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

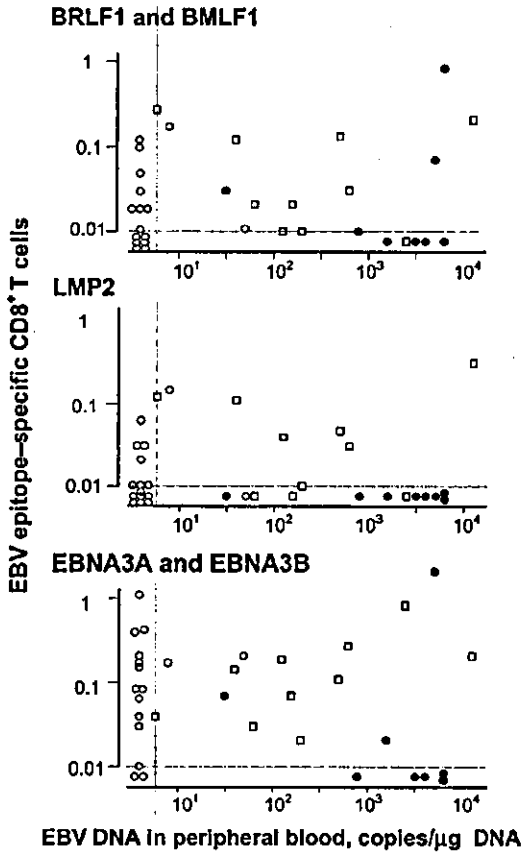

Figure 1. Association of Epstein-Barr virus (EBV) load in peripheral blood with the frequency of EBV epitope–specific $CD8^+$ T cells. EBV load was estimated using real-time polymerase chain reaction, and epitope-specific $CD8^+$ T cells were enumerated by tetramer binding. Data are shown separately 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.

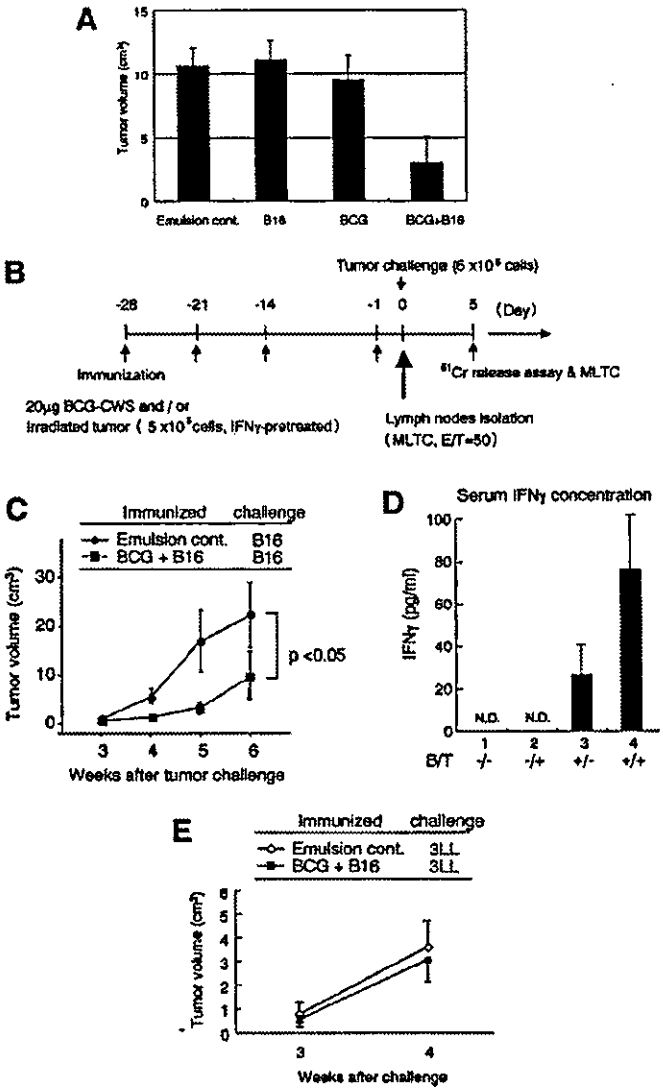

Fig. 1. Establishment of syngeneic mouse model to evaluate adjuvant potential for antitumor immunity. *A*, effect of solvents on implanted tumor progression. C57BL/6 mice ($n = 4$ in each group) were preimmunized four times with emulsion buffer (*Emulsion cont.*), B16 cell debris (*B16*), bacillus Calmette-Guérin 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

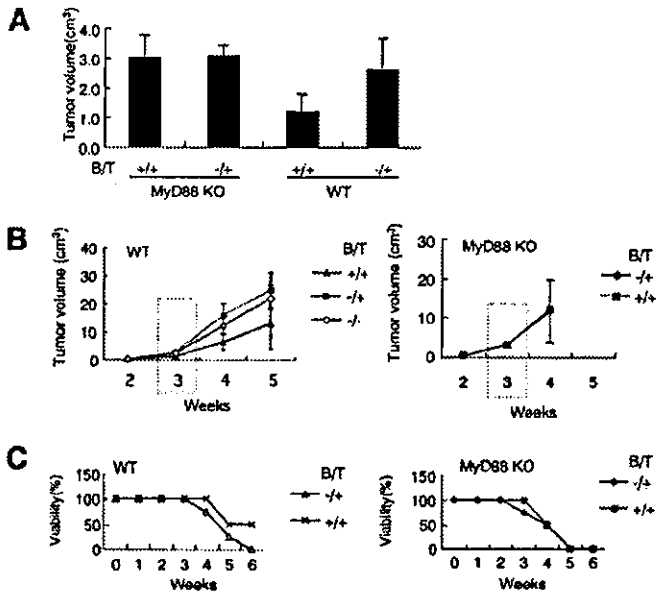

Fig. 2. Retarded tumor growth in wild-type but not MyD88−/− mice preimmunized with bacillus Calmette-Guérin (BCG)-emulsion-tumor. *A*, tumor volumes 3 weeks after tumor implantation. The experiments were performed according to the protocol shown in *Fig. 1B*. Tumor size was reduced by preimmunization 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.

