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### Article

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# Increased frequency of antigen-specific CD8<sup>+</sup> cytotoxic T lymphocytes infiltrating an Epstein-Barr virus-associated gastric carcinoma

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Gastric adenocarcinomas carrying Epstein-Barr virus (EBV) are known to be accompanied by massive lymphocyte infiltration. To characterize the tumor-infiltrating lymphocytes (TILs), we isolated and cultured such cells from a surgically resected EBV-associated gastric carcinoma. They were found to be positive for CD3, CD8, T-cell receptor  $\beta$  chain, and cytotoxic molecules. The isolated TILs consisted of human leukocyte antigen (HLA) class I-restricted CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), which killed autologous EBV-transformed cells (but not phytohemagglutinin blast cells) and recognized HLA-A24 as restriction molecules. However, the TILs did not recognize known EBV antigenic peptides presented by HLA-A24 molecules, nor HLA-A24<sup>+</sup> fibroblasts infected with vaccinia recombinant virus expressing each of the EBV latent proteins. EBV<sup>+</sup> gastric carcinomas do not express conventional target proteins of EBV-specific CTLs, and the data suggest that some cellular proteins may be involved in the strong T-cell response to EBV-associated gastric carcinoma. In addition, our data suggest that class I-restricted, antigen-specific CD8<sup>+</sup> CTLs are specifically expanded within EBV<sup>+</sup> gastric carcinoma tissue.

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## Introduction

Epstein-Barr virus (EBV) elicits a strong human leukocyte antigen (HLA) class I-restricted, antigen-specific CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) response in infected individuals (1). This response is believed to play an important role in controlling the virus during both primary infection and in the long-term carrier state. Markedly skewed epitopes are drawn mainly from EBNA3A, EBNA3B, and EBNA3C. Epitopes from 2 latent membrane proteins (LMP1 and LMP2A) and lytic-cycle antigens have also been reported (2-4).

It is well known that EBV is associated with several malignant diseases. These diseases include a proportion of Hodgkin's lymphomas, nasopharyngeal carcinomas, Burkitt's lymphomas, and immunoblastic lymphomas seen in immunocompromised hosts (5). EBV genomes have also been identified in some cases of T-cell lymphoma (6).

There is growing evidence that a subset of gastric carcinomas also carry EBV (7-14). Although gastric cancer is the most common malignant disease in Japan, the proportion associated with EBV is significantly higher in the United States (16%) than in Japan (6.9%) (8, 10). Involvement is significantly more frequent among males and in tumors in the upper part of the stomach. The incidence is highest in undifferentiated lymphoepithelioma-like carcinomas (80-90%), followed by moderately differentiated tubular adenocarcinomas and poorly differenti-

ated solid or medullary types (9-11). When EBV-encoded RNAs (EBERs) were applied for in situ hybridization, positive lesions were characterized by the presence of signals in almost all carcinoma cells and by the absence of signals in surrounding lymphocytes, other normal stromal cells, intestinal metaplasia, and normal gastric mucosa (8, 10, 11). PCR or Southern blot hybridization analysis revealed that a single genotype was present in each EBV-associated cancer (7, 9, 11), consistent with a clonal process, suggesting that EBV infection may occur before transformation and may be related to oncogenesis of EBV-associated gastric carcinomas (7, 9). EBV-associated gastric carcinoma expressed EBNA1 but not other latent infection proteins such as EBNA2, EBNA3A, EBNA3B, EBNA3C, and LMP1 (11), which are the target proteins of EBV-specific CTLs. There was no expression of lytic-infection gene products such as BZLF1, EA, or VCA (11, 14). Thus, the characteristic EBNA1-restricted EBV expression in gastric carcinoma is the same as that in Burkitt's lymphoma, which is believed to escape from surveillance by EBV-specific CTLs.

Saiki et al. (13) recently reported immunophenotypic characterization of EBV-associated gastric carcinoma using frozen tissue samples. They showed that (a) lymphocytes infiltrating EBV<sup>+</sup> tumor nests are predominantly CD8<sup>+</sup> T cells, many expressing perforin; (b) 4 times as many CD8<sup>+</sup> T cells infiltrate in EBV<sup>+</sup> cases as in EBV<sup>-</sup> cases; (c) the labeling index of Ki-67, a prolifera-

tion-associated antigen, in CD8<sup>+</sup> cells is 4 times higher in EBV<sup>+</sup> cases than in EBV<sup>-</sup> cases; (d) close contact between carcinomas and CD8<sup>+</sup> cells is evident; and (e) all EBV<sup>+</sup> and EBV<sup>-</sup> gastric carcinoma cells express MHC class I, whereas markers of immunological activation, such as MHC class II, ICAM-1, and Fas/Apo-1 expression, are more prominent in EBV<sup>+</sup> cases. Extensive lymphocyte infiltration is a consistent characteristic of EBV<sup>+</sup> gastric carcinoma, as supported by other authors (8, 9, 10, 12), and may be associated with the generally favorable prognosis of EBV<sup>+</sup> gastric carcinomas (12). The T cells may infiltrate, attracted with cytokines such as IL-1 secreted by the gastric cancer cells (15), or may clonally expand in response to some antigens presented by the carcinoma tissue. To determine whether the tumor-infiltrating lymphocytes (TILs) are antigen specific or nonspecific, functional studies are necessary. As far as we know, however, there has been no report of any functional analysis of TILs isolated from EBV<sup>+</sup> gastric carcinoma tissue.

We assume that the antigens recognized by gastric cancer TILs, of either viral or cellular origin, are shared by the EBV-transformed lymphoblastoid cell line (LCL), whereby the full array of EBV latent gene products are

translated. We address the question of whether the EBV<sup>+</sup> gastric cancer-associated antigens are expressed on autologous LCLs. In this paper, we demonstrate that the TILs isolated from an EBV<sup>+</sup> gastric carcinoma consist of HLA class I-restricted CD8<sup>+</sup> CTLs, which specifically recognize and kill autologous EBV-infected cells. The TILs did not kill HLA-A24<sup>+</sup> fibroblasts infected with vaccinia recombinant virus expressing each of the EBV latent proteins, suggesting that some cellular proteins may be recognized by them. Frequencies of such CD8<sup>+</sup> T cells in TILs were higher than those in PBMCs, indicating that class I-restricted, antigen-reactive CD8<sup>+</sup> CTLs are specifically expanded within an EBV<sup>+</sup> gastric carcinoma.

