rightward transcripts (BARTs), and small RNAs (EBERs). Immune response mediated by T-lymphocytes eliminates most latency III-infected cells; however, resting memory B lymphocytes provide a reservoir for latent virus. T-lymphocyte immunity to latency III-infected B lymphocytes persists for life and protects reactivation of latent virus from a reservoir [2].

However, in the absence of an effective immune response, reactivation of latent virus from a reservoir occurs and causes EBV-associated lymphoproliferative diseases. EBV-associated lymphoproliferative diseases occur with primary infection after transplantation or reactivation of latent virus as a consequence of immune suppression for organ transplantation and autoimmune diseases or acquired immune deficiency syndrome (AIDS) [3–6]. EBV-associated lymphoproliferative diseases are associated in the majority of cases with latency type III phenotype. The prognosis of EBV-associated lymphoproliferative diseases is variable; however, most of these are life-threatening and the prognosis of AIDS-associated lymphomas is extremely unfavorable, although introduction of highly active anti-retroviral treatment (HAART) decreased the incidence, increased the effectiveness of chemotherapy, and improved survival [5]. EBV infection of B-lymphocytes *in vitro* also results in latency III infection and sustained cell proliferation as lymphoblastoid cell lines (LCLs).

Activation of nuclear factor kappa B (NF-κB) has been connected with resistance against apoptosis and tumorigenesis [7]. Despite the diversity in clinical manifestations of EBV-associated lymphoproliferative diseases, strong and constitutive NF-κB activity is reported to be a common characteristic of this disease entity. LMP1 mimics signaling from tumor necrosis factor (TNF) receptor family members by association with tumor necrosis factor receptor-associated factors (TRAFs) and activates the IKK (IκB kinase)—NF-κB pathway [8].

NF-κB represents five cellular proteins: c-Rel, RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). The IκB inhibitory proteins consist of IκBα, IκBβ, IκBε, IκBγ, and Bcl-3. NF-κB forms homo- or heterodimers and exists as an inactive complex with IκB regulatory proteins in the cytoplasm. Various signaling pathways converge into IKK-mediated degradation of IκB proteins and subsequent release of uncomplexed NF-κB, which then migrates into the nucleus and activates the transcription of target genes [9].

Dehydroxymethylepoxyquinomicin (DHMEQ) is a new NF-κB inhibitor that is a 5-dehydroxymethyl derivative of the novel compound epoxyquinomicin C that has a 4-hydroxy-5,6-epoxycyclohexenone structure like panepoxydone. Panepoxydone had been found to inhibit TNF-α-induced activation of NF-κB [10]. We have shown that DHMEQ inhibits NF-κB at the level of nuclear translocation [11].

In this study, to investigate the possibility of NF-κB inhibition by DHMEQ as a strategy for the treatment and prevention of EBV-associated lymphoproliferative diseases, we investigated the effect of DHMEQ on apoptosis induction in four EBV-transformed LCLs as well as peripheral blood mononuclear cells (PBMC) in the early phase of EBV infection, and further examined the molecular mechanism of DHMEQ-induced apoptosis.

2. Materials and methods

2.1. Cells

B95.8 EBV-transformed LCLs were established by infection of lymphocytes from four healthy donors with culture supernatants of the virus producer B95.8 line as described previously [12], and are indicated in the text by the first two letters of the name of each donor. In all experiments to test the effects of DHMEQ treatment, LCLs were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

2.2. Chemicals

DHMEQ is an NF-κB inhibitor that blocks nuclear translocation of NF-κB [11]. DHMEQ was dissolved with dimethylsulfoxide (DMSO). DHMEQ or DMSO was used for experiments at indicated concentrations. Bisbenzimide H 33342 fluorochrome (Hoechst 33342) was purchased from Calbiochem (Bad Soden, Germany).

2.3. Electrophoretic mobility shift analysis

Electrophoretic mobility shift analysis (EMSA) was carried out according to the methods described previously [13]. For detecting NF-κB binding, a double-stranded oligonucleotide containing the κB site of the promoter for the mouse H-2Kb class I major histocompatibility antigen gene was used as a probe [14]. The nucleotide sequence is 5′-GAT CCG GCT GGG AAT CCC CGC TGG GAA TCC CCA TCT A-3′. For control EMSA, a double-strand oligonucleotide containing Oct-1 consensus sequence (Promega, Madison, WI, USA) was used as a probe. Antibodies used for supershift assays were as follows: NF-κB p50 (C-19) goat polyclonal antibody, rabbit polyclonal antibody for NF-κB p65 (C-20) and RelB (C-19), and mouse monoclonal antibody for c-Rel (B-6) and NF-κB p52 (C-5) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA). A mouse IgG antibody (Sigma, St. Louis, MO) served as a control.

2.4. Cell viability assay

The effects of DHMEQ on cell viability were assayed by color reaction with a tetrazolium salt, WST-8(4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenyl]-2*H*-5-tetrazolio]-1,3-benzene disulfonate sodium salt) (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). After incubation with DHMEQ or DMSO at the indicated concentrations and time points, cells were treated with Cell Counting Kit-8 according to the manufacturer's recommendations and the results were measured by a microplate reader (Bio-Rad, Richmond, CA) at a test wavelength of 450 nm and reference wavelength of 630 nm.

2.5. Analysis of apoptosis and caspase activities

To quantify apoptosis, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, Palo Alto, CA), then subjected to flow cytometric analysis. For analysis of nuclear DNA fragmentation, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay was done according to the manufacturer's recommendations (DeadEnd Fluorometric TUNEL Systems; Promega). Cells were analyzed using a FACS Calibur flow cytometer (BD Biociences) and fluorescence microscopy. Activities of caspase-3, -8, and -9 were determined by using green fluorochrome-labeled inhibitors of caspases (FLICA)-3, -8, and -9 (FLICA Apoptosis Detection Kit; Immunochemistry Technologies, Bloomington, MN). Cells from LCLs were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) for 8 h and fixed on slides; active caspases were detected by FLICA-3, -8, and -9. For detection of nuclear DNA, cells were stained with Hoechst 33342 and photographed through a UV filter and an Olympus BX50F microscope (Olympus, Tokyo, Japan).

2.6. In vivo effects of DHMEQ on NOG mice inoculated with LCLs

NOG mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The Ethical Review

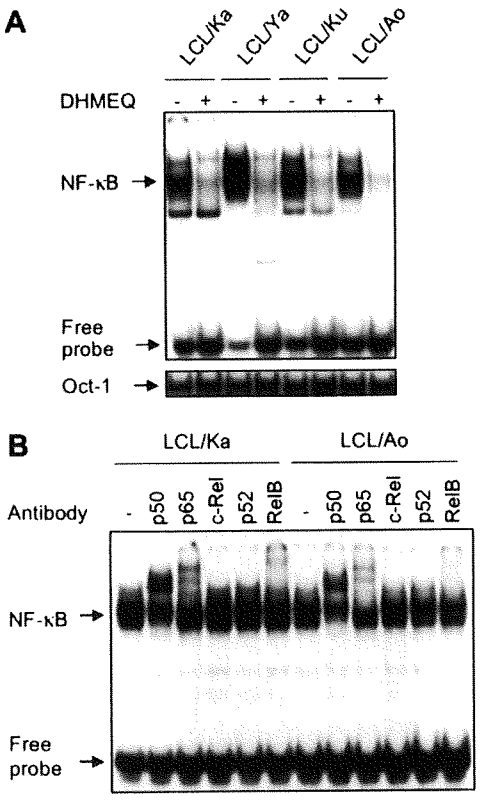

Fig. 1. Inhibition of constitutive NF-κB binding activity in LCLs by DHMEQ. (A) Inhibition of constitutive NF-κB activity in LCLs. LCLs were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) for 3 h. Nuclear extracts (2.5 μg) were examined for NF-κB binding activity by electrophoretic mobility shift analysis (EMSA) with a radiolabeled NF-κB-specific probe. Binding of Oct-1 served as a control. (B) Subcomponents of constitutive NF-κB activity in LCLs. Nuclear extracts (1 μg) of cells without DHMEQ treatment were subjected to supershift analysis with antibodies specific for NF-κB p50, p65, c-Rel, p52, and RelB or without antibody (−). The experiment using isotype matched IgG control showed the same result (data not shown). The position of shifted bands corresponding to NF-κB and free probes are indicated on the left.

