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Aberrant CD3- and CD28-Mediated Signaling Events in Cord Blood T Cells Are Associated with Dysfunctional Regulation of Fas Ligand-Mediated Cytotoxicity

Katsuaki Sato,¹ Hitomi Nagayama,¹ and Tsuneo A. Takahashi²

There have been numerous reports of decreased acute and chronic graft-vs-host disease (GVHD) in patients receiving HLA-matched or HLA-disparate umbilical cord transplants. However, little is known about the mechanisms underlying the low incidence of GVHD in umbilical cord blood transplantation (CBT). In this study, we examined CD3- and CD28-mediated functional properties and signaling events in CB T cells (CBTCs). Dual stimulation of peripheral blood TCs (PBTCs) and bone marrow TCs (BMTCs) with mAbs to CD3- and CD28-induced expressions of Fas ligand (FasL), as well as CD25 and CD154 (CD40L), whereas defective induction of these activation-associated cell surface molecules were observed in CBTCs. Engagement of both CD3 and CD28 induced FasL-mediated cytotoxicity in peripheral blood TCs (PBTCs) but not CBTCs; however, both of these tissue sources possess intrinsically similar proliferative responsiveness. Analysis of CD3- and CD28-induced signal transduction revealed a deficiency in signaling events that involved repressed tyrosine phosphorylation and enzymatic activities of a family of mitogen-activated protein kinases, extracellular signal-regulated kinase 2, stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), and p38α/β, as well as p56lck and ZAP-70 in CBTCs compared with those in PBTCs. These results suggest that CD3- and CD28-mediated signaling events blockage in CBTCs may be responsible for dysfunction of FasL-mediated cytotoxicity and lead to the low incidence of severe GVHD in CBT. The Journal of Immunology, 1999, 162: 4464–4471.

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3 Abbreviations used in this paper: BM, bone marrow; T, transplantation; CB, cord blood; allo, allogeneic; DCS, dendritic cells; ERK, extracellular signal-regulated kinase; L, ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; GVHD, graft-vs-host disease; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MNC, mononuclear cell; PB, peripheral blood; PTK, protein tyrosine kinase; R:S ratios, responder cell to stimulator cell ratios; SAPK/JNK, stress-activated protein kinase/c-jun N-terminal kinase; TCs, T cells.

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phosphorylation of ZAP-70 leading to its enzymatic activation. Following initial PTK activation, other biochemical signals are generated, including those derived from the phosphatidylinositol pathway and Ras/Rac/Rho-activated cascades of a family of mitogen-activated protein kinases (MAPKs) (13–16). MAPKs are activated following engagement of a variety of cell surface receptors via dual tyrosine and threonine phosphorylation and are thought to be involved in various cellular responses (17–20). The various members of the MAPK families differ in their substrate specificity and are activated by distinct upstream regulators and extracellular stimuli (17–20). Currently, the MAPK family is comprised of three subfamilies, namely: 1) the extracellular signal-related kinase (ERK) subfamily, including p42 and p44 MAPKs, 2) the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) subfamily, including the p46 and p54 SAPK/JNK isoforms and their variants; and 3) the p38 subfamily (17–20). Previous studies have shown that blockage in the MAPKs pathway leads to anergic states in several T cell clones (21–23). Although the cascade of signaling events that occur upon engagement of the TCR/CD3 complexes in mature T cells peripherally and in lymph nodes and several T cell clones have been demonstrated in previous studies, much less is known about the TCR/CD3 complex-mediated molecular events in CBTCs.

To examine the involvement of properties of TCs in the low incidence of GVHD in CBT, we investigated CD3- and CD28-mediated functional properties and signaling events in CBTCs. We demonstrated that dual stimulation of PBTCs and BMTCs with mAb to CD3 and CD28 induced FasL as well as CD25 and CD154, whereas CBTCs failed to induce these molecules following stimulation, and purification of TCs was performed by E-rosetting (9). T cell preparation was typically >90% pure, as indicated by anti-CD3 mAb staining (Becton Dickinson, Mountain View, CA). Cells were either untreated or stimulated with FITC-conjugated anti-CD3 mAb (Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD25 mAb (Becton Dickinson), PE-conjugated CD154 mAb (Phar-Mingen), or biotin-conjugated Fasl mAb (clone NOK2; Pharmingen) followed by PE-conjugated avidin (Becton Dickinson). Cells were also stained with the corresponding FITC- and PE-conjugated isotype-matched mAbs (Becton Dickinson). Analysis of fluorescence staining was performed with a FACS Calibur flow cytometer (Becton Dickinson) and CELLQuest Software (Becton Dickinson).