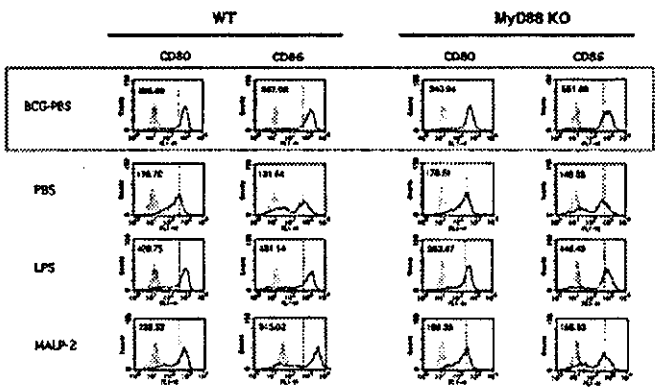

Fig. 3. Maturation marker profiles in bone marrow-derived dendritic cells (BM-DCs) treated with bacillus Calmette-Guérin (BCG) emulsion or BCG-PBS. Immature BM-DCs were cultured with various stimulators for 48 h. The cells were then washed and stained with FITC- or phycoerythrin-conjugated antibodies 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

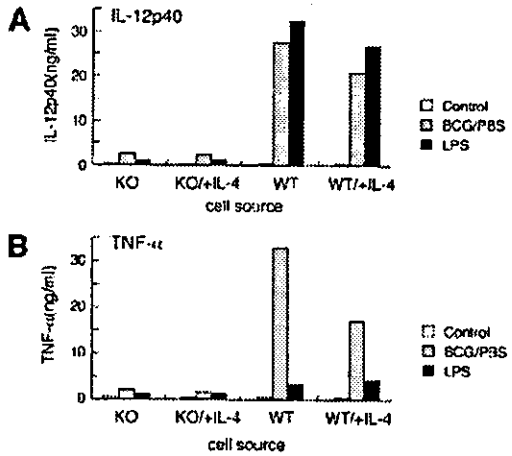

Fig. 4. Levels of cytokines induced by bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS) stimulation. Interleukin (IL)-12 p40 (*A*) and tumor necrosis factor (TNF)-α (*B*) production by bone-marrow derived dendritic cells (BM-DCs) stimulated with the indicated Toll-like receptor (TLR) agonists was 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.

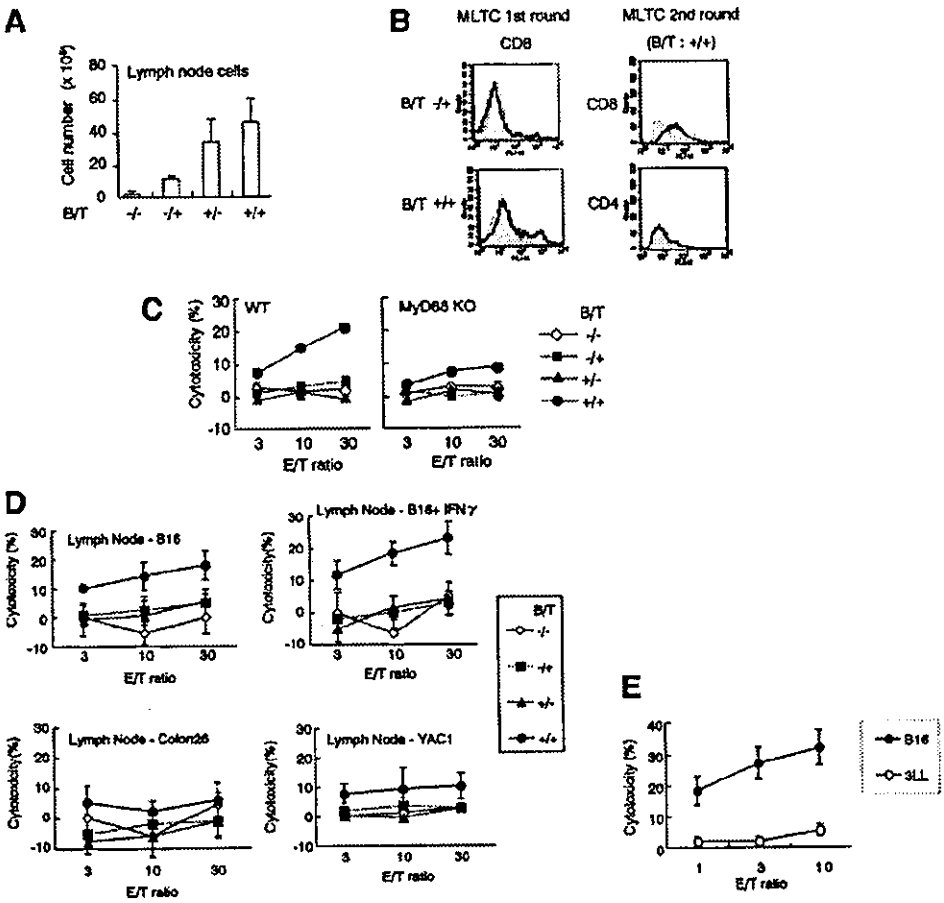

Fig. 5. CTL induction in lymph node cells in wild-type (*WT*) mice but not in MyD88−/− (*MyD88 KO*) mice. Lymph node cells were derived from mice 4 weeks after immunization with bacillus Calmette-Guérin (BCG)-emulsion-tumor (*B16*) or tumor debris only (control). Mice immunized with BCG emulsion with 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 I (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 I 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 antitumor 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 CD8αα$^+$ intestinal intraepithelial lymphocytes, CD8$^+$ thymocytes and CD8αα$^+$ 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.