## Methods

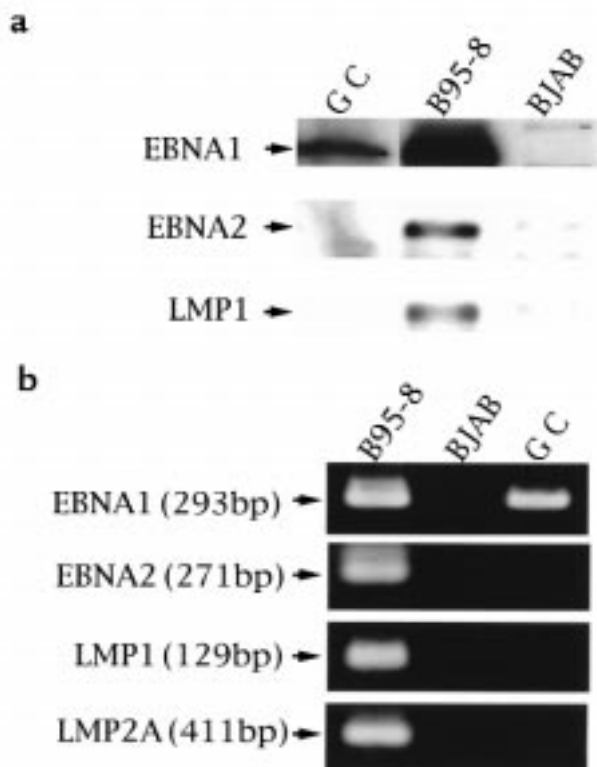
**Patient.** A 54-year-old Japanese man was diagnosed as suffering from a gastric tumor located in the cardia. The pathological diagnosis of a biopsy sample showed that it was a solid-type, poorly differentiated adenocarcinoma. Before starting the investigation, written informed consent was obtained from the patient after full explanation of the purpose and methods of the study. The study design had been approved by the Review Board of Aichi Cancer Center.

**PCR for EBV DNA.** DNA was extracted from the biopsy sample. B95-8 cells were the positive controls and BJAB cells were the negative controls. An EBV-specific primer was directed for amplification of 269 bp in the *Bam*HI-K region (16). PCR products were run on 0.9% agarose gel and stained with ethidium bromide.

**RNA preparation and RT-PCR.** Total RNA was extracted from cell pellets or tissue by using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The first-strand cDNA was synthesized from 1 µg of total RNA using the RNA LA PCR Kit (Takara Shuzo Co., Otsu, Japan). For the detection of EBV latent gene expression (EBNA1, EBNA2, LMP1, LMP2A), nested PCR was performed essentially as described previously (17). The conditions for the specific PCRs and primer sequences were described previously (17). Briefly, 2 µL of the cDNA was used as a template for the first PCR application using the PCR Amplification Kit (Takara Shuzo Co.). For nested PCR, 2 µL of the first PCR product was used as a template. The reaction was performed in an automated thermal cycler (Takara Personal; Takara Shuzo Co.). The nested PCR products were run on 1.8% agarose gel and stained with ethidium bromide.

**Immunoblotting.** Approximately 40 mg of frozen carcinoma tissue was lysed, sonicated, and boiled in 100 µL of lysis buffer. Two milligrams of the gastric carcinoma tissue was run on SDS-PAGE and analyzed by immunoblotting, using anti-EBNA1 polyclonal antibody and anti-EBNA2 and anti-LMP1 mAb's (DAKO Corp., Carpinteria, California, USA). Blots were then incubated with horseradish peroxidase-conjugated goat antibodies for the detection of bound antibodies. Immunoblots were developed using the ECL Western Blotting Detection System (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA). B95-8 ( $2 \times 10^5$ ) and BJAB cells ( $2 \times 10^5$ ) were run as EBV<sup>+</sup> and EBV<sup>-</sup> control cells, respectively.

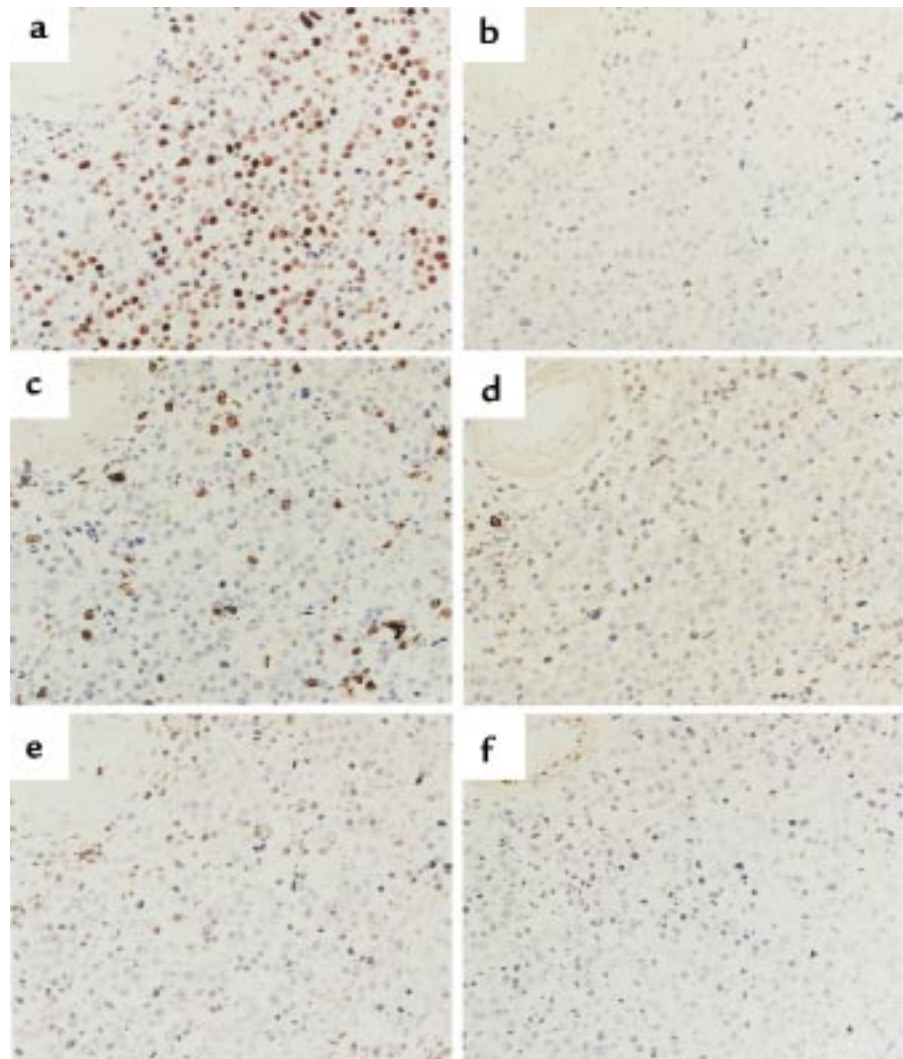
**Immunohistochemistry and in situ hybridization.** Immunostaining was performed on formalin-fixed paraffin sec-



