

Four color immunophenotyping of leukemic cells was performed at Moffitt Cancer Center flow cytometry laboratory on filtered fresh whole bone marrow aspirate after red blood cell lysis, using the following antibodies with the corresponding fluorochrome labeling accordingly: CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD19, CD20, CD23, CD33, CD34, CD45, CD56, CD61, CD117, cytoplasmic CD3 (cCD3), FMC-7, Glycophorin A, HLA-DR, MPO, and TdT.

Metaphase and interphase cytogenetic (G-banding and fluorescence in situ hybridization) and molecular studies by polymerase chain reaction. Metaphase cytogenetic studies were performed on the BM aspirate and analyzed using standard trypsin-Giemsa-banding techniques by Laboratory Corporation of America, NC and reported according to Human Cytogenetic Nomenclature (2005).

Fluorescence in situ hybridization (FISH), using unique sequence DNA probes for ABL and BCR gene region, chromosome 11q23 rearrangements/*MLL*, *RUNX1-ETO*, *CBFB-MYH 11*, *PML-RARA* and trisomy 8 was performed by Laboratory Corporation of America according to manufacturer's instructions (Vysis, Downers Grove, IL).

Molecular analysis by polymerase chain reaction (PCR) for *FLT3* internal Tandem duplication (ITD) and neophosphoin (*NPM1*) mutations were performed at ARUP Laboratories (Salt Lake City, UT).

Molecular studies by PCR for T-cell receptor, immunoglobulin heavy (IGH) and immunoglobulin light (IGL) chain gene rearrangements was performed at Tampa General Hospital (Tampa, FL).

Results

Morphology, histology, and immunophenotyping

The peripheral blood and the BM smears revealed 95% and 93% blast cells, respectively. Bone marrow trephine biopsy showed a hypercellular marrow space with essentially diffuse replacement by blasts (Fig. 1A). Blasts were variable in size, with immature large chromatin, visible nucleoli and scant cytoplasm without vacuoles or granules (Fig. 1B). By immunohistochemical stains, blasts were positive for CD3 (subset), CD79a (moderate), Pax-5 (strong), CD20 (subset), CD7 (subset), and TdT (strong). Blasts were negative for CD2, CD10, and MPO (data not shown). Immunophenotyping by 4-color parametric flow cytometry detected a single blast population with distinct immunoreactivity for both B-lymphoid and T-lymphoid antigens. The blasts expressed cCD3, moderately strong CD19, CD20 (subset), CD7 (subset-29%), and HLA-DR (see Fig. 2). They were negative for CD10, CD14, CD33, CD34, CD56, CD117, and MPO. A significant background population of mature T- or B-cells was not present.

Cytogenetics, FISH, and molecular analysis

Chromosomal analysis of unstimulated cultures revealed a subtle abnormal clone characterized by t(11;19) in nine of 20 cells examined. The genes involved in this fusion, were characterized as *MLL* at 11q23 and *MLLT1 (ENL)* at 19p13.3, which was confirmed by FISH analysis (data not shown).

Fluorescence in situ hybridization AML panel showed an apparent rearrangement of the *MLL* gene in 137 of 200 interphase cells analyzed. DNA probes specific for 5q7q, *RUNX1-RUNX1T1* [t(8;21)], *PML/RARA* [t(15;17)] and *CBFB* (inv16) as well as *BCR-ABL1* [t(9;22)] showed normal hybridization signals in all interphase cells examined.

There was no evidence of ITD or exon 20 point mutation in the *FLT3* gene or *NPM1* exon 12 mutation, by PCR. The mutational status of IGH and IGL chains, and T-cell receptor genes remain undetermined due to DNA of insufficient quantity (as per reference laboratory report).

Detection of *MLL* rearrangement and expression of cCD3, CD19, and CD79a meet the WHO criteria for classification as mixed phenotype acute leukemia with t(11;19)(q23;p13.3), B/T-lymphoid type.

Discussion

We describe a case of an adult male patient with biphenotypic acute leukemia in which blasts showed expression of both B- and T-lymphoid antigens, with a balanced translocation involving *MLL* at 11q23 and *MLLT1 (ENL)* at 19p13.3, resulting in *MLL-MLLT1* chimeric fusion protein.

The transforming capability of the chimeric protein that results from the respective translocations involving *MLL* has been explicitly demonstrated by a variety of approaches [14–19]. The most frequent translocation partner genes code for

transcription factors *MLLT2*, *MLLT3*, and *MLLT1* that belong to the same nuclear protein network involved in histone H3K70 methylation [13]. The role of *MLL-MLLT1* fusion protein in determination of lineage differentiation is still unclear. *MLL-MLLT1(ENL)* was shown to be required to initiate and maintain immortalization of myeloid progenitor cells mimicking the common myeloid type of *MLL*-associated leukemia [20]. When *MLL-MLLT1(ENL)* transduced primary hematopoietic cells were cultured under lymphoid growth conditions, a lymphoid/monocytoid biphenotypic cell was generated that resembled MLL leukemic cells [14]. Lavau et al., utilizing a similar experimental approach, and showed that *MLL-MLLT1(ENL)* fusion protein could also elicit an exclusively myeloid-type leukemia. These *MLL-MLLT1(ENL)* transformed cells were reversibly arrested in an early stage of development and could be forced into terminal differentiation by the addition of maturation promoting cytokines. Therefore, it is suggested that *MLL-MLLT1(ENL)* plays a role as a universal inhibitor of hematopoietic differentiation that blocks a common early maturation step. Our patient's leukemic blasts demonstrate an early lymphoid differentiation (CD19+, CD3+, TdT+, and CD10 negative) that could potentially differentiate toward T-lymphoid, B-lymphoid, or myeloid lineage by local cytokine and/or growth factor environment.

Some *in vitro* experiments showed that mouse cells transduced by *MLL-MLLT1(ENL)* are arrested at the pro-B phase of differentiation. At this stage, DJ κ rearrangement is initiated and one of the key transcription factors for the commitment to B-lymphoid lineage, Pax-5, is present and is required for progression beyond the pro-B cell stage [21,22]. In addition, Pax-5 suppresses the expression of genes that would initiate alternative lineage decisions through its interaction with co-repressors [23]. Therefore, Pax5 functions both as a transcriptional activator and as a repressor during normal B-cell development. Any interfering mechanism that results in a loss of Pax-5 activity, may lead to de-differentiation and cause an outgrowth of cells with bipotential behavior. In support of this hypothesis, pro-B cells that have lost Pax-5 by gene knockout show plasticity in development and can transdifferentiate into all cell lineages including T-cells [21,24]. By flow cytometry and immunostains, it is demonstrated that our patient's leukemic blasts expressed Pan-B cell markers (CD19, CD20, CD79a, and Pax-5) indicative of blasts with partial B-cell phenotype. Albeit no further investigation was carried out to evaluate Pax-5 activity, presumably, *MLL-MLLT1(ENL)* translocation in our patient occurred in an early pro-B stage of differentiation, in a cell with still some plasticity for bipotential differentiation.

Although rearrangements of the *MLL* in ALL and AML are generally associated with poor prognosis, cases with T-lymphoid lineage have a much better prognosis than their B-cell counterparts [25,26]. In particular, t(11;19)(q23;p13.3) resulting in *MLL-MLLT1(ENL)* fusion protein, is associated with favorable outcome in T-lineage ALL [27]. Cases of mixed phenotype leukemias B/T-lymphoid type are exceedingly rare and with the exception of the present case, none with t(11;19)(q23;p13.3) have been described. Our patient has responded well to intensive chemotherapy and subsequent allogeneic hematopoietic stem cell transplant and remains in complete remission 9 months following initial diagnosis. Albeit blasts display dual T and B markers in the present case, t(11;19)(q23;p13.3) could direct a less aggressive clinical course as seen in T-lymphoblastic leukemia. The influence of t(11;19)(q23;p13.3) on lineage differentiation remains uncertain and worthy of further exploring.

¹Department of Oncologic Science, Division of Hematopathology and Laboratory Medicine, Moffitt Cancer Center and Research Institute, Tampa, FL.²Malignant Hematology, Moffitt Cancer Center and Research Institute, Tampa, FL

*Correspondence to: Dr. Mojdeh Naghashpour, Department of Oncologic Science, Division of Hematopathology and Laboratory Medicine, Moffitt Cancer Center and Research Institute, Rm 2071, 12902 Magnolia Dr. HL MCC, Tampa 33612, FL. E-mail: mojdeh.naghashpour@moffitt.org

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Validation of the mantle cell lymphoma international prognostic index: A single-center retrospective analysis

Stephen D. Smith,^{1*} Eric Hsi,² Brian Bolwell,¹ Brad Pohlman,¹ Robert Dean,¹ Meagan Effinger,¹ Amanda Maggiotto,¹ and John Sweetenham¹

Mantle cell lymphoma (MCL) is a unique, recently recognized entity with a variable clinical course [1]. Its historical grouping as an indolent lymphoma belies its common behavior, which is generally aggressive and incurable, with a median survival of about 5 years with modern therapy [2]. In an effort to improve outcomes in MCL, intensified therapies, including the rituximab plus HyperCVAD regimen [3] and incorporation of hematopoietic progenitor cell transplantation (HPCT) [4–6], have been used. However, although capable of inducing remissions in most patients, there is little proof that intensive first-line therapy prolongs overall survival (OS), and a continual relapse pattern is commonly observed [4,7–10].