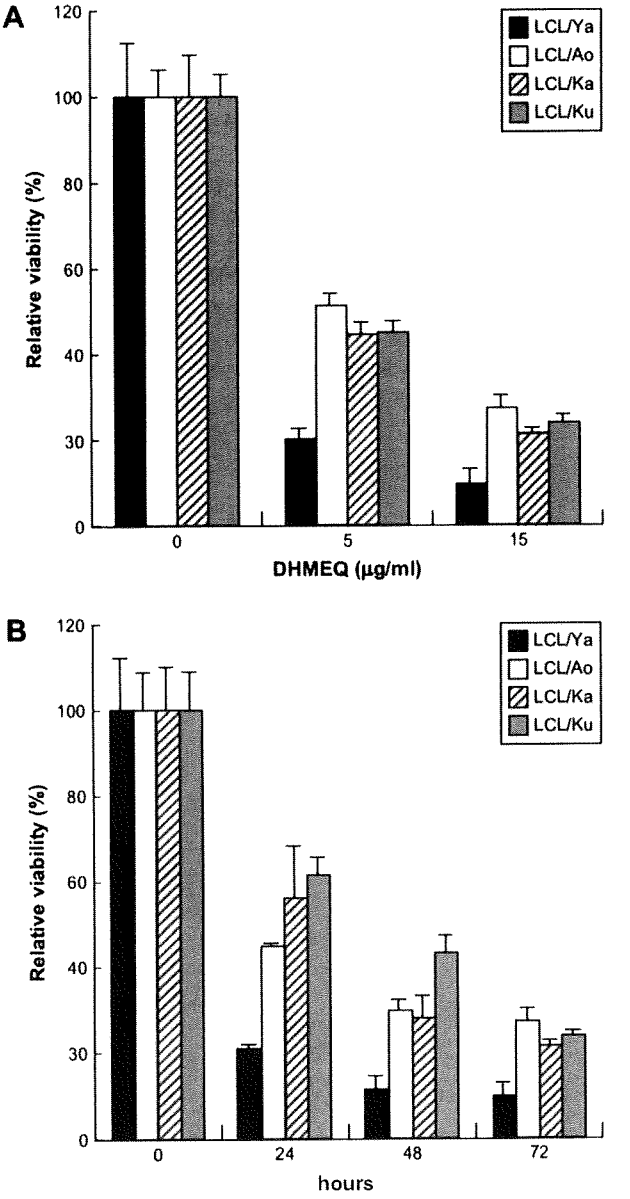

Fig. 2. DHMEQ inhibits proliferation of LCLs. The viability of the cells was determined by WST-8 assay and the relative levels compared with those of DMSO-treated cells are presented. Data represent the mean and standard deviation of triplicate experiments. (A) Results of dose–response experiments. LCLs were treated with 0, 5, or 10 μg/ml of DHMEQ for 72 h. ALL LCLs treated with 0, 5, or 10 μg/ml of DHMEQ showed statistical significance compared to DMSO-treated controls. (B) Results of time–response experiments. LCLs were treated with 10 μg/ml of DHMEQ for 24, 48, and 72 h. ALL LCLs treated for 24, 48, and 72 h except for LCL/Ku at the point of 24 h showed statistical significance compared to DMSO-treated controls.

Committee of the National Institute of Infectious Diseases approved the experimental protocol. 1×10^6 LCL cells were inoculated subcutaneously into the post-auricular region of NOG mice. DHMEQ was administered three times a week for 1 month into the post-auricular region of mice at a dose of 12 mg/kg, beginning on day 5 when tumors were palpable. The control mice were injected RPMI-1640 as was performed in our recent published papers [15,16]. Mice were killed 1 month after inoculation.

2.7. Immunohistochemistry

Cells were immunostained with antibodies and fluorescence signals were detected using confocal microscopy. Cytospin samples were prepared using 5×10^5 cells and cells were first washed three times with phosphate-buffered saline (PBS). Cells were then fixed with 100% cold acetone for 10 min at room temperature and washed three times in PBS. Samples were incubated with primary antibody at the concentration

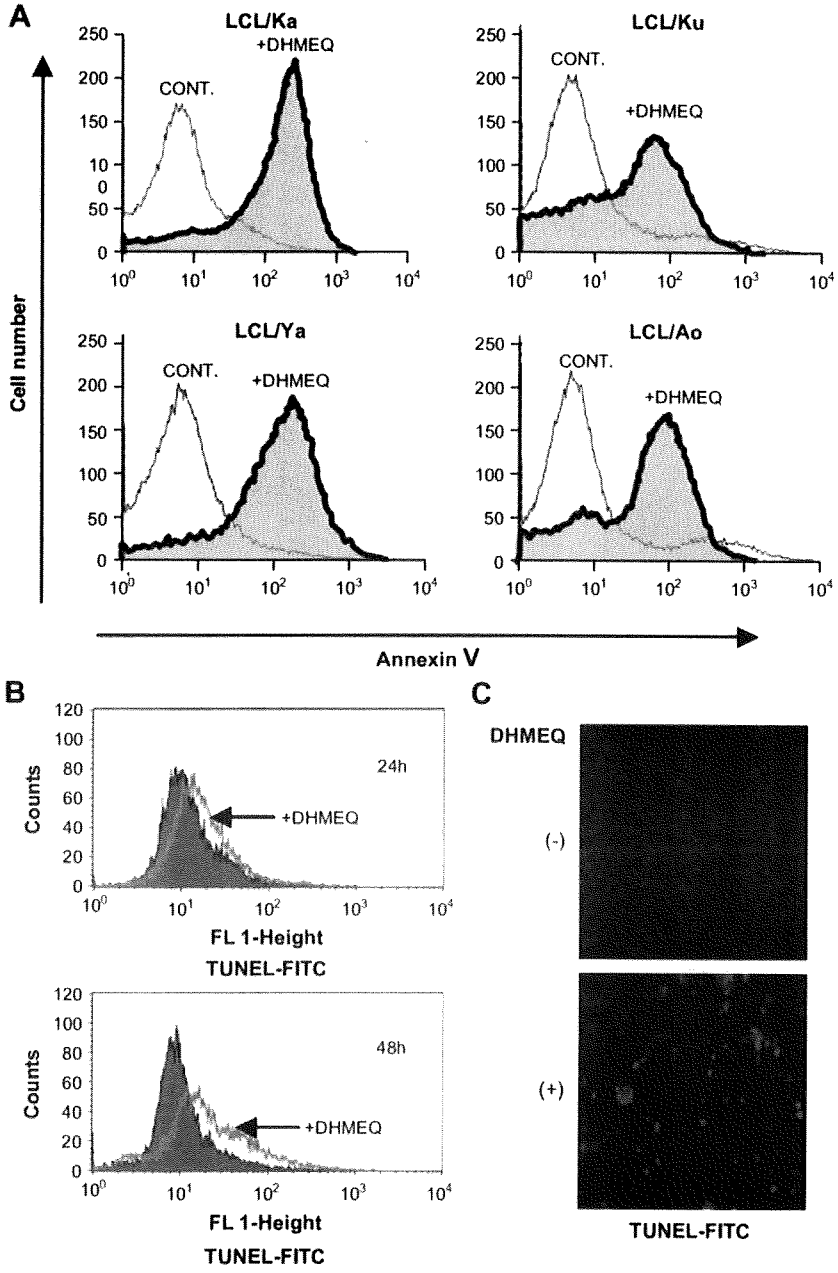

Fig. 3. DHMEQ induces apoptosis in LCLs. (A) Annexin V reactivity in LCLs after DHMEQ treatment. LCLs were treated with (filled curve) or without (open curve) 10 μg/ml of DHMEQ for 48 h, and the binding of FITC-conjugated Annexin V was analyzed by flow cytometry. (B) DNA fragmentation in LCL cells after DHMEQ treatment. DNA fragmentation in LCL cells was detected by TUNEL assay with flow cytometry. Representative flow cytometric profiles are shown for cells treated with 10 μg/ml of DHMEQ (open curve) or with DMSO alone (filled curve) for 24 h (upper panel) or 48 h (lower panel). (C) LCL cells were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) for 48 h, fixed on slides, and processed for TUNEL assay. A filter that selectively detects fluorescein isothiocyanate (FITC)-TUNEL fluorescence was used for the microscopic observation.

of 5 μg/ml at 4 °C overnight and washed with PBS three times. After incubation with fluorescence-labeled secondary antibody for 30 min at 37 °C, samples were washed three times in PBS and covered with a Perma Fluoro antifade reagent (Therme Shandon Co., Pittsburgh, PA). Fluorescence signals were detected using confocal microscopy (Radiance 2000) (Bio-Rad Laboratories. Antibodies used were as follows: anti-Epstein–Barr virus LMP clones CS, 1-4 mouse monoclonal antibody (Dako, Kyoto, Japan), and anti-p65 (c-20) goat polyclonal antibody (Santa Cruz Biotechnology Inc.

