

Production of proinflammatory cytokines without invocation of cytotoxic effects by an Epstein-Barr virus–infected natural killer cell line established from a patient with hypersensitivity to mosquito bites

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(Received 11 February 2010; revised 3 April 2010; accepted 14 June 2010)

Objective. Cumulative evidence supports that Epstein-Barr virus (EBV)–infected natural killer (NK) cells induce severe systemic and cutaneous inflammation in patients with hypersensitivity to mosquito bites (HMB). In order to understand the pathogenesis of HMB, we established an EBV-infected cell line and characterized the cytological profiles.

Materials and Methods. A novel EBV-infected NK-cell line, designated NKED, was established from a patient with HMB and used for the present study along with two other NK-cell lines, KAI3 and KHYG-1.

Results. NKED expressed the latency II-related transcripts. NKED cells were positive for CD2 and CD161 antigens, and negative for CD3, CD16, CD34, CD56, and T-cell receptor α/β and γ/δ antigens. Although NKED cells contained several cytotoxic molecules, the cells had an extremely poor cytotoxic activity. The majority of NKED cells were negative for perforin, major histocompatibility complex class I-restricted NK-cell receptors, CD94 and KIR2D, and an activating receptor, NKG2D. NKED cells, however, secreted higher levels of tumor necrosis factor- α . Stimulation with phorbol 12-myristate 13-acetate or tumor necrosis factor- α induced expression of BZLF1 messenger RNA in the NKED and KAI3 cells, indicating the transition from the latent- to the lytic-cycle infection.

Conclusions. These data suggested that NKED cells revealed a very low cytotoxic effect probably because of the low expression levels of perforin, but had the ability to release proinflammatory cytokines. NKED cells did not reflect the characteristics of HMB, as they were different from pathogenic NK cells proliferating in the HMB patient, but the difference indicated that pathogenic NK cells could change their character in the presence of interleukin-2. © 2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Most adults have been infected with the Epstein-Barr virus (EBV). Primary EBV infection occurs mostly in young children and is usually asymptomatic, but sometimes results in infectious mononucleosis and may also induce Gianotti-Crosti syndrome or virus-associated hemophagocytic syndrome [1–3]. Hypersensitivity to mosquito bites (HMB) is a disorder of natural killer (NK) cell lymphocytosis, characterized by abrupt onset of swollen, ulcerative skin lesions at the insect-bite sites, and associated with systemic symptoms, including high fever, lymphadenopathy, and hepatosplenomegaly, although the patients are

usually asymptomatic, despite the presence of circulating EBV-infected NK cells [4,5]. The EBV-DNA load in the plasma and peripheral blood mononuclear cells is usually highly elevated as compared with that of healthy volunteers. Furthermore, many patients with HMB have high antibody titers to lytic-cycle viral proteins, such as viral capsid antigens and early antigens, suggesting a booster phenomenon to the repetitive reactivation of EBV. The dermal infiltrates in the insect-bite sites are composed of a dense infiltration of cytotoxic T lymphocytes (CTLs), and a small number of EBV-encoded small nuclear RNA (EBER)-positive NK cells [6]. Some patients may present with clinical features of chronic active EBV infection in the clinical course, and may progress to fatal NK or T-cell lymphoma associated with EBV infection. Accumulating studies suggest that either the monoclonal or

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oligoclonal proliferation of EBV-infected NK and T cells plays a central role in the pathogenesis of HMB and chronic active EBV infection [7–9].

There has been controversy over whether EBV-infected T/NK cells are direct effectors that induce severe systemic as well as cutaneous symptoms in HMB, or host CTL responses against the EBV-infected cells induce such symptoms. It is noteworthy that patients with HMB are usually asymptomatic, despite the presence of a considerable number of circulating EBV-infected T/NK cells. These observations indicate that host CTLs are immunologically silent with respect to T/NK cells latently infected by EBV. After a mosquito or insect bite, however, both EBV-infected NK cells and CTLs migrate to the bite sites, and induce not only severe cutaneous reactions, but also systemic inflammations within a day.

In order to understand the cellular and molecular events leading to the abrupt onset of systemic inflammatory reactions in HMB, we have established an EBV-infected cell line from a patient and characterized its phenotype, NK-cell receptors, cytotoxic ability, and release of proinflammatory cytokines on stimulation.

Materials and methods

Patient

A 14-year-old Japanese girl had a 10-year history of vesicles, skin necrosis, and ulcers on mosquito-bite sites, frequently associated with high-grade fever, liver damage, and leukopenia due to hemophagocytosis. The percentage of CD56⁺ cells was increased in the peripheral blood (49.5% of the peripheral blood mononuclear cells). She showed an ordinary past-infection pattern in anti-EBV antibodies, but a larger number of EBV DNA copies (64,000 copies/μg DNA) in the peripheral blood cells. Histopathological examination showed a dense perivascular lymphocytic infiltrate containing CD56⁺ cells in the upper and mid dermis, and approximately 20% of the infiltrating cells were positive for EBER1 (Fig. 1). We diagnosed her as having HMB associated with EBV-infected NK-cell lymphocytosis.

Cell culture

The patient's peripheral blood mononuclear cells were suspended in AIM-V culture medium (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum under a protocol approved by the Ethics Committees of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. In order to establish the EBV-infected cell lines, limiting dilutions were performed under stimulation with interleukin-2 (rIL-2; Shionogi, Osaka, Japan; 50 U/mL). An EBV-positive NK cell line from CAEBV (KAI3 [10]), an EBV-negative NK cell line from aggressive NK-cell lymphoma (KHYG-1 [11]), and K562 target cells were obtained from Cell Bank (RIKEN BioResource Center, Ibaraki, Japan), and cultured in the same medium described here. Cells were cultured in a humidified atmosphere at 37°C with 5% CO₂ in air. One half of the medium was exchanged with fresh medium twice a week. The established cell line grew stably for >24 months in the presence of IL-2.