Progress in developing therapies and defining a first-line standard of care has been impeded by the absence of a disease-specific prognostic model for MCL. The International Prognostic Index (IPI) [11] and Follicular Lymphoma International Prognostic Index (FLIPI) [12] have been applied to MCL, but have limitations. Several studies have shown that the IPI as a whole fails to predict OS in MCL [13–16]. The FLIPI was shown superior to the IPI in a study of 92 patients from the Danish Lymphoma Registry [15]. In this study, number of nodal sites >4 was the strongest negative predictor of survival on multivariate analysis, though this was not validated in a more recent report [17]. The MIPI (Mantle Cell International Prognostic Index) was developed by Hoster et al. in 2008 to fill the void of disease-specific prognostication for MCL [17]. After analyzing 455 patients on prospective German and European studies, they found WBC count, age, and LDH and ECOG performance status as dominant prognostic factors. These parameters were combined into a formula to calculate the MIPI score. Three risk groups were identified at low,

intermediate, and high-risk for mortality with median survival not reached, 51, and 29 months, respectively at the time of analysis. A simplified MIPI, requiring no calculation, was also presented and had high concordance with the quantitative score.

The reports confirming the MIPI have since emerged but vary with respect to their methods, the characteristics of populations studied, and findings regarding impact on progression-free survival (PFS) and OS [6,18–24]. To further explore the MIPI, we reviewed outcomes of patients diagnosed with MCL from 1998 to 2008 at the Cleveland Clinic Taussig Cancer Institute (CCTCI). Both quantitative and simplified MIPI models were applied, as was the IPI, and analysis of event-free (EFS) and OS was undertaken.

Results

Characteristics of the patients and therapy are described in Table 1. Median follow-up of survivors is 5.4 years (range, 8 days–11.2 years). Most patients (68%) were treated with an anthracycline-based regimen, and rituximab was used in 62%. HyperCVAD was given in 16 (34%), and 11 (23%) underwent HPCT (1 allograft, 10 autograft) as part of first-line therapy.

Median OS and EFS among 47 study patients is 3.9 and 2.5 years, respectively (Supporting Information Figs. 1 and 2). Median OS of all MCL patients (n = 82) identified in the initial cohort was similar. 4.0 years. Univariate analysis showed an adverse impact of age > 60 (P = 0.02), LDH > upper limit of normal (P = 0.02), and ECOG score > 1 (P = 0.005) on OS. Improved OS on univariate analysis was seen with HPCT anytime (P = 0.005) and anthracycline anytime (P = 0.05). No impact of high-intensity (Hyper-

TABLE I. Patient and Treatment Characteristics (n = 47)

Parameter	Quantity
Median age, years (range)	61 (39–85)
Sex, no. male (%)	32 (72%)
ECOG 0–1 (%), ECOG 2–4 (%)	35 (74%), 12 (26%)
Blasticoid histology (%)	6 (13%)
Stage IV	45 (96%)
Median WBC count, 109/ μ L (range)	8.6 (2.8–522)
Elevated LDH (normal < 220/ μ L)	27 (57%)
Nonmarrow extranodal involvement	18 (38%)
IPI low/low-inter/high	8 (17%)/14 (30%)/12 (26%)/13 (28%)
MIPI low/intermediate/high	15 (32%)/12 (26%)/20 (43%)
Median time to treatment (range)	27 days (0–1,704)
Time to treatment > 90 days	6 (13%)
First-line treatment	
Anthracycline-based (CHOP, CNOP, HyperCVAD)	32 (68%)
CVP or chlorambucil	9 (19%)
Nucleoside analog	4 (9%)
Rituximab, single agent	2 (4%)
Rituximab during induction	29 (62%)
Rituximab at any point during therapy	36 (77%)
Autologous transplantation (autologous HPCT):	10 (21%); 5 (11%)
As induction; second line or later	
Allogeneic transplantation	4 (9%)
HyperCVAD induction/HyperCVAD then HPCT	16 (34%), 8 (17%)
"Intensive" induction therapy (involving HPCT or HyperCVAD)	18 (38%)
Primary refractory disease	5 (11%)

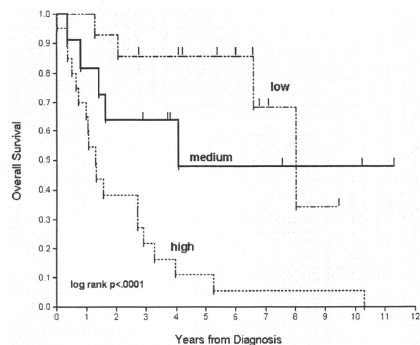


Figure 1. Overall survival by MIPI risk group (n = 47).

CVAD and/or HPCT) first-line therapy on OS was observed ($P = 0.1$). A trend toward an improved OS was seen with administration of rituximab at any point during therapy (rituximab "anytime," $P = 0.06$). Multivariate Cox regression analysis for OS was performed, taking into account age > 60, LDH > ULN, ECOG > 1, and HPCT at any time; no feature remained prognostic.

The MIPI delineated three unique groups (see Fig. 1), including a high-risk group with a 5-year Kaplan-Meier OS estimate of 11% versus 47% for intermediate and 86% for low-risk MIPI patients. The simplified MIPI also distinguished three distinct groups, but categorized six cases as intermediate risk that were designated low risk by the quantitative score (Supporting Information Fig. 3). Nonetheless, concordance was high with a weighted kappa value of 0.81 (95% CI 0.69–0.93) (Supporting Information Table I). The quantitative MIPI predicted EFS as well (log rank $P = 0.0002$). The MIPI maintained its prognostic capacity among 16 HyperCVAD-treated patients, despite few observations and noting that 8 of 16 also underwent HPCT (log rank $P = 0.01$). The MIPI also distinguished outcomes among those undergoing upfront HPCT (log rank $P = 0.03$). The IPI distinguished low- and high-risk groups, but low-int and high-int groups were closely approximated (Supporting Information Fig. 4).

Discussion

This study shows that the MIPI calculated at diagnosis is prognostic of OS and EFS in a cohort of MCL patients diagnosed at CCTCI from 1998 to 2008. The median survival of 3.9 years and continuous relapse risk (43%) observations from other series. A larger proportion of our patients (85%) fell into the high-risk group than most other studies. The median age, WBC count, and degree of LDH elevation herein resemble other MIPI studies [17,21,22,24], but a higher percentage (26%) of our patients had an ECOG score > 1, which may explain this finding. The MIPI identified three distinct prognostic groups, including a high-risk group with a dismal 5-year OS of 11%. Although the IPI also predicted OS in our series, intermediate-risk groups were poorly distinguished. This resembles findings of the initial MIPI publication [17]. The simplified MIPI was also predictive of OS and showed high concordance with the quantified MIPI score.

The small sample size and low statistical power limit the ability to define meaningful associations between treatment and outcomes. With this caveat, HPCT at any point did not impact OS on multivariate analysis (HR 0.4, $P = 0.09$). Although recent results from the European MCL Network showed first-relapse HPCT to prolong survival over CHOP-based regimens [8,18], and Nordic and American groups have reported high survival rates among patients receiving first-line immunochemotherapy and HPCT [5,6,25], the rapid emergence of effective novel agents and challenges in performing randomized studies of HPCT cast uncertainty on its optimal use.

The MIPI offers several advantages. First, it relies only on common laboratory studies and the ECOG performance score, a practical advantage for multicenter studies. The IPI and FLIPI depend on detection of extranodal disease sites and enumeration nodal sites (respectively), which in turn depend on the initial staging investigations applied. For example, radiographic imaging using PET, or routine biopsy of the gastrointestinal tract, would be expected to detect more sites of MCL involvement than CT imaging alone [26,27]. As it bypasses these studies, the MIPI may be a more universal applicable parameter. Second, other indices (not developed specifically for MCL) have demonstrated shortcomings in estimating prognosis in this disease [13–15]. Similar to the initial MIPI study [17], we found a lack of separation of the two intermediate-risk groups in the IPI. Despite its superiority to the IPI, the FLIPI failed to separate low- and intermediate-risk groups in a subsequent analysis [15,17]. The FLIPI was not assessed in this study given absence of data regarding nodal distribution of disease at diagnosis.

Lastly, the prognostic capacity of the MIPI in terms of OS has now been validated by various studies over the last 2 years. [6,7,19–22,24] Although a report of patients treated with rituximab and HyperCVAD failed to validate the MIPI, this brief report (published as correspondence [28]) used only the simplified MIPI, lacked statistical power, and reported a group with uniquely good short-term outcomes (see [29]). In contrast, in addition to the impact of the MIPI score on OS, our study and several others have recently found the MIPI predicts relapse parameters as well (EFS, PFS, or time to treatment failure) [6,18,21,22]. The quantitative and simplified MIPI showed good concordance, though the simplified MIPI identified a larger number of intermediate risk. In sum, our work confirms the value of the MIPI over the IPI and finds that quantitative and simplified forms have good concordance. In conjunction with other reports, this study suggests that MIPI is well suited for use for risk stratification in clinical trials of novel treatments and investigation of biomarkers toward the common goal of developing curative therapy for MCL.

Methods

Cases of MCL diagnosed at CCTCI from 1998 to 2008 were identified from our pathology database, yielding 85 unique patients. These subjects were retrospectively analyzed with approval of our Institutional Review Board. Forty-seven patients with advanced-stage disease, and for whom adequate data for assignment of both MIPI and IPI existed, were the subject of review. The MIPI score was calculated at diagnosis according to the quantitative algorithm of Hoster [17]. The simplified MIPI was also calculated. Immunohistochemical evaluation of proliferation index (Ki67) was not available for most cases, so was not included.

