Article

EBV-Induced Human CD8$^+$ NKT Cells Synergise CD4$^+$ NKT Cells Suppressing EBV-Associated Tumours upon Induction of Th1-Bias

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CD8$^+$ natural killer T (NKT) cells from EBV-associated tumour patients are quantitatively and functionally impaired. EBV-induced CD8$^+$ NKT cells drive syngeneic T cells into a Th1-bias response to suppress EBV-associated malignancies. IL-4-biased CD4$^+$ NKT cells do not affect either syngeneic T cell cytotoxicity or Th cytokine secretion. Circulating mDC1 cells from patients with EBV-associated malignancies impair the production of IFN-γ by CD8$^+$ NKT cells. In this study, we have established a human-thymus-SCID chimaera model to further investigate the underlying mechanism of EBV-induced CD8$^+$ NKT cells in suppressing EBV-associated malignancies. In the human-thymus-SCID chimera, EBV-induced CD8$^+$ NKT cells suppress EBV-associated malignancies in a manner dependent on the Th1-bias response and syngeneic CD3$^+$ T cells. However, adoptive transfer with CD4$^+$ NKT cells alone inhibits T cell immunity. Interestingly, CD4$^+$ NKT cells themselves secrete high levels of IL-2, enhancing the persistence of adoptively transferred CD8$^+$ NKT cells and T cells, thereby leading to a more pronounced T cell anti-tumour response in chimaeras co-transferred with CD4$^+$ and CD8$^+$ NKT cells. Thus, immune reconstitution with EBV-induced CD4$^+$ and CD8$^+$ NKT cells synergistically enhances T cell tumour immunity, providing a potential prophylactic and therapeutic treatment for EBV-associated malignancies. *Cellular & Molecular Immunology*. 2009;6(5):367-379.

Key Words: CD8$^+$ NKT cells, EBV, human-thymus-SCID chimaeras, IFN-γ

Introduction

Natural killer T (NKT) cells are unconventional, glycolipid-reactive, and semi-invariant αβT cells that are restricted to the antigen-presenting molecule CD1d and appear to bridge innate and adaptive immunity (1-3). Importantly, CD1d is mainly expressed on dendritic cells (DCs), macrophages, and B cells, implying that NKT cells primarily interact with antigen-presenting cells (APCs) rather than tissue cells (4).

As with conventional T cells, CD4 and CD8 molecules have been used to map the developmental pathway of NKT cells, but the pattern of these markers for NKT cells is more difficult to determine during development. CD1d-tetramer restricted NKT cells are generally CD4$^-$CD8$^-$ double negative or CD4$^+$ cells in mice and humans. However, CD8$^+$ NKT cells usually are present in humans in cases of infection and carcinoma.

A salient feature of NKT cells is their ability to rapidly secrete a variety of cytokines within a short time after activation. Copious production of prototypical Th1 and Th2 cytokines, IFN-γ and IL-4, respectively, has been thoroughly documented. Identification of the NKT cell subsets that might specialise in

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Abbreviations: DC, dendritic cell; EBV, Epstein-Barr virus; CTL, cytotoxic T lymphocytes; NPC, human nasopharyngeal epithelial cell lines; LEI, latent EBV-infection; LBC, human Hodgkin´s-derived EBV-associated B-cell lymphoma cell lines; PBMC, peripheral blood mononuclear cell.

Th1 versus Th2 functions has not been thoroughly investigated. The secretion of Th1 and Th2 cytokines by NKT cells is thought to underlie the regulatory properties of these cells (5, 6), which suggests a flexible role for NKT cells in immunoregulation, although a clear difference in cytokine production between NKT cell subsets cannot be resolved at this time. Although expression profiles of Th1/Th2 cytokines between mice and humans differ, the question remains as to how these cytokines lead to a regulated immune response *in vivo*. The cytokine profile of NKT cells is possibly influenced by the quality of the TCR signal, which is dependent on different antigens. An α-Galcer analogue with a shortened sphingosine stimulates a higher ratio of IL-4 to IFN-γ secretion, while a C-glycoside analogue of α-Galcer promotes a higher ratio of IFN-γ to IL-4 (7, 8). However, there is no structural basis for predicting how a compound will affect the cytokine profile produced by activated NKT cells.

As the first discovered human tumour virus, Epstein-Barr virus (EBV) is involved in the development of many malignancies, such as Hodgkin's lymphoma and nasopharyngeal carcinoma. EBV-induced cytotoxic T lymphocytes (CTLs) are thought to be the main effectors in cell-mediated immunity (9). Accumulating evidence indicates that EBV-induced CTLs, including $CD4^+$ and $CD8^+$ T cells, are able to recognise EBV-infected cells (10). Thus, adoptive T lymphocyte transfer following the administration of a nonmyeloablative chemotherapy regimen is proven to be an effective treatment in previous clinical trials (11). However, the mechanism by which these CTLs respond to EBV-associated malignant tumour cells *in vivo* is still unknown.

In this study, we further investigated the mechanism of the EBV-induced $CD8^+$ NKT cell response to EBV-associated malignancies based on our previous study, which demonstrated that EBV-induced $CD8^+$ NKT cells drive syngeneic T cells into a Th1-bias response to suppress EBV-associated malignancies and that IL-4-biased $CD4^+$ NKT cells do not affect T cell cytotoxicity. Here we found that EBV-induced $CD8^+$ NKT cells, alone or combined with syngeneic $CD4^+$ NKT cells, efficiently promote the Th1-bias response against Hodgkin's-derived EBV-associated B-cell lymphoma cell line (LBC)- or EBV-associated nasopharyngeal carcinoma cell line (NPC)-implanted HXTT H-T-S chimaeras. Adoptive transfer of EBV-induced cytotoxic NKT cells is a promising immunotherapy for the suppression of EBV-associated malignancies.

Materials and Methods

Patients, cells, and tetramer

Patients with Hodgkin's lymphoma were diagnosed according to the WHO criteria and staged according to the Ann Arbor classification (12). The patients with nasopharyngeal carcinomas (NPC) were diagnosed and staged according to the International Union Against Cancer and the American Joint Committee on Cancer staging manuals (13). Histopathology was based on the WHO international histological classification. Latent EBV-infection (LEI) or control normal subjects (CN) were healthy EBV seropositive or seronegative individuals, respectively (14). The clinical information of all patients and subjects was listed as in our previous paper (15). All patients were newly diagnosed and had no previous treatment. All patients and subjects provided informed consent according to institutional guidelines. Human fetal thymic cells and PBMCs were anonymously obtained from voluntarily elective pregnancy terminations (< 24-wk gestation; typing-matched HLA-A2 and HLA-DRB1(*03), mismatched HLA-A11 and HLA-DQ5). The possibility of lytic and latent EBV- and HTLV-I-infections in the mothers was excluded by Q-PCR and serologic determination (16). The fetal cells were isolated, aliquoted, cryopreserved and maintained in vapour-phase liquid nitrogen for further use. The viability of defrosted cells was evaluated by trypan blue dye exclusion before use. For transplantation, NKT cells were positively depleted from thymic cells with MACS beads in a protocol based on staining with the α-GalCer-loaded CD1d tetramer (ProImmune Inc., Bradenton, FL) (17). Human fetal peripheral $CD19^+$ B cells (NBC), a human Hodgkin's-derived EBV-associated B-cell lymphoma cell line (L54OCY, LBC), a human nasopharyngeal epithelial cell line (NP69, NPE), a human nasopharyngeal carcinoma cell line (CNE1, NPC) and the EBV^+ Raji B cell line were cultured and maintained in RPMI-1640 total medium. For functional studies, NKT cells were purified from PBMCs or organs by flow cytometry cell sorting or the MACS bead system based on staining with the α-GalCer-loaded CD1d tetramer (17, 18). The α-GalCer-loaded CD1d tetramers were synthesised and labelled as previously described (19, 20). All mAbs were purchased from R&D Systems Europe Ltd. (Abingdon, UK), except that the anti-Vβ11 mAb and anti-Vα24 mAb were from Coulter-ImmunoTech (Marseille, France) and the anti-CD161 pAb was from Santa Cruz Biotechnology (Santa Cruz, CA).