Flow cytometric analysis

Analysis of the cell surface markers was performed using the following anti-human fluorescent-conjugated monoclonal antibody (mAb) in direct immunofluorescence assays. Antibody concentrations were adjusted according to the protocol of the manufacturer. Cells were stained with fluorescein isothiocyanate (FITC) or with phycoerythrin-conjugated anti-CD2, -CD3, -CD4, -CD7, -CD8, -CD19, -CD-25, -CD30, -CD34, -CD56, -CD94, -TCR α/β, and -TCR γ/δ, -KIR2D, and -NKG2D mAb (Beckman Coulter, Fullerton, CA, USA), anti-CD16, and -CD161 (BD Pharmingen, San Diego, CA, USA), and anti-Fas-L and -tumor necrosis factor (TNF)-related apoptosis-inducing ligand mAb (eBioscience, San Diego, CA, USA). Flow cytometric analysis was performed by a FACS Caliber cytometer (BD Pharmingen) (Central Research Laboratory, Okayama University Medical School).

Reverse transcription polymerase chain reaction

Total RNA was extracted from purified cell populations using TRIzol Reagent (Invitrogen Life Technologies, San Diego, CA, USA) and then complementary DNA was generated using a Superscript First-Strand Synthesis System (Invitrogen Life Technologies). Reverse transcription polymerase chain reactions (RT-PCRs) for latent membrane protein (LMP)-1, EBV nuclear antigen (EBNA)-1 and EBNA2 were performed to determine the latency of EBV infection, and RT-PCR for BZLF1 was performed to recognize the induction of lytic replication. The primers for RT-PCR were as follows: latent membrane protein-1 (5'-GGCCTTGCTCTC CTTCTC-3' and 5'-CCAAAAGCTGCCAGATG-3'), EBNA1 (Q promoter-derived) (5'-GTGCGCTACCGGATGGCG-3' and 5'-CA TTTCCAGGTCCTGTACCT-3') [12], EBNA1 (C promoter- and W promoter-derived) (5'-TGGCGTGTGACGTGGTGTA-3' and 5'-CATTTCCAGGTCCTGTACCT-3') [12], EBNA2 (5'-ATTAGA GACCACTTTGAGCC-3' and 5'-AAGCGCGGGTGCTTAGAA GG-3') [12], and BZLF1 (5'-CATGTTTCAACCGCTCCGACT GG-3' and 5'-GCGCAGCCTGTCAATTTTCAGATG-3') [12]. We used IB4 cell complementary DNA as a positive control for the latency III-related transcripts, such as EBNA1 (Q promoter-derived/W promoter-derived) and EBNA2.

Quantification of EBV DNA

A real-time quantitative PCR assay was performed using a Light-Cycler EBV quantification kit (Roche Diagnostics, Mannheim, Germany) with a Light Cycler instrument (Roche Diagnostics) according to manufacturer's instructions. The number of EBV DNA copies was calculated as the number of virus copies per microgram DNA. The EBV detection limit in this assay was approximately 10 copy/μg DNA.

Immunohistochemistry assay for cytotoxic molecules

Immunostaining was performed as follows: preheated deparaffinized biopsy specimens were incubated with monoclonal antibodies to perforin (Novocastra, Newcastle upon Tyne, UK), granzyme B (Chemicon International, Temecula, CA, USA), granzyme B (Medical Biological Laboratories, Aichi, Japan), and T-cell intracellular antigen (TIA)-1 (Beckman Coulter) overnight at 4°C, then with biotin-labeled anti-mouse immunoglobulins for 60 minutes, and finally with horseradish peroxidase-labeled

