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難病・がん等の疾患分野の医療の実用化研究事業（がん関係研究分野）
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、臨床試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、リンパ球腫瘍における、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、共刺激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、プロトコール作成中である。

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、共刺激因子 CD137 がリンパ系腫瘍細胞に誘導的に発現することが明らかなとなった。この発現は抗腫瘍免疫に対して、影響を及ぼすとともに、腫瘍細胞の増殖にも関与すると考えられた。

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
難病・がん等の疾患分野の医療の実用化研究事業（がん関係研究分野）
分担研究報告書

成人 T 細胞白血病(ATL)に対する樹状細胞療法におけるデータセンターの体制整備

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研究要旨
「成人 T 細胞白血病(ATL)に対する新規複合的ワクチン療法（抗 CCR4 抗体を併用した樹状細胞療法第 I/II 相試験）」が医師主導治験として実施される。医師主導治験の実施にあたっては、自ら治験を実施する者となる医師が薬事法、および医薬品の臨床試験の実施の基準に関する省令(GCP)等の各種規制に従い様々な業務を行うことが義務付けられており、その一つがデータセンター業務である。医師主導治験の実施に必要なデータセンターの体制整備を推進した。

A．研究目的

今回実施される成人 T 細胞白血病（以下、ATL）に対する樹状細胞療法が、医師主導治験として世界に先駆けて実施される。この医師主導治験を遂行するために、GCP に準拠したデータの信頼性を確保するためのデータセンターの体制を整備することを目的とする。

B．研究方法

データセンターを、症例登録・割付部門、データマネジメント部門、統計解析部門の 3 部門を設置し、役割を明確化する。また、GCP で義務付けられている標準業務手順書(SOP)を作成するとともに、データを管理するためのバリデーションされた電子データ処理システムを導入する。

C．研究結果

ATL に対する樹状細胞療法の医師主導治験の実施に向けて、症例登録・割付部門、データマネジメント部門、統計解析部門の 3 部門を設置した。標準業務手順書(SOP) においては、各部門で作成し、病院長の承認を取得。また、バリデーションされ、監査証跡、データ入力証跡、修正証跡が残るようにデザインされていることが保証された電子データ処理システムを導入した。データのセキュリティ、およびバックアップについては、データセンター外に設置されている医療情報部門で管理する。

D．考察

今後は、医師主導治験開始前までに、GCP適格性調査（治験を支援するための体制、手順書の整備状況等の実地調査）を受け、医師主導治験を進めるのに十分な体制整備を完成させる必要がある。

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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Potential Contribution of a Novel Tax Epitope–Specific CD4$^+$ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Potential Contribution of a Novel Tax Epitope–Specific CD4$^+$ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8$^+$ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4$^+$ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4$^+$ as well as CD8$^+$ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4$^+$ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4$^+$ Th1-like cells, a major population of HTLV-1–specific CD4$^+$ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8$^+$ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101$^+$ patients post–allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4$^+$ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells in HLA-DRB1*0101$^+$–infected individuals and that Tax-specific CD4$^+$ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8$^+$ T cell responses. *The Journal of Immunology*, 2013, 190: 4382–4392.

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive CD4$^+$ T cell malignancy, adult T cell leukemia/lymphoma (ATL) (1, 2). This virus has infected 10–20 million people worldwide, especially in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (3). Approximately 5% of HTLV-1–seropositive individuals develop ATL, and another 2–3% develop a slow progressive neurologic disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or various chronic inflammatory diseases (4). The majority of HTLV-1–infected individuals remain asymptomatic throughout their lives.

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation (allo-HSCT), but not autologous HSCT, improved the outcome of ATL (5, 6). In previous clinical studies carried out by the ATL allo-HSCT Study Group, the overall survival rate within 3 y after allo-HSCT with reduced intensity conditioning (RIC) was 36% (7). HTLV-1 proviral load became and remained undetectable in some ATL patients with complete remission after allo-HSCT, suggesting that it is an effective treatment for ATL (7–9). In these studies, we reported that donor-derived HTLV-1 Tax-specific CD8$^+$ CTLs were induced in some ATL patients who achieved complete remission after allo-HSCT (10). These CTLs were able to lyse recipient–derived HTLV-1–infected T cells in vitro, suggesting potential contributions to graft-versus-leukemia effects. CD8$^+$ T cells, especially CTLs, generally play an important role in controlling viral replication in various infections, such as those

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Abbreviations used in this article: AC, asymptomatic carrier; allo-HSCT, allogeneic stem cell transplantation; ATL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1–associated myelopathy/tropical spastic paraparesis; HTLV-1, human T cell leukemia virus type 1; ILT, IL-2–dependent T cell line; LCL, lymphoblastoid B cell line; rhIL-2, recombinant human IL-2; RIC, reduced intensity conditioning; Treg, regulatory T.

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involving HIV, hepatitis B virus, and hepatitis C virus. In HTLV-1 infection, HTLV-1–specific $CD8^+$ T cells predominantly recognize the Tax Ag and are believed to contribute to controlling infected cells (11, 12). A high frequency of functional Tax-specific $CD8^+$ T cells can be detected in HAM/TSP patients and some asymptomatic carriers (ACs), whereas most ATL patients and a small population of ACs show severely reduced Tax-specific $CD8^+$ T cell responses (13, 14). The mechanism underlying the suppression of HTLV-1–specific $CD8^+$ T cell responses in these patients has not yet been fully elucidated.

For induction and maintenance of virus-specific CTLs, virus-specific $CD4^+$ Th cell responses are required in many virus infections (15–19). However, there are only a few reports of HTLV-1–specific Th cell responses (20–23), presumably because of their susceptibility to HTLV-1 infection in vivo and in vitro (24). Preferential HTLV-1 infection in HTLV-1–specific $CD4^+$ T cells could be one of the reasons for immune suppression in ATL patients. In addition, it has been reported that a higher frequency of $CD4^+FOXP3^+$ regulatory T (Treg) cells is observed in infected individuals compared with uninfected healthy donors. The frequency of Tax^- Treg cells, which are a major population of Treg cells in infected individuals, is negatively correlated with HTLV-1–specific CTL responses (25). HTLV-1 basic leucine zipper factor might also be involved in immune suppression, because HTLV-1 basic leucine zipper was constitutively expressed in infected cells (26) and inhibited the activity of IFN-γ promoters by suppressing NFAT and AP-1 signaling pathways, resulting in the impaired secretion of Th1 cytokines from $CD4^+$ Th cells in a transgenic mouse model (27) These reports suggest that both the dysfunction of HTLV-1–specific $CD4^+$ Th cells and the increased number of uninfected Treg cells might be implicated in the immunosuppression observed in ATL patients. Conversely, in HAM/TSP patients, $CD4^+$ T cells are predominantly found in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory, neurotoxic cytokines, such as IFN-γ and TNF-α (30), suggesting their contributions to the pathogenesis of HAM/TSP. However, the precise roles of HTLV-1–specific $CD4^+$ T cells in HTLV-1 infection remain unclear.

In some ATL patients who achieved complete remission after allo-HSCT, it has been suggested that donor-derived HTLV-1 Tax-specific CTLs may contribute to elimination of ATL cells (graft-versus-Tax effects) (10). We believe that $CD4^+$ T cells also play a critical role in the graft-versus-ATL effects because $CD4^+$ T cells are required for induction and maintenance of optimal CTL responses (15–19). It therefore is important to clarify the role of HTLV-1–specific $CD4^+$ T cells in the effects for understanding HTLV-1–specific T cell immunity in ATL patients after allo-HSCT and for developing new vaccine strategies to prevent recurrence of ATL.

Several studies have reported some HTLV-1–specific $CD4^+$ T cell epitopes restricted by different HLA haplotypes (20–23). The helper functions of these epitopes in HTLV-1–specific CTL responses in HTLV-1–infected individuals have not been well understood. However, Jacobson et al. (20) showed that $CD4^+$ T cells specific for Env gp46 196–209, an epitope restricted by HLA-DQ5 or -DRw16, exhibited a cytotoxic function by directly recognizing HTLV-1–infected cells. This observation raises the possibility that some HTLV-1–specific $CD4^+$ T cells may contribute to the graft-versus-ATL effects through their cytotoxic function in ATL patients after allo-HSCT.

In the current study, we demonstrated that both $CD4^+$ and $CD8^+$ Tax-specific T cell responses were induced in patients after allo-HSCT with RIC for ATL. To further analyze HTLV-1–specific $CD4^+$ T cell responses in ATL patients after allo-HSCT, we determined a novel HLA-DRB1*0101–resricted epitope, Tax155–167, recognized by HTLV-1–specific $CD4^+$ Th1-like cells, a major population of HTLV-1–specific $CD4^+$ T (T4) cell line, which was established from a patient in complete remission following allo-HSCT with RIC. Costimulation with oligopeptides corresponding to the Th1 epitope, Tax155–167, together with a known CTL epitope led to robust expansion of Tax-specific $CD8^+$ T cells in PBMCs from three HLA-DRB1*0101^+ patients after allo-HSCT tested. Furthermore, Tax155–167-specific $CD4^+$ T cells were found to be maintained in all HTLV-1–infected HLA-DRB1*0101^+ individuals tested, regardless of HSCT, by direct detection with newly generated HLA-DRB1*0101/Tax155–167 tetramers. Our results suggest that Tax155–167 may be a dominant epitope recognized by HTLV-1–specific $CD4^+$ T cells in HTLV-1–infected individuals carrying HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells may strengthen the graft-versus-ATL effects through efficient induction of Tax-specific CTL responses.

Materials and Methods

Subjects

A total of 18 ATL patients who underwent allo-HSCT with RIC regimen, and one HTLV-1–seronegative (#365) and two seropositive donors (one AC #310 and one HAM/TSP patient #294) carrying HLA-DRB1*0101 donated peripheral blood samples after providing written informed consent. Approximately one-half of these patients received allogeneic peripheral blood stem cell transplantation from HLA-A-, B-, and -DR–identical sibling donors. The other half received allogeneic bone marrow cells from HLA-A–, B–, and DR–identical seronegative unrelated donors (Table I). These patients were the participants of clinical studies organized by the ATL allo-HSCT Study Group, supported by the Ministry of Health, Welfare, and Labor of Japan. This study was also reviewed and approved by the Institutional Ethical Committee Review Board of the Tokyo Medical and Dental University.

