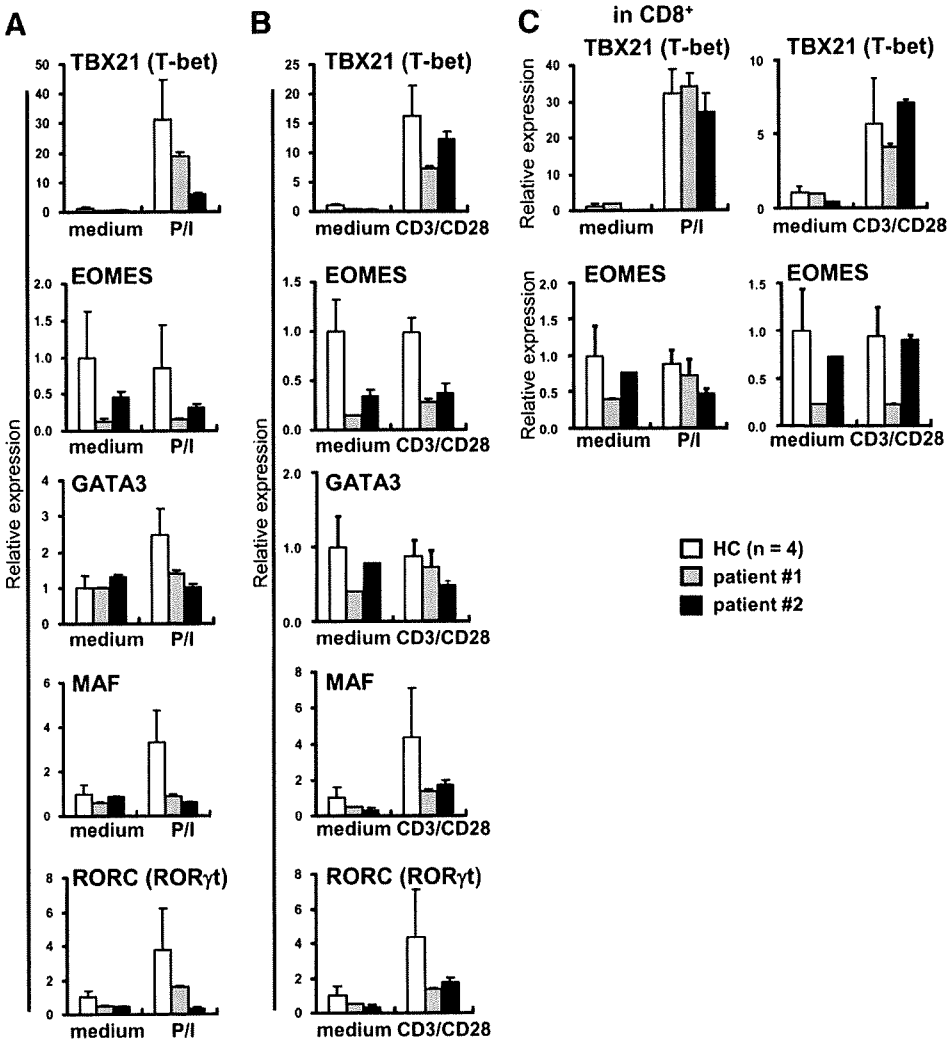

FIGURE 7. Impaired expression of effector-specific transcription factors for T cell differentiation. Purified CD4 T cells were activated by PMA/ionomycin or incubated in the medium for 4 h (*A*), or were incubated in the presence or absence of anti-CD3/anti-CD28 costimulation for 24 h (*B*). Expression levels of TBX21 (T-bet), GATA-3, MAF, RORC (ROR-γt), and EOMES mRNAs were determined in duplicate by real-time quantitative PCR. Similarly, purified CD8 T cells were activated by PMA/ionomycin or by anti-CD3/anti-CD28; and the expression levels of TBX21 and EOMES were assessed in healthy controls (HC). Patients 1 and 2 (*C*). Levels of each mRNA were expressed as relative expression compared with expression of 18S rRNA, and the mean mRNA expression level in cells from HC cultured in the medium was adjusted to 1.0. □, HC; ▨, patient 1; ■, patient 2. Error bars indicate the SD for HC (n = 5). The assay was performed three times for the patients; average and SEM are shown. P/I: PMA/ionomycin.

