as well as *in vitro*-generated HY-specific CTLs. TCR BV spectratyping showed a similar peak with same size in some BV family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific $CD8^+$ T cells. These findings suggest the circulation of a functional T-cell clone capable of eradicating lymphoblastic leukemia cells.

Materials and methods

Case report

A patient was a 15-year-old Japanese male (HLA-A*0201-positive) with chronic-phase CML. He had experienced no GVHD after receiving busulfan- and cyclophosphamide-conditioned bone marrow from his HLA-identical sister. At 40 months after bone marrow transfer, lymphoid blast crisis suddenly developed. After treatment with cyclophosphamide, adriamycin, vincristin and prednisolone, the hematologic relapse persisted. At 2 months after chemotherapy, the patient underwent PB stem cell transplantation (PBSCT) from the same donor following conditioning with cytarabine, cyclophosphamide and total body irradiation. An unmanipulated PB stem cell graft including a total of 5.5×10^6 CD34-positive cells/kg was infused. Cyclosporine, which is used to prevent GVHD, was withdrawn on day 21 to induce GVL effect. At 3 weeks after PBSCT, conversion to full donor chimerism was obtained. The patient developed grade II acute GVHD on day 29 after PBSCT, and the disease progressed to extensive chronic GVHD on day 80. At 20 weeks after PBSCT, no BCR/ABL transcripts were detected, and he remained in molecular remission until a relapse at 13 months after PBSCT. The patient died of veno-occlusive disease shortly after the third transplantation with cytarabine- and idarubicin-conditioned bone marrow from the same donor.

Peptide

An HLA-A201-restricted HY peptide was synthesized using a semiautomatic multiple peptide synthesizer based on the reported sequence.[5] The purity of the peptide was checked by reverse-phase high-pressure liquid chromatography.

Cell preparation

Cells were obtained from the post transplant patient and his stem cell donor. Peripheral blood mononuclear cells (PBMC) were prepared using density-gradient centrifugation. For the establishment of Epstein–Barr virus (EBV)-transformed LCLs, PBMC from the donor were depleted of T cells using the rosette formation method. A total of $2–3 \times 10^6$ non-T cells were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) medium containing 10% fetal calf serum (FCS; GIBCO) containing 10% culture medium from an EBV-producing cell line, B95-8, at 37°C for 2 h. The EBV-infected cells were cultured for 3 weeks until transformed LCL cells grew. LCL cells were maintained by changing the medium every 4 to 5 days.

Tetrameric HLA-A2/mHA HY peptide complexes

The generation of HLA-A2/mHA HY tetramers and tetramer labeling of HY-specific T cells was performed as described previously.[8]

Generation of dendritic cells (DCs) from monocytes

PB monocyte-derived DCs were generated as described previously.[9] Briefly, monocytes were isolated by adherence of donor PBMCs to plastic for 2 h. Monocytes were cultured in RPMI 1640 medium supplemented with 10% pooled AB serum, 10 ng/ml recombinant human interleukin-4 (IL-4) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (a gift from Kirin Brewery, Tokyo, Japan). On day 5, 100 U/ml recombinant human tumor necrosis factor (TNF)α was added. On day 8 or 9, the cells were harvested for use as monocyte-derived DCs for antigen presentation. Cultured cells expressed DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class I and class II.

Induction of HY peptide-specific CTLs

DCs were pulsed with an HY peptide for 90 min at 37°C in serum-free RPMI 1640. After washing, 1.0×10^6 peptide-(40 nM) pulsed DCs and 1.0×10^7 donor-derived (autologous) PBMC were cultured together in 24-well culture plates. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, minimal essential amino acids, sodium pyruvate and ampicillin (all from GIBCO) plus 10% autologous plasma. The cells were kept at 37°C in a humidified, 5% CO_2–air mixture. At days 7, 14 and 21, responder cells were restimulated with peptide-pulsed autologous DCs. From day 21, cultured T cells were suspended in 100 U/ml IL-2- (a gift from Shionogi, Osaka, Japan) containing culture medium and were restimulated weekly with peptide-pulsed autologous monocytes or DCs. T cells were harvested at day 35 and used for the cytotoxicity assay and RNA extraction for T-cell repertoire analysis.

Cytotoxicity (^{51}Cr release) assays

Donor-derived LCL cells and DCs as well as fibroblasts and leukemic cells of the patient were used as target cells in the standard 4-h ^{51}Cr release assay.[10] Specific lysis was calculated using the following formula: 100 × (cpm experimental release–cpm spontaneous release)/(cpm maximal release–cpm spontaneous release).

Preparation of target cells

Patient fibroblasts were isolated from a biopsy specimen and cultured in RPMI 1640 plus 10% FCS for 4 weeks. Single-cell suspensions were prepared by trypsinization. Donor-derived LCL cells and DCs and the patient's fibroblasts were suspended in 100 μl of ^{51}Cr solution containing an HY peptide at a concentration of 4 nM. In some experiments, concentrations of the peptide were changed as noted. Bone marrow mononuclear cells containing 98% BCR/ABL^+ cells were obtained from the patient

just before the second transplantation and were cryopreserved for use as leukemic cells. Thawed leukemic cells were cultured in RPMI 1640 plus 10% pooled AB serum for 24 h before use as a target in the CTL assay.

Blocking of cytotoxicity by monoclonal antibodies (MoAbs)

Polyclonal antibodies (control) or purified MoAbs were added to cultures of HY peptide- (4 nM) pulsed DCs in 96-well plates at a concentration of 10 μg/ml, and CTLs were immediately added to each well. MoAbs were HU-4 (anti-HLA-DR; kindly provided by Dr Akemi Wakisaka, Hokkaido University, Japan) and W6/32 (anti-HLA class I; American Type Culture Collection, Rockville, MD, USA).

Identification and isolation of IFNγ-producing CD8$^+$ T cells by flow cytometry

To detect circulating CD8$^+$ T-lymphocytes that recognize HY peptide, intracellular IFNγ was assessed by flow cytometry as described previously with slight modifications.[3,4] Briefly, donor-derived LCL cells were incubated for an hour with or without HY peptide. PBMCs were taken from the patient 12 weeks after the second transplantation. The CD8$^+$ T-lymphocytes were isolated from PBMCs using magnetic beads coated with an anti-CD8 monoclonal antibody (mAb) according to the manufacturer's instructions (Dynal AS, Oslo, Norway). A total of 10^6 CD8$^+$ cells were mixed with 10^6 autologous LCL cells in a culture tube in RPMI 1640 medium and cultured in a humidified 5% CO_2 incubator at 37°C for 1 h. Brefeldin A (Sigma, St Louis, MO, USA) was added at a final concentration of 10 μg/ml and the cells were cultured for an additional 5 h. After incubation, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with phosphate-buffered saline, cells were permeabilized with IC Perm (Biosourse International, Camarillo, CA, USA) and stained with PE-labeled anti-CD8 (Coulter, Miami, FL, USA) and FITC-labeled anti-human IFNγ (Biosourse International) MoAbs. Stained cells were analyzed and sorted on a FACScan (Becton Dickinson, San Jose, CA, USA).

RNA extraction and cDNA preparation

Total RNA was extracted from PBMCs or CTLs using a technique described elsewhere,[11] and was reverse transcribed into cDNA in a reaction primed with oligo(dT) using SuperScript II reverse transcriptase as recommended by the manufacturer (BRL, Bethesda, MD, USA).

Spectratyping of complementarity-determining region 3 (CDR3)

Conditions for the generation of the CDR3 size spectratyping have been reported previously.[6] Briefly, cDNA was polymerase chain reaction (PCR) amplified through 35 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) with a fluorescent BC primer and primers specific to 24 different BV subfamilies. Analyses of the pseudogenes BV10 and BV19 were excluded from this study.[12] A measure of 1 μl of each amplified products was mixed with 2.01 100% formamide, heated at 90°C for 3 min and electrophoresed on a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each BV subfamily was analyzed with an automatic sequencer (Applied Biosystems Division, Foster City, CA, USA) equipped with a computer program allowing the determination of the fluorescence intensity of each band. The results are given as peaks corresponding to the intensity of the fluorescence. Expansion of a limited number of T cells was judged when a prominent peak appeared in the CDR3 pattern with or without a reduced number of peaks (five peaks). Given the BV-NDN-BJ sequence of the identical CDR3 size between in HY-specific CD8$^+$ cells in PB and the CTLs, a more specific primer covering CDR3 and different BJ subfamilies[13] was designed specifically to amplify cDNA of the BV22$^+$ T-cell clone in both CTLs.

Direct sequencing of PCR products

BV22-BJ PCR products were purified and sequenced as described previously.[14]

Results

Cytolytic activity of in vitro*-generated HY-specific CTLs*

Cultured T female donor cells stimulated with autologous HY peptide-pulsed DCs pulsed were able to lyse HY peptide-pulsed autologous DCs and patient fibroblasts, but could not lyse untreated DCs (Figure 1a). Of note, the CTLs lysed nonpeptide pulsed leukemic cells of the patient more efficiently than his fibroblasts. CTLs showed cytotoxicity to HY peptide-loaded autologous DCs, in a peptide concentration-dependent manner (Figure 1b). Cytotoxicity mediated by the CTLs against HY peptide-pulsed autologous DCs was blocked to a similar degree by the addition of MoAb either against anti-class I or anti-CD3, but was not affected by the addition of anti-HLA-DR (Figure 1c). In addition, the CTLs could effectively lyse LCL cells of unrelated males who shared HLA-A*0201. In contrast, apparent cytotoxic activity was neither observed against allogeneic LCL cells that did not possess HLA-A2 nor against LCL cells of unrelated females (Figure 1d).

Tetramer staining of HY-specific CTLs

The patient in lymphoid crisis with CML relapse developed acute GVHD shortly after the second transplant, which progressed to extensive chronic GVHD. He achieved a molecular remission at 4 months after PBSCT despite the fact that leukemic cells accounted for more than 98% of his bone marrow cells when the conditioning regimen was started. This unusual clinical course appeared to suggest the induction of GVL reactions associated with GVHD.

Other than the HY antigens, there was no disparity in the minor histocompatibility alleles, including HA-1, HA-2,

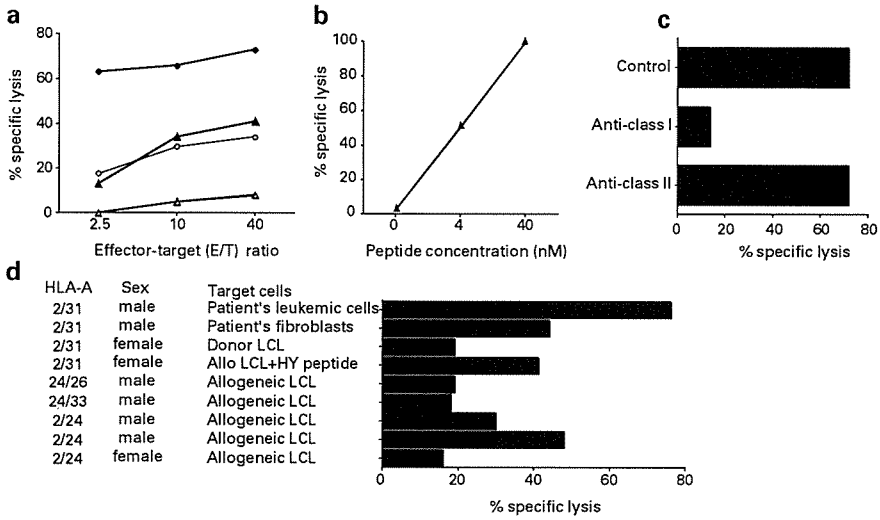

Figure 1 Cytotoxic activity of *in vitro*-generated HY-specific CTLs. (**a**) Cytotoxicity of cultured T cells stimulated by autologous DCs pulsed with HY peptide. The amount of peptide utilized in (a, b and c) was 4 nM. Target cells: autologous DCs without HY peptide (Δ); autologous DCs pulsed with HY peptide (▲); fibroblasts of the patient (○); leukemic cells of the patient (•). In (b, c, and d), cytotoxicity was determined at an E/T ratio of 10:1. (**b**) Effect of concentration of HY peptide on cytotoxicity of CTLs. Cytotoxicity of CTLs to autologous DCs loaded with various concentration of HY peptide was tested. (**c**) Antibody blockade of cytotoxicity against autologous DCs pulsed with HY peptide. Polyclonal antibodies (control), anti-class I MoAb or an anti-class II MoAb were added to cultures for testing blockade of cytotoxicity. (**d**) Cytotoxicity of HY peptide for 6 h. HY-specific CTLs against LCL cells of unrelated males who share HLA-A2 (A*0201).

CD31, CD49b and CD62L,[15,16] between the donor and the recipient. Tetramer staining demonstrated the expansion of HY peptide-specific T cells from undetectable prior to PBSCT to 15.9% of the circulating CD8$^+$ T cells 12 weeks after PBSCT (Figure 2a). Thereafter, frequencies of HY tetramer-positive T cells declined and disappeared from the PB, coinciding with molecular relapse. The *in vitro*-generated HY-specific CTLs were 90% HY tetramer-positive CD8$^+$ T cells (Figure 2b).