Generation of cell lines derived from patients and donors

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) density gradient centrifugation and stored in liquid nitrogen in Bambanker stock solution (NIPPON Genetics, Tokyo, Japan) until required. These were used in part to obtain HTLV-1–infected IL-2–dependent T cell lines (ILT) and EBV-transformed lymphoblastoid B cell lines (LCL). ILT-#350 was spontaneously immortalized during long-term culture of PBMCs from patient #350 before allo-HSCT and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 20% FCS (Sigma Aldrich, St. Louis, MO) and 30 U/ml recombinant human IL-2 (rhIL-2; Shionogi, Osaka, Japan). LCL-#307, -#341, and -#350 were established by maintaining PBMCs from ATL patients #307, #341, and #350, respectively, after allo-HSCT. These PBMCs were maintained in RPMI 1640 medium containing 20% FCS, following infection with the EBV-containing culture supernatant of the B95-8 cell line, LCL-Kan, derived from a healthy individual was also used.

Synthetic peptides

A total of 18 overlapping peptides, 12- to 25-mer in length, spanning the central region of Tax (residues 103–246) were purchased and used for epitope mapping (Scrum Tokyo, Japan) (Table II). HLA-A*2402–restricted CTL epitopes (Tax301–309, SFHSLHLLF) (10) were used for in vitro stimulation of Tax-specific CTLs (Hokudo, Sapporo, Japan).

GST–Tax fusion protein–based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST–fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST–Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1×10^6 cells/ml) were incubated with or without a mixture of GST–Tax-A, -B, and -C proteins (GST–TaxABC) in 200 μl RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined using an OptiEIA Human IFN-γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN-γ. $CD8^+$ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the

manufacturer's instructions. For cytokine profiling of a HTLV-1–specific $CD4^+$ T cell line, cells were stimulated with formaldehyde-fixed ILT-#350 for 48 h. Culture supernatant was collected, and various cytokines were measured using a Human Th1/Th2/Th17 Cytokine Kit for a Cytokine Beads Array (BD Biosciences).

Induction of HTLV-1–specific $CD4^+$ T cell line (T4 cells)

PBMCs (1×10^6 cells/ml) from patient #350, in complete remission at 180 d after allo-HSCT, were cultured for 2 wk with 100 nM Tax301–309 peptide in 96-well round-bottom tissue culture plate (BD Biosciences) in a final volume of 200 μl RPMI 1640 medium with 20% FCS and 10 U/ml rhIL-2. $CD4^+$ cells were then isolated by negative selection using a Human CD4 T lymphocyte Enrichment Set-DM (BD Biosciences) and maintained in RPMI 1640 medium with 20% FCS and 100 U/ml rhIL-2. Cells (1×10^6 cells/ml) were stimulated with formaldehyde-fixed ILT-#350 (2.5×10^5 cells/ml) every 2–3 wk. After multiple rounds of stimulation, the resulting $CD4^+$ T cell line was assessed for HTLV-1 specificity by comparing IFN-γ production against ILT-#350 to that against an HTLV-1–negative cell line, LCL-#350.

RT-PCR

Total RNA from cells was isolated using Isogen (Nippon Gene, Tokyo, Japan) and Turbo DNA-free (Life Technologies). First-strand cDNA was prepared from 0.5 μg RNA using ReverTra Ace and $Oligo(dT)_{20}$ primers provided in a ReverTra Ace-α-kit (Toyobo, Osaka, Japan). PCRs were performed in 50 μl reaction mixture containing ReverTra Dash (Toyobo), 0.5 μM of each HTLV-1 pX-specific primer (pX1, 5′-CCA CTT CCC AGG GTT TAG ACA GAT CTT C-3′ and pX4, 5′-TTC CTT ATC CCT CGA CTC CCC TCC TTC CCC-3′), and 2 μl cDNA. GAPDH-specific primers (GAPDH5′, 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH3′, 5′-TCC ACC ACC CTG TTG CTG TA-3′) were used as an internal control. The thermal cycling conditions comprised an initial activation step at 94°C for 1 min, followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 2 s), and extension (74°C, 30 s). The PCR amplicons were visualized by ethidium bromide staining following 2% (w/v) agarose gel electrophoresis.

Flow cytometry

For cell surface staining, the following fluorochrome-conjugated mouse anti-human mAbs were used: CD3-FITC (UCHT1; BioLegend, San Diego, CA), CD4-FITC (RPA-T4; BioLegend), CD8-FITC (RPA-T8; BioLegend), and CD8-PE-Cy5 (HIT8a; BD Biosciences, San Jose, CA). For tetramer staining, PE-conjugated HLA-A*0201/Tax11–19, HLA-A*1101/Tax88–96, HLA-A*1101/Tax272–280, and HLA-A*2402/Tax301–309 tetramers were purchased from Medical & Biological Laboratories (Nagoya, Japan). PE-conjugated HLA-DRB1*0101/Tax155–167 tetramer were newly generated through the custom service of Medical & Biological Laboratories. Whole-blood or cultured cells were stained with PE-conjugated Tax/HLA tetramer in conjunction with CD3-FITC and CD8-PE-Cy5 or CD4-PE-Cy5. For whole-blood samples, RBCs were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing. Samples were analyzed on a FACSCalibur (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Epitope mapping

T4 cells (3×10^5 cells/ml) were stimulated with LCL-#350, pulsed with various concentrations of synthetic peptides for 1 h at 37°C, at a responder/stimulator (R/S) ratio of 3. The culture supernatant was collected at 6 h poststimulation, and peptide-specific IFN-γ production from T4 cells was determined by ELISA.

HLA class II restriction assay

T4 cells (5×10^5 cells/ml) were cocultured for 6 h with ILT-#350 (1×10^5 cells/ml) in the presence or absence of anti-human HLA-DR (10 μg/ml; L243; BioLegend), anti-human HLA-DQ (10 μg/ml; SPVL3; Beckman Coulter, Fullerton, CA), or anti–HLA-ABC (10 μg/ml; W6/32; BioLegend). The IFN-γ in the supernatant was measured by ELISA.

To identify a HLA class II molecule responsible for Ag presentation to T4 cells, Tax155–167 peptide-specific IFN-γ responses were evaluated using various HLA-typed LCLs (LCL-#350, LCL-#341, LCL-#307, and LCL-Kan). These LCLs (1×10^5 cells/ml) were pulsed with 100 ng/ml Tax155–167 peptide for 1 h, fixed with 2% formaldehyde, and then cultured with T4 cells (3×10^5 cells/ml) for 6 h. The culture supernatant was collected, and IFN-γ in the supernatant was measured by ELISA.

Tetramer-based proliferation assay

PBMCs (1.0×10^6 cells/ml) were cultured for 13 or 14 d with or without 100 nM antigenic peptides in the presence of 10 U/ml rhIL-2. Cells were stained with HLA/Tax tetramer-PE, CD3-FITC, and CD8-PE-Cy5 or CD4-PE-Cy5 and then analyzed by flow cytometry.

Statistic analysis

Statistical significance was evaluated with the unpaired t test using Graphpad Prism 5 (Graphpad Software, La Jolla, CA). In all cases, two-tailed p values <0.05 were considered significant.

Results

Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC

We previously reported that Tax-specific $CD8^+$ T cells were induced in some ATL patients after allo-HSCT with RIC from HLA-identical sibling donors (10). In this study, we examined the Tax-specific T cell response in a larger number of ATL patients who received allo-HSCT with RIC. Table I provides a summary of the

Table I. Clinical information and summary for Tax-specific $CD8^+$ T cells in 18 ATL patients at 180 d post–allo-HSCT with RIC

ID (Age, Sex)	ATL Subtype	Type of Donor	Donor-HLA	Donor HTLV-1 Sero Status	Chimerism (%)[a]	Tetramer (%)[b]	Proviral Load[c]
239 (55, M)	Lymphoma	r-PB	A 26/33, DR 4/13	(−)	<5	NT	0.1
241 (61, F)	Acute	r-PB	A 2/26, DR 10/18	(−)	<5	0.00	0.1
247 (52, F)	Lymphoma	r-PB	A 24/−, DR 9/15	(−)	<5	0.07	0.1
270 (57, M)	Lymphoma	r-PB	A 24/33, DR 13/15	(−)	<5	0.00	0.0
300 (53, F)	Lymphoma	r-PB	A 24/26, DR 4/15	(+)	<5	1.34	4.8
301 (57, F)	Acute	ur-BM	A 24/33, DR 13/15	(−)	<5	0.72	0.0
307 (68, F)	Acute	r-PB	A 2/11, DR 14/15	(+)	<5	0.10	5.4
317 (60, M)	Acute	ur-BM	A 2/24, DR 14/15	(−)	<5	0.92	0.0
328 (62, M)	Acute	ur-BM	A 11/24, DR 8/9	(−)	<5	0.75	NT
340 (50, M)	Acute	r-PB	A 2/24, DR 4/8	(−)	<5	1.40	0.7
341 (61, F)	Acute	ur-BM	A 24/33, DR 1/15	(−)	<5	0.45	0.1
344 (58, M)	Lymphoma	ur-BM	A 2/24, DR 4/−	(−)	<5	0.44	0.0
349 (53, M)	Acute	r-PB	A 24/−, DR 8/15	(+)	<5	0.00	0.0
350 (60, F)	Acute	ur-BM	A 24/26, DR 1/14	(−)	<5	0.59	0.6
351 (57, F)	Acute	ur-BM	A 24/26, DR 9/12	(−)	<5	0.45	0.0
358 (63, F)	Lymphoma	r-PB	A 2/11, DR 4/14	(−)	<5	0.42	0.0
352 (61, M)	Acute	ur-BM	A 11/26, DR 8/15	(−)	<5	0.14	0.0
364 (52, M)	Acute	r-PB	A 24/26, DR 1/−	(−)	<5	0.11	0.0

[a]Indicates percentage of recipient-derived T cell chimerism.
[b]Indicates percentage of tetramer$^+$ cells among $CD8^+$ T cells in PBMCs.
[c]Indicates copy number per 1000 PBMCs.
F, Female; M, male; NT, not tested; r-PB, related donor-derived peripheral blood stem cell; ur-BM, unrelated donor-derived bone marrow cell.

results of Tax-specific $CD8^+$ T cell detection by flow cytometry, using the Tax/HLA tetramers, in the peripheral blood of 18 ATL patients at 180 d after allo-HSCT, together with clinical information. During this period, all patients achieved a complete chimera state consisting of >95% of donor-derived hematopoietic cells. By using four available tetramers (HLA-A*0201/Tax11–19, HLA-A*2402/Tax301–309, HLA-A*1101/Tax88–96, and HLA-A*1101/Tax272–280), Tax-specific $CD8^+$ T cells were found in 14 patients. Because the donors were uninfected individuals in the majority of cases (Table I), induction of the Tax-specific donor-derived $CD8^+$ T cells in recipients indicated the presence of newly occurring immune responses against HTLV-1 in the recipients. This evidence strengthens our previous observation (10, 32).