Despite poor IFN-γ production by CD8 T cells, we did not observe low T-bet induction in purified CD8 T cells when stimulated by PMA/ionomycin or by anti-CD3/anti-CD28 mAb (Fig. 7*C*). We then tested the expression of EOMES, a paralog of T-bet and a transcription factor required for CD8 effector function (50, 51). Although induction was negligible, there was a trend toward lower baseline expression of EOMES (0.23 and 0.69 for patients 1 and 2, respectively).

Expression of the E3 ubiquitin ligases that contribute to T cell anergy

Several lines of evidence indicate that E3 ubiquitin ligases (Grail, Cbl-b, and Itch) play important roles in the induction and maintenance of T cell tolerance (52–55). T cell stimulation without costimulation leads to up-regulation of these ligases (56). High expression of these E3 ubiquitin ligases is related to the absence of the expression of the effector-specific transcription factors (56, 57). There has been little research on the ligases in human systems. Because anti-CD3 stimulation did not induce appreciable up-regulation of the ligases, we examined the mRNA level of these molecules at baseline and after PMA/ionophore stimulation using a sensitive real-time PCR assay.

As shown in Fig. 8, baseline expression of the E3 ubiquitin ligases, with the exception of Grail, was detected in the controls and patients. The mRNA levels of Cbl-b and AIP4/Itch in the steady state were significantly elevated in patient 2 (2.5 and 4.3) compared with controls (1.0 ± 0.4 and 1.0 ± 0.6, n = 4) and patient 1 (1.0 and 1.3).

PMA/ionophore induced up-regulation of Cbl-b and Itch, but not Grail, in normal subjects, whereas the induction of Cbl-b and Itch mRNA above the baseline level was negligible in patient 2 (Fig. 8).

Of particular note was a paradoxical down-regulation of Itch, a regulator of NF-κB activation, upon PMA/ionophore stimulation, which was reproducibly observed only in patient 1.

RANKL induction was augmented in patient 2 with autoimmunity

Patient 1 had an autoimmune manifestation and immunodeficiency, whereas patient 2 had mild psoriasis-like cutaneous lesions and mild skin infections. We therefore attempted to uncover differences in T cell functions between two patients.

To explore the dissimilarities in their immune functions, we assessed mRNA expression levels in negatively selected, >97% pure CD4 T cells by comprehensive mRNA expression analysis using a GeneChip before and after stimulation through CD3/CD28.

The expression of most mRNAs from CD4 T cells poststimulation showed a remarkably high correlation between patients 1 and 2. However, we identified >100 genes that were differently expressed between the patients, and sought to identify the gene(s) that may explain the phenotypic difference from these genes.

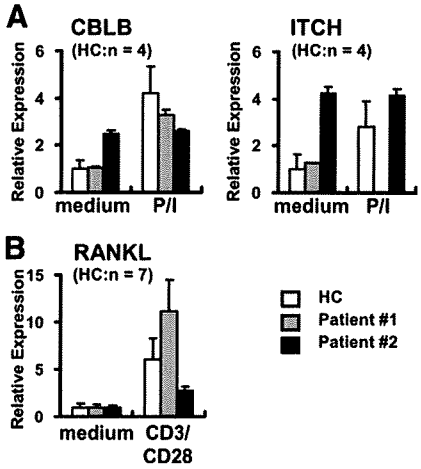

FIGURE 8. *A*, Expression levels of E3 ubiquitin ligases. Cbl-b and Itch mRNA levels were measured by real-time RT-PCR, as indicated in Fig. 5. Purified CD4 T cells from healthy controls (HC; $n = 4$) and patients (1 and 2) were activated with PMA/ionomycin or incubated in the medium only for 4 h. mRNA levels for Cbl-b and Itch were quantified in duplicate with 18S rRNA as a reference, and baseline expression in HC was adjusted to 1.0. Error bars indicate SD for HC and SEM for patients. Expression of Grail (RNF128) was not detectable before or after stimulation in this assay (data not shown). *B*, Level of RANKL mRNA expression. CD4 T cells from HC ($n = 7$) and patients (1 and 2) were stimulated with anti-CD3/anti-CD28 mAb, and RANKL mRNA expression was measured. Expression was quantified with 18S RNA as a reference, and the mean expression level of HC was adjusted to 1.0. □, HC; ▨, patient 1; ■, patient 2. Error bars indicate SD for controls and SEM for patients. P/I: PMA/ionomycin.