Detection of HY-reactive CD8$^+$ T cells by intracellular cytokine assessment

To demonstrate the HY peptide-driven expansion of CD8$^+$ T cells, we assessed intracellular accumulation of IFNγ in PB CD8$^+$ T cells by flow cytometry. At 12 weeks after PBSCT, 6.8% of PB CD8$^+$ T cells produced intracellular IFNγ in response to HY peptide-pulsed autologous LCL cells (Figure 3a), while IFNγ production was negligible in CD8$^+$ T cells in response to autologous LCL cells without the HY peptide (Figure 3b). These findings indicate that *in vivo* expansion and activation of HY peptide-reactive T cells occurred after the second transplantation. At that time, the proportion of circulating CD8$^+$ cells positive for HY-tetramer staining was 15.9% as show in Figure 2a. Although inducibility of IFNγ in HY-tetramer-positive CD8$^+$ T cells was not examined, there could be overlapping between CD8$^+$ T cells producing intracellular IFNγ in response to HY peptide and CD8$^+$ T cells stained with HY tetramer, because HY-tetramer staining must detect functional T cells reactive with HY peptide.

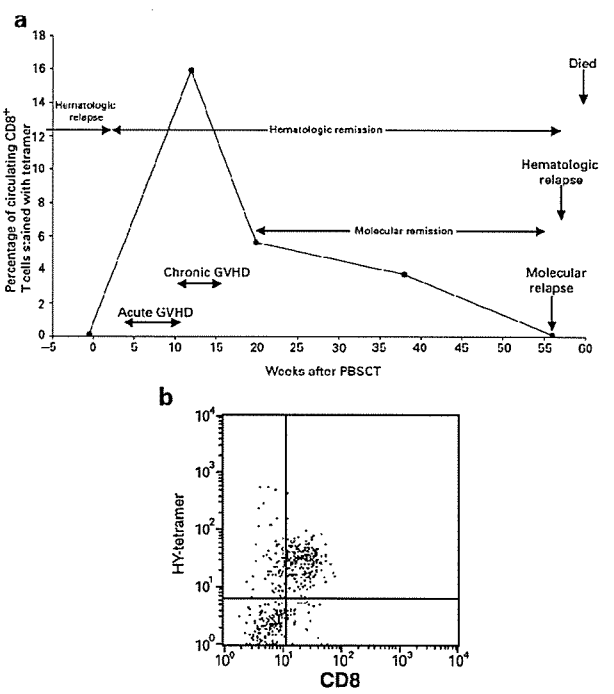

Figure 2 Monitoring and generation of HY-specific CD8$^+$ T cells. (**a**) Correlation between frequency of circulating HY-tetramer-positive CD8$^+$ T cells and clinical events. (**b**) HY-tetramer staining of the *in vitro*-generated HY-specific T cells, showing FITC-conjugated anti-CD8 antibody (*x*-axis) and PE-conjugated HY-tetramers (*y*-axis). Appropriate gates were set on vital lymphocytes according to their typical forward- and side-scattering characteristics.

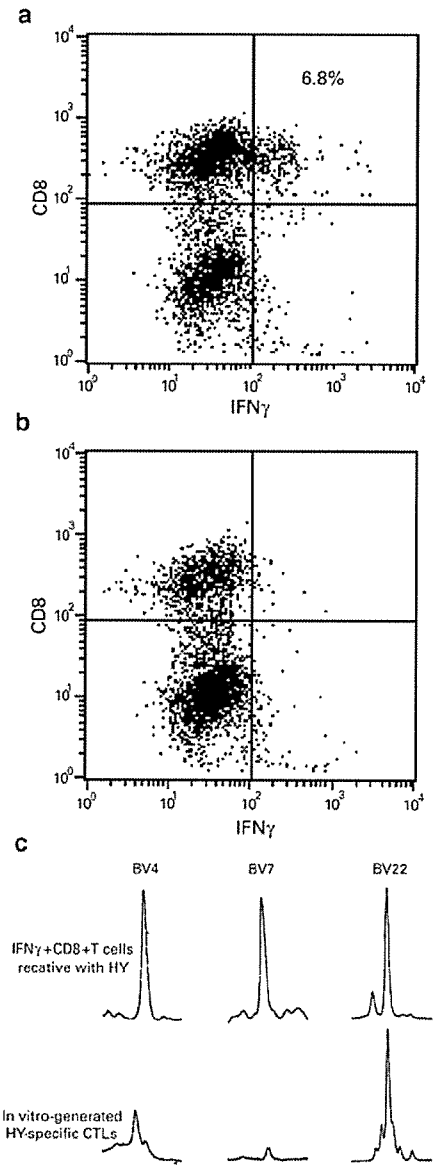

Figure 3 HY-specific IFNγ-producing CD8$^+$ T cells in PB. (**a**) PB CD8$^+$ T cells were incubated with autologous LCL cells pulsed with after fixation and permeabilization, the cells were stained for CD8 and IFNγ. The frequency of CD8$^+$ T cells producing IFNγ in response to HY peptide is shown as a percentage of total CD8$^+$ cells. (**b**) IFNγ production was negligible in CD8$^+$ T cells stimulated by autologous LCL cells not pulsed with the HY peptide. (**c**) CD8$^+$ IFNγ$^+$ T cells detected in Figure 3a were selected by fluorescence-activated cell sorting, and spectratyping was performed. CDR3 sizes of TCR BV subfamilies from CD8$^+$ IFNγ$^+$ T cells are shown together with the data from *in vitro*-generated HY-specific CTLs.

CDR3 size distribution of TCR BV cDNA of HY-specific T cells

Spectratyping of the TCR BV region was performed on *in vitro*-generated HY-specific CTLs and PB CD8$^+$ T cells that were stained with intracellular IFNγ MoAb in response to HY peptide stimulation and sorted as shown in Figure 3a. PB HY-reactive CD8$^+$ cells showed prominent skewing within BV4, BV7, BV22 and BV24, and the *in vitro*-generated HY-specific CTLs showed skewing within BV4, BV7, BV12, BV16 and BV22. The two T-cell populations shared the usage of BV4, BV7 and BV22 (Figure 3c), but only the BV22$^+$ T cells from *in vitro*-generated HY-specific CTLs and HY-responsive PB CD8$^+$ T cells had a similar peak with the same CDR3 size distribution.

Deduced amino-acid sequence of CDR3 of BV22 cDNA

To determine if HY-specific CTLs circulate in the patient, we subcloned the amplified cDNA of the *in vitro*-generated BV22$^+$ CTLs and BV22$^+$ T cells that produced intracellular IFNγ in response to HY peptide stimulation as shown in Figure 3a and determined the CDR3 sequence (Table 1). One of three N-D-N sequences of HY-specific CD8$^+$ cells from PB was identical to one of those of the CTLs. These findings indicate that the same cells isolated from the donor are expanding *in vivo*, and suggest that HY-specific CD8$^+$ T cells in PB of the patient have cytotoxic activity against leukemic cells.

Discussion

In mHAs HA-1- and HA-2-matched stem cell transfer between a female donor and a male recipient, we have observed the emergence of HY peptide-specific CTLs that result in the durable remission of relapsed leukemia. The present study utilized tetramer staining to show that HLA-A*0201-restricted HY peptide-specific CD8$^+$ T cells were present in the PB after the development of GVHD and led to the eradication of BCR/ABL transcript-positive leukemic cells. Further, these cells disappeared upon molecular relapse of disease. Assaying for the frequency IFNγ-producing cells by intracellular cytokine staining, we demonstrated the emergence of functional T cells in PB that were reactive with the HY peptide during the presence of HY tetramer$^+$ T cells. The data imply that HY-specific CTLs may have therapeutic potential as adoptive immunotherapy for relapsed leukemia after allogeneic SCT.

In this patient, leukemic relapse had occurred as full-blown disease of CML in lymphoid blast crisis. Although a beneficial effect of donor immunity was expected in the control of leukemic relapse, it would take months to start to work. Thus, we had chosen multiple transplants instead of donor leukocyte infusion (DLI) to reduce leukemic cells sufficiently.

Recently in mHAs HA-1 and/or HA-2 incompatible donor–recipient pairs an association between the emergence of HA-1 or HA-2 tetramer-positive CTLs and the complete disappearance of BCR/ABL$^+$ cells or of myeloma cells was reported.[17] Of the three reported patients, one who underwent female-to-male transplantation experienced an increase in HLA-B7-restricted HY-specific T cells as well as an increase in HA-2-specific T cells, but not that of

Table 1 Junctional amino-acid sequence of TCR BV22 of HY-specific T cells

V	*N-D-N*	*J*	*BJ family*
(a) BV22$^+$ T cells from in vitro-generated HY-specific CTLs			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	REGGRS	GYTFCSGTKLTV	1.2
CASS	KQKGNPPPI	SPLHFGNGTRLTVT	1.6
(b) Functional BV22$^+$ T cells from HY-reactive CTLs circulating in PB			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	RQSQGS	GYTFCSGTKLTV	1.2
CASS	RQGRGVSEF	SPLHFGNGTRLTVT	1.6

HLA-A2-restricted HY-specific T cells. However, the cell population(s) contributing to the GVL effect were unable to be identified. It is worth noting that this reported case with lymphoid blast crisis of BCR/ABL transcript-positive CML obtained molecular remission after the development of GVHD and the emergence of HY-specific CTLs as seen in the present case.

The *in vitro*-generated HY peptide-specific CTLs efficiently lysed leukemic cells of the patient, and were also to a lesser extent cytotoxic to the nonhematopoietic cells such as fibroblasts of the patient. Gratwohl *et al*[18] reported that male recipients with CML of female blood or marrow stem cell grafts are at a high risk of GVHD, but benefit from reduced incidence of disease recurrence. These findings provide evidence that HY-specific CTLs may be commonly induced in male patients given a stem cell graft from a female donor, leading to the development of GVL reactions and GVHD. This implies that the availability of a female blood or marrow graft may be beneficial to a leukemic male recipient at high risk of relapse.

In contrast to the ubiquitous expression of HY, HA-1 and HA-2 are exclusively expressed on hematopoietic cells.[1] *In vitro*-generated HA-1- and HA-2-specific CTLs specifically lyse leukemic cells, but not nonhematopoietic cells in a ^{51}Cr release assay.[1,19] Thus, upon HA-1- or HA-2-mismatched SCT and adoptive immunotherapy such as DLI, a low risk of GVHD would be expected. However, HA-1 disparity between a patient and a donor has been associated with the development of GVHD without reducing a rate of relapse.[15,20] Marijt *et al*[17] demonstrated the emergence of HA-1- and HA-2-specific CD8$^+$ T cells in PB of three patients after DLI preceding complete remission of relapsed leukemia. Relapse was associated with the development of GVHD in all three patients. A recent report showed that GVHD does not require alloantigen expression on host epithelium, and its development is primarily mediated by inflammatory cytokines such as TNFα and IL-1.[21] This may account for discrepancies between *in vitro* behavior of HA-1- and HA-2-specific CTLs and clinical observations. Based on these findings, we believe that in mHA-oriented allogeneic immunotherapy the ability of mHAs to induce powerful immune reactions is more important than restriction of mHAs to hematopoietic tissue, and so far it appears that GVHD is an inevitable consequence. In the future, selective blockade of cytokines mediating GVHD[21] may be a strategy to preserve GVL, while reducing toxicity of GVHD after mHA-oriented immunotherapy.

TCR BV spectratyping showed a similar peak with same size in a BV22$^+$ family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific CD8$^+$ T cells, and one shared N-D-N sequence. These findings suggest the expansion of a functional T-cell clone that participates in eradicating lymphoblastic leukemia cells positive for BCR/ABL transcripts, although we were not able to provide direct evidence demonstrating antileukemic activity of HY-specific CD8$^+$ T cells taken from PB of the patient. It would have been beneficial to sort the HY-tetramer-positive cells detectable in PB of the patient, to expand these cells using HY peptide-pulsed LCL cells, and to test their cytotoxicity against the leukemic targets. Restricted TCR BV usage for HA-1-specific CTLs has also been described.[7] Spectratyping could be beneficial in monitoring HY-specific CTLs *in vivo*, because spectratype analysis is more sensitive than tetramer analyses, and can be performed using as little as 500 cells.[6]

Compared with tetramer staining, a flow cytometric assay assessing intracellular IFNγ levels can be used to screen a large number of allogeneic peptides with a relatively little effort. In allogeneic SCT, this approach should be useful for initial screening of candidates for mHAs derived from polymorphic cellular proteins. Moreover, intracellular IFNγ assessment between CD8$^+$ T cells during the GVL effect or GVHD as a responder and hematopoietic cells or nonhematopoietic cells of a host as a stimulator may enable the detection of undefined mHAs-specific CTLs. As the IFNγ capture assay enables isolation of live T cells stained for surface-associated IFNγ,[22] further studies with regard to the function of responding effector T cells could elucidate their putative target antigens.

