

Therapeutic effects of Tax-targeted immunotherapy against full-blown ATL are still controversial. If HTLV-I-specific immunity following HSCT has any contribution to GVL effects in ATL, immunotherapy to reactivate Tax-specific CTLs may be worth attempting in post-HSCT patients with poor response, or even in ATL patients without HSCT, although its indication should be limited to cases in which ATL cells retain the ability to express HTLV-I Tax.

#### Five-year view

There are two issues remaining to be clarified which might affect the efficacy of immunotherapy against ATL: one is the nature of insufficient HTLV-I-specific CTL response in ATL patients and the other is the status of HTLV-I expression in ATL cells.

T-cell unresponsiveness to HTLV-I observed in orally infected rats suggests that primary oral infection in human babies is one of the causes, but probably not the only cause, of immune insufficiency in ATL. Although subcutaneous reimmunization of fixed syngeneic HTLV-I-infected cells was effective in breaking the immune tolerance in orally infected rats, caution is needed when translating such a finding to human

cases. Involvement of other suppressive mechanisms and the functions of antigen-presenting cells such as dendritic cells in ATL cases should be clarified.

The scarcity of HTLV-I expression in ATL cells has been raising controversy regarding the role of Tax in leukemogenesis and the rationale of Tax-mediated immune therapy of ATL. In our recent study, HTLV-I expression in ATL cells was irreversibly suppressed in around half of the ATL cases, while it was reversible in the other half [KURIHARA K, UNPUBLISHED DATA]. The reversible type of viral suppression commonly occurs in the PBMCs from asymptomatic HTLV-I carriers or HAM/TSP patients [18], where Tax-specific CTLs very likely contribute to antitumor surveillance. In this regard, Tax-specific CTLs might also potentially produce some antitumor effects in half the ATL cases retaining the ability to express viral antigens. It remains to be clarified where and when viral expression occurs *in vivo*.

The clinical efficacy of Tax-targeted immunotherapy in ATL will be indirectly predicted by further studies on the relationship between T-cell immunity and clinical outcome in post-HSCT ATL patients. A small-scale clinical trial of immunotherapy in ATL patients is also being planned.

#### Key issues

- Adult T-cell leukemia (ATL) cells are human T-cell leukemia virus Type I (HTLV-I)-infected T-cell clones that have survived and evolved *in vivo*.
- ATL occurs in approximately 5% of HTLV-I-infected individuals and is associated with mother-to-child infection (mainly via breastfeeding), the increase in infected cell number and insufficient HTLV-I-specific T-cell immunity.
- HTLV-I Tax, a crucial protein for cell growth and resistance to apoptosis in infected cells, is a major target of HTLV-I-specific cytotoxic T-lymphocytes (CTLs).
- HTLV-I Tax-specific CTLs contribute to controlling expansion of HTLV-I-infected cells at least in early HTLV-I leukemogenesis *in vivo*.
- Oral HTLV-I infection causes T-cell unresponsiveness to HTLV-I, allowing expansion of HTLV-I-infected cells in rats, suggesting that T-cell unresponsiveness to HTLV-I is a primary risk factor of ATL.
- T-cell unresponsiveness to HTLV-I by oral infection can be recovered by subcutaneous reimmunization with infected cells, suggesting that the risk of ATL can be reduced by vaccination.
- Tax-specific CTL response was strongly activated in some ATL patients who obtained complete remission following hematopoietic stem cell transplantation, supporting the notion that HTLV-I Tax might be one of the target antigens of graft-versus-leukemia effects in these patients.
- Tax-targeted immunotherapy might be worth consideration in ATL cases in which ATL cells retain the ability to express Tax.

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**Affiliations**

- Mari Kannagi  
Department of Immunotherapeutics,  
Tokyo Medical and Dental University,  
1-5-45 Yushima, Bunkyo-ku,  
Tokyo 113-8519, Japan  
Tel.: +81 358 035 798  
Fax: +81 358 030 235  
kann.impt@tmd.ac.jp
- Nanae Harashima  
Department of Immunotherapeutics,  
Tokyo Medical and Dental University,  
Tokyo, Japan
- Kiyoshi Kurihara  
Department of Immunotherapeutics,  
Tokyo Medical and Dental University,  
Tokyo, Japan
- Atee Utsunomiya  
Department of Hematology,  
Imamuwa Bun-in Hospital, Kagoshima, Japan
- Ryuji Tanosaki  
National Cancer Center Hospital, Tokyo, Japan
- Masato Masuda  
Second Department of Internal Medicine,  
University of the Ryukyus, Okinawa, Japan

## Potential immunogenicity of adult T cell leukemia cells *in vivo*

Kiyoshi Kurihara<sup>1</sup>, Nanae Harashima<sup>1</sup>, Shino Hanabuchi<sup>1</sup>, Masato Masuda<sup>2</sup>, Atee Utsunomiya<sup>3</sup>, Ryuji Tanosaki<sup>4</sup>, Masao Tomonaga<sup>5</sup>, Takashi Ohashi<sup>1</sup>, Atsuhiko Hasegawa<sup>1</sup>, Takao Masuda<sup>1</sup>, Jun Okamura<sup>6</sup>, Yuetsu Tanaka<sup>7</sup> and Mari Kannagi<sup>1\*</sup>

<sup>1</sup>Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

<sup>2</sup>Second Department of Internal Medicine, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

<sup>3</sup>Department of Hematology, Imamura Bun-in Hospital, Kagoshima, Japan

<sup>4</sup>National Cancer Center Hospital, Tokyo, Japan

<sup>5</sup>Department of Hematology, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan

<sup>6</sup>National Kyushu Cancer Center, Fukuoka, Japan

<sup>7</sup>Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan

Experimental vaccines targeting human T cell leukemia virus type-I (HTLV-I) Tax have been demonstrated in a rat model of HTLV-I-induced lymphomas. However, the scarcity of HTLV-I-expression and the presence of defective HTLV-I-proviruses in adult T cell leukemia (ATL) cells have raised controversy about the therapeutic potential of HTLV-I-targeted immunotherapy in humans. We investigated the expression of HTLV-I antigens in fresh ATL cells by using both *in vitro* and *in vivo* assays. In flow cytometric analysis, we found that 3 of 5 acute-type and six of fifteen chronic-type ATL patients tested showed significant induction of HTLV-I Tax and Gag in their ATL cells in a 1-day culture. Concomitantly with HTLV-I-expression, these ATL cells expressed co-stimulatory molecules such as CD80, CD86 and OX40, and showed elevated levels of antigenicity against allogeneic T cells and HTLV-I Tax-specific cytotoxic T-lymphocytes (CTL). Representative CTL epitopes restricted by HLA-A2 or A24 were conserved in 4 of 5 acute-type ATL patients tested. Furthermore, spleen T cells from rats, which had been subcutaneously inoculated with formalin-fixed uncultured ATL cells, exhibited a strong interferon gamma-producing helper T cell responses specific for HTLV-I Tax-expressing cells. Our study indicated that ATL cells from about half the patients tested readily express HTLV-I antigens including Tax *in vitro*, and that ATL cells express sufficient amounts of Tax or Tax-induced antigens to evoke specific T cell responses *in vivo*.

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**Key words:** cancer vaccine; human T cell leukemia virus type-I (HTLV-I); viral expression; co-stimulatory molecules; T cell immune response

Human T cell leukemia virus type-I (HTLV-I) is etiologically linked to adult T cell leukemia (ATL).<sup>1–3</sup> It is estimated that about 1 million people are infected with HTLV-I in Japan and 1–5% of infected subjects develop ATL.<sup>4,5</sup> Most other HTLV-I-carriers are asymptomatic throughout their lives and another small fraction of HTLV-I-carriers develop a chronic progressive neurological disorder termed HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP)<sup>6,7</sup> and other inflammatory disorders. Once patients develop acute-type ATL, leukemic cells resist anti-tumor chemotherapy, and the median survival time is 6.2 months.<sup>8</sup> Allogeneic hematopoietic stem cell transplantation (HSCT) has been applied recently in acute ATL patients and successful efficacy was obtained in some cases.<sup>9,10</sup> These effects may be attributed to a graft vs. leukemia reaction mediated by the donor-derived T cell immunity. There is also, however, a risk of graft vs. host reaction and its undesirable side effects are sometimes lethal. On this account, further improvement or new approaches are required for ATL treatment.

The precise mechanisms of HTLV-I-related diseases are not fully understood. HTLV-I viral protein Tax transactivates and interacts with many cellular proteins that regulate or dysregulate cell growth,<sup>11</sup> partly accounting for the mechanisms of HTLV-I-induced leukemogenesis.

In a rat model of HTLV-I-infected T cell lymphomas, uncontrollable expansion of tumor cells was highly associated with a

functional defect or suppression of HTLV-I-specific T cell immunity including cytotoxic T lymphocytes (CTL).<sup>12,13</sup> Vaccination with autologous HTLV-I-infected cells,<sup>12</sup> Tax-encoding DNA,<sup>14</sup> or oligopeptides corresponding to a CTL-epitope<sup>15</sup> elicited anti-tumor effects in this model. HTLV-I Tax serves as an immunodominant target antigen for HTLV-I-specific CTL not only in rats but also in humans.<sup>16,17</sup> HTLV-I-specific CTL have been detected in the peripheral blood of HTLV-I-infected individuals<sup>18</sup> and can be induced from healthy carriers and HAM/TSP.<sup>16,19,20</sup> HTLV-I-specific CTL, however, is induced infrequently from ATL patients.<sup>21,22</sup> Moreover, Tax-specific CTL are capable of killing short-term cultured ATL cells.<sup>22,23</sup> These observations indicated that immunotherapy directed against Tax might be effective for ATL.

It is controversial, however, whether HTLV-I-specific immunotherapy has any therapeutic advantages for ATL patients with advanced disease because of the scarcity of HTLV-I-expression in ATL cells. ATL cells sometimes contain mutations and deletions in HTLV-I proviral genome,<sup>24,25</sup> and the ATL cells may not be able to express Tax. It is also known that viral expression in freshly isolated peripheral ATL cells is transiently suppressed.<sup>26–28</sup>

The reasons for insufficient HTLV-I-specific T cell response in ATL patients are also unclear. We found recently that a strong Tax-specific CTL response was induced in ATL patients after HSCT from HLA-identical donors,<sup>29</sup> indicating that the immune insufficiency in these patients before transplantation was not HLA-related. Pique *et al.*<sup>30</sup> reported that HTLV-I-specific CTL do exist in ATL patients but insufficiently expand. This suggests involvement of some immune suppression or tolerance. Alternatively, the levels of viral expression in ATL cells may be too low to evoke T cell immunity *in vivo*.

Because these cells may be a vaccine candidate, we investigated HTLV-I-expression of fresh ATL cells from 5 acute-type

**Abbreviations:** ATL, adult T cell leukemia; CTL, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; HTLV-I, human T cell leukemia virus type-I; IFN- $\gamma$ , interferon-gamma; IL, interleukin; LTR, long terminal repeat; mAb, monoclonal antibody; MHC-II, Class II major histocompatibility complex; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PHA, phytohemagglutinin; SSC, side scatter.