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

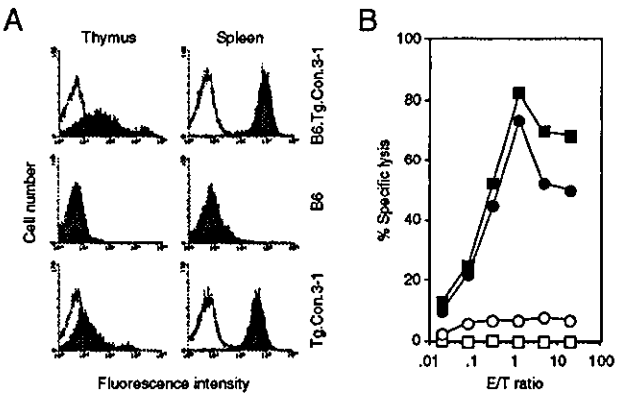

Fig. 1. TL expression on lymphocytes from B6 congenic Tg.Con.3-1 (C3H background), B6.Tg.Con.3-1. (A) TL expression on thymocytes and spleen cells. Cells were indirectly stained with rat anti-TL mAb (HD168) and FITC anti-rat IgG and analyzed on a FACSCalibur. (B) CTL susceptibility of TL$^+$ spleen cells. 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).

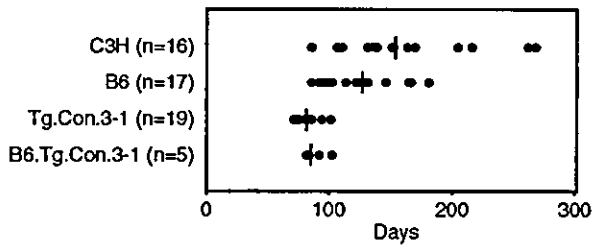

Fig. 2. Survival time of NBU-administered mice. Mice were orally given NBU for 60 days, and the development of lymphomas was followed. The first day of administration was designated as Day 0. All mice were confirmed to have died due to lymphoma development by macro- and microscopic examination. The survival 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.

presenting molecules.

Faster development of NBU-induced lymphomas is observed in TL transgenic mice. We administered NBU orally to B6 and C3H as well as to their $T3^b$-TL gene-transduced counterparts, B6.Tg.Con.3-1 and Tg.Con.3-1, respectively, to study the possibility that anti-TL immunity affects lymphoma development. As shown in Fig. 2, the survival times of B6 and C3H were prolonged, compared with their TL transgenic counterparts, in which TL-specific T cells are assumed to be eliminated and/or anergized, suggesting that anti-TL immunity protects against lymphoma growth *in vivo*. In addition, the survival time of C3H was found to be slightly longer than that of B6, reflecting slower development of lymphomas in C3H than in B6, although the difference is not statistically significant.

Grafted skins from B6.Tg.Con.3-1 on B6 are not rejected. To compare anti-TL immunity in B6 with that in C3H, skin-graft experiments were carried out with various combinations of transgenic, congenic and inbred strains of mice. As previously reported, skin grafts from Tg.Con.3-1 were rejected efficiently by recipient C3H mice, while (B6×C3H)F_1 mice were less potent at rejecting Tg.Con.3-1-derived skin, i.e., rejection was observed in 7 out of 9 grafts in this study, and 22 out of 29 grafts in the previous study.[15] In contrast to C3H, B6 could not reject skin grafts from B6.Tg.Con.3-1 (Table 1), although the expression level of TL in the skin of B6.Tg.Con.3-1 was comparable to that of Tg.Con.3-1 (data not shown). We also analyzed $CD11c^+$ Langerhans cells of both strains, but no significant difference in the cell number or TL expression was observed (data not shown). One possible explanation for the failure of B6 mice to reject B6.Tg.Con.3-1 skin grafts might be $H\text{-}2^b$-restricted active suppression by regulatory T cells. *In vivo* administration of anti-CD4 and/or CD25 mAbs, however, did not induce rejection of B6.Tg.Con.3-1 skin (data not shown), suggesting that at least $CD4^+CD25^+$ regulatory T cells (Treg)[21, 22] and type 3 helper T cells (Th3)[23, 24] are not responsible for the weak or absent responsiveness. When (B6×C3H)F_1 mice received skin from B6.Tg.Con3-1, graft crisis was evident in 4/10. The results also demonstrated that (B6×C3H)F_1 were less potent at rejecting B6.Tg.Con.3-1 than Tg.Con.3-1 skin grafts.

The number of TL tetramer$^+$ cells does not always correlate with skin graft rejection. Attempts were made to generate TL-specific CTLs from B6 mice receiving skin grafts from B6.Tg.Con.3-1, in the same way as employed for CTL induction in the C3H system,[9, 15, 19] but were unsuccessful, in concordance with the results of the skin-graft experiments described above. As shown in Fig. 3, less than 5% of $CD8^+$ cells were found to be positive for TL tetramer staining in primary MLC derived from B6.Tg.Con-1 skin-grafted B6 spleen, while 25–30% of those from Tg.Con.3-1 skin-grafted C3H were positive. In addition, only a TL tetramer-weakly positive population, considered to express low avidity TCR for TL,[6, 19] was induced from B6.