**Materials and Methods**

**Media and Reagents**

The medium used was RPMI 1640 supplemented with 2 mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, and 10% heat-inactivated FCS. Granulocyte-macrophage CSF (GM-CSF) was kindly provided by Kirin Brewery (Tokyo, Japan). IL-2 and IL-4 were purchased from PeproTech (London, U.K.). mAbs to CD3, CD28, and FasL (clone NOK2) were purchased from PharMingen (San Diego, CA). Con A was purchased from Sigma (St. Louis, MO). Metalloproteinase inhibitor KB-R8301 was kindly purchased from BioLabs (Beverly, MA).

**Preparation and culture of TCs**

Samples of PB, BM, and umbilical CB were obtained according to institutional guideline with informed consent from all healthy volunteers. Mononuclear cells (MNCs) from each sample were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density centrifugation, and purification of TCs was performed by E-rosetting (9). T cell preparations were typically >90% pure, as indicated by anti-CD3 mAb staining (Becton Dickinson, Mountain View, CA). Cells were either untreated or stimulated with a combination of immobilized mAbs to CD3 and CD28 (21, 22) in the presence or absence of 10 µM metalloproteinase inhibitor KB-R8301 (24, 25) for 16 h at 37°C, and cells were used for subsequent experiments. Con A blasts were prepared from PBMCs by cultivation with 5 µg/ml of Con A and 10 ng/ml of IL-2 for 48 h, followed by incubation in the presence of IL-2 alone for 5 days (25). Dead cells were removed by gradient centrifugation using Histopaque 1083 (Sigma).

**In vitro generation and culture of human dendritic cells (DCs)**

DCs were generated from PBMCs as described previously (26). Briefly, PBMCs were resuspended in culture medium and allowed to adhere to 6-well plates (Costar, Cambridge, MA). After 2 h at 37°C, nonadherent cells were removed, and adherent cells (~90% CD14+ cells) were cultured in 3 ml of medium supplemented with GM-CSF (50 ng/ml) and IL-4 (250 ng/ml). After 7 days of culture, DCs were harvested, washed, and used for subsequent experiments.

**Flow cytometry**

TCs were treated with 0.5% mouse serum (Dako, Glostrup, Denmark) for 15 min at 4°C to block the Fe receptor, and stained with FITC-conjugated anti-CD3 mAb (Becton Dickinson) and phycoerythrin (PE)-conjugated anti-CD25 mAb (Becton Dickinson), PE-conjugated CD154 mAb (Phar-Mingen), or biotin-conjugated Fasl mAb (clone NOK2; Pharmingen) followed by PE-conjugated avidin (Becton Dickinson). Cells were also stained with the corresponding FITC- and PE-conjugated isotype-matched mAbs (Becton Dickinson). Analysis of fluorescence staining was performed with a FACS Calibur flow cytometer (Becton Dickinson) and CELLQuest Software (Becton Dickinson).

**Assay for alloMLR**

TCs (10⁵) were cultured with various concentrations of irradiated (15 Gy from a 137Cs source) allogeneic monocyte-derived DCs (10⁵–10⁶) (26). Thymidine incorporation was measured on day 5 by a 18-h pulse with 0.5 µCi/well of ³H]thymidine (1 µCi/well; Amersham Life Science, Buckinghamshire, U.K.) (26).

**Cytotoxicity assay**

Unstimulated or stimulated TCs (10⁴–10⁵) were cultured with Na₂[⁵¹Cr]O₄ (100 µCi/10⁵ cells; New England Nuclear Life Science Products, Boston, MA)-labeled Con A blast (10⁴) for 4 h at various E:T ratios (1:10–1:100) in the presence or absence of 10 µg/ml control IgG (cont. IgG; Sigma) or antis-Fasl mAb (24, 25, 27, 28). The supernatants were harvested, radioactivity was counted, and percentage of specific lysis was calculated (27). Value of spontaneous release cpm was <10% of the total release cpm.