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\*Correspondence to: Department of Immunotherapeutics, Tokyo Medical and Dental University, Medical Research Division, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81-3-5803-0235.

E-mail: kann.impt@tmd.ac.jp

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and 15 chronic-type ATL patients to determine whether ATL cells themselves can be immunogenic and evoke HTLV-I-specific T cell response. We demonstrated that in nearly 50% of the ATL patients tested HTLV-I Tax was inducible after short-term culture. Nucleotide sequences of HTLV-I *tax* at representative CTL epitopes in these ATL cells were mostly conserved. Interestingly, rats inoculated with formalin-treated uncultured ATL cells successfully developed helper T cell responses specific for Tax-expressing cells *in vivo*, indicating that ATL cells may express a small but sufficient amount of HTLV-I antigens for T cell response *in vivo*. Our findings suggest that ATL cases may be divided into 2 groups depending on the ability to express HTLV-I antigens and in nearly 50% the cases of ATL patients, ATL cells may potentially be recognized by HTLV-I-specific T cells *in vivo*.

## Material and methods

### Patients and PBMC preparation

Heparinized peripheral blood samples were donated under informed consent from 19 patients diagnosed as acute-type or chronic-type ATL at Ryukyu University Hospital, Imamura Bun-in Hospital in Kagoshima, and Nagasaki University Hospital and from uninfected healthy volunteers. The clinical status of these patients is summarized in Table I. The diagnosis and clinical subtype of ATL were made according to Shimoyama's criteria.<sup>8</sup> The ATL patients did not receive any chemotherapy when tested. Two samples from Patient 1 were used in our study. The first sample (1-i) was taken at a chronic phase and the other (1-vi) was taken at the acute crisis after an 18-month interval. Otherwise, one sample per patient was used. PBMC were isolated by using Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density centrifugation and cryopreserved in liquid nitrogen until use.

### Animals

Inbred female F344/N Jcl-rnu/+ rats (F344 n/+; 4-week-old) were purchased from Clea Japan, Inc. (Tokyo, Japan). Rats were treated under the experimental protocol of the Animal Care Committee of our university.

### Cell lines

HTLV-I-negative human T cell line Molt-4<sup>31</sup> and HTLV-I-producing human T cell line MT-2<sup>32</sup> were maintained in 10% heat-inactivated FBS (Sigma, St. Louis, MO), 100 U/mL of pen-

icillin, 100 µg/mL of streptomycin in RPMI 1640 medium (Sigma) (10% FBS-RPMI).

ILT-Hod,<sup>33</sup> an IL-2-dependent HTLV-I-infected human T cell line was maintained in the presence of 10 U/mL of recombinant human IL-2 (rhIL-2; Shionogi Co., Osaka, Japan) in 10% FBS-RPMI. In addition, 2 other IL-15-dependent HTLV-I-infected human T cell lines, ILT-79 and ILT-85, were established from ATL Patients 79 and 85, respectively. To establish these lines, a CD4-positive cell-enriched fraction negatively separated from PBMC by using Dynabeads M-450 CD8 (DynaL, Oslo, Norway) and Dynabeads M-450 CD19 (DynaL) was stimulated with 1 µg/mL of phytohemagglutinin (PHA-p; Difco Laboratories, Detroit, MI) for 24 hr, washed and cultured in 10% FBS-RPMI containing 10 ng/mL of rhIL-15 (Sigma) for 1–3 months.

HLA-A24-restricted HTLV-I Tax-specific CD8<sup>+</sup> CTL line was induced from PHA-p-stimulated PBMC of a post-HSCT ATL patient by repeated stimulation with formalin-fixed autologous HTLV-I-infected cells established before the HSCT.<sup>29</sup> The CTL line was maintained in the presence of 100 U/mL of rhIL-2 with periodical stimulation with formalin-fixed autologous HTLV-I-infected cells at 10–14 day intervals.

HTLV-I-infected rat T cell line, FPM1,<sup>12</sup> derived from an F344 n/+ rat, were cultured in 10% FBS-RPMI. G14<sup>14</sup> is IL-2-dependent HTLV-I negative CD8<sup>+</sup> T cell line established from a F344 n/+ rat. G14-Tax<sup>14</sup> is a stable transfectant of G14 with HTLV-I Tax-expressing plasmids. G14 and G14-Tax were maintained in 10% FBS-RPMI containing  $5.5 \times 10^{-5}$  M of 2-mercaptoethanol and 10 U/mL of rhIL-2.

### Monoclonal antibodies

To detect intracellular HTLV-I antigens, mouse monoclonal antibodies (mAbs), Lt-4 (anti-p40 Tax, mouse IgG3),<sup>34</sup> NOR-1 (anti-p24 and p53 Gag; mouse IgG1),<sup>35</sup> GIN-7 (anti-p19, p28 and p53 Gag; mouse IgG2b)<sup>35</sup> and biotinylated GIN-7 were used.

For cell surface characterization, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse anti-human CD4, CD8, CD25, CD40, CD40L, CD86, OX40, HLA-A, B, C, HLA-DR (IgG1; BD Pharmingen Co., San Diego, CA), CD80 (IgG1; Immunotech, Marseille, France) and OX40L (TAG-34, IgG1)<sup>36</sup> mAbs were used. In addition, FITC-conjugated mouse anti-rat CD4 and PE-conjugated mouse anti-rat CD8 mAbs (IgG1; BD Pharmingen Co.) were used.

TABLE I—CLINICAL STATUS OF ATL PATIENTS TESTED

Patient ID	Age	Gender	Type of ATL	WBC number/ µL	Mononuclear cells/ WBC (%)	Abnormal lymphocytes/ WBC (%)
#1-vi <sup>1</sup>	60	F	Acute	42,000	63	49
#22	38	M	Acute	16,100	50	35
#80	39	M	Acute	141,000	>95	91
#85	77	M	Acute	67,400	74	67
#91	66	F	Acute	27,200	89	61
#1-i <sup>1</sup>	58	F	Chronic	21,500	87	55
#5	72	M	Chronic	12,200	64	25
#6	70	F	Chronic	14,400	71	33
#7	62	F	Chronic	8,300	69	34
#8	60	F	Chronic	17,400	70	47
#20	64	M	Chronic	14,700	48	28
#23	68	M	Chronic	14,200	74	58
#29	58	M	Chronic	6,000	59	21
#42	54	F	Chronic	8,800	48	15
#54	63	F	Chronic	10,700	73	43
#69	54	F	Chronic	13,300	68	36
#79	63	F	Chronic	19,500	64	6
#89	50	F	Chronic	28,000	41	27
#90	53	M	Chronic	7,700	30	8
#92	66	F	Chronic	16,900	83	76

<sup>1</sup>Pt. #1-i and Pt. #1-vi are the identical individual with an initial diagnosis of chronic ATL, whose diagnosis changed to acute ATL associated with elevated levels of serum LDH after 18 months.

#### Intracellular and surface staining and flow cytometric analysis

For intracellular HTLV-I-staining, cells were fixed with 1% paraformaldehyde in PBS containing 20  $\mu\text{g}/\text{mL}$  of lysolecithin (Sigma) for 2 min at room temperature. The cells were then centrifuged and resuspended in cold methanol. After incubation for 15 min at 4°C, the cells were centrifuged and incubated in 0.1% Triton-X in PBS for 5 min at 4°C. The cells were then washed with PBS containing 1% FBS and 0.1%  $\text{NaN}_3$  (staining buffer), and incubated with mouse mAbs to HTLV-I antigens or BALB/c control ascites, and subsequently with FITC-conjugated goat anti-mouse IgG + IgM mAbs (Immunotech) for 30 min at room temperature. The optimal concentrations of these mAbs were determined before use. Cells were washed twice, fixed with 1% formalin in PBS and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA). Live cells were gated based on a pattern of SSC and FSC for approximately  $1 \times 10^4$  cells.

An alternative permeabilizing method using saponin was also employed for intracellular staining. Briefly, cells were fixed with 4% formalin in PBS, then permeabilized with 0.5% saponin (Sigma) in staining buffer for 10 min at room temperature. Permeabilized cells were further incubated with mAbs to HTLV-I antigens as described above.

For surface staining, cells washed and stained with FITC- or PE-conjugated mAbs and appropriate isotype control mAbs. Cells were further stained with 7-ADD (BD Pharmingen Co.) and stained cells were gated out on FACS analysis to eliminate dead cells.

For two-color analysis of intracellular and cell surface antigens, cells were stained with FITC-conjugated mouse anti-human mAbs (CD80, CD86, OX40), fixed and permeabilized by saponin treatment. Permeabilized cells were further stained with biotinylated GIN-7, and subsequently with Cy-chrome streptavidin (BD Pharmingen Co.). After extensive washing, the cells were subjected to two-color flow cytometry.

#### Long PCR and nucleotide sequences

Genomic DNA was prepared from PBMC by sodium dodecyl sulfate-proteinase K digestion, followed by phenol-chloroform extraction and subjected to long PCR (Expand Long Template PCR system, Boehringer Mannheim, Mannheim, Germany) to detect deletion of HTLV-I provirus. The primers of HTLV-I long terminal repeat (LTR) were 5'-LTR (5'-GTCCACCCCTT-TCCCTTTCATTCACGACTGACTGC-3') and 3'-LTR (5'-GGC-TCTAAGCCCCGGGGGAT-3') as described before.<sup>37</sup> Each 500 ng of genomic DNA was subjected to 10 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min), and additional 20 cycles of denaturation (94°C, 10 sec), annealing (65°C, 30 sec) and extension (68°C, 8 min + 20 sec/cycle), then finalized by elongation of the product (68°C, 7 min). The PCR products were visualized by ethidium bromide staining after 0.6% agarose gel electrophoresis. The expected size of the amplified fragments with these LTR primers from a full-length HTLV-I provirus was 7.7 kbp. Long PCR products were partially sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) by using the Big Dye terminator and the primers pX4 (5'-GGGGAAGGAGGGGAGTCG-AGGGATAAGGAA-3') or pX12 (5'-TTGCCACCCACCCCTT-TTCCAGC-3') in accordance with the manufacturer's instructions. Amino acid sequences at CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, were then determined according to the nucleotide sequences.

#### Lymphocyte proliferation assay

For the mixed lymphocyte reaction (MLR), cryopreserved PBMC ( $2 \times 10^5$ /well) from a healthy volunteer were co-cultured with formalin-fixed ATL cells with or without preculture *in vitro* ( $5 \times 10^4$ /well) in 96-well U-bottom plates in triplicate at 37°C for 4 days. Cultures were pulsed with 37 kBq/well of [<sup>3</sup>H]-thymidine

([<sup>3</sup>H]-TdR) for an additional 16 hr to assess cell proliferation. Cells were harvested with a Micro 96 Harvester (Skatron, Lier, Norway) and [<sup>3</sup>H]-TdR uptake into cells was measured in a microplate  $\beta$  counter (Micro Beta Plus; Wallac, Turku, Finland). Proliferation of HTLV-I-specific CTL ( $1 \times 10^5$ /well) co-cultured with formalin-fixed ATL cells ( $5 \times 10^4$ /well) for 2 days, and proliferation of rat spleen T cells ( $1 \times 10^5$ /well) co-cultured with formalin-fixed various syngeneic rat cells ( $1 \times 10^5$ /well) for 3 days were also similarly measured.

