Analysis of a Chronic Myelogenous Leukemia Patient Vaccinated with Leukemic Dendritic Cells Following Autologous Peripheral Blood Stem Cell Transplantation

Shin-ichiro Fujii,[1, 4] Kanako Shimizu,[2] Koji Fujimoto,[1] Tetsuyuki Kiyokawa,[1] Taizo Shimomura,[2] Osamu Taniguchi,[3] Moritoshi Kinoshita[3] and Fumio Kawano[1]

[1]The Center for Bone Marrow Transplantation and Immunotherapy, Institute for Clinical Research, Kumamoto National Hospital, 1-5 Ninomaru, Kumamoto 860-0008, [2]Second Department of Internal Medicine, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-8556 and [3]Gene-Diagnostic Center, Otsuka Assay Laboratories, Otsuka Pharmaceutical Co., 224-18 Hiraishi Aza Imino, Kawauchi, Tokushima 771-0195

Dendritic cells (DCs) are believed to be the most potent antigen-presenting cells and may be important in the induction of anti-leukemia specific T cell responses. In this preliminary clinical study, a patient with chronic phase chronic myelogenous leukemia (CML) was vaccinated with autologous leukemic DCs following autologous peripheral blood stem cell transplantation (PBSCT). In an *in vitro* study, leukemic DCs were generated using granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α, and interleukin-4 from granulocyte colony-stimulating factor (G-CSF)-mobilized PBSC fraction of this patient, and were found to be Ph1$^+$, and to possess the morphologic and phenotypic characteristics of mature DCs. These cells could also elicit antigen-specific immune responses, including a vigorous cytotoxicity specific to CML cells. In the clinical experiment, we obtained evidence that infused leukemic DCs could induce T cell clones expressing the same T cell receptor usage as a cytotoxic T cell line, suggesting that the immune repertoire includes tumor-reactive T cells. These cytotoxic T lymphocytes are activated *in vivo*. The vaccination of leukemic DC caused a decrease in the number of Ph1$^+$ cells in the peripheral blood and bone marrow. These results indicate that the activity is an immunologically mediated phenomenon and vaccination therapy with leukemic DC following autologous PBSCT may be effective in treating CML.

Key words: Dendritic cells — Vaccination — Chronic myelogenous leukemia chronic phase — T cell receptor usage — Peripheral blood stem cell transplantation

While allogeneic bone marrow transplantation (allo-BMT) offers the only known possibility of a cure in patients with chronic myelogenous leukemia (CML), its use is generally restricted to individuals under the age of 45 years for whom there exist donors who are human leukocyte antigen (HLA)-identical. Thus, only a small percentage of patients with CML may obtain benefit from such treatment. In addition, this therapy may often be refused by patients because of the risk of life-threatening infections and of acute and chronic graft-versus-host disease (GVHD). A recent clinical study showed that the treatment of CML patients with interferon-α (IFN-α) was beneficial in some cases.[1] In particular, we demonstrated that in IFN-α-responsive patients, there is an enhancement of the accumulation of Vβ 9$^+$ and Vβ 20$^+$ T cells.[2] CML patients in the chronic phase who cannot undergo allo-BMT or who are unresponsive to IFN-α[3, 4] may benefit from autografting.[5] The success of auto-peripheral blood stem cell transplantation (auto-PBSCT) has been shown to depend on the proportion of malignant progenitors to nor-

mal cells. Peripheral blood stem cells (PBSCs) from these patients may require the *ex vivo* purging of the malignant progenitors or the *in vivo* selection of Ph1$^-$ PBSCs by priming and mobilization with antileukemia agents,[6–8] prior to harvesting and transplantation. While Ph1$^-$ graft cells for transplantation have been harvested from PBSC following intensive chemotherapy,[8] the complete elimination of residual clonogenic malignant cells from the marrow after PBSCT is too difficult, since auto-PBSCT is mainly associated with the absence of a graft-versus-leukemia (GVL) reaction.

Cure for CML by auto-PBSCT requires the elimination of residual CML cells following transplantation. A critical component in the generation of antileukemic cytotoxic T lymphocytes (CTLs) is the presentation of leukemia-associated antigens by potent antigen-presenting cells.[9, 10] Dendritic cells (DCs) are the most effective antigen-presenting cells, being capable of inducing primary responses from naive T cells.[11] In the present study, we generated a large number of leukemic DCs, i.e., DCs of leukemic origin, by using granulocyte colony-stimulating factor (G-CSF) to mobilize PBSC,[12] and we investigated whether these cells effectively present leukemia antigens to T lymphocytes

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[4] To whom correspondence should be addressed.
 E-mail address: fujii1018@aol.com

and activate antileukemic CTL without the addition of exogenous antigens.[13] We also characterized the efficacy of this novel procedure for treating patients with CML.

MATERIALS AND METHODS

Patient and clinical course We studied a 47-year-old Japanese woman with chronic phase CML within one year of diagnosis. This patient was selected for study because she had responded poorly to IFN-α and was unwilling to undergo allo-BMT. Cytogenetic analysis of bone marrow (BM) cells from this patient showed that 100% of the analyzed metaphases carried the t(9;22) translocation. After we had obtained informed consent, samples of peripheral blood (PB) and BM were collected from this patient and an HLA-matched sibling donor (HLA-A(2, 24), B(35, 51), Cw(3), DRB1(04), DQB1(04)). As a vaccination study, one and a half month after PBSCT subsequent to high-dose chemotherapy (ICE therapy), this patient was given four injections of leukemic DCs at weekly intervals (Fig. 1). The protocol was reviewed and approved by the institutional review board of the Kumamoto National Hospital. To prevent undesirable effects due to remaining cytokines

in the DC suspension prepared for infusion, we measured the remaining cytokines by enzyme-linked immunosorbent assay (ELISA) in the supernatant of cultured cells after washing out the cytokine with cytokine-free medium three times. We also evaluated the side effects by measuring the anti-autologous antibody, for example, anti-nuclear antibody, anti-thyroglobulin antibody, and immunoglobulins (IgG, IgM) as well as delayed-type hypersensitivity (DTH) reaction every week.

Generation of dendritic cells PBSCs were harvested three times from a G-CSF mobilized CML patient. An aliquot of each PBSC preparation diluted 1:3 in RPMI-1640 (GIBCO Laboratories, Grand Island, NY), was centrifuged using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and the PBSCs from the interface were washed with serum-free medium. Leukemic DCs were generated from our patient by culturing PBSCs (2×10^6/ml) at 37°C in endo-toxin-free 75-cm^2 tissue culture flasks (Iwaki Inc., Chiba) in RPMI-1640 that contained 100 U/ml penicillin, 100 μg/ml kanamycin, 2 mM L-glutamine, and 10% heat-inactivated autologous serum (complete medium (CM)), supplemented with 100 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Gen-
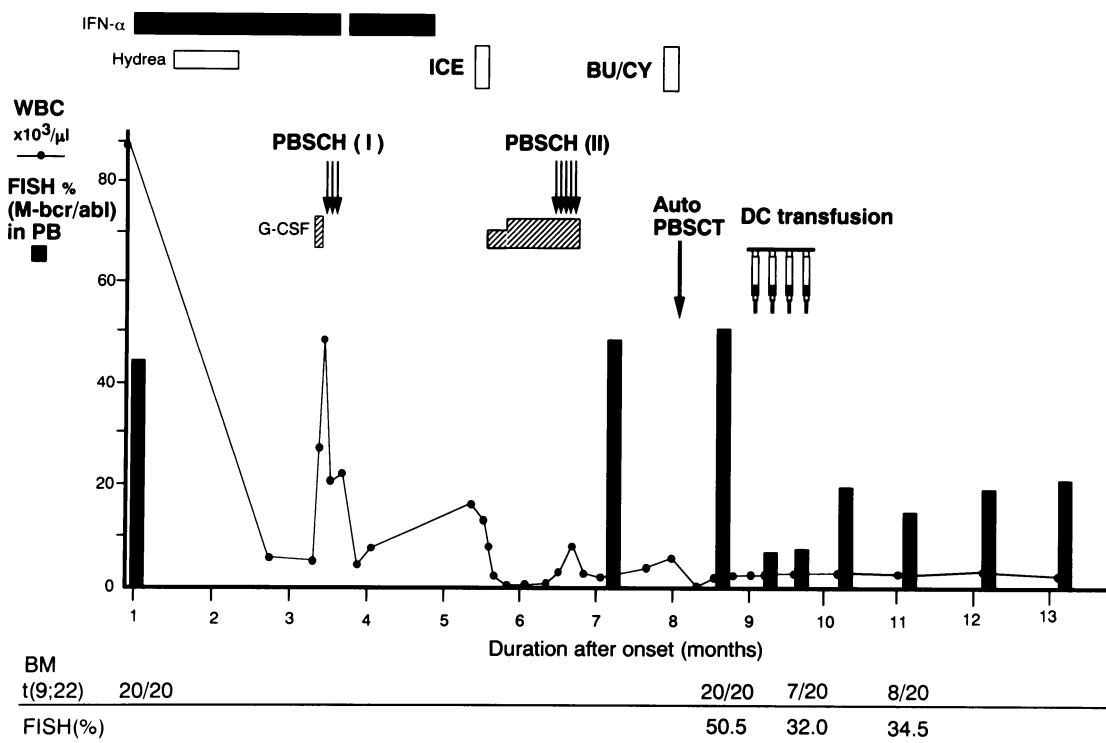