Human-thymus-SCID chimaeras and humanised xenogeneic tumour-transplanted human-thymus-SCID chimaeras

To establish the human-thymus-SCID chimaeras (referred to as hu-thym-SCID chimaeras), 8-wk-old female SCID mice (NOD/LtSz-prkdc^{scid}/prkdc^{scid} strain, the Jackson Laboratory) were irradiated (300 cGy/mouse) prior to cell-transplantation. Human fetal thymic cells (containing 1×10^7 thymocytes, thymocytes: thymic epithelial cells and other stromal cells = 1 : 1; immature and mature NKT cells were positively depleted) (17, 18) were transplanted into thymi of anaesthetised SCID mice. The chimaeras were then intrathymically challenged with EBV (10^7 pfu) (14, 21). The challenge was repeated once after 6 days. The hu-thym-SCID chimaeras were maintained for 4 weeks (unless otherwise stated) (22). In some cases, $CD4^+$ or/and $CD8^+$ NKT cells (1×10^5 cells/mouse) were purified from thymi of EBV-exposed hu-thym-SCID chimaeras (at week 4 post-establishment), mixed with syngeneic $CD3^+CD56^-CD161^-$ T cells (2×10^6 cells/mouse) purified from the spleens of EBV-exposed hu-thym-SCID chimaeras and antigen presenting cells (DCs, 0.5×10^6 cells/mouse), and i.v. adoptively transferred into

Table 1. The sequences of primers and probes for mRNA and DNA detection in Q-PCR and Southern blot assays

Target	Sense	Antisense
IL-2	5'-TGC AAG GGA CTC AGG TGA TG-3'	5'-CTG CTT ATT TAG GAT ACC TAT TAA-3'
IFN-γ	5'-GCT AAA ACA GGG AAG CGA AAA A-3'	5'-GGA CAA CCA TTA CTG GGA TGC T-3'
IL-4	5'-CAC AGG CAC AAG CAG CTG AT-3'	5'-GCC AGG CCC CAG AGG TT-3'
IL-13	5'- GAG TGT GTT TGT CAC CGT TG-3'	5'-TAC TCG TTG GCT GAG AGC TG-3'
IL-10	5'-GTG ATG CCC CAA GCT GAG A-3'	5'-TCC CCC AGG GAG TTC ACA-3'
TGF-β	5'-TCA GAG CCA CAA ATC CTG AAA G-3'	5'-CAC CAA GTG TAC CCC GAA AGA-3'
LMP1	5'-TCC TCC TCT TGG CGC TAC TG -3'	5'-TCA TCA CTG TGT CGT TGT CC-3'
LMP1, probe	5'-GAA CAG CAC AAT TCC AAG GAA CAA -3'	
EBNA1	5'-GAT GAG CGT TTG GGA GAG CTG-3'	5'-TCC TCG TCC ATG GTT ATC AC-3'
EBNA1, probe	5'-AGA CCT GGG AGC AGA TTC AC-3'	
BZLF1	5'-AGG TGC CTT TTG TAC AAG CT-3'	5'-ATA ATG GAG TCA ACA TCC AG-3'
BZLF1, probe	5'-ATA CAA GAA TCG GGT GGC TT-3'	
BALF2	5'-GGA CTT TTT GGG CAA CTT TCT C-3'	5'-TGA CCA GGT CCT TGT AGG TG-3'
BALF2, probe	5'-TGA ACA CGC TCT TCT TTA GGC-3'	
RAZ	5'-AGG CCT AAA AAG GAT GGC TT-3'	5'-ACC AAT GTC TGC TAG CTG TT-3'
RAZ, probe	5'-GGT GCT GCA TAA GCT TGA TA-3'	

primary SCID mice. At day 4 post-cell transfer, human Hodgkin's-derived EBV-associated B-cell lymphoma cells (L54OCY, 1×10^6 cells/chimaera) or nasopharyngeal carcinoma cells (CNE1, 1×10^6 cells/chimera) were s.c. xenogeneically transplanted into the flanks of different EBV-sensitised hu-thym-SCID chimaeras (referred to as humanised xenogeneic-tumour-transplanted human-thym-SCID chimaeras and abbreviated as HXTT H-T-S chimeras) and maintained for further use at the indicated times. In some cases, to detect the therapeutic effects of EBV-induced NKT cells on EBV-associated malignancies, tumour-transplanted SCID mice (at week 3) were i.v. adoptively transferred with EBV-exposed $CD4^+$ or/and $CD8^+$ NKT cells (1×10^5 cells/mouse) plus syngeneic spleen $CD3^+CD56^-$ $CD161^-$ T cells (2×10^6 cells/mouse) and DCs (0.5×10^6 cells/mouse) at day 21 post-transplantation (when the tumours were largely established) and maintained for the indicated times. The mice were housed in a pathogen-free environment in the Animal Research Institute at Wuhan University. The Wuhan University Ethical Committee approved all protocols in this study in accordance with the current Chinese laws.

Flow cytometry

The α-GalCer-loaded CD1d tetramer was used to define total NKT cells. The appropriate isotype Abs and empty CD1d tetramer conjugated with a fluorochrome were used to gate out the NKT cells. For tetramer staining, the cells were incubated with the fluorochrome-labelled tetramer at 37°C for 15 min. For the cellular proliferation study, carboxyfluorescein diacetate succimidyl ester (CFSE) was used to label the cells, and the intensity of the CFSE fluorescence was subsequently analysed (23). All analyses were performed with a flow cytometer (FACSCalibur, BD Biosciences). Four-color analysis was done with CellQuest software.

Real time quantitative RT-PCR

All real time quantitative RT-PCR (Q-PCR) reactions were performed as described elsewhere (24). Briefly, total RNA from purified cells (1×10^4, purity > 99%) or cell lines was prepared with the Quick Prep® total RNA extraction kit (Pharmacia Biotech) according to the manufacturer's instructions. RNA was reverse transcribed using oligo $(dT)_{12\text{-}18}$ and Superscript II reverse transcriptase (Life Technologies, Grand Island, USA). The real time quantitative PCR was performed in special optical tubes in a 96-well microtiter plate (Applied Biosystems, Foster City, CA) with an ABI PRISM® 7700 Sequence Detector System (Applied Biosystems). Using the SYBR® Green PCR Core Reagents Kit, fluorescence signals were determined for each PCR cycle via the 5' to 3' endonuclease activity of AmpliTaq Gold to provide real time quantitative PCR information. The primers and probes used in Q-PCR are listed in Table 1.

Cytokine assays

For intracellular cytokine detection, the protocol from the Cytofix/Cytoperm Plug with Golgiplug Kit (BD PharMingen) was employed. NKT cells were exposed to various stimuli in the presence of Golgiplug and APCs at 37°C for the indicated times. The cells were then intracellularly stained and subjected to flow cytometry. For ELISPOT cytokine detection, NKT cells were exposed to different stimuli in the presence of APCs, and the plates were stained with streptavidin-horseradish peroxidase (Mabtech), diluted to 1:100, and Nova Red substrate according to the manufacturers' instructions. The cytokine production of purified NKT cells was assessed by sandwich ELISA (PeproTech EC) according to the manufacturer's instructions.