Tetramer analysis was also conducted with (B6×C3H)F_1 mice grafted with Tg.Con.3-1 or B6.Tg.Con.3-1 skins, and the results are summarized in Table 2. We found that 7.5 to 15% of $CD8^+$ cells in primary MLC with the spleens from Tg.Con.3-1 skin-grafted F_1 mice were TL tetramer-positive, being lower than that with Tg.Con.3-1 skin-grafted C3H mice (25–30%) as shown in Fig. 3, in accordance with the results of skin grafting, which showed that immunity against TL in F_1 mice is generally weaker than that of C3H (Table 1). The proportion of TL tetramer$^+$ cells in $CD8^+$ T cells in primary MLC with the spleen cells from B6.Tg.Con.3-1 skin-grafted F_1 mice was lower than in the Tg.Con.3-1 skin-grafted F_1, being in general agreement with the results of skin grafting. These results together suggest that the precursor frequencies of TL-specific CTL in B6 are lower than in C3H with intermediate values for (B6×C3H)F_1. When examined at the level of individual mice, however, the proportion of TL tetramer$^+$ cells does not always correlate with the fate of the grafted skins.

TL-specific CTL is negatively selected and/or anergized to a certain extent by reacting with H-2K^b. In our previous study, it was of in-

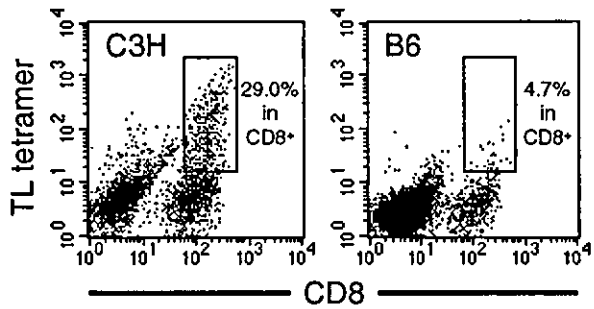

Fig. 3. Tetramer analysis of primary MLC. MLC was performed with spleen cells from skin-grafted mice 8 weeks after transplantation as described previously.[9, 15, 19] Spleen cells from C3H and B6 that had received skin grafts from Tg.Con.3-1 and B6.Tg.Con.3-1, respectively, were stimulated once with γ-irradiated spleen cells from the respective TL transgenic strains. Cells from MLC were incubated with the PE-labeled $T3^b$-TL tetramer (1 μg/ml) at room temperature for 30 min and then with an FITC-labeled anti-CD8α mAb at 4°C for 30 min.

Table 2. Frequency of CTL precursors against TL in skin-grafted (B6×C3H)F_1 mice

Mouse	Skin grafting		MLC/TL tetramer staining[1] (%)	
	Skin graft	Result	Stimulator	TL tetramer$^+$/$CD8^+$ cells
1	Tg.Con.3-1	Rejected	Tg.Con.3-1	15.4
			B6.Tg.Con.3-1	14.0
2		Rejected	Tg.Con.3-1	7.4
			B6.Tg.Con.3-1	7.5
3		Crisis	Tg.Con.3-1	15.0
			B6.Tg.Con.3-1	14.3
4	B6.Tg.Con.3-1	Crisis	Tg.Con.3-1	8.5
			B6.Tg.Con.3-1	7.9
5		Accepted	Tg.Con.3-1	6.5
			B6.Tg.Con.3-1	8.7
6		Accepted	Tg.Con.3-1	9.0
			B6.Tg.Con.3-1	7.5

1) Spleen cells were obtained from the F_1 mice at 8 weeks after skin grafting, and stimulated with spleen cells from TL transgenic mice from 5 days. Cells from such primary MLC were stained with PE-TL tetramers and FITC-labeled anti-CD8α mAb (see "Materials and Methods" and also the legend of Fig.3).

terest that the immune responses against TL⁺ skin grafts were weaker in H-2K^b transgenic mice (C3H background) than in C3H mice (rejection of Tg.Con.3-1 grafts, 51/52 in C3H versus 6/10 in Tg.H-2K^b-1),[15] suggesting that H-2K^b expression in the thymus and/or periphery might result in negative selection and/or tolerance induction of a certain population of TL-specific T cells, especially CTL. To explore this possibility, we employed two unique TL⁺ B lymphomas (110501 and 110201b) established from Tg.Con.3-1 mice by oral administration of NBU in the same way as described previously.[12,16] As shown in Fig. 4, these lymphomas were defined as of B-cell origin, since they expressed B220 but not Thy-1. A higher level of TL (5–10 times more as compared with TL⁺ T lymphomas, such as C3NB1 (C3H origin)[12] and ERLD (B6 origin)) and MHC class I were expressed, but little or no expression of MHC class II (I-A^k and I-E^k), CD40, CD80 (data not shown) or CD86 was observed. When these TL⁺ B lymphomas were transplanted into C3H nude mice or Tg.Con.3-1 mice, they grew progressively, as with TL⁺ T lymphomas, but when they were transplanted into C3H mice, they grew to a certain extent, but were finally rejected in most mice (Figs. 5 and 6), suggesting that T lymphocytes were involved in the rejection. Unexpectedly, when the tumor cell numbers were increased, they were rejected more readily. *In vivo* antibody-depletion experiments showed that CD8 cells are predominantly involved in TL⁺ B lymphoma rejection (data not shown). When TL⁺ B lymphomas were transplanted into (B6×C3H)F$_1$ or Tg.H-2K^b-2 mice (C3H background) expressing H-2K^b ubiquitously, they were not rejected as expected from our previous study on skin grafting,[15] as described above, supporting the possibility that TL-specific effector T cells are negatively selected and/or anergized to a certain extent in these mice by interacting with H-2^b molecules in the thymus and/or periphery. Of those, H-2K^b molecules seem to be more important than H-2D^b, since the frequency of TL-specific T lymphocytes in Tg.H-2K^b-2 mice determined by TL tetramer analysis was 7.5–15%, being comparable to that of (B6×C3H)F$_1$ (data not shown).