**Figure 1** Immunoblot analysis of EBV latent protein expression. Protein blots were probed with an anti-EBNA1 polyclonal antibody and mAb's for EBNA2 and LMP1. Whole-cell lysate of the gastric cancer tissue (GC) was run simultaneously with that of B95-8 cells as an EBV<sup>+</sup> control and that of BJAB cells as an EBV<sup>-</sup> control. (b) EBV gene expression in the gastric carcinoma tissue, as assessed by RT-PCR analysis. Total RNA extracted from B95-8 cells, BJAB cells, and the gastric carcinoma tissue (GC) was reverse transcribed and subjected for nested PCR amplification with primers specific for EBNA1, EBNA2, LMP1, or LMP2A.

**Figure 2**

CD8<sup>+</sup> T cells with  $\alpha\beta$  T-cell receptors and cytotoxic molecules infiltrating into the EBV<sup>+</sup> gastric carcinoma. Corresponding areas of serial sections of tissue can be seen in **a-f**. Carcinoma cells were positive for EBER1 by in situ hybridization (**a**) and were negative for the ZEBRA protein by immunostaining (**b**). The infiltrating lymphocytes were positive for CD8 (**c**), the  $\beta$  chain of the T-cell receptor (**d**), and TIA-1, a cytotoxic molecule (**e**); they were negative for CD57, a natural killer cell marker (**f**).



tions, using an avidin-biotin peroxidase complex method (18) with specific mAb's. The mAb's were purchased from the following companies: CD3, CD4, CD56, and CD57 were from Becton Dickinson Immunocytometry systems (San Jose, California, USA); CD8, CD20, CD79a, and the ZEBRA protein of EBV were from DAKO Corp.; and TIA-1 and the  $\beta$  chain of the T-cell receptor were from Immunotech (Marseilles, France). Detection of EBERs was performed using FITC-labeled EBER oligonucleotides and a hybridization kit (DAKO Corp.). Hybridization products were detected using an anti-FITC mouse mAb (DAKO Corp.) and a VectaStain ABC kit (Vector Laboratories, Burlingame, California, USA).

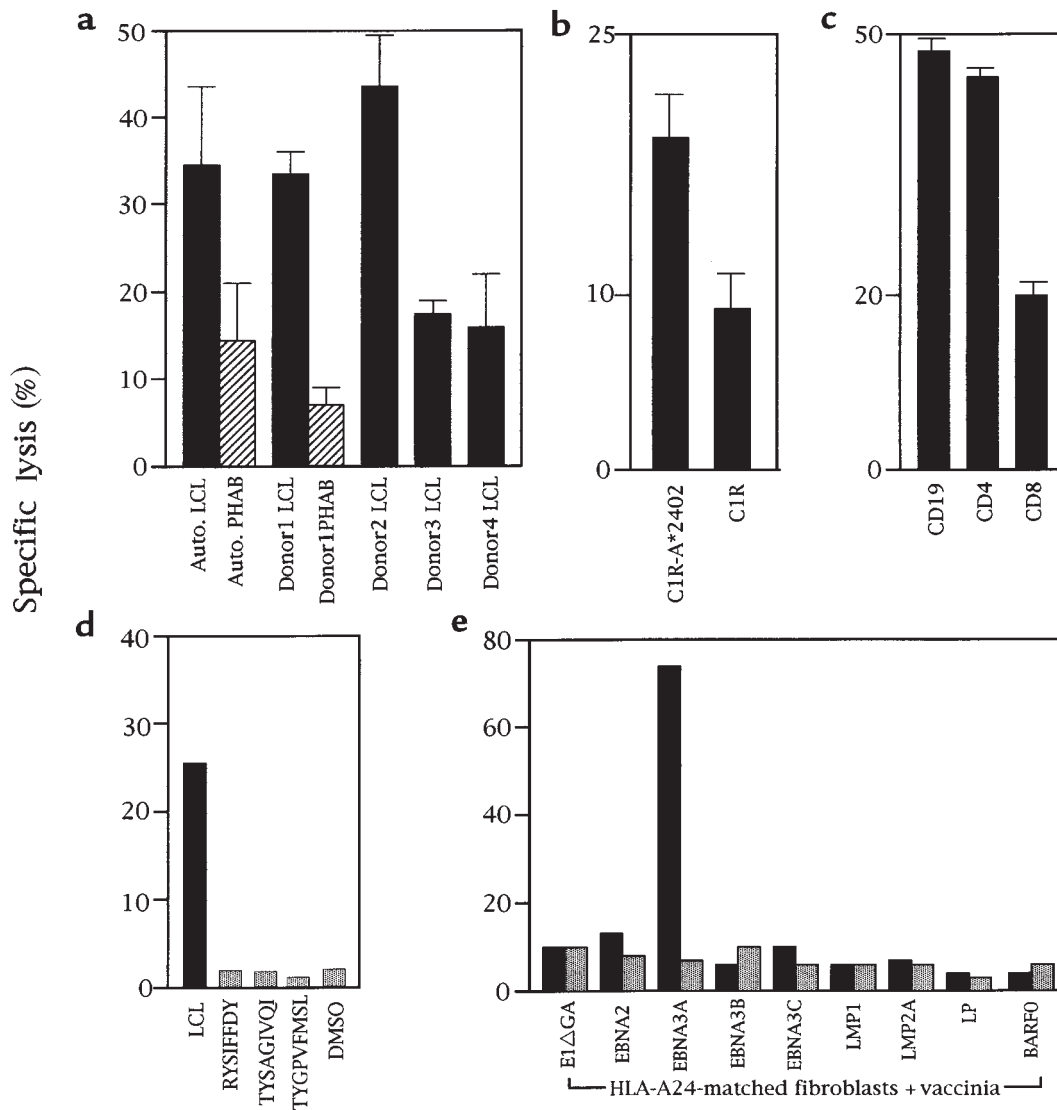
**Isolation and culture of TILs.** Surgically resected tumor tissues were finely minced with a disposable blade, and mononuclear cells were purified by centrifugation on Ficoll-Hypaque density gradients (Pharmacia Biotech AB, Uppsala, Sweden). After 3 washes in PBS, cells were seeded in 24-well plates (Corning-Costar Corp., Cambridge, Massachusetts, USA) and cultured in Iscove's modified Dulbecco's medium (GIBCO BRL, Grand Island, New York, USA) supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin,  $5 \times 10^{-5}$  M  $\beta$ -