Among them, TNFSF11 (RANKL) showed >2-fold higher expression in patient 1 after stimulation. In addition, RANKL expression was >50% higher in patient 1 than in the controls.

Because RANKL was identified as a candidate key molecule involved in the pathogenesis of RA (58, 59), we quantified RANKL mRNA in CD3/CD28-stimulated CD4 T cells by real-time PCR. The result shown in Fig. 8*B* demonstrates that compared with healthy controls, RANKL induction was higher in patient 1, but lower in patient 2.

Discussion

In this study, we describe broad defects in T cell function in two siblings with a novel deficiency of human ICOS. Most of the abnormalities presented in this study have not been reported in humans, and some have not been reported in the murine model of ICOS deficiency.

The marked decline in two T cell subpopulations, memory CD4 T cells and CTLA-4$^+$CD45RO$^+$ Tregs, can be explained, at least in part, by a recent observation that the ICOS-ICOS-L interaction plays an important role in the expansion and survival of these effector T cells (23, 60).

With regard to CD4 memory T cells, we observed significant reductions in the numbers of both TCMs and TEMs in the steady state, which were not observed in the previously reported cases of human ICOS deficiency (31–33). A reduction in the number of TEMs, but not of TCMs, was demonstrated in ICOS knockout mice by Burmeister et al. (23). TEMs were decreased up to 4-fold in the steady state; the decrease was more pronounced in older mice. TEMs and TCMs display significant and intermediate ICOS expression, respectively (23). Through detailed research on expansion, differentiation, and survival of effector T cells in the absence of ICOS, they suggested that ICOS controls the pool size of effector T cells. These data suggest that both memory subsets may require ICOS for proliferation and survival in humans. Therefore, a decline in total memory cells may be observed in ICOS-deficient mice over a longer observation period and/or after recurrent infections. Alternatively, it is equally plausible that the ICOS-ICOS-L interaction plays a pivotal role in commitment to memory T cells.

CTLA-4$^+$CD45RO$^+$ICOS$^+$CD4$^+$CD25$^+$ Tregs, commonly observed in adults, were virtually absent in our ICOS-deficient patients. The reduction was seemingly counterbalanced by an increased number of CTLA-4$^-$CD45RO$^-$ Tregs. Although the contribution of ICOS to the expansion and maintenance of Tregs as a whole has been previously reported (23, 41), our observation addresses the role of ICOS in the maintenance of an IL-10-producing memory subset of Tregs, but not TGF-β-producing CTLA-4$^-$CD45RO$^-$ICOS$^-$ naive Tregs. Supporting this is the observation that in mice, ICOS$^+$ Tregs display a strict propensity to undergo rapid apoptosis in culture unless signaled by ICOS-L (23).

The decrease in the number of CTLA-4$^+$ Tregs may be alternatively explained by defective induction of a gene that regulates Treg development. A recent study in mice has demonstrated that ROR-γt controls the development of IL-10-producing Tregs that coexpress ICOS in addition to CCL20 (61). This finding may suggest that the decrease in CTLA-4$^+$CD45RO$^+$FoxP3$^+$ Tregs is a consequence of reduced induction of ROR-γt/RORC, as observed in the present study.

Another notable T cell defect in our patients was the impaired capacity of their T cells to mount Th1, Th2, and Th17 responses. Reduced cytokine production was observed not only when the patients' CD4 T cells were activated by costimulatory signals, but also when they were stimulated by PMA/ionomycin.