#### ELISA

Human and rat interferon-gamma (IFN- $\gamma$ ) production in 100  $\mu\text{L}$  of culture supernatants was measured by Human IFN- $\gamma$  ELISA kit (Endogen, Woburn, MA) and Rat IFN- $\gamma$  ELISA kit (BioSource Inc., Camarillo, CA), respectively. Absorbances were measured at 450 nm using microplate reader (BioRad, Hercules, CA) and analyzed with Microplate Manager III software.

#### Inoculation of ATL cells in rats

Ten million formalin-fixed PBMC from ATL patients or uninfected healthy volunteers, with or without pre-culture *in vitro*, were subcutaneously administered to 4-week-old female F344n/+ rats twice with a 2-week interval. The rats were sacrificed at 1 month after second immunization. Spleen T cells from these rats were enriched through a nylon-wool column, and their IFN- $\gamma$  production and proliferation against formalin-fixed syngeneic G14, G14-Tax or FPM1 cells were examined by IFN- $\gamma$  ELISA and a [<sup>3</sup>H]-TdR uptake assay, respectively, as described elsewhere.<sup>38</sup>

#### DNA-vaccination to rats

Plasmids containing wild-type *tax* cDNA controlled under the human  $\beta$ -actin promoter (p $\beta$ MT-2 Tax) and its control plasmid p $\beta$ APR.1-neo vector<sup>39</sup> were coated on Au particles and inoculated into rats by using Gene Gun as described previously.<sup>14</sup> Immunization was carried out 3 times with a 1-week interval. One week after final immunization, rats were sacrificed and spleen cells were collected.

#### Cytotoxicity assay

Spleen cells ( $5 \times 10^6$  cells) from immunized rats were used as effector cells after 7 days of co-culture with formalin-fixed G14-Tax ( $2 \times 10^6$  cells) in 24-well plate. Target cells (G14 or G14-Tax) were incubated with 370 kBq of [<sup>3</sup>H]-TdR per  $10^6$  cells for 12 hours at 37°C, followed by extensive washing. These target cells ( $1 \times 10^4$ /well) and effector cells ( $1 \times 10^5$ /well) were plated in 96-well U-bottom plates at the effector/target ratio of 10. After 6 hr of incubation at 37°C, cells were harvested to glass filters and radioactivities remaining in the target cells were measured in a microplate  $\beta$  counter. The percentage of specific cell lysis was calculated as [(cpm without effector - cpm with effector)/cpm without effector]  $\times$  100.

#### Statistical analysis

Results are expressed as the mean  $\pm$  SD. Differences between the 2 groups were analyzed for significance by Student's *t*-test. Differences among 3 groups were evaluated by Dunnett's *t*-test using SPSS Base 11.0J (SPSS Inc., Chicago, IL); *p*-values <0.05 were considered to be statistically significant.

## Results

#### Detection of intracellular HTLV-I antigens in cell lines

Initially, to detect intracellular HTLV-I antigens by flow cytometry, the conditions of cell permeabilization and staining methods were determined using established cell lines. We used lysolecithin-paraformaldehyde, methanol and Triton-X to fix and permeabilize the cell membranes, and stained the cells with mAbs to HTLV-I p40 Tax (Lt-4), p24 Gag (NOR-1), and p19 and 28 Gag (GIN-7). The staining patterns under the optimal conditions are shown in Figure 1. HTLV-I-producing human T cell line MT-2, but not HTLV-I-negative Molt-4 cell line, was strongly stained with all of

these mAbs (Fig. 1a,b). Under the same conditions, an IL-2-dependent ILT-Hod cell line established previously from an ATL patient, exhibited 2 peaks, consisting of a large population weakly expressing HTLV-I antigens and a small population expressing substantial levels of the HTLV-I antigens (Fig. 1c). The levels of intracellular HTLV-I antigens, especially p40 Tax, fluctuated and were influenced by the culture conditions (data not shown). We

also used saponin to permeabilize the cell membranes and compared the staining efficiency for detecting intracellular antigens with the methods using Triton-X. As shown in Figure 1d, saponin-treated ILT-Hod cells could also be stained with mAbs to HTLV-I but the detection levels were significantly lower than those in Triton-X-treated ILT-Hod cells. Thereafter, the permeabilization method using Triton-X was used primarily.

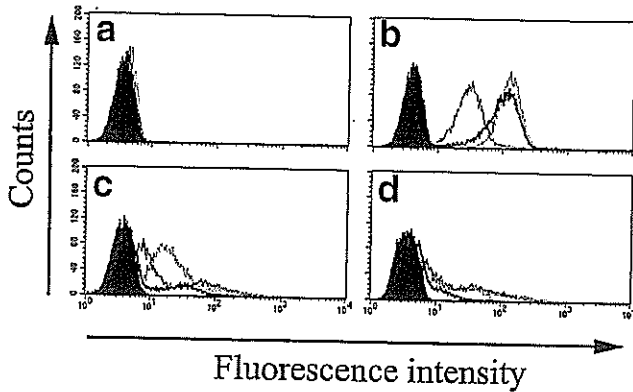


FIGURE 1 - Detection of intracellular HTLV-I antigens in cell lines. Molt-4 (a), MT-2 (b), and ILT-Hod (c,d) cells were permeabilized with Triton-X (a-c) or saponin (d) treatment (see Material and Methods), stained with control ascites (closed histogram), anti-p40 Tax mAb (Lt-4, solid lines), anti-p24 Gag mAb (NOR-1, thin lines) or anti-p19 and p28 Gag mAb (GIN-7, dashed lines) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The cells were fixed with 1% formalin-PBS and analyzed using a flow cytometer.

Induction of HTLV-I antigens in PBMC from ATL patients

The clinical status of the ATL patients tested is summarized in Table I. Expression of HTLV-I antigens in ATL cells from 5 acute and 15 chronic ATL patients who had not received chemotherapy were investigated. Cryopreserved PBMC from ATL patients were permeabilized, and stained with mAbs against HTLV-I antigens, Lt-4, NOR-1 and GIN-7 immediately (Day 0) or after *in vitro* 1-day cultivation. When viral expression was detected in 1 day, cells were kept in culture for 3-9 days if available. Representative data of intracellular HTLV-I-expression in the PBMC from an acute ATL patient (Patient 85) is shown in Figure 2. Although HTLV-I antigens were not detectable in the PBMC of the ATL patients before culture, a large number of live cells strongly expressed HTLV-I antigens in a 1-day incubation. The HTLV-I-positive cell number increased with further incubation, whereas the live cell number decreased (data not shown). The intensity of p40 Tax in the positive population nearly reached the maximal level in 1 day, whereas the intensity of p24 or p19 and p28 Gag antigens was further enhanced in 3 days of incubation. Similar induction was observed in the PBMC of 3 (Patients 22, 85, 91) of 5 acute-type ATL patients tested, although the proportion of HTLV-I-

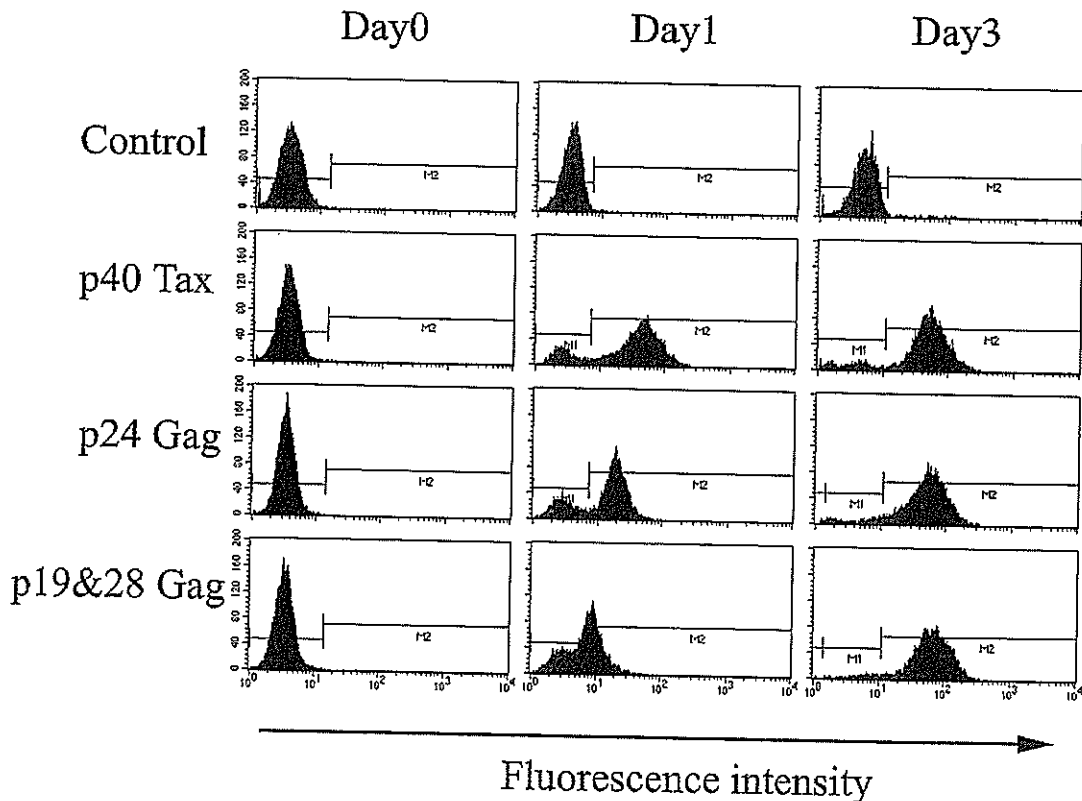


FIGURE 2 - Induction of HTLV-I antigens in PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were incubated for the indicated periods in 10% FBS-RPMI, and permeabilized with Triton-X for intracellular staining with control ascites, Lt-4 (p40 Tax), NOR-1 (p24 Gag) or GIN-7 (p19 & p28 Gag) and subsequently with FITC-conjugated anti-mouse IgG + IgM. The M2 region shown in each histogram is regarded as positive.

expressing cells differed among individuals (Table II). In Patient 80 (acute ATL), only a small percentage of the cells expressed HTLV-I antigens after 9 days of incubation that probably arose from a minor population of the PBMC. The PBMC of Patient 1-vi who converted to acute-type ATL from chronic-type ATL (Patient 1-i) did not show any detectable levels of HTLV-I expression during 1 day of incubation.

In the chronic ATL patients, the results were more variable. In the PBMC of 3 (Patients 7, 79, 90) of 15 chronic ATL patients tested, more than 30% (range = 35.4–66.5%) of live cells expressed detectable levels of HTLV-I antigens in 1 day of incubation. In 3 chronic ATL patients (Patients 42, 54, 69), HTLV-I antigens were also induced but at a lower proportion (range = 6.8–11.3%) of the PBMC. No detectable levels of HTLV-I expression were observed in the PBMC from the rest of the chronic ATL patients tested.