**RT-PCR**

RNA from each sample was isolated using Trizol LS reagent (Life Technologies, Gaithersburg, MD). The first strand cDNA kit (SuperScript Pre-amplification System; Life Technologies) was used to make cDNA from 5 µg of each RNA. Amplification of each cDNA was performed with a SuperTag Premix kit (Sawady Technology, Tokyo, Japan) using specific primers (14).

**Western blot analysis and immune complex kinase assay**

TCs were starved in serum-free medium for 16 h at 37°C, and subsequently kept for 4 h on ice to reduce the basal levels of tyrosine phosphorylation of intracellular proteins. Cells (2 × 10⁵) were untreated or stimulated with a combination of immobilized mAbs to CD3 and CD28 for 5 min at 37°C, washed twice in cold PBS, resuspended in 100 µl of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate), and cell lysates were obtained. Total cell lysates or the immunoprecipitates with Abs to CD3ε, p56lk, or ZAP-70 (13, 14, 21–23) were fractionated by 12% SDS-PAGE, transferred onto PVDF membranes (Millipore, Bedford, MA), and probed with HRP-conjugated anti-phosphotyrosine mAb (clone RC20). Blots were visualized by enhanced chemiluminescence (ECL) (New England Biolabs). To ensure similar amounts of respective proteins in each sample, the same membrane was stripped off, reprobed with stated Abs, and developed with HRP-conjugated secondary Abs by ECL. For immune complex kinase assay, the immunoimmunoprecipitates with Abs to p56lk or ZAP-70 were washed three times with lysis buffer and twice with kinase buffer before resuspending in 20 µl of kinase buffer containing 10 µM ATP (New England Biolabs), 5 µCi [γ-³²P]ATP (New England Nuclear Life Science Products), and 5 µg enolase (Sigma). The mixtures were incubated at 30°C for 5 min, and the reactions were terminated, separated by 12% SDS-PAGE, transferred onto PVDF membrane, immunoblotted, and subjected to autoradiography.
Results

FasL expressions were induced in PBTCs and BMTCs but not in CBTCs following dual stimulations with anti-CD3 mAb and anti-CD28 mAb. Engagement of TCR/CD3 complexes and adhesion/costimulatory molecules with their respective counter ligand or mAbs results in activation of signals that drive induction of various functions, including secretion of several cytokines and expression of adhesion/costimulatory molecules on their surface in mature TCs (13, 14, 21–23). Previous studies have shown that several functions were impaired in CBTCs following various stimulations (5–12). Therefore, we examined the cell surface expression levels of CD3 and CD28 in PBTCs, BMTCs, and CBTCs by flow cytometry using the respective mAbs. Flow cytometric analysis revealed similar expression levels of CD3 and CD28 among PBTCs, BMTCs, and CBTCs (Fig. 1A), and the ratio of the cell subpopulations expressing CD3 and/or CD28 was not different in these TC subsets (Fig. 1B).

To analyze CD3- and CD28-mediated functional consequences in PBTCs, BMTCs, and CBTCs, we examined phenotypic changes following dual stimulation with mAbs to CD3 and CD28 (Fig. 2). Engagement of both CD3 and CD28 by specific mAbs induced significant cell surface expression of CD25 and CD154 in PBTCs and BMTCs, although these cells had low expression levels of CD25 and did not constitutively express CD154 in their normal states. On the other hand, treatment of CBTCs with mAbs to both CD3 and CD28 induced a lower expression level of CD25 compared with PBTCs and BMTCs, whereas CD154 was not induced.

Previous studies revealed that FasL expressed on activated PBTCs and NK cells mediates functional Fas-expressed target cell lysis by these effector cells (24, 25, 28). The Fas/FasL system has also been implicated in the pathogenesis of GVHD as well as autoimmune diseases, fulminant hepatitis and AIDS (3, 24, 25, 28). To examine the capacities of PBTCs, BMTCs, and CBTCs to express FasL, the cells were stimulated with mAbs to CD3 and
CD28, and the expression of FasL transcripts and its product on the cell surface were analyzed. Dual stimulation of PBTCs and BMTCs with mAbs to CD3 and CD28 induced FasL transcript expression, although not constitutively (Fig. 2B), and these results are concomitant with cell surface expression (Fig. 2A). On the other hand, ligation of CD3 and CD28 with their respective mAbs in CBMCs failed to induce FasL transcript expression or its product on the cell surface. These results indicate that CD3- and CD28-mediated activation-associated phenotypic changes are impaired in CBTCs.