mercaptoethanol, and 10% heat-inactivated FCS (culture medium; HyClone Laboratories, Logan, Utah, USA) in the presence of 100 U/mL recombinant IL-2. Simultaneously, wells supplemented with 1  $\mu$ g/mL of cyclosporine A instead of IL-2 were prepared in an attempt to establish cancer cell lines and/or spontaneous LCL, and were cultured for up to 4 weeks.

**Flow cytometry.** TILs were incubated with FITC- or phycoerythrin-labeled mAb's specific to CD3, CD4, CD8, or CD16 antigens (Immunotech). Stained cells were analyzed by FACScan (Becton Dickinson Immunocytometry Systems) using Lysis II software.

**Preparation of LCLs, an EBV-specific CTL line, and PBMCs activated by PHA and IL-2.** LCLs were prepared from the patient and several volunteers by transforming PBMCs with B95-8 cell culture supernatant as described previously (19). An EBV-specific CTL line was established by stimulating PBMCs repeatedly with irradiated autologous LCLs as described previously (19). Aliquots of PBMCs were activated with a 6,000:1 dilution of phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, Michigan, USA) in culture medium as targets for CTL assays. Aliquots of PHA-activated cells from the patient's PBMCs were further cultured with 100 U/mL IL-2 for 2





**Figure 3**

Characterization of TILs. (a) The TILs recognized and killed autologous LCLs, but not PHA blast cells. HLA-A24<sup>+</sup> allogeneic LCLs (donors 1 and 2) were killed more efficiently than HLA-A24<sup>-</sup> LCLs (donors 3 and 4). (b) The TILs killed HLA-A\*2402-transfected EBV<sup>+</sup> C1R cells more efficiently than untransfected C1R cells. (c) Cytotoxicity of TILs is largely mediated by the CD8<sup>+</sup> population. Aliquots of TILs were reacted with anti-CD19<sup>-</sup>, anti-CD4<sup>-</sup>, and anti-CD8<sup>-</sup>-coated magnetic beads, and unbound cells were resuspended in the same volume and used as effector cells. Autologous LCLs were used as targets. The effector/target ratio was 20:1 before the magnetic bead treatment. Results are mean  $\pm$  SD in a-c. (d) TILs did not recognize known antigenic peptides presented by HLA-A24 molecules. The peptide derived from EBNA3A (RYSIFFDY), EBNA3B (TYSAGIVQI), or LMP2A (TYGPVFMSL) was pulsed on <sup>51</sup>Cr-labeled HLA-A24<sup>+</sup> PHA blast cells used as targets. Filled and dotted bars indicate EBV-transformed cells and PHA blast cells, respectively. (e) TILs did not recognize any EBV latent proteins presented by HLA-A24 molecules. HLA-A24<sup>+</sup> fibroblasts were infected with each of the vaccinia recombinants expressing the EBV latent proteins. E1ΔGA is a recombinant virus that expresses a glycine-alanine repeat-deleted form of EBNA1. LP represents the EBV leader protein. Filled bars indicate results using EBV-specific CTLs as effectors. Dotted bars indicate results using the TILs. The effector/target ratio was 20:1 in a, d, and e, and 10:1 in b.

weeks (IL-2-activated PBMCs) and used as effectors for several assays as controls for TILs. Antigen-specific CTLs can proliferate, retaining their specificity for antigens, upon stimulation with PHA and IL-2 (20).

**HLA class I typing.** Class I tissue typing was performed on lymphocytes using classic serological methods. The results of the typing are summarized in Table 1.

**Peptides.** EBV peptides known to be presented by HLA-A24 molecules to CTLs were synthesized and analyzed by Sawady Technology Co. (Tokyo, Japan). The amino acid sequences were as reported earlier (1, 2): RYSIFFDY

(EBNA3A), TYSAGIVQI (EBNA3B), and TYGPVFMSL (LMP2A). Each peptide was >95% pure. The peptides were dissolved first in DMSO, and CTL assays were carried out in the presence of the peptide (20  $\mu$ g/mL). The same concentration of DMSO used for peptide solution was added as a control of target-cell toxicity.

**Vaccinia virus recombinants and fibroblast infection.** Vaccinia virus recombinant coding for each of the EBV latent proteins (1-3) were kind gifts from A.B. Rickinson (University of Birmingham, Birmingham, United Kingdom). E1ΔGA is a recombinant virus that expresses a

glycine-alanine repeat-deleted form of EBNA1 (21). A previous work has suggested that the presence of the repeat prevents the protein from being presented to the CD8<sup>+</sup> CTL repertoire (22). For cytotoxicity assays, HLA-A24<sup>+</sup> fibroblasts obtained by skin biopsy were infected for 2 hours with recombinant vaccinia virus at an moi of 10, and were incubated in culture medium for an additional 16 hours before being trypsinized and used as targets in a cytotoxic assay.