Anti-TL antibody activity is higher in C3H than B6 mice receiving TL⁺ skin grafts. We previously demonstrated by antibody-depletion experiments that CD4⁺ T cells are indispensable for rejection of TL⁺ skin from Tg.Con.3-1.[25] Such observations suggest the possibility that the weaker immune responsiveness against TL in B6 than in C3H is due to not only negative selection and/or anergy of CD8⁺ CTLs, but also the lack of Th activity. We evaluated helper T cell (Th) activity in anti-TL immunity by measuring anti-TL antibodies in the sera from skin-grafted mice in both B6 and C3H strains, due to the lack of a direct assessment system of Th function for CTL induction. As shown in Fig. 7A, anti-TL antibody activity was much higher in sera

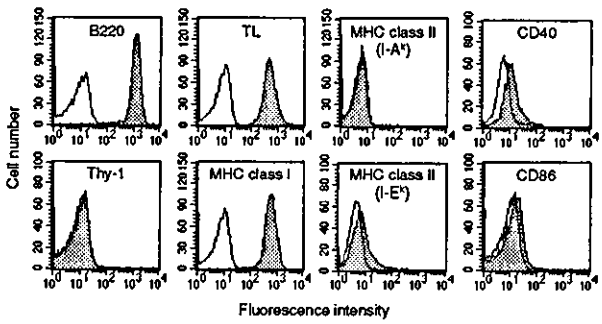

Fig. 4. Surface phenotype of NBU-induced B lymphomas derived from Tg.Con.3-1. NBU-induced B lymphoma cells (110501) were stained and analyzed on a FACSCalibur. Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

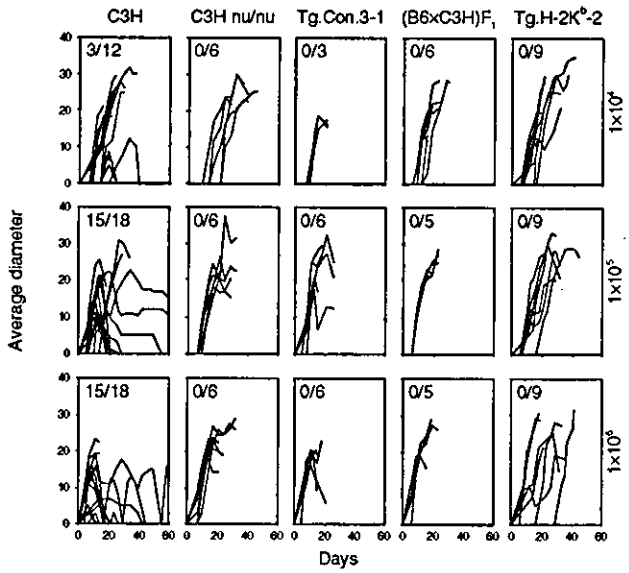

Fig. 5. Growth of NBU-induced B lymphomas *in vivo*. Mice were injected subcutaneously (s.c.) with NBU-induced B lymphoma cells (110501). The number of surviving mice in each group at day 60 is shown (surviving/total mice), and cell numbers injected are given on the right. Tumor size values are the averages of the longest and the shortest diameters. Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

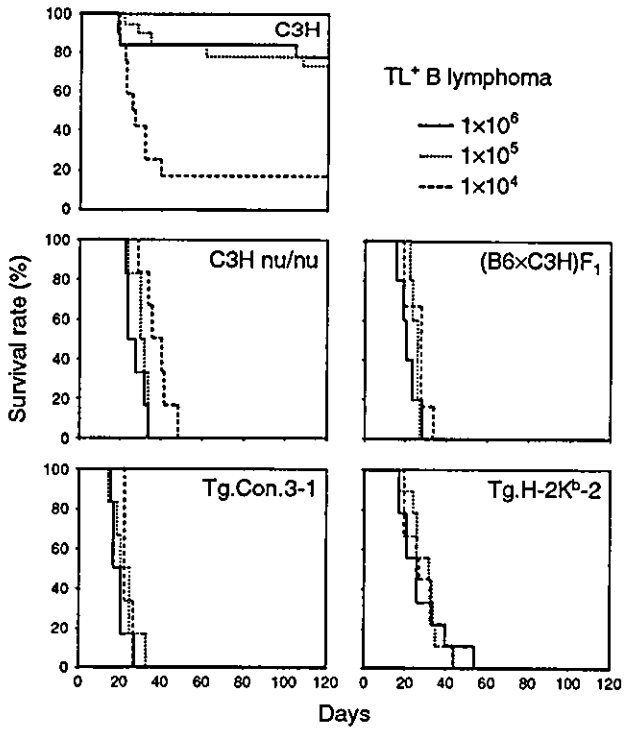

Fig. 6. Survival time of mice transplanted with NBU-induced B lymphomas. Mice were injected s.c. with NBU-induced B lymphoma cells (110501), and the survival time was determined. The cell numbers injected were 1×10^4 (broken line), 1×10^5 (dotted line), and 1×10^6 (unbroken line). Similar results were also obtained with another NBU-induced B lymphoma 110201b from Tg.Con.3-1.