We also used a GST–Tax fusion protein-based assay to evaluate Tax-specific T cell responses. The tetramer-based assay was limited to four kinds of epitopes and restricted by three HLA alleles but did not detect T cells directed to other epitopes or HLAs. The GST–Tax fusion protein-based assay can detect both $CD4^+$ and $CD8^+$ T cell responses, irrespective of HLA types. However, this sensitivity is not as good as single-cell analysis by flow cytometry (31). As shown in Fig. 1A, there was a wide variation in the IFN-γ responses to the Tax protein in the PBMCs among the 16 patients tested. In five patients (#247, #270, #328, #340, and #349), IFN-γ production of PBMCs against GST–TaxABC proteins was very low or not specific for the Tax protein. PBMCs from the other 11 patients (#239, #241, #301, #317, #341, #344, #350, #351, #352, #358, and #364) produced higher amounts of IFN-γ in response to GST–TaxABC proteins compared with GST. However, the levels of IFN-γ production varied among the patients.

We also evaluated the extent to which Tax-specific $CD4^+$ T cells were responsible for IFN-γ in the GST–Tax-based immunoassay system. We used PBMCs from patients #350 and #341, who showed high Tax-specific T cell responses. $CD8^+$ cell–depleted PBMCs from patient #350 and #341 showed a reduced but still significant level of Tax-specific IFN-γ–producing response compared with whole PBMCs (Fig. 1B). These results indicate that not only $CD8^+$ but also $CD4^+$ T cells against Tax are present in the peripheral blood from patient #350 and #341 after allo-HSCT with RIC.

Induction of an HTLV-1–specific $CD4^+$ T cell line from patient #350

We next attempted to induce HTLV-1–specific $CD4^+$ T cells from the PBMCs of patient #350 at 180 d after allo-HSCT, using an HTLV-1–infected T cell line (ILT-#350) as APCs. Freshly isolated PBMCs were stimulated for 2 wk with Tax301–309, a dominant CTL epitope presented by HLA-A*2402, to eliminate HTLV-1–infected cells, which potentially existed in PBMCs. The $CD4^+$ cells were then isolated from the cultured cells and stimulated with formaldehyde-fixed ILT-#350 every 2–3 wk. The established cell line was found to be a $CD4^+$ T cell line (designated as T4 cells thereafter) because cells expressed CD3 and CD4 but not CD8

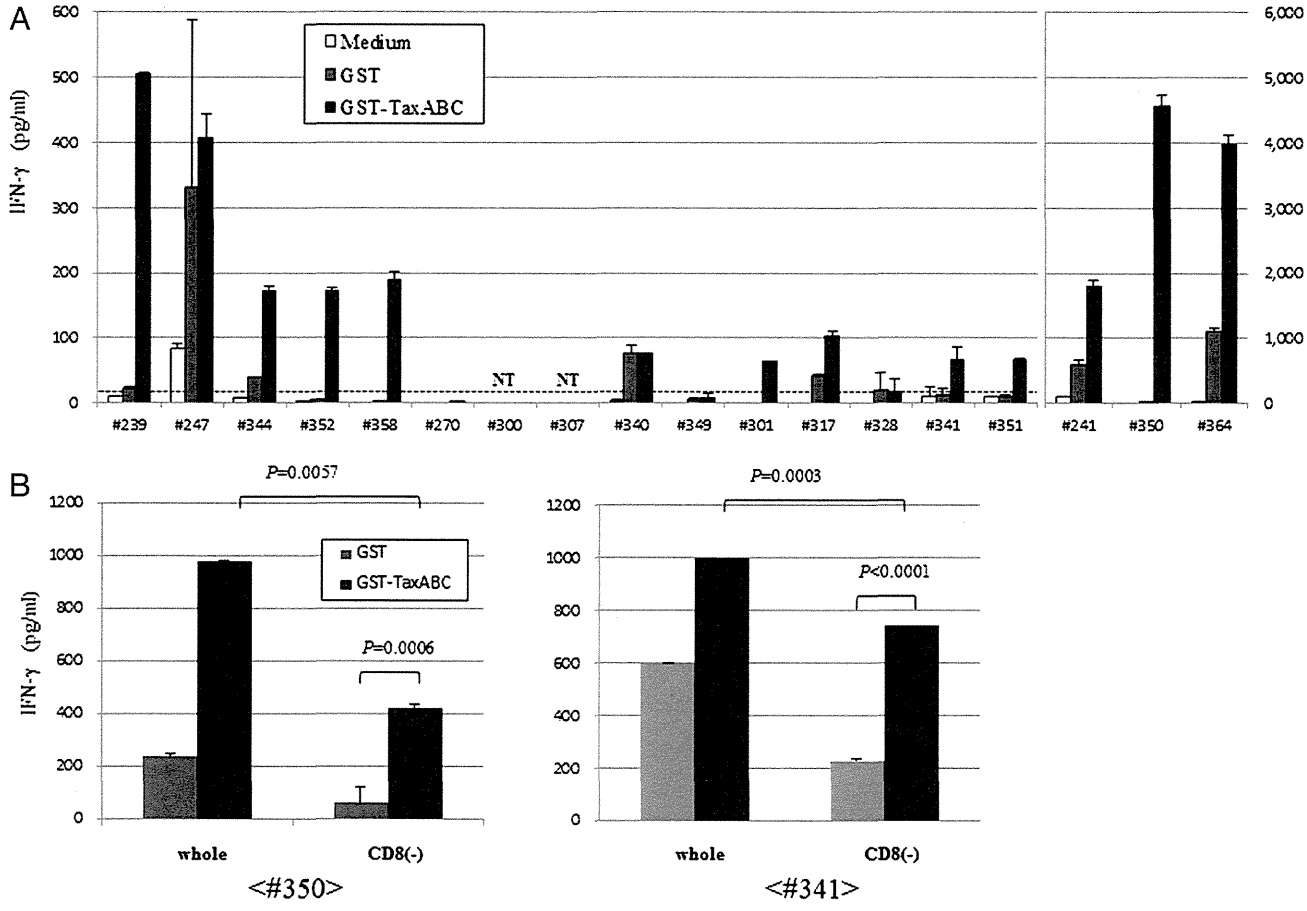


FIGURE 1. Diversity of Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC. (**A** and **B**) PBMCs from 18 ATL patients at 180 d after allo-HSCT (A) or whole and $CD8^+$ cell–depleted PBMCs from two patients at 540 d after allo-HSCT (#350 and #341) (B) were cultured for 4 d in the absence (open square) or presence of GST (gray square), or GST–Tax (black square) proteins. The concentration of IFN-γ in the supernatant was determined by ELISA. The *y*-axis on the right side indicates the results from three patients (#241, #350, and #364). The dotted horizontal line indicates the detection limit (23.5 pg/ml). The error bars represent SD of duplicated wells. The representative result of two independent experiments is shown in (B).

(Fig. 2A). Because HTLV-1 has been shown to preferentially infect $CD4^+$ T cells in vivo and in vitro (24), we examined HTLV-1 expression in T4 cells by RT-PCR (Fig. 2B). As expected, the T4 cells did not express HTLV-1 Tax, indicating that the cells were not infected with HTLV-1. We assessed expression of various cytokines in T4 cells (Fig. 2C). The T4 cells were stimulated with formaldehyde-fixed ILT-#350 or LCL-#350. The cells produced large amounts of IFN-γ and TNF-α and small amounts of IL-2, IL-4, and IL-10 in response to ILT-#350 but not against LCL-#350. IL-6 and IL-17A were not detected in the culture supernatant. These data indicate that T4 cells are mainly HTLV-1–specific $CD4^+$ Th1-like cells but contain minor populations to produce Th2 cytokines.

Determination of the minimum epitope recognized by T4 cells

Freshly isolated PBMCs in the patient #350 produced IFN-γ in response to GST–Tax (Fig. 1A). We expected that the epitope recognized by the T4 cells should be present in the Tax protein. We therefore examined whether the T4 line responded to Tax using LCL-#350 pulsed with GST–Tax proteins as APCs. As shown in Fig. 3A, the T4 cells produced significantly higher amounts of IFN-γ in response to GST–TaxABC and GST–Tax-B (residues 113–237) (31) but not GST–Tax-A (residues 1–127) (31) and -C (residues 224–353 (31), when compared with the GST control protein, indicating that the T4 cells recognized the central region (residues 113–237) of the Tax Ag. We next synthesized eight overlapping 25-mer peptides spanning the central region of Tax (residues 103–246) and analyzed their abilities to stimulate T4 cells (Table II). The cell line produced high amounts of IFN-γ only when stimulated with Tax154–178 (Fig. 3B). We then prepared four overlapping 15-mer peptides, covering residues 154–178 of Tax, to examine the IFN-γ responses of the T4 cells (Table II). Both Tax151–165 and Tax156–170-stimulated cells to induce IFN-γ responses but not at a comparable level to Tax154–178 (Fig. 3C). These results suggest that the epitope recognized by T4 cells might be present in the N-terminal half of Tax154–178. We therefore stimulated the cells with Tax154–168, Tax155–169, or Tax156–170. The cells showed higher IFN-γ responses against Tax154–168 and Tax155–169 than Tax156–170, indicating that the minimum epitope might be within residues 155–168 of Tax (Fig. 3D). To identify the minimum epitope recognized by T4 cells, we next synthesized three overlapping peptides of 12- to 14-mer lengths beginning at residue 155 of Tax (Table II). Tax155–167 induced IFN-γ responses in cells at a similar level to Tax155–169 and Tax155–168, although Tax155–166 did not (Fig. 3E). Moreover, IFN-γ production of cells in response to various concentrations of Tax155–167 was comparable to that against Tax155–169 and Tax155–168 (Fig. 3F). These data clearly show that the minimum epitope recognized by the T4 cells is Tax155–167.