Although the ICOS-ICOS-L interaction was important in vivo in the generation and/or maintenance of effector memory and central memory cells, the absence of an ICOS signal through ICOS-L did not seem to contribute to the T cell effector defects observed in our ex vivo experiments. First, ICOS-L expression was not induced in purified T cells upon CD3/CD28 costimulation (supplemental Fig. 3*A*).[3] In addition, blocking potential ICOS-ICOS-L interaction in the controls did not result in decreased cytokine production or in decreased up-regulation of MAF and RORC (supplemental Fig. 3, *B* and *C*).[3]

Additional experiments indicated that there was an abnormality at the level of transcriptional regulation of Th1, Th2, and Th17 polarization, and decreased induction of the master regulators T-bet, GATA-3, MAF, and RORC in the patients. Previous research on mice has shown that ICOS regulates MAF expression and GATA-3 induction (62), and our present study points to an additional role of ICOS in the complete induction of T-bet and RORC.

One major factor contributing to the poor effector T cell responses in the patients could be the decrease in total memory CD4 T cells. This is particularly likely in the case of IL-4 and IL-17 production, because the memory T cells had only mild defect in producing IL-4 and IL-17. Although the CD4$^+$CD45RO$^+$ T cells in the patients displayed a significantly reduced ability to produce IFN-γ, the decreased response may be explained by pronounced reduction in TEMs in the patients. To determine whether the memory T cell compartment in our patients is functionally defective or intact on a per cell basis, we would need further analysis of various parameters of the T cell effector functions in naive T cells, TCMs, and TEMs.

Nurieva et al. (56) demonstrated that murine ICOS$^{-/-}$ CD4 T cells showed defective induction of T-bet, GATA-3, and EOMES in the absence of CD28 costimulation because of up-regulation of E3 ubiquitin ligases: Grail, Cbl-b, and Itch. It is uncertain whether the augmented baseline expression of E3 ubiquitin ligases is relevant to the observed effector T cell dysfunction, because this was

confirmed only in patient 2. It is rather unlikely that the different expression of the E3 ubiquitin ligases contributed to the T cell defects in the patients because augmented induction of these ligases was not detected in the patients.

In contrast to the global impairment in cytokine synthesis, IL-2 production was only marginally affected. Supporting this observation, induction of transcription factors for IL-2 (c-Jun/c-Fos) was normal in the patients (data not shown). Similarly, induction of IL-21 and TGF-β was also unaffected in the CD4 T cells of the patients, although their induction was modest under costimulatory conditions (data not shown). It should be noted that production of IL-22, a Th17 cytokine fundamental for the development of psoriasis, showed normal induction, and that both the patients had psoriatic cutaneous lesions (63). Although whether IL-17A and IL-22 are produced by the same Th17 subset is still unclear (64, 65), our data suggest an IL-22-producing CD4 T cell subset is not functionally impaired in the patients.

Previous studies in mice have shown that ICOS is necessary for optimal CD8 T cell responses (34). ICOS can directly stimulate CD8 T cells (35); and ICOS-Ig-treated mice displayed diminished IFN-γ production by CD8 T cells. Our study has demonstrated that CD8 memory cells are reduced in ICOS deficiency, and that CD8 T cells in the absence of ICOS can mount a very low IFN-γ response, for the first time in humans. ICOS is induced on terminally differentiated $CD28^-$CD8 effector T cells (11), and thus, may play a role in maintaining the CD8 subset. Therefore, a decrease in the number of IFN-γ-producing CD8 T cells could be ascribed to the reduction in $CD45RO^+$ memory CD8 T cells or in $CD28^-$CD8 T cells (data not shown) in our ICOS-deficient patients. IFN-γ production is regulated by T-bet and EOMES cooperatively or redundantly in CD8 T cells (50, 51). T-bet induction was normal when stimulated with PMA/ionomycin or anti-CD3/anti-CD28, whereas a baseline expression of EOMES was decreased in CD8 T cells in the patients. Although this may explain the impaired production in part, further research on the CD8 T cells stimulated with various common γ-chain cytokines would be necessary to assess whether the transcriptional regulation of CD8 effector functions is impaired in the absence of ICOS.