Fig. 1. Clinical course of a patient treated with PBSCT and DC infusion. Initially harvested PBSCs were used for the induction of leukemic DCs and the PBSCs subsequently harvested were used for auto-PBSCT. The patient was treated with PBSCT and subsequently vaccinated with manipulated leukemic DCs (2×10^6 cells/body) four times at weekly intervals. PBSCH, peripheral blood stem cell harvest; ICE, idarubicin-cytarabine-etoposide; BU/CY, busulfan/cyclophosphamide.

zyme Corp., Cambridge, MA), 2.5 ng/ml recombinant tumor necrosis factor-α (TNF-α; Genzyme Corp.) and 1000 U/ml interleukin-4 (IL-4; Genzyme Corp.).

Characterization of leukemic DC in an *in vitro* study

Flow cytometric analysis: Fluorescein- or phycoerythrin-conjugated mouse monoclonal antibodies against CD54 (Immunotech, Marseille, France), HLA-DR, CD14 (Ortho Diagnostics, Raritan, NJ), CD1a, CD11c, CD80, and CD86 (PharMingen, San Diego, CA), as well as fluorochrome-labeled isotype controls (Becton Dickinson & Co., Mountain View, CA), were utilized in flow cytometric analysis, using a CYTORONABSOLUTE flow cytometer and ImmunoCount II software (Ortho, Raritan, NJ).

Fluorescence in situ hybridization (FISH): DCs for FISH analysis were enriched using anti-CD1a antibody and immunomagnetic separation. Briefly, PBSCs from this patient, cultured for 7 days under the above-described conditions, were washed and treated for 30 min at 4°C with mouse monoclonal anti-CD1a antibody (IgG2a) (Cosmo Bio Corp, Tokyo) at a concentration of 1 μg/10^6 cells. The cells were washed and incubated for 30 min with rat anti-mouse IgG2a-coated magnetic beads (Dynal, Oslo, Norway) at a ratio of 20 Dynabeads/target cell (1×10^7 beads/ml cell suspension). Rosetted cells were separated using a magnet, washed, and briefly treated with 4 μl of releasing buffer (50 U/μl) to detach the beads, and the beads were removed magnetically.

To confirm that the immunomagnetically enriched CD1a$^+$ DCs arose from leukemic progenitors, dual-color *bcr/abl* translocation DNA probes (Oncor, Gaithersburg, MD) were used to examine interphase nuclei. Four hundred cells were examined in each sample by FISH to quantitate the percentage of cells bearing the *bcr/abl* translocation.[14] DCs were immunostained with the mouse anti-CD83 antibody and the indirect immunoalkaline phosphatase method and colored red with an alkaline phosphatase substrate kit I (Vector red; Vector Laboratories, Inc., Burlinghame, CA), as described previously.[15]

IL-12 production by leukemic DC: Five million leukemic or normal DCs were cultured in 10 ml of CM. The medium was removed after 48 h and cytokine expression was measured by an ELISA, using a human IL-12 testing kit (R&D Systems, Minneapolis, MN) and a microplate reader (SOFTmax; Molecular Devices Corp., Menlo Park, CA).

In vitro phagocytosis of latex particles: FluoSpheres (Molecular Probes, Eugene, OR) are carboxylate-modified microspheres, 1 μm in diameter, that emit a green fluorescence; 0.03% (vol/vol) of them were added to a suspension of 1.5×10^5 cells/ml in CM on culture day 7. After culture for 4 h in a 6-well dish (Nunc, Roskilde, Denmark), the cells were subjected to two-color FACS analysis for phycoerythrin-conjugated anti-DR and FluoSpheres.

Allo-mixed lymphocyte reaction (allo-MLR): Peripheral blood mononuclear cells (PBMCs) from a healthy volunteer (5×10^4/100 μl) were cultured for 6 days in 96-well U-bottomed culture microplates (Costar, Cambridge, MA) together with up to 2×10^4 30-Gy irradiated stimulator cells, (e.g. DCs or PBMCs originated from other normal individuals or DCs from our patient). For the final 8 h of culture, [^{3}H]thymidine (1 μCi/well; Amersham Life Science, Buckinghamshire, UK) was added to each well. Results are presented as the mean±SD of triplicate measurements.

Antigen-specific CD4$^+$ lymphocyte proliferation assay: Antigen-specific lymphocyte proliferation responses to keyhole limpet hemocyanin (KLH) protein and tuberculin purified protein derivative (PPD) were measured using Premix WST-1 Cell Proliferation Assay System (Takara, Otsu). Various numbers of isolated leukemic DCs treated with KLH (50 μg/ml) (Calbiochem, Bad Soden, Germany) or PPD (2.5 μg/ml) (Nihon BCG Inc., Tokyo) or untreated were irradiated and cocultured with CD4$^+$ T cells (5×10^4/well) selected from previously established antigen-specific T cell lines by using anti-CD4 moAb Dynabeads M-450 and CD4/CD8 DETACHaBEADs (Dynal, Oslo, Norway). T cell proliferation was measured by Premix WST-1 assay using a microplate reader at 450 nm with a reference wavelength of 690 nm during the last 4 h of the 3-day culture as described previously.[32]

Establishment of antileukemic CTL line PBMCs from this patient with CML (1×10^6 cells/ml) were cocultured with 1×10^6 irradiated mature leukemic DCs, previously cultured for 7 days in CM supplemented with 100 U/ml of IL-2. Cells were resuspended in fresh medium every 3 to 4 days. On days 7, 14, 21, and 28, additional aliquots of irradiated leukemic DCs (3:1) were added. On day 35, the CTL line was assayed for antileukemic activity.