Southern blots

For DNA detection in Southern blot analyses, specific TCR

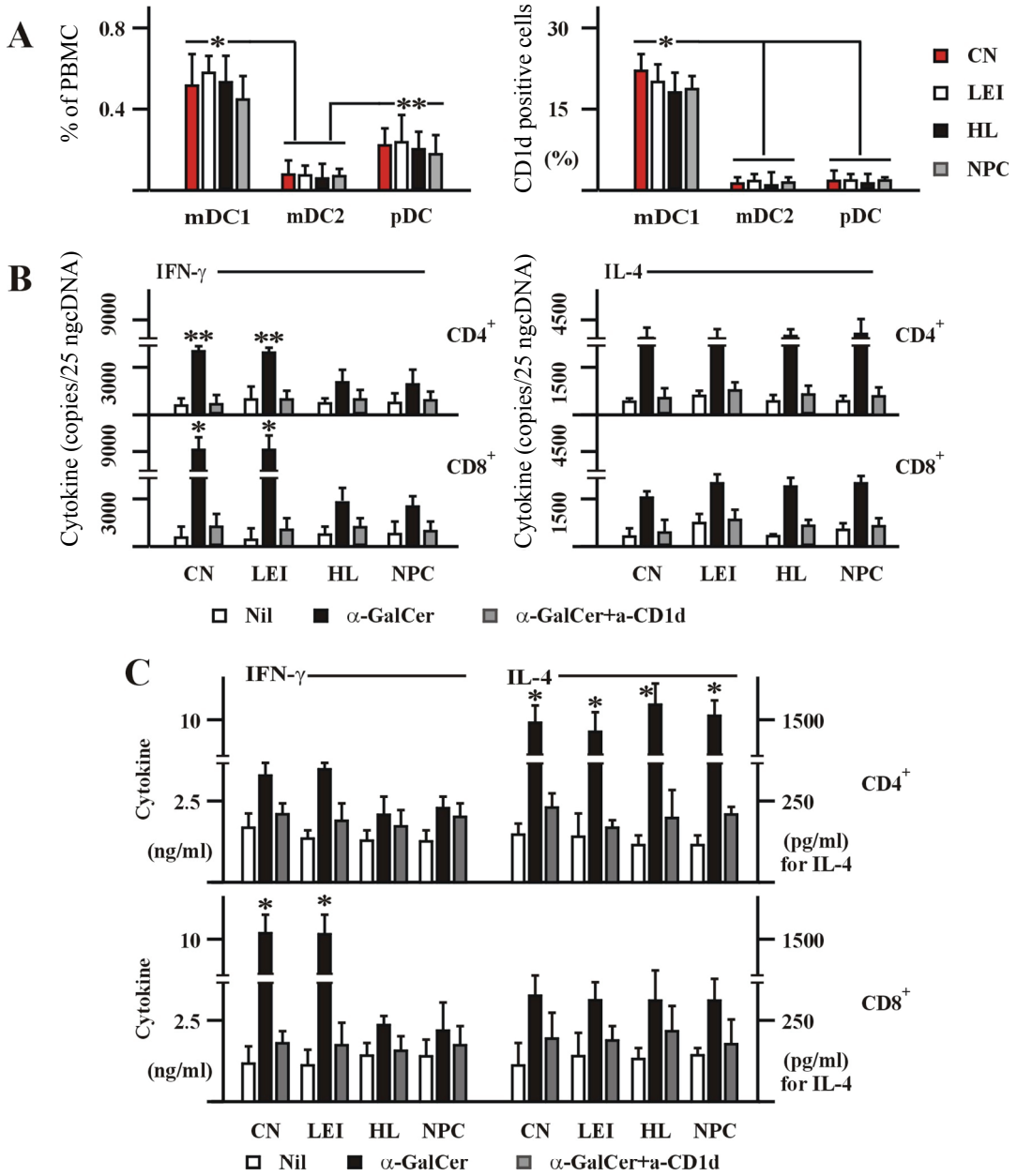

Figure 1. Frequencies and CD1d expressions of circulating DCs from patients with EBV-associated malignancies, and their interactions with NKT cell lines. (A) Frequencies and CD1d expression of circulating DCs. Frequencies (left panel) and CD1d expressions (right panel) of circulating myeloid dendritic cells 1 and 2 (mDC1, mDC2), plasmacytoid dendritic cells (pDC) in PBMC from normal control (CN) and latent EBV-infection subjects (LEI), different patients with HL and NPC. PBMC were stained by three different markers (BDCA-1 for mDC1, BDCA-2 for pDC, and BDCA-3 for mDC2) and CD1d, respectively (25). Data were mean ± SD (n = 6). *$p < 0.01$. **$p < 0.05$. (B) Cytokine expression by $CD4^+$ and $CD8^+$ NKT cell lines. Data showed IFN-γ and IL-4 expressions in $CD4^+$ and $CD8^+$ NKT cell lines. The $CD4^+$ and $CD8^+$ NKT cell lines were generated from PBMC from latent EBV-infection subjects as described previously (25) since their high capacity to produce IFN-γ and IL-4. The different type 1 mDCs were purified from normal control (CN) and latent EBV-infection subjects (LEI), different patients with HL and NPC, co-cultured with $CD4^+$ or $CD8^+$ NKT cell lines in presence of α-GalCer (1 μg/ml) or α-GalCer (1 μg/ml) plus CD1d mAb (5 μg/ml) for 24 h. The culture medium was negative control (Nil). The NKT cell lines were re-purified using MACS bead system based on staining with α-GalCer-loaded CD1d tetramer after co-culture. The expressions of IFN-γ (left panels) and IL-4 (right panels) by $CD4^+$ and $CD8^+$ NKT cell lines were assessed by Q-PCR. Data were mean ± SD (n = 6). *$p < 0.001$; **$p < 0.01$. (C) IFN-γ and IL-4 expressions in $CD4^+$ (upper panel) and $CD8^+$ (lower panel) NKT cell lines derived from the PBMC from different patients. The cell co-culture system was set up as described above, and stimulated in presence of α-GalCer (1 μg/ml) or or α-GalCer (1 μg/ml) plus CD1d mAb (5 μg/ml) for 48 h, The culture supernatants were assessed for cytokine expression by ELISA after 48 hour-stimulatons. Data were mean ± SD (n = 6). *$p < 0.001$. CN and LEI *vs.* HL and NPC; $CD4^+$ *vs.* $CD8^+$ NKT cells.

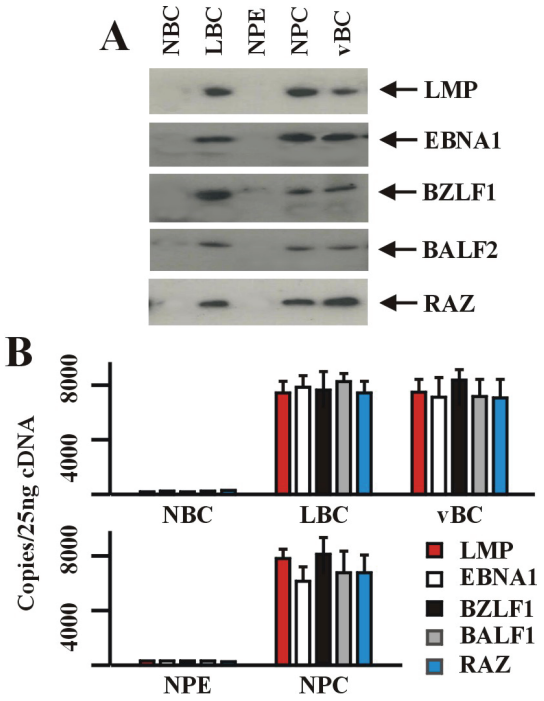

Figure 2. EBV-infection in human EBV-associated tumor cell lines. Southern blot (A) and Q-PCR (B) analyses for detections of diverse EBV genomic DNA and mRNA transcripts, respectively. The used primers and probes were shown in Table 1. The applied methods were detailed in the reference (32). Human fetal peripheral CD19$^+$ B cells (NBC), human Hodgkin-derived EBV-associated B-cell lymphoma cell line (L54OCY, LBC), human naso-pharyngeal epithelial cell line (NP69, NPE), human nasopharyngeal carcinoma cell line (CNE1, NPC) were examined. The EBV$^+$ Raji B cell line was as positive control (vBC). Data were representatives or mean ± SD (n = 5).

DNA probes, labelled with α-[^{32}P]dCTP, were generated by PCR amplification of the sequence listed in Table 1. DNA (5 μg) was electrophoresed under denaturing conditions, followed by transfer onto a nylon membrane. The membrane was then hybridised overnight with 1 × 10^6 cpm/ml of ^{32}P-labelled probe, followed by autoradiography.

Statistical analysis

Statistical analyses were performed using paired or unpaired Student's *t* test. A *p* value < 0.05 was considered statistically significant.