CBTCs exhibited selective low sensitivity for proliferative response in alloMLR

The in vitro proliferative responsiveness of PBTCs and CBTCs generated in alloMLR have been previously reported. Most studies comparing the ability of PBTCs and CBTCs to proliferate in response to alloAg have been performed by using bulk cultures or sets of purified TCs as responder cells and irradiated B cell lines as stimulator cells at relatively high responder cell to stimulator cell ratios (R:S ratios, 10:1) (27, 29). DCs have been shown to be unique professional major APCs capable of stimulating resting TCs in the primary immune response and are more potent APCs than monocytes/macrophages, B cells, or their respective cell lines (26). Therefore, we cultured PBTCs or CBTCs (10^5) with irradiated allogeneic monocyte-derived DCs (10^2–10^5) to clarify the differences between the proliferative responsiveness of PBTCs and CBTCs to alloAg (Fig. 3A). The capacity of CBTCs to proliferate following stimulations with alloAg were significantly weaker than those of PBTCs at lower stimulation levels (R:S ratios, 10^3:2 × 10^3), whereas comparable responses to higher stimulation were observed (R:S ratios, 10^2:1). These results indicate that CBTCs possess proliferative responsiveness to alloAg, although this sensitivity is lower than that of PBTCs.

CBTCs had impaired CD3- and CD28-induced FasL-mediated cytotoxicity

Previous studies have demonstrated a decrease in the cytotoxic function of CBTCs generated by alloMLR compared with PBTCs (27). However, there are conflicting reports that the cytotoxic activity of CBTCs against alloAg-expressing cells is comparable to that of PBTCs (30). To directly examine potential cytotoxic capacities, PBTCs or CBTCs were unstimulated or stimulated with a combination of anti-CD3 mAb plus anti-CD28 mAb, and the cytotoxicities of these cells against allogeneic Con A blasts expressing functional Fas on their cell surface (25) were examined. Treatment of PBTCs with a combination of mAbs to CD3 and CD28 induced cytotoxicity against allogeneic Con A blasts (Fig. 3B), which was partially blocked by anti-FasL mAb (Fig. 3D), indicating that the Fas/FasL system is involved in PBTC-mediated cytotoxicity. In contrast, little or no cytotoxicity was induced in CBTCs following these stimulations (Fig. 3, B and C). These results are consistent with the deficient expression of inducible FasL.

FIGURE 3. CBTCs demonstrated impaired FasL-mediated cytotoxicity but exhibited proliferative responsiveness. A, AlloMLR of PBTCs and CBTCs with monocyte-derived DCs. PBTCs or CBTCs (10^5) were cultured with the indicated numbers of the irradiated allogeneic monocyte-derived DCs. Proliferative response was measured on day 5. Values are the mean ± SD obtained for triplicate cultures. B and C, Induction of FasL-mediated cytolytic activity was seen in PBTCs but not in CBTCs after dual stimulation with anti-CD3 mAb and anti-CD28 mAb. The cytolytic activity of the resulting cells prepared as described in Fig. 2 against allogeneic Con A blasts were analyzed by a 4-h ^51Cr-release assay at an E:T ratio from 10 to 100 (B) or at an E:T ratio of 50 in the presence or absence of 10 μg/ml control IgG or anti-FasL mAb (C). The results are representative of five experiments done with similar results.
(Fig. 2, A and B). These results indicate that CBTCs impair the CD3- and CD28-mediated capacity of killing target cells via Fas/FasL system.

**Aberrant CD3- and CD28-mediated signaling events in CBTCs**

FasL expression has been shown to be regulated by TCR/CD3 complex-mediated signaling events involving p56<sup>lck</sup>, ZAP-70 and Ras signaling pathways in T cell clones (13–15). However, CD3- and CD28-mediated signaling events in CBTCs remains poorly understood. Therefore, we examined signaling events involving PTK-mediated cascades triggered by CD3 and CD28 in CBTCs. Dual stimulation with mAbs to CD3 and CD28 initiated tyrosine phosphorylation of several intracellular proteins in PBTCs (Fig. 4A). On the other hand, a similar pattern of tyrosine phosphorylation of several target proteins was also observed in CBTCs following these stimulations, although the degree of phosphorylation was significantly lower than that of PBTCs (Fig. 4A).