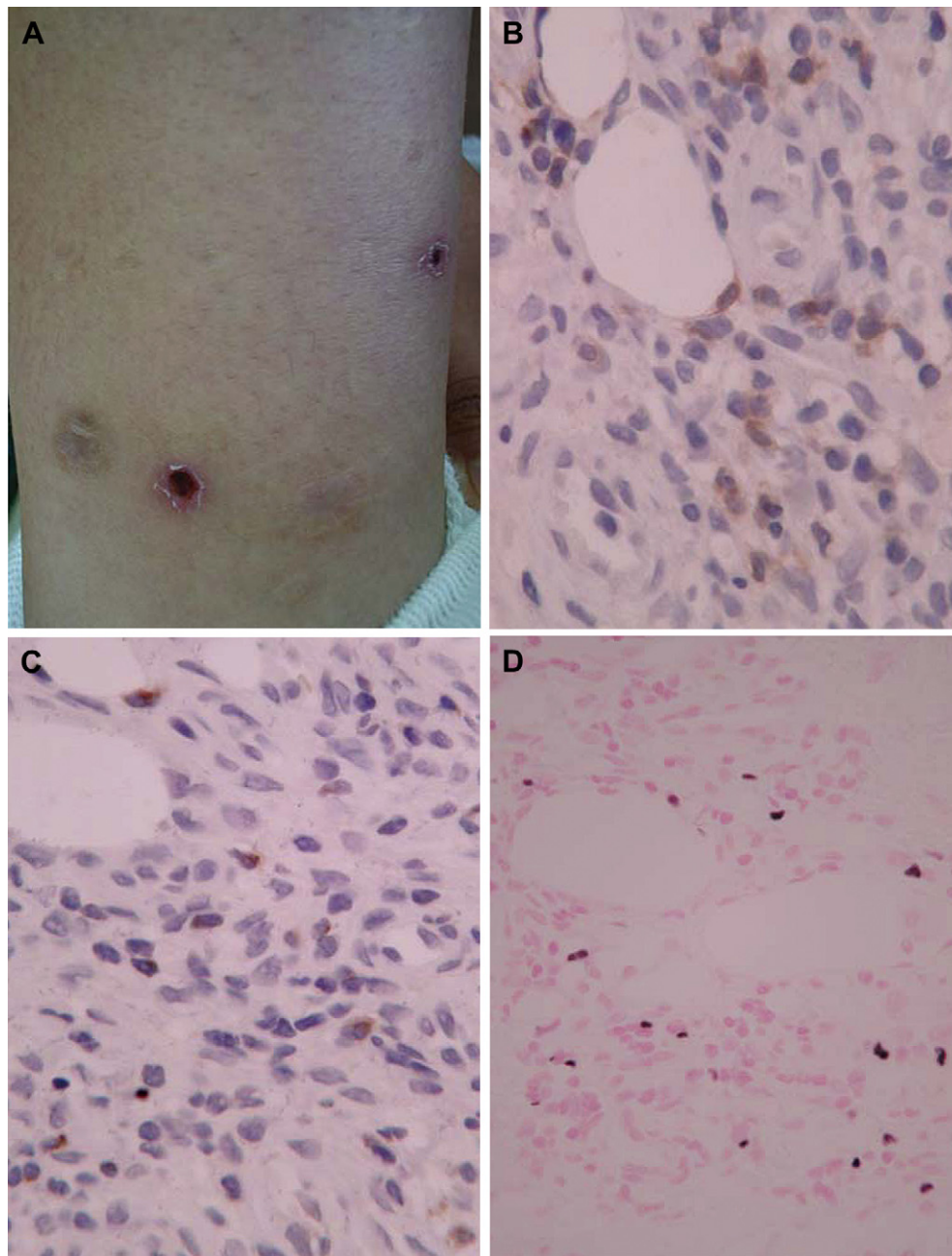


Figure 1. Clinical finding of the patient with hypersensitivity to mosquito bites (HMB). She had skin necrosis, ulcers, and scars on mosquito-bite sites (A). Histological findings of infiltrating cells in the specimen from skin lesion. Immunohistochemistry assay for CD56 (B) and for granzyme B (C), and in situ hybridization with the fluorescein isothiocyanate-labeled Epstein-Barr virus–encoded small nuclear RNA (EBER) probe (D).

streptavidin for 30 minutes at room temperature. The peroxidase activity was visualized by incubation with diaminobenzidine in the presence of hydrogen peroxide.

In situ hybridization

Lymphoid cells containing EBER-1 were detected by in situ hybridization on paraffin-embedded sections, using FITC-labeled nucleotide probes (Novocastra). EBER1 is synthesized in large quantities in the nuclei of EBV latently infected cells. Deparaffinized sections were treated with proteinase K (10 mg/mL) for 10 minutes at 37°C,

and acetylated for 10 minutes with 0.1 M triethanolamine containing 0.025% acetic acid. After prehybridization with salmon sperm DNA, sections were hybridized with the FITC-labeled probe overnight at 37°C. Specimens were rinsed with 1× saline sodium citrate (SSC) (150 mM sodium chloride and 15 mM sodium citrate [pH 7.0]) for 30 minutes, and incubated with alkaline phosphatase–labeled anti-FITC antibody (Novocastra) for 30 minutes. Color development was performed by incubation with a substrate solution containing 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium chloride.

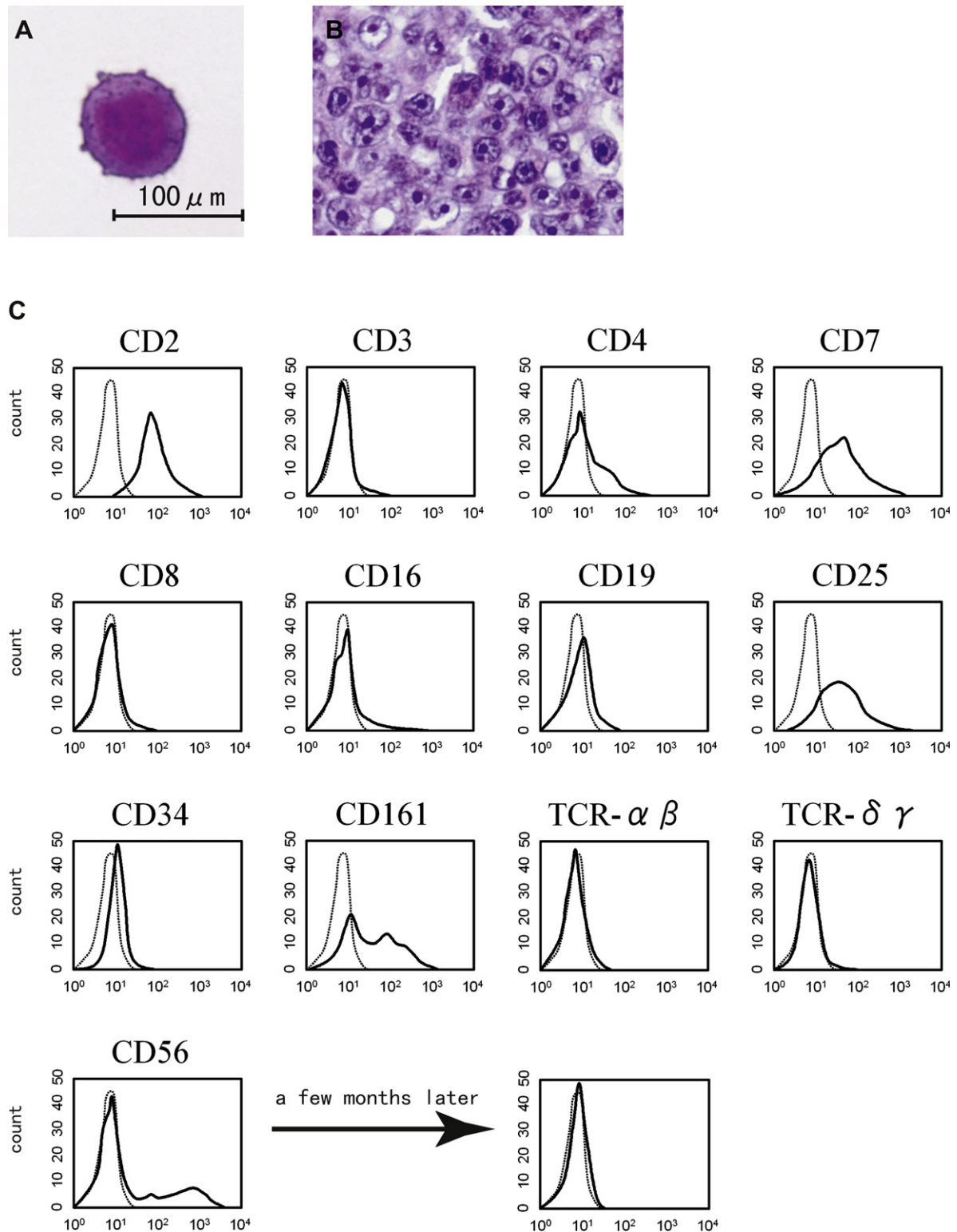


Figure 2. (A) Morphology of NKED cells on May-Giemsa staining (original magnification, $\times 400$). (B) Hematoxylin-eosin staining using a paraffin-embedded section of NKED (original magnification, $\times 400$). (C) Flow cytometric analysis for surface cell markers of the established cell line, NKED. The solid line and dotted lines represent staining with specific monoclonal antibodies and isotype controls. The vertical axis denotes the cell number counted and the horizontal axis denotes fluorescence intensity (log scale).