2.8. Real-time quantitative PCR

The expression level of anti-apoptotic genes was quantified by real-time reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was extracted from the cells by ISOGEN reagent (Nippon Gene Co., Toyama, Japan) and treated according to the manufacturer's instructions. cDNA was synthesized using oligo dT and random primers synthesized with a PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). Amplification was performed with SYBR premix Ex Taq (Takara Bio Inc.) and the primer sets for c-IAP1, Bfl-1, BCL-XL, and c-FLIP (Takara Bio Inc.). The viral DNA load in EBV-infected PBMC was determined by real-time PCR with slight modifications of a previously described method [17]. DNA samples were extracted from the cells with a DNeasy tissue kit (Qiagen, Hilden, Germany). Amplification with SYBR premix Ex Taq (Takara Bio Inc.) and primers for BALF5 gene encoding the viral DNA polymerase (5′-CGG AAG CCC TCT GGA CTT C-3′ and 5′-CCC TGT TTA TCC GAT GGA ATG-3′) was performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc.) and analyzed using the manufacturer's software.

2.9. Statistical analysis

Differences between mean values were assessed by *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. DHMEQ efficiently blocks constitutive NF-κB activity in LCLs

We first examined the effects of DHMEQ against constitutive NF-κB activity in established LCLs. Treatment with DHMEQ at a concentration of 10 μg/ml abrogated constitutive NF-κB binding activity in these cell lines (Fig. 1A). Components of NF-κB that are constitutively activated in LCLs were analyzed by

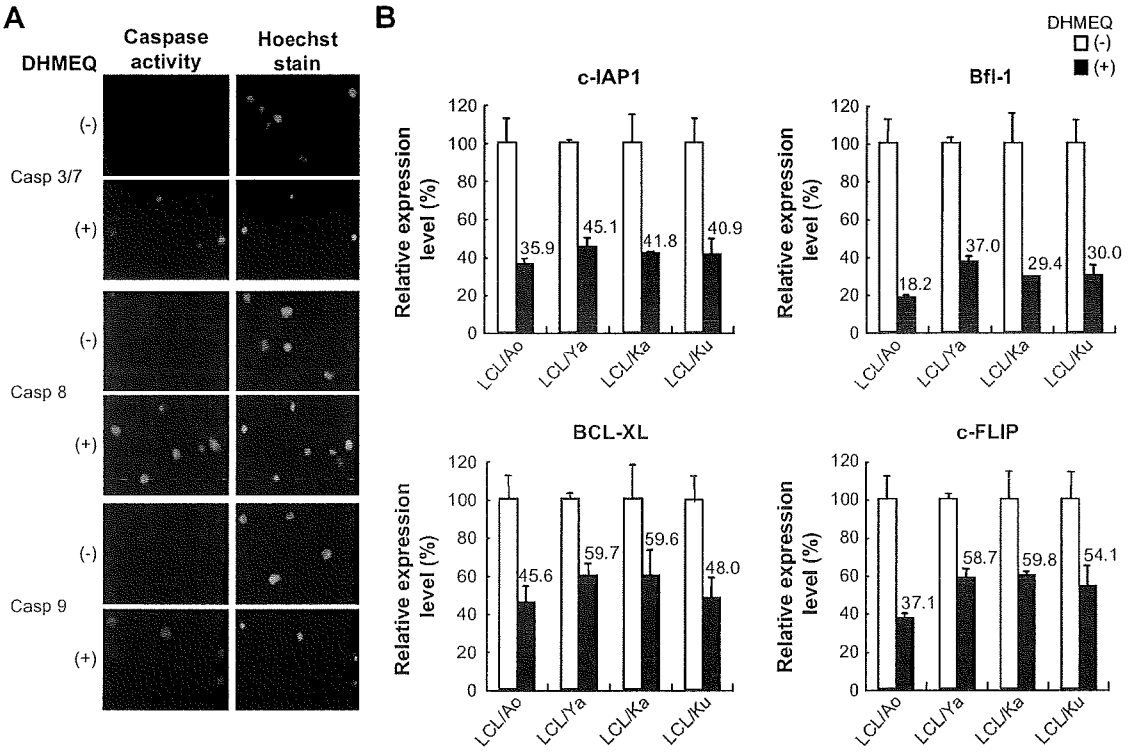

Fig. 4. Activation of caspase-3, -8, and -9. (A) LCL cells were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) for 8 h and fixed on slides. Caspase-3/7, -8, and -9 activities in LCLs after DHMEQ treatment were detected by green fluorochrome-labeled inhibitors of caspases (FLICA)-3/7, -8, and -9 (left panels) and nuclear DNA was stained with Hoechst 33342 (right panels). (B) Effects of DHMEQ on genes regulating apoptosis in LCLs. Quantification of the gene expression by real-time PCR. LCLs were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) for 4 h. The expressions of c-IAP1, Bfl-1, BCL-XL, and c-FLIP were quantified by real-time PCR. The data are means with standard deviation of triplicate experiments. The numbers above the bar graphs indicate the means of each gene expression after DHMEQ treatment. The reduction of the expressions of c-IAP1, Bfl-1, BCL-XL, and c-FLIP was statistically significant.

supershift assays. The results revealed that the NF-κB components consist of p50, p65, and RelB (Fig. 1B).

3.2. DHMEQ induces apoptosis of LCLs

To study the significance of NF-κB activation in the growth of LCLs, we examined the effects of DHMEQ on cell viability. Results of WST-8 assays showed that DHMEQ treatment reduced the cell viability of all four LCLs in a dose- and time-dependent manner (Fig. 2A and B).

NF-κB plays a key role in resistance to apoptosis [18]. Thus, we next examined whether DHMEQ induces apoptosis of LCLs by analyzing Annexin V reactivity and DNA fragmentation. Flow cytometric analysis showed a significant increase in the number of Annexin V-positive cells after DHMEQ treatment (Fig. 3A). Fragmentation of the nuclei of LCLs was clearly demonstrated after DHMEQ treatment by the TUNEL assay (Fig. 3B and C).

3.3. DHMEQ-induced apoptosis involves activation of caspases 3, 8, and 9

To confirm that the induction of apoptosis in LCLs by DHMEQ is caused by activation of the caspase pathway, we first examined activation of caspase-3/7 by immunostaining, using an antibody that recognizes a cleaved form of caspase-3/7. Results clearly showed cleavage of caspase-3/7, confirming that DHMEQ-induced apoptosis is associated with activation of the caspase pathway (Fig. 4A, top). To differentiate the membranous and mitochondrial pathways, we next examined the activation of caspases 8 and 9, which are upstream of caspase-3/7, by immunostaining. DHMEQ-treated LCL cells showed activation of both caspase-8 and caspase-9 (Fig. 4A, middle and bottom).

To understand the molecular mechanisms of apoptosis induction of LCLs after NF-κB inhibition by DHMEQ, we next examined by quantitative RT—PCR the changes in the expression levels of anti-apoptotic genes c-IAP1, Bfl-1, Bcl-XL, and c-FLIP, reportedly under the control of NF-κB, after DHMEQ treatment. The results demonstrated down-regulation of all of these genes (Fig. 4B).

3.4. DHMEQ shows a potent inhibitory effect on the growth of LCL cells in NOG mice

Because results *in vitro* suggested potential efficacy of DHMEQ for the treatment of patients with EBV-associated lymphoproliferative diseases, we next examined whether DHMEQ treatment can suppress the growth of xenografted LCL cells in a NOG mouse model. The gross appearance of resected tumors in mice treated with DHMEQ showed reduction of the tumor mass 1 month after inoculation of LCL cells (Fig. 5A and B). A decrease in the size of tumors in mice treated with DHMEQ was demonstrated when compared with controls 1 month after the injection of LCL cells (Fig. 5C).