Results

Circulating mDC1 cells from patients with EBV-associated malignancies impaired IFN-γ production of CD8$^+$ NKT cells

In our previous study, we found that the frequencies of total and CD8$^+$ NKT cells in PBMCs from LEI and CN subjects were significantly higher than those from HL and NPC patients. Cytotoxic assays demonstrated that CD8$^+$ NKT cells from LEI subjects strongly targeted α-GalCer-loaded HL and NPC cell lines, resulting in a high level of cell death, while CD4$^+$ NKT cells from various subjects either lacked or showed very weak cytotoxicity to both α-GalCer-loaded tumour cell lines and non-tumour cells (15). To understand the relationship between NKT cells and APCs, we examined circulating DCs from patients with EBV-associated malignancies and healthy subjects. The frequencies of mDC1 cells were comparable among HL and NPC patients and LEI and CN subjects. Furthermore, we determined the expression profile of CD1d and found that CD1d was predominantly expressed on mDC1 cells compared with mDC2 cells and pDC cells. The expression levels of CD1d on mDC1 cells were comparable among HL and NPC patients and LEI and CN subjects (Figure 1A). Due to the high capacity of NKT cells to produce IFN-γ and IL-4, these cytokines were detected in both CD4$^+$ and CD8$^+$ NKT cell lines from PBMCs derived from various EBV-infection subjects. Coexisting with circulating DCs, CD4$^+$ and CD8$^+$ NKT cells from LEI and CN subjects highly expressed IFN-γ upon pre-stimulation with α-GalCer, while IL-4 levels were approximately equal in the various subjects (Figure 1B). The levels of cytokines produced by purified NKT cells were confirmed by means of an ELISA assay. After stimulation with α-GalCer alone, expression levels of IFN-γ were significantly higher in CD8$^+$ (lower panel) NKT cell lines derived from the PBMCs from CN and LEI subjects than patients, while the IL-4 levels were generally high in CD4$^+$ (upper panel) NKT cells derived from the PBMCs from both patients with HL and NPC and CN and LEI subjects (Figure 1C). Circulating mDC1 cells from HL or NPC patients impaired IFN-γ production by CD8$^+$ NKT cell lines, but did not affect IL-4 production (Figure 1B), in agreement with a previous observation (25).

EBV-induced CD8$^+$ NKT cells promote a Th1-bias response that suppresses EBV-associated tumour cells in vitro

We previously found that the frequencies of total and CD8$^+$ NKT cells in PBMCs from HL and NPC patients (< 3%) were significantly lower than those from LEI and CN subjects (15-30%). By contrast, the frequency of CD4$^+$ NKT cells was comparable among various patients and subjects, as was the frequency of CD4$^-$CD8$^-$ (DN) NKT cells, although this frequency was low in all groups (15). CD4$^+$ NKT cells in PBMCs of HL and NPC patients and LEI and CN controls produced similar low levels of IFN-γ and high levels of IL-4, IL-13, IL-10 and TGF-β1. CD8$^+$ NKT cells from LEI and CN subjects expressed very high levels of IFN-γ and IL-2, but low levels of IL-4, IL-13, IL-10, and TGF-β1. CD8$^+$ NKT cells from HL and NPC patients produced much less IFN-γ than cells from the control subjects. CD8$^+$ NKT cells from LEI and CN subjects efficiently killed α-GalCer-loaded EBV-associated HL and NPC cell lines, but not non-tumour cells, while CD8$^+$ NKT cells from HL and NPC patients displayed only moderate cytotoxicity against these α-GalCer-loaded EBV-associated cell lines. CD8$^+$ NKT cells

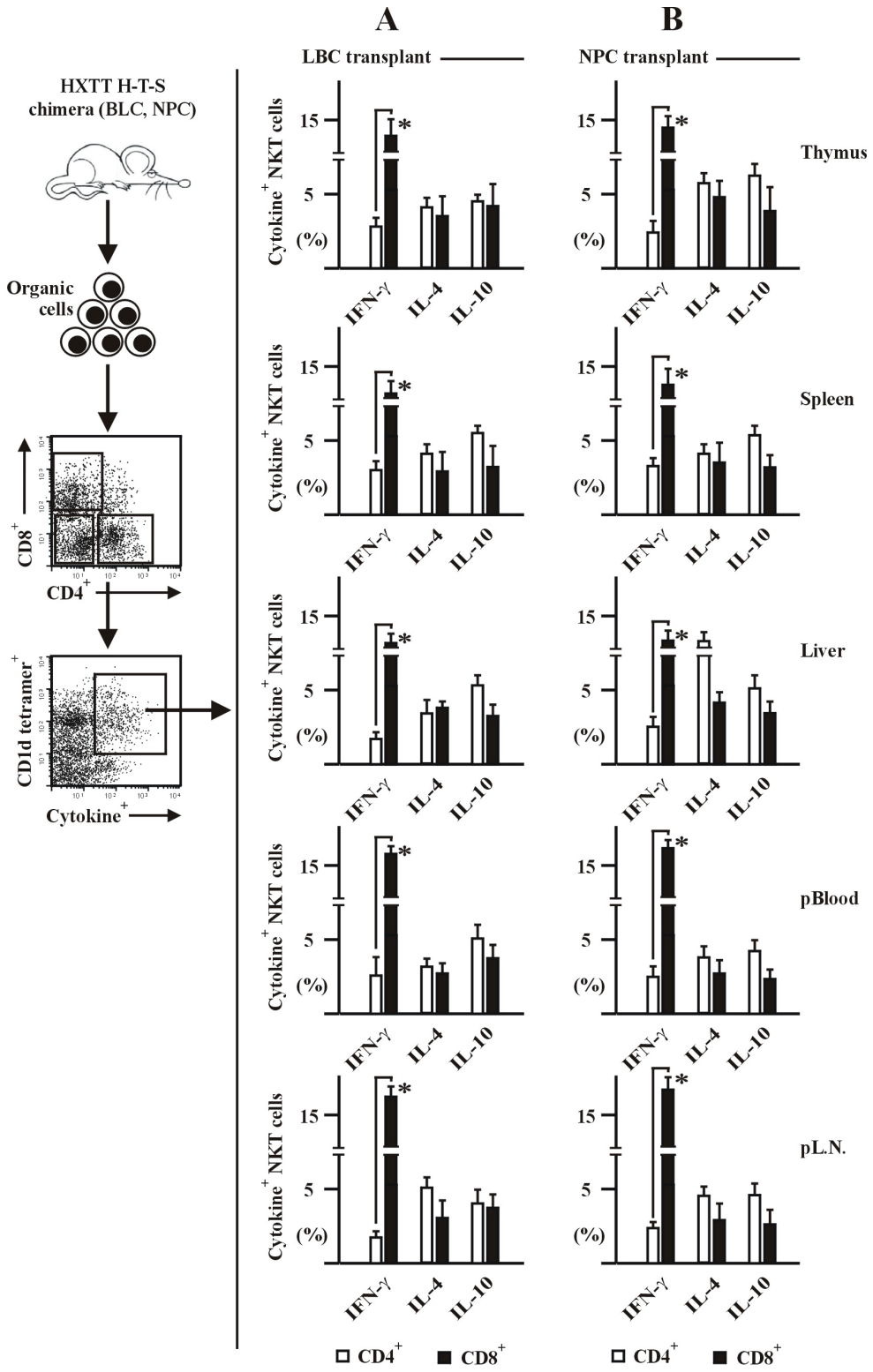

Figure 3. Cytokine expression by CD4$^+$ and CD8$^+$ NKT cells *in vivo* in HXTT H-T-S chimeras. The HXTT H-T-S chimeras were implanted with LBC (A) or NPC (B) as described in Methods. The NKT cells from the unchallenged hu-thym-SCID chimera were used as a control (data not shown). The animals were euthanized at week 12 post-tumor-implantation. The various organs as indicated were collected and single cell suspensions were prepared. pBlood, peripheral blood. The cells were *in vitro* stimulated with α-GalCer (1 μg/ml) for 12h. The indicated cytokines in NKT cells were examined by tetra-color (cytokine Ab, CD4 Ab, CD8 Ab, CD1d tetramer) intracellular flow cytometry. An experimental and analysis schemes were illustrated in the leftmost panel. Data were mean ± SD (n = 5). * $p < 0.001$.

from LEI subjects strongly targeted α-GalCer-loaded HL and NPC cell lines, resulting in a high level of cell death. $CD4^+$ NKT cells from various patients and subjects lacked or showed very weak cytotoxicity to both α-GalCer-loaded tumour cell lines and non-tumour cells (15).