HTLV-I-induction was observed 3 of 5 acute-type and 6 of 15 chronic-type ATL patients, when HTLV-I-expression in >5% of the 1-day cultured PBMC was regarded as positive.

*Conservation of representative CTL epitopes in ATL cells*

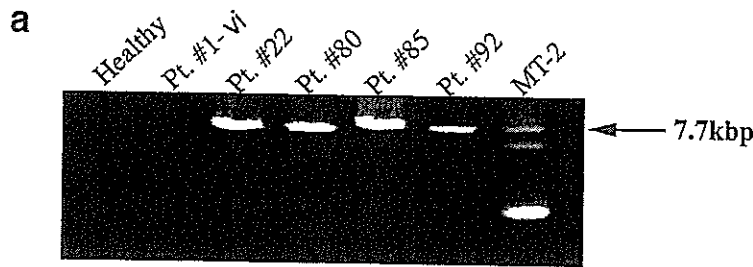
Because HTLV-I Tax is a major target antigen of HTLV-I-specific CTL,<sup>16,17</sup> we investigated whether ATL cells possessed mutations at the CTL epitopes in Tax. At first, the HTLV-I proviral genome integrated in the PBMC from 5 acute-type ATL patients was amplified by a long PCR method using LTR primers. As shown in Figure 3a, in 4 of 5 samples tested, comparable sizes of DNA fragments with a full-length provirus were amplified. No PCR product was obtained from the remaining case (Patient 1-vi). The DNA fragments amplified from the 3 patients were then examined for their nucleotide sequences at the regions corresponding to Tax 11–19 and Tax 301–309, representative CTL epitopes restricted by HLA-A2 and A24, respectively (Fig. 3b). Of the 4 acute-type ATL patients tested, 2 had HLA-A2, and all 4 had HLA-A24. The nucleotide sequences at Tax 11–19 were conserved in all patients regardless of the presence of HLA-A2. The sample from Patient 91 had a single mutation resulting in the substitution of serine (S) to asparagine (N) at the position 304. In the other 3 patients, nucleotide sequences at the Tax 301–309 region were identical to the prototype HTLV-I.<sup>40</sup>

TABLE II—INDUCTION OF HTLV-I ANTIGENS IN PBMC OF ATL PATIENTS FOLLOWING *IN VITRO* CULTIVATION

Patient	Type of ATL	Culture period (days) <sup>1</sup>	Percentage of positive cells for			
			Control antibody	Tax (L1-4)	p24 (NOR-1)	p19&p28 (GIN-7)
#1-vi	Acute	0	0.1	0.0	0.0	0.1
		1	0.0	0.1	0.1	0.0
#22	Acute	0	0.0	0.0	0.0	0.0
		1	0.0	15.0	17.8	10.2
		3	0.0	26.4	56.4	38.1
#80	Acute	0	0.0	0.1	0.1	0.1
		1	0.0	0.5	0.3	0.2
		9	0.0	0.1	3.2	0.3
#85	Acute	0	0.1	0.0	0.0	0.0
		1	0.3	80.9	74.9	45.6
		3	0.6	91.4	93.9	97.3
#91	Acute	0	0.0	0.0	0.0	0.0
		1	0.2	31.4	25.8	22.5
		1	0.0	0.0	0.0	ND <sup>2</sup>
#1-i	Chronic	0	0.0	0.0	0.0	ND
		1	0.0	0.1	0.1	ND
#5	Chronic	0	0.0	0.0	0.0	0.0
		1	0.2	1.2	1.5	0.8
		9	0.1	0.9	7.0	6.7
#6	Chronic	0	0.2	0.1	0.1	ND
		2	0.1	0.2	0.1	ND
		0	0.1	0.0	0.1	0.1
#7	Chronic	0	0.1	0.0	0.0	62.1
		1	0.2	52.3	66.5	0.0
		0	0.1	0.1	0.1	0.1
#8	Chronic	0	0.1	0.2	0.3	0.1
		1	0.1	0.2	0.3	0.1
		0	0.0	0.1	0.0	0.0
#20	Chronic	0	0.3	0.5	0.4	0.4
		1	0.0	0.0	0.1	0.0
		0	0.0	0.5	0.4	0.2
#23	Chronic	0	0.0	0.0	0.0	0.2
		1	0.0	0.0	0.0	0.1
		0	0.1	0.3	0.4	0.0
#29	Chronic	0	0.0	0.0	0.0	0.0
		1	0.1	0.3	0.4	0.1
		0	0.0	0.0	0.0	0.0
#42	Chronic	0	0.0	0.0	0.0	0.1
		1	0.0	11.3	0.2	0.1
		0	0.0	0.2	0.1	0.0
#54	Chronic	0	0.0	9.7	10.5	5.4
		1	0.1	0.3	0.2	0.1
		0	0.0	6.0	6.8	3.3
#69	Chronic	0	0.4	0.1	0.0	0.0
		1	0.0	63.4	59.2	33.2
		0	0.0	0.0	0.0	0.1
#79	Chronic	0	0.0	0.0	0.0	0.6
		1	0.1	1.3	1.1	0.0
		0	0.1	0.1	0.1	0.0
#89	Chronic	0	0.1	35.4	32.0	15.9
		1	0.0	0.0	0.0	0.0
		0	0.0	0.0	0.0	0.0
#90	Chronic	0	0.0	0.0	0.0	0.0
		1	0.3	0.9	1.1	0.8
		1	0.3	0.9	1.1	0.8

<sup>1</sup>Cryopreserved PBMC were thawed and expression of intracellular HTLV-I antigens were analyzed immediately (Day 0) or following incubation for the indicated days in 10% FBS RPMI. When the cells were cultured for longer than 3 days, 100 U/mL of IL-2 was added to the culture medium to maintain cell viability. <sup>2</sup>ND, not done.





**b**

Patients	HLA		Amino acid sequences	
	A2	A24	Tax11-19	Tax301-309
Pt. #1-vi	+	+	(not amplified)	(not amplified)
Pt. #22	+	+	LLFGYPVYV	SFHSLHLLF
Pt. #80	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #85	-	+	LLFGYPVYV	SFHSLHLLF
Pt. #91	+	+	LLFGYPVYV	SFH <sup>*</sup> NLHLLF
Prototype HTLV-I			LLFGYPVYV	SFHSLHLLF

**FIGURE 3**—Conservation of representative CTL epitopes in the proviruses from acute ATL cells. (a), Detection of full-length HTLV-I provirus in ATL cells. Genomic DNA (500 ng) extracted from PBMC of a healthy volunteer (lane 1), five acute ATL patients (lane 2–6), or MT-2 cells (lane 7) were amplified by a long PCR system with specific primers for 5' and 3' HTLV-I LTR. PCR products were visualized by ethidium bromide. The expected size of the fragments amplified from a full-length HTLV-I provirus was 7.7kbp in this system. (b) Nucleotide sequences of the long PCR products from acute ATL cells prepared in (a) were determined and the sequences at the regions corresponding to representative CTL epitopes, Tax 11-19 and Tax 301-309 restricted by HLA-A2 and A24, respectively, are shown as amino acid sequences. \*Indicates the site of a single mutation (agt → aat) found.

#### Induction of co-stimulatory molecules in PBMC from ATL patients

It has been reported that HTLV-I-infected cell lines express a variety of surface molecules of activated T cells.<sup>28</sup> We investigated the expression of co-stimulatory molecules in ATL cells. It is known that typical ATL cells usually express CD4, CD25 and HLA-DR.<sup>28</sup> Besides these antigens, we assessed the expression of co-stimulatory molecules such as CD40, CD40L, CD80, CD86, OX40 and OX40L that participate in the interaction between antigen-presenting cells and T cells for efficient T cell-mediated immunity.<sup>41</sup> As shown in Figure 4, positive control ILT-Hod cells, an HTLV-I-infected T cell line established previously from an ATL patient, clearly expressed CD4, CD25, CD80, CD86, HLA-A, B, C and HLA-DR, and partially expressed OX40 and OX40L. The PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patient, that significantly expressed HTLV-I antigens after *in vitro* cultivation, were then analyzed before and after culture (Fig. 4). CD4, CD25 and HLA-A, B, C were detectable in uncultured PBMC from both patients. ATL cells from Patient 85 were double positive for CD4 and CD8. HLA-DR was detected in Patient 85 but not in Patient 79. In addition, small but detectable levels CD86 (14%) and OX40 (11%) were expressed in uncultured PBMC of Patient 85 and Patient 79, respectively (Day 0, closed histogram). After 1–3 days cultivation, expression of CD25, CD80, CD86 and OX40 was increased significantly. Similar induction of co-stimulatory molecules was also observed in other ATL cells with HTLV-I induction by culture (data not shown).

The results of two-color staining for HTLV-I Gag antigens and co-stimulatory molecules are shown in Figure 5. In 1-day cultured PBMC of Patient 85, the cells expressing intracellular HTLV-I Gag antigens partially expressed CD80 (23%) and CD86 (26%), and exclusively expressed OX40 (93%). This clearly indicated that HTLV-I and co-stimulatory molecules were co-expressed in ATL cells at the single cell level.

Among ATL cases without viral induction, the samples from Patient 1 expressed CD86 and OX40, and the sample from Patient 6 spontaneously expressed OX40 before culture (data not shown). The other samples tested did not express detectable levels of these co-stimulatory molecules. CD40 and CD40 ligand were not detectable in the PBMC of any ATL patients tested.