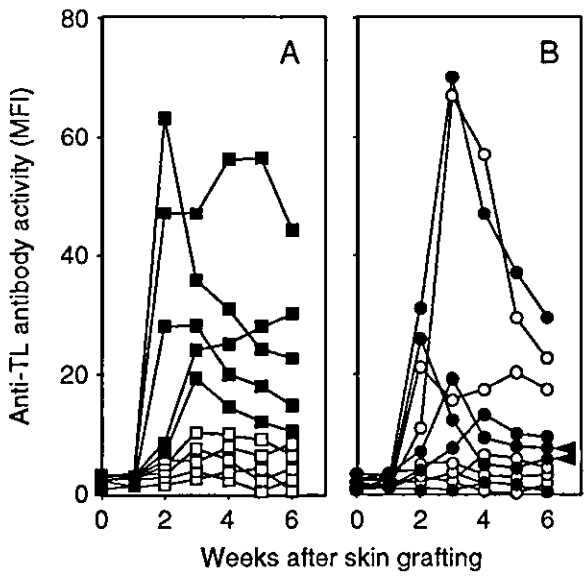

Fig. 7. Antibody activity against TL in the sera from skin-grafted mice. $T3^b$-TL transfected and untransfected RMA-S cells were indirectly stained with sera plus FITC anti-mouse immunoglobulins and analyzed on a FACSCalibur. Relative antibody activity against TL was calculated by use of the following equation: mean fluorescence intensity (MFI) of stained $T3^b$-TL transfected RMA-S cells−MFI of stained RMA-S cells. (A) C3H and B6 mice were transplanted with skins from Tg.Con.3-1 (n=5, closed squares, all mice rejected the grafts) and B6.Tg.Con.3-1 (n=5, open square, all mice accepted the grafts), respectively. (B) (B6×C3H)F_1 mice were transplanted with skins from Tg.Con.3-1 (n=5, closed circles) or B6.Tg.Con.3-1 skin (n=5, open circles). All B6.Tg.Con.3-1 skin-transplanted F_1 mice accepted the grafts. Three F_1 mice rejected Tg.Con.3-1 skin, while 2 showed crisis (as indicted by arrowheads).

from C3H than B6 animals, suggesting the possibility that the Th activity in generation of anti-TL CTLs is also higher in the former. In general, anti-TL antibody activity in (B6×C3H)F_1 mice was slightly weaker than in the C3H strain, although there were some exceptional individuals (Fig. 7B). No apparent differences were observed between B6.Tg.Con.3-1 skin- and Tg.Con.3-1 skin-grafted mice, implying that the skins from both strains have similar antigenicity in inducing antibody production against TL.

Discussion

Recent findings that susceptibility to chemically induced and spontaneous tumorigenesis is enhanced in immunodeficient mice strongly support the concept of cancer immune surveillance (reviewed in Ref. 26). In the present study, we observed that NBU-induced lymphoma developed more slowly in C3H than the TL transgenic counterpart strain, Tg.Con3-1, in which TL-reactive lymphocytes cannot be detected by conventional assays such as TL tetramer and cytoplasmic IFN-γ staining (data not shown), and we suggested that TL-specific immunity plays a certain role in protection against lymphoma development *in vivo*, at least when NBU is used as a carcinogenic agent. In addition, we recognized that the development of lymphomas was slightly slower in C3H than in B6. In the previous study, we found that the T/B ratio and TL expression of NBU-induced lymphomas were somewhat different between C3H and B6.[12] A half of C3H-derived lymphomas (8/16) expressed TL (all of them were of T-cell origin), while the rest (3 T lymphomas and 5 B lymphomas) were TL-negative. On the other hand, all B6-derived lymphomas (17/17) were of T-cell origin, and almost all (16/17) expressed TL. The relatively high frequency of TL^- lymphoma development in C3H may reflect cancer immunoediting[26,27] due to a tumor escape mechanism from immunity against TL. Moreover, it is of interest that primary T lymphomas derived from B6 strains tend to show heterogeneous TL expression profiles when studied by flow cytometry, suggesting multi-clonal T cell origin (data not shown). These findings together favor the possibility that the immune responses against TL in C3H play a more pivotal role than in B6, conferring protection against TL^+ lymphoma development; this view seems to be supported by the results of the skin graft experiments, in which C3H rejected TL^+ skin from Tg.Con3-1, whereas B6 could not reject B6.Tg.Con3-1 skin, although differences in host factors other than immunity between these two strains may also be involved.[28-30] It should also be noted in this regard that lymphoma development in B6 was significantly slower than in the TL transgenic counterpart strain, B6.Tg.Con.3-1, suggesting that immune surveillance against TL, albeit weaker than in C3H, is also working in the B6 strain. To our knowledge, these experimental results are the first to suggest that tumor antigen-specific immune surveillance is actually working *in vivo*.

In this and previous skin-graft experiments, we showed that the immune responses against TL are stronger in C3H than in B6, (B6×C3H)F_1 or H-$2K^b$ transgenic mice. The stronger response against TL in C3H mice is not due to the allogenicity of $T3^b$-TL, since the amino acid sequences of $T3^b$-TL (B6) and $T3^k$-TL (C3H) are identical. When (B6×C3H)F_1 mice were grafted with TL transgenic skins, they rejected the grafts less efficiently than C3H, suggesting that the frequency of TL-specific CTL precursors is lower in F_1 mice than in C3H, which is supported in part by the results of TL tetramer experiments (Fig. 3 and Table 2). In this study, we showed that even naïve C3H mice rejected MHC class II negative B lymphomas expressing a high level of TL, but most of (B6×C3H)F_1 and Tg.H-$2K^b$-2 could not (Figs. 5 and 6). These results together suggest that TL-specific CTL are deleted and/or tolerized to a certain extent in these strains expressing H-2^b, especially H-$2K^b$. We previously demonstrated that TL-specific CTL recognize the framework of the α1/α2 domains of TL.[18] We therefore searched for specific amino acid sequences common between $T3^{b\,(or\,k)}$-TL and H-$2K^b$, but not H-$2K^k$ or D^k, which might be responsible for the negative selection and/or tolerance induction, but none could be elucidated. These results suggest that H-2^b molecules, especially H-$2K^b$, presenting antigenic peptides may form antigenic determinants which resemble TL (free of peptides) and work as restriction elements for negative selection and/or tolerance induction. In addition, there may be a difference in TL-specific Th activity between B6 and C3H contributing to the difference in CTL induction of these strains, since $CD4^+$ T cells have been shown to be necessary for graft rejection of TL^+ skin.[25] In this regard, we obtained the interesting result in this study that (B6×C3H)F_1 mice rejected Tg.Con.3-1 skin more efficiently than B6.Tg.Con.3-1 skin (Table 1), suggesting that activation of H-2^k-restricted Th cells may be necessary for graft rejection. The results that humoral immune response against TL was weaker in B6 and (B6×C3H)F_1 than in C3H may also support this possibility, although there were some exceptional individuals among (B6×C3H)F_1 mice (Fig. 7).