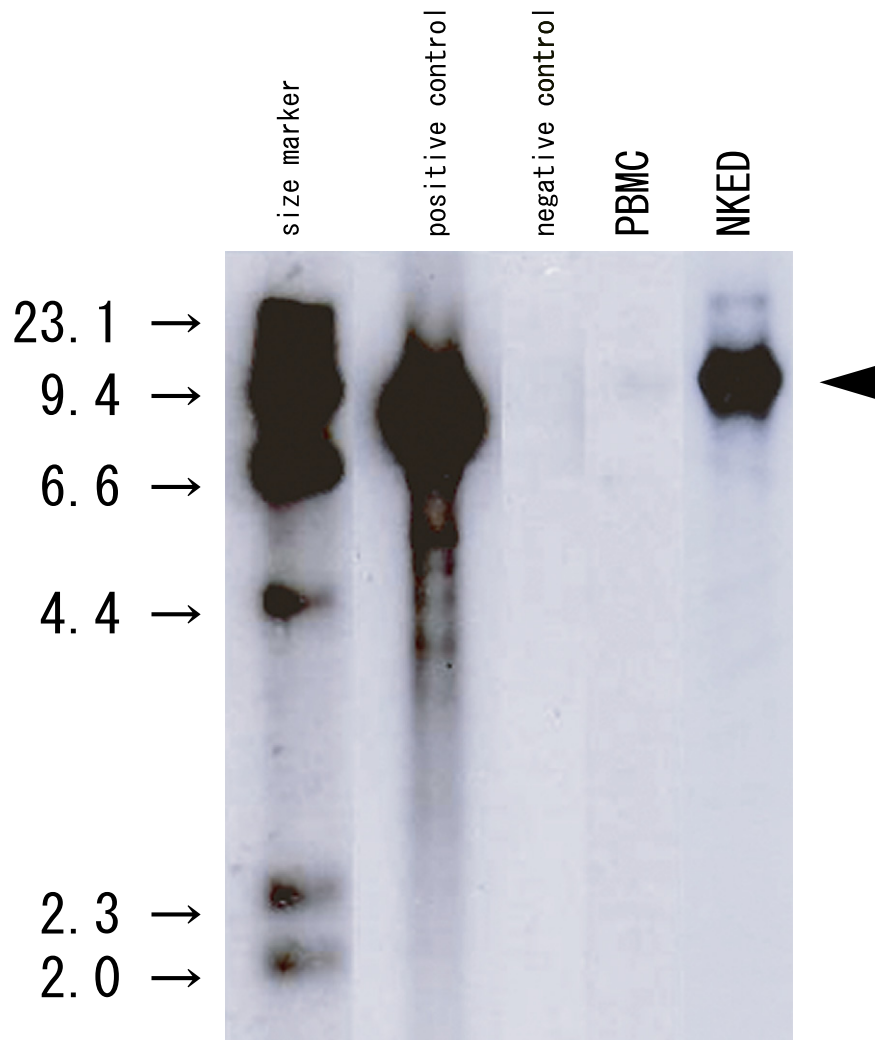


Figure 3. Southern blotting analysis for determination of the clonality of Epstein-Barr virus (EBV) with a terminal repeat probe. Positive control was obtained from B95-8 (lane 2). Negative control was obtained from KHYG-1 (lane 3). DNA was digested with Bam HI and hybridized with the ^{32}P -labeled XhoI probe. Arrow indicated clonal EBV episome band. PBMC = peripheral blood mononuclear cells.

Southern blotting

The clonality of EBV was determined by Southern blotting using a terminal repeat probe. Genomic DNA extracted from the peripheral blood mononuclear cells or cultured cells was digested with BamHI, subjected to gel electrophoresis, transferred to a nylon membrane, hybridized with a ^{32}P -labeled XhoI fragment from the terminal region of EBV, and visualized by autoradiography. Raji and B95-8 cells were used as controls for monoclonal cell expansion with a single EBV–terminal repeat and polyclonal cell proliferation with EBV replication, respectively.

Analysis of cytokine levels by cytometric bead array

After 48 hours of incubation with rIL-2 at a concentration of 5×10^5 cells/mL, the soluble cytokine (IL-5, IL-6, IL-10, TNF- α , and interferon- γ) levels in the cell culture supernatants were measured by a cytometric bead array kit (BD Pharmingen) according to the manufacturer's instructions.

Cytotoxicity assay

^{51}Cr -release cytotoxicity assays were performed using K562 target cells labeled with $100 \mu\text{Ci } ^{51}\text{Cr}$ and incubated with effector cells at effector-to-target ratios of 10:1 to 1.25:1 in round-bottom 96-well plates (Asahi Glass, Tokyo, Japan) for 4 hours. Spontaneous release and maximal release were determined by incubating K562 target cells in medium alone or in 5% Triton X-100, respectively. The assay was always performed in triplicate. The percentage of specific lysis was determined with this formula: % specific lysis = $[\text{mean cpm (experimental release)} - \text{mean cpm (spontaneous release)}] / [\text{mean cpm (total release)} - \text{mean cpm (spontaneous release)}] \times 100$.

Induction of lytic replication

To induce the lytic replication, we treated the EBV-infected NK cell lines, NKED and KAI3, with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and TNF- α (0.1, 1, 10, 100 ng/mL) at a concentration of 5×10^5 cells/mL. Cells were harvested at 12, 24, and 48