Survival was identified from medical records and confirmed using a public social security database. For purposes of estimating EFS, an event was defined as failure to achieve at least partial remission, relapse, or death from any cause. Outcomes were compared using log-rank analysis of Kaplan-Meier survival analyses. Univariate analysis was used to assess individual

candidate prognostic factors using Pearson's χ^2 test. Factors independently affecting OS were sought using a Cox proportional hazards regression model. Concordance between the quantitative and simplified MIPI scores was assessed via weighted kappa analysis. JMP v8.0 was used for statistical calculations.

¹Cleveland Clinic Department of Hematologic Oncology and Blood Disorders;

²Cleveland Clinic Department of Clinical Pathology

*Correspondence to: Stephen D. Smith, Taussig Cancer Institute R35,

Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195

E-mail: smiths11@ccf.org

Additional Supporting Information may be found in the

online version of this article.

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High Incidence of Human Herpes Virus 6-Associated Encephalitis/Myelitis following a Second Unrelated Cord Blood Transplantation

Yasuo Mori,^{1,2} Toshihiro Miyamoto,¹ Koji Nagafuji,¹ Kenjiro Kamezaki,¹ Asataro Yamamoto,¹
Noriyuki Saito,^{1,2} Koji Kato,¹ Katsuto Takenaka,¹ Hiromi Iwasaki,² Naoki Harada,¹
Yasunobu Abe,³ Takanori Teshima,² Koichi Akashi^{1,2}

Human herpes virus (HHV)6-associated limbic encephalitis and/or myelitis is one of the life-threatening central nervous system complications following allogeneic hematopoietic stem cell transplantation (HSCT). Recent reports have shown significant correlations of these complications with unrelated cord blood transplantation (UCBT). We retrospectively analyzed 228 allogeneic HSCT recipients in our single institution; 13 patients (5.7%) were diagnosed with HHV6-associated encephalitis/myelitis. This complication was documented in 8 of 51 UCBT recipients (15.7%) and 5 of 177 recipients (2.8%) transplanted with bone marrow or peripheral blood stem cells, indicating a higher incidence of this complication occurring in UCBT recipients ($P = .0005$). In addition, HHV6-associated encephalitis/myelitis occurred more frequently in recipients who underwent 2 or more HSCTs (7 of 59 recipients [11.9%]), compared to those who received only 1 HSCT (6 of 169 recipients [3.6%], $P = .018$). Of note, the incidence of this complication increased to 28.6% (6 of 21 recipients), when the analysis was restricted to a second or more UCBT recipients. All 13 patients presented preengraftment immune response prior to the onset of encephalitis. Two patients manifested typical symptoms at the onset of HHV6-associated encephalitis/myelitis, such as memory dysfunction, disorientation, and consciousness disturbance. However, 4 patients presented only with dysesthesia and pruritus, described as typical manifestations of patients with calcineurin-inhibitor-induced pain syndrome (CIPS), and the remaining 7 showed both symptoms, indicating that CIPS-like symptoms might be manifestations of HHV6-associated myelitis. Thus, physicians should be alert to this rare but often fatal complication, particularly for those who receive 2 or more HSCTs using UCBT.

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INTRODUCTION

Human herpes virus (HHV)6 causes roseola infantum in infants, acute nonspecific febrile illness in younger children, and can give rise to an infectious mononucleosis-like syndrome in adults [1]. HHV6

can be reactivated under conditions of severe immunosuppression, and is recognized as an opportunistic and potentially life-threatening pathogen for allogeneic hematopoietic stem cell transplantation (HSCT) recipients [2-8]. Many investigators have reported various clinical manifestations of HHV6 reactivation in the central nervous system (CNS), such as short-term memory dysfunction, disorientation, consciousness disturbance, hyponatremia [6,9], or diabetes insipidus [10]. Diagnosis of these complications in the CNS can be made using specific techniques, such as abnormal high-intensity signals in bilateral temporal lobes (limbic area) on T2-weighted and/or fluid-attenuated inversion recovery sequences from magnetic resonance imaging (MRI), and detection of HHV6 DNA in the cerebrospinal fluid (CSF) by the polymerase chain reaction (PCR) method.

Recent reports have suggested that HHV6 reactivation is more likely to occur in recipients of unrelated cord blood transplantation (UCBT) than in those who

From the ¹Department of Medicine and Biosystemic Science; ²Center for Cellular and Molecular Medicine; and ³Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

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Correspondence and reprint requests: Toshihiro Miyamoto, MD, PhD, Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan (e-mail: toshmiya@intmed1.med.kyushu-u.ac.jp).

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receive allogeneic bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT) [6,11,12]. These findings may be because of the absence of primed HHV6-specific T cells and the immunologic immaturity of UCBT [11-14]. Because of the increasing preference for UCBT procedures because of the rapid availability of stored transplantable units, we should pay more attention to HHV6-associated encephalitis/myelitis for UCBT procedures.

In this study, we described 13 patients with HHV6-associated encephalitis/myelitis among 228 allogeneic HSCT recipients. We found a higher incidence of HHV6-associated encephalitis/myelitis for UCBT recipients (8 of 51, 15.7%) than for BMT and PBSCT recipients (5 of 177, 2.8%, $P = .0005$). In addition, the incidence of HHV6-associated encephalitis/myelitis increased to 28.6% for recipients who received 2 or more UCBTs (6 of 21). We also identified the factors associated with the onset of HHV6-associated encephalitis/myelitis in this study.

PATIENTS AND METHODS

Patients

The medical records of all patients who underwent allogeneic HSCT at the Kyushu University Hospital between January 2002 and October 2009 were reviewed. A total of 228 patients (136 men, 92 women) (median age = 47 years), were studied. Patients' characteristics are listed in Table 1. Primary diseases included myelodysplastic syndrome (MDS)/acute myelogenous leukemia (AML) ($n = 92$), chronic myelogenous leukemia (CML) ($n = 13$), acute lymphoblastic leukemia (ALL) ($n = 31$), malignant lymphoma (ML) ($n = 57$), aplastic anemia (AA) ($n = 14$), and others ($n = 21$). A total of 101 cases were considered early state defined as follows: acute leukemia (AML and ALL) in remission; CML in chronic phase (CP); MDS classified as refractory anemia (RA) or RA with ringed sideroblasts. All others ($n = 127$) were considered as nonearly state. This study was approved by the institutional review board of Kyushu University Hospital.

Transplantation Procedures

A total of 133 patients had received conventional preparative regimens comprised of either total body irradiation (TBI)/cyclophosphamide (Cy) ($n = 91$), or busulfan (Bu)/Cy ($n = 42$) (Table 1). The remaining 95 cases had received purine analog-based reduced-intensity conditioning (RIC) regimen comprised of either fludarabine (Flu)/Cy ($n = 16$), Flu/Bu ($n = 48$), or Flu/melphalan ($n = 31$). Low-dose TBI (2-4 Gy), antithymocyte globulin (ATG), and alemtuzumab were administered in 49, 3, and 3 cases, respectively.

The sources of stem cells were related granulocyte colony-stimulating factor (G-CSF)-mobilized PB ($n = 71$), related BM ($n = 15$), unrelated BM ($n = 91$), or unrelated CB ($n = 51$). HLA-matching varied from haploidentical (3/6) to identical (6/6). A total of 59 recipients had received 2 or more HSCTs as listed in Table 1: the source of stem cells for the first transplant were 24 autologous and 35 allogeneic ones. The reasons for a second or more HSCT were disease relapse ($n = 50$) and graft failure ($n = 9$). Graft-versus-host-disease (GVHD) prophylaxis was as follows: 20 received calcineurin-inhibitor (CI) alone; 193 received CI plus short-term methotrexate (MTX); and 15 received CI plus mycophenolate mofetil (MMF).

Diagnosis of HHV6-Associated Encephalitis/Myelitis

HHV6-associated encephalitis/myelitis was directly proved when HHV6 viral DNA was detected in the CSF, as previously reported [15]. For those patients who were unable to undergo lumbar puncture because of severe thrombocytopenia or a deteriorated general condition, we diagnosed HHV6-associated encephalitis/myelitis if they satisfied more than 2 of the following 3 criterion: (1) typical clinical manifestations; (2) detection of HHV6 viral DNA in PB, previously reported as a predictive marker for developing HHV6-associated encephalitis/myelitis [16]; or (3) limbic encephalopathy based on the selective involvement of the medial temporal lobe on MRI. Routine monitoring of HHV6 viral load of PB by PCR was not performed in this study.

Statistical Analysis

The aim of this study is to clarify the factor correlated with developing HHV6-associated encephalitis/myelitis. For univariate comparisons, we examined categorical variables, including age, sex, underlying diseases, disease status, conditioning regimen, stem cell source, HLA matching, GVHD prophylaxis, and prior HSCT using the chi-square test. The incidence of HHV6-associated encephalitis/myelitis is generally very low. The risk of type 1 error has been known to increase when the event per variable is <10 ; we narrowed an analysis to 1 risk factor selected by -2 log likelihood method to avoid it. Odds ratios were calculated using logistic regression analysis. Survival following allogeneic HSCT was measured from the date of stem cell infusion until the date of death. The survival period was calculated using the Kaplan-Meier method. P -values $< .05$ were considered to indicate statistical significance. All statistical analyses used SPSS 17.0 program (SPSS Japan Inc., Tokyo, Japan).