Another interesting finding obtained in this study is that even naïve C3H mice were able to reject large numbers of two TL^+ B lymphomas expressing a very high level of TL (derived from Tg.Con.3-1), while conventional TL^+ T lymphomas were not rejected,[12] suggesting that a higher expression of TL plus expression of certain co-stimulatory molecules on B lymphomas (not on T lymphomas) may be important to activate anti-TL immunity, although the TL^+ B lymphomas are not strong expressers of CD40, CD80 and CD86 (Fig. 4 and not shown). This

observation supports the report by Schultze *et al.* that human B lymphocytes are a very good source of antigen-presenting cells (APC), at least *in vitro*.[31] When B lymphocytes are activated with CD40 ligand (or anti-CD40 mAb) plus IL-4, they proliferate vigorously, whereas mature dendritic cells (DC), which are known as the best APC population, do not. Using activated B lymphocytes as APC, we have also established CTL clones and succeeded in defining many new epitopes of cytomegalovirus pp65.[32,33] Prophylactic tumor suppression experiments against TL$^+$ conventional T lymphoma by immunization with TL$^+$ B cells activated with anti-CD40 mAb plus IL-4 are now under way in an attempt to establish more effective and convenient immunization protocols than those employed earlier using the skin or DC.[12]

A further interesting finding is that the number of TL tetramer$^+$ cells generated in primary MLC does not always correlate with skin-graft rejection at the level of individual mice. In melanoma patients, it is also reported that immunological responses monitored *in vitro* against certain tumor antigens do not always correlate with clinical outcomes.[34,35] Furthermore, anti-TL antibody activity does not always correlate with skin-graft rejection either, as shown in Fig. 7B. These findings altogether suggest that *in vitro* monitoring methods so far established are not sufficient to determine exactly the functional anti-tumor activity *in vivo*. In this regard, more reliable and sensitive methods need to be developed to establish more effective immunization protocols.

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bjh research paper

Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant

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Summary

Minor histocompatibility antigens (mHAs) are major histocompatibility complex (MHC)-associated peptides, which trigger T-cell responses that mediate graft *versus* host disease (GVHD) and graft *versus* leukaemia effects. We recently identified a new mHA epitope, termed ACC-1, which is presented by HLA-A*2402 and encoded by *BCL2A1*, whose expression is restricted to haematopoietic cells including leukaemic cells. HLA-A24/ACC-1 tetramer detected the presence of ACC-1-specific $CD8^+$ cells in the peripheral blood of a patient up to 7 months following transplantation, and these tetramer-positive cells were expandable *in vitro* by ACC-1 peptide stimulation. A retrospective analysis of 320 patients with HLA-A*2402 who had received a human leucocyte antigen (HLA) genotypically matched unrelated donor through the Japan Marrow Donor Programme was conducted to determine whether ACC-1 disparity is associated with adverse clinical outcomes such as GVHD. Among these patients, ACC-1 disparity was detected in 55 (17·2%) donor/recipient pairs. After adjusting for known risk factors, the hazard ratios or odds ratios of acute and chronic GVHD, relapse and disease-free survival were not statistically different between patients receiving ACC-1 compatible and incompatible transplantation. These data suggest that disparity of haematopoietic cell-specific mHA, ACC-1, is unlikely at least to augment GVHD, and that T cells specific for ACC-1 may also be used for immunotherapy of recurring leukaemia without GVHD.

Keywords: minor histocompatibility antigen, ACC-1, graft *versus* leukaemia effect, graft *versus* host disease.

Allogeneic haematopoietic cell transplantation (allo-HCT) is an effective treatment for various types of haematological malignancies. The curative graft *versus* leukaemia (GVL) effect of allo-HCT is mainly mediated by donor-derived T cells that are specific for minor histocompatibility antigens (mHAs) (Goulmy, 1997; Warren *et al*, 1998), which are major histocompatibility complex (MHC)-associated peptides that originate from polymorphisms in the genome. However, relapse of the original disease has still been one of the major causes of treatment failure even with the beneficial GVL effect following allo-HCT (Weiden *et al*, 1981; Horowitz *et al*, 1990). Donor lymphocyte infusion (DLI) has been developed for patients with relapsing leukaemia (Kolb *et al*, 1990) and shown to induce long-lasting complete remissions in chronic myeloid leukaemia (CML) in chronic phase (Mackinnon *et al*, 1995; Dazzi *et al*, 2000). The powerful immunotherapeutic effect of mHA-specific T cells eradicating relapsing leukaemia by DLI has been shown using human leucocyte antigen (HLA)-tetramer technologies (Marijt *et al*, 2003). It is generally believed that mHAs, exclusively expressed on recipient haematopoietic cells including leukaemic cells, such as HA-1 and HA-2 (Mutis *et al*, 1999) or those on lineage-specific