In our previous study, we also found that EBV-induced human NKT cells rapidly responded to EBV-associated tumour cells through the secretion of cytotoxic cytokines, such as IFN-γ and IL-10 (15). In order to verify the specificity of the cytotoxic killing of the target cells, we first demonstrated the existence of EBV in different cell lines. Five transformation-associated EBV-genes including LMP1, EBNA1, BZLFl, BALF2 and RAZ were examined in human EBV-associated tumour cell lines and non-tumour cell lines. Using Southern blot and Q-PCR analysis, these viral genes and their mRNA transcripts were detected in EBV-associated tumour cell lines (L54OCY and CNE1), but not in non-tumour cells (fetal $CD19^+$ B cells and a nasopharyngeal epithelial cell line NP69) (Figure 2).

To elucidate the mechanism by which NKT cells inhibit EBV-associated malignancies *in vivo*, we established hu-thym-SCID chimaeras (22). Our cytotoxicity assay demonstrated that $CD8^+$ NKT cells from various organs of EBV-exposed hu-thym-SCID chimaeras displayed a strong proliferative capacity and cytotoxicity against α-GalCer-primed EBV-associated tumour cell lines *in vitro*. Transferred thymic $CD8^+$ NKT cells (plus spleen syngeneic T cells and DCs, referred to as +T+DC) from EBV-exposed hu-thym-SCID chimaeras efficiently functioned as protective effectors against human EBV-associated HL and NPC (15). We next examined the cytokine production of NKT cells *ex vivo* in HXTT H-T-S chimaeras transferred with EBV-induced $CD4^+$ and $CD8^+$ NKT cells (Figure 3). A high frequency of $CD8^+$ NKT cells, purified from various organs from the tumour-bearing HXTT H-T-S chimaeras (at week 12), expressed IFN-γ and IL-2, but few cells expressed IL-4, IL-13, IL-10 and TGF-β1 (Figures 3A and 3B; some data not shown). Particularly, a very high frequency of $CD8^+$ NKT cells, purified from the peripheral blood and lymph nodes, produced IFN-γ (Figures 3A and 3B). $CD4^+$ NKT cells from the various organs expressed moderate levels of IL-4, IL-13, IL-10 and TGF-β1 and low levels of IFN-γ (Figures 3A and 3B; some data not shown). The differential cytokine production patterns of $CD4^+$ and $CD8^+$ NKT cells were confirmed by Q-PCR (data not shown).

EBV-induced $CD4^+$ NKT cells synergised with $CD8^+$ NKT cells to promote a Th1-bias response against EBV-associated tumours

In our previous study, we established several virus-exposed hu-thym-SCID chimaeras and demonstrated for the first time that EBV promotes the generation of IFN-γ-biased $CD8^+$ NKT cells in human-thymus-SCID chimaeras. Thymic $CD8^+$ NKT cells from EBV-exposed hu-thym-SCID chimaeras promoted Th1 immunity, but $CD4^+$ NKT cells displayed a Th2-bias response (15). The synergistic effect of EBV-exposed thymic $CD4^+$ and $CD8^+$ NKT cells significantly suppressed EBV-associated malignancies and prolonging animal survival (15). In a semi-permeable transwell membrane co-culture system, which prevented direct cell-cell contact between syngeneic spleen $CD3^+CD56^-CD161^-$ T cells and NKT cells (24), the NKT cells lost their ability to direct biased cytokine secretion by syngeneic T cells (data not shown), strongly suggesting that this NKT cell activity is cell-cell contact dependent.

To further investigate the mechanism of the synergistic effect between EBV-exposed $CD4^+$ and $CD8^+$ NKT cells in suppressing EBV-associated malignancies, we established EBV-sensitised chimaeras (non-tumour-bearing model) by transfer with EBV-exposed $CD4^+$ or/and $CD8^+$ NKT cells plus syngeneic $CD3^+CD56^-CD161^-$ T cells, and re-challenged them with EBV. Both $CD4^+$ and $CD8^+$ NKT cells showed comparable levels of proliferation in various chimaeras in response to the EBV re-challenge (Figure 4A). $CD4^+$ NKT cells mainly produced IL-4, and a high frequency of $CD8^+$ NKT cells expressed IFN-γ in response to EBV re-challenge (Figure 4B). In the chimaeras transferred with EBV-exposed $CD4^+$ NKT cells alone, the proliferation of $CD3^+$ T cells was inhibited following the EBV re-challenge, compared with that for chimaeras transferred with EBV-exposed $CD8^+$ NKT cells alone (Figure 4C). The $CD3^+$ T cells predominantly produced IL-4 in the chimaeras transferred with EBV-exposed $CD4^+$ NKT cells alone, while the T cells expressed a high level of IFN-γ in the chimaeras transferred with EBV-exposed $CD8^+$ NKT cells alone (Figure 4D). Vigorous proliferation of $CD3^+$ T cells and a high frequency of IFN-γ producing cells were observed in the chimaeras transferred with EBV-exposed $CD4^+$ and $CD8^+$ NKT cells together, in response to EBV re-challenge (Figure 4C, 4D).

EBV-induced $CD4^+$ NKT cells promote persistence of $CD8^+$ NKT cells in response to EBV-associated malignancies

To better understand the synergistic anti-tumour effect of $CD4^+$ NKT cells, we analysed the persistence of $CD4^+$ and $CD8^+$ NKT cells and the cytokine production profile in LBC- or NPC-bearing HXTT H-T-S chimaeras. Frequencies of total NKT cells varied from 0.03-0.05% in livers and 2-4% in peripheral blood from both of the chimaeras transplanted with $CD4^+$ or/and $CD8^+$ NKT cells (+T+DC) directly following establishment, respectively (Figure 5A, some data not shown). Frequencies of hepatic and blood total NKT cells dropped sharply (< 0.008% or < 0.03%) in HXTT H-T-S chimaeras transplanted with $CD4^+$ NKT cells (+T+DC) at week 3 post-establishment. In HXTT H-T-S chimaeras transplanted with $CD8^+$ NKT cells (+T+DC), the frequencies of total NKT cells persisted longer and then decreased (~0.02% in livers at week 12 and ~0.04% in blood at week 15) (Figure 3A, some data not shown). The frequencies of hepatic and blood total NKT cells were rather stable in HXTT H-T-S chimaeras transplanted with $CD4^+$ and $CD8^+$ NKT cells (+T+DC) within 15 weeks (Figure 5A, some data not shown). The persistence patterns of hepatic and blood $CD4^+$ and $CD8^+$ NKT cells were similar to those of total NKT cells in LBC- or NPC-bearing HXTT H-T-S chimaeras