**CTL assay.** CTL assays were performed using <sup>51</sup>Cr release as described previously (19). Briefly, effector cells were washed once and adjusted to the desired cell concentration in fresh culture medium. Radiolabeled target cells (2,500 cells/100 μL) were seeded in 96-well, V-bottom plates (Corning-Costar Corp.). Each assay was performed in triplicate. After a 5-hour incubation in a humidified incubator at 37°C, the supernatant was harvested and its radioactivity counted with a gamma counter. Percent specific lysis was calculated as follows: percent specific lysis = (experimental lysis - minimum lysis) × 100/(maximum lysis - minimum lysis). Minimum lysis was obtained by incubating the target cells with the culture medium alone. Maximum lysis was obtained by exposing the target cells to 1% Nonidet P-40. For deletion of parts of effector cells, immunomagnetic beads were used according to the manufacturer's instructions (Dynabeads M-450 CD4, CD8, and CD19; DYNAL, Oslo, Norway). As target cells, EBV-carrying C1R and C1R-A\*2402 cells (HLA-A\*2402-transfected C1R cells; kindly provided by M. Takiguchi, Kumamoto University, Kumamoto, Japan) (23) were employed.

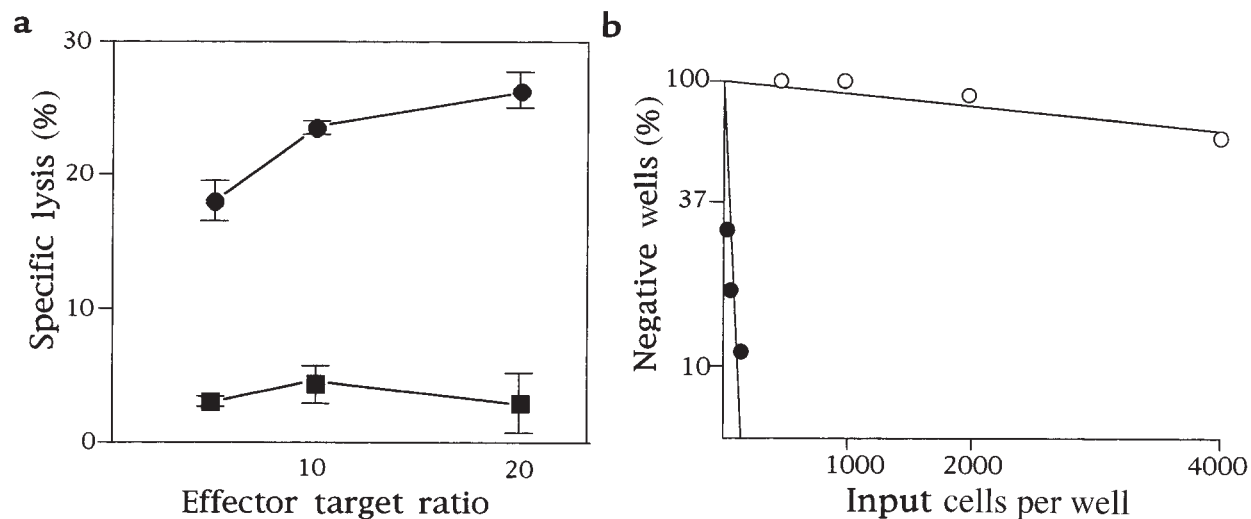
**Detection of precursor frequency of CTLs by limiting dilution assay.** Before the assays were performed, CD8<sup>+</sup> T cells were isolated by positive selection with M-450 CD8 immunomagnetic beads (DYNAL). Detachment of immunomagnetic beads from isolated cells was achieved by using DETACHaBEAD CD4/CD8 (DYNAL). Isolated cells were >95% CD8<sup>+</sup> according to FACS analysis. Lim-

**Table 1**  
HLA class I types of the patient and donors

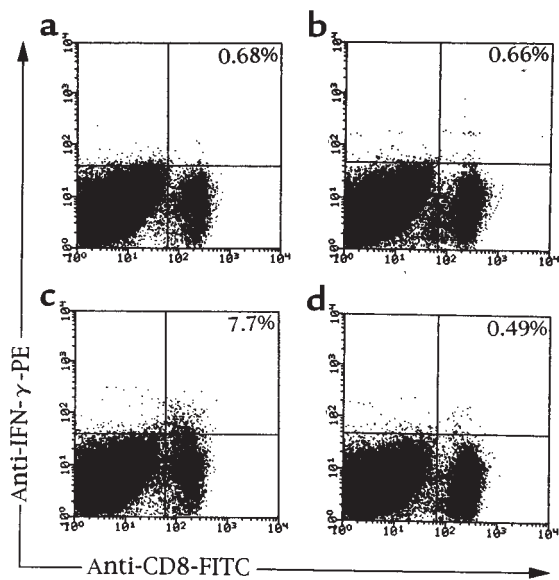
Patient	HLA class I type		
	A24	B52 B62	Cw1
Donor 1	A2 A24	B46 B61	Cw1 Cw3
Donor 2	A24 A26	B52 B62	Cw3
Donor 3	A11 A26	B62	Cw3 Cw4
Donor 4	A2 A31	B51	Cw3

iting dilution assays (LDAs) were performed using a modification of the methods reported previously (4). Twenty-four wells were used per dilution of isolated CD8<sup>+</sup> cells in 96-well, U-bottom plates (Corning-Costar Corp.) with 2 × 10<sup>4</sup> irradiated autologous LCLs and 1,000 PBMCs per well, containing a total volume of 200 μL in the presence of IL-2 (40 U/mL). Twenty-four wells of irradiated LCLs and 1,000 PBMCs were used only as a control. On day 7, an additional IL-2 was added. CTL assays were carried out on day 12. To assay CTL precursor frequency, 100 μL of each well was transferred into a well containing <sup>51</sup>Cr-labeled 2,500 LCLs. After a 5-hour incubation, the supernatant was harvested and its radioactivity was counted. Percent specific lysis of each well was calculated using mean spontaneous release for target cells from 24 wells containing no effector cells as minimum lysis. Wells were scored as positive for CTL recognition if the level of specific lysis exceeded 3 SDs above the mean spontaneous release for target cells. Frequency values were determined from the cell input number at which 37% of the wells were negative, using the method of maximum likelihood (4).

**Detecting IFN-γ-producing CD8<sup>+</sup> T cells in response to LCLs by flow cytometry.** For determination of CD8<sup>+</sup> antigen-specific T-lymphocyte frequency, intracellular cytokine staining using flow cytometry was performed as described previously, with slight modifications (24).