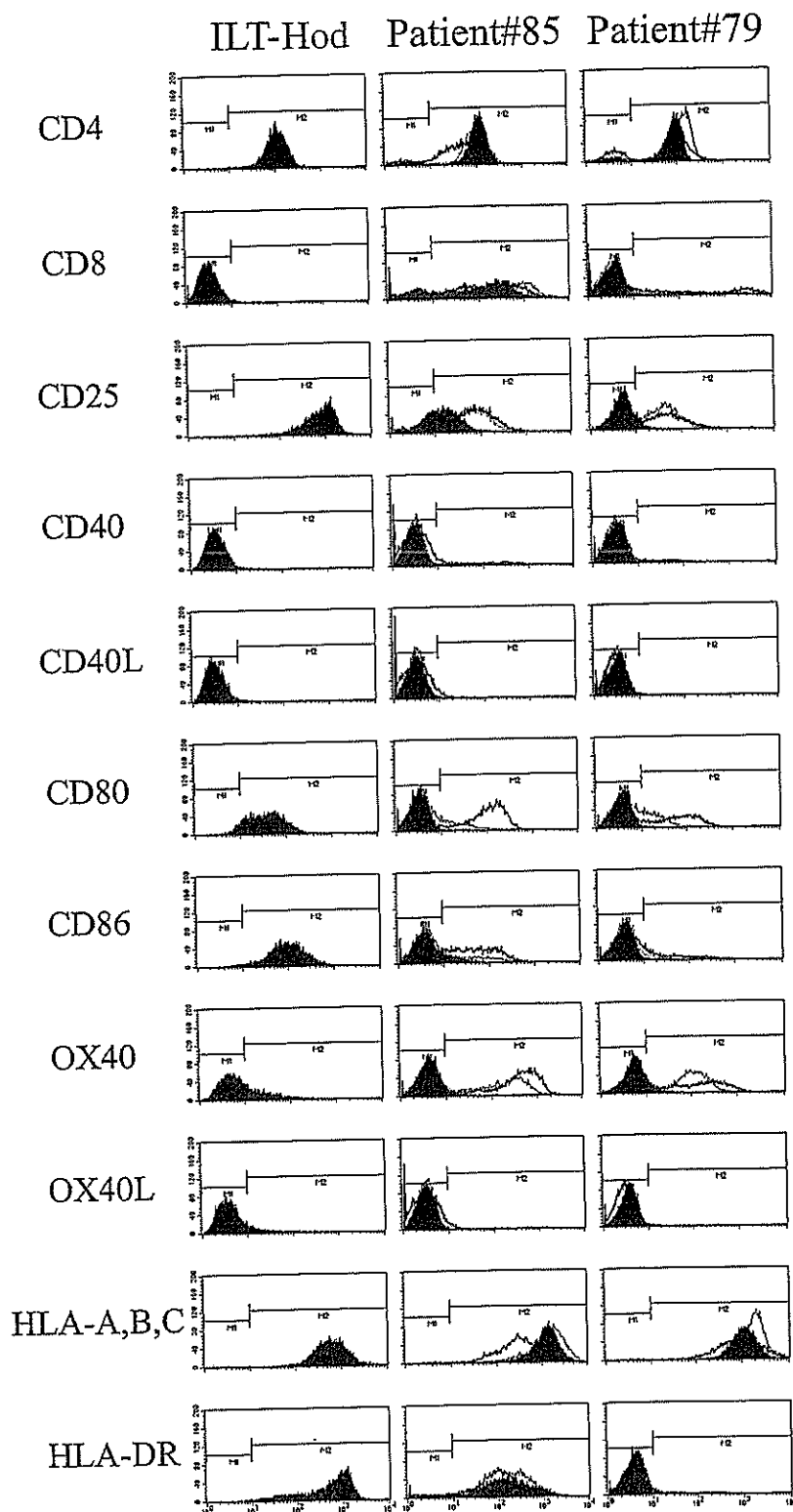
#### Augmentation of immunogenicity of ATL cells *in vitro*

We assessed the immunogenicity of short-term cultured ATL cells by *in vitro* MLR and HTLV-I-specific CTL assays. The results of MLR using allogeneic responder T cell and formalin-fixed PBMC from an acute (Patient 85) and a chronic (Patient 79) ATL patients were shown in Figure 6a. The levels of responder T cell proliferation were significantly enhanced upon mixing with 1-day or 3-day cultured ATL cells. Long-term cultured HTLV-I-infected T cell lines (ILT-85 and ILT-79) derived from these patients also induced significant levels of allogeneic MLR. It is of note that the ATL cells of Patient 79 expressed CD80 and OX40 but not HLA-DR (Fig. 4), suggesting that enhancement of MLR with ATL cells from this patient was not due merely to augmented HLA-DR. The levels of MLR against T cell-enriched fractions from 2 healthy volunteers were not markedly enhanced by preculture (Fig. 6b).

We assessed whether ATL cells could activate HTLV-I-specific CTL *in vitro* by mixing HLA-A24-restricted HTLV-I-specific CD8<sup>+</sup> CTL with formalin-fixed PBMC from HLA-A24-positive ATL patients (Patient 85 and Patient 1-vi) or a healthy volunteer. The results are shown in Table III. One- to three-day precultured, but not uncultured, PBMC of Patient 85 accelerated [<sup>3</sup>H]-TdR incorporation into the CTL, and induced marked levels of IFN- $\gamma$  production in the CTL. In contrast, PBMC from a healthy volunteer or Patient 1-vi, in which HTLV-I antigens were not inducible, failed to enhance DNA synthesis or IFN- $\gamma$  production in the CTL. Thus, short-term cultured ATL cells could be a specific stimulator as well as a target for HTLV-I-specific CTL in these *in vitro* assays.

#### *In vivo* induction of HTLV-I-specific T cell responses by inoculating ATL cells in rats

We investigated whether ATL cells could evoke HTLV-I-specific T cell immune responses *in vivo*. Twice with a 2-week interval, immunocompetent adult rats were subcutaneously inoculated with 10<sup>7</sup> cells/head of uncultured or 3 days-cultured PBMC from an acute ATL Patient 91 after formalin-treatment. Figure 7a shows the results of IFN- $\gamma$ -production of spleen T cells from these rats 1 month after the last immunization. Surprisingly, in 2 of 2 rats inoculated with uncultured ATL cells, spleen T cells produced high levels of IFN- $\gamma$  against stimulation with Tax-expressing syngeneic rat G14-Tax and HTLV-I-infected FPM1 cells but not with HTLV-I-negative G14



**FIGURE 4** – Induction of co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from an acute ATL Patient 85 and a chronic ATL Patient 79 were thawed and stained immediately (closed lines), or after incubation for 1 day (thin lines) or 3 days (solid lines) in 10% FBS-RPMI, FITC- or PE-conjugated anti-human mAbs (CD4, CD8, CD25, CD40, CD40L, CD80, CD86, OX40, OX40L, HLA-A, B, C, HLA-DR) as indicated. Cell surface antigen expression on ILT-Hod, an IL-2-dependent HTLV-I-infected cell line was analyzed by flow cytometry as a positive control. Live cells were gated and are shown as histograms. Each M1 region indicates where the cells stained with the isotype control mAb distributed (not shown). Accordingly, each remaining M2 region is regarded as positive.

cells (Fig. 7a; Rats 1 and 2). Similar or higher levels of HTLV-I-specific T cell responses were observed in the rats inoculated with 3-days cultured ATL cells from the same patient (Rats 3 and 4). T cells from control rats inoculated with PBMC from uninfected healthy human volunteers produced minimal levels of IFN- $\gamma$ .

T cells from the rats inoculated with ATL cells also showed strong proliferative response against stimulation with G14-Tax and

FPM1 cells but not with G14 cells (Fig. 7b). There was no significant difference between T cell response of the rats inoculated with uncultured and cultured ATL cells. CD4 positive cells became a dominant population in the spleen T cells from immunized rats after co-culture with formalin-fixed G14-Tax cells, whereas initially CD8 positive cells dominated before co-culture (Fig. 7c).

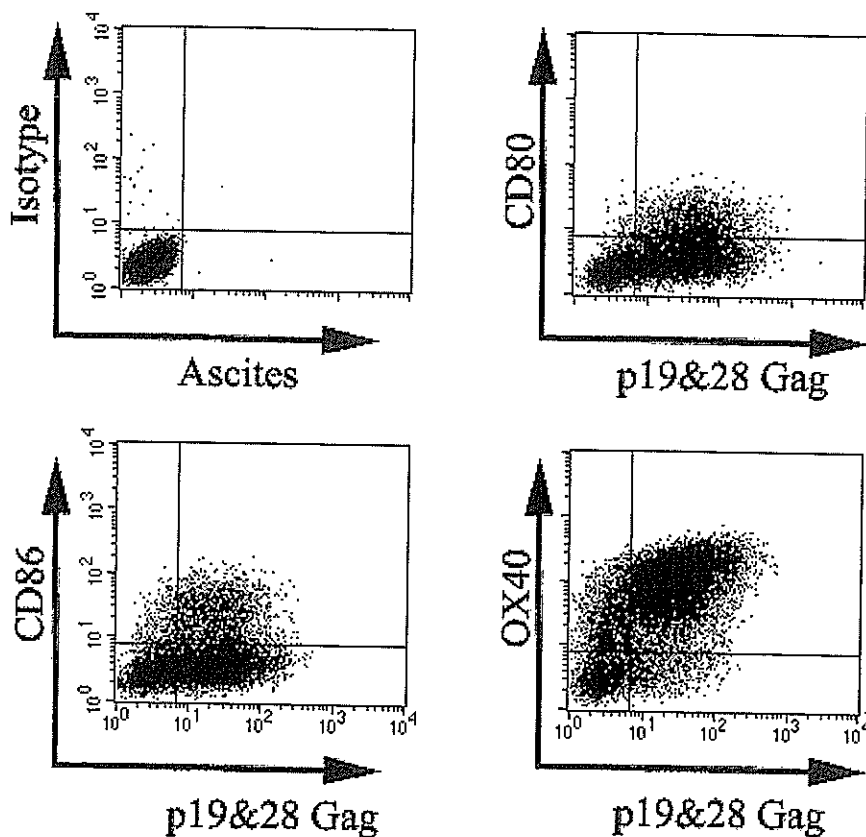


FIGURE 5 – Double induction of HTLV-I and co-stimulatory molecules in the PBMC of ATL patients after *in vitro* cultivation. Cryopreserved PBMC from Patient 85 (acute ATL) were thawed and incubated for 1 day in 10% FBS-RPMI, and then subjected to a two-color analysis of cell surface antigens (CD80, CD86 or OX40) detected by FITC-conjugated mAbs and intracellular HTLV-I p19 and 28 Gag antigens detected by biotinylated GIN-7 mAb with Cy-Chrome-streptavidin after permeabilization with saponin (see Material and Methods). Live cells were gated and are shown as dot plots (FL-1/FL-3).

We tested cytotoxicity of the spleen cells from these rats inoculated with ATL cells after 7 days of *in vitro* co-culture with formalin-fixed G14 Tax cells. The results were shown in Figure 7d. The spleen cells from rats inoculated with ATL cells did not significantly kill Tax-expressing cells. In contrast, the positive control spleen cells from Tax-coding DNA-vaccinated rats showed strong cytotoxicity for G14-Tax but not for G14 cells.

These results suggest that not only precultured but also uncultured PBMC of ATL patients possessed sufficient amounts of antigens to evoke Tax-specific helper T cell response *in vivo*.

## Discussion

We demonstrated that ATL cells retained the ability to express HTLV-I antigens including Tax in nearly 50% of the cases of ATL patients. Although the viral antigens became detectable in ATL cells by flow cytometric analysis only after short-term culture, the experiments with direct inoculation of the ATL cells to rats showed that uncultured ATL cells were also able to induce HTLV-I-specific T cell response *in vivo*.

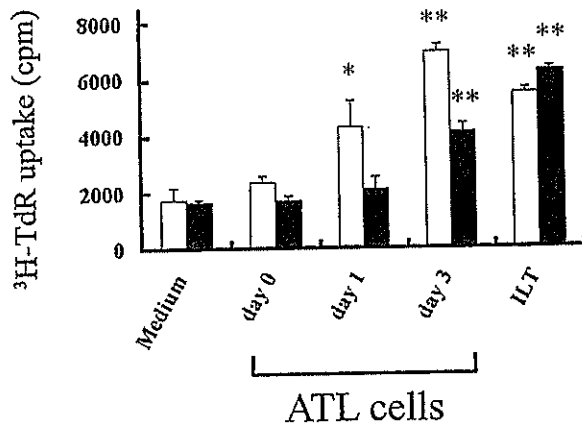
It has been noted that HTLV-I expression is inducible in ATL cells from some, but not all, ATL patients.<sup>26,28</sup> In our present study, induction of HTLV-I Tax and Gag in ATL cells was observed in 3 of 5 acute ATL, 6 of 15 chronic ATL, and 9 of 19 ATL cases tested (Table II). In one case with chronic ATL (Patient 79), HTLV-I expression was induced in many more cells than the number of morphologically identified abnormal lymphocytes, indicating that many peripheral HTLV-I-infected cells could appear as normal lymphocytes. It is intriguing that HTLV-I-expression was induced more frequently in acute-type ATL cells than in chronic-type ATL cells, despite the fact that acute-type ATL is supposed to be at a more advanced stage than chronic-type ATL. During *in vivo* evolution of HTLV-I-infected cells toward ATL, modification of viral expression may not be an absolute requirement.