Table 1. Characteristics of the 228 Patients

Characteristics	Total	HHV6 Encephalitis/Myelitis		P-value
		Yes (n = 13)	No (n = 215)	
Age, median (range)	47 (16-68)	48 (29-65)	47 (16-68)	NS
Sex (male/female)	136/92	10/3	126/89	NS
Underlying disease				NS
MDS/AML	92	7	85	
CML	13	0	13	
ALL	31	1	30	
ML	57	4	53	
AA	14	1	13	
Others	21	0	21	
Disease status at transplantation				NS
Early*	101	4	97	
Nonearly	127	9	118	
Conditioning regimen				.038
Conventional	133	4	129	
Reduced-intensity	95	9	86	
Stem cell source				.0005
Related PB	71	2	69	
Related BM	15	0	15	
Unrelated BM	91	3	88	
Unrelated CB	51	8	43	
Times and sources of HSCT				.018
First	169	6	163	
non-UCB	139	4	135	
UCB	30	2	28	
Second / first source	55	6	49	
non-UCB / Auto	18	1	17	
/ non-UCB	14	0	14	
/ UCB	4	0	4	
UCB / Auto	4	0	4	
/ non-UCB	7	4	3	
/ UCB	8	1	7	
Third / second source	4	1	3	
Non-UCB / Auto	1	0	1	
/ non-UCB	1	0	1	
UCB/ non-UCB	2	1	1	
HLA matching				.012
≤4/6	52	6	46	
5/6	47	4	43	
6/6	129	3	126	
GVHD prophylaxis				<.0001
CI alone	20	2	18	
CI plus sMTX	193	5	188	
CI plus MMF	15	6	9	
IgG-antibody for HHV6 (titer)				NS
negative	0	0	0	
×10-×20	45	3	42	
×40-×80	125	7	118	
×160-	26	1	25	
unknown	32	2	30	

CI indicates calcineurin-inhibitor, including cyclosporine and tacrolimus; NS, not significant; CML, chronic myelogenous; MDS/AML, myelodysplastic syndrome/acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; AA, aplastic anemia; MTX, methotrexate; MMF, mycophenolate mofetil; PB, peripheral blood; BM, bone marrow; CB, cord blood; HSCT, hematopoietic stem cell transplantation; UCB, unrelated CB; GVHD, graft-versus-host disease; non-UCB included related PB, related BM, and unrelated BM.

*Early stage is defined as: acute leukemia in remission; CML in chronic phase; MDS classified as refractory anemia or refractory anemia with ringed sideroblasts; lymphoma in remission. All others are considered nonearly stage.

RESULTS

Incidence of HHV6-Associated Encephalitis/Myelitis

In our series, a total of 13 patients among 228 allogeneic HSCT recipients (5.7%) developed HHV6-associated encephalitis/myelitis after allogeneic HSCT. Median onset was 23 days after transplantation, in line with the previous multicenter retrospective research in Japan [17]. HHV6 viral DNA was detected in CSF samples of 9 patients, whereas the remaining 4 were diagnosed with HHV6-associated encephalitis/myelitis by satisfying the criteria for clinical features and MRI findings (n = 1), clinical features and PCR assay of PB (n = 2), or all 3 criteria (n = 1). There was no statistical difference among the underlying diseases. A previous report has demonstrated a close association between a lower titer of anti-HHV6 IgG (<×40) and development of HHV6 reactivation [14], however, in our study, there was no significant relationship between them (Table 1).

Seven of 59 (11.9%) patients who underwent 2 or more HSCTs developed HHV6-associated encephalitis/myelitis, which was significantly more frequent than in the 6 of 169 (3.6%) patients who received a first HSCT (P = .018). A high incidence of HHV6-associated encephalitis/myelitis was also found in patients who received UCBT compared to those who received a non-UCBT (8 of 51, 15.7% versus 5 of 177, 2.8%; P = .0005). Moreover, the incidence of HHV6-associated encephalitis/myelitis increased to 28.6% (6 of 21), when the analysis was restricted to those patients who received a second or more UCBT, which was significantly more frequent than those who received a second non-UCBT (1 of 38; P = .003). Patients who received HSCTs from HLA-mismatched donors developed HHV6-associated encephalitis/myelitis more frequently than those with HLA-matched donors (10 of 99, 10.1% versus 3 of 129, 2.3%; P = .012). This may reflect the fact that most UCBT recipients received transplants using HLA-mismatched cord blood. We also found a relatively high incidence of HHV6-associated encephalitis/myelitis in those patients who underwent Flubased RIC (9 of 95) or CI and MMF for the prevention of GVHD (6 of 15), as UCBT recipients preferentially received RIC and GVHD prophylaxis with CI and MMF in our institution.

Clinical Features of HHV6-Associated Encephalitis/Myelitis

Clinical and laboratory findings for the 13 patients at the onset of HHV6-associated encephalitis/myelitis are shown in Table 2. The median onset of clinical symptoms was 23 days (range: 14-614 days). Symptoms occurred within 10 days of their neutrophil engraftments (>0.5 × 10⁹/L) in 10 patients, whereas

the remaining 3 patients developed clinical manifestations more than 10 days after their engraftments. Prior to the onset of HHV6-associated encephalitis/myelitis, immunosuppressive treatments had commenced for 7 patients which comprised of >1 mg/kg of methylprednisolone against GVHD or hemophagocytic syndrome (HPS).

The cardinal features of HHV6-associated encephalitis are well documented: short-term memory dysfunction, disorientation, consciousness disturbance, and seizures [2-7]. In our study, these typical symptoms were found in 2 of the 13 patients, whereas 4 developed only systemic pruritus with no apparent skin rash and intermittent pain in the extremities, which were similar to the reported manifestations of CI-induced pain syndrome (CIPS) [18,19]. Both encephalitis and CIPS-like symptoms were found in the remaining 7 patients.

Positive results for head MRI scans (limbic encephalitis) were observed in 7 of 9 patients (77.8%) who developed encephalitis-type symptoms, which were not found in the 4 patients who only had CIPS-like symptoms; only 1 presenting with CIPS-like symptoms had positive results for spinal MRI. Interestingly, all patients who developed HHV6-associated encephalitis/myelitis had noninfectious fevers before their engraftments known as a "preengraftment immune reaction" (PIR) or "hyperacute GVHD" [13,20], and 3 of them subsequently developed HPS (shown in Table 2).

HHV6 viral DNA was detected in CSF samples of all 9 patients who were assessed. The viral copy levels varied from 2×10^2 to 2×10^5 copies/ μ L (Table 2); however, these did not correlate with the degree of sequelae or the presence of definite abnormal findings on MRI.

Treatment and Outcomes

Nine of 13 patients were treated with antiviral combination therapy using gancyclovir (GCV; 5-10 mg/kg/day) and foscarnet (FCV; 60-180 mg/kg/day). Monotherapy with FCV was initiated for 2 patients, ACV for 1, and GCV for the other. Clinical features were relieved in all but 1 patient treated with GCV plus FCV. However, mild neurologic sequelae remained in all 3 survivors, such as memory impairment, CIPS-like dysesthesia, dysuria, or dyschezia (Table 2).

Ten patients died thereafter; the causes included HHV6-associated encephalitis ($n = 1$), disease progression ($n = 4$), GVHD ($n = 2$), infection ($n = 2$), and donor-derived secondary MDS ($n = 1$). In our study, the 5-year survivals after HSCT were $17.3\% \pm 14.6\%$ for the patients with HHV6-associated encephalitis/myelitis, and $37.2\% \pm 3.9\%$ for the patients without this complication. Although no significant difference in survival was noted ($P = .31$), patients with HHV6-

associated encephalitis/myelitis tended to have poorer outcomes, as previously reported [21,22].

Risk Factors for HHV6-Associated Encephalitis/Myelitis

From the univariate analysis using logistic regression, HHV6-associated encephalitis/myelitis were strongly associated with the stem cell source (CBT versus others; $P < 0.001$). Multivariate analysis confirmed that a second or more UCBT was the only independent risk factor for HHV6-associated encephalitis (odds ratio = 13.58; 95% confidence interval = 3.45-53.5; $P < .001$) (Table 3).

DISCUSSION

A second HSCT may be the only way to ensure survival in some patients with hematologic malignancies that relapse after the first HSCT. UCB has become increasingly preferred as the source of graft for a second HSCT because of the rapid availability of stored transplantable units. However, using UCB presents an increased risk of graft failure and infectious complications. HHV6-associated encephalitis/myelitis is one of the most life-threatening CNS complications after UCBT. In this study, HHV6-associated encephalitis/myelitis occurred more frequently for UCBT recipients (15.7%) compared to BMT and PBSCT recipients (2.8%), and the incidence of HHV6-associated encephalitis/myelitis increased to 28.6% for recipients who received 2 or more UCBTs, although this observation should be interpreted with caution and confirmed in a prospective study because of the limited number of patients. We should also pay attention to the possibility that this result might reflect the selection bias; the patients requiring a second UCBT had more aggressive disease than those who require second non-UCBT and were, by nature, more susceptible to various infections including HHV6 reactivation. In addition to the absence of primed HHV6-specific T cells with UCBT, the high incidence of HHV6-associated encephalitis/myelitis after 2 or more UCBTs may be explained by a prolonged immune deficiency caused by multiple HSCTs as well as by HLA mismatches between donors and recipients [11-14]. CMV antigen tests were positive in 10 of 13 patients with HHV6-associated encephalitis/myelitis (data not shown), which might also suggest the impaired immune function as well. In our study, the all-cause mortality within 100 days for second or more UCBT recipients who developed this complication reached 50%, indicating that HHV6-associated encephalitis/myelitis is a common serious problem, especially following a second or more UCBT.