To address the mechanism underlying the repression of CD3- and CD28-mediated tyrosine phosphorylation events in CBTCs, we examined tyrosine phosphorylation of CD3<sub>e</sub>. Fig. 4B shows that ligation of CD3 and CD28 induced lower levels of tyrosine phosphorylation of CD3<sub>e</sub> in CBTCs than PBTCs, even though the total amounts of CD3<sub>e</sub> were equivalent.

Faith et al. (16) previously suggested that defective TCR stimulation in anergized type 2 Th cells may correlate with abrogated p56<sup>lck</sup> and ZAP-70 tyrosine kinase activities. To further evaluate the role of p56<sup>lck</sup> and ZAP-70 in defective CD3- and CD28-mediated tyrosine phosphorylation events in CBTCs, their tyrosine phosphorylation levels and kinase activities were examined (Fig. 4, C and D). Unlike PBTCs, dual stimulation of CBTCs with mAbs to CD3 and CD28 did not significantly induce tyrosine phosphorylation and the up-regulation of tyrosine kinase activities of p56<sup>lck</sup> and ZAP-70.

Recent studies have shown that blockage in the Ras/Raf/ERK2, SAPK/JNK, and p38<sup>mapk</sup> pathway leads to anergic states in mature Tcs (21–23). To clarify the potential involvement of ERK2, SAPK/JNK, and p38<sup>mapk</sup> in the functional deficiencies in CBTCs, cells were unstimulated or stimulated with dual stimulation using mAbs to CD3 and CD28, and the level of MAPKs phosphorylation was assessed by immunoblotting with antityrosine-phosphorylated MAPK mAbs (Fig. 5, A–C). Dual stimulation of PBTC increased tyrosine phosphorylation of ERK2, SAPK/JNK, and p38<sup>mapk</sup> compared with those of unstimulated cells. On the other hand, dual stimulation with mAbs to CD3 and CD28 resulted in a significant reduction in the tyrosine-phosphorylated forms of these MAPKs when compared with those of PBTCs. The total amounts of these MAPKs were unchanged following stimulations in PBTCs or CBTCs (Fig. 5, A–C).

Enzymatically activated ERK2, SAPK/JNK, and p38<sup>mapk</sup> phosphorylate their respective transcription factors as substrates (Elk-1, c-Jun, or activating transcription factor-2, respectively) (17–23). A series of previous studies have revealed that MAPK-mediated phosphorylation of these transcription factors result in the interactions with their respective enhancer elements and regulation of various gene expression leading to functional activation of mature Tcs (17–23). Therefore, we examined whether any kinase activities were associated with changes in the amounts of the tyrosine phosphorylation forms of these MAPKs (Fig. 5, A–C). Indeed, the kinase assays clearly showed that engagement of both CD3 and CD28 triggered activation of ERK2, SAPK/JNK, and p38<sup>mapk</sup> in PBTCs. Conversely, dual stimulation with mAbs to CD3 and CD28 resulted in lower kinase activities of these MAPKs in CBTCs than those of PBTCs. These results agree with the levels of the tyrosine phosphorylation forms of these MAPKs (Fig. 5, A–C).
Discussion

Allogeneic BMT is indicated for selected genetic and hematologic diseases and for hematopoietic reconstitution in cases of iatrogenic or accidental ablation of BM. HLA-identical sibling donors offer the best graft compatibility, but HLA-identical unrelated donors may also provide acceptable graft (1–3). However, the successful outcome of allogeneic BMT is significantly limited by the risk of GVHD, leading to morbidity and mortality (1–3). Recent studies have shown that the Fas/FasL pathway is involved in the incidence of GVHD, and TCR/CD3 complex-mediated signaling events potentially regulate this system (3, 4, 13–15, 24, 25). On the other hand, placental CB, as an alternative source of hematopoietic stem cells for BM reconstitution, has recently been shown to yield successful HLA-identical or HLA-disparate sibling and unrelated donor CB grafts in children and adults (1, 2). A remarkable attribute of CB as donor tissue for BM replacement has been a lesser GVHD (1, 2). Thus, CB has the potential to overcome some of the limitations for sibling and unrelated BMT, and there is increasing clinical interest in CBT as alternative new therapeutic protocol for BM reconstitution worldwide. Although the use of CB has been characterized by an apparent lower incidence of severe GVHD, little is known about scientific basis for this phenomenon. Our findings provide the information that CD3- and CD28-mediated signaling events blockage in CBTCs may be responsible for dysregulation of the Fas/FasL system (Table I), which may be involved in the lower incidence of severe GVHD in CBT.