*HLA-DRB1*0101 restriction of Tax-specific T4 cells*

To analyze HLA class II molecules involved in the presentation of the minimum epitope, T4 cells were stimulated with ILT-#350 in the presence or absence of anti–HLA-DR, -DQ, and anti-HLA class I blocking Abs. As shown in Fig. 4A, the addition of an anti–HLA-DR blocking Ab abrogated IFN-γ responses of the T4 cells against ILT#-350, indicating that the epitope was HLA-DR restricted.

We further investigated the HLA-DR alleles responsible for the presentation of the minimum epitope by using four HLA-typed LCLs displaying different HLA-DRs. As shown in Fig. 4B, the T4 cells responded by producing IFN-γ when Tax155–167 was presented by autologous LCL-#350 (DR1/14) and allogeneic LCL-#341 (DR1/15). These results clearly indicate that this epitope is presented by HLA-DRB1*0101 on APCs. We searched for a known HLA-DRB1*0101 motif in the identified epitope Tax155–167 and found that this epitope contained the HLA-DRB1*0101 motif (Fig. 4C) (33).

Enhancement of Tax-specific $CD8^+$ T cell expansion by Tax155–167-specific $CD4^+$ T cell help

As T4 cells were established from PBMCs of an HTLV-1–infected patient #350, it is suggested that Tax155–167-specific $CD4^+$ T cells may be maintained in the $HLA\text{-}DRB1*0101^+$ patient #350.

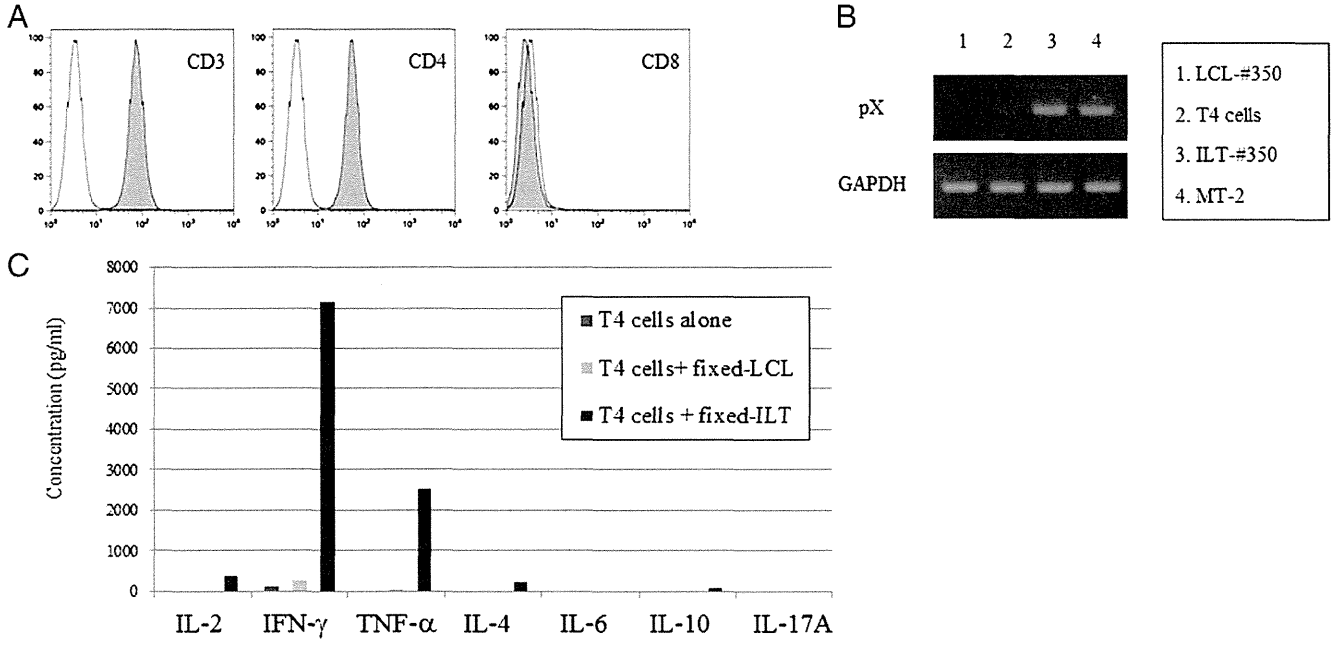


FIGURE 2. Phenotype and function of $CD4^+$ T cell line (T4) generated from patient #350. (**A**) Cell surface phenotype of T4 cells was analyzed by flow cytometry. (**B**) Total RNA was extracted from LCL-#350 (*lane 1*), T4 cells (*lane 2*), ILT-#350 (*lane 3*), and MT-2 (*lane 4*). Tax mRNA expression for each cell type was analyzed by RT-PCR. GAPDH was used as an internal control. (**C**) T4 cells were stimulated for 24 h with or without formaldehyde-fixed ILT-#350 or LCL-#350 cells. The concentration of indicated cytokines in the supernatants was measured using a cytometric bead array system.

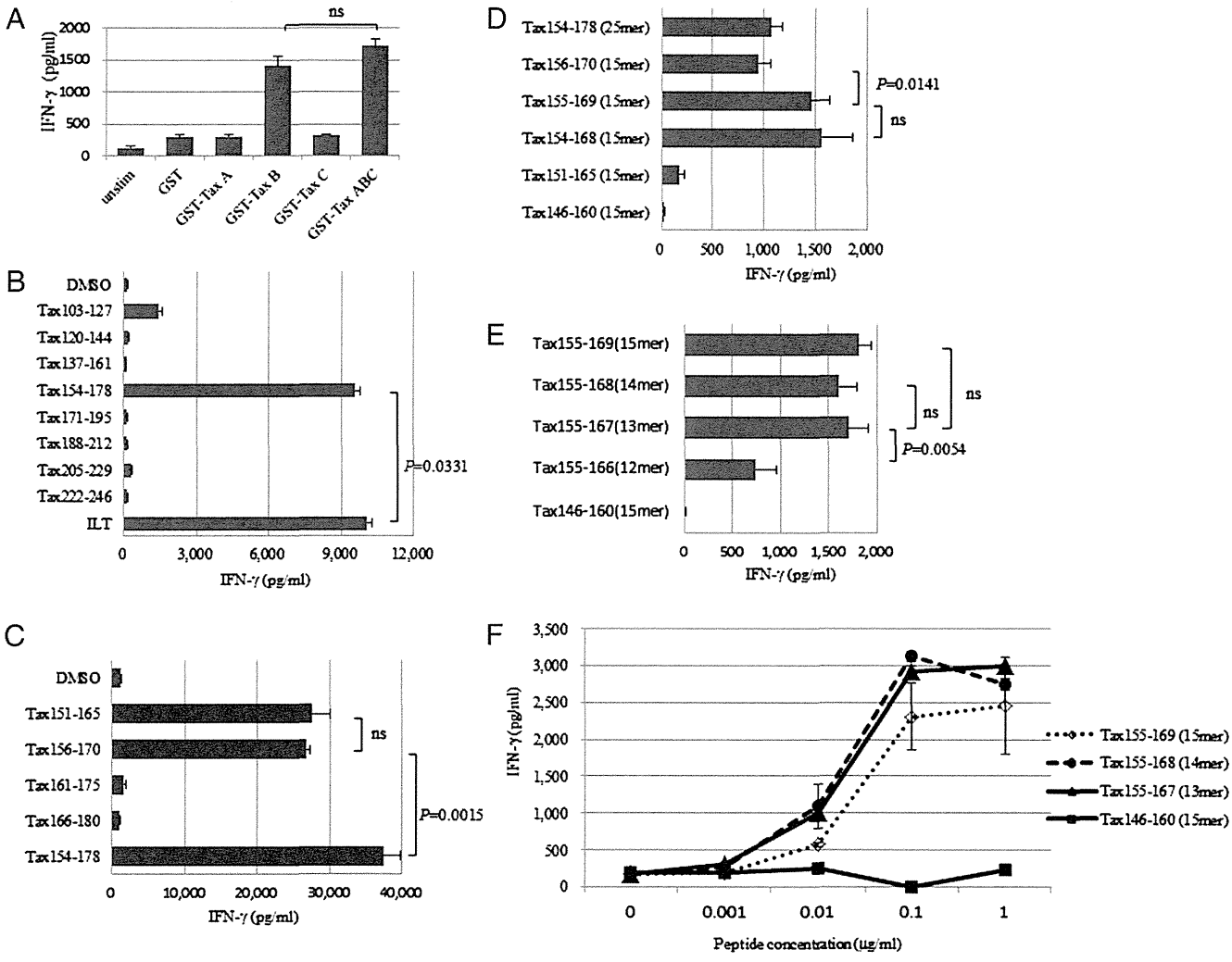


FIGURE 3. Identification of the dominant Tax-derived epitope recognized by established T4 cells. (**A**) Donor-derived LCL-#350 was pulsed with GST, GST–Tax-A, GST–Tax-B, GST–Tax-C, or a mixture of GST–Tax-A, -B, and -C (GST–TaxABC) for 24 h and then cocultured for 24 h with the T4 cells at a responder/stimulator (R/S) ratio of 3. IFN-γ production from T4 cells was analyzed by ELISA. (**B** and **C**) LCL-#350 was pulsed with the indicated overlapping 25-mer-long (B) or 15-mer-long (C) synthetic peptides (10 μg/ml) within the Tax-B region for 1 h. Formaldehyde-fixed ILT-#350 cells were cocultured with T4 cells for 6 h. IFN-γ in the supernatant was measured by ELISA. (**D** and **E**) IFN-γ responses of T4 cells were assessed using the indicated overlapping 12- to 25-mer-long synthetic peptides (100 ng/ml). (**F**) IFN-γ responses of T4 cells against indicated concentrations of 13- to 15-mer-long peptides were assessed as in (B) and (C). (A–F) Results are representative of two or three independent experiments. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired *t* test.