T-cell cytotoxicity assay The CTL line (10^5 cells) and target cells (10^4 cells) were cocultured, and IFN-γ production was determined by ELISA. The CTL line (CD4:CD8=2:3) was tested for T-cell cytotoxicity against CML patient-derived bone marrow mononuclear cells (BMMNCs), or Epstein-Barr (EB) virus-transformed B cell lymphoblastoid lines (BLCLs),[16] or against HLA-matched healthy donor-derived BLCLs in a lactate dehydrogenase (LDH) release assay at an effector:target (E:T) ratio of 1:1, 3:1, 5:1, 25:1, or 50:1.[17] Target cells were cocultured with effector T cells for 6 h in 96-well U-bottomed plates (Nunc, Roskilde, Denmark) in phenol red-free RPMI (GIBCO BRL, Gaithersburg, MD) containing 0.5% autologous serum. Spontaneous release by effector and target cells was measured in separate incubations of the individual cell lines. Maximal LDH release was measured after lysis of the target cells with 0.5% Triton X-100 (Sigma Chemical Co., St Louis, MO). Cell-free supernatants were incubated in a separate 96-well plate (Nunc) with LDH substrate for 30 min before measuring the
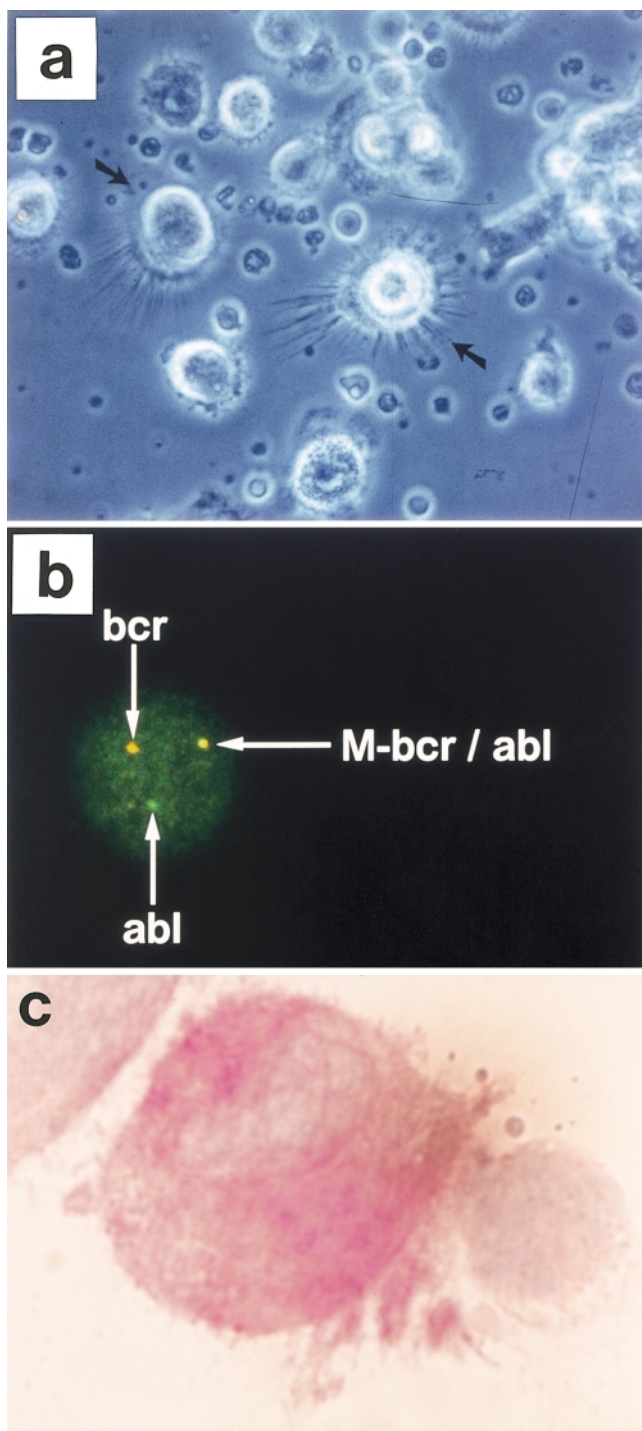
Fig. 2. Morphology of leukemic DC. a. Phase-contrast micrographs and b. *bcr/abl* genotypes of immunomagnetically enriched leukemic DC were assayed after 7 days of culture in the presence of GM-CSF, IL-4 and TNF-α. Leukemic DC displayed one yellow (*bcr* gene), one green (*abl* gene), and one yellow/green (fused *bcr-abl* gene) hybridization signal. c. Immunocytochemical staining showed CD83 expression in leukemic DC.

absorbance at 490 nm, with a 650 nm reference, using a microplate reader (SOFTmax; Molecular Devices Corp.). The percentage of cytotoxicity was calculated as $[E-(St+Se)]/[M-St]\times100$, where E is the release of LDH by effector-target coculture, St is the spontaneous release by target cells, Se is the spontaneous release by effector cells and M is the maximal release by target cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *bcr-abl* fusion gene Total RNA was isolated from each PBMC preparation using TRIzol Reagent (GIBCO BRL), and cDNA was synthesized from each RNA by incubation with Superscript II reverse transcriptase (GIBCO BRL) and 100 pmol of random hexamer oligonucleotide primers (GIBCO BRL) at 42°C for 50 min. The major bcr-abl fusion cDNA was amplified using oligonucleotide primers (5′primer, 5′-ACAGAATTCGCT-GACCATCAATAAG-3′ and 3′primer, 5′-TGTTGACTG-GCGTGATGTAGTTGCTTGG), which yielded a 385 bp product for b3a2.[18] Samples were subjected to 30 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s) and extension (72°C for 1 min), followed by 15 min of extension at 72°C. Amplified products were analyzed by 1% agarose gel electrophoresis.

Analysis of the clinical response

DTH to DC-based vaccination: DTH skin test was performed using leukemic DC pulsed with PPD (2.5 μg/ml) before vaccination, and during follow-up. One million leukemic DCs were injected intradermally into the forearm. A positive skin-test reaction was defined as >5-mm diameter erythema and induration 48 h after intradermal injection.