**Figure 4**  
(a) Cytotoxicity toward autologous LCLs of TILs (filled circles) and IL-2-activated PBMCs (filled squares). (b) Precursor frequency of antigen-specific CD8<sup>+</sup> CTLs reactive to autologous LCLs in TILs (filled circles) and in PBMCs (open circles), as determined by LDA.



**Figure 5** Intracellular IFN- $\gamma$  staining with flow cytometry. The TILs (a and c) and PBMCs (b and d) were cultured for 5 hours either with (c and d) or without (a and b) autologous LCLs, and were stained for intracellular IFN- $\gamma$ . Unstimulated cells were mixed with the same number of autologous LCLs before staining with mAb's. The percentage of CD8<sup>+</sup> T cells positive for the IFN- $\gamma$  stain is shown in the top right corner of each panel.

Briefly, TILs or PBMCs were resuspended at a concentration of  $2 \times 10^6$ /mL in culture medium. Autologous and HLA-disparate LCLs were resuspended at a concentration of  $10^6$ /mL in the culture medium. Aliquots of the responder cells (0.5 mL) and LCLs (0.5 mL) were mixed in culture tubes ( $16 \times 125$  mm; Becton Dickinson Labware, Lincoln Park, New Jersey, USA) in the presence of 1  $\mu$ L of IC Block (BioSource International, Camarillo, California, USA) and were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 5 hours. As an unstimulated control, the same number of responder cells and LCLs were separately incubated in the presence of the IC Block and mixed thereafter. The cells were fixed with IC Fix (BioSource International), permeabilized with IC Perm (BioSource International), and stained with phycoerythrin-labeled anti-IFN- $\gamma$  and FITC-labeled anti-CD8 mAb's (BioSource International). Stained cells were analyzed by FACScan using the Lysis II software. Live gating of lymphocytes was performed, and up to 50,000 events were acquired for each analysis.

**Enzyme-linked immunospot assay.** An enzyme-linked immunospot assay (ELISPOT) was performed as described previously, with slight modifications (25), using 96-well MultiScreen-HA plates with a nitrocellulose base (Millipore Corp., Bedford, Massachusetts, USA) coated with 10  $\mu$ g/mL of anti-IFN- $\gamma$  mAb (Genzyme Pharmaceuticals, Cambridge, Massachusetts, USA). After blocking each well with culture medium, irradiated autologous LCLs ( $10^5$ ) were seeded into each well, and isolated CD8<sup>+</sup> T cells (as described above) were seeded in graded numbers of 10,000, 50,000, 2,500, 1,250, 625, 313, and 156 per well in the presence of 50 U/mL IL-2. Each

dilution was seeded in triplicate. To determine spontaneous IFN production, 10,000 CD8<sup>+</sup> cells were incubated without LCL, which yielded <3 spots per well. The plates were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 28 hours and extensively washed with PBS containing 0.05% Tween-20. The detection antibody, polyclonal rabbit anti-IFN- $\gamma$  (250:1; Genzyme Pharmaceuticals), was then added to individual wells. After incubation at room temperature for 90 minutes, the plates were washed and further incubated with 1:800 dilution of peroxidase-conjugated goat anti-rabbit IgG (Genzyme Pharmaceuticals) at room temperature for 90 minutes. To view IFN- $\gamma$ -specific spots, 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved in *N,N*-dimethylformamide (Sigma Chemical Co.) diluted in 0.1 M sodium acetate buffer (pH 5.0) with 0.015% H<sub>2</sub>O<sub>2</sub>, and 100  $\mu$ L was added to each well. After 30–40 minutes, the reaction was stopped by washing with water, and the plates were dried. The bottom membranes were photographed and spots were counted. Diffuse large spots were counted as specific because some wells containing only LCLs produced tiny spots. Wells that yielded 20–100 specific spots were counted to calculate the percent frequency of antigen-specific CD8<sup>+</sup> T cells; percent frequency = number of spots  $\times$  100/number of input CD8<sup>+</sup> cells.

## Results

**Virological and histological features of the gastric carcinoma.** DNA extracted from the gastric carcinoma tissue was positive for EBV sequences by PCR analysis (data not shown). Immunoblot analysis revealed that the carcinoma tissue expressed EBNA1 protein, but not EBNA2 or LMP1 protein (Figure 1a). RT-PCR analysis showed that the gastric carcinoma tissue expressed EBNA1 mRNA, but not EBNA2, LMP1, or LMP2A mRNAs (Figure 1b). This pattern of the EBV latent gene expression (latency I) in gastric carcinoma tissues is consistent with results reported previously (11, 14).

Using surgical tissue sections, definite labeling for EBERS was observed in nuclei of almost all cancer cells (Figure 2a). No lymphocytes or stromal cells were positive for EBERS. There was no signal when ZEBRA protein, an EBV lytic protein, was stained with a specific mAb, indicating that almost all the carcinoma cells were latently infected with EBV (Figure 2b). Most TILs infiltrating within both stromal and cancer tissues were positive for CD8 and the  $\beta$  chain of the T-cell receptor (Figure 2, c and d). A cytotoxic molecule, TIA-1, was found to be present (Figure 2e), suggesting cytotoxic potential. There were some CD4<sup>+</sup> T cells and B cells (data not shown), but only a few cells were positive for the natural killer cell markers CD57 (Figure 2f) and CD56 (data not shown).