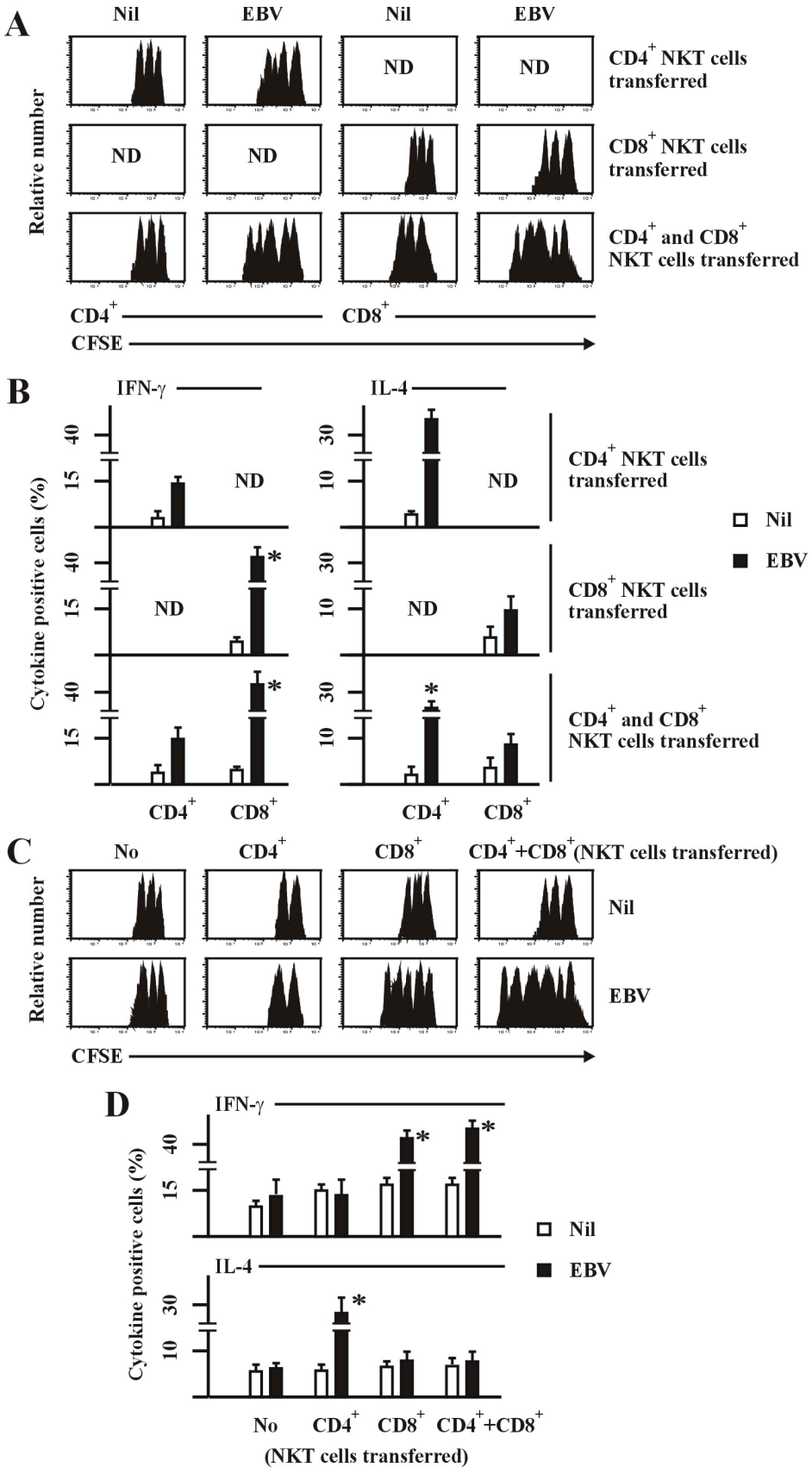

Figure 4. Proliferation and cytokine expression by $CD4^+$ and $CD8^+$ NKT cells, and T cells from EBV-sensitized chimeras *in vivo* in response to EBV re-challenge. The SCID mice were adoptively transferred *i.v.* with the different combinations of the immune cells purified from EBV-exposed hu-thym-SCID chimeras ($CD4^+$, $CD8^+$ NKT cells, $CD4^+$ + $CD8^+$ NKT cells transferred) as described in Methods. On day 3 post-reconstitution, EBV-sensitized chimeras were re-challenged *i.v.* with EBV (10^7 pfu) and maintained for further 4 days, or left unchallenged (Nil). (A) Proliferation of chimeric $CD4^+$ and $CD8^+$ NKT cells. In this experiment, the purified thymic $CD4^+$ or $CD8^+$ NKT cells from EBV-exposed chimeras were labelled with CFSE prior to the transfer into the SCID mice. After EBV re-challenge, EBV-sensitized chimeras were euthanized. The PBMC were stained with appropriate tetramer/Abs, and analyzed by flow cytometry. ND, no determination. Data were representative (n = 6). (B) IFN-γ and IL-4 expression by $CD4^+$ and $CD8^+$ NKT cells. As described above, EBV-sensitized chimeras were euthanized after EBV re-challenge. The indicated cytokines in NKT cells were examined by tetra-color (cytokine Ab, CD4 Ab, CD8 Ab, CD1d tetramer) intracellular flow cytometry. The experimental and analysis schemes were illustrated in the leftmost panel of Figure 3 (without tumor-implantation). ND, no determination. Data were mean ± SD (n = 5). *p < 0.001. (C) Proliferation of EBV-specific $CD3^+CD56^-CD161^-$ T cells. The EBV-sensitized chimeras were established as described above. The purified spleen $CD3^+CD56^-CD161^-$ T cells were labelled with CFSE prior to transfer into the SCID mice. The EBV-sensitized chimeras were euthanized after EBV re-challenges, and PBMC were stained with appropriate tetramer/Abs, then analyzed by flow cytometry for cell proliferation. Data were mean ± SD (n = 5). *p < 0.001. (D) IFN-γ and IL-4 expression by $CD3^+$ T cells. Analysis was performed as described above. The indicated cytokines in $CD3^+$ T cells were examined by intracellular flow cytometry. Data were mean ± SD (n = 5). *p < 0.001.