Analysis of T cell receptor (TCR) repertoire: RT-PCR followed by single-strand conformation (RT-PCR-SSCP) analysis, as applied to TCR analysis, was performed as described.[19] Briefly, each cDNA was mixed with 50 pmol of each primer set (i.e. a Vβ primer and a constant region β-chain primer[19]), and amplification was performed using dNTPs and 0.2 U of *Taq* DNA polymerase (GIBCO BRL) for 35 cycles (94°C for 1.5 min, 56°C for 2 min and 72°C for 3 min). The amplified DNA was diluted 1:20 and heat-denatured at 90°C for 2 min, and 2 μl of each diluted sample was electrophoresed on a non-denaturing 4% polyacrylamide gel. The DNA was subsequently transferred to Hybond N membranes (Amersham, Little Chalfont, Buckinghamshire, UK) and hybridized with the biotinylated Cβ internal probe [5′-A(A,C)AA(C,G)GTGTTCCCACCCGAGG-TCGCTGTGTT] at 42°C for 16 h, followed by incubations with streptavidin, biotinylated alkaline phosphatase and a chemiluminescent substrate system (Phototope-Star Detection Kit for Nucleic Acid; New England Biolabs Inc., Beverly, MA) to visualize the products. The bands on the films were measured only if they were clearly delineated from the background. The total number of bands in each lane was determined after counting three times.
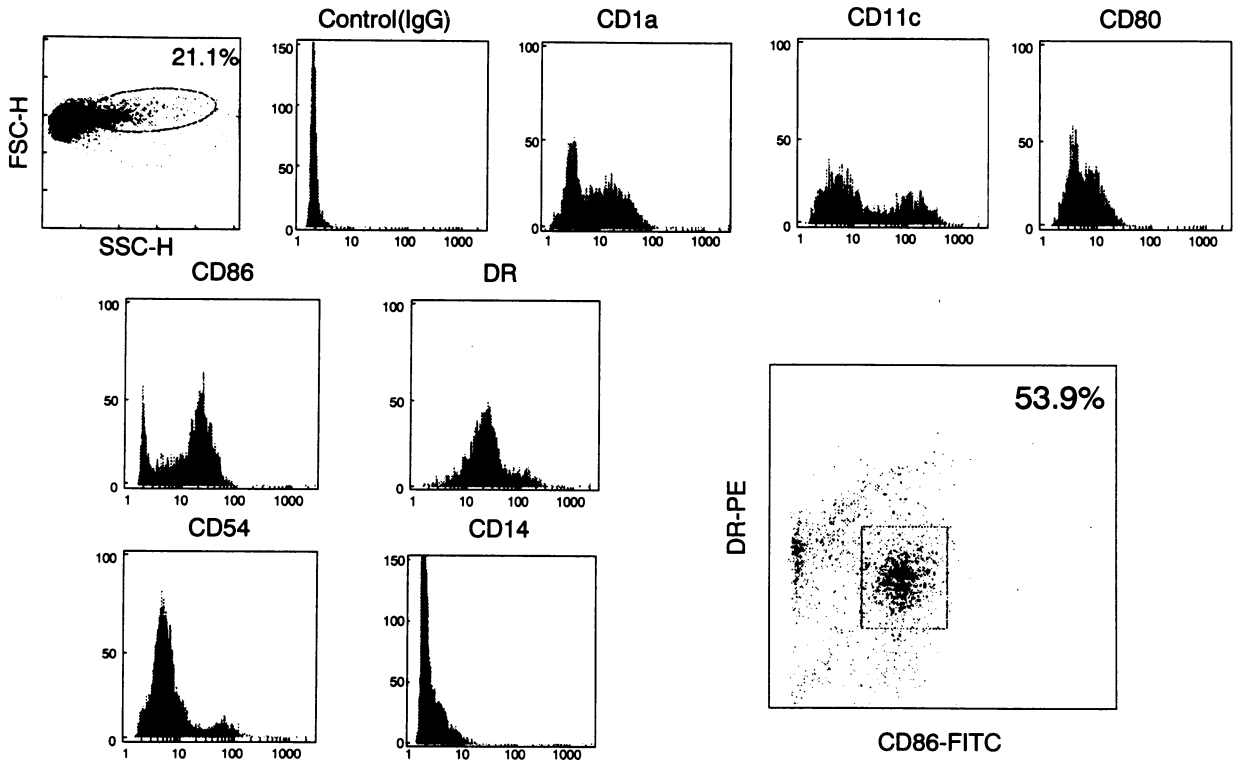
Fig. 3. Phenotypic analysis of leukemic DCs generated from the patient's PBSCs on day 7. The gated DC population (21.1% of total cells) contained the typical DC markers, CD1a$^+$, CD11c$^+$, CD80$^+$, CD86$^+$, DR$^+$, CD54$^+$, and CD14$^-$. Two-color flow cytometric analysis showed that 53.9% of these mature leukemic DCs were DR$^+$CD86$^+$. Similar results were observed in cells derived from 3 other patients with CML. FSC-H, forward scatter; SSC-H, side scatter.
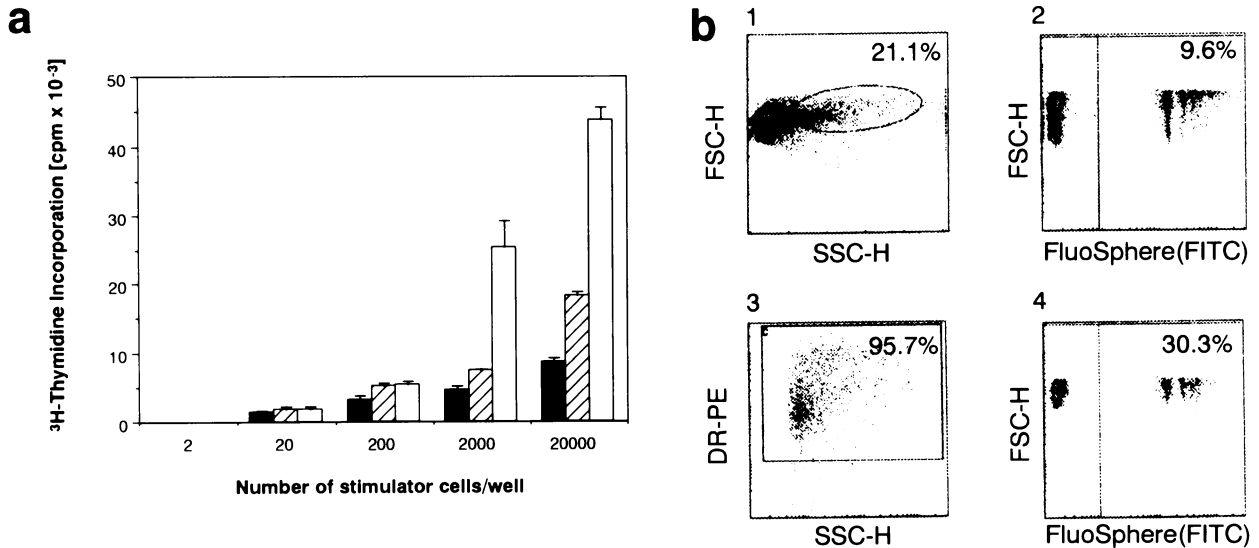

Fig. 4. Functional analysis of leukemic DCs generated from the patient's PBSCs on day 7. a. Allo-MLR T cell immune response. The responder cells were PBMC obtained from a healthy volunteer. Stimulator cells, included DCs from the treated patient (▨), and PBMC (■) and ordinary DC (□) from another healthy volunteer. b. Phagocytic capability of leukemic DCs. Phagocytic cells were 9.6% of total cells. In the DC fraction (21.1% of total cells), 95.7% of cells expressed DR$^+$ (4b-3), and 30.3% of them possessed phagocytic capability (4b-4). FluoSphere, fluorescent microsphere; FITC, fluorescein isothiocyanate.

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The detectable DNA fragments were subcloned into the pCR2.1 vector (Invitrogen, Carlesbad, CA) at the TA cloning site and were sequenced with an automatic sequencer (ABI3700; Applied Biosystems, Foster City, CA).

Intracellular cytokine analysis: Flow cytometric determination of IFN-γ and IL-4 in the cytoplasm of peripheral
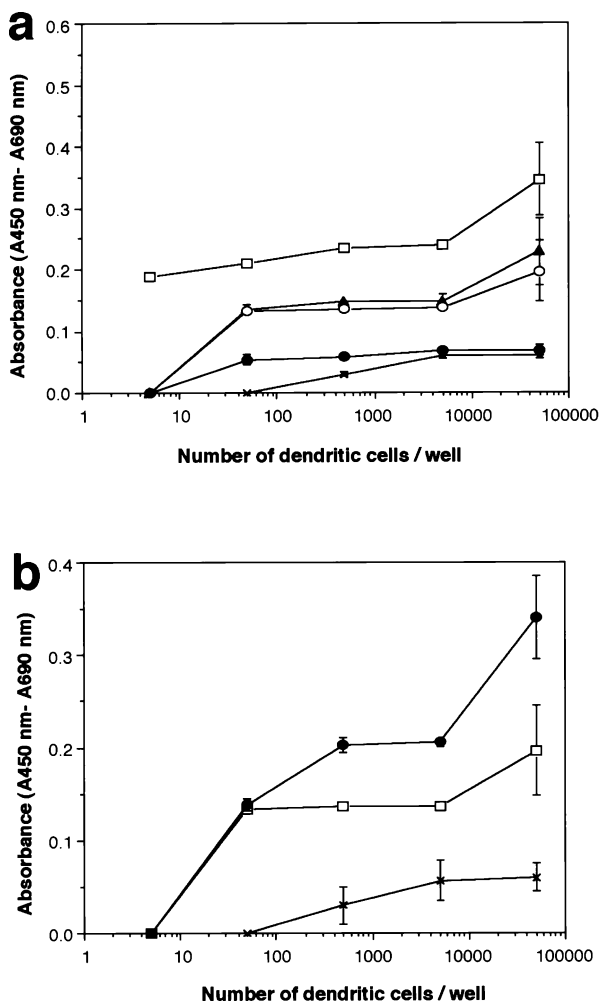