Nucleotide sequences at 2 representative CTL epitopes, Tax 11–19 and Tax 301–309 restricted by HLA-A2 and A24, respectively, were highly conserved in proviruses from 4 acute ATL patients tested (Fig. 3). We chose these epitopes because they were predominantly recognized by CTL in 2 ATL patients after hematopoietic stem cell transplantation,<sup>29</sup> and also because genomic frequencies of HLA-A2 and A24 in Japanese are 24.7% and 35.6%, respectively.<sup>42</sup> ATL cells retained their ability to express viral antigens in 3 of 4 patients with conserved epitopes. These observations suggest that CTL escape mutants may not be the main reason for ATL-development in these patients.

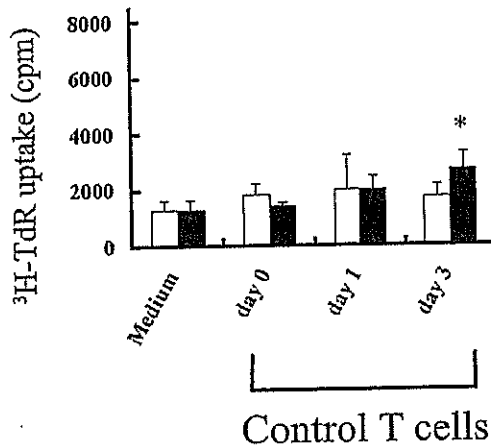
Direct inoculation of fresh ATL cells into naive rats efficiently induced HTLV-I-specific helper T cell response *in vivo* (Fig. 7), despite the apparent absence of HTLV-I antigens in these cells by flow cytometry. Because ATL cells were derived from human, the inoculated rats might potentially respond to xenogenic antigens. Because we used syngeneic rat target cells for the cytokine production assay to evaluate immune response in the rats, however, reactions against xenogenic antigens should not be picked up by this assay. In addition, because we treated uncultured ATL cells with formalin before inoculation to rats, immune response of the rats was not due to further induction of viral antigens in the ATL cells or secondary HTLV-I-infection *in vivo* either. Spleen T cells from the rats inoculated with ATL cells reacted with syngeneic HTLV-I-infected or Tax-expressing rat cells but not uninfected cells, indicating that these T cells recognized HTLV-I antigens including Tax or Tax-induced antigens.

The *in vivo* HTLV-I-antigenicity of fresh ATL cells described above contrasted with the observation that only precultured but not uncultured ATL cells activated HTLV-I-Tax-specific CTL line *in vitro* (Table 3). This may be partly explained by the different sensitivity of the responding T cells between *in vivo* and *in vitro* detection systems. The Tax-specific CTL line used in the *in vitro* assay has been established by a long-term culture, whereas the spleen T cell population of immunized rats would be more heter-

a



b



**FIGURE 6** – Augmentation of antigenicity for allogeneic mixed lymphocyte reactions (MLR) in precultured PBMC from ATL patients. (a) PBMC from a healthy donor as responder cells were mixed with formalin-fixed PBMC from Patient 85 (acute ATL, open bar) and Patient 79 (chronic ATL, closed bar) that had been uncultured (Day 0) or precultured for the indicated periods (Day 1 or 3), or with formalin-fixed ILT cells derived from Patient 85 (open bar) and Patient 79 (closed bar). After 4 days of culture, [<sup>3</sup>H]-TdR incorporated into the cells was measured. (b) Similar allogeneic MLR were carried out with responder PBMC from a healthy donor and formalin-fixed T cell-enriched PBMC fractions from two other healthy volunteers (open and closed bar) that had been precultured for the indicated periods. The results were expressed as the mean ± SD in counts per minutes (cpm) obtained from triplicate cultures. Differences between the 2 groups were analyzed for significance (\**p* < 0.05, \*\**p* < 0.001, compared with Day 0) by Student's *t*-test. Similar results were obtained in another set of independent experiments.

ogeneous. Antigens of formalin-fixed ATL cells inoculated to rats were presumably processed and presented mainly on MHC-II by professional antigen-presenting cells, which would favor CD4 positive helper T cell response *in vivo*. The amounts of antigen required for priming T cells *in vivo* might be smaller than those for activating the CTL line *in vitro*. In addition, the presence of co-stimulatory molecules on ATL cells might have been advantageous to induce T cell response *in vivo*.<sup>41,43</sup>

Short-term cultured ATL cells significantly expressed co-stimulatory molecules including CD80, CD86, and OX40 as well as HTLV-I antigens such as Tax and Gag at the single cell level. The levels of

**TABLE III** – PROLIFERATIVE RESPONSE AND IFN- $\gamma$  PRODUCTION OF HTLV-I-SPECIFIC CTL IN RESPONSE TO ATL CELLS WITH OR WITHOUT *IN VITRO* PRECULTIVATION<sup>1</sup>

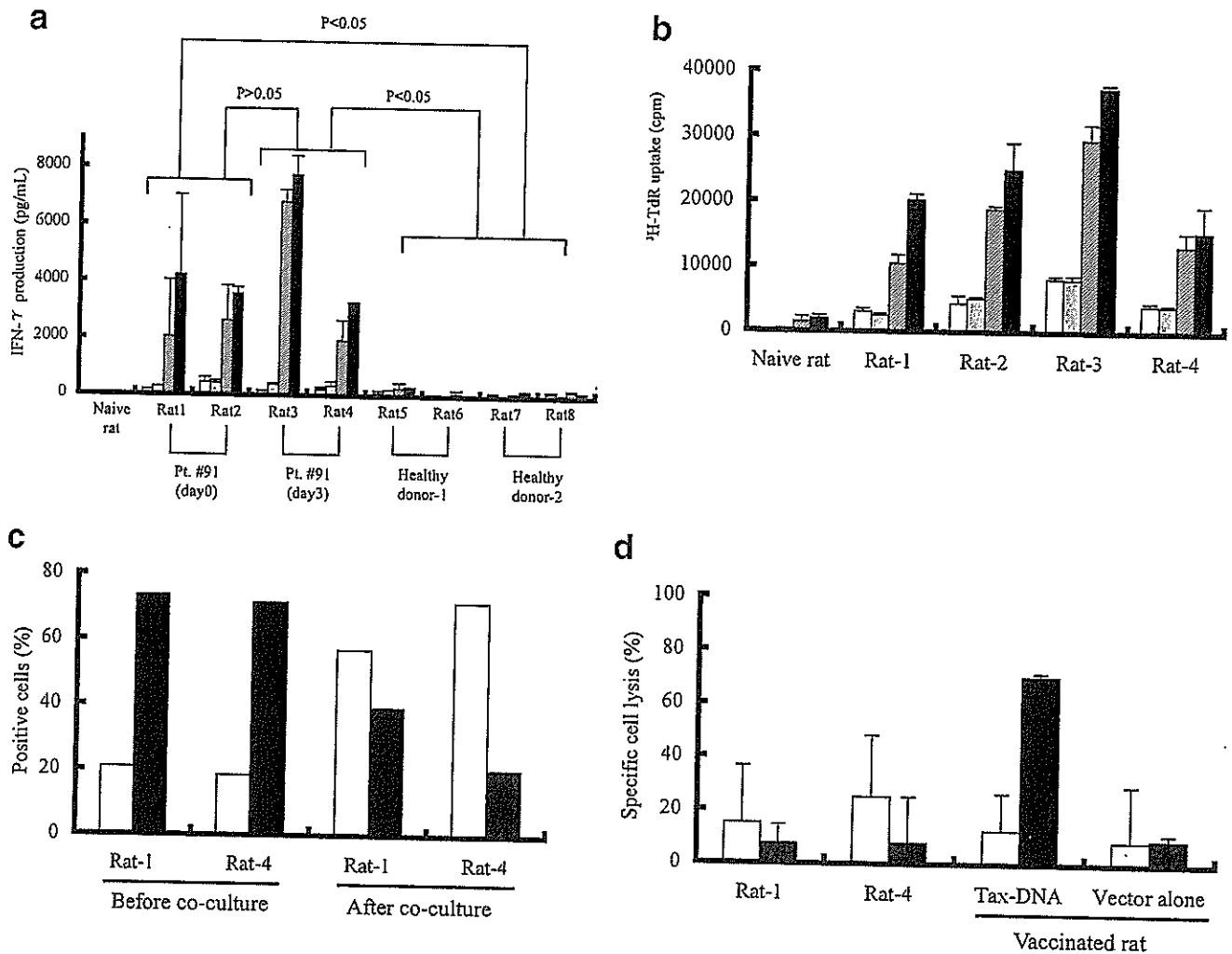
Subject	Stimulator PBMC		Responses of HTLV-I-specific CTL	
	Preculture period (Day)		[ <sup>3</sup> H]-TdR uptake (cpm)	IFN- $\gamma$ production (pg/mL)
Pt. #85	0		2,469 ± 246	Undetectable
	1		3,350 ± 423 <sup>2</sup>	673 ± 45 <sup>3</sup>
	3		4,498 ± 296 <sup>3</sup>	534 ± 79 <sup>3</sup>
Pt. #1-vi	0		2,493 ± 84	Undetectable
	1		2,470 ± 366	Undetectable
Healthy	0		2,737 ± 215	Undetectable
	1		2,554 ± 78	Undetectable
	3		2,748 ± 195	Undetectable
None	—		2,611 ± 115	Undetectable

<sup>1</sup>HLA-A24-restricted HTLV-I-specific CTL (1 × 10<sup>5</sup>/well) were cocultured for 24 hr with formalin-fixed cryopreserved PBMC (5 × 10<sup>4</sup>/well) from HLA-A24-positive ATL patients or healthy volunteers pre-incubated for the indicated periods (0, 1 or 3 days), and IFN- $\gamma$  production in the culture supernatant and [<sup>3</sup>H]TdR uptake for an additional 16 hr were measured. The results are expressed as the mean ± SD. Differences between the 2 groups were analyzed for significance. <sup>2</sup>*p* < 0.05. <sup>3</sup>*p* < 0.001, compared with Day 0 by Student's *t*-test. Similar results were obtained in another set of independent experiments.

Tax-expression in ATL cells reached the maximum in 1 day, whereas expression of Gag and co-stimulatory molecules increased with further incubation (3 days) (Fig. 4), suggesting that Tax was involved in the activation of the other molecules. A number of previous studies pointed to the potential transactivation of CD25,<sup>44</sup> OX40<sup>45</sup> and OX40L<sup>46</sup> by HTLV-I Tax. Induction of CD80 and CD86 in HTLV-III-infected cells has also been reported.<sup>47</sup> In our present study, spontaneous expression of OX40 and CD86 was sporadically observed in ATL cells before or without HTLV-I-induction. This indicates that Tax expression under detectable levels or some other mechanisms may be involved in activating co-stimulatory molecules in these ATL cells.