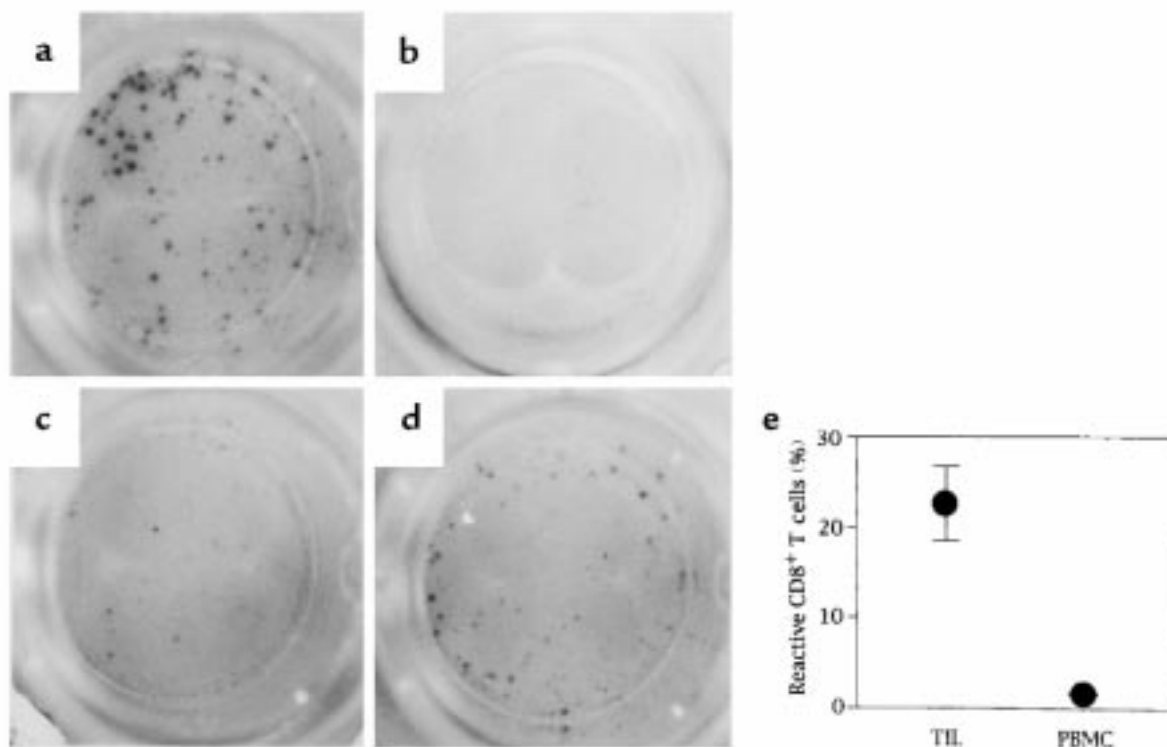
**Expansion of TILs from the EBV-associated gastric carcinoma.** Around day 3 after starting TIL culture, it was observed that clumps of cancer cells were surrounded by lymphocytes with the appearance of a rosette formation. Around 1–3 weeks after starting the culture, the TILs had expanded well. No tumor cell line or LCL was established from wells supplemented with cyclosporine A instead of IL-2, indicating that there

were few free virions produced by the EBV<sup>+</sup> gastric cancer cells to transform infiltrating B cells. During the culture, tumor cells disappeared gradually from the wells. The surface phenotype of the TILs at day 15 was as follows: CD3 = 83.2%; CD4 = 34.5%; CD8 = 52.6%; and CD16 = 3.8%.

**Characterization of the TILs.** Data for cytotoxicity toward autologous and allogeneic LCLs are shown in Figure 3a. PHA blast cells were also tested as targets negative for EBV. The TILs had significant cytotoxicity toward the autologous LCLs, compared with HLA class I incompatible LCLs and autologous PHA blast cells (Figure 3a and Table 1). Allogeneic LCLs with HLA-A24 molecules were more sensitive to TIL-mediated cell lysis than those without the molecules. Allogeneic PHA blast cells positive for HLA-A24 were weakly killed by the TILs. To confirm the HLA-A24 restriction, a CTL assay using HLA-A24-transfected EBV<sup>+</sup> lymphoblastoid cells (C1R-A\*2402) was performed. Cytotoxicity toward C1R-A\*2402 cells was significantly higher than toward untransfected C1R cells (Figure 3b). To determine the effector subpopulation mediating the target-cell lysis, cell depletion of TILs was accomplished using mAb-coupled magnetic beads. The results showed that the CD8<sup>+</sup> population had the main cytotoxic activity (Figure 3c). These observations showed that a part of the TILs consisted of HLA class I (A24)-restricted CD8<sup>+</sup> CTLs specifically recognizing EBV-infected cells.

It is well known that the EBV latent proteins such as EBNA3A, EBNA3B, EBNA3C, and LMP2A are major target proteins of EBV-specific CTLs (1, 2). However, EBV<sup>+</sup> gastric carcinomas expressed only EBNA1 protein. To examine the possibility that the TILs recognize epitopes in LMP2A, EBNA3A, and EBNA3B proteins presented by HLA-A24, we tested these peptides for their ability to sensitize HLA-A24<sup>+</sup> and EBV<sup>-</sup> target cells for the TIL-mediated target-cell lysis. Donor 1 PHA blast cells were used as the target cells. As shown in Figure 3d, all the peptides failed to sensitize the target cells at a concentration of 20 μg/mL. Then, to identify the EBV latent proteins recognized by the TILs, HLA-A24<sup>+</sup> fibroblasts infected with recombinant vaccinia virus expressing each of the EBV latent genes were used for the TIL-mediated target-cell lysis. The target cells infected with recombinant vaccinia virus expressing EBNA3A proteins were recognized by a polyclonal EBV-specific CTL line obtained from the patient, confirming EBV antigen presentation on the target cells (Figure 3e). As shown in Figure 3e, however, the recombinant vaccinia virus failed to sensitize the target cells, therefore indicating that none of the latent proteins were recognized by the TILs.

**Antigen-specific CD8<sup>+</sup> T cells are specifically activated in TILs.** Because the patient's PBMCs contained EBV-specific CTL precursors, the TILs and PBMCs were compared for their cytotoxic activity against the autologous



**Figure 6**

ELISPOT results. A higher frequency of antigen-specific CD8<sup>+</sup> T cells reactive to autologous EBV-transformed cells was found in TILs than in PBMCs. IFN-γ-specific spots are evident in wells into which 313 CD8<sup>+</sup> TILs (a), no T lymphocytes (b), 313 CD8<sup>+</sup> peripheral T lymphocytes (c), and 2,500 CD8<sup>+</sup> peripheral T lymphocytes (d) were introduced. Each well contained 10<sup>5</sup> irradiated autologous EBV-transformed cells as antigen-presenting cells. (e) The mean percentage of the antigen-specific T cells in CD8<sup>+</sup> cells. Bars indicate SD.