Several reports have found that PIR often preceded engraftment and that HPS frequently co-occurred in those UCBT recipients who were treated with RIC

Table 2. Clinical, Laboratory, and Radiographic Characteristics of HHV6-Associated Encephalitis/Myelitis

Patient	Graft Source	Prior HSCTs		Conditioning	Prior		Onset (Day)	Types of Clinical Manifestations	HHV6 Viral Load in CSF/PB (Copies/mL)	Localization on MRI	Antiviral Treatment	Outcome
		(Indication of Current HSCT)	—		PRK / HPS	PRK / HPS						
1	1st RBSC	—	—	Conv	+/-	6/14	encephalitis	2 × 10 ⁴ / N/A	limbic	GCV	survive with memory impairment	
2	1st RBSC	—	—	Conv	+/+	24	combined	N/A / 7 × 10 ³	limbic	GCV + FCV	dead by relapsed AML	
3	1st URBM	—	—	Conv	+/+	61	encephalitis	2 × 10 ⁴ / N/A	None	GCV + FCV	dead by GVHD	
4	1st URBM	—	—	Conv	+/-	19	combined	2 × 10 ⁴ / N/A	limbic	GCV + FCV	dead by GVHD	
5	1st URBM	—	—	RIC	+/+	23	combined	2 × 10 ⁴ / N/A	limbic	GCV + FCV	dead by sepsis	
6	1st URBM	—	—	RIC	+/+	19	CIPS-like	5 × 10 ⁴ / N/A	None	FCV	survive with CIPS-like dyesthesia	
7	2nd URBM	Auto (relapse)	—	RIC	+/-	22	combined	N/A / 4 × 10 ³	None	GCV + FCV	dead by sepsis	
8	2nd URBM	URBM (relapse)	—	RIC	+/+	35	combined	N/A / negative	limbic	ACV	dead by HHV6 encephalitis	
9	2nd UCB	RBSC (relapse)	—	RIC	+/+	27	combined	9 × 10 ³ / 3 × 10 ³	limbic	GCV + FCV	dead by donor-derived MDS	
10	2nd UCB	UCB (relapse)	—	RIC	+/+	19	combined	2 × 10 ⁷ / 1 × 10 ⁴	limbic	GCV + FCV	dead by relapsed AML	
11	2nd UCB	URBM (relapse)	—	RIC	+/+	23	CIPS-like	N/A / 8 × 10 ³	None	FCV	dead by relapsed AML	
12	2nd UCB	URBM (relapse)	—	RIC	+/+	14	CIPS-like	1 × 10 ⁷ / 8 × 10 ³	None	GCV + FCV	survive with CIPS-like dyesthesia	
13	2nd UCB	Auto / URBM (relapse)	—	RIC	+/-	17	CIPS-like	2 × 10 ⁷ / N/A	None	GCV + FCV	dead by relapsed AML	

RBSC indicates related peripheral blood stem cell; UCB, unrelated cord blood; URBM, unrelated bone marrow; Conv, conventional; RIC, reduced-intensity conditioning; PRK, preengraftment immune reaction; HPS, hemophagocytic syndrome; CIPS, calcineurin-induced pain syndrome; CSF, cerebrospinal fluid; N/A, not assessed; FLAIR, fluid-attenuated inversion recovery; ACV, acyclovir; GCV, gancyclovir; FCV, foscavir; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; AML, acute myelogenous leukemia; MRI, Magnetic resonance imaging; PB, peripheral blood.

(RIC-UCBT), and that PIR and HPS were characterized by symptoms possibly induced by hypercytokinemia [23-25]. In the setting of RIC-UCBT, an HLA disparity as well as residual recipients' immunocompetent cells may augment allo-immune mediated events, such as pro-inflammatory cytokine storms, which result in the development of PIR and/or HPS [6,23-25]. Our study also demonstrated that RIC-UCBT recipients developed PIR more frequently (23 of 31, 74.2%) than UCBT recipients who underwent conventional conditioning (5 of 20, 25%). Several investigators have also found a higher incidence of HHV6-associated encephalitis/myelitis that has been documented for RIC-UCBT recipients with complications like PIR or HPS [6,25]. In fact, in our study, all 13 patients with HHV6-associated encephalitis/myelitis had suffered from PIR prior to the onset of encephalopathy, and 3 of them subsequently developed severe HPS. In contrast, among 43 UCBT recipients who did not develop HHV6-associated encephalitis/myelitis, PIR and HPS were observed less frequently in 20 (46.5%) and 0 (0%), respectively, indicating that the development of HHV6-associated encephalitis/myelitis might be closely associated with PIR or HPS. In line with our observations, Ogata et al. [26] recently reported that episodes of PIR or GVHD were closely correlated with the development of HHV6-associated encephalitis. In addition, they also demonstrated a significant increase of serum interleukin (IL)-6 levels, particularly in those patients preceding HHV6 reactivation and progression to encephalopathy, suggesting that a high IL-6 level might be critical for the development of HHV6-associated encephalitis/myelitis. Several reports previously demonstrated high levels of IL-6 in the sera and cerebral spinal fluids of patients with aseptic meningitis/encephalitis, indicating the possible participation of IL-6 in the pathogenesis of meningitis/encephalitis [27-29]. The contributions of IL-6 to the development of meningitis/encephalitis may include epithelial and endothelial injuries, increased permeability in the blood-brain barrier, or apoptosis in the cerebrum resulting from a hyper immune reaction [26,30,31]. We also observed increased levels of IL-6 in the majority of the patients with PIR (unpublished data). Thus, PIR or HPS may augment excessive immune reactions when combined with these observations, such as high IL-6 secretion, which partially contribute to the subsequent development of HHV6-associated encephalitis/myelitis.

CIPS is a newly established disease entity that is characterized by severe pain, typically in the lower limbs. CIPS is hypothesized to result from calcineurin-induced vascular changes that disturb bone perfusion and permeability, which leads to intrasoone vasoconstriction and BM edema [18,19]. This rare syndrome has been described in organ transplant recipients [32-35], where CIs must be

Table 3. Results of a Logistic Regression Analysis

Characteristics	Univariate		Multivariate (stepwise)	
	Odds Ratio (95% Confidence Interval)	P-Value	Odds Ratio (95% Confidence Interval)	P-Value
Age	1.000 (0.954-1.048)	.988		
Sex	1.799 (0.464-6.982)	.396		
Underlying disease	1.312 (0.388-4.443)	.662		
Disease status	1.385 (0.393-4.876)	.612		
Conditioning regimen	2.491 (0.707-8.777)	.155		
Stem cell source				
non-CB	1 (reference)	—	1 (reference)	—
CB (previous HSCT -)	1.052 (0.119-9.313)	.964	1.052 (0.119-9.313)	.964
CB (previous HSCT +)	13.58 (3.447-53.52)	<.001	13.58 (3.447-53.52)	<.001
HLA	1.933 (0.542-6.889)	.309		
GVHD prophylaxis				
CI alone	1 (reference)	—		
CI plus sMTX	0.197 (0.035-1.107)	.065		
CI plus MMF	2.800 (0.427-18.38)	.283		

GVHD indicates graft-versus-host disease; CB, cord blood; HSCT, hematopoietic stem cell transplantation; MTX, methotrexate; MMF, mycophenolate mofetil; CI, calcineulin-inhibitor.

maintained at a high concentration, whereas several cases of CIPS have been reported for patients undergoing HSCT [18,19,36-39]. Moreover, in our study, 11 of 13 patients with HHV6-associated encephalitis/myelitis developed CIPS-like dysesthesia and/or pruritus. Although CI concentrations in these patients were maintained within the targeted ranges and reduction in concentrations was ineffective, HHV6 DNA was directly detected in CSF samples and administration of antiviral agents improved their symptoms, indicating the relation of the symptoms to the reactivation of HHV6. We found that 7 cases that developed CIPS following HSCT have been reported to date [18,19,36-39]; 3 developed CIPS after UCBT, and 4 developed CIPS after a second HSCT: 2 for UCBT and 2 for BMT, respectively. In addition, Kida et al. [38] showed that no possible cases of CIPS were observed among 189 allogeneic BMT and 65 allogeneic PBSCT recipients, whereas 2 of 34 cases that underwent UCBT developed CIPS. As shown in our study, patients who receive UCBT or a second HSCT have a high risk of occurrence of HHV6-associated encephalitis/myelitis. Based on these observations, we hypothesize that HHV6 reactivation might contribute to aggravation of CIPS, and therefore, CIPS might be one of the early manifestations of HHV6-associated encephalitis/myelitis. HHV6 reactivation, localized in the posterior horn of the spinal cord, where signals for both itch and pain sensations are transmitted via α -fibers and $A\delta$ -fibers [40], may cause distinctive manifestations like CIPS. Indeed, all 4 of the cases who only developed CIPS-like dysesthesia and/or pruritus had positive results for HHV6 in their CSF samples, but not for head MRI (limbic encephalitis), and only 1 case with MRI scanning of the spine (case #13) showed multiple abnormal T2-high lesions. Because HHV6-associated encephalitis/myelitis and CIPS can often occur at 2 to 4 weeks posttransplantation, it is important to make a differential diagnosis of these 2 syndromes.

In summary, our results have clearly shown that 2 or more RIC-UCBT presents a high risk for the development of HHV6-associated encephalitis/myelitis, although there are the limitations of the retrospective single center analysis. Earlier diagnosis of and intervention for this complication with combination antiviral therapy may provide prolonged survival and neurologic benefits. Therefore, transplantation physicians should be aware that CIPS-like dysesthesia and pruritus might be early manifestations that correlate with the reactivation of HHV6, especially for patients who develop myelitis. These “high-risk” recipients can be potential candidates for prophylactic administration of antiviral agents in future investigations.