In addition to the reduced numbers of effector T cells, which either potentiate or inhibit T cell responses, the present study demonstrates for the first time an aberrant induction of negative costimulatory molecules on activated T cells in ICOS-deficient patients. CTLA-4 and BTLA are induced upon activation and transmit an inhibitory signal to T cells to regulate the balance between T cell activation, tolerance, and immunopathology (3–5). Costimulatory and coinhibitory molecules are normally induced in the absence of ICOS in mice and humans (23, 31). In our patients, however, induction of CTLA-4 and BTLA following CD3/CD28 signaling was impaired. Although the molecular basis of the defective expression is still not known, this may be ascribed to the decreased memory T cell subset in the patients. At all events, our findings imply that an inhibitory signal to suppress activated T cells could not be appropriately induced in the patients.

Collectively, these data highlight the positive contribution of ICOS to the maintenance of, or commitment to, effector T cells and a subset of Tregs, and the induction of negative costimulatory receptors on activated T cells. The immunodeficiency in our ICOS-deficient patients, although mild, can be understood by the defects in their effector T cell functions as well as in T cell-dependent B cell help, but a reasonable explanation is still required for the development of autoimmunity, RA, IBD, IP, and psoriasis in ICOS-deficient patients.

Most studies have depicted ICOS as a positive costimulator in the immune reaction. For example, research in ICOS-deficient mice suggests that ICOS is critically involved in autoimmune development and allogeneic reactions (21, 25–29). There are, however, some results indicating that abrogation of the ICOS-ICOS-L interaction aggravates the disease process. For example, in some initial studies on ICOS-null mice, experimental autoimmune encephalomyelitis was unexpectedly exacerbated and allergen-dependent airway sensitivity was augmented (14, 66, 67). What is the role of ICOS in autoimmune development?

Burmeister et al. (23) reported that ICOS supports the expansion and survival of Th1 or Th2 responder cells, Th17 cells, and $FoxP3^+$ regulatory effector cells. They hypothesized that the absence of ICOS function in a particular mouse model would result in a phenotype reflecting a deficiency of the dominant effector T cell type. Thus, blockade of the ICOS-ICOS-L interaction could mainly affect Treg subsets and lead to the development of autoimmune disorders.

Our observations in human ICOS deficiency may fit the concept of ICOS as an agonist molecule. In our ICOS-deficient patients, defects in T cell function leading to termination of the activated T cell response may have been dominant.

Another question remains, as we observed a wide range of autoimmune diseases in patient 1, but not in patient 2. Although phenotypic variation in siblings with the same mutation is not uncommon in human genetic disorders, there might have been some contributing factor(s).

First, our analysis showed paradoxical down-regulation of Itch expression after PMA/ionophore stimulation in patient 1. Because Itch induction is important in the control of NF-κB activation (55), an activation signal in the absence of CD28 costimulation may have led to continuous inflammation in patient 1.

Second, although the T cell immune functions and stimulation-induced mRNA expression pattern of CD4 T cells were strikingly similar between the siblings, we found that the T cells of patient 1 showed exaggerated induction of RANKL expression and poorer production of IFN-γ. Previous studies demonstrated that T cells, which contribute to the development of RA and IBD, were characterized by poor IFN-γ production, production of inflammatory cytokines including IL-17A and TNF, and RANKL expression (59). Although IL-17A induction was not increased, the augmented RANKL expression and poor IFN-γ production in T cells may have contributed to the autoimmune disease progression. Characterization of RANKL-expressing IL-17A-negative T cells requires further investigation.

Third, we surmised that a major infectious episode may have upset the subtle balance between effector T cells and Tregs in our patients. In fact, patient 1 developed a series of autoimmune disorders after a severe bacterial infection.

Finally, the reason for the apparent discrepancy in T cell functions between the ICOS-deficient patients presented in this study and the ICOS deficiency described in previous reports (31–33) is elusive. One possibility that explains the difference in cytokine production in our patients and ICOS deficiency in previous reports could be different stimulation condition (dose of mAb and incubation period). However, it is unlikely because effective cytokine synthesis from the T cells was not observed in our patients even with an increased dose of anti-CD28 mAb and with longer time periods (supplemental Fig. 4).[3] This indicates the presence of other intrinsic factor(s).