3.5. DHMEQ inhibits outgrowth of EBV-infected peripheral blood B-lymphocytes

EBV-infected B lymphocytes under immunocompromised conditions acquire latency III infection, which may lead to proliferation and transformation into lymphoproliferative diseases including lymphomas [2,3]. Previous data link NF-κB activation by LMP-1 to transformation; however, they also indicate that NF-κB activation is not sufficient for transformation and should coordinate with other signals like mitogen-activated protein kinases [19]. Roles of NF-κB activity in EBV-infected lymphocytes for their survival during the early phase of infection are not fully understood. Therefore, to investigate the roles of NF-κB activation on the survival of EBV-infected lymphocytes during the early phase of infection, we examined the effect of NF-κB inhibition by DHMEQ on their survival and the EBV viral load in PBMC infected with EBV. Lymphocytes infected with EBV under immunosuppressive conditions already show constitutive NF-κB activation as well as LMP1 expression. Treatment of these cells with DHMEQ inhibited translocation of NF-κB into the nucleus (Fig. 6A). DHMEQ treatment also eliminated LMP1-expressing lymphocytes from PBMC (Fig. 6B). Finally, DHMEQ treatment prevented the outgrowth of lymphocytes infected

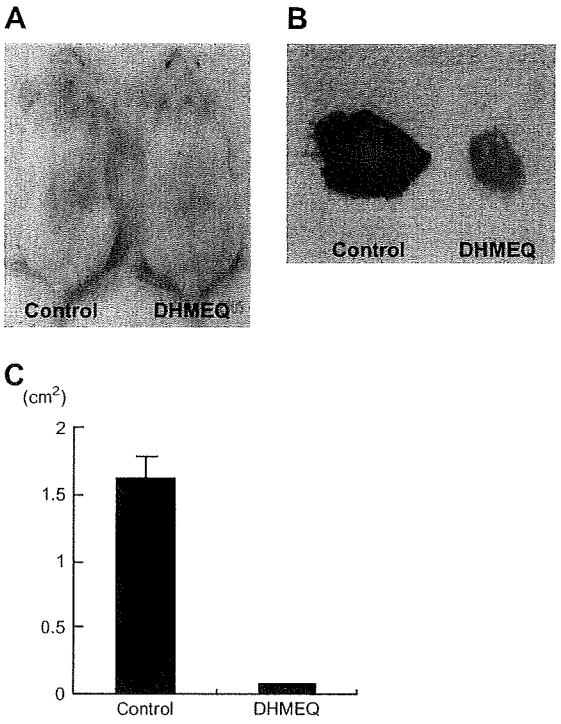

Fig. 5. DHMEQ inhibited the tumor growth of LCL cells *in vivo*. NOG mice were inoculated with LCL cells and administered DHMEQ (12 mg/kg) ($n = 5$) or control medium ($n = 5$) subcutaneously in the post-auricular region three times a week for up to 1 month. (A) Photograph of the backs of mice. (B) Photograph of a tumor at the site of LCL cells inoculation. (C) Subcutaneous tumor volume of mice inoculated with LCL cells and administered DHMEQ or control medium 1 month after inoculation.

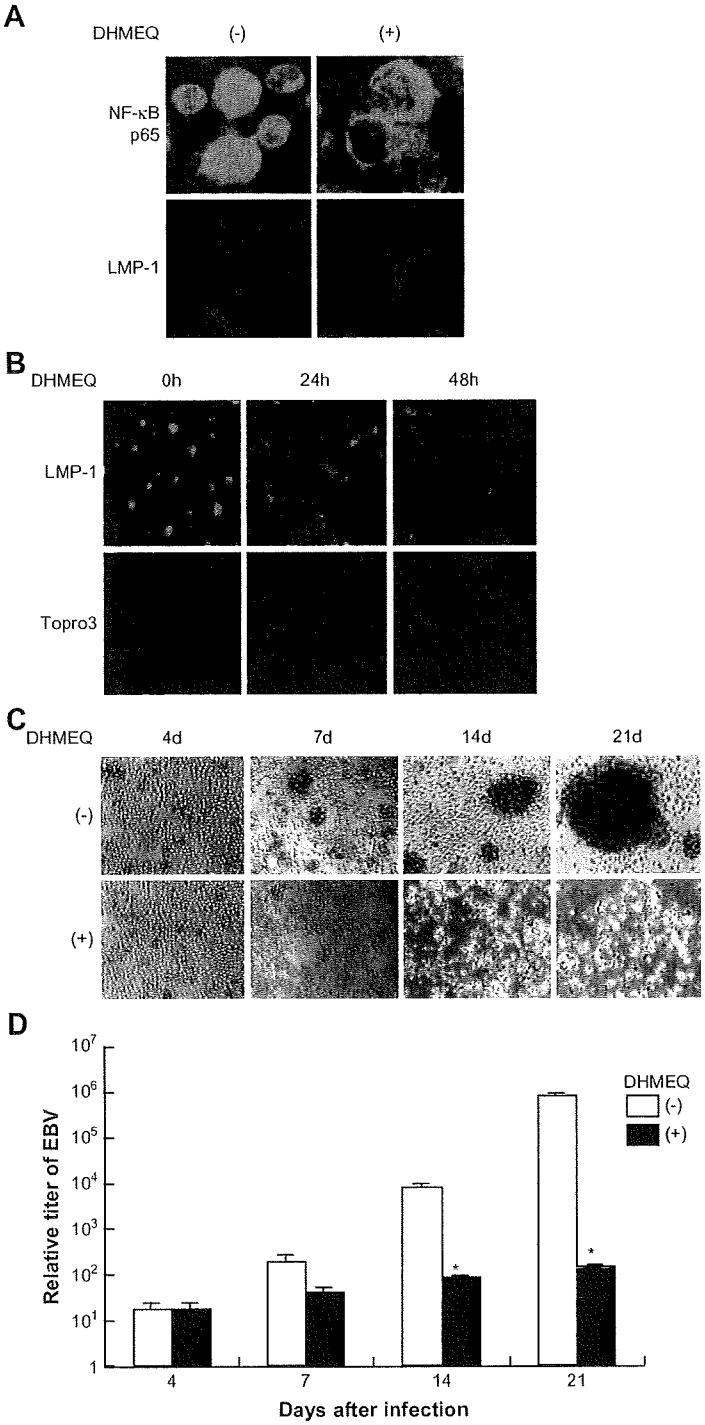

Fig. 6. Effects of DHMEQ on PBMC infected with EBV. 8×10^5/ml of PBMC from a healthy donor infected with EBV using supernatant of B95.8 line were cultured in RPMI 1640 medium supplemented with 10% FBS and 200 ng/ml cyclosporine A. Cells were harvested 4 days and 14 days after infection and served for experiments. (A) Inhibition of NF-κB and expression of LMP1 in lymphocytes. At the point of 4 days after infection, cells were treated with or without 10 μg/ml of DHMEQ for 1 h and immunostained with antibodies for LMP1 and NF-κB p65. DMSO-treated cells served as a control. (B) DHMEQ treatment eliminated LMP1 expressing cells from PBMC. At the point of 14 days after infection, cells were treated with or without 10 μg/ml DHMEQ for the indicated number of hours. Cells stained with anti-LMP1 antibody and topro 3 were observed by confocal microscopy. DMSO-treated cells served as a control. (C, D) Photographs of EBV-infected PBMC and quantification of viral load by real-time PCR. Cells cultured for 4 days were treated with 10 μg/ml of DHMEQ (+) or with DMSO alone (−) thereafter twice a week. Cells were observed by microscopy at the indicated days (C). Cells were harvested on the indicated days and genomic DNA was isolated. The viral load was quantified by real-time PCR as described in Section 2. The data are means and standard deviations of triplicate experiments (D). The asterisks indicate statistical significance.

with EBV and decreased the EBV viral load in PBMC (Fig. 6C and D).

4. Discussion

In the present study, we showed that the NF-κB inhibitor DHMEQ blocked strong and constitutive NF-κB activity, reduced viability, and induced apoptosis in LCLs. Induction of apoptosis by DHMEQ in LCLs is associated with inhibition of NF-κB, which is followed by down-regulation of NF-κB regulated anti-apoptotic genes. These observations, combined with our previous study about the mechanisms of action of DHMEQ [11], indicate that apoptosis induction of LCLs by DHMEQ is mediated by inhibitory effect of DHMEQ against NF-κB. DHMEQ appears to be more specific to NF-κB pathway compared with IκB kinase (IKK) inhibitor, Bay 11-7082 used in the previous studies [20,21], because DHMEQ inhibits downstream of IKK and Bay 11-7082 has been reported to be apparently not specific for NF-κB pathway [22]. Therefore our study provides further evidence for the importance of NF-κB in the survival of LCLs and indicates effectiveness of DHMEQ in the treatment of EBV-infected transformed lymphocytes.