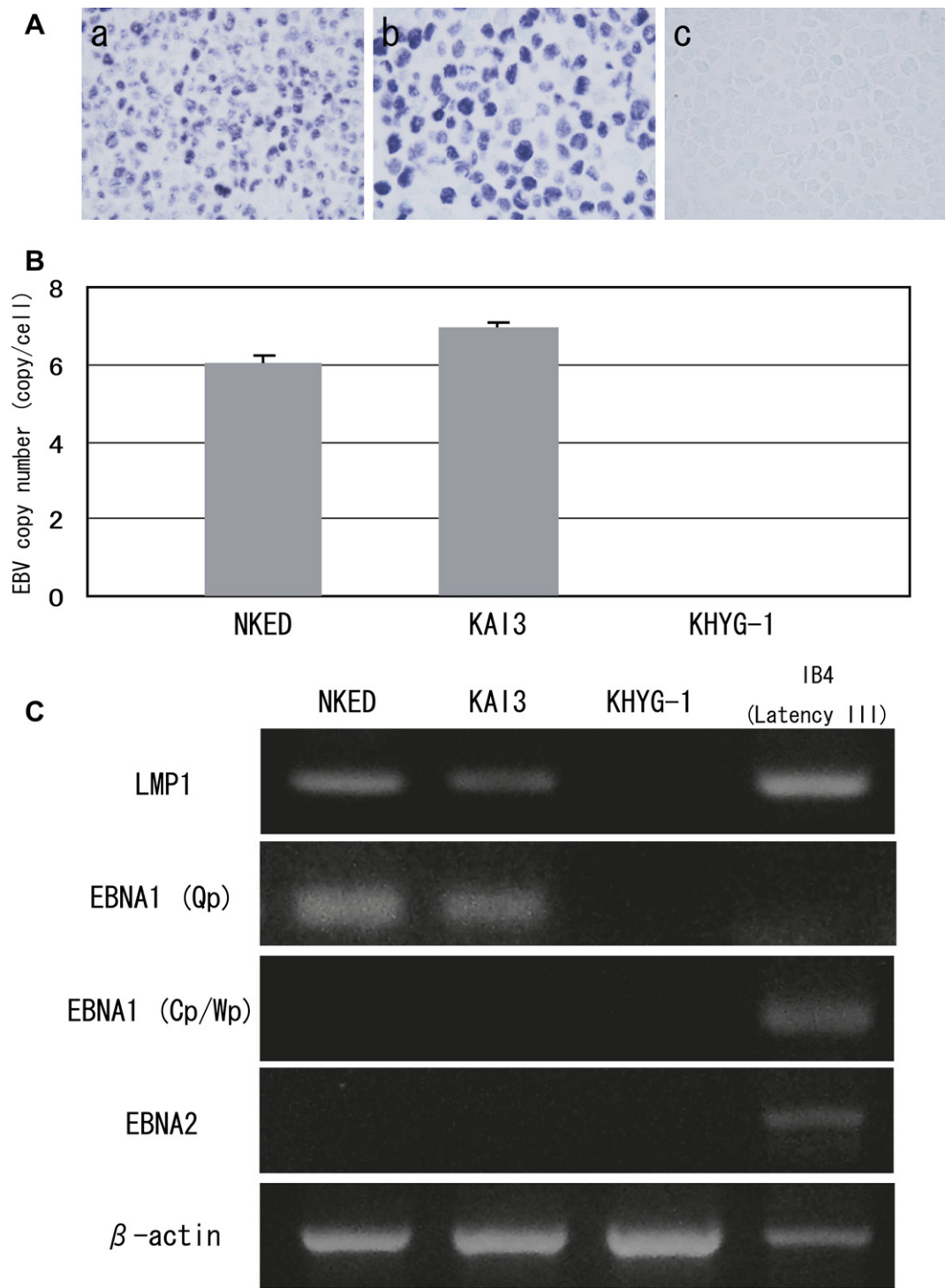


Figure 4. (A) In situ hybridization with the fluorescein isothiocyanate (FITC)-labeled Epstein-Barr virus (EBV)-encoded small nuclear RNA (EBER) probe (original magnification, $\times 400$) for NKED (a), KAI3 (b), and KHYG-1 cells (c). (B) The copy number of EBV genomes in cell lines with real-time PCR method. The copy number of EBV genomes was calculated with the formula: $1 \mu\text{g DNA} = 1.7 \times 10^5 \text{ cells}$. (C) Reverse transcription polymerase chain reaction analysis for EBV-encoded transcripts for latent infection in NK cell lines, NKED, KAI3, and KHYG-1. Positive control for latency III-related transcripts was obtained from IB4. LMP = latent membrane protein; EBNA = EBV nuclear antigen.