LCLs. For this purpose, IL-2-activated PBMCs were prepared as a control; they had minimal cytotoxicity toward the autologous LCLs (Figure 4a). The frequency of the antigen-specific CD8<sup>+</sup> T cells was evaluated by LDA (Figure 4b), intracellular IFN- $\gamma$  staining with flow cytometry (Figure 5), and the ELISPOT assay (Figure 6). Precursor frequencies of CD8<sup>+</sup> CTL determined by the LDA were 1 in 51.4 (1.9%) and 1 in 8,000 (0.013%) for the TILs and PBMCs, respectively (Figure 4b). Intracellular IFN- $\gamma$  staining to detect antigen-specific CD8<sup>+</sup> cells showed ~7% of CD8<sup>+</sup> T cells in TILs to be reactive to the autologous LCLs (Figure 5). This reaction was class I restricted because the number of IFN- $\gamma$ -producing CD8<sup>+</sup> cells stimulated by HLA class I-incompatible LCLs was reduced to 1.6% (graphical data not shown). Finally, the ELISPOT assay revealed the highest frequency: 22.8% of CD8<sup>+</sup> cells in TILs were antigen-specific versus 1.5% in PBMCs, as determined for specific IFN- $\gamma$  production (Figure 6). Data on antigen-specific T-cell frequencies are summarized in Table 2. The findings indicated that class I-restricted CD8<sup>+</sup> T cells reactive to autologous EBV-infected cells expanded and were activated to present a cytolytic phenotype in the gastric carcinoma tissue.

## Discussion

The present study revealed class I-restricted CD8<sup>+</sup> T cells reactive to EBV-infected cells infiltrating in gastric carcinoma tissue. The frequency of antigen-specific CD8<sup>+</sup> T cells was found to be elevated in TILs, relative to the patient's PBMCs, as shown by LDA, intracellular IFN- $\gamma$  staining, and ELISPOT assay. Differences in values with these 3 assays probably reflect different sensitivities. These T cells were supposed to be stimulated by the cancer cells directly. Because no spontaneous LCLs were established in wells supplemented with cyclosporine A, it was unlikely that the TILs were stimulated by such LCLs *in vitro*. To support this, no EBV latent protein presented by HLA-A24 molecules was recognized by the TILs (Figure 3, d and e). Our results have extended the immunohistological observation of frozen samples of EBV<sup>+</sup> and EBV<sup>-</sup> gastric carcinomas reported by Saiki et al. (13). The authors demonstrated that higher numbers of activated CD8<sup>+</sup> T cells infiltrated in EBV<sup>+</sup> carcinomas than in EBV<sup>-</sup> carcinomas, and that EBV<sup>+</sup> carcinomas expressed HLA class II molecules more prominently than EBV<sup>-</sup> carcinomas. IFN- $\gamma$  upregulates HLA class II molecules in gastric cancer cell lines (26). We showed that the TILs secreted IFN- $\gamma$  upon antigenic stimulation, and this could be one of the sources that upregulate HLA class II expression on gastric cancer cells *in vivo*.

One puzzling aspect regarding EBV-associated gastric carcinomas is the contrast between massive infil-

tration of CD8<sup>+</sup> T cells and the very limited expression of EBV latent genes (11, 14). In our case, immunoblot analysis revealed that only EBNA1 protein was expressed; RT-PCR analysis showed that gene expression was restricted to EBNA1. This expression pattern is essentially the same as the results reported previously (11, 14), and similar to the latency I represented by Burkitt's lymphoma (7). There are only 3 viral genes expressed in latency I: EBERs, EBNA1, and BARF0. First, EBERs are not considered to be translated, so it is unlikely that antigenic peptides are encoded in the EBV genes. Second, EBNA1 is reported to escape from the endogenous antigen-presentation pathway because of unknown functions of its glycine-alanine repeats (22). Finally, expression of BARF0 protein is too low to be recognized by the CD8<sup>+</sup> CTL repertoire (27). Thus, it is not likely that any of the 3 gene products effectively stimulate the TILs. Indeed, in our study, the TILs did not kill fibroblasts expressing glycine-alanine repeat-deleted EBNA1 or BARF0 (Figure 3e) proteins. Another possibility is that some other EBV latent proteins, even if not detectable by Western blotting, might be expressed in amounts sufficient to elicit the CTL response. This possibility is purely speculative, however; as far as we tested, no EBV latent protein was recognized by the TILs through HLA-A24 molecules (Figure 3, d and e).

There are 2 other possible explanations for the aggressive CTL response to EBV<sup>+</sup>-infected cells. First, some cellular proteins, which are upregulated as a result of 1 or more combinations of the type I EBV latent gene expression and are necessary for cancer cell maintenance, may be recognized by the TILs. In this case, the level of protein expression should exceed the threshold that breaks the immunological tolerance or anergy. Second, infection of tumor cells with EBV may increase their ability to elicit immunity against their tumor-specific antigens through the procedure known as tumor xenogenization (28, 29). For example, a poorly immunogenic tumor transfected with an influenza virus hemagglutinin gene was shown to generate effective transplantation immunity against the original tumor cells (28). Cytokines produced by either virus-specific T cells or tumor cells upon infection should create an environment that enables T lymphocytes of lower affinity to respond to tumor-rejection antigens (30).

Together, our data indicated expansion of class I-restricted, antigen-specific CD8<sup>+</sup> CTLs within EBV<sup>+</sup> gastric carcinoma tissue. Because EBV<sup>+</sup> gastric carcinomas do not express conventional target proteins of EBV-specific CTLs, the results suggest that some cellular proteins may be involved in the strong CD8<sup>+</sup> T-cell response to EBV-associated gastric carcinoma. Molecular cloning of such genes may explain why massive CD8<sup>+</sup> T-cell accumulation occurs selectively in EBV<sup>+</sup> gastric cancer tissue, where conventional target proteins of EBV-specific CTLs are absent or scarce. Identification of such antigens will be important in establishing immunotherapy for EBV<sup>+</sup> gastric cancer, Burkitt's lymphomas, nasopharyngeal carcinomas, and T-cell lymphomas where EBV latent gene expression is limited.

**Table 2**  
Summary of data for frequencies of CD8<sup>+</sup> antigen-specific T cells

Effector cells	LDA	Intracellular IFN- $\gamma$	ELISPOT
TILs	1.9 <sup>A</sup>	7.0	22.8
PBMCs	0.013	ND	1.5

<sup>A</sup>Percent frequency. ND, not detected.

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