We also showed that DHMEQ inhibits constitutive NF-κB activation in B lymphocytes expressing LMP1, eliminates these cells from PBMC, and inhibits the outgrowth of lymphoblastic cells. The results indicate that B lymphocytes become dependent on NF-κB for proliferation and survival within several days after EBV infection. Although previous data indicate that not only NF-κB but also other signals like mitogen-activated protein kinases are involved in transformation of lymphocytes to LCL cells [19], the results in this study indicates that abrogation of constitutive NF-κB activity appears to be sufficient to prevent transformation of EBV-infected lymphocytes. Previous reports underscored constitutive NF-κB activity as a molecular target in LCL cells [20,21,23]. Our study shows a new insight that constitutive NF-κB activity is a common molecular target in EBV-infected transformed and untransformed lymphocytes.

Recent reports showed that EBV viral load is a useful marker for disease status of lymphoproliferative diseases or lymphomas in patients with immunosuppression [24]. We showed that DHMEQ treatment prevented the increase of EBV viral load in PBMC. The reduction of EBV viral load in PBMC by DHMEQ indicates not only that the elimination of lymphocytes infected by EBV contributes to the reduction, but also that the replication of EBV virus may depend on NF-κB activity. However, previous studies showed that NF-κB activity does not promote replication of EBV virus, but rather inhibits its replication [25]. Therefore, reduction of viral load in lymphocytes infected with EBV treated with DHMEQ appears to be due to the elimination of lymphocytes infected with EBV. Collectively, early detection of the increase of EBV viral load and purging infected cells under transformation by a NF-κB inhibitor may contribute to the preventive intervention against lymphoproliferative diseases in patients with profound immunosuppression.

Our results suggest that the effects of DHMEQ depend on the down-regulation of NF-κB-dependent genes that control apoptosis. Down-regulation of c-FLIP, involved in anti-apoptosis blocking caspase-8, as well as Bfl-1, Bcl-XL and c-IAP, involved in the anti-apoptosis blocking caspase-9, by DHMEQ may result in activation of membranous and mitochondrial pathways, respectively [26]. This implies the possibility that in EBV-infected lymphocytes, the induction of anti-apoptotic genes is counteracting the apoptotic pressure and preventing these cells from undergoing apoptosis.

The mice treated with DHMEQ in 1% DMSO did not show any relevant signs of toxicity such as body weight loss in this experiment. The dose of DHMEQ administered in this experiment was 12 mg/kg three times a week, far less than the LD_{50} of DHMEQ, 180 mg/kg (Naoki Matsumoto, K.U., unpublished observation, July 1999). Results of our in vivo model suggest that DHMEQ may be feasible and less toxic at an effective dose, although the pharmacokinetics has not yet been elucidated. In our NOG mice model, the results indicate that local administration of DHMEQ can prevent primary tumor growth without significant signs of toxicity. Additional experiments, which include intraperitoneal and intravenous administration of DHMEQ, will further confirm efficacy of DHMEQ against LCLs *in vivo*.

Our recent study also indicates that DHMEQ has little effect on the viability of PBMC or purified B cells *in vitro* under almost the same experimental condition as this study [27]. These *in vitro* and *in vivo* results suggest a favorable toxic profile and potent NF-κB inhibitory effect by DHMEQ. Thus, DHMEQ appears to be a candidate for the treatment of EBV-associated lymphoproliferative diseases as well as for their chemoprevention.

In conclusion, our study indicates that the unique NF-κB inhibitor DHMEQ is a potential compound that targets constitutive activation of NF-κB in EBV-infected transformed and untransformed B cells. Because EBV-associated lymphoproliferative diseases are life-threatening and the prognosis of AIDS-associated lymphomas is extremely unfavorable, our results support preventive intervention with a NF-κB inhibitor as a new strategy in patients with immunosuppression.

Acknowledgments

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Potential Role of NK Cells in the Induction of Immune Responses: Implications for NK Cell–Based Immunotherapy for Cancers and Viral Infections

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Natural killer (NK) cells recognize tumor cells and virus-infected cells and attack without being sensitized to antigens. The development of the antitumor/antivirus activities of NK cells is controlled by multiple mechanisms such as direct cytotoxic activity against target cells, antibody-dependent cell-mediated cytotoxicity, secretion of Th1-type cytokines, and interactions with dendritic cells. The development of these activities plays a significant role in both innate and adaptive immunities. Considering the recent progress made in elucidating the molecular and cellular biology of NK cells, we summarize the current situation and discuss future possibilities with regard to NK cell–based adoptive immunotherapy.

Keywords adoptive immunotherapy, cancer immunotherapy, immunotherapy, NK cell, viral immunotherapy

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INTRODUCTION

Natural killer (NK) cells are lymphocytes that recognize tumor cells and infected cells and destroy them immediately without involving any specific antigen recognition mechanisms. Furthermore, they induce subsequent immune responses as the first-line immune cells of *in vivo* defense. At the present time, no single cell-surface marker for the identification of human NK cells has been determined, and detection of these cells is usually based on lymphocytes that are negative for CD3 and positive for CD16 or CD56. Although the ratio of NK cells varies according to age, sex, and so forth, these cells represent about 5–20% of peripheral blood lymphocytes in healthy adults.

During the past few years, many new findings have been reported in the field of the molecular and cellular biology of NK cells, including NK cell receptors, expression of major histocompatibility complex class I antigens on target cells, antibody-dependent cell-mediated cytotoxicity (ADCC), cross-talk with dendritic cells, and NK cell subsets as described in this review. In addition, we recently developed a feasible and safe method for *ex vivo* NK cell expansion. Based on these findings, the possibility of clinical use of NK cell–based adoptive immunotherapy is discussed.

NK Cell Receptors

NK cells were originally discovered as a group of cells with natural killing ability against tumor cells [1,2]. However, the mechanism by which NK cells recognize their target cells remained unknown for a long time. Kärre suggested the hypothesis of "missing self," in which NK cells are considered to recognize and attack tumor cells with decreased or absent expression of self-markers, namely major histocompatibility complex (MHC) class I molecules [3]. This hypothesis was proved at the molecular level in previous studies [4,5]. At the same time, it was clarified that not only a lack of inhibitory signals by missing-self molecules but also activating signals from the target cells are required for the recognition and killing action.

NK cells have many receptors, as summarized in Table 1. Many of these are paired receptors comprising activating and inhibitory receptors with similar extracellular domains but different functions. Inhibitory receptors have long intracellular domains with inhibitory signal motifs, whereas activating receptors have short intracellular domains and combine with adaptor molecules that possess signal motifs. The y chains of high affinity IgE receptors (FcεRIγ) and two killer cells activating receptor-associated proteins/DNAX-activating

TABLE I Human NK Cell Receptors

Receptor	Structure	Ligand	Adaptor	Signal type
CRACC	IgSF	CRACC	None	Activating
DNAM-1	IgSF	PVR, nectin-2	None	Activating
FcγRIII (CD16)	IgSF	IgG Fc region	FcεRIγ/CD3ζ	Activating
KIR2DL, KIR3DL	IgSF	HLA-A,B,C	None	Inhibitory
KIR2DS, KIR3DS	IgSF	Unknown	DAP12	Activating
LAIR-1	IgSF	Collagen	None	Inhibitory
LILR	IgSF	HLA-A,B,C,E,F,G	None	Inhibitory
NKp30 (CD337)	IgSF	Unknown	FcεRIγ/CD3ζ	Activating
NKp44	IgSF	Unknown	DAP12	Activating
NKp46 (CD335)	IgSF	Influenza HA	FcεRIγ/CD3ζ	Activating
NTB-4	IgSF	NTB-4	None	Activating
2B4 (CD244)	IgSF	CD48	None	Activating
KLRG1	C-lectin	Cadherins (E-,N-,R-)	None	Inhibitory
CD94-NKG2A	C-lectin	HLA-E	None	Inhibitory
CD94-NKG2C	C-lectin	HLA-E	DAP12	Activating
CD94-NKG2E	C-lectin	Unknown	DAP12	Activating
NKG2D	C-lectin	MICA/B,ULBP1-4	DAP10	Activating
NKR-P1	C-lectin	LLT	None	Inhibitory
NKR-PIA	C-lectin	LLT1	FcεRIγ/CD3ζ	Activating
LFA-1 (CD11a/CD18)	β2-integrin	ICAM-1,2,3,4	None	Activating

IgSF, immunoglobulin superfamily; HA, hemagglutin.

proteins of 12 kDa (DAP12) and 10 kDa (DAP10) have been identified as adaptor molecules. However, some activating receptors of NK cells do not require adaptor molecules, as the receptors themselves transmit the activating signals. Many of the inhibitory receptors ligate with MHC class I molecules. The paired receptors detect molecules expressed on the target cells and send activating or inhibitory signals for cytotoxic activity. The development of the cytotoxic activity of NK cells is then adjusted by the balance between the positive and negative signals transmitted inside the cells.