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TIM-3 Is a Promising Target to Selectively Kill Acute Myeloid Leukemia Stem Cells

Yoshikane Kikushige,¹ Takahiro Shima,¹ Shin-ichiro Takayanagi,² Shingo Urata,¹ Toshihiro Miyamoto,¹ Hiromi Iwasaki,¹ Katsuto Takenaka,¹ Takanori Teshima,¹ Toshiyuki Tanaka,³ Yoshimasa Inagaki,² and Koichi Akashi^{1,*}

¹Department of Medicine and Biosystemic Sciences, Kyushu University Graduate School of Medicine, Fukuoka 812-8582, Japan

²Innovative Drug Research Laboratories Kyowa Hakko Kirin Co., Ltd., Tokyo 194-8538, Japan

³School of Pharmacy, Hyogo University of Health Sciences, Kobe 650-8530, Japan

*Correspondence: akashi@med.kyushu-u.ac.jp

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SUMMARY

Acute myeloid leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), an ultimate therapeutic target for AML. Here we identified T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed on LSCs in most types of AML except for acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). TIM-3⁺ but not TIM-3⁻ AML cells reconstituted human AML in immunodeficient mice, suggesting that the TIM-3⁺ population contains most, if not all, of functional LSCs. We established an anti-human TIM-3 mouse IgG2a antibody having complement-dependent and antibody-dependent cellular cytotoxic activities. This antibody did not harm reconstitution of normal human HSCs, but blocked engraftment of AML after xenotransplantation. Furthermore, when it is administered into mice grafted with human AML, this treatment dramatically diminished their leukemic burden and eliminated LSCs capable of reconstituting human AML in secondary recipients. These data suggest that TIM-3 is one of the promising targets to eradicate AML LSCs.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). LSCs self renew and generate leukemic progenitors that actively divide to produce a large number of immature clonogenic leukemic blasts (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994). This hierarchical stem cell-progenitor-mature cell relationships in AML appears to simulate normal hematopoiesis that originates from hematopoietic stem cells (HSCs) with self-renewal activity. We have shown that like normal HSCs, AML LSCs are quiescent *in vivo* and appear to reside at the endosteal "osteoblastic" niche in the bone marrow based on our analysis in a xenograft model (Ishikawa et al., 2007). AML LSCs are resistant to chemotherapeutic reagents that usually target cycling malignant cells. In the majority (~90%) of AML patients, the conventional chemotherapies can diminish the leukemic clones to achieve remission. However,

~60% of such remission patients still relapse, and the recurrence of AML in these patients should originate from LSCs that survive the intensive chemotherapies. Therefore, the LSC should be the ultimate cellular target to cure human AML.

To eradicate the AML LSC without killing normal HSCs, it is critical to isolate a molecule that is expressed or functions specifically at the AML LSC stage (Krause and Van Etten, 2007). It has been shown that the AML LSCs mainly reside within the CD34⁺CD38⁻ fraction of leukemic cells and can reconstitute human AML in immunodeficient mice (Lapidot et al., 1994), although recent studies have suggested that LSCs can exist also in CD34⁺CD38⁺ (Taussig et al., 2008) or CD34⁻ blastic fractions at least in some types of AML (Martelli et al., 2010; Taussig et al., 2010). Normal HSCs with long-term reconstitution activity also have the CD34⁺CD38⁻ phenotype (Bhatia et al., 1997; Ishikawa et al., 2005). However, the expression pattern of other surface molecules in the CD34⁺CD38⁻ fraction of AML cells is different from that of normal controls. For example, CD34⁺CD38⁻ AML cells possess many phenotypic characteristics analogous to normal granulocyte/macrophage progenitors (GMPs) (Yoshimoto et al., 2009). Previous studies have reported molecules preferentially expressed in AML cells. Such molecules include CLL-1 (van Rhenen et al., 2007), CD25, CD32 (Saito et al., 2010), CD33 (Florian et al., 2008; Hauswirth et al., 2007), CD44 (Florian et al., 2006; Jin et al., 2006), CD47 (Jaiswal et al., 2009; Majeti et al., 2009), CD96 (Hosen et al., 2007), and CD123 (Jin et al., 2009; Yalcintepe et al., 2006). However, in our hands, some of these molecules are expressed in LSCs at a level insufficient for clear distinction, are expressed also in normal HSCs at a considerable level, or are found only in a fraction of AML cases. It is therefore critical to isolate ideal targets for AML LSCs with sufficient specificity and sensitivity.

Here we report a new surface molecule that might be useful to eradicate AML LSCs leaving normal HSC intact. We performed differential transcriptional profiling of AML LSCs and HSCs and extracted the T cell immunoglobulin mucin-3 (TIM-3) as a promising AML LSC-specific target surface molecule. TIM-3 is originally found as a surface molecule expressed in CD4⁺ Th1 lymphocytes in mouse hematopoiesis and is an important regulator of Th1 cell immunity and tolerance induction (Monney et al., 2002; Sabatos et al., 2003; Sánchez-Fueyo et al., 2003). Murine TIM-3 is also expressed in CD11b⁺ macrophages and CD11c⁺ dendritic cells and recognizes apoptotic cells' phosphatidylserine through its IgV domain to mediate phagocytosis (Nakayama et al., 2009).

We found that human TIM-3 was expressed in the vast majority of CD34⁺CD38⁻ LSCs and CD34⁺CD38⁺ leukemic progenitors in AML of most FAB types, except for acute

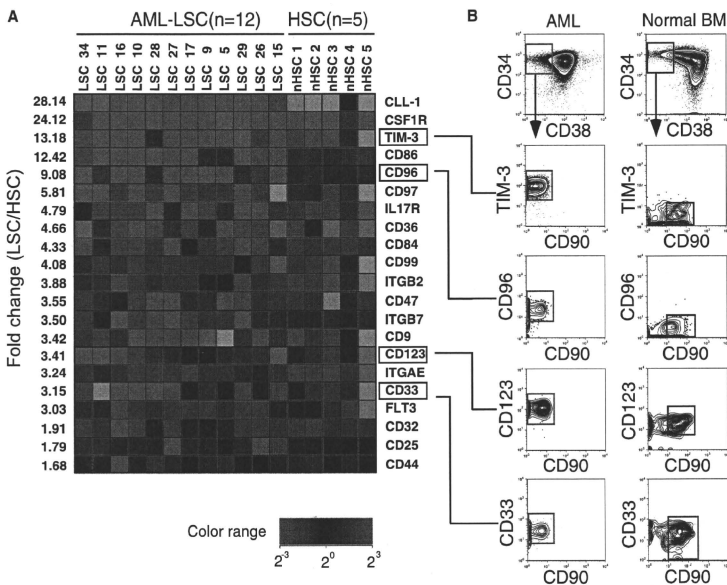


Figure 1. The Expression of LSC-Specific Surface Molecules in AML
 The CD34⁺CD38⁻ adult bone marrow HSCs and CD34⁺CD38⁻ AML LSCs were purified and tested for their surface molecule expression.
 (A) Results of cDNA microarray analysis of LSCs and AML LSCs. Representative genes coding surface molecules that are expressed highly in AML LSCs are shown. TIM-3 is expressed specifically in LSCs at high levels in the majority of AML patients. Patient numbers correspond to those in Table S1.
 (B) The expression of representative surface proteins in HSCs and AML LSCs on FACS.

promyelocytic leukemia (M3). TIM-3 was not expressed in CD34⁺CD38⁻ normal HSCs or the vast majority of CD34⁺CD38⁻ normal progenitors. Administration of anti-human TIM-3 mouse antibodies with a complement-dependent cytotoxicity (CDC) and an antibody-dependent cellular cytotoxicity (ADCC) selectively inhibited engraftment and development of human AML in xenograft models. Our data strongly suggest that the use of TIM-3 to target AML LSCs is a promising approach for the improvement of leukemia therapy.

RESULTS

TIM-3 Is Expressed in the CD34⁺CD38⁻ Fraction of AML Patients' Bone Marrow Cells

In most types of AML, LSCs are concentrated in the CD34⁺CD38⁻ fraction of AML cells (Ishikawa et al., 2007; Lapidot et al., 1994), whose phenotype is common to normal adult HSCs. Patients' characteristics are shown in Table S1 available online. To search for the AML LSC-specific molecules, 10,000 each of purified CD34⁺CD38⁻ AML cells and CD34⁺CD38⁻ normal HSCs were subjected to cDNA microarray analysis. We extracted 256 genes with >4-fold change between normal

HSCs and CD34⁺CD38⁻ AML cells and then selected 197 differentially expressed genes with <0.01 of a cut-off p value (Table S2). Genes coding surface molecules that are expressed highly in CD34⁺CD38⁻ AML cells were selected for this study. Figure 1A shows the mRNA levels of candidate LSC-specific surface molecules in purified CD34⁺CD38⁻ AML cells and normal HSCs. The molecules expressed in CD34⁺CD38⁻ AML cells at levels >8-fold higher as compared to normal HSCs included TIM-3 and previously identified LSC-specific molecules such as CLL-1 (van Rhenen et al., 2007), CSF1R (Aikawa et al., 2010), and CD96 (Figure 1A; Hosen et al., 2007). As shown in Figure 1B, TIM-3 protein was highly expressed in CD34⁺CD38⁻ AML cells but not in normal HSCs. We focused on TIM-3 not only because it is expressed specifically in CD34⁺CD38⁻ AML cells at high levels, but also because it is expressed in the majority of patients with most AML types.