Another advantage of the flow cytometric cytokine production assay is that it is possible to assess the production of multiple cytokines on an antigen-specific, single-cell basis. It has already been demonstrated by Nazaruk *et al*[23] that a subset of EBV-specific CD8$^+$ T-cell lines produce IL-4 or IL-13 in addition to IFNγ upon stimulation with phorbol myristate acetate and ionomycin. Such a technique could be utilized in determining the cytokine production capabilities of mHA-specific CTLs in PBSCT.

In conclusion, the present data provide evidence that the emergence and activation of HY-specific CD8$^+$ T cells may participate in eradicating lymphoblastic leukemic cells. This implies that *in vitro*-generated HY-specific CTLs may have therapeutic potential for relapsed leukemia after allogeneic SCT.

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Quantitative Analysis of Epstein-Barr Virus (EBV)–Specific CD8$^+$ T Cells in Patients with Chronic Active EBV Infection

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To clarify the pathogenesis of chronic active Epstein-Barr virus (EBV) infection, EBV-specific CD8$^+$ T cells were enumerated, by use of human leukocyte antigen (HLA)–A*2402–restricted tetramers, in 8 patients with chronic active EBV infection, 10 patients with infectious mononucleosis, and 16 EBV-seropositive healthy control subjects. In most of the patients with chronic active EBV infection, EBV-specific CD8$^+$ T cells were not detected. Of note, latent membrane protein 2–specific CD8$^+$ T cells were not detectable in any patients with chronic active EBV infection. In contrast, EBV-specific CD8$^+$ T cells were detected in patients with infectious mononucleosis and in healthy control subjects. Low frequencies of EBV-specific CD8$^+$ T cells may be one of the immunological features of chronic active EBV infection.

Epstein-Barr virus (EBV) is a ubiquitous virus that infects >90% of the human population. Primary infection often occurs during childhood and is generally asymptomatic, although it sometimes progresses to infectious mononucleosis. In immunocompetent hosts, EBV-specific immunity—especially, EBV-specific CD8$^+$ T cells—controls the virus both during primary infection and during the long-term carrier state that follows [1]. Chronic active EBV infection, which is characterized by chronic or recurrent infectious mononucleosis–like symptoms that persist for a long time, is seen in otherwise immunocompetent hosts. Patients with chronic active EBV infection have an unusual pattern of anti-EBV antibodies and high virus loads in their peripheral blood [2, 3]. Although the precise pathogenesis of chronic active EBV infection is unclear, recent studies have shown that clonal expansion of EBV-infected T cells and NK cells may be associated with development of the disease [3, 4]. A national survey conducted in Japan showed that the majority of patients had chronic active EBV infection that involved either T cells or NK cells, and only 2 of 82 patients had a main infection that involved B cells [4].

EBV-specific CD8$^+$ T cell responses have been well documented in subjects with primary infection and in carriers of EBV [5], but they have been less well studied in subjects with chronic active EBV infection. In the present study, we examined the frequency of EBV-specific CD8$^+$ T cells in 3 groups of subjects (patients with chronic active EBV infection, patients with infectious mononucleosis, and healthy control subjects who were EBV seropositive), using a set of tetrameric complexes (tetramers) of major histocompatibility complex (MHC) class I associated with specific peptides. For comparison, frequencies of cytomegalovirus (CMV)–specific CD8$^+$ T cells in CMV-seropositive individuals were also studied. Because HLA-A24 is one of the most common alleles in Japan, being the most frequently encountered HLA class I allele, and because this allele is almost exclusively of the genotype A*2402, we used HLA-A*2402–restricted tetramers in the present study. In addition, we measured EBV loads to evaluate the association with the frequency of EBV-specific CD8$^+$ T cells.

Patients and methods. Eight patients with chronic active EBV infection, 10 patients with infectious mononucleosis, and 16 EBV-seropositive healthy control subjects were enrolled in the present study. Informed consent was obtained from all subjects or their parents. All subjects were HLA-A24 positive. The mean patient age was 18.5 years for the group of patients with chronic active EBV infection, 6.5 years for the group of patients with infectious mononucleosis, and 19.7 years for the group of seropositive control subjects. Patients with infectious mononucleosis were younger than the other study subjects, because they were mainly identified in pediatric wards. All patients with chronic active EBV infection fulfilled the diagnostic criteria described elsewhere [2–4]. Blood samples were obtained, either at diagnosis or before initiation of immunosuppressive treatment, from 6 of 8 patients with chronic active EBV infection. The other 2 patients had received immunochemotherapy, which consisted of etoposide and steroids, but

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their blood samples were obtained at least 1 month after therapy. For 7 of 8 patients with chronic active EBV infection, the EBV-infected cell fractions were identified by magnetic cell sorting and quantitative polymerase chain reaction (PCR), as described elsewhere [3]; 6 patients had EBV-infected NK cells, and 1 had infected T cells. All patients with infectious mononucleosis had symptoms typical of the disease. Serological examination showed that they had either anti–viral capsid antigen (VCA) IgM antibodies or anti-VCA IgG in the absence of anti-EBV nuclear antigen (EBNA) antibodies, indicating that they had primary infections. Blood samples were obtained during the early convalescent phase of infectious mononucleosis. All seropositive control subjects had both anti-EBV VCA IgG and anti-EBNA antibodies, indicating previous EBV infection. Anti-CMV IgG antibody titers were measured in all subjects.

The MHC/peptide tetramers were produced as described elsewhere [6]. In brief, recombinant HLA-A*2402 heavy chain and human β_2-microglobulin proteins were folded in the presence of 1 of 7 viral peptides: TYPVLEEMF (derived from EBV BRLF1), DYNFVKQLF (derived from BMLF1), IYVLVMLVL and TYGPVFMCL (derived from latent membrane protein [LMP]–2), RYSIFFDYM (derived from EBNA3A), TYSAGIVQI (derived from EBNA3B), and QYDPVAALF (derived from CMV pp65 protein) [6–8]. Purified MHC complexes were biotinylated and mixed with phycoerythrin-labeled streptavidin (Molecular Probes).

For tetramer staining, blood was incubated with 10 μg/mL each tetramer and a Tri-color–labeled anti-CD8 monoclonal antibody (Caltag) at 37°C for 15 min. Then, red blood cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). After undergoing 2 washings, stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). For each analysis, $CD8^+$ T cells were gated, and 30,000–100,000 events were acquired. Data were analyzed using CellQuest software (BD Biosciences). The limit of detection was 0.01% of $CD8^+$ T cells.

To quantify EBV DNA, real-time quantitative PCR assays with a fluorogenic probe were performed as described elsewhere [3]. DNA extracted from peripheral blood mononuclear cells (PBMCs) was used for PCR of 50 cycles performed, at 95°C for 15 s and at 62°C for 1 min, with a 7700 Sequence Detector (Applied Biosystems). For each sample, a threshold cycle value was calculated by determining the point at which fluorescence exceeded 10 times the SD of the value at baseline. A plasmid pGEM-BALF5 that contained the BALF5 gene was used as a positive control [3]. The threshold cycle values for clinical samples were plotted on a standard curve, and copy numbers were calculated and expressed per microgram of DNA. The limit of detection of this assay was ~5 copies/μg DNA. The logarithmic mean was used to compare the virus load. CMV DNA was also detected using the real-time PCR assay, as described elsewhere [9].

Statistical analysis was conducted using StatView software (version 5.0; SAS Institute). Data for patients with chronic active EBV infection or infectious mononucleosis and for seropositive subjects were analyzed using the χ^2 test, to assess significant differences.

Results. EBV DNA was detected in PBMCs from all 8 patients with chronic active EBV infection. The virus load was $10^{1.5}$–$10^{3.8}$ copies/μg DNA (mean, $10^{3.2}$ copies/μg DNA). EBV DNA was also detected in all 10 patients with infectious mononucleosis; the virus load was $10^{0.7}$–$10^{4.1}$ copies/μg DNA (mean, $10^{2.4}$ copies/μg DNA). By contrast, EBV DNA was detected in

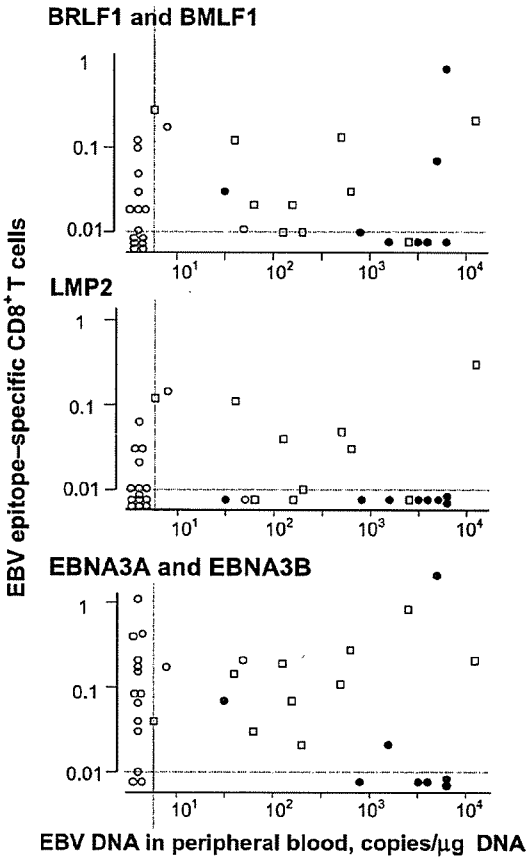

Figure 1. Association of Epstein-Barr virus (EBV) load in peripheral blood with the frequency of EBV epitope–specific $CD8^+$ T cells. EBV load was estimated using real-time polymerase chain reaction, and epitope-specific $CD8^+$ T cells were enumerated by tetramer binding. Data are shown separately for 3 categories: lytic proteins (i.e., BRLF1 and BMLF1), latent proteins presumably expressed in chronic active EBV infection (i.e., latent membrane protein–2 [LMP2]), and latent proteins not expressed in chronic active EBV infection (i.e., EBNA3A and EBNA3B). The frequency of each epitope-specific $CD8^+$ T cell was added together and combined in each category. For LMP2, 2 sets of tetramers specific to different epitopes were used. Dotted lines denote limits of detection for each parameter. ○, EBV-seropositive healthy control subjects; □, patients with infectious mononucleosis; ●, patients with chronic active EBV infection.

Table 1. Rate of detection of Epstein-Barr virus (EBV)– and cytomegalovirus (CMV)–specific CD8⁺ T cells, by tetramer binding.

Protein(s)	Rate of detection of specific CD8⁺ T cells, % (no. of subjects who had such cells detected/no. of subjects tested)			
	In patients			
	With chronic active EBV infection	With infectious mononucleosis	In healthy EBV-seropositive control subjects	P[a]
BRLF1 + BMLF1	50 (4/8)	90 (9/10)	63 (10/16)	.16
LMP2	0 (0/8)[b]	70 (7/10)	50 (8/16)	.01[c]
EBNA3A + EBNA3B	38 (3/8)[b]	100 (10/10)	88 (14/16)	.003[c]
CMV pp65[d]	20 (1/5)[b]	75 (3/4)	88 (7/8)	.04[c]

NOTE. EBNA, EBV nuclear antigen; LMP2, latent membrane protein–2.

[a] By use of the χ^2 test.

[b] Significantly lower than the other groups.

[c] Statistically significant.

[d] Only CMV-seropositive subjects were tested.

only 2 of 16 seropositive control subjects, for whom virus loads were $10^{0.9}$ copies/μg DNA and $10^{1.7}$ copies/μg DNA. No CMV DNA was detected in PBMCs from any patient or control subject.

The association of the EBV load with the presence of EBV epitope–specific CD8⁺ T cells is shown in figure 1. In most of the seropositive control subjects, EBV DNA was undetectable, although specific CD8⁺ T cells varied in frequency for each epitope. Patients with infectious mononucleosis who had higher virus loads tended to have higher frequencies of specific CD8⁺ T cells. Most patients with chronic active EBV infection had high virus loads, but few had specific CD8⁺ T cells; of note, CD8⁺ T cells specific to LMP2 were not detected in any of the patients with chronic active EBV infection. Because of the small sample size of the present study, we could not find any association between EBV-specific CD8⁺ T cells and severity of chronic active EBV infection, subject age at onset of infection, duration of infection, or type of EBV-infected cell.

The rates of detection of EBV- and CMV-specific CD8⁺ T cells in the 3 groups of subjects studied are summarized in table 1. CD8⁺ T cells specific to the BRLF1 and BMLF1 epitopes were detected in all 3 groups of subjects, with the rate of detection highest in the group of patients with infectious mononucleosis. LMP2-specific CD8⁺ T cells were detected in most of the patients with infectious mononucleosis and in one-half of the seropositive control subjects, but they were not detected in any of the patients with chronic active EBV infection. CD8⁺ T cells specific to EBNA3 proteins were detected less frequently in patients with chronic active EBV infection than in the other 2 groups of subjects. Between the 3 groups, there were significant differences in the rates of detection of CD8⁺ T cells specific to LMP2 and EBNA3 proteins. For comparison, we also measured CMV pp65–specific CD8⁺ T cells in CMV-seropositive subjects. CMV pp65–specific CD8⁺ T cells were detected less frequently in patients with chronic active EBV infection than in patients with infectious mononucleosis and in EBV-seropositive control subjects.