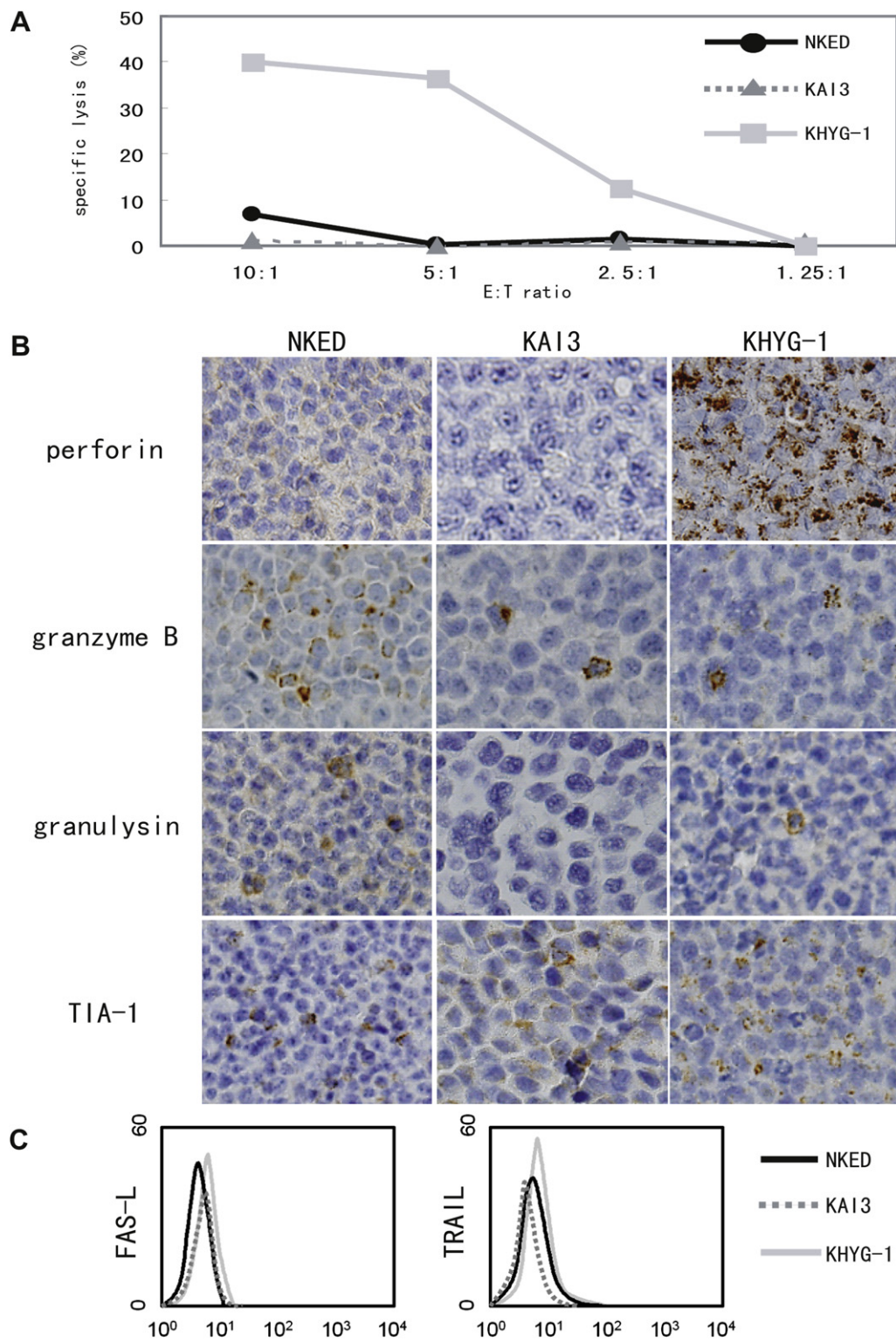


Figure 5. (A) The natural killing activity of natural killer (NK) cell line cells against K562 cells. (B) Immunostaining assay for perforin, granzyme B, granulysin, and T-cell intracellular antigen 1 (TIA-1). Paraffin-embedded sections of NKED, KAI3, and KHYG-1 cells were stained with monoclonal antibody by an immunoperoxidase technique (original magnification, $\times 400$). (C) Flow cytometric analysis for Fas-L and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) of NKED, KAI3, and KHYG-1.

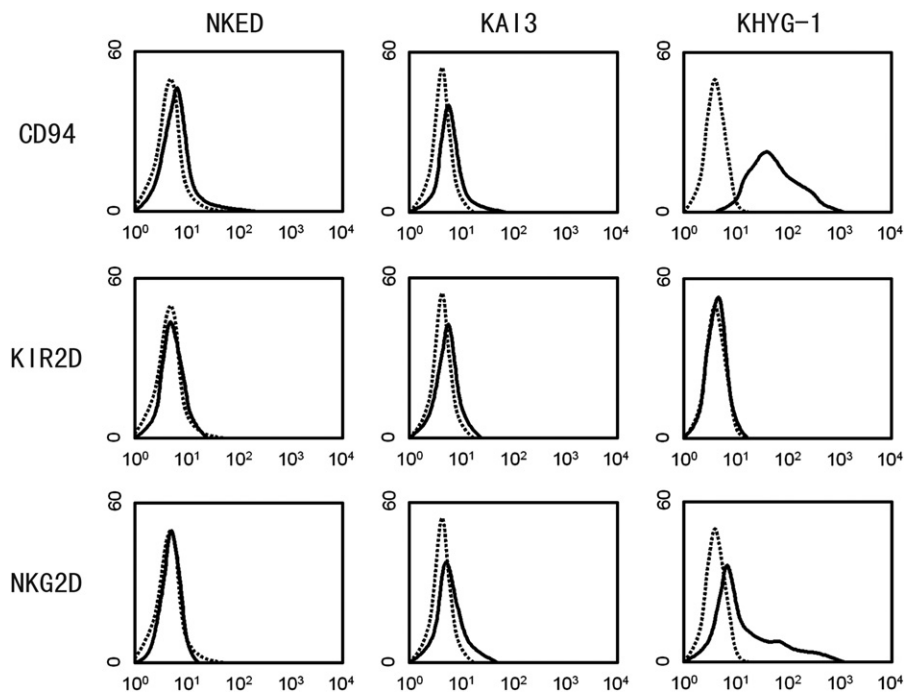


Figure 6. Flow cytometric analysis for natural killer (NK) cell receptor, KIRs and CD94 and NKG2D. To detect KIRs, monoclonal antibody for CD158 + p50.3 was used. Monoclonal antibody for CD158 recognizes KIR2DL, KIR2DS1, and KIR2DS2, and monoclonal antibody for p50.3 recognizes KIR2DS4.

hours later. To recognize the induction of lytic replication, RT-PCR for BZLF1 was performed as described here.

Results

A novel EBV-infected NK cell line established from a patient with HMB

An IL-2–dependent cell line, designated NKED, was established from a patient with HMB and maintained for >24 months in the presence of recombinant human IL-2. NKED cells appeared with large mononuclear cells with a high cytoplasm-to-nucleus ratio. Nuclei were round and had fine chromatin and many mitotic figures with many azurophilic granules (Fig. 2A, B). Flow cytometric analysis showed that NKED cells were positive for CD2, CD7, CD25, CD30, and CD161, and negative for CD3, CD4, CD8, CD16, CD19, CD34, TCR α/β , and TCR γ/δ (Fig. 2C). NKED cells lost the expression of CD56 during the culture period, although the same clone expressing CD56^{dim/bright} had been present for a few months in the early culture. Despite the partial reversion of the CD56^{dim/bright} subset to CD56^{neg} phenotypes, CD161 expression persisted without any T-cell markers. These phenotypes suggest that the phenotype of NKED cells mimics that of immature NK cells, which are characterized by a phenotype of CD34⁺, CD161⁺, and CD56⁺ [13,14].

Southern blotting analysis to detect the EBV terminal repeat showed that NKED cells were composed of monoclonal EBV-infected cells, while no dominant clone was

detected in the peripheral blood (Fig. 3). EBV1 was detected in almost all NKED and KAI3 cells by in situ hybridization (Fig. 4A). The EBV genome copy numbers determined by real-time PCR assay were 6 and 7 copies/cell for NKED and KAI3 cells, respectively (Fig. 4B). Both NKED and KAI3 cells expressed EBNA1 and latent membrane protein-1 messenger RNA (mRNA), but did not express EBNA2 mRNA, indicating the latent infection, type II (Fig. 4C).