We evaluated the TIM-3 protein expression on cell surface of AML cells by FACS analysis. As shown in Figure 2, the vast majority of the CD34⁺CD38⁻ LSCs as well as CD34⁺CD38⁻ progenitor fractions in AML M0, M1, M2, and M4 types expressed TIM-3 at a high level in virtually all cases studied. In AML M5, M6, and M7, a considerable fraction of CD34⁺CD38⁻ cells

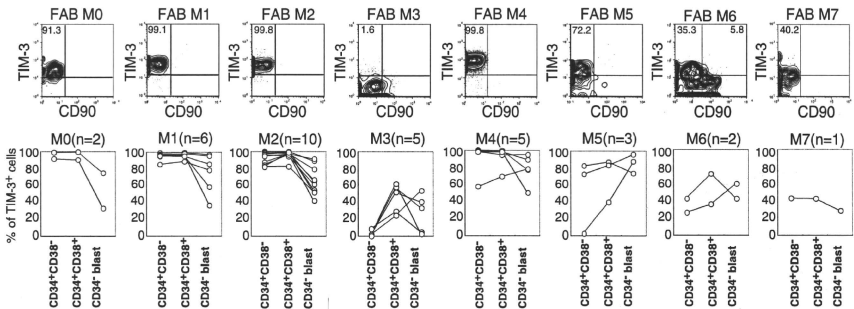


Figure 2. The Expression of TIM-3 in Stem and Progenitor Populations of AML of Each FAB Type

Expression of TIM-3 in each FAB type of AML. The representative expression pattern of TIM-3 in the CD34⁺CD38⁻ LSC fraction (top) and distribution of TIM-3 in CD34⁺CD38⁻ LSCs, CD34⁺CD38⁺ leukemic progenitors, and CD34⁻ leukemic blasts (bottom) are shown.

expressed TIM-3. TIM-3 was, however, not expressed in the CD34⁺CD38⁻ population in all five M3 cases tested. In general, TIM-3 was expressed in both CD34⁺CD38⁻ LSCs and CD34⁺CD38⁺ leukemic progenitor fractions, but its expression tended to decline at the CD34⁻ leukemic blast stage (Figure 2, bottom).

The TIM-3-Expressing of AML Fraction Contains the Vast Majority of Functional LSCs in a Xenograft Model

Recent studies have suggested that at least in some AML cases, LSCs that are capable of initiating human AML in xenograft models reside not only within the CD34⁺CD38⁻ fraction but

also outside of this population including CD34⁺CD38⁺ (Taussig et al., 2008) or CD34⁻ (Martelli et al., 2010; Taussig et al., 2010) AML cells. To evaluate whether functional AML LSCs express TIM-3, 10⁶ cells of human TIM-3⁺ and TIM-3⁻ AML populations were transplanted into sublethally irradiated immunodeficient mice. We used NOD.Cg-Rag1^{tm1Mom}/Il2rg^{tm1Wjl}/SzJ (NRG) mice for the xenogeneic transplantation experiments, by which higher chimerism of human hematopoietic cells was observed in xenotransplantation assays (Pearson et al., 2008). Recipients transplanted with TIM-3⁺ and TIM-3⁻ AML cells were sacrificed 8–10 weeks after transplantation. As shown in Figure 3, human CD45⁺CD33⁺ AML cells were

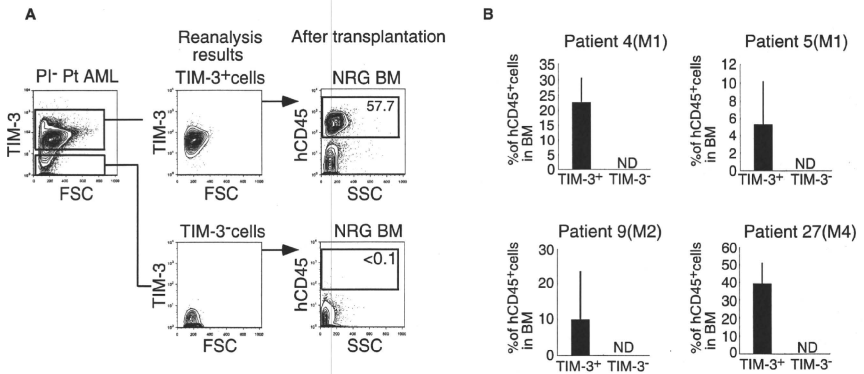


Figure 3. The TIM-3⁺ AML Population Contains the Vast Majority of Functional LSC Activity

(A) Representative analysis of xenotransplantation of purified TIM-3⁺ or TIM-3⁻ AML cells from patient 27 into NRG mice. Only TIM-3⁺ cells reconstitute hCD45⁺ AML cells after transplantation.

(B) Summarized data of four independent experiments. Only TIM-3⁺ (not TIM-3⁻) AML cells reconstituted human AML cells in xenotransplantation experiments in all experiments, suggesting that most functional LSCs reside in the TIM-3⁺ AML fraction.

Cell Stem Cell

TIM-3 Targets AML Stem Cells

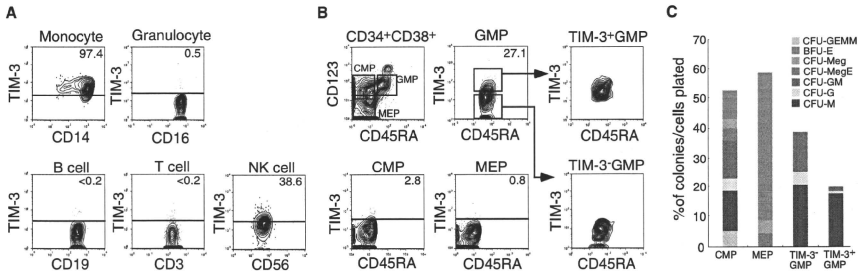


Figure 4. TIM-3 Is Expressed in Monocytes and Their Progenitors in Normal Hematopoiesis
 (A) TIM-3 expression in normal mature blood cells.
 (B) TIM-3 expression in normal hematopoietic progenitors. A fraction of GMPs but not other myeloid progenitors express TIM-3.
 (C) Results of clonogenic assays of myelo-erythroid progenitors including single TIM-3⁺ GMPs out of five independent experiments. The vast majority of TIM-3⁺ GMPs gave rise to macrophage colonies (CFU-M).

reconstituted only in mice transplanted with TIM-3⁺ AML cells, whereas TIM-3⁻ AML cells failed to reconstitution in all four AML cases tested (Figure 3B). Thus, all 23 mice injected with TIM-3⁺ AML cells reconstituted human AML, whereas 11 mice injected with TIM-3⁻ AML never developed human AMLs after transplantation. These results strongly suggest that LSCs exclusively reside within the TIM-3⁺ fraction in human AML at least in these patients.

TIM-3 Is Not Expressed in Normal Adult HSCs, and Its Expression Begins after Cells Are Committed to the Monocyte Lineage

Murine TIM-3 is expressed in a fraction of Th1 cells, monocytes, dendritic cells, and mast cells (Anderson et al., 2007; Monney et al., 2002; Nakae et al., 2007). The expression of human TIM-3 protein in normal steady-state human hematopoiesis is shown in Figures 4A. In periphery, TIM-3 was expressed in monocytes and a fraction of NK cells, but not in granulocytes, T cells, or B cells (Figure 4A). In the bone marrow, TIM-3 was not expressed in normal HSCs (Figure 1) or the vast majority of the CD34⁺CD38⁺ progenitor population. Within the CD34⁺CD38⁺ fraction, TIM-3 was expressed only in a fraction of GMPs at a low level, but not in common myeloid progenitors (CMPs), megakaryocyte/erythrocyte progenitors (MEPs) (Figure 4B), or common lymphoid progenitors (CLPs) (not shown). In clonogenic colony-forming unit (CFU) assays, the vast majority of purified TIM-3⁺ GMPs gave rise to CFU-M, whereas colonies derived from TIM-3⁻ GMP contained CFU-GM as well as CFU-G and CFU-M (Figure 4C). These data strongly suggest that TIM-3 up-regulation mainly occurs in concert with the monocyte lineage commitment at the GMP stage in normal hematopoiesis.

Anti-Human TIM-3 Antibodies Did Not Impair Development of Normal Hematopoiesis

To selectively eliminate TIM-3-expressing AML LSCs in vivo, we developed a monoclonal antibody against TIM-3 that has an efficient interaction with cellular Fc receptors on innate immune effector cells. It has become clear that the ADCC activity is one of

the most important factors to eliminate target cells in antibody therapies (Nimmerjahn and Ravetch, 2007). A TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably expressing human TIM-3 and soluble TIM-3 protein. The variable portion of the VH regions of the cloned hybridoma that recognize TIM-3 were then grafted onto IgG2a Fc regions, because IgG2a subclass is most efficient to induce ADCC activity in mice (Nimmerjahn and Ravetch, 2005; Uchida et al., 2004). The established clone, ATIK2a, possessed CDC activities in EoL-1 and L929 cells transfected with TIM-3 (Figure 5A), as well as Kasumi-3, an AML cell line that spontaneously expresses TIM-3 (not shown). Importantly, ATIK2a displayed strong ADCC activity against TIM-3-expressing EoL-1 and L929 cells in vitro (Figure 5B).