We therefore evaluated the helper function of Tax155–167-specific $CD4^+$ T cells on the expansion of dominant Tax-specific CTLs in fresh PBMCs of the patient #350. Freshly isolated PBMCs from patient #350 (A24/26, DR1/14) at 540 d after allo-HSCT were stimulated for 13 d with the HLA-A24–restricted CTL epitope peptide (Tax301–309) in the presence or absence of the HLA-DRB1*0101–restricted $CD4^+$ Th epitope peptide (Tax155–167), and Tax-specific $CD8^+$ T cell expansion was evaluated using the HLA-A*2402/Tax301–309 tetramer. As shown in Fig. 5, Tax301–309-specific $CD8^+$ T cells proliferated to 9.26% of $CD8^+$ T cells when stimulated with Tax301–309 alone. Surprisingly, a highly elevated frequency (62.3%) of tetramer-binding $CD8^+$ T cells was detected by in vitro costimulation with Tax301–309 and Tax155–167, suggesting the presence of Tax155–167-specific $CD4^+$ Th cells in patient #350.

We examined whether Tax155–167-specific $CD4^+$ T cells existed and functioned as helper cells in the other two HTLV-1–infected HLA-DRB1*0101^+ patients after allo-HSCT (day 360 for patient #341 and day180 for #364). These patients had detectable levels of HLA-A*2402/Tax301–309 tetramer-binding $CD8^+$ T cells in the peripheral blood (Fig. 5). In patients #341 and #364, the tetramer-binding cells expanded to 7.7 and 0.849% of $CD8^+$ T cells at 13 d of culture when stimulated with the CTL epitope peptide, Tax301–309, alone. Costimulation of PBMCs with both peptides Tax155–167 and Tax301–309 led to a vigorous proliferation of tetramer-binding $CD8^+$ T cells (59.6% for patient #341 and 15.5% for patient #364) as observed in patient #350 (Fig. 5). These results indicate that Tax155–167-specific $CD4^+$ T cells may be present and contribute to enhancing $CD8^+$ T cell responses in HTLV-1–infected HLA-DRB1*0101^+ individuals after allo-HSCT.

*Tax155–167-specific $CD4^+$ T cells were maintained in HTLV-1–infected HLA-DRB1*0101^+ individuals*

We next generated the HLA-DRB1*0101/Tax155–167 tetramer to directly detect Tax155–167-specific $CD4^+$ T cells and examined the presence of Tax155–167-specific $CD4^+$ T cells in the PBMCs freshly isolated from two HLA-DRB1*0101^+ patients after allo-HSCT (day 180 for patient #350 and day 360 for patient #364).

Table II. Synthetic oligopeptides used in this study

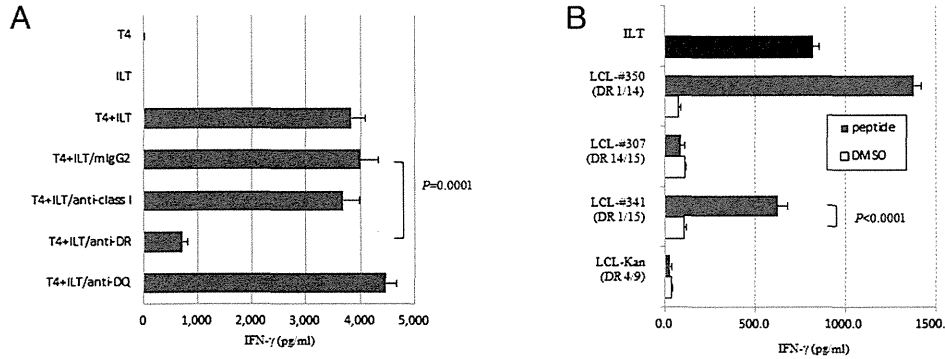
Peptide	Sequence
Tax103–127	P S F L Q A M R K Y S P F R N G Y M E P T L G Q H
Tax120–144	M E P T L G Q H L P T L S F P D P G L R P Q N L Y
Tax137–161	G L R P Q N L Y T L W G G S V V C M Y L Y Q L S P
Tax154–178	M Y L Y Q L S P P I T W P L L P H V I F C H P G Q
Tax171–195	V I F C H P G Q L G A F L T N V P Y K R I E E L L
Tax188–212	Y K R I E E L L Y K I S L T T G A L I I L P E D C
Tax205–229	L I I L P E D C L P T T L F Q P A R A P V T L T A
Tax222–246	R A P V T L T A W Q N G L L P F H S T L T T P G I
Tax146–160	L W G G S V V C M Y L Y Q L S
Tax151–165	V V C M Y L Y Q L S P P I T W
Tax154–168	M Y L Y Q L S P P I T W P L L
Tax155–169	Y L Y Q L S P P I T W P L L P
Tax156–170	L Y Q L S P P I T W P L L P H
Tax161–175	P P I T W P L L P H V I F C H
Tax166–180	P L L P H V I F C H P G Q L G
Tax155–168	Y L Y Q L S P P I T W P L L
Tax155–167	Y L Y Q L S P P I T W P L
Tax155–166	Y L Y Q L S P P I T W P

Tax155–167-specific CD4$^+$ T cells were detected ex vivo in the patient #350 (0.11%) and proliferated to 11.6% among CD4$^+$ T cells at 13 d poststimulation with Tax155–167 peptide. In the patient #364, tetramer-binding CD4$^+$ T cells were undetectable in fresh PBMCs but expanded to 0.37% by in vitro stimulation with Tax155–167 peptide (Fig. 6A). In an HLA-DRB1*0101$^+$–seronegative donor #365, Tax155–167-specific CD4$^+$ T cells were not found in fresh PBMCs and did not become detectable at 13 d after stimulation with Tax155–167 peptide (Fig. 6A). This result indicates that Tax155–167-specific CD4$^+$ T cells are maintained and possesses the abilities to proliferate in response to HTLV-1 Tax in these patients.

We further examined whether Tax155–167-specific CD4$^+$ T cells existed in two HTLV-1–infected individuals carrying HLA-DRB1*0101, an AC #310 and a HAM/TSP patient #294, and detected 0.18 and 0.31% of tetramer-binding cells in peripheral CD4$^+$ T cells, respectively (Fig. 6B). These results suggest that Tax155–167-specific CD4$^+$ T cells are maintained in HTLV-1–infected individuals expressing an HLA-DRB1*0101 allele, regardless of HSCT.

Discussion

In this study, we demonstrated Tax-specific CD4$^+$ T cell responses in some ATL patients post–allo-HSCT and identified a novel HLA-DRB1*0101–restricted CD4 T cell epitope, Tax155–167, which was recognized by HTLV-1–specific CD4$^+$ T cells and consequently led to robust Tax-specific CD8$^+$ T cell expansion. We also found that Tax155–167-specific CD4$^+$ T cells existed in all HTLV-1–infected HLA-DRB1*0101$^+$ individuals tested, regardless of HSCT, by newly generated HLA-DRB1*0101/Tax155–167 tetramers. These results suggest that Tax155–167 might be a dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells



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	Relative position												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Tax155-167	**Y**	**L**	**Y**	**Q**	**L**	**S**	**P**	**P**	**I**	**T**	**W**	**P**	**L**
Anchor residues	Y,V, L,F, I,A M,W	*	*	L,A, I,V, M,N, Q	*	A,G, S,T, P	*	*	L,A, I,V, N,F, Y				

FIGURE 4. HLA-DRB1*0101 restriction of Tax155–167 recognition by established T4 cells. (**A**) T4 cells were cocultured for 6 h with ILT-#350 in the presence or absence of the following blocking Abs (10 μg/ml): anti-human HLA-DR; anti-human HLA-DQ; anti–HLA-class I; or isotype control. IFN-γ production from T4 cells was measured by ELISA. (**B**) The T4 cells were cocultured for 6 h with autologous (#350) or allogeneic (#307, #341, and Kan) LCLs pulsed with (closed bar) or without (open bar) Tax155–167 for 1 h or with recipient-derived ILT-#350. The HLA-DR alleles of each LCL line are indicated in parentheses. IFN-γ production of T4 cells was assessed by ELISA. (A and B) Representative data of three independent experiments are shown. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired *t* test. (**C**) The amino acid sequence between residues 155 and 167 of Tax contained a putative HLA-DRB1*0101 anchor motif (33).

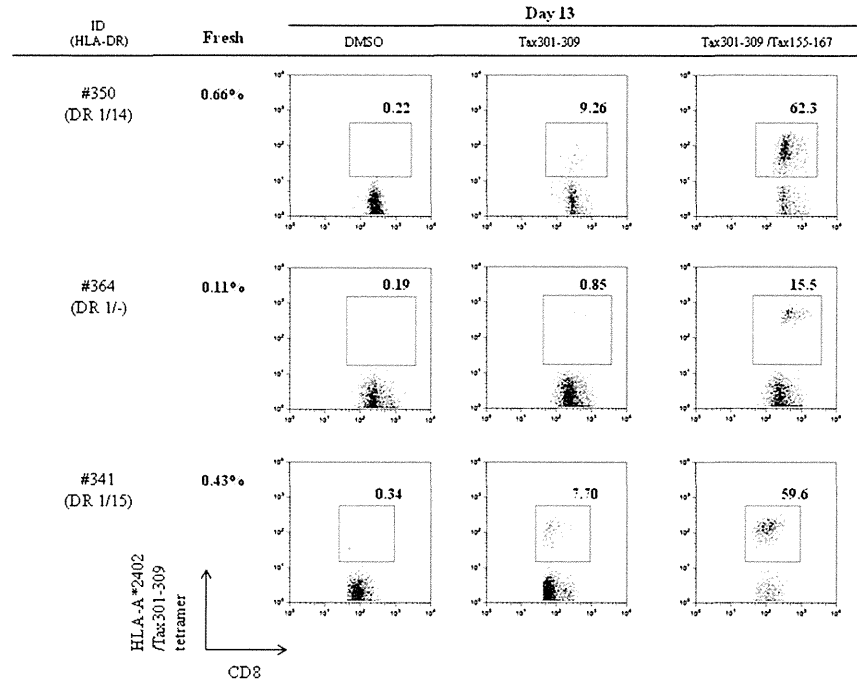

FIGURE 5. Augmentation of Tax-specific $CD8^+$ T cell expansion by costimulation with CTL epitope and Tax155–167 peptides. PBMCs from HLA-DRB1*0101– and HLA-A24–expressing ATL patients (#350, #364, and #341) who underwent allo-HSCT with RIC were cultured for 13 d in the presence of DMSO, 100 nM CTL epitope (Tax301–309), or a mixture of Tax301–309 (100 nM) and Tax155–167 (100 nM) peptides. Data indicate percentages of HLA-A*2402/Tax301–309 $tetramer^+$ cells among $CD3^+CD8^+$ T cells. Fresh indicates frequency of HLA-A*2402/Tax301–309 $tetramer^+CD8^+$ T cells detected in fresh peripheral blood.

in HTLV-1–infected individuals expressing HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells might efficiently induce HTLV-1–specific CTL expansion to strengthen the graft-versus-ATL effects in ATL patients after allo-HSCT.