Another possibility is the difference in the mutation site of the ICOS gene. Our patients harbored a homozygous single-base deletion at codon 285 located in the extracellular domain, whereas other ICOS deficiencies have homozygous deletion in exons 2 and 3 of the ICOS gene (33). In addition to defective ICOS expression,

this mutation may result in the expression of a 120-aa ICOS protein that affects immune function, for example, by binding to ICOS-L on B cells, monocytes, or a subset of T cells. Despite extensive investigation, however, we have been unable to demonstrate the expression of a truncated ICOS protein in our patients' lymphocytes.

In summary, the present study on T cell functions in two novel ICOS-deficient patients has shown that the interaction between ICOS and ICOS-L is critical for the development and maintenance of multiple types of effector cells and Tregs, and that the defects are at least in part due to diminished memory T cells and/or impaired induction of master regulators. Collectively, the results of our study highlight a major role of ICOS as a coordinator of T cell immune responses and T cell maintenance.

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Disclosures

The authors have no financial conflict of interest.

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Ex Vivo-Expanded Donor CD4$^+$ Lymphocyte Infusion Against Relapsing Neuroblastoma: A Transient Graft-Versus-Tumor Effect

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High-risk neuroblastoma has a poor prognosis despite multimodal treatment including high-dose chemotherapy. A 7-year-old male with neuroblastoma received ex vivo-expanded donor CD4$^+$ T lymphocyte infusion (CD4$^+$ DLI) after recurrence in the bone marrow following allogeneic hematopoietic stem cell transplantation from his HLA-identical mother. The disease transiently responded to CD4$^+$ DLI with reduction of tumor cells and a decrease of serum neuron-specific enolase. The response was associated with development of continued high fever and an increase of cytotoxic T lymphocytes in peripheral blood. This case suggests a possibility of a graft-versus-tumor effect against neuroblastoma. Pediatr Blood Cancer 2009;52:895–897.

Key words: CD4$^+$ donor lymphocyte infusion; graft-versus-tumor effect; neuroblastoma

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can exert an immune graft-versus-tumor (GVT) effect mediated by donor lymphocytes, which plays a therapeutic role in the treatment of hematologic malignancies. The GVT effect was directly confirmed by the observation that donor lymphocyte infusion (DLI) can successfully induce remission of chronic myelogenous leukemia, which relapse after allo-HSCT [1]. Several small studies have also suggested GVT effects following allo-HSCT in patients with solid tumors [2–6]. Although allo-HSCT has been applied in a considerable number of patients with neuroblastoma (NBL) [6], there are few reports describing a GVT effect against this malignancy. Here, we describe a patient with relapsing NBL showing transient tumor regression after ex vivo-expanded donor CD4$^+$ lymphocyte infusion (CD4$^+$ DLI).

CASE REPORT

A 4-year-old male was diagnosed with stage 4 NBL (International NBL Staging System: INSS) who developed as a retroperitoneal mass with metastases to the bone marrow (BM), cervical lymph nodes and bone (orbit). Pathological studies showed poorly differentiated NBL (International NBL Pathology Classification: INPC) with Shimada's unfavorable histology without amplified N-myc expression. He was initially treated with combination chemotherapy consisting of cyclophosphamide, vincristine, pirarubicin (THP-adriamycin), cisplatin, and etoposide. He then received high-dose chemotherapy (HDC) consisting of thio-TEPA and melphalan with autologous peripheral blood stem cell transplantation (auto-PBSCT), followed by surgical removal of primary tumor [7,8].

The disease recurred in the BM, right mandible, bilateral cervical lymph nodes, and right iliac and inguinal lymph nodes at 6 years of age, 13 months after HDC with auto-PBSCT. Following combination chemotherapy consisting of topotecan, cyclophosphamide, and cisplatin, he received an allogeneic bone marrow transplantation (allo-BMT) from his HLA-identical mother. The conditioning regimen consisted of busulfan (16 mg/kg) and fludarabine (180 mg/m^2) preceded by topotecan (30 mg/m^2). Prophylaxis for graft-versus-host disease (GVHD) was short-term methotrexate and tacrolimus. Engraftment was prompt and no acute GVHD was observed. He was also treated with radiotherapy to lymph nodes of the neck and pelvis after allo-BMT, which led to successful renewed remission. However, he developed a recurrence in BM with elevation of serum neuron-specific enolase (NSE) 1 month after completion of radiotherapy, for which he received two courses of conventional DLI [1–5 × 10^6/kg CD3$^+$ T-lymphocytes] from his mother (Fig. 1). However, tumor cells in BM increased and

