of the proviral load in the peripheral blood mostly remained below 100 copies per 1000 PBMCs at least for 1 year after vaccination, with the exception of sporadic small spikes (Fig 3A).

Discussion

Although various therapeutic trials have been conducted, the prognosis of ATL remains dismal. According to the simplified ATL prognostic index (ATL-PI) (Katsuya *et al*, 2012), the median survival time is only 4·6, 7·0 and 16·2 months, while the 2-year overall survival rate is 6%, 17% and 37%, for patients in high-, intermediate- and low-risk groups, respectively. According to the ATL-PI, Patients 1 and 2 were classified as intermediate-risk, while Patient 3 was classified as high-risk. Therefore, it is quite unique and surprising that all three patients remained in a favourable condition, without the need for any additional anti-tumour therapy, for at least 24, 14 and 19 months respectively, after only three administrations of the Tax-DC vaccine. In particular, Patients 1 and 3 obtained PR by 8 weeks after the initiation of the Tax-DC vaccine therapy.

Although these results are exciting, we cannot completely rule out the persisting effects of lenalidomide and/or mogamulizumab, which were previously administered in each patient prior to the Tax-DC vaccine therapy. These previous treatments may also have positively contributed to the present results via their immunomodulatory effects. According to recent reports, mogamulizumab has been shown to decrease the level of $CCR4^+$ regulatory T cells (Ishida & Ueda, 2011), and lenalidomide has immunomodulatory effects indirectly enhancing the activity of natural killer and T cells (Wu *et al*, 2008; De Keersmaecker *et al*, 2012).

The biopsy specimen of a residual surface lymph node from Patient 2 contained HTLV-I proviruses, although the viral expression was not inducible in the isolated cells even after *in vitro* culture (Fig 4). In general, induction of Tax expression after short-term culture is observed in approximately 50% of ATL cases (Kurihara *et al*, 2005). In the other 50% of ATL cases, the ATL cells lack the ability to express Tax, presumably due to the genomic and epigenetic changes in the HTLV-I proviruses (Takeda *et al*, 2004). Given that the viral expression was inducible in PBMCs of Patient 2 obtained prior to vaccination, the absence of viral induction