In HTLV-1 infection, analysis of virus-specific $CD4^+$ T cell responses appears to be limited because $CD4^+$ T cells are preferentially infected with HTLV-1 (24, 34, 35), and HTLV-1 Ags are produced from infected cells at a few hours postculture (34, 36). In this study, we used blood samples from 18 ATL patients after allo-HSCT with RIC and from HLA identical–related or unrelated donors and found that these recipients had undetectable or very low proviral loads (Table I), as previously shown (7–9). We previously reported that Tax-specific CTLs were induced in some patients with complete remission after allo-HSCT for ATL and might contribute to the graft-versus-leukemia effect (10). In the current study, Tax-specific T cell responses or tetramer-binding $CD8^+$ T cells were detected in 68.8% (11 of 16) or 82.4% (14 of 17) of patients tested, respectively (Fig. 1A, Table I). In addition, helper function of Tax-specific $CD4^+$ T cells to enhance Tax-specific $CD8^+$ T cell expansion was observed in PBMCs from all three HLA-DRB1*0101$^+$ patients tested (Fig. 5). These data suggest that both $CD8^+$ and $CD4^+$ Tax-specific T cell responses might contribute to elimination of remaining leukemic and/or infected cells in some patients having T cell responses against Tax. However, given the fact that not all ATL patients who achieved complete remission after allo-HSCT had Tax-specific $CD8^+$ T cells, graft-versus-host reaction may mainly contribute to achieve complete remission after allo-HSCT. It is of note that Tax-specific

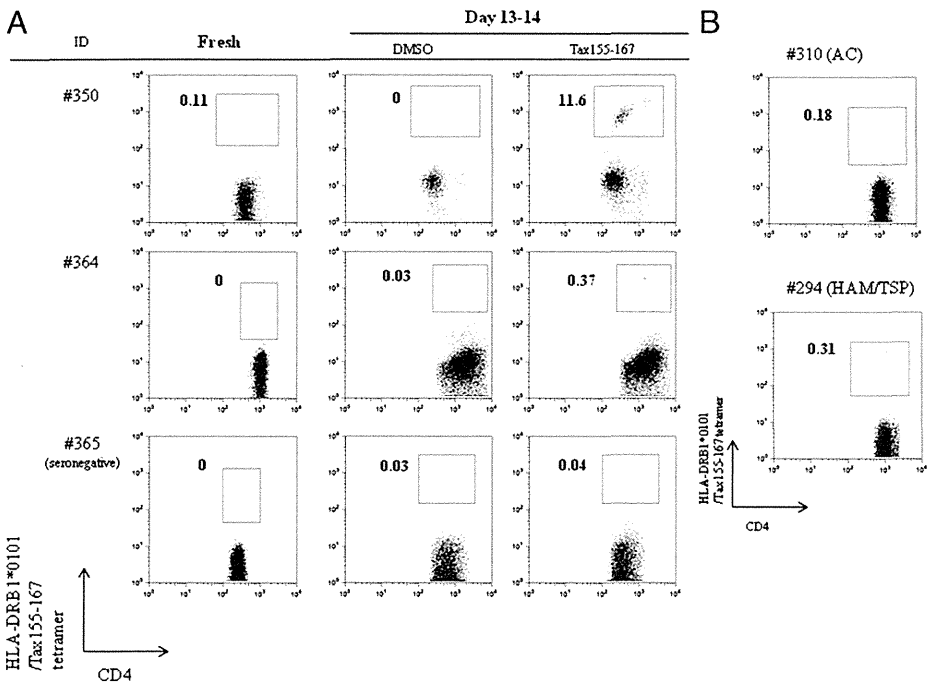

FIGURE 6. Detection of Tax155–167-specific $CD4^+$ T cells in HTLV-1–infected HLA-DRB1*0101$^+$ individuals. (**A**) In two ATL patients after allo-HSCT (#350 and #364) and an HLA-DRB1*0101$^+$–seronegative donor (#365), frequency of HLA-DRB1*0101/Tax155–167 tetramer-binding $CD4^+$ T cells was analyzed in fresh PBMCs and PBMCs cultured for 13–14 d in the presence of Tax155–167 (100 nM) peptide. Data indicate percentages of $tetramer^+$ cells in $CD3^+CD4^+$ T cells. (**B**) Frequency of HLA-DRB1*0101/Tax155–167 tetramer-binding $CD4^+$ T cells in fresh PBMCs from an AC #310 and an HAM/TSP patient #294 was analyzed. Data indicate percentages of $tetramer^+$ cells in $CD3^+CD4^+$ T cells.

T cell responses were detected in 57.1% (four of seven) or 87.5% (seven of eight) of the patients after allo-HSCT with RIC from HTLV-1–seronegative sibling or unrelated donors, respectively. A Tax-specific T cell response was not detected in three patients who underwent allo-HSCT from seropositive donors (Fig. 1, Table I).

It has been proposed that CTLs are the main effector cells against many pathogenic viruses, including HTLV-1. To date, many CTL epitopes recognized by HTLV-1–specific CTLs have been identified, some of which are thought to be the candidates of peptide-based T cell immunotherapy (10, 20, 32, 37–40). $CD4^+$ T cells have also been known to be critical for induction and maintenance of Ag-specific $CD8^+$ T cells (15–19). With respect to HTLV-1 infection, there are several reports identifying HLA-DRB1*0101–restricted epitopes recognized by $CD4^+$ T cells against Env or Tax (Env380–394 (21), Env436–450, Env451–465, Env456–470 (23), and Tax191–205 (22)), which were established by stimulating PBMCs from uninfected or infected individuals with synthetic peptides. In this study, for determination of an epitope recognized by HTLV-1–specific $CD4^+$ T cells, we established an HTLV-1–specific $CD4^+$ T cell line from the patient #350 at 180 d after allo-HSCT by several stimulations with an HTLV-1 Ags-expressing T cell line (ILT-#350) from the same patient. In addition, we found that Tax155–167-specific $CD4^+$ T cells were present in peripheral blood from patient #350 at 180 and 540 d after all-HSCT, indicating that the epitope, Tax155–167, identified in this study is naturally presented on HTLV-1–infected cells and predominantly recognized by HTLV-1–specific $CD4^+$ Th cells in the patient #350 at least within 540 d after allo-HSCT. Another HLA-DRB1*0101–restricted Tax epitope, Tax191–205, has been reported previously (22). In this study, the amino acid sequence within this region was revealed to be conserved in the infected T cell line, ILT-#350 established from the patient #350 (data not shown), indicating that Tax191–205 can be presented on APCs and Tax191–205-specific $CD4^+$ T cells may be induced in patient #350. However, Tax155–167-specific but not Tax191–205-specific $CD4^+$ T cells were revealed to predominantly appear in the HTLV-1–specific T4 cell line, established from PBMCs in the patient #350 at 180 d after allo-HSCT. This suggests that in the case of patient #350 at 180 d after allo-HSCT, Tax191–205-specific $CD4^+$ T cells may not be the most frequent population among HTLV-1–specific $CD4^+$ T cells.

It has been known that Ag-specific effector and memory $CD4^+$ T cells are typically present at much lower frequencies than their $CD8^+$ counterparts and that MHC class II tetramer might have a weak TCR–MHC affinity (41). Although this limited affinity of MHC class II tetramer might preclude detection of Ag-specific low-affinity $CD4^+$ T cells, the low-affinity $CD4^+$ T cells, below detection with MHC class II tetramers, were also proved to be critical effectors in Ag-specific responses (42). In the current study, MHC class II tetramer analysis revealed that Tax155–167-specific $CD4^+$ T cells were present in HLA-DRB1*0101$^+$ HTLV-1–infected individuals: two ATL patients after allo-HSCT (day 180 for #350 and day 360 for #364), an AC #310, and a HAM/TSP patient #294 (Fig. 6). Because of a shortage of blood sample from patient #341, we could not perform the direct detection for Tax155–167-specific $CD4^+$ T cells by the MHC class II tetramers. However, enhanced expansion of Tax301–309-specific $CD8^+$ T cells was observed in patient #341 at 360 d after allo-HSCT when PBMCs were stimulated with Tax301–309 in the presence of Tax155–167 (Fig. 5). So far, Tax155–167-specific $CD4^+$ T cells were detected in fresh and/or Tax155–167-stimulated PBMCs of all HTLV-1–infected HLA-DRB1*0101$^+$ individuals tested, although their frequencies were various. These results suggest that Tax155–167 may be the dominant epitope recognized by Tax-specific $CD4^+$ T cells in HTLV-1–infected HLA-DRB1*0101$^+$ individuals. In ATL patients after HSCT, the donor-derived T cells reconstituted in recipients will first encounter HTLV-1 Ags, because HTLV-1 still persists in the patients even though proviral loads become undetectable in the peripheral bloods. Indeed, we found that donor-derived Tax155–167-specific $CD4^+$ T cells were present in three ATL patients after allo-HSCT from seronegative donors. This finding also suggests that Tax155–167-specific naive $CD4^+$ T cells may pre-exist in HLA-DRB1*0101$^+$ individuals and can be primed with HTLV-1 Ags during the primary infection. In this study, Tax155–167-specific $CD4^+$ T cells were also detected in an AC and a HAM/TSP patient (Fig. 6B), suggesting that Tax155–167-specific $CD4^+$ T cells may be maintained in some HLA-DR1$^+$ individuals during the chronic phase of HTLV-1 infection. However, it has been reported that epitope hierarchies may change because of T cell escape mutants (43, 44) and unresponsiveness or deletion of epitope-specific T cells because of prolonged Ag stimulation during chronic infection (45, 46). Further longitudinal studies with a number of samples will be required to confirm that Tax155–167 is a dominant epitope of HTLV-1–specific $CD4^+$ T cells in HLA-DRB1*0101$^+$–infected individuals in the course of HTLV-1 infection.