NKED cells show low cytotoxicity against K562 cells due to their low expression of perforin

In order to assess the cytotoxic ability of NKED cells, we first examined their killing activity against K562 target cells lacking the major histocompatibility complex class I molecules. NKED and KAI3 cells did not exhibit killing activity. Only KHYG-1 cells showed cytotoxicity against K562 cells, depending on the effector-to-target cell ratio (Fig. 5A). Next, in order to clarify the reason for the low killing activity of NKED cells, we examined the expression levels of the cytotoxic molecules perforin, granzyme B, granulysin, and TIA-1 by immunohistochemistry. While KHYG-1 cells, which showed cytotoxicity against K562 cells, expressed all four molecules, NKED cells expressed granzyme B, granulysin, and TIA-1, but not perforin (Fig. 5B), even though the levels of perforin mRNA expression were roughly identical between NKED and KHYG-1 cells in the real-time PCR assay (data not shown). In addition, we examined the death receptors Fas-L and

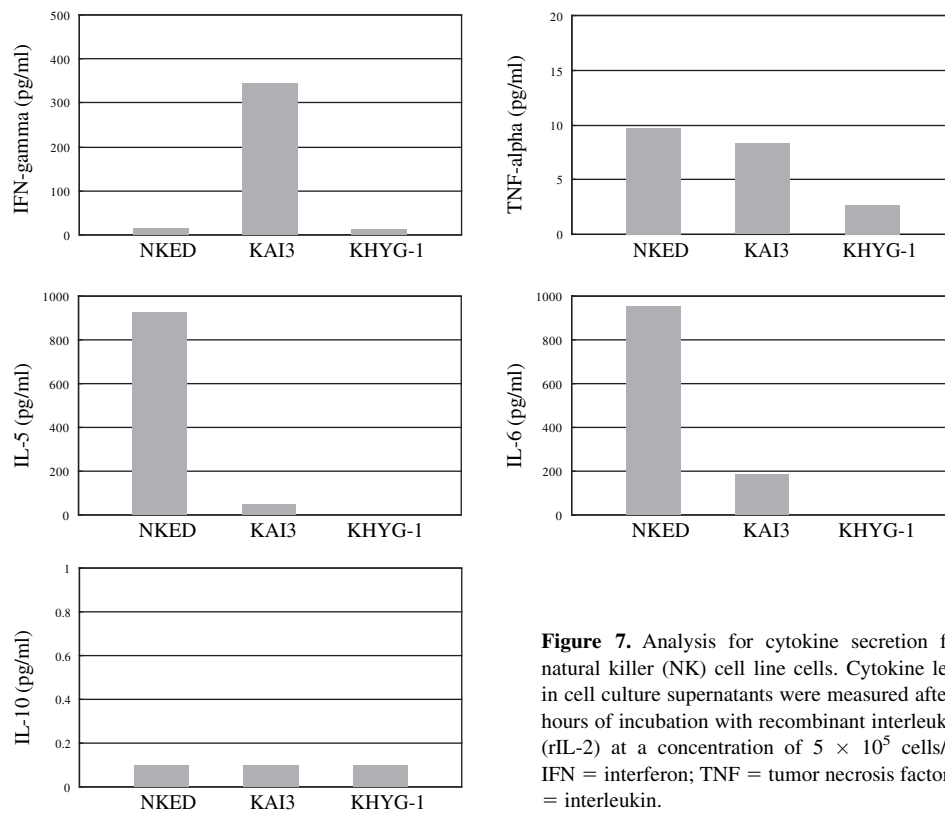


Figure 7. Analysis for cytokine secretion from natural killer (NK) cell line cells. Cytokine levels in cell culture supernatants were measured after 48 hours of incubation with recombinant interleukin-2 (rIL-2) at a concentration of 5×10^5 cells/mL. IFN = interferon; TNF = tumor necrosis factor; IL = interleukin.

TNF-related apoptosis-inducing ligand by flow cytometric analysis. Results showed that there was no expression of these death receptors in any of the three cell lines (Fig. 5C).

Because the expression levels of CD56 might be closely associated with the cytotoxic effects [15,16], we assessed the cytotoxicity in each cell fraction with different CD56 expression levels, i.e., the CD56^{neg}, CD56^{dim}, and CD56^{bright} cell fractions sorted by FACS Aria (BD Pharmingen) (Central Research Laboratory, Okayama University Medical School). The results demonstrated that there was no significant difference in the killer activities of the fractionated cells from NKED and KHYG-1 (data not shown).

NK-cell receptor expression

NK-cell receptors can inhibit or activate NK-cell functions, such as cytotoxicity and cytokine production. In order to confirm the low cytotoxic effect of NKED cells from another viewpoint, we examined the expression levels of both inhibitory and activating NK-cell receptors, such as major histocompatibility complex class I–restricted inhibitory receptors killer cell immunoglobulin-like receptor (KIR) family and CD94/NKG2 family, and major activating receptor NKG2D, which does not associate with CD94 unlike other NKG2 family members.

Flow cytometric analysis showed that the majority of NKED and KAI3 cells were negative for all the NK-cell

receptors, including NKG2D, while many KHYG-1 cells capable of sufficient killer activities were positive for NKG2D as well as CD94 (Fig. 6).

Cytokines secretion

NKED cells secreted high levels of TNF- α (approximately 10 pg/mL), IL-5 (approximately 900 pg/mL), and IL-6 (approximately 950 pg/mL) into the culture medium, as compared with other NK cell line cells (Fig. 7). In addition to TNF- α , KAI3 cells secreted interferon- γ (approximately 350 pg/mL). By contrast, KHYG-1 cells did not release cytokines into the culture medium except for a low level of TNF- α (approximately 2.5 pg/mL). Therefore, both NKED and KAI3 cells with their relatively low killer activities had a greater ability to secrete certain proinflammatory cytokines than KHYG-1 cells with their high cytotoxicity.

BZLF1 mRNA expression

Treatments with PMA (50 ng/mL) induced BZLF1 mRNA expression in NKED and KAI3 cells after 48 hours of stimulation, indicating the invocation of the lytic-cycle infection. TNF- α also induced BZLF1 mRNA expression in both cell lines, but the required concentrations were different between the cell lines; 0.1 ng/mL TNF- α was sufficient to induce BZLF1 mRNA for KAI3 cells and 100 ng/mL was required for NKED cells (Fig. 8).