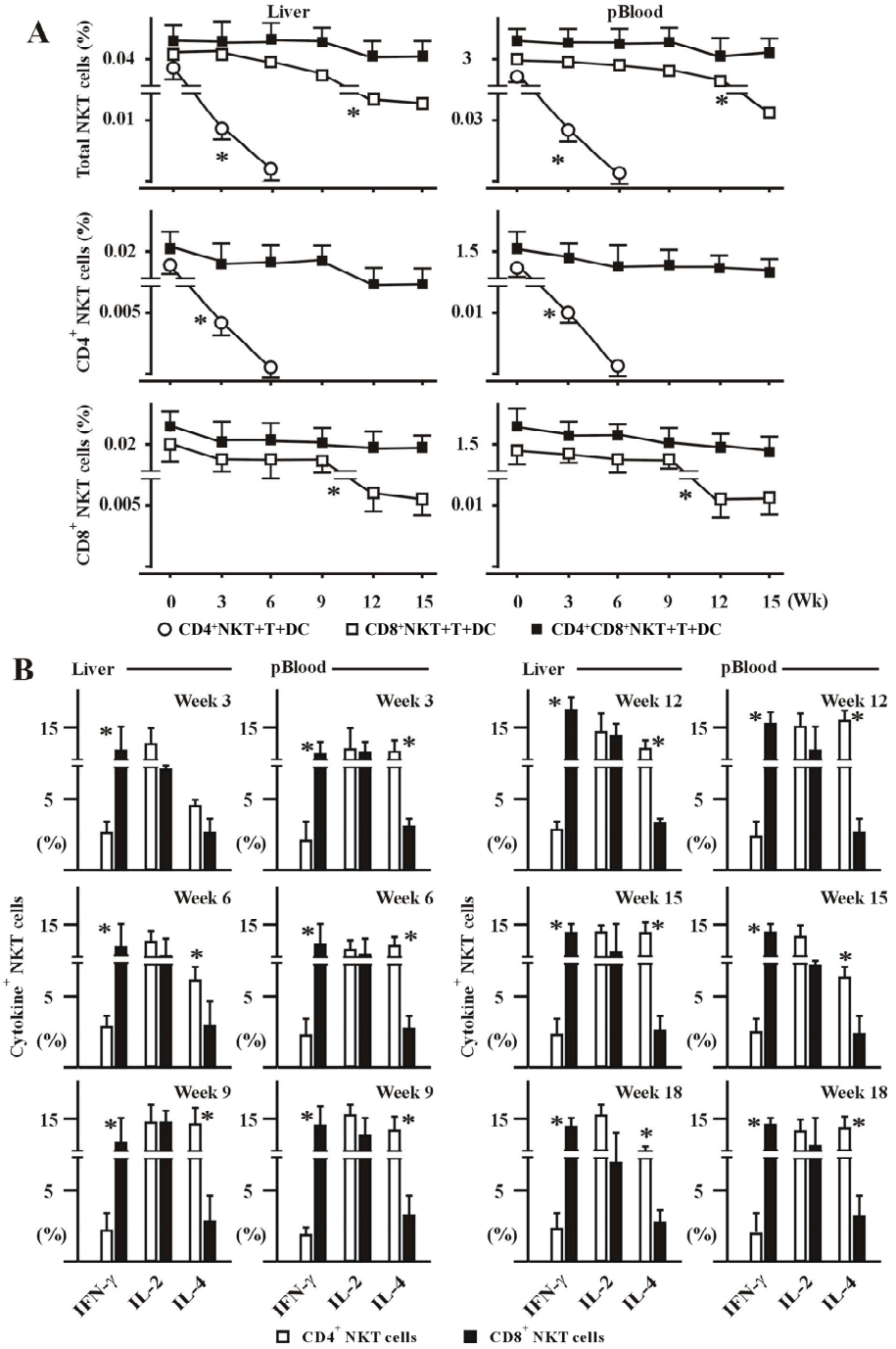

Figure 5. Frequencies and cytokine expression by $CD4^+$ and $CD8^+$ NKT cells in LBC-bearing HXTT H-T-S chimeras. The SCID mice were adoptively transferred *i.v.* with the different combinations of immune cells purified from EBV-exposed hu-thym-SCID chimeras as described in Methods (CD4 or/and CD8 NKT+T+DC). The HXTT H-T-S chimeras were then implanted with LBC cells. The animals were euthanized at the indicated time points, and, livers and peripheral blood (pBlood) were collected, and single cell suspensions were prepared. (A) Frequencies of total, $CD4^+$ and $CD8^+$ NKT cells. Data illustrate the frequencies of total, $CD4^+$ and $CD8^+$ NKT cells in the livers and peripheral blood from the LBC-bearing HXTT H-T-S hu-thym-SCID chimeras, as assessed by flow cytometry. The experimental and analysis schemes were illustrated in the leftmost panel of Figure 3A. UD, under detectable level. ND, no determination. Data were mean ± SD (n = 3). *$p < 0.001$. $CD4^+$ or $CD8^+$ NKT cell-transferred chimeras *vs.* $CD4^+$ + $CD8^+$ NKT cell-transferred chimeras. (B) Cytokine expression by $CD4^+$ and $CD8^+$ NKT cells *in vivo* in LBC-bearing HXTT H-T-S chimeras transferred with EBV-exposed $CD4^+$ and $CD8^+$ NKT cells (+T+DC). The cells were *in vitro* stimulated with α-GalCer (1 μg/ml) for 12h. Data showing expression of the various cytokines in NKT cells were determined by tetra-color (cytokine Ab, CD4 Ab, CD8 Ab, CD1d tetramer) intracellular flow cytometry. The experimental and analysis schemes were illustrated in the leftmost panel of Figure 3. Data were mean ± SD (n = 3). *, $p < 0.001$. $CD4^+$ *vs.* $CD8^+$ NKT cells.

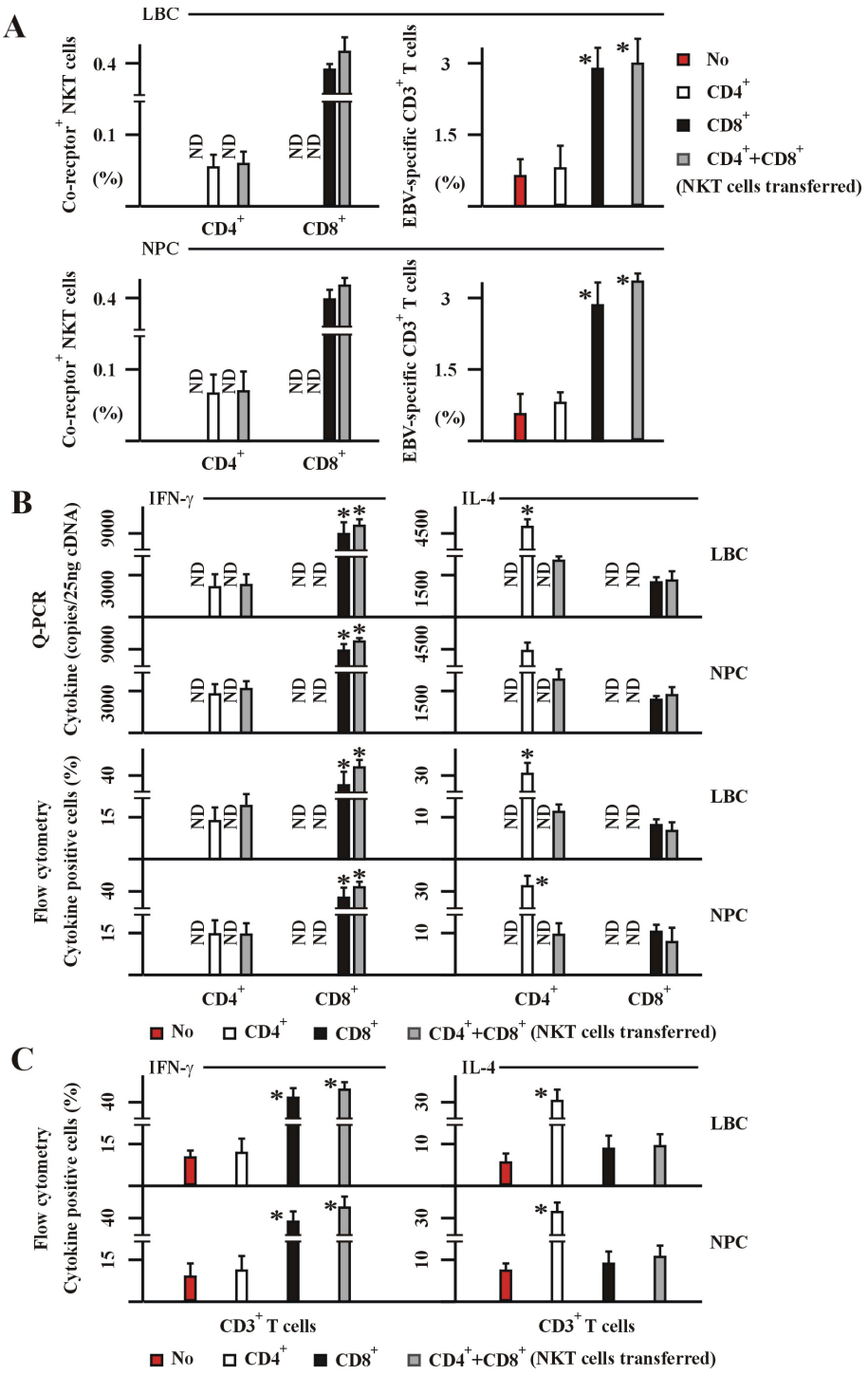

Figure 6. Frequency and cytokine expression by infiltrating chimeric CD4$^+$ and CD8$^+$ NKT cells and EBV-induced CD3$^+$ T cells into tumor tissues. The EBV-sensitized chimeras were established by adoptive transfer of the different combinations of the immune cells from EBV-exposed hu-thym-SCID chimeras as described in Methods (CD4$^+$, CD8$^+$ NKT cells, CD4$^+$+CD8$^+$ NKT cells transferred), followed by LBC or NPC tumor cell transplantation (HXTT H-T-S chimeras). The cell transfer of immune cells without NKT cells was as control (No). The HXTT H-T-S chimeras were eradicated at day 21 post-transplantation. The visualized tumors were dissected. The tumor tissues were finely minced, and digested. The single cell suspensions from different tumor tissues were obtained. (A) Frequencies of chimeric CD4$^+$ and CD8$^+$ NKT cells, and CD3$^+$ T cells. Data were assessed by flow cytometry. ND, no determination. Data were mean ± SD (n = 5). *, $p < 0.001$. (B and C) Cytokine expression by chimeric CD4$^+$ and CD8$^+$ NKT cells, and CD3$^+$ T cells. IFN-γ and IL-4 expression by CD4$^+$ and CD8$^+$ NKT cells (B), and in CD3$^+$ T cells (C) were assessed by Q-PCR and flow cytometry. ND, no determination. Data were mean ± SD (n = 5). *, $p < 0.001$.