Once NK cells become activated, they damage the target cells through release of cytotoxic granules such as perforin or granzyme, expression of death-inducing ligands such as tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) or FasL, and secretion of Th1-type cytokines or chemokines.

As mentioned above, many receptors have been identified on NK cells. This situation probably arises because NK cell receptors are encoded by embryonic genes, and numerous activating receptors are

required in order to maintain the variety in antigen recognition, unlike T-cell or B-cell receptors that create various patterns by random gene rearrangements.

In association with viral infections, NKp30, NKp44, and NKp46 have been identified as activating receptors that belong to the immunoglobulin superfamily. Although their ligands on tumor cells are still unknown, NKp44 and NKp46 have been reported to combine with influenza hemagglutinin and activate NK cells [6,7]. On the other hand, the activity of NKp30 is inhibited by ligation with pp65 of human cytomegalovirus, and this is an interesting escape mechanism for this virus from the immune system [8]. In HIV infection, the activating killer Ig-like receptor (KIR) allele KIR3DS1, in combination with human leukocycte antigen-B alles that encode molecules with isoleucinat at position 80 (HLA-B Bw4-80Ile) is associated with delayed progression to AIDS [9], whereas allelic combinations of KIR3DL1 and HLA-B Bw4 are associated with both AIDS progression and plasma HIV RNA abundance [10]. There are also ample indications that NK cells can eradicate malignant cells in patients with a favorable combination of HLA and KIR genes [11]. Selecting these individuals for specific treatments should provide insights into the feasibility of successful antitumor or antiviral therapy mediated through NK cells.

EXPRESSION OF MHC CLASS I MOLECULES ON TARGET CELLS

The balance between activating and inhibitory signals is important in the activation of NK cells. For this, the amounts of ligands expressed on the target cells are crucial, in addition to the amounts of activating and inhibitory receptors expressed on NK cells. Although tumor cells and virus-infected cells with decreased or absent MHC class I molecule expression can escape from attack by cytolytic T-lymphocytes (CTL), the cells are usually damaged by NK cells. As summarized in Table 2, many cases have been reported regarding tumor cells with downregulated or absent expression of MHC class I molecules. The frequency of loss or downregulation of MHC class I expression in metastatic tumors is higher than in the primary ones [12,13]. For the same types of tumor cases, the survival rate of patients is significantly low, and their relapse rate after treatment is significantly high [13,14]. Although certain tumors such as breast, colorectal, prostate, and cervical cancer have a high frequency of abnormal MHC class I expression, these types of tumors do not have the poorest prognosis. Further investigations are necessary to clarify how the level of NK cells and CTL activity in blood,

TABLE II Frequencies of Loss or Reduction in Expression of Major Histocompatibility Complex Class I Molecules in Surgically Removed Tumor Lesions

Tumor	Low or negative cases/total cases	Percentage (%)	Reference
Melanoma, primary	66/414	16	[12]
Melanoma, metastases	287/495	58	[12]
Head and neck carcinoma	20/41	49	[59]
Laryngeal carcinoma	25/70	36	[60]
Breast carcinoma	356/439	81	[61]
Lung carcinoma	35/93	38	[62]
Hepatocellular carcinoma	24/57	42	[63]
Colorectal carcinoma	63/87	72	[64]
Colorectal carcinoma, primary	144/455	32	[65]
Renal cell carcinoma	17/45	38	[14]
Bladder carcinoma	18/72	25	[66]
Prostatic carcinoma	311/419	74	[67]
Cervical carcinoma	27/30	90	[68]
Ovary carcinoma, primary	19/51	37	[69]
Bone and soft tissue sarcoma	46/74	62	[13]
Osteosarcoma, primary	13/25	52	[13]
Osteosarcoma, metastases	7/8	88	[13]

and the balance between activating and inhibitory ligands of NK cell receptors expressed on the surface of tumors, besides the level of MHC class I expression, contribute to the prognosis of each type of tumor.

On the contrary, T lymphocytes infected by HIV are not destroyed by NK cells, regardless of their abnormal expression of MHC class I molecules [15–17]. In such cases, expression of HLA-C or HLA-E, the ligands of inhibitory receptors, is hardly inhibited, although HLA-A or HLA-B expression is inhibited. Therefore, HIV-infected cells can escape from attack by CTL or NK cells [16–18]. It is necessary to clarify whether such cases also occur with tumor cells.

ADCC

NK cells not only work nonspecifically with antigens but also remove tumor cells and infected cells on which antigen-specific antibodies are bound by ADCC. This process occurs through the activating receptor CD16, which is also used to identify NK cells among lymphocytes. CD16 is a low-affinity activating Fc receptor that allows NK cells to interact with antibody-coated cells, thereby inducing ADCC as well as secretion of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, or T-cell recruiting chemokines [19,20]. CD16 is expressed on monocytes, macrophages,

granulocytes, and NK cells among all immune cells. Macrophages and granulocytes express both activating and inhibitory CD16, whereas NK cells only express activating CD16.

Because of the recent development of antibody therapies for cancers, monoclonal antibodies such as trastuzumab (Herceptin®, F. Hoffman–La Roche Ltd., Basel, Switzerland), rituximab (Rituxan®, F. Hoffman–La Roche Ltd., Basel, Switzerland), and anti-EGF receptor mAbs, which are effective against cancers, have been used clinically. NK cells, which possess Fc receptors, play a significant role in the mechanism of these therapies [21,22].

Regarding HIV infection, HIV-infected cells bound by anti-gp120 antibodies are destroyed by NK cells [17]. It has also been reported that higher ADCC activities specific for the HIV envelope protein are associated with lower disease stages of HIV infection [23]. Furthermore, it has become possible to destroy HIV-infected cells through ADCC with an IgG-IgA chimera-bound protein with strong binding ability for CD16 [24].

In addition to further development of pharmaceutical antibody products, the development of specific and powerful treatment methods against tumor cells and virus-infected cells based on the ADCC of NK cells, such as the concomitant use of *ex vivo*–expanded NK cells, is expected.

CROSS-REGULATION OF DENDRITIC CELLS AND NK CELLS

Dendritic cells (DCs) are key cells that lead antigen-specific immune reactions as potent antigen-presenting cells. NK cells induce innate immune responses through reciprocal interactions with DCs [25,26]. Furthermore, these interactions initiate T-cell responses [27].

Interleukin (IL)-12 and IL-2, the cytokines produced by mature DCs, induce the production of IFN-γ by NK cells [27,28]. On the other hand, it is considered that activated NK cells mainly produce Th1-type cytokines, such as IFN-γ, TNF-α, or granulocyte-macrophage colony-stimulating factor, induce the maturation and activation of DCs, and are also involved in the shift from innate immunity to adaptive immunity [25,29].

NK cells also produce and secrete large quantities of CC-chemokines such as CC-chemokine ligand 3 (CCL3; generic name, MIP1α), CCL4 (generic name, MIP1β), and CCL5 (generic name, RANTES), which are the ligands of CC-chemokine receptor 5 [30,31]. It is considered that migration of immature DCs, as well as CTL and Th1-type T cells, to tumor cells or lymphatic nodes is induced by these chemokines.

Reciprocal DC-NK cell interactions that normally occur after an inflammatory insult are affected during HIV-1 infection [32]. The amounts of secreted IFN-γ, a potent inducer of DC differentiation, are decreased when NK cells are exposed to autologous mature DCs generated from viremic but not aviremic HIV-1–infected individuals [32]. Although the ability to promote DC maturation is essentially confined to NK cells expressing a $KIR^{neg}/NKG2A^{dull}/NKp30^{pos}$ phenotype [33], freshly purified NK cells from HIV-1 viremic individuals express increased levels of KIRs and downregulate levels of NKG2A and NKp30 [34]. These data suggest that impaired maturation of DCs may occur due to functional impairment of NK cells in patients with poorly controlled viremia.

The functional cross-talk between NK cells and DCs implies a critical role for NK cells in the initiation and regulation of cellular immunity. Therefore, decreased NK cell activity detected *in vivo* may indicate that innate and adaptive immune reactivities against cancers or viral infections are both decreased.

NK Cell Subsets

At least two subsets of NK cells exist in the peripheral blood. The main subsets are $CD3^-CD56^{dim}$ NK cells whose main function is cytotoxic activity and $CD3^-CD56^{bright}$ cells that produce large amounts of Th1-type cytokines such as IFN-γ [35].

NK cells expanded by our recently developed culture method [36,37] were found to possess not only the $CD3^-CD56^{bright}$ phenotype with high cytokine production ability but also high cytotoxic activity (Fig. 1). Because NK cells showing high immune reactivity are not commonly observed in the peripheral blood, the roles of such cells *in vivo* need to be further discussed in the future.