Discussion. EBV establishes a lifelong infection in humans, with distinct virus latency patterns occurring during the acute and chronic phases of infection. During primary infection, EBV-infected B cells express the full spectrum of EBNAs and LMPs, in addition to BARF0 [1]. This expression pattern, which has been called "latency III," is also seen in lymphoblastoid cell lines in culture [1]. In the majority of patients with chronic active EBV infection, EBV also infects either T cells or NK cells [3]. EBV-infected T cells or NK cells express only a few EBV-related antigens; this expression pattern is known as "latency II". Imai et al. [10] established 4 EBV-infected T cell lines from patients with chronic active EBV infection; these lines expressed EBNA1, latent membrane protein–1 [LMP1], and LMP2, but not EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA leader protein. Such restricted patterns of latent gene expression, whereby LMP1 and LMP2, in addition to EBNA1, are variably expressed, have also been demonstrated in affected tissues from patients with chronic active EBV infection [11]. We have detected the expression of LMP2 in PBMCs from nearly one-half of the patients with chronic active EBV infection studied, including some of the case patients in the present study (authors' unpublished data).

A previous study demonstrated defective EBV-specific cytotoxic T lymphocyte (CTL) activity in patients with chronic active EBV infection [12], but no study has examined the frequency of CD8⁺ T cells specific to each latent protein. In the present study, for the first time, to our knowledge, we examined CD8⁺ T cells by use of a set of tetramers that were specific to both lytic and latent EBV proteins. Patients with chronic active EBV infection had lower frequencies of EBV-specific CD8⁺ T cells than did the other subjects studied. Of note, LMP2-specific CD8⁺ T cells were not detected in any of the patients with

chronic active EBV infection. LMP2 is immunologically subdominant, and the frequencies of LMP2-specific CTLs may border the limits of detection of the tetramer assay. Therefore, minor global suppression might result in the complete lack of LMP2-specific CTLs in patients with chronic active EBV infection. A more sensitive, functional assay, such as an enzyme-linked immunospot assay, would help to solve the problem. Nevertheless, because LMP2 is thought to be expressed in EBV-infected cells in patients with chronic active EBV infection, this finding raises the possibility that EBV-infected cells can escape detection by EBV-specific CTLs, which could allow the virus to attain persistently high virus loads in patients with chronic active EBV infection.

We also examined CMV pp65–specific $CD8^+$ T cell responses among the 3 groups of subjects studied. Of interest, CMV pp65–specific $CD8^+$ T cells were also detected less frequently in patients with chronic active EBV infection. The reason why few patients with chronic active EBV infection had CMV-specific $CD8^+$ T cells is not clear. We considered 2 hypotheses. One hypothesis is that the CMV-specific $CD8^+$ T cell response is secondarily suppressed after the development of chronic active EBV infection. EBV has a number of strategies to suppress and evade the immune system. For example, a recent study showed that LMP1, which is considered to be expressed by EBV-infected cells in chronic active EBV infection, has immunosuppressive effects on T cells via interleukin-10 [13]. Another hypothesis is that patients with chronic active EBV infection have a defect in regulating lymphocyte activation or proliferation, resulting in a decrease in the number of both EBV- and CMV-specific $CD8^+$ T cells. In the latter case, CMV infection may be sufficiently controlled by other mechanisms, such as innate immunity.

Chronic active EBV infection is a disease associated with high rates of morbidity and mortality and many complications. Effective standard treatments have not yet been established. Recently, allogeneic stem cell transplantation has been used in the treatment of selected patients with chronic active EBV infection [3]. Transplantation may eliminate EBV-infected cells and reconstitute EBV-specific cellular immunity. However, transplantation constitutes a substantial risk to patients with chronic active EBV infection, resulting in a higher risk of treatment-associated complications, such as multiple-organ failure [3]. Alternatively, transfer of EBV-specific CTLs has been attempted in some patients with chronic active EBV infection [14, 15]. The findings of the present study should help to establish the use of such adoptive immunotherapy against chronic active EBV infection.

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Adjuvant-Mediated Tumor Regression and Tumor-Specific Cytotoxic Response Are Impaired in MyD88-Deficient Mice

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ABSTRACT

The *Mycobacterium bovis* bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS) activates Toll-like receptor (TLR) 2 and TLR4, but unlike the typical TLR4 agonist bacterial lipopolysaccharide barely induces type 1 IFN. BCG-CWS has been used for adjuvant immunotherapy for patients with cancer. We investigated the adjuvant potential of BCG-CWS for induction of CTLs subsequent to TLR-mediated dendritic cell (DC) maturation, using a syngeneic mouse tumor model (B16 melanoma in C57BL/6). We evaluated the retardation of tumor growth and cytotoxic response in wild-type and MyD88−/− mice immunized with tumor debris and/or BCG-CWS. Delays in tumor growth and cytotoxic response were induced by immunization with a mixture of BCG-CWS emulsion and the tumor. BCG-CWS was capable of activating DCs *ex vivo* by the criteria of CD80/CD86 up-regulation and cytokine (interleukin-12, tumor necrosis factor-α) induction. Efficient tumor suppression and *ex vivo* cytokine induction did not occur in MyD88-deficient mice and cells, suggesting that the MyD88 adapter is crucial for induction of tumor cytotoxicity. Because TLR4 is involved in both MyD88-dependent and -independent pathways and the latter affects DC maturation, our findings indicate that both pathways cooperate to induce CTL-based tumor immunity.

INTRODUCTION

Microbial components that activate the host immune system have been designated as adjuvants. Adjuvants have often been used for immunization with pure antigen for potential induction of antibody production and CTL and natural killer (NK) cell activation (1, 2). Variations in the output responses appear to depend on the properties of each adjuvant and the target immunocompetent cells. One representative adjuvant is dead mycobacteria conjugated with mineral oil, which is called Freund's complete adjuvant (3).

Because cancers become established and clinically detectable presumably by circumventing the host immune surveillance, tumor cells generally possess poor immunogenicity by themselves (4, 5). Enhancing host *in vivo* immunity and/or increasing tumor antigenicity has been a goal in the design of immunotherapy. Selective manipulation of immune cells, particularly dendritic cells (DCs), has been attempted for immunotherapy with vectors and reagents (6). Although DCs sensitized with a targeting antigen migrate to lymphoid tissues and induce a strong and efficient T-cell response, this tailored therapy requires cell purification and culture. Furthermore, manipulation of patient tumor cells or DCs and the identification of CTL-defined tumor-associated antigens are highly crucial for the routine application of this method to patients. In this regard, cell-free vaccines would be more suitable for clinical purposes. Adjuvants should be an alternative tool for tuning up host immunocompetent cells for cancer immunotherapy.

The role of adjuvants in effective immune potentiation had not been identified at the molecular level until Toll-like receptor (TLR) was discovered in mammals (7). TLR is a receptor family consisting of >10 members in humans and mice (8, 9). At present, the evidence is accumulating that each TLR is a receptor for a specific adjuvant. Adjuvants, here named pathogen-associated molecular patterns based on the nature of their receptors (10), have been found to interact with professional antigen-presenting cells (11, 12), including DCs, via TLRs on their membranes.

We have conducted immunoadjuvant therapy by s.c. administration of agonists of TLRs. Bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS), which has been used as a potent adjuvant therapy in patients with cancer, has been identified as an agonist of TLR2 and TLR4 (13, 14). BCG-CWS exerted tumor regression activity within a dose that demonstrates no toxicity. The application trial of BCG-CWS to >600 patients with postoperative cancer largely (>60%) brought about good prognosis for the patients in our hospital (15, 16). In *ex vivo* analysis of the patients' blood mononuclear cells, IFN-γ was produced in response to exogenously added BCG-CWS in most of the patients with good prognosis (15–17). The mechanism of BCG-CWS-mediated host immune activation is at least in part attributable to the maturation of DCs, which is induced through TLR2 and TLR4 (13, 14) and putative BCG-CWS uptake receptors (16) on DCs prepared from blood from normal volunteers. The costimulators CD80/CD83/CD86 and the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-12 p40 are up-regulated in human DCs by experimental stimulation with BCG-CWS (13). None of the lymphocyte populations (NK, NKT, B, and T cells) are directly activated in response to BCG-CWS (13, 17). Hence, BCG-CWS acts as a potent inducer of *ex vivo* DC maturation via its TLR agonist activity. However, the mechanisms by which BCG-CWS induces *in vivo* effector activation, including IFN-γ production and CTL response to tumor-associated antigens, and the resultant suppression of tumor growth have not been identified.

TLR signaling pathways, on the other hand, are being elucidated. The cytoplasmic domain of each TLR recruits distinct sets of adapter molecules that in turn activate specific downstream signaling molecules (8–11). To date, four adapters have been identified, and selection of the adapters appears to determine the particular TLR signaling pathway leading to the activation of specific TLR-defined transcription factors such as nuclear factor-κB, c-Jun (AP-1), or IRF-3 (8, 9, 18). MyD88 is a pivotal adapter that activates nuclear factor-κB, leading to induction of the cytokines TNF-α, IL-6, IL-8, and IL-12 (19, 20). However, the up-regulation of costimulators and induction of IFN-β are largely independent of MyD88 (21, 22). Lipopolysaccharide (LPS)-stimulated TLR4 reportedly activates both MyD88-dependent and -independent pathways (21, 22). BCG-CWS, despite

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acting as an agonist of TLR4 (13), poorly induces LPS-like MyD88-independent responses. The molecular mechanism whereby BCG-CWS serves as a TLR4 agonist with properties distinct from those of LPS and has the potential for exerting antitumor immunity also remains unresolved.

Here we show evidence, using MyD88-deficient mice, that MyD88 is a critical adapter for induction of tumor-specific cytotoxicity and subsequent active immunity for tumor suppression by BCG-CWS. These results provide insight into the mechanism of the BCG-based antitumor potential (23) and may be useful for testing adjuvant immunotherapies for cancer presently under study.

MATERIALS AND METHODS

BCG-CWS. BCG-CWS was prepared in our laboratory as described previously (24). The lot used in this study (Lot 10-2) consisted of mycolic acid, arabinogalactan, and peptidoglycan with >97% purity and no LPS contamination: the results of chemical analysis of this lot were published previously (14, 24). Minimal amounts of phospholipid (~0.2%) and amino acids (<2%) contaminated this preparation. No mannan or glucose was detected. Because BCG-CWS is insoluble in water and organic solvents, oil-in-water emulsion forms of BCG-CWS micelles (BCG emulsion) were used throughout the *in vivo* study. Dried BCG-CWS was resuspended at a concentration of 1 mg/ml in emulsion buffer (PBS containing 1% drakeol and 1% Tween 80) with a Potter homogenizer and sterilized by heating for 30 min at 60°C (24). In some *in vitro* experiments, we also used BCG-CWS that was homogenized in PBS without oil or solubilizer (BCG-PBS), which acted more potently on DCs in culture than the BCG emulsion.

Mice and Cell Lines. Breeding pairs of MyD88−/− mice were provided as reported previously (21). Wild-type male and female C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). Mice were maintained in our institute under specific pathogen-free conditions. All animal experiments were approved by the committee in our institute.

B16D8 was established in our laboratory as a subline of the B16 melanoma cell line (25). This subline was characterized by its low or lack of metastatic properties when injected s.c. into syngeneic C57BL/6 mice (25). Mouse cell lines 3LL, EL-4 (C57BL/6 origin), Colon-26 (BALB/c origin), and YAC-1 (BALB/c origin) were provided by Sumitomo Co. Ltd., as described previously (26). These cell lines were cultured in RPMI 1640 containing 10% FCS.

Reagents and Antibodies. The following materials were obtained as indicated: Fetal bovine serum was from Bio Whittaker (Walkersville, MD), mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and mouse IL-2 were from PeproTech EC, Ltd. (London, United Kingdom), polymyxin B and LPS (*Escherichia coli* O111:B4) were from Sigma (St. Louis, MO), [^{51}Cr]sodium chromate was from Amersham Biosciences (Piscataway, NJ), and Lympholyte-M was from Cedarlane (Ontario, Canada). The ELISA kits for IL-12 p40 and TNF-α were from Amersham Biosciences.

The following antibodies were used: FITC-conjugated goat antirat, rabbit, mouse IgG F(ab′)$_2$ from American Qualex (San Clemente, CA), antimouse CD40 (phycoerythrin labeled), antimouse CD80 (FITC), antimouse CD86 (FITC) and isotype control antibodies [American hamster IgG (FITC), rat IgG2a (FITC), rat IgG2a (phycoerythrin), and rat IgG1 (FITC) from eBioscience (San Diego, CA)], antimouse CD8 (FITC) and antimouse CD4 (phycoerythrin) from Immunotech (Marseille, France), anti-H-2K^b (FITC) and anti-H2D^b (FITC) from Cedarlane, and anti-Qa-Ib from PharMingen (San Diego, CA).