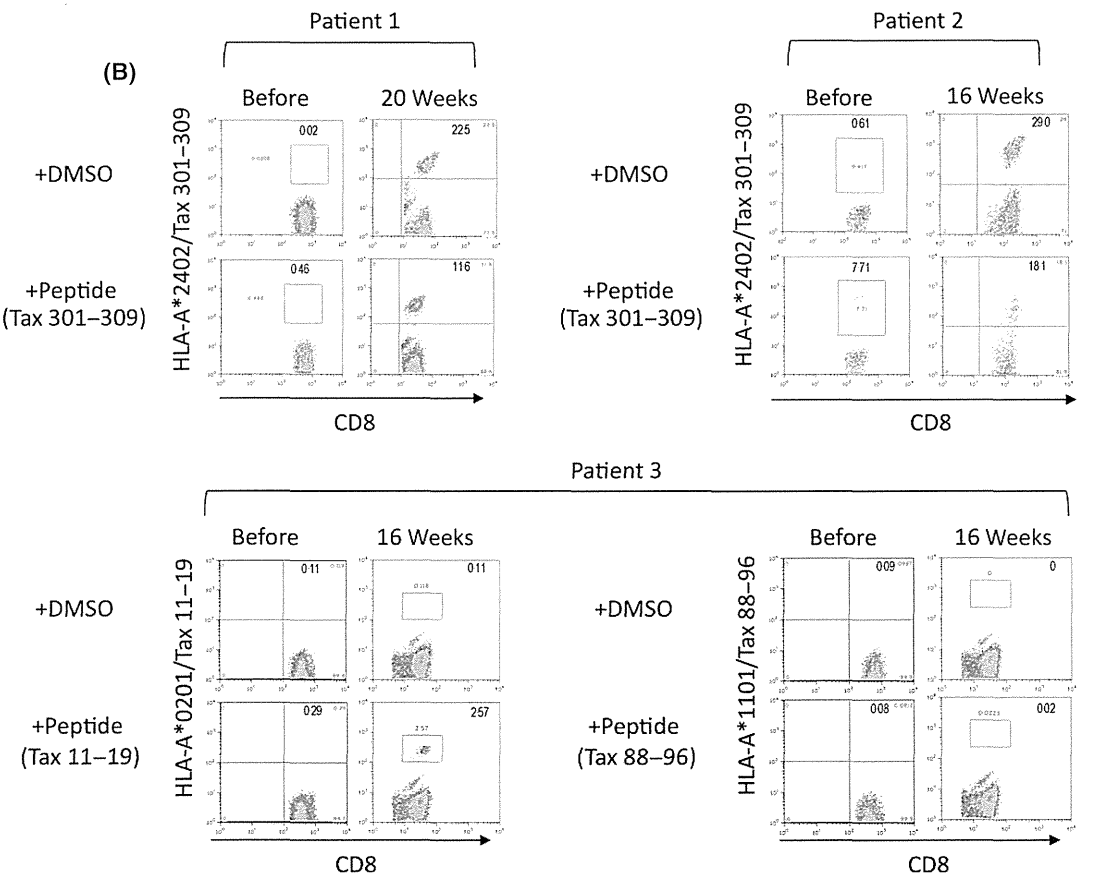


Fig 3. Immunological responses in the three patients after the Tax-DC vaccine therapy. (A) Long-term kinetics of the Tax-specific cytotoxic T cells (CTLs; % CD8+ cells, blue solid line), and γ-interferon (IFN-γ)-producing Tax-specific CTLs (% CD8+ cells, blue broken line), and human T cell leukaemia virus type-I proviral load (HTLV-I PVL) [copies/1000 peripheral blood mononuclear cells (PBMCs), red] in the peripheral blood of the three patients. Each arrow indicates administration of the vaccine. (B) The proliferative ability of the Tax-specific CTLs was evaluated using flow cytometry following incubation of the PBMCs for 13–15 d *in vitro* with cognate Tax peptide (100 nmol/l) or dimethyl sulfoxide (DMSO) in the presence of 10 u/ml of recombinant human IL2. The cells were stained with HLA/Tax tetramer-PE, anti-human CD8-PE-Cy5 mAb and anti-human CD3-FITC mAb. The values represent the percentage of tetramer$^+$ cells/CD3$^+$ CD8$^+$ cells.