Fig. 5. Antigen-specific CD4+ T cell proliferation induced by leukemic DCs. a. In these assays, a T cell line established with leukemic DC and a T cell line with KLH-pulsed leukemic DC were incubated with irradiated leukemic DC (○ , ●) and KLH pulsed leukemic DC (▲, □), respectively. As a negative control, a CD4+ T cell response (×), using DCs from healthy volunteer as stimulators, is also shown. b. Irradiated PPD-pulsed leukemic DCs were incubated with a PPD-DC stimulated T cell line (●) or with primary CD4+ T cells from the patient following vaccination with PPD-pulsed DC (□). As a negative control, an autologous CD4+ T cell response (×), using DCs from the healthy volunteer, is also shown.
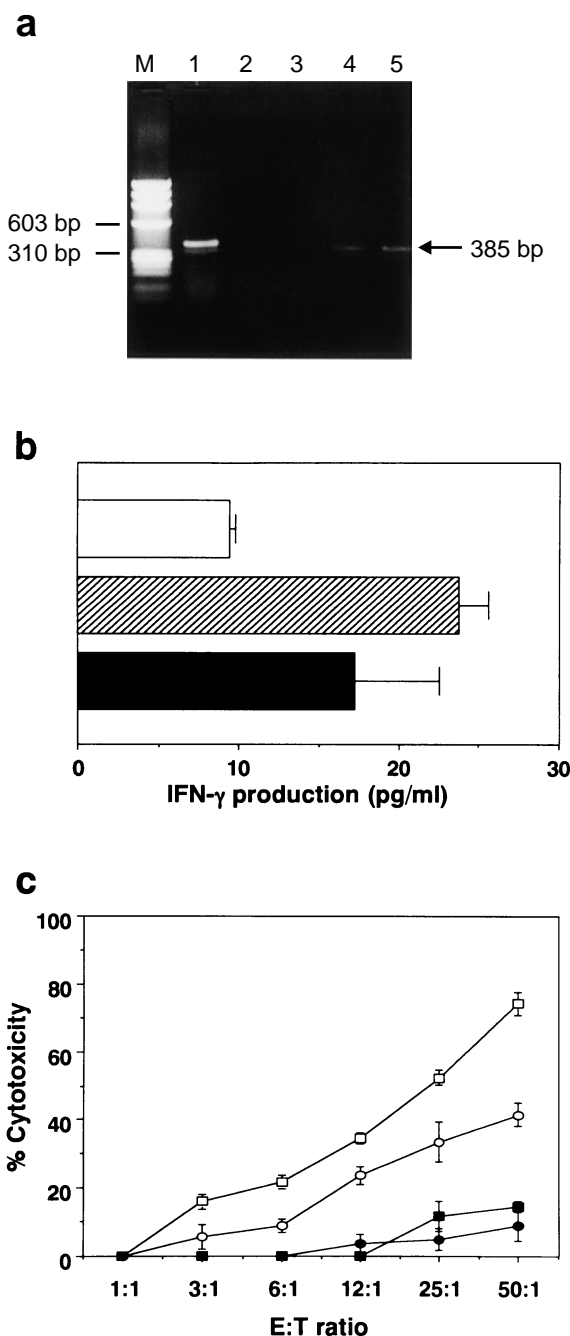
Fig. 6. Induction of anti-leukemic CTLs by leukemic DCs. a. RT-PCR analysis of *bcr-abl* fusion gene expression in K562 cells (lane 1), CEM cells as a negative control (lane 2), HLA-matched healthy donor-derived LCL (lane 3), and patient-derived LCL (lane 4), and BMMNC (lane 5). b. IFN-γ production by co-culturing a CTL line (1×10^5 cells/ml) with 10^4/ml donor-derived LCL (white bar), patient-derived LCL (hatched bar), or patient-derived BMMNC (black bar). c. Cytotoxicity assay of a CTL cell subline against CML-derived LCL (□), CML-derived BMMNC (○), HLA-matched donor-derived LCL (■) and K562 cells (●).

CD4^{+} or CD8^{+} T cells was performed as described.[20] Briefly, the patient's CD4^{+} or CD8^{+} T cells were continuously treated with FACS lysing and permeabilization solutions (Becton Dickinson Immunocytometry System; San Jose, CA). The cells were subsequently incubated with FITC-anti-IFN-γ (Becton Dickinson) and PE-anti-IL-4 (Becton Dickinson) in 0.1% BSA-PBS; FITC-mouse IgG2a and PE-mouse IgG1 (Becton Dickinson) were used as controls. The percentages of cells positive for IFN-γ and IL-4 were counted and evaluated by FACS.

RESULTS

Characterization of leukemic DCs in an *in vitro* study

Generation and phenotypic analysis of leukemic DCs: The PBSCs that were freshly isolated from the CML patient were observed to contain monocytes, lymphocytes, and stem cells. Beginning with an initial cell concentration of 2.0×10^6/ml (total volume; 40 ml), $3.8 \pm 0.7 \times 10^6$/ml viable cells were recovered on day 7, and $4.5 \pm 0.6 \times 10^6$/ml on day 14. After 3 to 4 days of culture, the number of imma-

ture DCs expressing major histocompatibility complex (MHC) class I and class II antigens increased and appeared to be higher in the nonadherent clusters having short projections. After 7 days, the population mainly consisted of mature DCs with a typical dendritic morphology (Fig. 2a). Mature DCs on day 7 to 14 expressed the costimulatory molecules B7-1 (CD80), and B7-2 (CD86), as well as CD1a and HLA-DR, and showed slightly decreased secretion of IL-12 (7.2 ± 1.8 pg/5×10^5 cells/ml, compared with 18.3 ± 1.6 pg/5×10^5 cells/ml by control cells) (Figs. 2, 3). The number of mature DCs increased to $20 \pm 3\%$ after 7 days and to $28 \pm 5\%$ after 14 days in culture. These cells were maintained for one month by transferring every 3 days into a fresh medium that contained GM-CSF, IL-4, and TNF-α.

Detection of t(9;22) in leukemic DC: To assess whether the mature DCs were of leukemic origin, they were immunomagnetically purified on day 7 with anti-CD1a antibody. The presence of the *bcr-abl* fusion gene in the CD1a^{+} DCs was determined by interphase fluorescence *in situ* hybridization (FISH). While normal cells displayed a
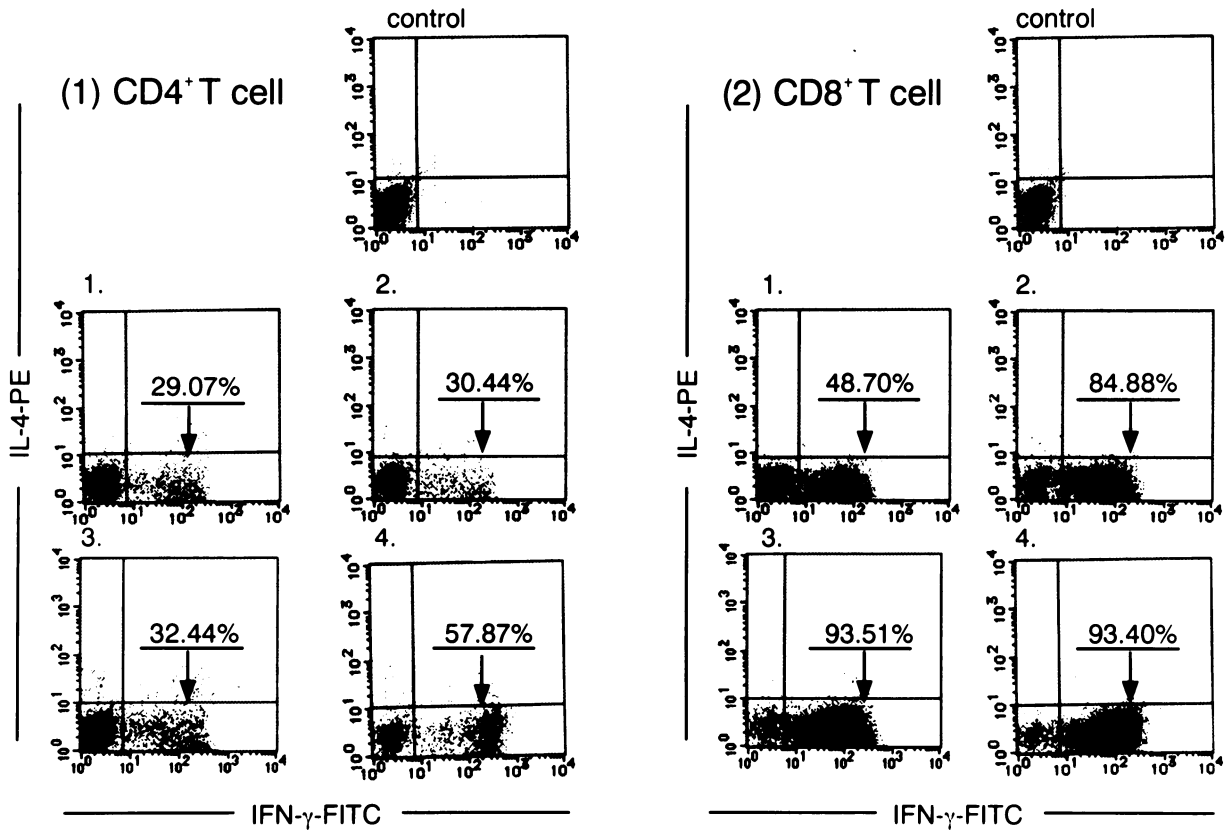