A peptide of melanocyte differentiation antigen tyrosinase-related protein-2 [SVYDFFVWL; TRP-2 (180–188)] was obtained from Biologica Co. (Aichi, Japan). TRP-2 (180–188) contains the epitope for rejection of B16 melanoma (27) and is mounted on H-2K^b.

Flow-Activated Cell-Sorting (FACS) Cytometric Analysis of Cell Surface Antigens and ELISA. The practical methods were described previously (13). Briefly, for FACS analysis, cells were suspended in PBS containing 0.1% sodium azide and 1% FCS and then incubated for 30 min at 4°C with fluorescently labeled monoclonal antibodies. Cells were washed, and the fluorescence intensity was measured by FACS.

For ELISA, culture supernatants of DCs were collected after removal of insoluble material by centrifugation and stored at −30°C. The levels of IL-12 p40 and TNF-α were measured by commercially available ELISAs.

Bone Marrow-Derived DCs (BM-DCs) of Mice. BM-DCs were prepared by a reported method (21, 28) with minor modifications. Briefly, BM cells were cultured overnight in 24-well plates at 0.5–1 × 10^6 cells/2 ml/well in RPMI 1640 containing 50 μM 2-mercaptoethanol, 10 mM HEPES, and 10% FCS. Nonadherent cells were harvested, resuspended in the same medium supplemented with 10 ng/ml GM-CSF, and cultured in the GM-CSF-containing medium. On day 3, adherent cells were cultured in fresh medium with 10 ng/ml GM-CSF. On day 6, nonadherent cells and loosely adherent cells were harvested and used for experiments as immature DCs. Immature DCs were resuspended in fresh RPMI 1640 containing 10 ng/ml GM-CSF and cultured for 24 h for ELISA or 48 h for FACS. To exclude the possible effect of contaminating LPS, BCG-CWS and macrophage-activating lipopeptide (MALP)-2 were pretreated with polymyxin B at 37°C for 60 min (14). The stimulating reagents were then added to the culture medium of immature DCs as indicated in the text (final concentration, 15 μg/ml BCG-CWS, 100 ng/ml LPS, 100 nM MALP-2, 10 μg/ml polymyxin B).

For *ex vivo* DC stimulation, we used BCG-PBS in place of BCG emulsion because BCG emulsion was not suitable for culture cell stimulation because of its micelle formation (data not shown), which interferes with the easy access of BCG-CWS to cells.

Immunization and Tumor Challenge. On days −28, −21, −14, −1, and +7 relative to the day of B16D8 challenge (Fig. 1*B*), 5 × 10^5 B16D8 cells (in 10 μl) were irradiated in PBS to prepare "debris," and the debris was mixed with 20 μl of 1 mg/ml BCG-CWS in emulsion buffer (BCG-emulsion-tumor). Wild-type and MyD88−/− mice each received s.c. immunizations containing 30 μl of this mixture at the base of the tail. The administration protocol is shown in Fig. 1*B*. As controls, tumor debris only or emulsion only, but not BCG-CWS in emulsion buffer (BCG emulsion), was used for the reason described in the "Discussion." We also checked the activity of this reagent for induction of tumor cytotoxicity. At tumor challenge, C57BL/6 mice were shaved at the flank and received s.c. injections of 300 μl of 6 × 10^5 syngeneic B16D8 melanoma cells in PBS. After 3 weeks, tumor volumes were measured at regular intervals by a caliper. The mouse 3LL cell line was used as an irrelevant control tumor. Tumor volume was calculated using the formula: tumor volume (cm^3) = long diameter (cm) × short diameter (cm) × short diameter (cm) × 0.4.

Generation of Tumor-Specific CTLs by *in Vitro* Tumor Stimulation. Immunized mice were sacrificed on day 0 (Fig. 1*B*), and lymph node cells were isolated by use of Lympholyte-M. Lymph node cells (5 × 10^6) were cultured with 1 × 10^5 irradiated (160 Gy) B16D8 cells (27, 29), pretreated with or without 100 units/ml IFN-γ for 24 h, in a 24-well culture plate in 2 ml of RPMI 1640 supplemented with 50 μM 2-mercaptoethanol, 10 mM HEPES, and 10% FCS. Mouse IL-2 was not added except where indicated. After 5 days, the cytolytic activity of tumor cells was tested with the cultured lymph node cells. In some cases, the lymph node cells were restimulated with irradiated B16D8 cells and 20 units/ml mouse IL-2.

Tumor-specific cytotoxic activity was enhanced by an alternative method. Splenocytes from naïve C57BL/6 mice were homogenized, incubated with 100 μg/ml of the TRP-2 (180–188) peptide for 4 h at 37°C, and irradiated with 30 Gy. For *in vitro* stimulation, these peptide-pulsed, irradiated splenocytes (2 × 10^6 cells) were mixed with lymph node cells or splenocytes (2 × 10^6 cells) isolated from BCG-emulsion-tumor-immunized mice in 2 ml of medium (see above) and cultured for 5 days at 37°C. Repetitive restimulation was performed an additional four times with these cells, and effector cells were prepared with use of Lympholyte-M. The cytotoxic activity of the effector cells toward B16 melanoma cells was evaluated by the ^{51}Cr release assay (29).

Assessment of *in Vitro* Cytolytic Activity. Target cells were labeled with ^{51}Cr for 3 h at 37°C, then washed and coincubated with effector cells at the indicated lymphocyte-to-target cell ratio in V-bottomed 96-well plates in a total volume of 200 μl of RPMI 1640. Cytotoxicity was determined by measuring the ^{51}Cr radioactivity released in 100 μl of the supernatant harvested from the plate after 8 h of incubation at 37°C (29). The percentage of specific lysis was calculated using the formula: specific lysis (%) = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

RESULTS

Establishment of Mouse Model for Evaluation of Adjuvant Potential. The B16 melanoma cell line (1×10^3–3×10^6 cells) was implanted in a syngeneic C57BL/6 host to determine an appropriate tumor burden. The dose of 6×10^5 cells/8-week-old female mouse was determined to be appropriate based on the injection of different numbers of cells s.c. into wild-type mice (not shown). This dose yielded 100% tumor manifestation within 4 weeks from the time of tumor injection in mice, with 93% survival of the mice for >6 weeks. We next examined immunotherapy protocol conditions with various doses of tumor debris and/or oil-in-water emulsion containing BCG-CWS (BCG emulsion; Fig. 1, *A* and *B*). Emulsion buffer or BCG emulsion only was used as a control. These controls elicited minimal tumor regression in mice (Fig. 1*A*). We decided to use emulsion or BCG emulsion as a control for subsequent experiments. In initial trials, we found that four repeats of immunization with B16 tumor debris conjugated with BCG emulsion (BCG-emulsion-tumor) were required for suppression of tumor growth in mice: three immunizations with BCG-emulsion-tumor or more than four immunizations with tumor debris or emulsion alone was insufficient for suppression of tumor progression (data not shown).

A large-scale study was then performed according to the protocol established (Fig. 1*B*). The tumor indices in each group are shown in Fig. 1*C*. Tumor cells grew progressively in mice treated with emulsion buffer ($n = 30$). Tumors grew similarly when tumor debris alone was used instead of emulsion (data not shown). Compared with the control, the growth kinetics of the tumor were significantly retarded in mice immunized with BCG-emulsion-tumor ($n = 30$; Fig. 1*C*). In the mice treated with emulsion control and BCG-emulsion-tumor, 30 of 30 and 18 of 30 died, respectively, of tumor progression 7 weeks after tumor inoculation.

The levels of IFN-γ in mice treated with tumor debris, BCG emulsion, or both were measured by ELISA (Fig. 1*D*). IFN-γ was detected in mouse serum at low levels in the BCG-emulsion-only group and at high levels in the BCG-emulsion-tumor group. No IFN-γ was detected in mice treated with saline or tumor debris only. These results to some extent resemble those observed in BCG-CWS-treated patients with cancer (15, 16).

Mice vaccinated with B16 debris in BCG emulsion showed progression of implanted 3LL cells, an irrelevant syngeneic tumor line (Fig. 1*E*). In mice preimmunized with BCG emulsion, specificity appeared to be exerted on the tumor species initially immunized as antigens.

Antitumor Response in Wild-Type and MyD88-Deficient Mice. TLR2 and TLR4 activate nuclear factor-κB and p38 mitogen-activated protein kinase through two adapters, Mal/TIRAP and MyD88 (30, 31). MyD88 is the relevant effector because it directly binds IRAK family proteins via their death domains (20). Using the same protocol, we examined tumor growth in age-matched wild-type ($n = 8$) *versus* MyD88−/− mice ($n = 8$). The reduction in tumor growth achieved by immunization with BCG-emulsion-tumor was almost abolished in MyD88−/− mice (Fig. 2*A*). The tumor sizes of the wild-type mice were significantly smaller than those of MyD88−/− mice, with the effect being evident at early time points. Tumor growth was delayed in wild-type mice immunized with BCG-emulsion-tumor compared with the MyD88−/− mice, in which tumor grew irrespective of immunization (Fig. 2*B*). All MyD88−/− mice died within 6 weeks after tumor challenge (Fig. 2*C*), although 50% of the wild-type mice were surviving at the 6-week time point. It is notable that in wild-type mice, delayed hypersensitivity-like skin reactions developed only in the group immunized with BCG-emulsion-tumor, whereas no skin lesions were observed in MyD88−/− mice.

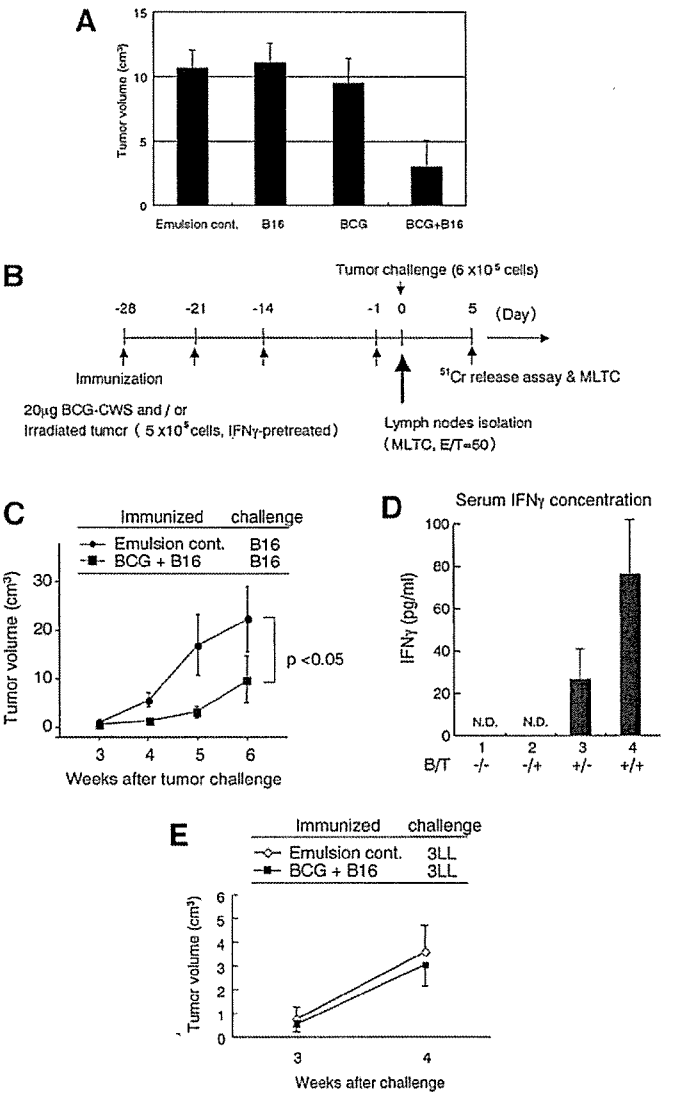

Fig. 1. Establishment of syngeneic mouse model to evaluate adjuvant potential for antitumor immunity. *A*, effect of solvents on implanted tumor progression. C57BL/6 mice ($n = 4$ in each group) were preimmunized four times with emulsion buffer (*Emulsion cont.*), B16 cell debris (*B16*), bacillus Calmette-Guérin emulsion (*BCG*), or BCG-emulsion-tumor (*BCG + B16*), and live B16 melanoma cells were implanted according to the protocol described in *B*. Tumor size was measured 5 weeks after tumor inoculation. *B*, experimental protocol for tumor implantation into mice. Mice (C57BL/6) were preimmunized four times with irradiated tumor debris and BCG emulsion or emulsion only before they were challenged with live B16 cells. One additional immunization was performed after the B16 challenge. The indicated mixtures (30 μl) were injected s.c. to form a bleb. Tumor volume was measured every week after the challenge. For *in vitro* experiments, mice were sacrificed on day 0. Lymph node cells were prepared from excised lymph node tissue. Lymph node cells were then mixed with irradiated B16 cells and cultured. After 5 days in culture, CTL response was tested by the ^{51}Cr release assay, and a portion of the cells was restimulated with irradiated B16 cells and cultured to analyze the ratio of CD8-positive cells by fluorescence-activated cell sorting *BCG-CWS*, bacillus Calmette-Guérin cell-wall skeleton; *MLTC*, mixed lymphocyte and tumor cell culture. *C*, tumor growth was delayed in mice preimmunized with BCG-emulsion-tumor. Control mice were immunized with emulsion buffer only. Each point represents the mean ± SE (*bars*; $n = 30$). *D*, measurement of serum IFN-γ concentrations on day 0. IFN-γ was measured by ELISA with serum from mice immunized with BCG-CWS (*B*) and/or tumor debris (*T*). Each *column* represents the mean ± SE (*bars*). *N.D.*, not detected. *E*, test for specificity of the antitumor effect in mice receiving B16 vaccinations. 3LL cells were injected in mice ($n = 8$) immunized with B16 cell debris in BCG emulsion, and 3LL tumor growth was measured 3–4 weeks after challenge. *BCG + B16*, mice preimmunized with BCG-emulsion-tumor (B16).