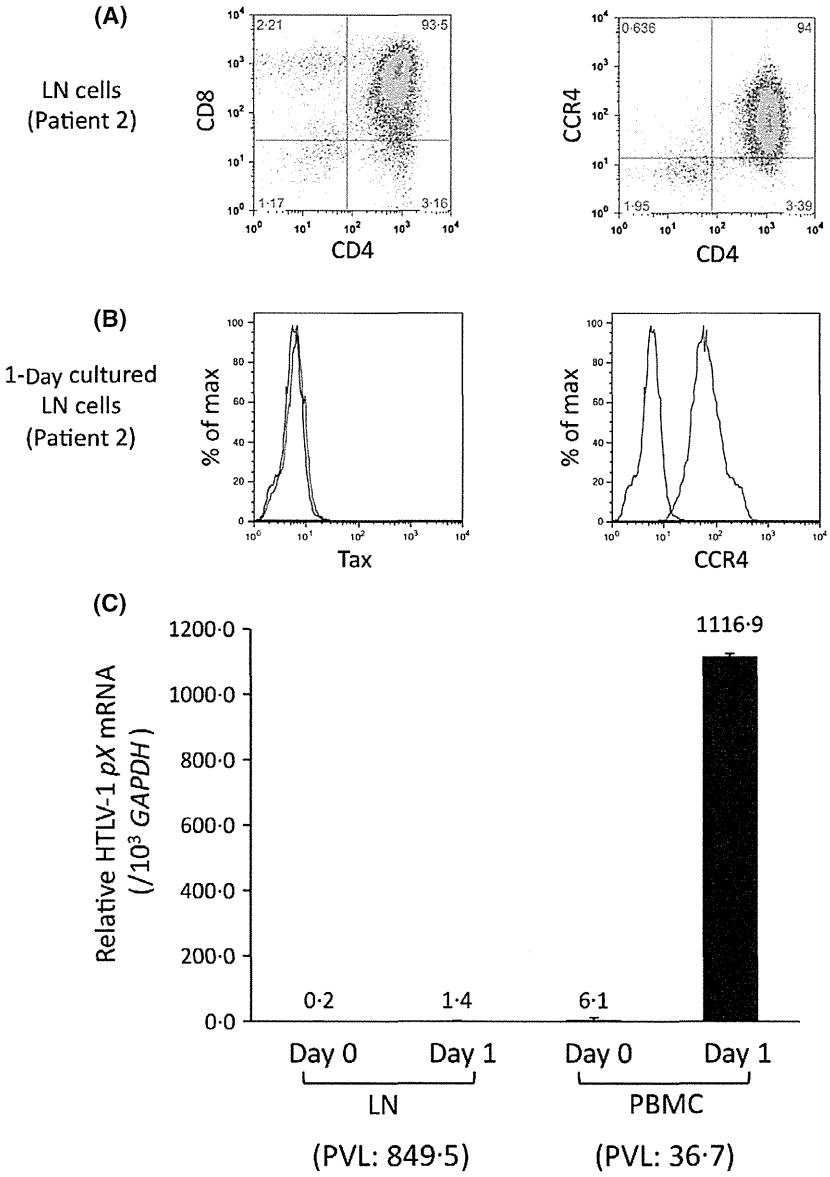


Fig 4. Absence of HTLV-I expression in the lymph node cells obtained from Patient 2. Cells were isolated from a biopsy specimen of the inguinal lymph node (LN) from Patient 2 at 9 weeks after the initiation of the Tax-DC vaccine therapy and subjected for characterization. (A) The cell surface phenotype of the LN cells immediately after isolation was analysed following staining with the indicated mAbs. (B) The intracellular Tax and CCR4 expression levels (red) in the LN cells after a 1-d culture *in vitro* were analysed following fixation of the cells with methanol. The blue histogram indicates the results of control antibody staining. (C) The HTLV-I *pX* mRNA expression levels in the LN cells before and after a 1-d culture *in vitro* were evaluated by quantitative reverse transcription polymerase chain reaction. The viral mRNA expression in peripheral blood mononuclear cells (PBMCs) obtained from the same patient before vaccination was similarly analysed as a positive control. The relative values standardized by *GAPDH* mRNA copy numbers were indicated as the means and standard deviations of duplicate samples. The proviral load (PVL) in the samples (copies/1000 cells) is indicated in parenthesis.

in the lymph node cells suggests that these tumour cells had escaped from Tax-specific CTLs.

Intriguingly, the Tax-specific CTLs demonstrated a vigorous proliferative response *in vitro* in all three patients at approximately 16–20 weeks after the initiation of the Tax-DC vaccine therapy. In particular, in Patients 1 and 2, the CTLs proliferated spontaneously without stimulation (Fig 3B). Similar phenomena have been reported in patients with HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis (Jacobson *et al*, 1990; Takamori *et al*, 2011) and occasionally in ATL patients post-HSCT (Harashima *et al*, 2005), interpreted to be the result of a normal CTL response against HTLV-I-infected cells *in vivo*. In the present study, although it is unclear whether the Tax-DC vaccine newly induced CTLs or simply activated pre-existing CTLs, Tax-specific CTLs appear to survey infected cells, at least for several months after the Tax-DC vaccine therapy, in responding to the dynamic activity of HTLV-I-infected cells *in vivo*.

In Patient 3, the Tax-specific CTLs emerged after vaccination and exhibited a clear proliferative response that peaked at 16 weeks. This response was preferentially directed toward the HLA-A2-restricted Tax epitope used for the therapy, not the HLA-A11-restricted epitope, suggesting the contribution of the Tax-DC vaccine therapy to CTL induction.

Although active CTL responses were observed in the first several months in all three patients, the responses diminished thereafter. At later time points (6 months or later) the sIL2R levels gradually increased in Patients 1 and 2 (Fig 2B). This finding suggests the need for a boosting vaccination or additional treatment to decrease the degree of immune suppression in order to maintain long-lasting anti-tumour effects.

In conclusion, the Tax-DC vaccine therapy is a safe and feasible treatment for ATL patients in stable condition. The promising clinical outcomes observed in the present study imply that the Tax-DC vaccine therapy has the potential to be an effective second-line treatment for ATL, although the anti-tumour effects of this vaccine therapy must be confirmed in further clinical trials with an increased number of patients. To our knowledge, this is the first clinical report to show the significance of a therapeutic vaccine targeting viral antigens as a new treatment modality for HTLV-I-induced malignancies. Given that Tax-specific CTL responses are impaired in patients with smouldering types of ATL and also in a small subset of asymptomatic HTLV-I carriers (Takamori *et al*, 2011), the vaccine therapy may be beneficial in these populations as well. The present study thus provides important information in a new era of anti-ATL immune therapies with the potential to be extended for prophylaxis of the disease in the future.

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Authorship

Y.S. designed the study, prepared the protocol, administered the Tax-DC therapy in patients and analysed the data. A.H. designed the study, prepared the protocol, established the method of Tax-DC preparation and analysed the data. T.I. administered the Tax-DC therapy in the patients. A.S., R.T., A.T., I.C., T.F., O.M. and T.T. participated in the protocol preparation. M.M. performed the provirus analysis. N.W. and A.T. performed the flow cytometric analysis. S.T. and K.A. supervised the institutional cell processing. M.K. proposed the initial idea and concept, designed the study, prepared the protocol and analysed the data. N.U. and J.O. supervised and coordinated the clinical and basic studies. M.K., Y.S., A.H. and J.O. wrote the manuscript. All co-authors approved the final version of the manuscript.

Disclosure

Tokyo Medical and Dental University holds a patent for the Tax epitope for HLA-A*11:01, of which M. Kannagi and R. Tanosaki are included in the inventors. This epitope was not used for a vaccine in the present study. S. Takaishi receives grants and personal fees from the MEDINET Co. Ltd., outside the submitted work.

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