Fig. 7. Intracellular cytokine analysis in CD4^{+} and CD8^{+} T cells from the vaccinated patient. Panels: 1, just after PBSCT; 2, just after 1st DC injection; 3, just after the 4th DC injection; 4, a week after the 4th DC injection. In both CD4^{+} and CD8^{+}, type-1 T cells (IFN-γ releasing) proliferated time-dependently.

random distribution of two red (*bcr* gene) and two green (*abl* gene) hybridization signals, the leukemic cells also displayed a yellow/green signal that resulted from the fusion of the *bcr* and *abl* genes (Fig. 2b), suggesting that these DCs, which were generated during the *in vitro* differentiation of CML cells, were of leukemic origin. Of the $CD1a^+$ population, $86.7 \pm 7.0\%$ were *bcr-abl* fusion-positive cells. Moreover, by immunostaining, these DCs were clearly shown to express mature dendritic cell marker, CD83 antigen (Fig. 2c).

Allo-MLR: Generally, DCs are strong stimulators of MHC-mismatched peripheral blood lymphocytes. We found that mature leukemic DCs were more potent stimulators of allogenic lymphocytes than freshly isolated CML cells, although they were less potent than ordinary DCs (Fig. 4a).

Phagocytic capability and T cell immune response: In the presence of exogenous antigens, mature leukemic DCs have the potential to stimulate autologous lymphocytes. We therefore assayed whether leukemic DCs are antigen-specific stimulators of $CD4^+$ T lymphocytes. Prior to anti-gen stimulation, about 10% of the leukemic DCs showed active ingestion of latex particles. FACS analysis of initially-injected DCs showed that 95.7% of the DC fraction (21.1% of total cells) was DR^+ (Fig. 4b-3), and 30.3% of these DR^+ cells were actively phagocytic (Fig. 4b-4). After taking up exogenous antigens, such as KLH, mature DCs showed antigen-specific stimulation of T cells (Fig. 5a). An *in vitro* analysis of the T cell-immune response to PPD showed specific $CD4^+$ T cell proliferation (Fig. 5b). It is suggested that leukemic DCs could induce antigen-specific $CD4^+$ T cell proliferation *in vitro* as well as *in vivo*.

Cytotoxicity of DC-stimulated T cells against autologous CML targets: When we assayed the patient's cells for expansion of *bcr-abl* fusion mRNA, we found that, during IFN-α treatment, this message was positive in BMMNCs and BLCLs from this patient, but was absent from an HLA-matched subject (Fig. 6a).

When we generated a T cell line by coculturing T cells with irradiated autologous leukemic DCs supplemented with IL-2 (100 U/ml), we found that $63.0 \pm 19.0\%$ of these cells were $CD8^+CD4^-$ and $CD45RO^+$, and $33.0 \pm 16.3\%$
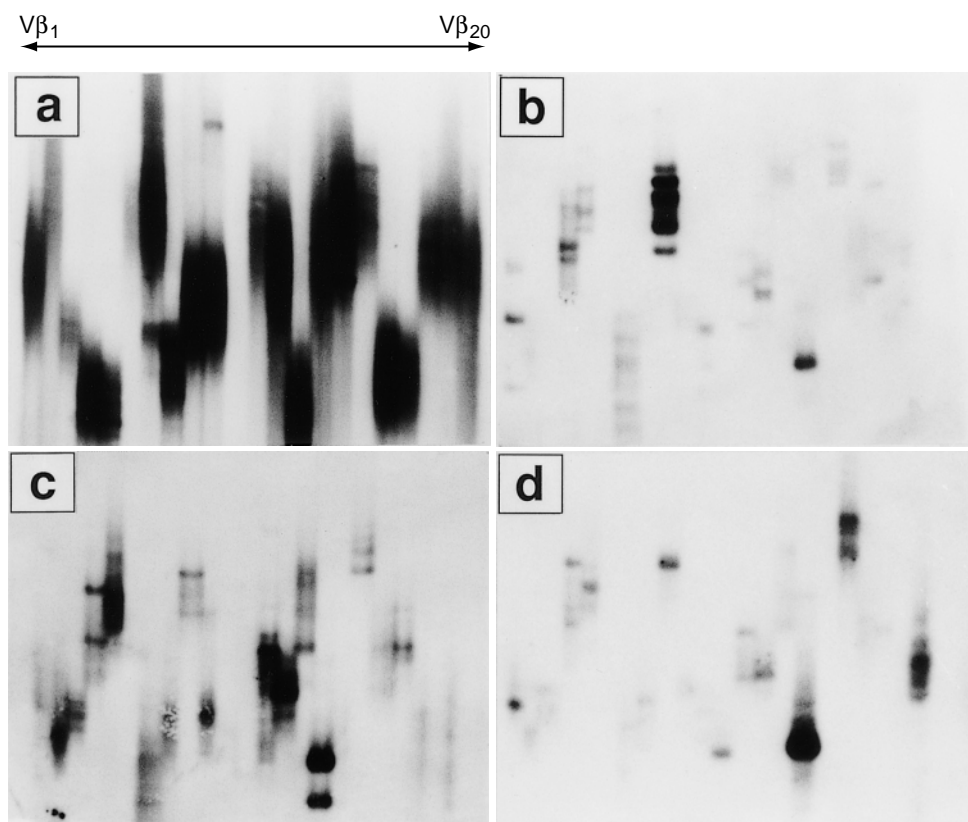

Fig. 8. Analysis of T cell receptor repertoire. *TCR Vβ* gene patterns were assayed in a normal healthy volunteer (a), and in the patient during IFN-α treatment (b), just after the PBSCT (c), just after the DC treatment (d). Some novel sharp bands were seen after DC infusion, suggesting the presence of T cell oligoclonal proliferation.
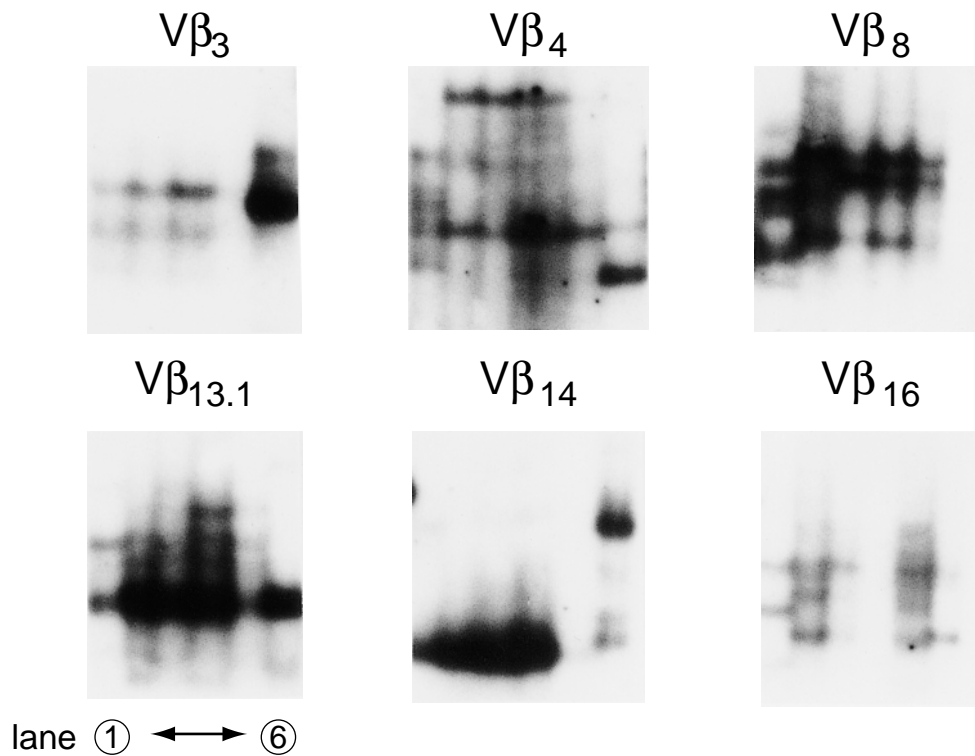
lane ① ⟷ ⑥