transplanted with $CD4^+$ or/and $CD8^+$ NKT cells (+T+DC) (Figure 3A; some data not shown). The persistence patterns of spleen and blood T cells were similar to those of NKT cells in both of the tumour-bearing chimaeras transplanted with $CD4^+$ or/and $CD8^+$ NKT cells (+T+DC) (data not shown). A very high frequency of hepatic and blood $CD8^+$ NKT cells expressed IFN-γ and IL-2, but few cells expressed IL-4, IL-13, IL-10, and TGF-β1 in both chimaeras co-transferred with $CD4^+$ and $CD8^+$ NKT cells (+T+DC) within 18 weeks, as assessed by intracellular flow cytometry (Figure 5B; some data not shown). Hepatic or blood $CD4^+$ NKT cells in the LBC- or NPC-bearing HXTT H-T-S chimaera expressed very high frequencies of IL-2 and IL-4, moderate frequencies of IL-13, IL-10 and TGF-β1, and low frequencies of IFN-γ (Figure 5B; some data not shown). The cytokine production patterns of $CD4^+$ and $CD8^+$ NKT cells were confirmed by Q-PCR (data not shown). Data indicated that the persistence of $CD8^+$ NKT cells and T cells was comparatively reduced in the chimaeras that did not receive $CD4^+$ NKT cells. Adoptively transferred $CD4^+$ NKT cells may sustain greater persistence of $CD8^+$ NKT cells and T cells (Figure 5A; some data not shown) through their secretion of a high level of IL-2 by $CD4^+$ NKT cells (Figure 5B), thereby leading to a more pronounced anti-tumour effect.

EBV-induced $CD8^+$ NKT cells and syngeneic $CD3^+$ T cells suppressed EBV-associated malignancies upon Th1-bias response

To understand the mechanism of the anti-tumour effect of EBV-exposed NKT cells, we examined the frequency and cytokine expression profiles of infiltrating chimaeric $CD4^+$ NKT cells, $CD8^+$ NKT cells, and $CD3^+$ T cells in solid tumour tissues in different HL- and NPC-transplanted HXTT H-T-S chimaeras (Figure 6). A few $CD4^+$ NKT cells and $CD3^+$ T cells infiltrated into the solid HL and NPC tumours in the chimaeras transferred with EBV-exposed $CD4^+$ NKT cells alone (Figure 6A). The tumour-infiltrating $CD4^+$ NKT cells and $CD3^+$ T cells mainly produced IL-2 and IL-4 (Figures 6B and 6C; some data not shown). In contrast, a large number of $CD8^+$ NKT cells and $CD3^+$ T cells infiltrated into the HL and NPC tumour tissues in the HXTT H-T-S chimaeras transferred with EBV-exposed $CD8^+$ NKT cells alone (Figure 6A). The tumour-infiltrating $CD8^+$ NKT cells and $CD3^+$ T cells mainly produced IFN-γ (Figures 6B and 6C). Interestingly, the highest numbers of $CD8^+$ NKT cells and $CD3^+$ T cells infiltrated into the HXTT H-T-S chimaeras transferred with EBV-exposed $CD4^+$ and $CD8^+$ NKT cells together (Figure 6A). The tumour-infiltrating $CD8^+$ NKT cells and $CD3^+$ T cells produced the most IFN-γ (Figures 6B and 6C).

Thus, EBV-induced $CD8^+$ NKT cells were necessary and sufficient to enhance T cell immunity against EBV-associated malignancies. Combined with $CD4^+$ NKT cells, EBV-induced $CD8^+$ NKT cells efficiently promote a Th1-bias response against EBV-associated malignancies in LBC- or NPC-bearing HXTT H-T-S chimaeras. Thus, $CD4^+$ NKT cells were synergistic, but not required, for $CD8^+$ NKT cells to enhance T cell immunity.

Discussion

Evidence strongly suggests that NKT cells are reactive to a self-antigen bound to CD1d expressed on APCs, including DCs. CD1d molecules on the surface of DCs play a critical role in the presentation of glycolipid antigens to NKT cells. Many of the diverse effects of activated NKT cells may be mediated by their communication with $CD1d^+$ DCs. Activation of NKT might result from CD1d-mediated presentation of endogenous ligands by DCs in the case of cellular stress. On the contrary, activated NKT cells can induce DCs to release IL-12 through the interaction of CD40L expressed on NKT cells and CD40 expressed on DCs (26).

An effective cancer immunosurveillance process associated with $CD8^+$ NKT cells exists in subjects with latent EBV infection, but without pathogenesis, due to different frequencies of $CD8^+$ NKT cells in different subjects. Various cytokines and cell populations are also involved in the cancer immunosurveillance process. IFN-γ-deficient mice exhibited an increased incidence of carcinoma, suggesting that IFN-γ-dependent immune responses might be effective in the promotion phase of carcinogenesis (27). IFN-γ and IL-4 are typical Th1 and Th2 related cytokines, respectively. These two cytokines can be used to evaluate Th1/Th2 differentiation. Previous studies showed that activated NKT cells regulate immune responses, including tumour rejection, by producing IFN-γ and IL-4 (28). NKT activation after α-GalCer treatment can alter the balance of secreted cytokines from a Th2-bias to a Th1-bias (29). Similarly, our data demonstrated that EBV-induced $CD8^+$ NKT cells promoted a Th1-bias response to suppress EBV-associated tumour cells *in vivo*.

IFN-γ is an essential cytokine in the viral cell-mediated immune response. IFN-γ production is a sensitive indicator of target cell recognition by responsive lymphocytes that are reactive to virus-associated antigens. Antitumor responses and responses to infectious agents depend on IFN-γ secreted by NKT cells, but NKT cells are not the sole, or even the major, source of responsive cytokines. Therefore, activation of NKT cells results in a responsive activation cascade. The synergistic antitumor effect of EBV-exposed $CD4^+$ and $CD8^+$ NKT cells is quite impressive in this study because the combined transfer of EBV-induced $CD4^+$ and $CD8^+$ NKT cells leads to a significant proliferation of $CD4^+$ and $CD8^+$ NKT cells and higher production of IFN-γ. We have observed that EBV-induced $CD8^+$ NKT cells are necessary and sufficient to enhance T cell immunity to suppress EBV-associated malignancies in human-thymus-SCID chimaeras (15). The necessary outcome is an increased Th1-bias response to EBV-associated malignancies when EBV-exposed $CD4^+$ NKT cells cooperated with $CD8^+$ NKT cells in LBC- or NPC-bearing HXTT H-T-S chimaeras. The EBV-exposed $CD4^+$ NKT cells that are centrally important for persistence of $CD8^+$ NKT *in vivo* exert a synergistic effect to

suppress EBV-induced malignancies.

Activation of NKT cells also affects nearly every hematopoietic cell type, including DCs, NK cells, B lymphocytes, and T lymphocytes. As reported, NK cells are stimulated to secrete IFN-γ within a few hours after α-GalCer stimulation (30). The intensity of the immune response of a particular glycolipid may depend on how much it stimulates communication between NKT cells and other cell types. Therefore, EBV exposure is a major contributor to Th1- biased immunity in this study. However, the identity of these viral antigens and the mechanism in which they affect the pattern of cytokines and immune responses *in vivo* must be further investigated. In addition, the integration of weak TCR signals and IL-12R-mediated signals may lead to a Th1-biased cytokine production by NKT cells (18). The cytokine profile of activated NKT cells can be influenced by the generation of signals from various types of receptors. Other cellular signals may contribute to Th1-biased cytokine profiles produced by EBV-induced $CD8^+$ NKT cells alone or together with synergistic EBV-induced $CD4^+$ NKT cells. Adoptive transfer of donor-derived EBV-induced CTLs has been successful in the prevention and treatment of EBV-associated post-transplant lymphoproliferative disorders (PTLDs) in the context of allogeneic stem cell transplantation (31). Therefore, the adoptive transfer of EBV-induced $CD8^+$ NKT cells alone or combined with syngeneic EBV-induced $CD4^+$ NKT cells is a promising immunotherapy for the suppression of EBV-associated malignancies.

Acknowledgements

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