Among three patients (#241, #350, and #364) showing high T cell responses against recombinant Tax protein, two patients (#350 and #364) were found to carry HLA-DRB1*0101 and have efficient $CD4^+$ Th cell responses against Tax155–167. Intriguingly, it has been reported that HLA-DRB1*0101 is associated with susceptibility to HAM/TSP (47, 48). In addition, $CD4^+$ T cells have been shown to be the dominant cells infiltrating in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory cytokines (30). These observations suggest that HLA-DRB1*0101 might be associated with susceptibility to HAM/TSP via an effect on high $CD4^+$ T cell activation. Further studies are needed to clarify whether HLA-DRB1*0101 is associated with high Tax-specific $CD4^+$ T cell responses in HTLV-1–infected individuals.

Early studies using lymphocytic choriomeningitis virus showed that $CD4^+$ T cell help is critical for maintenance of $CD8^+$ T cell function during chronic infections (18). It has also been suggested that $CD4^+$ T cells are required for optimal CTL responses during HTLV-1 infection (49). Aubert et al. (50) showed that both Ag-specific naive and effector $CD4^+$ T cell help rescued exhausted $CD8^+$ T cells in vivo, resulting in a decrease in viral burden. In the current study, we determined a novel HLA-DRB1*0101–restricted Th epitope, Tax155–167, which was capable of augmenting Tax-specific $CD8^+$ T cell expansion by stimulating Tax155–167-specific $CD4^+$ T cells. This epitope would be a useful tool for investigating the roles of HTLV-1–specific $CD4^+$ T cells in antitumor immunity and in pathogenesis of HTLV-1–related inflammatory diseases such as HAM/TSP and developing novel vaccines to prevent progression or recurrence of ATL.

Disclosures

The authors have no financial conflicts of interest.

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Interferon-α (IFN-α) suppresses HTLV-1 gene expression and cell cycling, while IFN-α combined with zidovudin induces p53 signaling and apoptosis in HTLV-1-infected cells

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Abstract

Background: Human T-cell leukemia virus type-1 (HTLV-1) is the causative retrovirus of adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 gene expression is maintained at low levels *in vivo* by unknown mechanisms. A combination therapy of interferon-α (IFN-α) and zidovudin (AZT) shows therapeutic effects in ATL patients, although its mechanism is also obscure. We previously found that viral gene expression in IL-2-dependent HTLV-1-infected T-cells (ILTs) derived from ATL patients was markedly suppressed by stromal cells through a type I IFN response. Here, we investigated the effects of IFN-α with or without AZT on viral gene expression and cell growth in ILTs.

Results: ILTs expressed variable but lower amounts of HTLV-1 Tax protein than HTLV-1-transformed HUT102 cells. Following the addition of IFN-α, the amounts of HTLV-1 p19 in the supernatants of these cells decreased in three days, while HTLV-1 gene expression decreased only in ILTs but not HUT102 cells. IFN-α also suppressed the spontaneous HTLV-1 induction in primary ATL cells cultured for 24 h. A time course study using ILTs revealed that the levels of intracellular Tax proteins decreased in the first 24 h after addition of IFN-α, before the reduction in HTLV-1 mRNA levels. The initial decreases of Tax protein following IFN-α treatment were observed in 6 of 7 ILT lines tested, although the reduction rates varied among ILT lines. An RNA-dependent protein kinase (PKR)-inhibitor reversed IFN-mediated suppression of Tax in ILTs. IFN-α also induced cell cycle arrest at the G0/G1 phase and suppressed NF-κB activities in these cells. AZT alone did not affect HTLV-1 gene expression, cell viability or NF-κB activities. AZT combined with IFN-α markedly induced cell apoptosis associated with phosphorylation of p53 and induction of p53-responsive genes in ILTs.

Conclusions: IFN-α suppressed HTLV-1 gene expression at least through a PKR-mediated mechanism, and also induced cell cycle arrest in ILTs. In combination with AZT, IFN-α further induced p53 signaling and cell apoptosis in these cells. These findings suggest that HTLV-1-infected cells at an IL-2-dependent stage retain susceptibility to type I IFN-mediated regulation of viral expression, and partly explain how AZT/IFN-α produces therapeutic effects in ATL.

Keywords: ATL, HTLV-1, IFN-α, PKR, Innate immunity, Anti-viral therapy, AZT, p53

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Background

Human T-cell leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma (ATL) [1-3], a malignant lympho-proliferative disorder resistant to chemotherapy. The virus is also responsible for HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4,5], a chronic inflammatory demyelinating disorder. Despite such severe clinical outcomes, levels of HTLV-1 gene expression are thought to be very low *in vivo*. HTLV-1 mRNA, but not proteins, are detectable in peripheral blood mononuclear cells (PBMCs) of HTLV-1-infected individuals [6]. Although undetectable, a low level of HTLV-1 proteins must be present *in vivo*, as HTLV-1-infected individuals maintain antibodies against HTLV-1 structural proteins and Tax protein-specific cytotoxic T lymphocytes.

Recent therapeutic approaches, such as allogeneic hematopoietic stem cell transplantation (allo-HSCT) [7,8], a humanized antibody therapy targeting CCR4 [9,10], or anti-viral therapy with interferon (IFN)-α and zidovudin (AZT) [11-13] partly improved ATL prognosis. *Ex vivo* studies have indicated that graft-versus-tumor responses including anti-Tax cytotoxic T-cells were potentially involved in the therapeutic mechanisms of allo-HSCT [14], and that the CCR4-antibodies were capable of inducing antibody-dependent cellular cytotoxicities [15]. However, combining AZT/IFN-α hardly affects HTLV-1-infected cells *in vitro* [16], and the mechanisms of its therapeutic effects remain unclear. A recent report indicated that the triple combination of arsenic trioxide/IFN-α/AZT demonstrated more favorable therapeutic effects in ATL patients [17]. The combination of arsenic trioxide and IFN-α has been reported to induce proteolysis of Tax in HTLV-1-infected cells *in vitro* [18,19]. As IFN-α is indispensable in AZT/IFN-α, arsenic trioxide/IFN-α or arsenic trioxide/IFN-α/AZT therapies, ATL cells might be susceptible to IFNs *in vivo*.

It is well established that HTLV-1-infected cells are resistant to type I IFNs *in vitro*. For example, IFN-α reduced the virus release but not viral protein synthesis in HTLV-1-transformed HUT102 or MT-2 cells [20]. The mechanisms of the resistance to type I IFNs in HTLV-1-infected cells include reduction in the phosphorylation of Tyk2 and STAT2 [21], Tax-mediated competition with CREB binding protein/p300 [22], Tax-mediated up-regulation of SOCS1 [23,24], and up-regulation of IRF4 [25], all of which result in inhibition of IFN signaling. This may explain why IFN-α combined with AZT does not affect HTLV-1-infected cells *in vitro*, while conflicting with the clinical effects of AZT/IFN-α therapy in ATL patients. This discrepancy between *in vivo* and *in vitro* systems can be partially attributed to differences in status of HTLV-1-infected cells between the two systems.

We previously found that HTLV-1-infected cells could induce type I IFN responses in co-cultured stromal cells [26]. We also found that viral expression in HTLV-1-infected T-cells is markedly suppressed at both mRNA and protein levels through type I IFN responses mediated by stromal cells co-cultured [26]. This observation again conflicts with the previous notion of HTLV-1-mediated resistance to type I IFNs *in vitro*. Our experimental system differed from previous studies in two ways. First, we used IL-2-dependent HTLV-1-infected T-cells (ILTs) derived from ATL patients, while previous studies used IL-2-independent HTLV-1-transformed cell lines such as HUT102. Second, we used stromal cells as effectors; these mediated the type I IFN response, but could have also produced multiple factors other than IFNs.

In the present study, we investigated whether purified type I-IFNs can affect viral expression and cell growth of HTLV-1-infected cells by using various ILTs. Here we report a novel finding that IFN-α suppresses intracellular Tax expression at a translational level at least through PKR. We further demonstrate that IFN-α activates p53 pathways in cooperation with AZT, partly explaining the mechanisms of the therapeutic effects of AZT/IFN-α in ATL.

Results

Effects of IFN-α on HTLV-1 p19 release and viral transcription

We evaluated the baseline levels of HTLV-1 gene expression in HUT102, ILT-Hod and ILT-#29 cell lines (Figure 1A). Relative levels of HTLV-1 mRNA in ILT-Hod and ILT-#29 cells were comparable with those in HUT102 cells. However, the levels of Tax protein in ILT-Hod and ILT-#29 cells were much lower than those of HUT102, and were barely detectable by immunoblotting only after stimulation of ILTs with phorbol 12-myristate 13-acetate (PMA). Flow cytometry results also indicated that ILT-Hod and ILT-#29 cells expressed smaller amounts of intracellular Tax protein than HUT102 cells. In addition, our analyses often identified Tax-negative cell populations in ILTs, with the ratio of these populations fluctuating during culture. These cells are also HTLV-1-infected, as all the cells in ILT-Hod and ILT-#29 cultures express HTLV-1 Gag protein after stimulation with PMA (Figure 1A insert), suggesting a dynamic turnover of HTLV-1 proteins in ILTs. Tax expression in HUT102 cells was apparently stable (Figure 1A).