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haematopoietic cells such as HB-1 (Dolstra *et al*, 1999) may result in a GVL effect in the absence of severe graft *versus* host disease (GVHD). In line with the same concept, we have sought and identified two novel haematopoietic lineage-specific mHA epitopes using linkage analysis; one is restricted by HLA-A24 (designated as ACC-1^Y, corresponding to adenine at nucleotide position 56) and another is by HLA-B44 (designated as ACC-2^D, corresponding to adenine at nucleotide position 245), both spanning two of the three polymorphic amino acids on the *BCL2A1* gene (Akatsuka *et al*, 2003). The ACC-1^Y- and ACC-2^D-specific $CD8^+$ cytotoxic T-lymphocyte (CTL) clones were originally derived from the peripheral blood mononuclear cells (PBMC) of patients receiving a HLA-identical sibling transplant for acute myeloid leukaemia in partial remission and CML in accelerated phase respectively (Akatsuka *et al*, 2002). These patients did not develop acute GVHD and have not suffered a leukaemia relapse for more than 2 years.

Before proceeding with clinical trials, there would be several ways to test whether a newly identified mHA is safe for immunotherapeutic intervention (e.g. it will not cause severe GVHD). One such approach is an *in situ* skin model recently reported by Dickinson *et al* (2002), in which CTLs specific for mHAs encoded by ubiquitously expressed genes, but not those expressed only on haematopoietic tissues, damaged cultured dermal epithelia. Another approach is a statistical analysis to examine whether donor/recipient disparity of a mHA of interest will increase severe GVHD or decrease relapse, as applied for HA-1, the most extensively studied mHA, whose expression is limited to haematopoietic cells (Goulmy *et al*, 1996; Tseng *et al*, 1999; Gallardo *et al*, 2001; Lin *et al*, 2001; Socie *et al*, 2001).

In this study, we showed that T cells reactive with a synthetic HLA-A24 tetramer incorporating the ACC-1^Y peptide were detectable in the recipient PBMC up to 200 d following bone marrow transplantation (BMT) and that these T cells could be easily expanded *in vitro*. Secondly, we questioned whether donor/recipient disparity of ACC-1 was associated with an increased risk of GVHD in recipients of HLA-identical unrelated BMT through the Japan Marrow Donor Programme (JMDP) by genotyping the ACC-1 locus in DNA samples from 320 donor/recipient pairs.

Materials and methods

Patient selection

This study was approved by the Institutional Review Boards of the Aichi Cancer Centre and the JMDP. Between March 1993 and April 1998, 444 patients with haematological malignancies received non-T cell-depleted BMT from an HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 genotypically matched unrelated donor identified through the JMDP (Morishima *et al*, 2002). Of these, we selected 320 patient/donor pairs on the basis of the presence of HLA-A24, which is the restriction HLA molecule for ACC-1. GVHD prophylaxes used were short-term methotrexate and either cyclosporine ($n = 283$) or tacrolimus ($n = 37$); antithymocyte globulin was not used. Patients were assessed for acute and chronic GVHD according to previously published criteria (Glucksberg *et al*, 1974; Shulman *et al*, 1980). The patient characteristics are summarized in Table I.

Detection of ACC-1^Y-specific T cells in post-HCT PBMC was applied to those patients who received an HLA-identical, HLA-A24 positive, ACC-1 disparate transplant in a separate study approved by the Institutional Review Boards of the Aichi Cancer Centre. An Epstein–Barr virus transformed B lymphoid cell line (LCL) was established by infecting an aliquot of PBMC with B95-8 supernatant (ATCC, Rockville, MD, USA).

Genotyping of ACC-1 polymorphism

Genotyping was performed blind to the recipient's clinical information. We performed allelic discrimination to detect the *BCL2A1* G56A polymorphism using fluorogenic 3′-minor groove binding (MGB) probes in a polymerase chain reaction (PCR) assay. Primers flanking the +56 polymorphic region were: 5′-ATTTACAGGCTGGCTCAGGACTA-3′ (forward) and 5′-GGACCTGATCCAGGTTGTGGTAT-3′ (reverse), and MGB probes complementary to the polymorphic region were: 5′-FAM-CTGCAGTGCGTCCT-MGB-3′ for the 'G' allele and 5′-VIC-TCTGCAGTACGTCCTA-MGB-3′ for the 'A' allele (Applied Biosystems, Tokyo, Japan). The PCR was conducted

Table I. Patient characteristics.

	ACC-1		
	Compatible	Incompatible	*P*-value
No. of pairs	264	55	
Median patient age, years (range)	25 (1–50)	26 (7–50)	0·40
Median donor age, years (range)	34 (20–50)	33 (21–49)	0·43
Sex (donor/recipient), *n* (%)			0·99
Male/male	102 (39)	22 (40)	
Male/female	66 (25)	14 (25)	
Female/male	59 (22)	11 (20)	
Female/female	38 (14)	8 (15)	
Disease, *n* (%)			0·83
Standard risk leukaemia*	118 (45)	22 (40)	
High risk leukaemia†	115 (43)	26 (47)	
Others	32 (12)	7 (13)	
Preconditioning, *n* (%)			0·86
TBI regimen	224 (85)	47 (86)	
Non-TBI regimen	41 (15)	8 (14)	
GVHD prophylaxis, *n* (%)			0·27
Cyclosporine based	232 (88)	51 (93)	
Tacrolimus based	33 (12)	4 (7)	

TBI, total body irradiation; GVHD, graft *versus* host disease.
*Acute leukaemia in first complete remission and chronic myeloid leukaemia in first chronic phase.
†More advanced stage than standard risk leukaemia.