ATL may be categorized into at least 2 groups by the ability of HTLV-I-expression in their ATL cells. In our present study, HTLV-I expression was inducible in about half the ATL cases, and the other half showed irreversible viral silencing in their ATL cells. Although the irreversible silencing of HTLV-I may be due to various genomic changes in ATL cells,<sup>24,25</sup> HTLV-I expression is not completely silent in the other inducible type of viral suppression. This is supported by previous and recent reports that HTLV-I mRNA is detectable by RT-PCR in fresh ATL cells.<sup>27,48</sup> The inducible type of suppression is commonly seen in PBMC from HTLV-I-carriers and HAM/TSP patients.<sup>22,49</sup> Despite such suppression of viral expression *in vivo*, HTLV-I Tax-specific CTL are highly activated in HAM/TSP patients and some HTLV-I-carriers,<sup>16,19</sup> implying the presence of sufficient levels of antigen-presentation *in vivo* for priming and maintaining CTL. This is consistent with the observation in our present study that sub-detectable amounts of viral expression induced HTLV-I-specific T cell response *in vivo* but not fully activated Tax-specific CTL line *in vitro*. Such marginal levels of viral expression may partly explain how HTLV-I persists *in vivo* in the presence of HTLV-I-specific CTL. Nevertheless, active HTLV-I-specific CTL responses are associated with tumor-free state in human<sup>16,21,29</sup> and limited proviral loads in rats,<sup>38</sup> still suggesting contribution of HTLV-I-specific CTL to controlling expansion of HTLV-I-infected cells *in vivo*. It remains to be clarified where and when HTLV-I-specific CTL can affect infected cells *in vivo*.

Our results indicated that, in respect of the ability of viral expression, ATL has diversity even within the acute type ATL. In about half the ATL cases, ATL cells retained the ability of viral expression. Among these patients, fresh ATL cells from one case could induce Tax-specific helper T cell response *in vivo* despite their undetectable viral expression in *in vitro* assays. These imply that ATL cells may express low but sufficient levels of Tax or Tax-induced antigens to be recognized by T cells *in vivo*.



**FIGURE 7** - *In vivo* induction of HTLV-I-specific T cell response by inoculation of fresh ATL cells in rats. Immunocompetent naive rats were subcutaneously immunized with either ten million of formalin-fixed ATL cells from Patient 91 that had been uncultured (Rats 1, 2) or 3 day-precultured (Rats 3, 4), or with the same number of formalin-fixed uncultured PBMC from 2 healthy donors (Rats 5-8), twice with a 2-week interval. One month after second immunization, spleen cells were collected. (a) IFN- $\gamma$  production of spleen T cells from these rats were measured by ELISA after incubation without (open bar) or with formalin-fixed syngeneic G14 (gray bar), G14-Tax (hatched bar) or FPM1 (closed bar) cells for 6 days. Similar results were obtained also at the third day of incubation when half the medium was changed. (b) Proliferation of spleen T cells from immunized rats was monitored by a [ $^3$ H]-TdR-uptake assay after 3 days of incubation with various stimulator cells that were set up similarly to (a). (c) CD4 (open bar) or CD8 (closed bar) positive cell number in the spleen T cells from Rats 1 and 4 was evaluated by flow cytometry before and after 7 days of co-cultivation with formalin-fixed G14-Tax. Values indicate percent positive against viable cell number. (d) Cytotoxicities of spleen cells from Rats 1 and 4 were measured after 7 days of pre-culture with formalin-fixed G14-Tax. Similarly pre-cultured spleen cells from rats vaccinated with plasmids containing *tax* cDNA (Tax-DNA) or vector plasmids alone served as positive and negative controls, respectively. These effector cells were mixed with [ $^3$ H]-TdR-labeled G14 (open bar) and G14-Tax (closed bar) cells at the effector/target cell ratio of 10. After 6 hr incubation, cells were harvested and radioactivities remaining in the target cells were measured. Specific cell lysis were calculated (see Material and Methods) and expressed as the mean  $\pm$  SD of triplicate cultures.

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Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects

Christophe Borg<sup>1</sup>, Magali Terme<sup>1</sup>, Julien Taïeb<sup>1</sup>, Cédric Ménard<sup>1</sup>, Caroline Flament<sup>1</sup>, Caroline Robert<sup>1</sup>, Koji Maruyama<sup>1,2</sup>, Hiro Wakasugi<sup>2</sup>, Eric Angevin<sup>1</sup>, Kris Thielemans<sup>3</sup>, Axel Le Cesne<sup>4</sup>, Véronique Chung-Scott<sup>1</sup>, Vladimir Lazar<sup>1</sup>, Isabelle Tchou<sup>1</sup>, Florent Crépineau<sup>1</sup>, François Lemoine<sup>5</sup>, Jacky Bernard<sup>6</sup>, Jonhantan A. Fletcher<sup>7</sup>, Ali Turhan<sup>8</sup>, Jean-Yves Blay<sup>9</sup>, Alain Spatz<sup>10</sup>, Jean-François Emile<sup>11</sup>, Michael C. Heinrich<sup>12</sup>, Salah Mécheri<sup>13</sup>, Thomas Tursz<sup>1</sup> and Laurence Zitvogel<sup>1</sup>

<sup>1</sup>Department of Clinical Biology, Equipe de Recherche Mixte 0208, INSERM, Institut Gustave Roussy, Villejuif, France.

<sup>2</sup>Pharmacology and Immunology Unit, National Cancer Center, Tokyo, Japan.

<sup>3</sup>Laboratory of Molecular and Cellular Therapy, Medical School of the Vrije Universiteit Brussel, Brussels, Belgium.

<sup>4</sup>Department of Medical Oncology, Institut Gustave Roussy, Villejuif, France.

<sup>5</sup>Centre d' Etudes et de Recherches en Virologie et Immunologie, Hôpitaux Pitié Salpêtrière, Paris, France.

<sup>6</sup>Centre Jean Godinot, Reims, France.

<sup>7</sup>Harvard Medical School, Boston, Massachusetts, USA.

<sup>8</sup>Translational Research Unit, Institut Gustave Roussy, Villejuif, France.

<sup>9</sup>Hospices Civils de Lyon, Hôpital Edouard Hériot, Lyon, France.

<sup>10</sup>Department of Pathology and Translational Research, Institut Gustave Roussy, Villejuif, France.

<sup>11</sup>Department of Pathology, Paul Brousse Hospital, Assistance Publique-Hôpitaux de Paris, Villejuif, France.

<sup>12</sup>Oregon Health and Science University Cancer Institute, Portland, Oregon, USA.

<sup>13</sup>Immuno-Allergy Unit, Pasteur Institute, Paris, France.

**Address correspondence to:** Laurence Zitvogel, ERM0208 INSERM, Department of Clinical Biology, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cedex, France. Phone: 33-1-42-11-50-41; Fax: 33-1-42-11-60-94; E-mail: [zitvogel@igr.fr](mailto:zitvogel@igr.fr).

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**Abstract**

Mutant isoforms of the KIT or PDGF receptors expressed by gastrointestinal stromal tumors (GISTs) are considered the therapeutic targets for STI571 (imatinib mesylate; Gleevec), a specific inhibitor of these tyrosine kinase receptors. Case reports of clinical efficacy of Gleevec in GISTs lacking the typical receptor mutations

prompted a search for an alternate mode of action. Here we show that Gleevec can act on host DCs to promote NK cell activation. DC-mediated NK cell activation was triggered in vitro and in vivo by treatment of DCs with Gleevec as well as by a loss-of-function mutation of KIT. Therefore, tumors that are refractory to the antiproliferative effects of Gleevec in vitro responded to Gleevec in vivo in an NK cell-dependent manner. Longitudinal studies of Gleevec-treated GIST patients revealed a therapy-induced increase in IFN- $\gamma$  production by NK cells, correlating with an enhanced antitumor response. These data point to a novel mode of antitumor action for Gleevec.

## Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract. Somatic gain-of-function mutations of the c-kit protooncogene are found in 85% of GISTs (1), and recently, mutations of the PDGFR $\alpha$  chain were reported in 35% of the GISTs lacking the KIT mutations (2). The 2-phenylaminopyrimidine compound imatinib mesylate (STI571; Gleevec) was initially designed to specifically block the ATP-binding site of break point cluster region/Abelson leukemia virus (BCR/ABL) tyrosine kinase, and it also inhibits the kinase activity of 3 related kinases: BCR/ABL, PDGFR, and KIT (3-5). Gleevec administration results in objective (partial or complete) response or stabilization in about 80% of GIST patients (6). Clinical response to Gleevec correlates with the mutational status of the c-kit gene. GISTs harboring an exon 11 mutation (76% of GISTs) exhibit the highest objective response rate and the longest time to progression (7).

However, several lines of evidence indicate that Gleevec might mediate antitumor effects by an alternate mode of action instead of having a direct effect on tumoral c-kit mutations. Indeed, the pharmacokinetics of Gleevec have no predictive value for clinical responses, and some GISTs with very low expression of KIT have been shown to respond to Gleevec (8, 9). We therefore hypothesized that, in addition to its cell-autonomous antitumor effects, Gleevec might act indirectly on host cells outside of the tumor. The validity of this hypothesis relied on case reports of GISTs devoid of mutations that we isolated in the cohort of patients responding to Gleevec. To demonstrate this novel mode of action of Gleevec, we selected mouse tumor models that were resistant to the antiproliferative effects of Gleevec in vitro but responded in vivo to long-term exposure to Gleevec or to short-term exposure to Gleevec combined with a DC growth factor, fms-like tyrosine 3 kinase ligand (FL) (10). Here we show that Gleevec acts on host DCs to promote NK cell activation and NK cell-dependent antitumor effects in mice. We also report that most GIST-bearing patients that were treated with Gleevec acquired NK cell activation, which positively correlated with clinical outcome (time to progression). This novel mode of action of Gleevec opens new fields of investigation for immunotherapeutic approaches.