Properties of DCs Matured in Response to BCG-CWS. We then analyzed the surface expression of costimulatory molecules and the

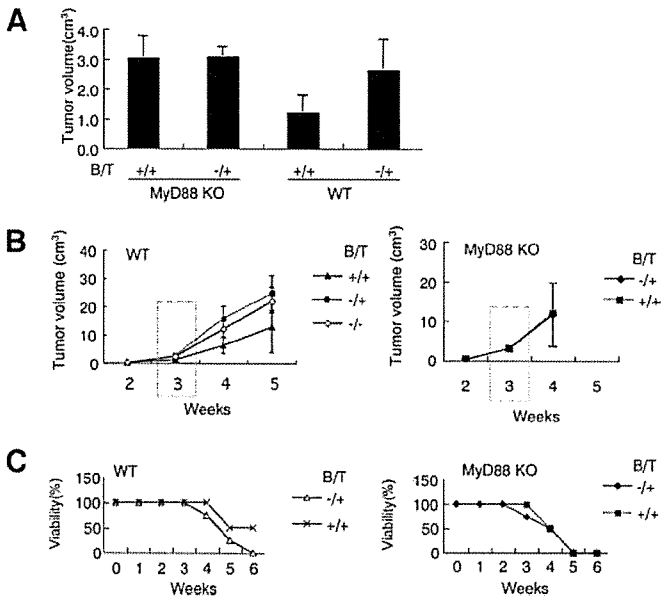

Fig. 2. Retarded tumor growth in wild-type but not MyD88−/− mice preimmunized with bacillus Calmette-Guérin (BCG)-emulsion-tumor. *A*, tumor volumes 3 weeks after tumor implantation. The experiments were performed according to the protocol shown in *Fig. 1B*. Tumor size was reduced by preimmunization with BCG-emulsion-tumor in wild-type mice. In contrast, tumor size was barely reduced by the same treatment in MyD88−/− mice. Each *column* represents the mean ± SE [*bars*; $n = 8$, four males and four females; sex differences were not observed (not shown)]. *B*, effect of preimmunization on tumor growth in wild-type and MyD88−/− mice. Mice were preimmunized with the indicated materials. Tumor growth was monitored thereafter. All wild-type mice survived >5 weeks, whereas four of eight MyD88−/− mice died within 5 weeks. Data are representative at least three experiments. BCG emulsion had almost no effect on tumor growth (not shown). *C*, antitumor response induced by BCG-emulsion-tumor depends on MyD88. Survival rate of tumor-bearing wild-type (*WT*) *versus* MyD88−/− mice (*MyD88 KO*) that had been immunized with BCG-emulsion-tumor or emulsion only is shown. Terminally moribund mice were killed. Data are representative of two experiments. *B/T*, bacillus Calmette-Guérin cell-wall skeleton/tumor debris.

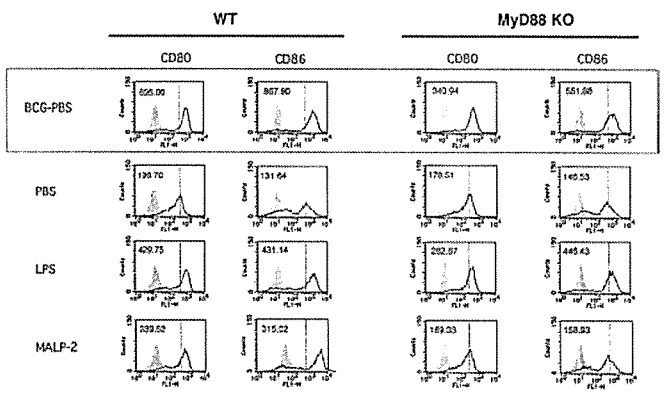

Fig. 3. Maturation marker profiles in bone marrow-derived dendritic cells (BM-DCs) treated with bacillus Calmette-Guérin (BCG) emulsion or BCG-PBS. Immature BM-DCs were cultured with various stimulators for 48 h. The cells were then washed and stained with FITC- or phycoerythrin-conjugated antibodies to measure the levels of the indicated DC maturation markers by fluorescence-activated cell sorting. Isotype-matched labeled nonimmune IgG was used as a control. BCG-PBS could induce the maturation of immature DCs derived from wild-type (*WT*) and MyD88−/− (*MyD88 KO*) mice. Lipopolysaccharide (*LPS*) and macrophage-activating lipopeptide (MALP)-2 were used as positive controls for Toll-like receptor (TLR)4 and TLR2 agonists, respectively, that activate DCs. Note that MALP-2 barely up-regulates the markers in MyD88−/− BM-DCs. One representative of three experiments is shown.

responses to cytokines of BM-DCs prepared from wild-type and MyD88−/− mice and of BM-DCs that were stimulated with BCG-CWS (Fig. 3). LPS and MALP-2, representative ligands for TLR4 and TLR2, respectively (21, 32), were used as control DC maturation inducers. The appropriate doses of these TLR stimulators were determined by the ability to induce IL-12 p40, and 15 μg/ml BCG-CWS was found to functionally correspond to 100 nM MALP-2 and 100 ng/ml LPS. Wild-type BM-DCs responded to LPS and MALP-2 by showing up-regulation of surface CD40 (not shown), CD80, and CD86 (Fig. 3, *left panel*). BCG-CWS enhanced the surface expression of these maturation markers similarly to the TLR2 and TLR4 stimulators.

The effects of the TLR stimulators on MyD88−/− BM-DCs were next tested (Fig. 3, *right panel*). LPS, but not MALP-2, induced costimulator up-regulation in MyD88−/− cells with a FACS profile similar to that of MyD88+/+ cells. Enhancement of the costimulator levels was also induced by BCG-PBS in MyD88−/− BM-DCs as well as in wild-type BM-DCs.

In general, costimulators were induced on the DC surface by stimulation with either LPS or MALP-2. In MyD88−/− cells, only the MALP-2-mediated DC maturation was abrogated. These profiles are in accord with previous findings (30, 31) that MyD88 is the only adapter that governs TLR2-dependent DC maturation, whereas TLR4 additionally activates a MyD88-independent pathway that may participate in DC maturation. Because BCG-CWS up-regulated DC maturation markers even in MyD88−/− cells, BCG-CWS-mediated activation of TLR2 and TLR4 appears to induce BM-DC maturation via the MyD88-dependent and -independent pathways. If this is the case, MyD88 and other adapters may participate in functional maturation of BM-DCs. Although the outputs are different between BCG-CWS- and LPS-stimulated DCs, particularly with respect to induction of IFN-inducible genes (33), both of these stimulators rely on either MyD88 or an alternative adapter for the DC maturation signal.

The cytokine production profiles of BCG-CWS-stimulated BM-DCs are shown in Fig. 4; LPS was used as a positive control. BCG-PBS induced IL-12 p40 and TNF-α production in wild-type BM-DCs but not MyD88−/− DCs (Fig. 4). Hence, MyD88−/− cells lose the ability to produce cytokines but retain the ability to up-regulate DC maturation markers in response to BCG-CWS. This difference was also observed in LPS-stimulated wild-type *versus* MyD88−/− DCs, which is consistent with a finding reported by Kaisho *et al.* (21). Addition of IL-4 to the cells barely affected this

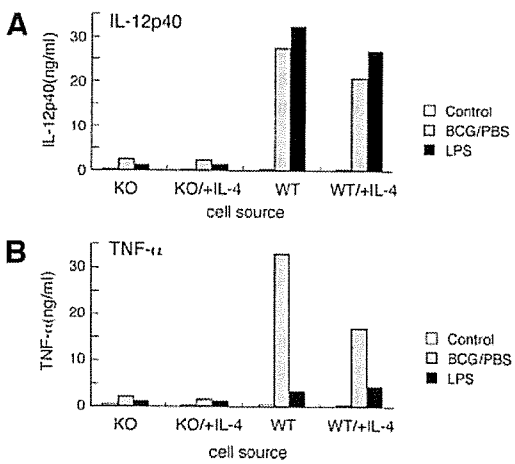

Fig. 4. Levels of cytokines induced by bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS) stimulation. Interleukin (IL)-12 p40 (*A*) and tumor necrosis factor (TNF)-α (*B*) production by bone-marrow derived dendritic cells (BM-DCs) stimulated with the indicated Toll-like receptor (TLR) agonists was determined by ELISA. MyD88−/− DCs (*KO*) did not produce IL-12 p40 or TNF-α in response to BCG-PBS, whereas wild-type DCs (*WT*) stimulated with BCG-PBS efficiently produced these cytokines. Lower levels of TNF-α (*B*) were induced by lipopolysaccharide (LPS) than by BCG-PBS in wild-type DCs, which may reflect the fact that BCG-CWS induces the continuous production of TNF-α but LPS does not (33). Similar results were obtained in three additional experiments.

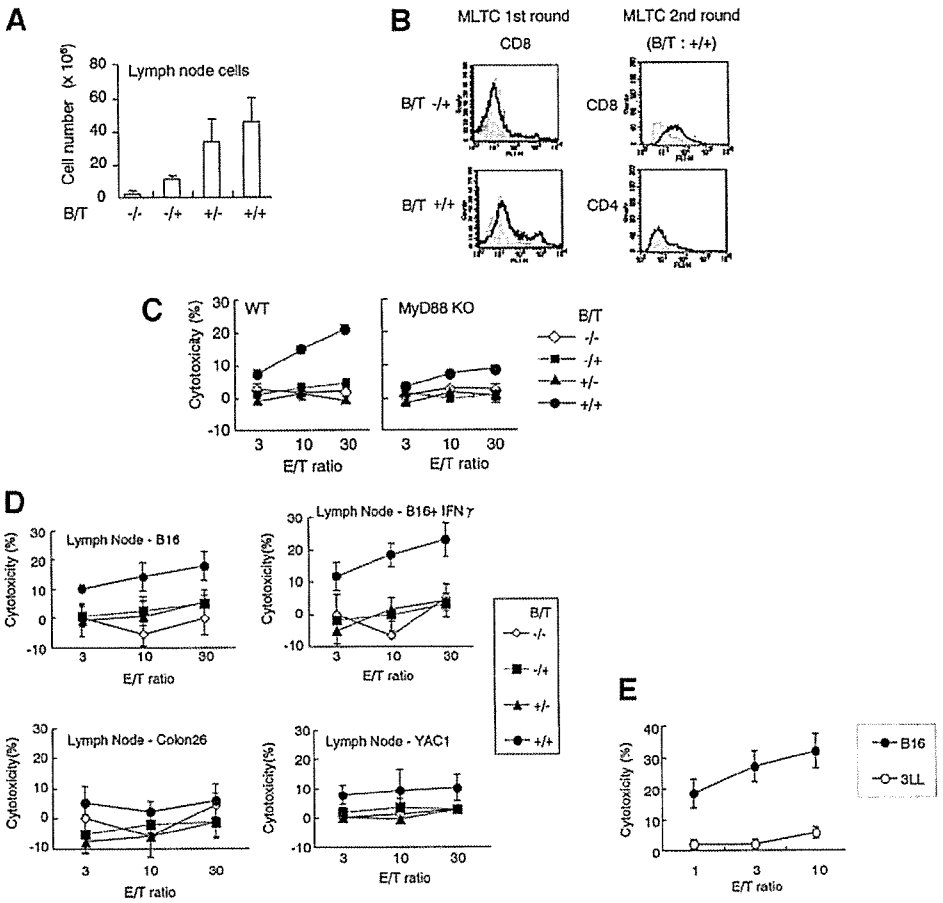

Fig. 5. CTL induction in lymph node cells in wild-type (*WT*) mice but not in MyD88−/− (*MyD88 KO*) mice. Lymph node cells were derived from mice 4 weeks after immunization with bacillus Calmette-Guérin (BCG)-emulsion-tumor (*B16*) or tumor debris only (control). Mice immunized with BCG emulsion with or without B16 debris showed lymph node swelling. The numbers of collected lymph node cells and spleen cells (not shown) were counted by use of a hematocytometer. The numbers of cells collected from lymph nodes are shown in *A*. Cells were stimulated *in vitro* one round with tumor debris (*B, left panel*). The CD8+ population was 17 or 3% after one round of stimulation in cells harvested from mice with BCG-emulsion-tumor or with tumor debris only, respectively. In a similar way, after two rounds of stimulation, CD8- and CD4-positive cell populations were measured by fluorescence-activated cell sorting (*B, right panel*). MLTC, mixed lymphocyte and tumor cell culture. The CTL response directed against B16 melanoma parent cells was assessed with cells derived from mice that had been treated with the indicated reagent combinations (*C*). B16-specific CTL activity was induced in wild-type mice immunized with BCG-emulsion-tumor (B16), but only a very low CTL response was detected with MyD88−/− cells. The specificity of the detected CTL response was examined with various tumor cells as targets (*D*). A strong CTL response was induced through one round of stimulation in wild-type lymph node cells against B16 melanoma cells and B16 cells treated with IFN-γ. The CTL response was not directed against other syngeneic tumor cell lines, Colon26 and YAC-1. Virtually no CTL activity appeared in lymph node cells from MyD88−/− mice even with IFN-γ-treated target cells (not shown). Natural killer cells may induce slight cytolysis against YAC-1 cells. The results are representative of six similar experiments. Finally, specific CTL response against B16 melanoma was confirmed with a CTL-rich fraction repetitively stimulated with the tyrosinase-related protein-2 peptide mounted on the MHC on splenocytes (see "Materials and Methods" for details; *E*). 3LL was used as a control target. *B/T*, bacillus Calmette-Guérin cell-wall skeleton/tumor debris.