Secretion of cytokines such as IL-2, IL-12, IFN-γ, and TNF-α by donor-derived activated mature TCs may be involved in the development of GVHD in allogeneic BMT (10–12). A series of previous studies have shown CBTCs impaired capacity to produce these cytokine following several stimulations (10–12). We (5) and others (7–9) have previously reported that CBTCs exhibit low levels of IL-2R and IL-12R complexes on their cell surface, and stimulation of these cells failed to induce cell surface expression of CD154. On the other hand, previous studies have shown that CBTCs exhibited similar proliferative capacity to PBTCs in response to allostimulations when CBTCs or PBTCs were cultured with irradiated allogeneic B cell lines at relatively high stimulation (R:S ratios, 10:1) (27, 29). We showed that the proliferative response of CBTCs was lower than that of PBTCs when CBTCs or PBTCs were cultured with allogeneic DCs at lower stimulation (R:S ratios, 10^3:2 × 10^5) compared with PBTCs, whereas similar proliferative capacities were observed at higher stimulation (R:S ratios, 10^2:1) (Fig. 3A), suggesting that CBTCs may exhibit lower sensitivity in response to alloAg, whereas these cells may possess intrinsically similar proliferative capacity compared with that of PBTCs. These phenomena imply that the defective capacities of CBTCs to exhibit several functions, including secretion of various cytokines, expression of adhesion/costimulatory molecules, and proliferative responses to alloAg may contribute to low incidence of GVHD, although the direct involvement of these deficiencies in tissue destruction remains unclear.

The Fas/Fasl system as well as perforin lytic pathway are believed to be involved in donor-derived mature TC-mediated tissue damage in the pathogenesis of GVHD in recipients (3, 24, 25, 28). We showed that engagement of CD3 and CD28 by their respective mAbs induced FasL transcript and its product on the cell surface in PBTCs and BMTCs (Fig. 2, A and B). Furthermore, dual stimulation of PBTCs with mAbs to CD3 and CD28 induced cytotoxicity against allogeneic Con A blasts (Fig. 3B), which was partially impaired in CBTCs.
intracellular signaling leading to aberrant activation of p56

block by mAb to FasL (Fig. 3C). These results suggest that the Fas/FasL system and other killing mechanisms, such as the perforin lytic pathway, may be involved in PBTC-mediated cytotoxicity (3, 4, 24, 25, 28). We also showed that CBTCs failed to induce cytotoxic capacity against these target cells (Fig. 3, B and C). These results are consistent with the deficient expression of inducible FasL (Fig. 2, A and B) and the lack of constitutive perforin expression in CBTCs (4). These results suggest that the defective CD3- and CD28-induced capability of killing allogeneic target cells via FasL-dependent or -independent manners may be correlated with low incidence of tissue damage in GVHD in CBT.

Ligation of TCR/CD3 complexes and adhesion/costimulatory molecules with their respective counter ligand induce various intracellular signaling events leading to the induction of a variety of TC functions (13–16). We showed that dual stimulation of CBTCs induced lower tyrosine phosphorylation of CD3e, p56\(lck\), and ZAP-70, as well as MAPks (ERK2, SAPK/JNK, and p38\(\text{mapk}\)) and their enzymatic activation compared with those of PBTCs (Figs. 4 and 5). Also, Porcu et al. (29) recently reported that alloAg-stimulated CBTCs were preferentially induced into a state of anergy following restimulation.

In summary, our results provide possible reasons for an advantage of CB as a donor tissue in allogeneic CBT in terms of low frequencies of severe GVHD. Although we suggest that a defect in inducible FasL expression as well as other functional deficiencies in CBTCs may contribute to the low frequency of severe GVHD in CBT, other mechanisms involving cells, such as B cells and NK cells, may also participate in these phenomena. Defining the precise mechanisms of immunological properties of CB may facilitate use of CB as a donor tissue for sibling and unrelated CBT.

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