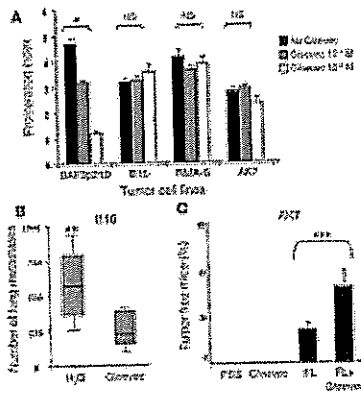


## Results

*GISTs devoid of c-kit/PDGFR mutations respond to Gleevec.* According to Heinrich and colleagues (7), the mutational status of c-kit predicts the clinical response of the GIST to Gleevec; they report objective (partial or complete) responses only in cases involving a mutation in the genes encoding c-kit or the PDGFR $\alpha$  chain. In this previous study (7), activating mutations of c-kit or PDGFR $\alpha$  were found in 88% and 5% of GISTs, respectively. In patients with GISTs harboring the exon 11 c-kit mutation, the partial response rate was 83%, whereas patients with GISTs harboring the exon 9 c-kit mutation and those with no detectable mutation of c-kit or PDGFR $\alpha$  had a partial response rate of 48% and 0%, respectively (7). However, here we report the first 6 cases (3 in a phase I/II French study and 3 in a phase II US study [ref. 7]) of GISTs that did not display the target mutations of Gleevec but still exhibited objective tumor responses. We analyzed the genomic DNA in these 6 paraffin-embedded primary GISTs and did not find any mutations in the following Gleevec targets: c-kit exons 9, 11, 13, and 17, and PDGFR $\alpha$  exons 12, 14, and 18. However, 2 patients presenting with liver, stomach, or lung metastases did exhibit complete responses to Gleevec with 26 months of disease-free survival. One patient presenting with liver metastases displayed a partial response with 24 months of progression-free survival (PFS), and 3 patients exhibited stable disease (7, 15, and 17 months of PFS) (see Supplemental Table 1A; supplemental material available at <http://www.jci.org/cgi/content/full/114/3/379/DC1>). This finding prompted the search for an alternate mode of action of Gleevec that is not cell autonomous.

*In vivo efficacy of Gleevec in tumors resistant to Gleevec in vitro.* Accordingly, we identified several mouse tumor models resistant to the antiproliferative effects of Gleevec in vitro (Figure 1A) but sensitive to Gleevec in vivo. While B16F10 melanoma cell proliferation was not inhibited by micromolar concentrations of Gleevec in vitro, the establishment of B16 lung metastases in C57BL/6 mice was significantly hampered by oral feeding with Gleevec (Figure 1B). Gleevec also induced significant antitumor effects against the AK7 mesothelioma and the MCA102 fibrosarcoma models (data not shown). The Gleevec-mediated antitumor effects could be potentiated by the adjunction of FL, a hematopoietic growth factor endowed with immunostimulatory capacities (10). Indeed, a short administration of Gleevec (4 days) combined with FL (FL+Gleevec) promoted synergistic antitumor effects against established AK7 mesothelioma with up to 45% tumor-free mice using FL+Gleevec versus 14% using FL alone or 0% using Gleevec alone (Figure 1C). Similarly, the combination of FL and Gleevec prevented 100% of mice from developing the transporter associated with antigen processing-deficient (TAP-deficient) RMA-S lymphoma, whereas neither FL nor Gleevec alone prevented this lymphoma (Figure 2A). It is noteworthy that tumor-free mice were not immune to autologous tumor cells, since rechallenge was lethal in most cases (data not shown). Therefore,

Gleevec alone or in combination with FL significantly curtails tumor progression in tumor models resistant to Gleevec in vitro, which suggests a non-cell autonomous mode of action.



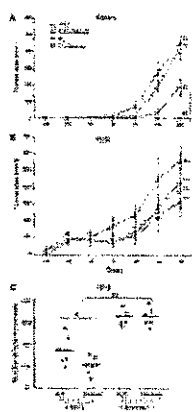
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Figure 1

Gleevec prevented tumor progression in vivo in tumor models resistant to the Gleevec antiproliferative effects in vitro. (A) Mouse tumor models resistant to Gleevec in vitro. AK7, B16F10 (B16), RMA-S, MCA 102, or BAF3p210 cells (bearing the BCR/ABL translocation) were incubated for 24 hours with the indicated doses of Gleevec, and the absolute number of surviving cells was determined by trypan blue exclusion assay. Proliferation indexes are shown. The Wilcoxon two-sample rank sum test was used to compare the proliferation indexes ( $*P < 0.05$ ). (B) Gleevec prevents establishment of B16F10 lung metastases. We injected  $5 \times 10^5$  B16F10 tumor cells in the tail vein at day 0. Oral feeding with Gleevec (150 mg/kg bid) or H<sub>2</sub>O (200  $\mu$ l) was administered on days 5–11 and mice were sacrificed for the enumeration of lung metastases on day 11. The data from 3 independent experiments, each including 5–7 mice per group, were pooled and are depicted. The Wilcoxon two-sample rank sum test was used to compare the number of lung metastases ( $**P < 0.05$ , Gleevec versus H<sub>2</sub>O). (C) FL and Gleevec synergize to eradicate AK7. We inoculated  $3 \times 10^6$  AK7 tumor cells in the abdominal flank of C57BL/6 mice on day 0. FL was started at day 11 when AK7 tumors reached a diameter of  $20 \pm 20$  mm<sup>2</sup>. FL was continued for 10 days and combined with Gleevec the day before FL arrest and for 8 consecutive days (same doses as in B). Each experiment included 5–7 mice/group and was repeated twice with similar results. The Kruskal Wallis multiple comparison test was used for statistical analyses and significant effects are signified by triple asterisks.



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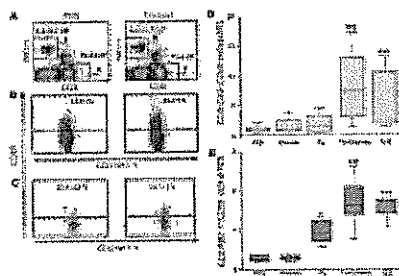
**Figure 2**

Gleevec promoted NK cell-dependent antitumor effects. (A and B) FL and Gleevec synergize to prevent RMA-S tumor establishment. In one abdominal flank of C57BL/6 mice, TAP-deficient RMA-S cells were injected (A), whereas TAP-sufficient RMA cells were injected into the other flank of the same mice (B). The same number of cells of each type were injected ( $10^6$  cells). From 6 days before injection (day -6) to day 3, FL ( $10 \mu\text{g}/\text{day}$ ) or PBS ( $200 \mu\text{l}$ ) was injected intraperitoneally. From day 1 to day 4, Gleevec ( $150 \text{ mg}/\text{kg bid}$ ) or  $\text{H}_2\text{O}$  ( $200 \mu\text{l}$ ) was administered orally. The number of tumor-free mice at the end of the experiment is indicated in parentheses. Each experiment included 5 mice per group and was repeated twice with similar results. (C) The antitumor effects of Gleevec against B16F10 lung metastases are mediated by NK cells. Neutralizing anti-NK1.1 mAb ( $300 \mu\text{g}$  of PK136 mAb/mouse) or normal mouse serum (NMS) were administered intraperitoneally at days -4, -2, 0, and 4 in C57BL/6 mice. At day 0,  $5 \times 10^5$  B16F10 tumor cells were inoculated into the tail vein. Oral feeding with Gleevec ( $150 \text{ mg}/\text{kg bid}$ ) or  $\text{H}_2\text{O}$  ( $200 \mu\text{l}$ ) was administered on days 5-11, and mice were sacrificed for the enumeration of lung metastases at day 11. The results of one representative experiment (of 2) including 5-7 mice per group are shown. The Kruskal Wallis multiple comparison test was used to compare the number of lung metastases ( $*P < 0.05$  between Gleevec and  $\text{H}_2\text{O}$  in NMS groups;  $**P < 0.01$  between NMS and anti-NK1.1 mAb in Gleevec groups; there was no significant difference between  $\text{H}_2\text{O}$  and Gleevec in anti-NK1.1 mAb groups).

*Gleevec promotes NK cell-dependent antitumor effects.* The combination of FL and Gleevec was unable to protect mice against the development of TAP-sufficient RMA tumors (Figure 2B). Since TAP-deficient tumor cells are known to be elective NK cell targets (11), and since immunosurveillance against AK7 and B16 involved NK cells (10, 12), we hypothesized that NK cells could be the critical effectors of the Gleevec-mediated antitumor effects. Indeed, the neutralization of NK1.1<sup>+</sup> cells in C57BL/6 mice inoculated with B16F10 melanoma cells abrogated the Gleevec-mediated antimetastatic effects (Figure 2C). Similar results were achieved in the RMA-S tumor model, in which anti-NK1.1 monoclonal antibodies significantly hampered the antitumor effects induced by the combination of FL and Gleevec (data not shown).

To confirm that long-term exposure to Gleevec or short-term administration of Gleevec in combination with FL promoted NK cell activation, we examined the upregulation of the activation marker CD69 on NK cells in various mouse strains (that is, in athymic Swiss<sup>nu/nu</sup> and in immunocompetent C57BL/6 mice). Prolonged (15-21 days) oral feeding with Gleevec-induced loss of splenic T cells (Figure 3A) but selectively allowed NK cell survival and/or

expansion and activation (Figure 3, B and C). In contrast, shorter exposure (4 days) to Gleevec did not promote NK cell activation. However, the combination of FL with low dosages of Gleevec (FL+Gleevec for 4 days) activated NK cells, supporting the antitumor effects observed (Figure 1). Indeed, the number of CD3<sup>-</sup>/DX5<sup>+</sup>/CD69<sup>+</sup> NK cells in the spleen (Figure 3D) and in the liver (see Supplemental Figure 1, A and B) of Swiss<sup>nu/nu</sup> mice treated with FL+Gleevec was augmented compared with that in littermates receiving either agent alone, reaching a level comparable to that obtained after injection of recombinant interleukin-2. Moreover, only NK cells harvested from FL+Gleevec-treated or IL-2-treated mice secreted IFN- $\gamma$  following ex vivo stimulation (data not shown). The synergy of FL+Gleevec triggered NK cell activation in the C57BL/6 genetic background (Figure 3E). We therefore conclude that NK cells are the critical effectors involved in the non-tumor-cell-autonomous mode of action of Gleevec.



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Figure 3

Gleevec alone or combined with FL induced NK cell activation in vivo. (A) Long-term exposure to Gleevec in C57BL/6 mice induced reduction of the splenic T lymphocyte counts but selectively maintained the NK cell subset. After red blood cell removal and an adherence step, splenocytes were enumerated after 15–21 days of oral feeding with Gleevec (150 mg/kg bid) or H<sub>2</sub>O (200 µl) and analyzed by flow cytometry using anti-CD3 and anti-NK1.1 mAb's. The absolute numbers of CD3<sup>+</sup>/NK1.1<sup>-</sup> T cells and CD3<sup>+</sup>/NK1.1<sup>+</sup> NK cells were deduced from the percentages obtained in 2 independent experiments and are indicated in the boxes (B). T lymphocytes were not activated during Gleevec oral feeding. In the CD3<sup>+</sup>/NK1.1<sup>-</sup> T cell gate, the CD69 expression is shown. (C) NK lymphocytes were activated during therapy with Gleevec. In the CD3<sup>+</sup>/NK1.1<sup>+</sup> NK cell gate, the CD69 expression is shown. Swiss<sup>nu/nu</sup> mice (D) and C57BL/6 littermates (E) were injected intraperitoneally with 10 µg of FL or 100 µl of PBS each day for 10 days. From day 7 to day 10, mice received either Gleevec (150 mg/kg bid) or H<sub>2</sub>O (200 µl). At day 11, all mice were sacrificed to analyze the expression of the NK activation marker CD69 on NK1.1<sup>+</sup> or DX5<sup>+</sup>/CD3<sup>-</sup> splenocytes. Positive controls included mice treated with rhIL-2 (1 × 10<sup>5</sup> IU intraperitoneally,