NK Cell Immunotherapy of Human Malignancies

As mentioned above, NK cells are immune cells that show cytotoxic activity from the early stage of the *in vivo* defense mechanism against tumor cells and virus-infected cells without being sensitized to the antigens and play a significant role in association with DCs when adaptive immunity starts to become active. In fact, epidemiologic data indicate that cancer is more likely to develop if NK cell activity is low [38]. In addition, NK cell activity in cancer patients is significantly decreased compared with that in healthy individuals [39,40]. NK cell infiltration is a positive prognostic parameter in several cancer types, including

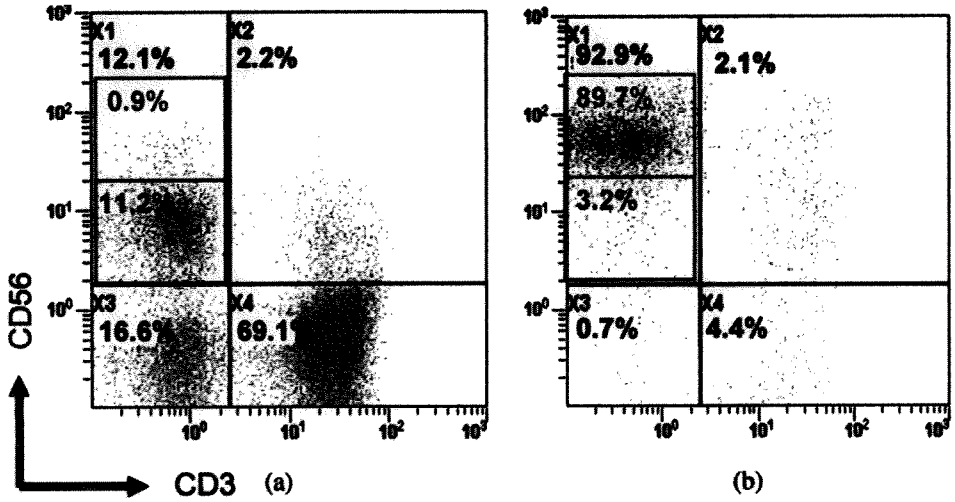

FIGURE 1 Flow cytometric analyses (Cytomics FC500; Beckman Coulter, Miami, FL) reveal the percentages of $CD3^{-}CD56^{bright}$ NK cells (red squares) and $CD3^{-}CD56^{dim}$ NK cells (blue squares) in (a) blood and (b) among cells cultured for 14 days.

gastric carcinoma, squamous cell lung carcinoma, and colorectal cancer [41–43]. In addition, we found that human malignant cells diminish and metastasis is inhibited after administration of human NK cells to living mice using the NOG/SCID γc(null) (NOG) mouse model [36,37]. These results suggest a role for NK cells in tumor immunosurveillance and their beneficial effects for many experimentally successful immunotherapy strategies.

Clinical trials using autologous NK cells in metastatic renal cell carcinoma patients resulted in remarkable tumor regressions [44]. A positive treatment effect has also been reported for brain tumors [45]. On the other hand, another report indicated that the efficacy of adoptive transfer of autologous NK cells is limited [46]. The efficacies of NK cell treatments for solid tumors vary among these reports because the activities and numbers of NK cells used differ among the studies. It has also been difficult to culture sufficient numbers of highly active NK cells. Therefore, stable clinical data are not available, and it is necessary to develop a practical NK cell expansion methodology.

In recent reports [36,37], we described a feasible and safe culture method that enables the generation of activated NK cells expanded *ex vivo* by several hundred- to thousand-fold for 2 weeks under good manufacturing practice–conformant conditions (Fig. 2). These cultured $CD3^{-}CD56^{bright}$ NK cells not only show high cytotoxicity but also express the activation marker CD69 and produce large amounts of IFN-γ

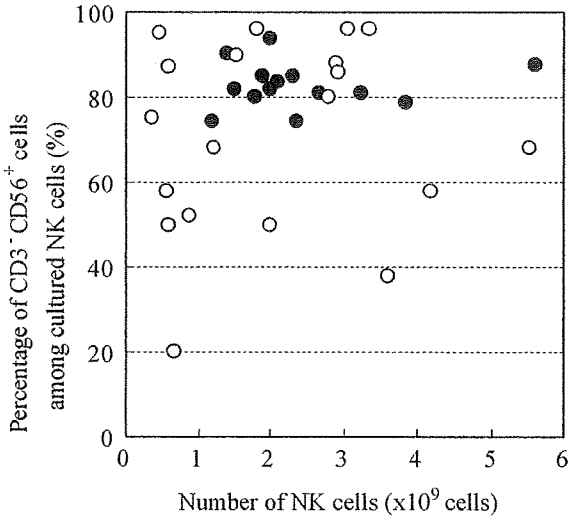

FIGURE 2 The absolute numbers and percentages of NK cells among *ex vivo*–expanded cells from 50-mL blood samples obtained from healthy donors (closed circle) and patients with various stage IV advanced cancers (open circle) after 14 days in culture.

(Fig. 3). The absolute number and cytotoxic activity of these NK cells were 10 times higher than those of cultured lymphokine-activated killer (LAK) cells. Therefore, higher antitumor activity can be expected compared with previous methods. Furthermore, when these NK cells were infused into healthy volunteers, not only NK cells but also T and B cells accumulated in the peripheral blood, and the NK cell activity also increased in the peripheral blood without any adverse effects (Fig. 4). The absolute numbers of $CD3^{-}CD56^{bright}$ and $CD3^{-}CD56^{dim}$ NK cells increased several times for a period of 2 weeks after NK cell infusion. The proportion of $CD3^{-}CD56^{bright}$ NK cell subsets slightly increased in volunteers' NK cells after the infusion of cultured cells. These data suggest that these cells function effectively in tumor immunosurveillance *in vivo*. When we infused autologous NK cells into patients, CT scanning revealed some clinical effects in two cancer patients (Fig. 5). In addition, when the *in vivo* kinetics of infused NK cells were investigated by labeling them with radioactive isotopes, it was confirmed that the cells accumulated in the lungs immediately after infusion and then became distributed to the liver, spleen, and bone marrow. In patients with tumors, infused NK cells were absorbed into the tumors [47]. It is necessary to accumulate a large amount of clinical data for NK cell therapy in order to evaluate NK cell usefulness for cancer and viral immunotherapy.