tendency. Of note, expected levels of IL-12 p40 and lower levels of TNF-α were detected in LPS-treated BM-DCs compared with BCG-PBS-treated DCs. In conclusion, BCG-CWS induces up-regulation of costimulators but fails to induce TNF-α and IL-12 p40 in MyD88−/− DCs.

Tumor-Specific CTL Induction in Wild-Type and MyD88-Deficient Mice. The BCG-CWS-based vaccines elicited cytotoxic responses, presumably CTLs, against B16 melanoma cells (Fig. 5). To test the relationship between the BCG-CWS-based cytotoxic response and antitumor potential, lymph node cells were recovered from individual wild-type mice administered BCG-emulsion-tumor (B16 cells) and restimulated with irradiated B16 cells, and their ability to lyse B16 targets was assessed. For logistical reasons, it was not possible to quantify the CTL response in all mice from each experiment. A total of 12 mice in two experiments were tested according to the protocol (Fig. 1*B*).

We measured the number of draining lymph node cells from wild-type mice to evaluate the effect of immunization *in vivo*. The number of lymph node cells recovered was increased in response to BCG-CWS immunization (Fig. 5*A*), although the number of spleen cells was not affected (not shown). Lymph node cells from mice immunized with tumor debris only, BCG emulsion only, or BCG-emulsion-tumor were stimulated *in vitro* one round with tumor debris and stained with CD8 for FACS analysis (Fig. 5*B, left panel*). CD8+ cells were augmented only in lymph node cells stimulated with BCG-emulsion-tumor in response to the *in vitro* tumor challenge. No cytolytic activity against B16 tumor cells was found in lymph node cells stimulated with BCG emulsion only (Fig. 5, *C* and *D*). After a second round of *in vitro* tumor challenge, CD8+ T cells in lymph node cells of the BCG-emulsion-tumor group were further increased, whereas CD4+ cells did not expand (Fig. 5*B, right panel*). However, CD8+ T cells were only marginally expanded in similarly treated MyD88−/− cells (data not shown).

A significant cytotoxic response was detected in lymph node cells from wild-type mice challenged with BCG-emulsion-tumor after the first round of *in vitro* stimulation with tumor (Fig. 5*C, left panel*). In contrast, only a minimal cytotoxic response was detected in lymph node cells from MyD88−/− mice preimmunized with BCG-emulsion-tumor (Fig. 5*C, right panel*). No other wild-type or MyD88−/− groups pretreated with either BCG emulsion or tumor debris exhibited

a cytolytic response (Fig. 5*C*). The results paralleled those of tumor regression in wild-type *versus* MyD88-deficient mice. Thus, tumor immunity appeared to be linked with cytotoxicity in conjunction with signaling via MyD88.

We next examined whether the BCG-CWS-induced tumor lysis was specific to the tumor, an initially challenged subline of B16 melanoma. The lymph node cells harvested from the four groups of wild-type mice immunized with BCG emulsion and/or B16 tumor debris were treated as in Fig, 5*C*. The cytolytic response toward various types of syngeneic tumor cells was measured in these four groups (Fig. 5*D*). A significant response was detected only in the group stimulated with BCG-emulsion-tumor. Cytolysis was enhanced if IFN-γ was added to the B16 target cells (Fig. 5*D*). The cytotoxicity was directed against B16, but not Colon 26 or YAC1 cells. Thus, the BCG-CWS-induced tumor lysis is likely to be CTL dependent. CTLs induced by BM-DCs via TLR-MyD88 signaling thus appear to be specific for the antigens used for the initial sensitization.

To show tumor reactivity, specificity, and MHC restriction of the CTLs more clearly, we tried to enrich the CTL population specific to B16 melanoma by repetitive restimulation of lymph node cells from mice immunized with B16 debris. However, when we repetitively stimulated lymph node cells from wild-type mice immunized with BCG-emulsion-tumor, harvested lymphocytes largely died, as reported previously (34). Thus, we used an alternative way to expand the CTL population toward B16 melanoma. Finally, we obtained a CTL-rich fraction through peptide-pulsed restimulation: lymph node cells from immunized mice restimulated were four times with splenocytes pulsed with TRP-2 (180–188). CTLs against B16 melanoma proliferated and exhibited robust cytotoxic activity (Fig. 5*E*). Thus, initially immunized tumor confers specificity on CTLs in terms of proliferation and cytotoxicity.

DISCUSSION

In the present study using a mouse syngeneic model and MyD88−/− mice, we demonstrated that (*a*) immunizing a tumor with BCG-CWS induces effective tumoricidal response; (*b*) the response is specific to the immunized tumor species, suggestive of CTL induction; (*c*) BCG-CWS-mediated TLR2/4 stimulation leads to the induction of both DC maturation markers and cytokines (IL-12 p40 and TNF-α), only the latter being impaired in MyD88−/− cells; and (*d*) BCG-CWS-dependent CTL induction and tumor regression are abolished in MyD88−/− mice. Hence, MyD88-dependent cellular responses involve BCG-CWS-mediated cytokine production and tumor cytotoxicity induced by DCs. The results may reflect the finding that TLR2 and TLR4 share the same adapter, MyD88 (30, 31), which is activated by BCG-CWS.

BCG in emulsion buffer (BCG emulsion) elicited minimal regression of the control tumor in mice (Fig. 1*A*), which was inconsistent with the nature of this adjuvant in humans (15). Because we have kept the mice under high specific pathogen-free conditions, the mice may not be sensitized to human pathogens. At present, we consider that most Japanese are vaccinated with BCG, leading to robust response to BCG emulsion and eliciting antitumor immunity. Other reasons, including differences between the human and mouse immune systems or the properties of the tumors implanted, may have led to this discrepancy. In most experiments, we used emulsion-only as a control.

The first and second of our findings provide molecular-based evidence for adjuvant activity that corroborate previous experimental findings about BCG-mediated antitumor immune potential (15, 16). Human DC maturation is induced by BCG-CWS in a TLR2/4-dependent manner (13, 14). Immunizing tumor debris with BCG adjuvants subsequently induced a specific cytolytic response to the immunized tumor species (Fig. 5). These findings suggest that CTLs are responsible for the antitumor cytotoxicity induced by BCG-CWS. Final confirmation of this issue, however, will be needed to show the parallelism between CD8+ T-cell depletion and loss of antitumor responses *in vivo*. CTL induction toward specific tumors has not been experimentally verified in the BCG therapy. We therefore first demonstrated that TLR signaling in DCs participates in inducing tumor-specific cytotoxicity, most likely reflecting CTLs.

The third and fourth findings demonstrate that the TLR adapter MyD88 plays a key role in DC-mediated CTLs. TLR2 recruits MyD88, whereas TLR4 recruits other adapters in addition to MyD88. Hence, DC maturation is supported by MyD88 in TLR2 and by both the MyD88-dependent and -independent pathway in TLR4. Because BCG-CWS is a ligand for TLR2/4 (13), our interpretation is that the surface markers of DC maturation are up-regulated by either the MyD88-dependent or -independent pathway in BCG-CWS-stimulated cells. The BCG-CWS-mediated DC maturation should be crucial for tumor-specific CTL induction.

The pathways sustaining CTL response appear to differ from those supporting the allostimulatory mixed lymphocyte response. The mixed lymphocyte response is provoked even in MyD88−/− DCs if they are stimulated with LPS or BCG-CWS (Ref. 21).[6] For CTL induction, in contrast, MyD88-dependent cellular responses are essential in addition to MyD88-independent responses. In line with this, TLR4-mediated pathways including MyD88 and other adapters (35, 36) are important for induction of the LPS-mediated lymphoproliferative response. Therefore, activation of TLR4 by BCG-CWS in MyD88-dependent and -independent manners would be essential for CTL induction. Alternatively, unidentified receptors for the uptake of BCG-CWS (14, 16) may participate in the observed antitumor response.

What molecule is responsible for the MyD88-independent DC activation response is the next question to be addressed. It has been accepted that at least four adapters, MyD88, Mal/TIRAP, TICAM-1, and TICAM-2, are linked to TLR4 to deliver signals leading to the activation of nuclear factor-κB, c-Jun (AP-1), and IFN-β (18, 30, 31, 36, 37). These signaling pathways are known to mature DC in different ways and stages. IFN-β expression is a major outcome of the MyD88-independent pathway in TLR3 and leads to a unique DC maturation via the TICAM-1 adapter (37). TICAM-1 recruited to the TLR4–TICAM-2 complex was identified as an effective adapter in TLR4-mediated IFN-β promoter activation (36, 38). Unexpectedly, however, BCG-CWS activates TLR4, but no IFN-inducible genes are induced (33). As reported recently, IFN type 1 (a main product of the TICAM-1 pathway) and STAT-1, rather than TNF-α-mediated cellular responses (39), cause LPS-mediated endotoxic shock, and LPS acts on TLR4 in a manner that activates both MyD88 and TICAM-1 (36, 38). This may be the reason that BCG-CWS is far less toxic than LPS and suitable for clinical use as an adjuvant.

The important point is that TICAM-1-mediated DC activation, unlike the MyD88-dependent DC response, sustains DC motility to lymph nodes, which is supported by CCR7 (40, 41). Thus, factors induced by TICAM-1 (37) cause the maturation of DCs in a fashion distinct from those induced by MyD88 (42). It remains possible that TICAM-1 contributes to full DC maturation in concert with MyD88-dependent signaling. This issue could be clarified by testing TICAM-1 knockout mice in the future.

It has been accepted that MyD88 is shared as an adapter with receptors for IL-1β, IL-18, and some members of the TLR family (18–20, 43). For example, type 1 IFN directly activates the gene

[6] Our unpublished data.

expression of IL-18 receptor components (AcPL), IL-1 receptor-related protein, and MyD88 in NK and T cells (44). MyD88 may support the so-called danger signal induced by tumors or tumor-disrupted tissues (45). This interpretation is reminiscent of the properties of the danger signal in the suppression of tumor cell progression. It seems possible that most danger signals cause the delay of tumor growth through activation of the adapter MyD88.

BCG-CWS was found to induce the expression of IL-23 but not IL-12 p70 simultaneously with DC maturation (33). IL-23 participates in the production of IFN-γ in lymphocytes and is relatively weakly induced in the activation of NK cells (33, 46). *In vitro* analysis suggests that BCG-CWS activity induces DC maturation and IL-23 production, leading to effective Th1 polarization. The importance of IL-23 has not been clarified, but we favor the interpretation that the resultant induction of IFN-γ directly activates CD4+ T cells or cancels CD4+/CD25+ regulatory T-cell activity (47). Recent studies using Serex suggested that CTL induction is sustained in the absence of regulatory T-cell activity (48). In fact, IFN-γ-positive patients who underwent repetitive administration of BCG-CWS have enhanced long-term survival, which may reflect the induction of memory T function, presumably attributable to IL-23 (49). Of course, possible differences between the mouse and human TLR systems need to be determined in this regard.