Fig. 9. Analysis of specific *TCR Vβ* gene patterns in primary T cells during DC treatment and in the patient's CTL line. Lane 1, prior to DC injection; lane 2, just after the 1st DC injection; lane 3, just after the 2nd DC injection; lane 4, just after the 3rd DC injection; lane 5, just after DC treatment; lane 6, the patient's CTL line. Infused DC induced Vβ 3+, 4+, 13.1+, 14+, and 16+ T cells time-dependently. T cell clones expressing Vβ 3, 8, 13.1 were common.

were CD4+CD8− and CD45RO+, whereas fewer than 2.0% of these cells were CD4+CD8+ or CD56+. Assay of IFN-γ production by an E:T mixture (10:1), a measure of CTL activity by the T cell line, showed enhanced IFN-γ production by patient's BLCLs (23.77 pg/ml) and BMMNCs (17.19 pg/ml) as compared with HLA-matched, but Ph1-negative BLCLs (9.40 pg/ml) (Fig. 6b). These DC-stimulated T cells exhibited potent cytotoxic activity against CML cells; at an effector:target ratio of 25:1, 52.3±2.2% cytotoxicity was observed against Ph1+-BLCL as well as BMMNCs, but they exhibited very low cytotoxicity toward HLA-matched healthy donor-derived BLCL cells and K562 cells (Fig. 6c). To investigate the roles of CD8 and MHC class I in the recognition of the target cells, blocking studies were performed as previously described.[15] Cytotoxicity against Ph1+-BLCL and BMMNCs using anti-MHC class I (Becton Dickinson) as well as anti-CD8 monoclonal antibody (Ancell Corp., Bayport, MN) was decreased to less than 6%.

Similar results were seen with CD8+ T cells selected from this population (at an effector:target ratio of 25:1, 45.5±2.8% cytotoxicity), suggesting that cytotoxicity is

Table I. Major Deduced Amino Acid Sequences of TCR β Chains That Carried Dominant Vβ Gene Segments in the Vβ 3, Vβ 13.1 and Vβ 16 Families

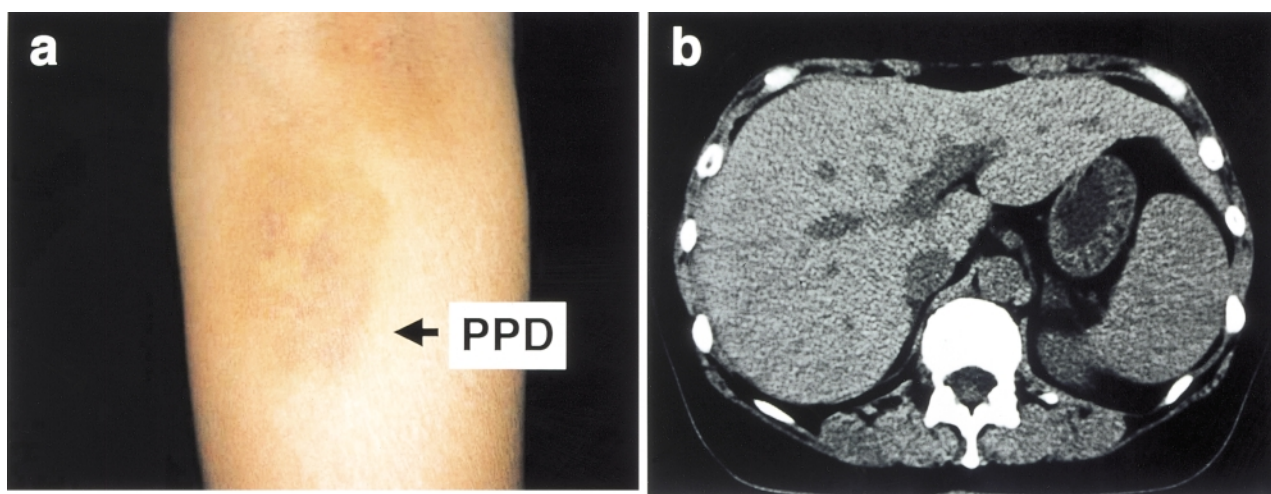|	Frequency (%)
a Vβ 3	
V S R E K K E R F S L I L E S A S T N Q T S M Y L C A S S T A G G S A F F G Q G T R L T V V E D L K V F P P E V A V F E P S E	11/12 (92) *n*=12
b Vβ 13.1	
Q G E V P N G Y N V S R S T T E D F P L R L L S A A P S Q T S V Y F C A S S Y S G E G E T Q Y F G P G T R L L V L E D L K N V F P P E V A V F E P S E	9/12 (75) *n*=12
c Vβ 16	
K E S K Q D E S G M P N N R F L A E R T G G T Y S T L K V Q P A E L E D S G V Y F C A S S H S P G L A G Y R E Q Y F G P G T R L T V T E D L K N V F P P E V A V F E P S E	12/14 (86) *n*=14

Fig. 10. Immune reaction by leukemic DC. a. DTH reaction toward PPD. Skin test with the second injection of PPD-pulsed leukemic DCs was positive. b. Mild splenomegaly emerged following the fourth injection of DCs, which may also indicates the T cell response in this patient.

mediated by an effector T-cell population and is directed at MHC-matched, leukemic cells. However, cytotoxic activity of CTLs including CD4$^+$ T cells was shown to be slightly stronger than that of isolated CD8$^+$ T cells alone (data not shown).

Analysis of clinical responses

DTH to DC-based vaccination: DTH reactions were also used to detect antigen-specific immunity. Vaccination of this patient with PPD-stimulated leukemic DCs induced an immune response as well as T cell immune response to PPD in an *in vitro* study, whereas, prior to vaccination, there was no DTH reactivity to the antigens tested (Fig. 5b).

Intracellular cytokine analysis: Cytokine expression by memory/effector T-cell populations has been shown to follow two patterns pertaining to the type-1 and type-2 T cell immune responses. When we assayed the enhanced intracellular levels of cytokine (IFN-γ and IL-4) in CD4$^+$ and CD8$^+$ T lymphocytes, we observed production of IFN-γ in both subsets, which is consistent with a type-1 cytokine-releasing T cell immune response. The percentages of IFN-γ-releasing CD4$^+$ and CD8$^+$ T lymphocytes suggest a time-dependent increase in the ratio of the type-1 subsets of T cells, such as Th1 type helper T cells or cytotoxic T cells (Fig. 7).

Analysis of T cell receptor repertoire: Prior to the transfusion of leukemic DCs, the *TCR Vβ* gene pattern in this patient showed multiple sharp bands on a smeared background, suggesting the presence of T cell oligoclonal proliferation together with a heterogeneous repertoire of *TCR β* genes (Fig. 8c). In contrast to bands during the IFN-α treatment or after the PBSCT, following the fourth injec-

tions of DCs, novel sharp bands were observed (Fig. 8, b–d). In Vβ 3, 4 and 16 lanes, bands prior to DC injection were distinct from those during and after the DC treatment. In Vβ 8, and 14 lanes, bands were common in pre- and post-DC treatment, but they were enhanced. Some Vβ repertoire usages (Vβ 3, 13.1, and 16) in peripheral blood are identical to those of the CTL line generated *in vitro* (Fig. 9), suggesting that leukemic DC induced functional T cell clones *in vivo*. These DNA bands were subcloned into pCR2.1 vector and were sequenced as shown in Table I. These major sequences of bands from primary PBMC were identical with those from CTL lines.