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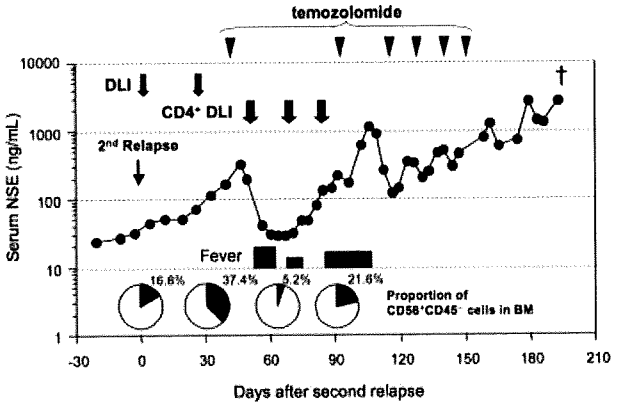

Fig. 1. Clinical course and changes in serum NSE. NSE, neuron-specific enolase; BM, bone marrow. DLI indicates donor lymphocyte infusion: 1st dose, 1×10^6/kg and 2nd dose, 5×10^6/kg $CD3^+$ T lymphocytes. $CD4^+$ DLI indicates ex vivo-expanded donor $CD4^+$ lymphocyte infusion: 1st and 2nd dose, 1×10^7/kg; and 3rd dose, 5×10^7/kg. The purity of CD4-single positive cells was 93.4%, 95.6%, and 90.9%, respectively. The majority of contaminating cells were $CD4^+CD8^+$. Temozolomide was administered at 150 mg/m^2 daily for five consecutive days for each cycle.

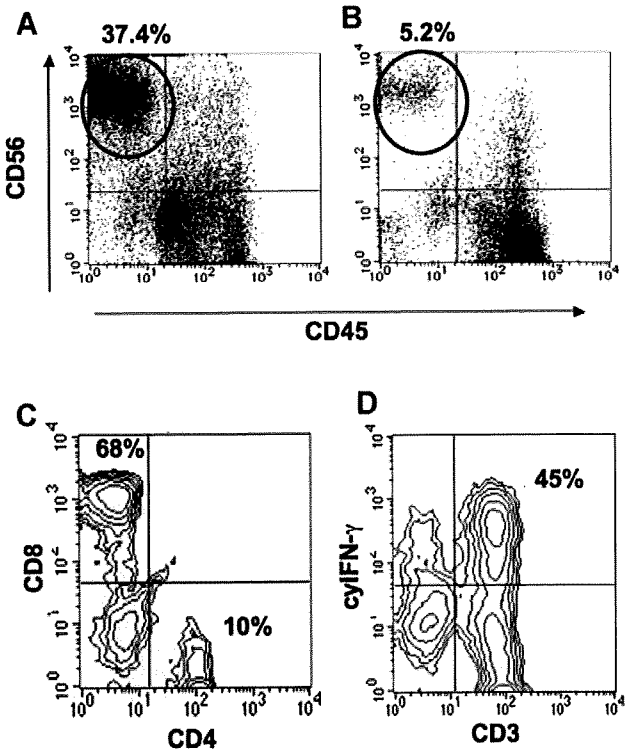

Fig. 2. Flow cytometric analysis. Tumor cells ($CD56^+CD45^-$) in iliac bone marrow before (**A**) and 12 days after (**B**) the first $CD4^+$ donor lymphocyte infusion (DLI). Proportion of $CD4^+$ or $CD8^+$ T lymphocytes (**C**) and $CD3^+$ T lymphocytes producing cytoplasmic IFN-γ (**D**) in peripheral blood mononuclear cells after $CD4^+$ DLI.

associated with increased serum NSE but without development of GVHD. We therefore infused ex vivo-expanded donor $CD4^+$ T lymphocytes ($CD4^+$ DLI) with the aim of accelerating allogeneic immunoreaction without eliciting GVHD.