Live BCG has been reported to be effective for reduction of bladder tumor growth by activating host immunity (23). BCG-CWS has also been administered to patients for postoperative treatment of cancer, producing good prognoses (15, 17, 18). In addition to being a cost-effective therapy, such BCG-CWS treatment is simple, highly useful, and applicable to patients with various cancers. Patients with postoperative lung cancer receiving this therapy exhibited a high quality of life index of ~70% and 5-year survival of ~60% in our clinic (15, 17). The most intriguing idea is to use these adjuvant functions to establish an optimal immunotherapy strategy for the host: combined activation of CTLs and NK cells ought to more potently elicit anti-tumor potential because both MHC-negative and -positive tumor cells can be eliminated as their targets (50, 51). Such a therapy could provide immune system-activating signals, eliminate inhibitory factors, and avoid the emergence of immunoresistant phenotypes. These aims may be achieved by a combination of adjuvant modalities that induce CTL- and NK-mediated tumor elimination.

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Immunity against mouse thymus-leukemia antigen (TL) protects against development of lymphomas induced by a chemical carcinogen, *N*-butyl-*N*-nitrosourea

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Mouse thymus-leukemia antigens (TL) are aberrantly expressed on T lymphomas in C57BL/6 (B6) and C3H/He (C3H) mice, while they are not expressed on normal T lymphocytes in these strains. When *N*-butyl-*N*-nitrosourea (NBU), a chemical carcinogen, was administered orally to B6 and C3H strains, lymphoma development was slower than in T3^b-TL gene-transduced counterpart strains expressing TL ubiquitously as self-antigens, suggesting that anti-TL immunity may play a protective role. In addition, the development of lymphomas was slightly slower in C3H than in B6, which seems to be in accordance with the results of skin graft experiments indicating that both cellular and humoral immunities against TL were stronger in C3H than B6 mice. The interesting finding that B lymphomas derived from a T3^b-TL transgenic strain (C3H background) expressing a very high level of TL were rejected in C3H, but not in H-2K^b transgenic mice (C3H background), raises the possibility that TL-specific effector T cell populations are eliminated and/or anergized to a certain extent by interacting with H-2K^b molecules. (Cancer Sci 2004; 95: 914–919)

Expression of mouse thymus leukemia antigens (TL) belonging to the major histocompatibility complex (MHC) class Ib family is restricted to the intestines in all mouse strains, as well as to the thymus of TL$^+$ strains (e.g., A-strain and BALB/c).[1–4] Recent studies by us and others showed that TL molecules bind with CD8$\alpha\alpha^+$ intestinal intraepithelial lymphocytes, CD8$^+$ thymocytes and CD8$\alpha\alpha^+$ peripheral T lymphocytes.[5–8] In addition, their possible roles *in situ* have been elucidated to a certain extent,[7–9] though many issues remain to be clarified. Although TL$^-$ strains (e.g., C57BL/6 (B6) and C3H/He (C3H)) do not express TL in the thymus, a proportion of T lymphomas originating in these mice express TL as a serologically defined tumor antigen.[1,10–12]

In a previous study, we demonstrated that a certain fraction of *N*-butyl-*N*-nitrosourea (NBU)-induced lymphomas in C3H and B6 mice express TL, but the expression profiles are somewhat different between these two strains.[12] TL is expressed by only a half of the C3H-derived lymphomas (8/16), but most B6-derived lymphomas are TL-positive (16/17). In another study, we derived Tg.Con.3-1 and Tg.Con.3-2 transgenic mice (C3H background) expressing T3^b-TL ubiquitously[13,14] and demonstrated that TL can serve as a transplantation antigen by grafting the skin of these mice onto syngeneic C3H.[15] We also showed that immune responses against grafted skins are weaker in (B6×C3H)F$_1$ than in C3H mice. Especially, when skins from Tg.Con.3-2 (a lower expresser of TL) were grafted on F$_1$ mice, none was rejected, in contrast to the case of C3H. These observations together suggest that immunity against TL is weaker in B6 than in C3H, and that anti-TL immunity may contribute to the resistance to lymphoma development in these mice.

To further examine this possibility, in the present study, we administered NBU orally to C3H and B6, as well as T3^b-TL gene-transduced counterparts, Tg.Con.3-1 and B6.Tg.Con.3-1. The observed faster lymphoma development in the T3^b-TL gene-transduced mice, in which TL-reactive lymphocytes are most likely to be eliminated and/or anergized, provided support for anti-TL immunity playing a protective role against lymphoma growth *in vivo*. Furthermore, the onset of lymphoma development in C3H is slightly slower than that of B6, which may be in line with the results of skin-graft experiments showing that cellular and humoral immunities against TL were weaker in B6 than C3H. In addition, using B lymphomas (derived from Tg.Con.3-1) expressing a very high level of TL, we compared *in vivo* anti-tumor immunity between H-2K^b transgenic (Tg.H-2K^b-2, C3H background) and C3H mice, and observed that the lymphomas were not efficiently rejected by the former, suggesting that TL-specific T cells may be eliminated and/or anergized in these transgenic mice by interacting with H-2K^b molecule.

Materials and Methods

Mice. The derivation of the transgenic mouse strains used in this study has been described previously.[13,14] Tg.Con.3-1, having a chimeric gene in which the *T3^b* gene from B6 is driven by the *H-2K^b* promoter, expresses T3^b-TL ubiquitously. Another strain, Tg.H-2K^b-2, having the *H-2K^b* transgene with its own promoter, similarly expresses H-2K^b. Both strains were generated on a C3H background. To prepare a congenic strain expressing T3^b-TL ubiquitously on a B6 background, Tg.Con.3-1 mice were back-crossed 16 times with B6, and homozygotes for the TL transgene were finally derived by intercrossing of heterozygotes. The strain thus established was designated as B6.Tg.Con.3-1. B6, C3H, and (B6×C3H)F$_1$ mice were purchased from Japan SLC (Hamamatsu, Japan).

NBU-induction of lymphomas. Female mice at 5 weeks of age were orally given NBU (Nacalai Tesque, Kyoto, Japan) as described previously,[12,16] and the development of lymphomas was observed. Two Tg.Con.3-1-derived B lymphomas (110501 and 110201b) were established, maintained *in vivo* and used for further experiments.

Flow cytometric analysis. A mAb against TL (HD168) was generated as described previously.[17] MAbs against mouse MHC class I (M1/42) and H-2E^k (M5/114) were purchased from ATCC (Manassas, VA). A fluorescein isothiocyanate

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(FITC)-labeled mAb against mouse CD8α (53-6.7) and biotinylated mAbs against mouse H-2A^k (11-5.2), B220 (RA3-6B2), Thy-1.2 (30-H12) and CD86 (GL1) were purchased from BD Biosciences Pharmingen (San Diego, CA) and phycoerythrin (PE)-labeled mAb against mouse CD40 (3/23) from Serotech (Oxford, UK). FITC-labeled goat anti-rat IgG (Chemicon International, Inc., Temecula, CA), FITC-labeled rabbit anti-mouse immunoglobulin (DAKO-PATTS, Glostrup, Denmark), or PE-labeled streptavidin (BD Biosciences Pharmingen) was used as the second reagent. PE-labeled T3^b-TL tetramers were prepared as described previously.[6,18] Stained cells were analyzed on a FACSCalibur using CellQuest software (BD Biosciences Immunocytometry Systems, San Diego, CA).

Skin graft and test bleed. Skin grafting was performed as previously described,[9,15,19] and test bleeds from the tail vain were performed weekly. Antibody activity against TL in the sera was assayed by indirect flow cytometric analysis using T3^b-TL transfected and untransfected RMA-S as indicator cells.[20]

Cytotoxic T lymphocyte (CTL) assay. TL-specific CTL lines were established from the spleens of skin-grafted C3H mice and their cytotoxic activity was measured by ^{51}Cr release assay using concanavalin A (Con A) blasts from TL transgenic animals and their background strains as target cells. All procedures were performed as previously described.[9,15,19]

Tetramer analysis. Mixed lymphocyte culture (MLC) was performed with spleen cells from skin-grafted mice 8 weeks after transplantation as described previously,[9,15,19] and cells from MLC were stained with T3^b-TL tetramers, which were prepared as described previously.[6] Briefly, spleen cells from skin-grafted mice were stimulated with γ-irradiated spleen cells from TL transgenic strains for 5 days and used for the experiments. Cells from MLC were incubated with the PE-labeled T3^b-TL tetramers (1 μg/ml) at room temperature for 30 min and then with an FITC-labeled anti-CD8α mAb at 4°C for 30 min to enumerate TL tetramer-positive cells.

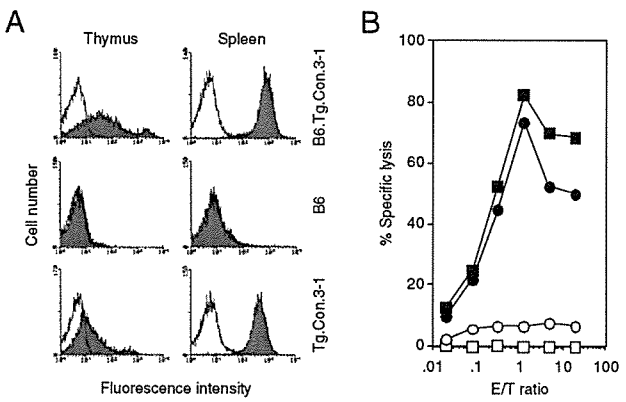

Fig. 1. TL expression on lymphocytes from B6 congenic Tg.Con.3-1 (C3H background), B6.Tg.Con.3-1. (A) TL expression on thymocytes and spleen cells. Cells were indirectly stained with rat anti-TL mAb (HD168) and FITC anti-rat IgG and analyzed on a FACSCalibur. (B) CTL susceptibility of TL$^+$ spleen cells. Con A blasts were prepared from spleen cells of B6.Tg.Con.3-1 and Tg.Con.3-1 and also their background strains, and their susceptibility to a TL-specific CTL line generated from Tg.Con.3-1 skin-grafted C3H was tested by ^{51}Cr-release assay. Con A blasts were derived from B6.Tg.Con.3-1 (closed rectangles), Tg.Con.3-1 (closed circles), B6 (open rectangles), and C3H (open circles).

Results

The B6 congenic TL transgenic strain, B6.Tg.Con.3-1, is derived from Tg.Con.3-1 (C3H background). To explore anti-TL immunity in B6, we derived a new congenic strain, B6.Tg.Con.3-1, expressing T3^b-TL ubiquitously by crossing Tg.Con.3-1 (C3H background) with B6. B6.Tg.Con.3-1 accepted B6 skin grafts (data not shown), in line with the histocompatibility between these two strains, except for the narrow region containing the transgene, *TL*. As shown in Fig. 1A, the expression levels of TL on lymphocytes from Tg.Con.3-1 and B6.Tg.Con.3-1 strains were similar, although thymocytes from B6.Tg.Con.3-1 were stained slightly more intensely with anti-TL mAb. The expression profile resembled that of H-2K^b (data not shown), indicating that *T3^b* gene expression is controlled by the *H-2K^b* promoter.

We next tested whether TL molecules expressed on cells from B6.Tg.Con.3-1 are recognized by TL-specific CTL lines generated from Tg.Con.3-1 skin-grafted C3H. As shown in Fig. 1B, Con A blasts from both B6.Tg.Con.3-1 and Tg.Con.3-1 TL transgenic strains were equally susceptible to these CTL lines, whereas those from B6 and C3H were not, confirming that TL molecules expressed on the cells of B6.Tg.Con.3-1 can be recognized by TL-specific CTLs without involvement of H-2 as

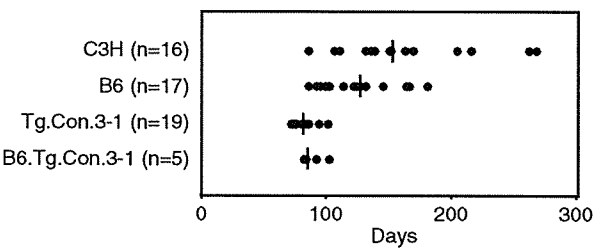

Fig. 2. Survival time of NBU-administered mice. Mice were orally given NBU for 60 days, and the development of lymphomas was followed. The first day of administration was designated as Day 0. All mice were confirmed to have died due to lymphoma development by macro- and microscopic examination. The survival times of B6 and C3H were significantly longer than those of their counterparts ($P<0.01$ and $P<0.001$ in the Mann-Whitney test, respectively). Vertical bars show median values.

Table 1. Transplantation of Tg.Con.3-1 and B6.Tg.Con.3-1 skins expressing T3^b-TL

Donor	Recipient	Number of grafts[1]				Rejection time (days)
		Total	Rejected[2]	Crisis[3]	Accepted	
Tg.Con.3-1	C3H	10	10	0	0	16.4±7.5
	(B6×C3H)F$_1$	9	7	2	0	23.4±1.7
	Tg.Con.3-1	10	0	0	10	—
B6.Con.3-1	B6	10	0	0	10	—
	(B6×C3H)F$_1$	10	0	4	6	—
	B6.Tg.Con.3-1	10	0	0	10	—

1) Grafts were observed daily for at least 100 days.
2) Rejection was defined as a loss of >95% of the grafted tissue.
3) Grafts showed transitory signs of rejection and became smaller.