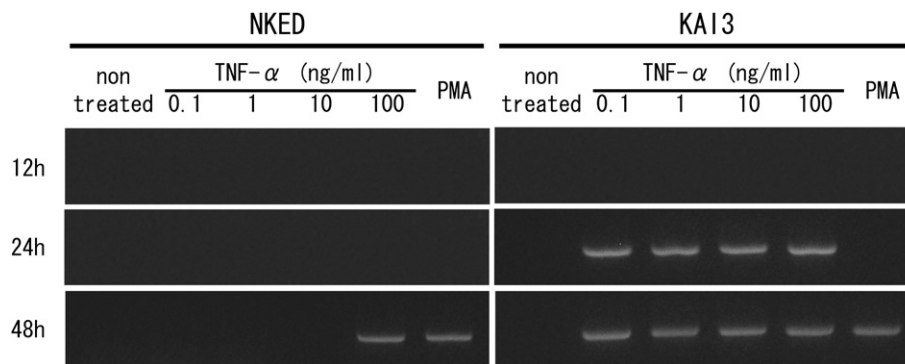


Figure 8. Reverse transcription polymerase chain reaction analysis for BZLF1 expression of NKED (left) and KAI3 (right) by treatment with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) or tumor necrosis factor- α (TNF- α) (0.1–100 ng/mL); 5×10^5 cells were harvested after 12, 24, and 48 hours of incubation with PMA or TNF- α .

Discussion

NK cells are important effectors of the innate immune system and play a critical role in early host defense against invading pathogens, while adaptive immune responses are being activated [17]. In NK-cell development, common lymphoid progenitors subsequently differentiate into the following subsets: stage 1 progenitor NK cells, stage 2 precursor NK cells, stage 3 immature NK cells, stage 4 mature NK cells (CD56^{bright}), and finally stage 5 mature NK cells (CD56^{dim}) [13,14]. EBV-infected NKED cells were positive for CD2, CD7, CD25, and CD161 antigens, and negative for CD3, CD4, CD8, CD16, CD19, CD34, CD56 and T-cell receptor α/β and γ/δ antigens. This phenotype is consistent with that of stage 3 immature NK cells.

NK cells do not require antigen-specific recognition to kill target cells, and they mediate cell killing through a variety of mechanisms, including perforin/granzyme granule-mediated exocytosis or signaling through the death receptor pathways, such as the Fas-FasL or TNF-related apoptosis-inducing ligand pathway [18,19]. Granule exocytosis is the major mechanism of killing used by NK cells, and it is well-known that perforin plays a crucial role in granule-mediated cytotoxicity, although the exact role of perforin has not been precisely defined. Our cytotoxicity assay demonstrated that NKED and KAI3 cells had poor cytotoxicity against K562 cells, despite the presence of cytotoxic molecules granzyme B, granulysin, and TIA-1, whereas the KHYG-1 cells exhibited cytotoxic effects. This discrepancy was explained by the fact that NKED and KAI3 cells did not express perforin, while KHYG-1 cells did.

NK-cell function, such as cytotoxicity and cytokine production, is tightly regulated by a balance between positive and negative signals provided by a diverse array of cell surface receptors [16,20]. Inhibitory receptors, such as the KIR family and the lectin-like CD94/NKG2A receptor family, play the most prominent role in regulating NK cells. When NK-cell inhibitory receptors bind to major

histocompatibility complex class I molecules, their effector functions are blocked. KIRs also act as activating receptors because they exist as sets of paired inhibitory and activating receptors; however, KIRs represent a relatively minor component of the activating NK-cell repertoire, unlike their inhibitory counterparts. The C-type lectin-like receptor NKG2D, which does not associate with CD94, unlike other NKG2 family members, plays a key role in innate and adaptive immune responses in activating receptors [21,22]. In the present study, NKED and KAI3 cells did not express sufficient numbers of NK-cell receptors because they were stage 3 immature NK cells. KHYG-1 cells expressed CD94 more dominantly than KIRs, which was compatible with the character of stage 4 mature NK cells. KHYG-1 cells also expressed NKG2D, indicating that they were in an activating state.

In addition to the cytotoxic effects, certain subsets of NK cells are capable of releasing proinflammatory cytokines, which trigger an adaptive or antigen-specific immune response [19]. NKED cells secreted higher levels of IL-5, IL-6, and TNF- α into the culture medium under the presence of recombinant IL-2 than the other NK cell lines tested. This observation indicates that NKED cells may have the potential to induce systemic inflammation with the subsequent activation of an adaptive immune system. As previously reported, CD56[−] CD161⁺ immature NK cells cultured with IL-2 could produce IL-5 [23]; however, IL-5 function in NK-cell differentiation or NK-cell lymphoma progression has not been defined. IL-6 released from NKED cells could partly contribute to the maintenance of the NKED cells themselves [24]. Alternatively, IL-6 produced by NKED or KAI3 cells might affect the cytotoxic effects of bystander NK cells, as reported previously [25]. The transition from the latent- to the lytic-cycle infection of EBV can be induced by PMA in EBV-infected B cell lines [26–28]. We demonstrated that the lytic-cycle infection was induced in NKED and KAI3 cells by treatment with PMA or TNF- α . Therefore, it is

possible that TNF- α secreted from NKED cells can cause NKED cells to have a lytic-cycle infection.

Both mature NK-cell subsets with a CD56^{bright} phenotype and immature NK cells with a CD34[−], CD161⁺, CD56[−] phenotype play a pivotal role in cytokine secretion, but are less effective in cytotoxicity [16,29]. Taken together with the CD34[−], CD161⁺, CD56[−] phenotype of NKED cells, their functions including their low cytotoxicity and high production of cytokines are consistent with those of immature NK cells.

In the HMB patient, proliferating NK cells were stage 4 NK-cell phenotype and have high levels of NK-cell activity with NK1 cytokine secretion [30–32]. NKED cells did not reflect the characteristics of HMB, as they were different from NK cells proliferating in the HMB patient in the phenotype, cytotoxicity, and cytokine secretion. Perhaps the difference was caused by the exposure of cells to IL-2 for a long time, but it also indicated that pathogenic NK cells could change their character into that with low cytotoxicity and high cytokine production in the presence of IL-2.

Acknowledgments

Supported by a Grant-in-Aid for Scientific Research (B) (No. 20390307) from Japan Society for the Promotion of Science (Tokyo, Japan), and by a Grant-in-Aid for Scientific Research on Priority Areas (C) (No. 20591321) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Tokyo, Japan).

Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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