Clinical parameters: This patient was treated with irradiated leukemic DCs (2×10^6 DCs/body) once a week for four weeks (Fig. 1). The tumor burden of Ph1$^+$ clones in the PB and BM of this patient showed a progressive decrease three months after the injection of DCs. By FISH analysis, the percentage of tumor cells in PB decreased from 50.5 to 20.0%, whereas that in BM decreased from 50.5 to 32.0%. The cell number with Ph1$^+$ chromosome decreased from 20/20 cells to 7/20 cells in BM (Fig. 1), suggesting major cytogenetic and hematological responses. A typical DTH reaction to PPD was observed in this patient after vaccination with leukemic DCs, i.e., erythema and induration (15×20 mm) at the site of injection were observed 48 h after the injection of irradiated leukemic DCs pulsed with PPD at the second vaccination, in accordance with the *in vitro* study (Figs. 5, 10a). Mild splenomegaly was also observed one month after the vaccination with leukemic DCs (Fig. 10b).

DISCUSSION

We have described the vaccination of a CML patient with dendritic cells derived from leukemic clones. First, we characterized the leukemic DCs. Furthermore, we showed that this vaccination was effective for our patient and induced a T cell response against tumor cells.

T-cell immunotherapy may be an effective method to reduce the tumor burden of patients with CML who are unresponsive to IFN-α or chemotherapy alone.[9] Patients with CML who received non-T cell depleted allografts and who developed both acute and chronic GVHD have been found to experience a 75% reduction in risk of relapse, while recipients who developed only acute GVHD did not experience a statistically significant reduction in relapse.[21] These findings suggest that the T cell-mediated GVL effect can be distinguished from the alloimmune effects that cause acute GVHD.[22] In relapse patients with CML, approximately 82% and 73% of the allo-BMT recipients infused with donor-derived T cells have been reported to enter cytogenetic and molecular remission, respectively.[9, 23]

Clinical trials with DCs in treating tumor-bearing patients have achieved success, as has been the case in animal models.[24–27] DCs are antigen-presenting cells that can enhance the generation of antigen-specific helper and cytotoxic T cells from naive T cells. In a previous study, we showed that cord blood-derived DC pulsed with irradiated leukemic cells can present antigens to T cells, and induce anti-leukemic cytotoxic T cells.[28] In agreement with the present findings, the DCs generated from the CML progenitors have been shown to carry t(9;22), which suggests they are leukemic in origin.[13, 29] In addition, the DR⁺CD86⁺ phenotype and the morphology of these mature leukemic DCs are similar to those of typical mature DCs.[13]

In this *in vitro* study, we showed that leukemic DCs can take up exogenous particles, such as latex beads, and KLH protein. They can also function as potent accessory cells in the proliferation of KLH antigen-specific CD4⁺ T cells, as well as in stimulating an allo-T cell response. These findings indicate that exogenous protein antigen (KLH) was taken up, processed and loaded onto MHC class II molecules by leukemic DCs for effective presentation to helper T cells. However, it appeared that leukemic DCs have slightly weak allo-reactivity and produce less IL-12 than normal DCs. Thus, we found that leukemic DCs can be differentiated from CML cells and that they can behave as antigen-presenting cells similar to normal dendritic cells, although perhaps less efficiently. We found that these leukemic DCs pulsed with *bcr-abl* fusion peptides enhanced cytotoxic effects against CML cells (data not shown). These potentials of leukemic DCs suggest that the antitumor effects might be further increased, if we pulsed leukemic DCs with tumor antigen exogenously.

In a clinical application, we further investigated whether DC vaccination could induce antigen-specific immunity. We found that our patient vaccinated with PPD-pulsed DCs exhibited a positive immune response to PPD, including efficient generation of PPD-specific memory CD4⁺ T cells (Fig. 5b). In evaluating the vaccine effect, we detected a slight DTH reaction even using leukemic DCs alone, but the reaction using DCs pulsed with PPD was enhanced, so we employed this method. This reflected the normal interaction between DCs and CD4⁺ T cells, because we could not detect the reaction when we simply used macrophages pulsed with PPD. Tumor peptides in association with MHC molecules have been shown to be recognized by effector T cells during DTH reactions, and an association between clinical response and DTH reactivity has also been described.[26] Our finding, that leukemic DCs can generate leukemia-specific memory CD4⁺ T cells from naive T cells, means that antigen presentation by mature leukemic DCs may sustain the expression of leukemic antigens initially presented on the surface of the CML cells.[13, 30] A T cell line activated by leukemic DCs *in vitro* showed high production of IFN-γ and specific cytotoxicity against the patient's BMMNCs and BLCLs, which express *bcr-abl* fusion mRNA, but not against those from a HLA-matched healthy subject, suggesting that injected leukemic DCs had anti-leukemic effects in this patient. These findings indicate that leukemic DCs apparently display an adjuvant effect in antitumor immunity. Since CD8⁺ T cytotoxic and CD4⁺ T helper 1 cells are involved in antitumor immunity, leukemic DCs may act primarily as the best immunostimulators of these cells.

The analysis of T cell receptor (TCR) clonality and intracellular cytokine synthesis in this patient's T cells revealed a specific oligoclonal T cell expansion, releasing the cytokine from type-1 helper T cells (IFN-γ is dominant, while IL-4 is recessive). Moreover, when we compared the accumulation of TCR Vβ in peripheral blood, we observed the enhancement of accumulation of the Vβ 3, 4, 8, 13.1, 14, and 16 families. In the post-DC treatment, novel bands emerged in Vβ 3, 4, and 16, suggesting that Vβ 3⁺, 4⁺, and 16⁺ T cells were newly introduced, while bands in the Vβ 8 and 14 families were enhanced, suggesting that infused leukemic DCs could enhance these T cell clones. These findings indicate that some T cell clones were enhanced, and other T cell clones were newly induced by leukemic DCs. In particular, in Vβ 3, 13.1, and 16, bands from TCR usage *in vivo* were identical to those of the CTL line *in vitro* (Fig. 9, Table I), indicating that the immune repertoire includes leukemia-reactive CTLs activated *in vivo*.

In our previous study, we demonstrated that in IFN-α responsive CML patients, the expression of Vβ 9 and 20

families was predominant.[2] Therefore, our findings may suggest that DCs induce T cell repertoires different from those associated with IFN-α activated T cells. In other words, the mediating immune mechanism may be different between DC and IFN-α. We demonstrated that leukemic DC can stimulate the anti-leukemic T cells and that this phenomenon is apparently immune mechanism-mediated. These findings suggest that leukemic DCs might present as-yet unidentified antigens associated with malignancy, as well as a range of leukemia-associated antigens derived from the *bcr-abl* fusion protein, to T cells both in patients and *in vitro*.[30, 31] In general, the generation of an effective cytotoxic T cell response probably requires both a strong helper T cell response and the production of cytokines in the microenvironment of the lymphoid tissue. In the four weeks after immunotherapy, mild splenomegaly was also observed, which may reflect an immune T cell response by the injected leukemic DCs.

Our main objective was to assess the clinical response to vaccination with autologous leukemic DCs in our patient. In fact, the administration of autologous PBSC to patients following high-dose chemotherapy failed to improve the percentage of benign progenitors as a whole.[8] The subject had not responded well to auto-PBSCT alone, because the same percentage of Ph1$^+$ cells as before was still detected in the bone marrow as well as in the periph-

eral blood soon after transplantation. In contrast, we found that Ph1$^+$ colony-forming progenitors were replaced by Ph1$^-$ progenitors through infusion of leukemic DCs (data not shown), and CML cells were reduced in cytogenetic level, suggesting that the number of malignant progenitor clones, as well as well-differentiated CML cells, was decreased by DC therapy. Leukemic DC therapy resulted in complete hematological remission and a major cytogenetic response (>65% Ph1$^-$) in this patient, and this was maintained for at least six months after vaccination.

By employing our methods described here, cells harvested from G-CSF mobilized PBSC can generate a sufficient number of leukemic DCs for use in the cellular immunotherapy of CML. The elimination of residual leukemic cells using leukemic DCs may work in co-operation with conventional therapy to offer a superior and better tolerated treatment for CML as compared with that currently used.

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