We added IFN-α at various concentrations (300, 3000, and 30000 IU/ml) on HUT102, ILT-Hod, and ILT-#29 cells (Figure 1B). The amounts of HTLV-1 p19 released in supernatants significantly decreased after 72 h in culture for all the cell lines tested. Gag mRNA levels were also decreased in ILT-Hod and ILT-#29 in 3 days of culture (Figure 1B). These suppressive effects were observed at all doses of IFN-α used, indicating that 300 IU/ml of IFN-α was sufficient to produce these effects.

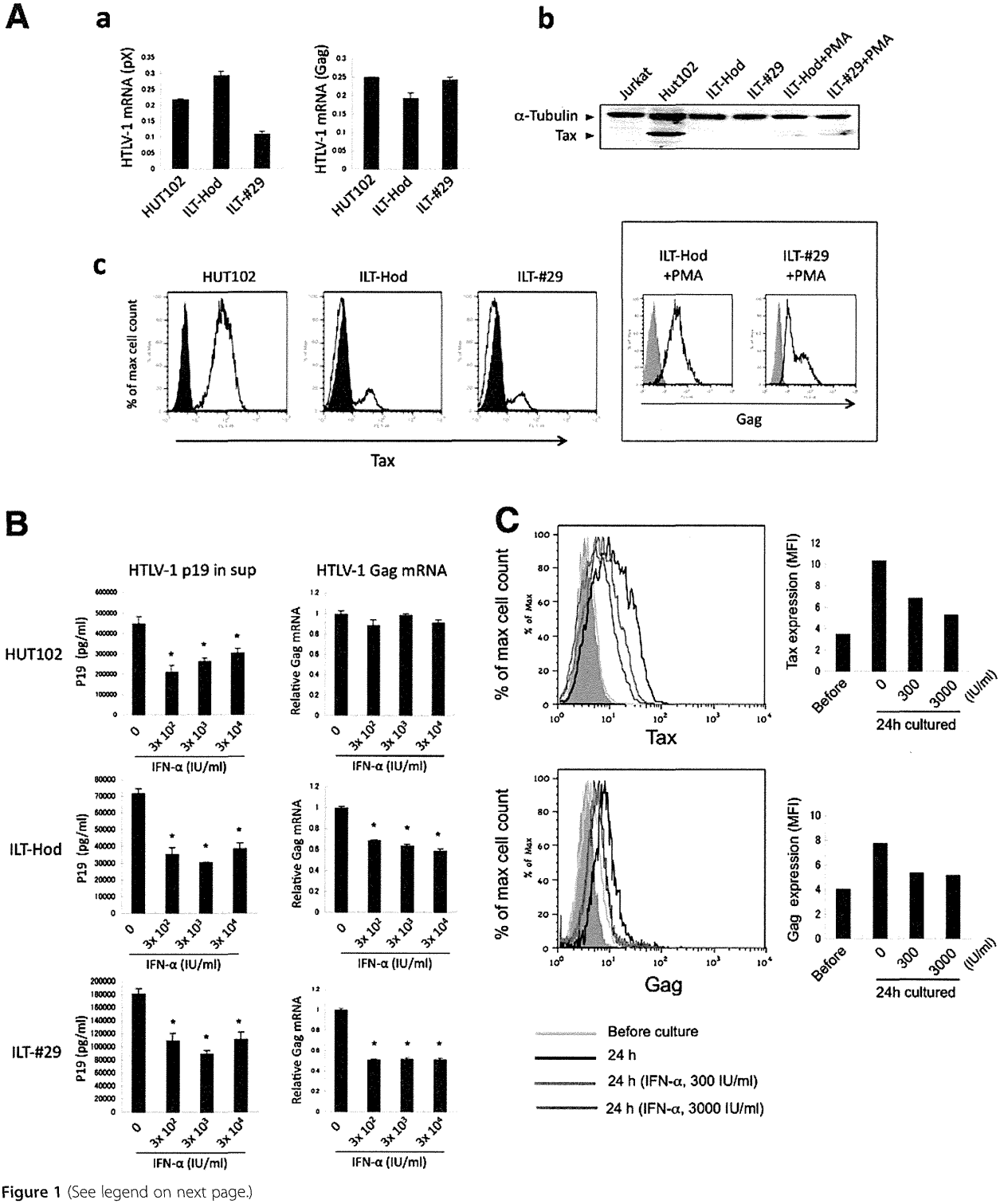

Figure 1 (See legend on next page.)

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Figure 1 Effects of IFN-α treatment on HTLV-1 p19 release and viral transcription in various HTLV-1-infected cell lines. A. Expression of HTLV-1 mRNAs (**a**) and proteins (**b**, **c**) were evaluated by quantitative RT-PCR (**a**), immunoblotting (**b**), and flow cytometry (**c**), respectively, in HTLV-1-infected HUT102, ILT-Hod and ILT-#29 or uninfected Jurkat cell lines. **a**. The mRNA copy numbers measured by using pX or Gag primers were standardized to those for GAPDH and indicated as the means and standard deviations (SD) of duplicate samples. **b**. Cell lysates from indicated cell lines were subjected to an immunoblotting assay with antibodies to Tax (40 kDa) and α-Tubulin (50 kDa). The lysates in lanes 5 and 6 were prepared from ILT-Hod and ILT-#29 cells stimulated with PMA (50 ng/ml) overnight, respectively. **c**. Intracellular Tax proteins in permeabilized cells were stained with Alexa Fluor 488-labeled anti-Tax mAb (open histogram) and mouse IgG3 isotype control antibody (closed histogram). The inserted box indicates Gag expression in ILT-Hod and ILT-#29 cells stimulated with PMA (50 ng/ml) for 17h. **B**. HUT102 (top), ILT-Hod (middle) and ILT-#29 (bottom) cells were cultured for 3 days with or without three doses of IFN-α indicated. HTLV-1 p19 concentrations in the supernatants (left) and Gag mRNA levels were measured by ELISA and quantitative RT-PCR, respectively. Data are presented as the means and SD of duplicate samples. **C**. Frozen stored primary ATL cells were thawed and analyzed for intracellular Tax (top) or Gag (bottom) proteins by flow cytometry immediately (green line) or 24 h after culture with no (black line), 300 IU/ml (red line) or 3000 IU/ml (blue line) of IFN-α in the presence of IL-2 (30 IU/ml). The closed histogram represents samples stained with isotype controls. The mean fluorescence intensity (MFI) of each histogram was indicated in the bar graphs.

In HUT102 cells, IFN-α suppressed HTLV-1 p19-release but not viral transcription, which is in agreement with previous reports [20].

We also examined the effects of IFN-α in primary ATL cells (Figure 1C). In the absence of IFN-α, intracellular expression of HTLV-1 proteins was spontaneously induced in ATL cells within 24 h after the initiation of culture. IFN-α suppressed the induction of Tax expression in these cells at a concentration of 3000 IU/ml more efficiently than 300 IU/ml. IFN-α also suppressed induction of Gag protein expression but equally at two doses.

Because HTLV-1 mRNA expression was suppressed in ILT-Hod and ILT-#29 cells as well as primary ATL cells following IFN-α treatment, we used these ILTs for further study on the effects of IFN-α at a dose of 3000 IU/ml hereafter.

IFN-α reduced Tax protein expression before reduction of pX mRNA

We next examined the time-course of IFN-α effects on Gag and Tax expression at protein and mRNA levels in ILT-Hod and ILT-#29 cells. Expression of intracellular Tax protein decreased within 1 day after addition of IFN-α to both cell lines. Intracellular Tax expression was maintained at lower levels than the control without IFN-α for at least 8 days (Figure 2A, top panels). Intracellular Gag protein expression in IFN-α-treated cells became lower than untreated cells at later time points (3–8 days), although the levels of viral expression fluctuated during culture (Figure 2A, bottom panels). Expression of HTLV-1 mRNAs in both cell lines were comparable to untreated cells or slightly increased in 1 day after IFN-α treatment, despite the reduction in Tax protein. At later time points (3–8 days), HTLV-1 mRNA levels were significantly decreased (Figure 2B). Thus, in IFN-α-treated ILTs, Tax protein was reduced first without apparent reduction in viral transcription, followed by reduction in viral mRNA and other viral protein expression.

We compared the levels of HTLV-1 proteins and mRNAs at 1 day after IFN-α treatment in these ILTs in several experiments, and confirmed that, at this time point, IFN-α reproducibly suppressed Tax protein levels in both cell lines, whereas the effects of IFN-α were inconsistent on Gag protein levels and not suppressive on HTLV-1 mRNA levels measured by using two different primer sets specific for pX and one for Gag regions (Figure 2C).

We further examined the effects of IFN-α on Tax protein and pX mRNA expression in several other ILT lines derived from ATL and HAM/TSP patients (Figure 2D). Although the suppression rates varied among cell lines, IFN-α suppressed intracellular Tax expression in 6 of 7 ILT cell lines tested in 24 h after IFN-α treatment. In ILT-#294 and HUT102 cells, Tax expression was not suppressed by IFN-α. HTLV-1 mRNA levels were not markedly suppressed or even enhanced in some cell lines in 24 h. Transient enhancement of HTLV-1 mRNA levels were sometimes observed also in ILT-Hod or ILT-#29 in 1 day after IFN-α treatment (Figure 2B, C). The effects of IFN-α on cell growth were limited, with mild reductions observed in some ILT lines after 3–4 days of culture (Figure 2E).

PKR was involved in IFN-α-mediated reduction of Tax protein expression

Since the reduction in intracellular Tax protein levels was induced by IFN-α at an earlier stage than for mRNA in ILT cells, we assumed that some post-transcriptional mechanisms such as PKR-induced translational suppression might be involved. We therefore treated ILT-Hod and ILT-#29 cells with IFN-α in the presence of a chemical PKR-inhibitor or its negative-control (Figure 3A). The otherwise decreased levels of Tax protein in both ILTs in the presence of IFN-α were markedly augmented by the PKR-inhibitor. In both ILTs, the negative-control inhibitor did not alter the Tax protein levels. Interestingly, the PKR-inhibitor increased Tax expression in the absence of IFN-α as well especially in ILT-#29 cells (Figure 3A). The enhancement of Tax expression by PKR-