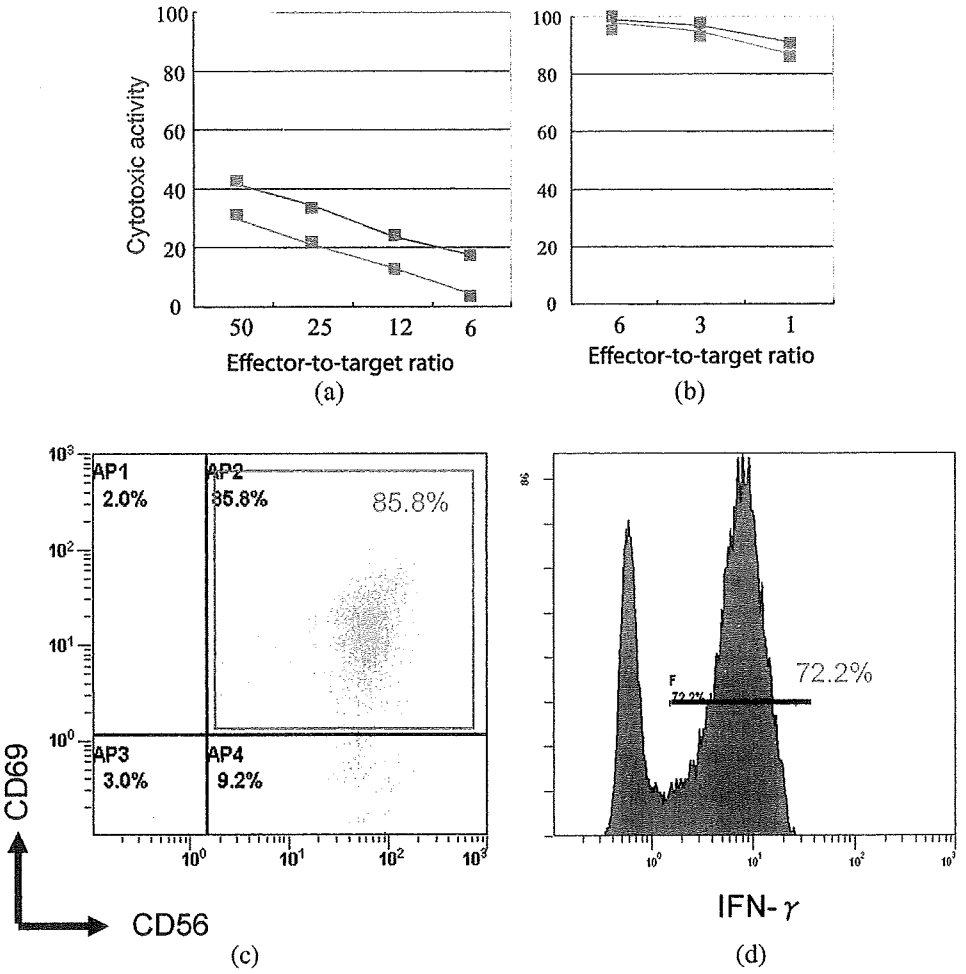

FIGURE 3 Characterization of *ex vivo*–expanded NK cells after 14 days in culture. The spontaneous cytotoxic activities of (a) freshly isolated peripheral blood mononuclear cells from two volunteers and (b) *ex vivo*–expanded NK cells cultured for 14 days were measured against K562 cells at different effector-to-target ratios in a calcein-AM release assay using a TERASCAN VP (Minerva Tech., Tokyo, Japan) as previously described (35). (c) Increased expression levels of CD69, an activation marker for NK cells that is not expressed on NK cells in blood, and (d) induction of intracellular IFN-γ after incubation with 50 μg phorbol 12-myristate 13-acetate, 1 μM ionomycin, and 1 μM monensin for 6 h in *ex vivo*–expanded NK cells were measured by flow cytometry.

LYMPHODEPLETION IN NK CELL THERAPY

Recently, performance of systemic lymphodepleting chemotherapy prior to concomitant therapy of adoptive immunotherapy with tumor-reactive T cells and IL-2 administration was reported to make the

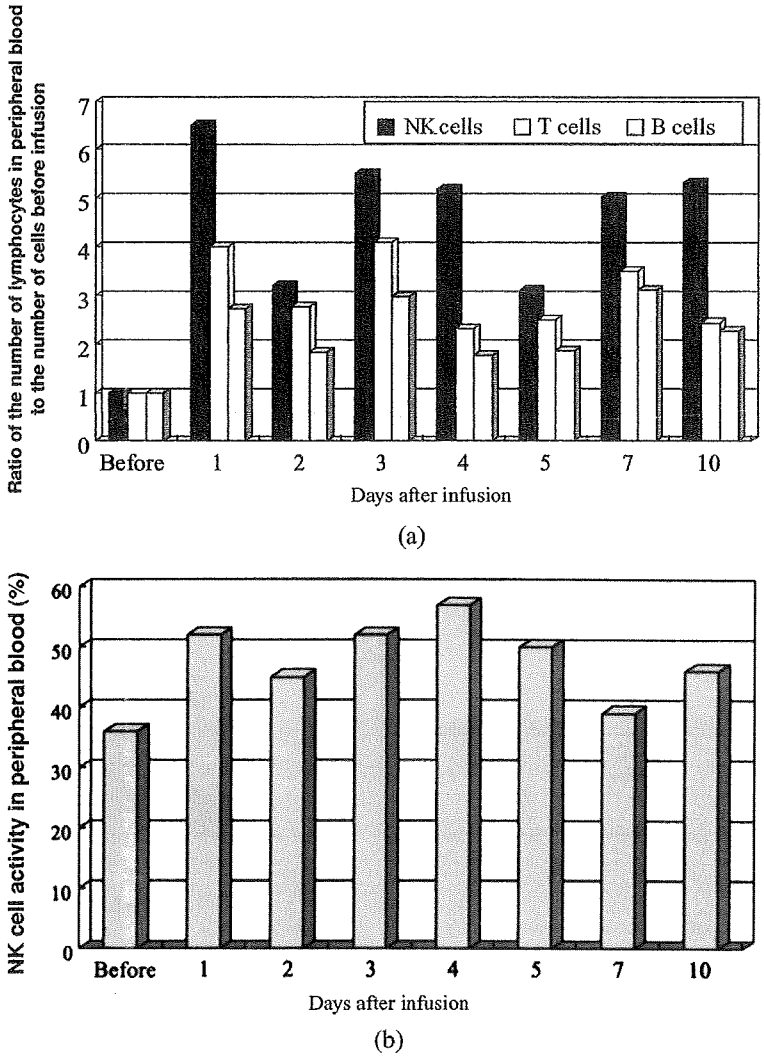

FIGURE 4 Safety and immune responses of autologous activated NK cells. Autologous activated NK cells (2×10^9) were transfused into two healthy donors, and blood was collected at various time points. (a) The changes of the number of lymphocytes in the peripheral blood are shown as the ratio of cells relative to the number of cells before infusion. The number of cells before infusion was set as the baseline value. (b) The NK cell activities in peripheral blood lymphocytes were measured as described in Figure 3.

concomitant therapy more efficient, and this finding is drawing attention [48]. Removal of $CD4^+CD25^+$ regulatory T cells (Treg) that show inhibitory actions in immune responses accounts for the effectiveness of lymphodepletion. In cancer patients, increased Treg inhibit not only the function of tumor-reactive T cells but also the function of NK cells

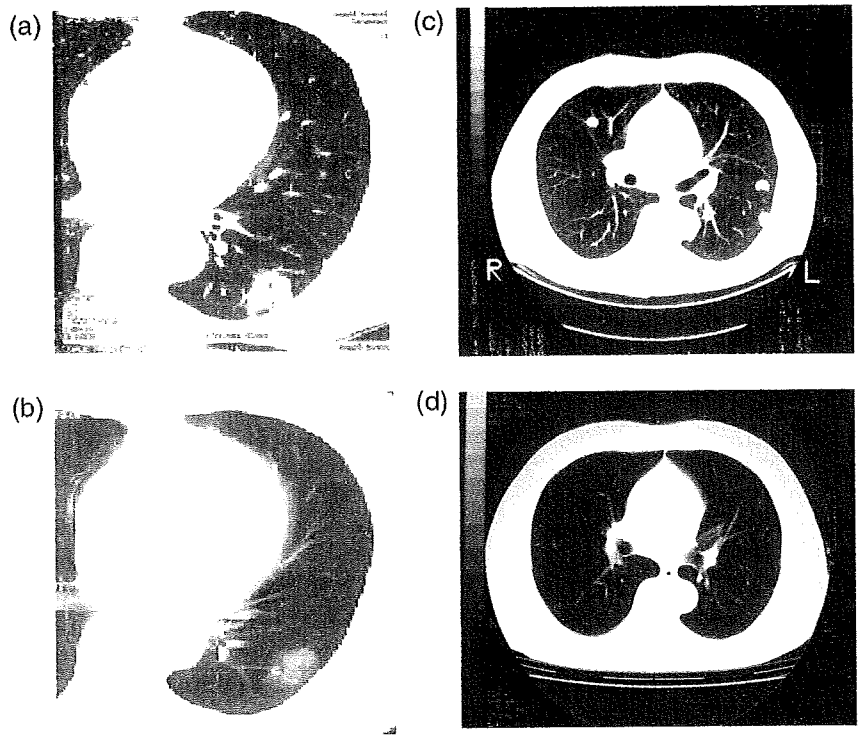

FIGURE 5 Antitumor effects of autologous activated NK cells. (a, b) Images for a 61-year-old female patient with pulmonary adenocarcinoma. (c, d) Images for a 58-year-old male patient with lung metastasis of renal cell carcinoma. Both patients refused standard treatments such as surgical operations, chemotherapy, and radiotherapy. After receiving informed consent from the patients, autologous activated NK cells (2×10^9 cells/injection) were transfused into the patients via 6 injections at 2-week intervals. The effects of the infusions were evaluated clinically by computed tomography scanning before the first cell infusion (a, c) and after the last cell transfusion (b, d).

[49,50]. Current clinical immunosuppressive agents have little effect on NK cell function [51]. We recently reported the possibility of Treg depletion using mild hyperthermia [52]. The results suggest that the effect of NK cell–based adoptive immunotherapy can be intensified by removing Treg or inhibiting their function using an immunosuppressant or hyperthermia, without performing lymphodepletion [51–54].

ALLOGENIC NK CELL THERAPY

Allogenic NK cells, which do not accept inhibitory signals, damage tumor cells more effectively than do autologous NK cells. Acute myeloid leukemia (AML) relapse can be completely controlled by transplants