Mononuclear cells were isolated from his mother. $CD4^+$ T lymphocytes were purified by CD4 monoclonal antibody (mAb)-coated magnetic beads and cultured for 1 week in the presence of recombinant IL-2 (350 IU/ml; Proleukin, Chiron BV, Amsterdam, The Netherlands) in a flask with immobilized anti-CD3 mAb, OKT3 (5 μg/ml; Jansen-Kyowa, Tokyo, Japan) [9]. This trial and culture procedure were approved by the Institutional Review Boards of Tokyo Medical and Dental University, and Osaka University Hospital. Written informed consent was obtained from the parents of the patient. The patient, then 7 years of age, was treated with $CD4^+$ DLI following administration of temozolomide (Fig. 1). Shortly after the first $CD4^+$ DLI (1×10^7/kg) with 93.4% purity of CD4-single positive cells, he developed high fever of 40°C without other GVHD signs such as skin rash, jaundice, or diarrhea. High fever continued for 2 weeks with reduction of serum NSE levels from 325.5 to 29.2 ng/ml. Iliac BM aspiration showed a decrease in the ratio of the tumor cells ($CD56^+CD45^-$ cells) from 37.4% to 5.2% (Fig. 2A,B). Twelve days after $CD4^+$ DLI, $CD8^+$ T lymphocytes with IFN-γ production predominated in peripheral blood (Fig. 2C,D). However, serum NSE increased after the second $CD4^+$ DLI. Despite the third $CD4^+$ DLI at an increased dose of 5×10^7/kg, the disease continued to progress. He then received temozolomide but without response and died 7 months after the second relapse.

DISCUSSION

The prognosis of high-risk NBL, characterized by an older age, metastases, N-myc amplification, and unfavorable histologic findings, remains poor [10,11]. More than half of these high-risk patients relapse despite strategies involving HDC followed by auto-HSCT, which indicates a need for novel strategies to eradicate residual disease. Allo-HSCT has been already used for adult patients with solid tumors [4,6], in particular renal cell carcinoma [2,5] and breast cancer [3,5]. Recent trials using allo-HSCT, mostly following non-myeloablative preconditioning, showed a response rate of up to 57% against renal cell carcinoma [2,3,5].

A dramatic reduction of tumor cells was observed in our patient following $CD4^+$ DLI. The clinical response with the development of high fever immediately after $CD4^+$ DLI combined with an increase of IFN-γ-producing $CD8^+$ T lymphocytes, that is, cytotoxic T lymphocytes (CTLs), suggests a GVT effect. Moreover, we observed no increase of NK cells in peripheral blood nor increase of expression of HLA-A24 (the patient's and the donor's HLA-A type) on residual tumor cells (data not shown). Taken together, the immunoreaction against NBL cells was presumably caused by CTLs, not by NK cells. $CD8^+$ T lymphocytes (CTLs) were increased following $CD4^+$ DLI. Expanded and activated $CD4^+$ helper T lymphocytes might have produced cytokines that stimulated CTL differentiation and enhanced the ability of antigen-presenting cells to stimulate CTL differentiation through a CD40-CD40L interaction [12].

An immunological response due to lymphocytes might be attributable in our case to scattered tumor cells in BM, which were abundant in bloodstream, as is more frequently seen in leukemia. Although the administration of temozolomide shortly before $CD4^+$ DLI might have affected the clinical response, there was no response

during the second course of temozolomide during the final course of the disease, which suggests that the first course was not associated with a reduction of tumor cells.

In 1994 Matthay et al. [13] reported no advantage of allo-HSCT over auto-HSCT in patients with NBL and few reports suggest a GVT effect against NBL. Inoue et al. [14] reported a case showing the disappearance of NBL within 3 years after allo-HSCT from an HLA haploidentical donor. Although a considerable number of patients with NBL has been treated with allo-HSCT [6], detailed analysis has not been performed regarding its efficacy. Dykes et al. [15] have recently used $CD3^+$ T-cell depleted allo-PBSCT from HLA-haploidentical donor to patients with NBL.

The response in our patient suggests a transient GVT effect against NBL cells. Immunotherapy with allogeneic lymphocytes might open new avenues for overcoming the dismal prognosis of high-risk NBL.

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