We first tested the effect of ATIK2a treatment on reconstitution of normal HSCs in a xenograft model. The major effectors in ADCC reaction are NK cells. Because NRG mice do not have NK cells because of γ c mutation (Pearson et al., 2008), we used NOD-SCID mice for this experiment to potentiate ADCC activity of ATIK2a antibodies. NOD-SCID mice were sublethally irradiated and were transplanted with 10⁵ CD34⁺ adult human bone marrow cells. 15 μ g of ATIK2a was intraperitoneally injected to mice 12 hr after transplantation, which was followed by further injections of 15 μ g of ATIK2a once a week until mice were sacrificed at 12 weeks after transplantation. Injection of ATIK2a did not affect reconstitution of normal hematopoiesis: The percentage of human cells were equal (~1%), and human B and myeloid cells were normally reconstituted irrespective of ATIK2a treatment in three independent experiments (not shown). We also tested the effect of this ATIK2a treatment in NOD-SCID mice transplanted with 10⁵ CD34⁺ cord blood cells. Cord blood cells have potent reconstitution activity in NOD-SCID mice, and percentage of hCD45⁺ human cells reached ~50% after transplantation (Figure 5C). Again, the chimerism of human cells was equal, and CD19⁺ B cells and CD33⁺ myeloid cells were reconstituted irrespective of ATIK2a treatment (Figure 5C, left). In mice injected with ATIK2a, however, human TIM-3⁺ monocytes were removed (Figure 5C, right). These data suggest that

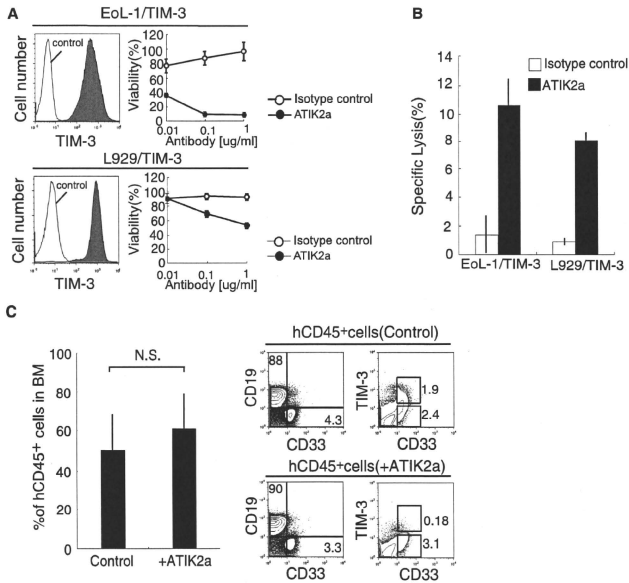


Figure 5. ATIK2a, a New Monoclonal Antibody against TIM-3, Has CDC and ADCC Activities and Does Not Harm Normal Hematopoietic Reconstitution

(A) CDC assays to evaluating the killing effect of ATIK2a antibodies on EoL-1 and L929 cell lines with enforced expression of human TIM-3. (B) ADCC activities of ATIK2a on TIM-3-expressing EoL-1 and L929 cell lines.

(C) The effect of ATIK2a treatment on human hematopoietic reconstitution in NOD-SCID mice transplanted with 10^5 CD34⁺ human cord blood cells. 15 μg of ATIK2a was intraperitoneally injected to mice 12 hr after transplantation, which was followed by further injections of 15 μg of ATIK2a once a week until mice were sacrificed at 12 weeks after transplantation. In this experiment, percentages of human cells in 10 each of mouse groups treated with control or ATIK2a antibodies were equivalent at 12 weeks after transplantation.

targeting TIM-3 does not affect development of normal hematopoiesis but remove TIM-3-expressing monocytes.

Anti-Human TIM-3 Antibodies Effectively Blocked Development of AML LSCs but Not that of Normal HSCs

We then tried to test the effect of ATIK2a in AML LSCs. We transplanted 10^5 bone marrow cells of AML patients (patients 6, 9, 15, 18, and 26) into NOD-SCID mice. The bone marrow of patients 6, 9, 15, and 26 were completely occupied by AML clones, and normal HSCs were not seen on FACS. Samples of each patient were transplanted into six mice, and three mice each were treated with 15 μg of ATIK2a or control IgG 12 hr after transplantation and with the same dose of antibodies once a week (Figure 6A). Mice were sacrificed 16 weeks after xenotransplantation. As shown in Figure 6B, the chimerism of AML cells were low in the NOD-SCID xenotransplant system. Nonetheless, ATIK2a injection significantly blocked AML reconstitution in these mice. In all of these patients, mice injected with control IgG showed reconstitution of CD34⁺TIM-3⁺ cells that contained primitive AML stem or progenitors as well as CD33⁺ AML blasts

(Figure 6B). In contrast, in mice treated with ATIK2a, the leukemic clone was barely detectable, and did not contain detectable numbers of CD34⁺ cells (not shown), displaying significantly lower chimerisms as compared to control mice in all four independent experiments (Figure 6C).

The bone marrow of patient 18 possessed a small fraction of CD34⁺CD38⁻CD90⁺TIM-3⁻ cells that was phenotypically normal HSCs, in addition to the major fraction of CD34⁺CD38⁻CD90⁻TIM-3⁺ AML LSCs (Figure 6D). Interestingly, in mice transplanted with the bone marrow from this patient, ATIK2a injection induced reconstitution of normal myeloid and B cells, whereas control mice developed AML. These data strongly suggest that the ATIK2a treatment selectively inhibited development of human AML, presumably by targeting LSCs, instead allowing normal HSCs to reconstitute human hematopoiesis *in vivo*.

TIM-3 Targets Leukemic Stem Cells

In testing the inhibitory effect of ATIK2a on established human AML in a xenotransplant system, we used the NRG mice to increase engraftment efficiency of human AML cells. Eight

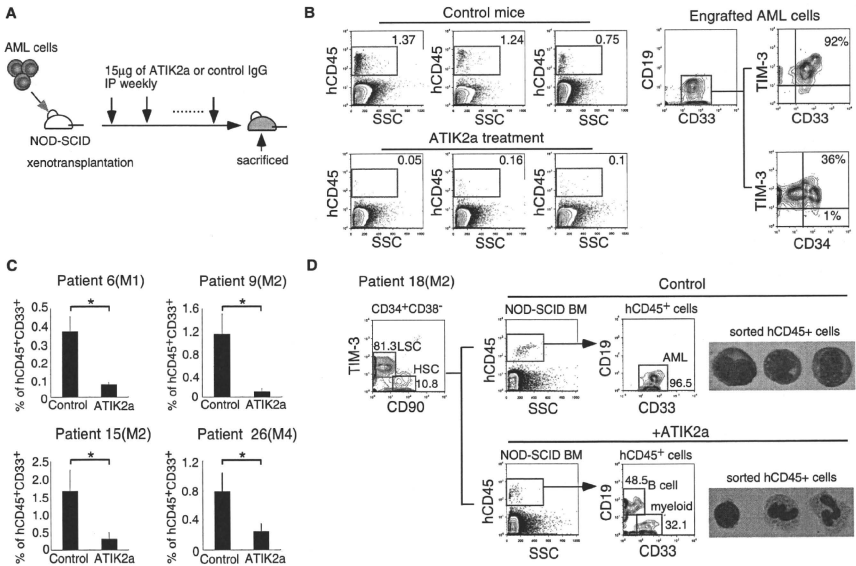


Figure 6. ATIK2a Antibodies Blocked AML Reconstitution in NOD-SCID Mice

(A) Schedule of ATIK2a administration in NOD-SCID experiment. ATIK2a treatment was started 12 hr after the transplantation. (B) Analysis of mice transplanted with AML bone marrow cells at 16 weeks after transplantation. Three control mice (left top) showed reconstitution of human CD45⁺ cells, and the majority of these cells were TIM-3⁺CD33⁺ AML cells that contained CD34⁺ leukemic progenitor or stem cell populations (right). In contrast, mice treated with ATIK2a (left bottom) have only a small number of hCD45⁺ cells. Representative data of patient 9 are shown. (C) Summary of four independent experiments to test the effect of ATIK2a on reconstitution of AML bone marrow cells from patients 6, 9, 15, and 26. In all experiments, ATIK2a treatment significantly inhibited the AML reconstitution. Three mice per group were analyzed. (D) Selective inhibition of AML reconstitution by ATIK2a in mice reconstituted with the bone marrow of patient 18, which contained both normal HSCs and AML LSCs (left). Injection of the bone marrow cells resulted in AML development in control mice (right top), whereas mice treated with ATIK2a developed normal hematopoiesis (right bottom).

weeks after injection of 10^6 AML cells, engraftment of human AML cells were confirmed by blood sampling. In NRG mice, ATIK2a cannot fully exert its ADCC effects because of a lack of NK cells. Therefore, we injected a high dose (80 μ g) of ATIK2a to maximize its CDC effects on AML cells in vivo. These mice were treated with ATIK2a or control IgG, 3 times a week for 4 weeks (Figure 7A). In all four cases tested (patients 7, 14, 27, and 28), ATIK2a treatment significantly reduced human CD45⁺ AML burden in vivo: ATIK2a strongly suppressed or eliminated the TIM-3⁺ AML fraction (Figure 7B, left) that contains all functional LSCs in our hand (Figure 3B), as well as the CD34⁺CD38⁻ LSC fraction (Figure 7B, right, and Figure 7C), suggesting that reduction of leukemic burden by ATIK2a was achieved at least in part by killing LSCs.

In patients 7 and 27, in order to verify the anti-AML LSC effect of ATIK2a treatment, 10^6 human CD45⁺ AML cells from the primary NRG recipients were further retransplanted into secondary NRG recipients. In patients 14 and 28, however,

reduction of AML cells by ATIK2a in primary recipients was very severe, and we could not harvest sufficient numbers of AML cells to transplant into secondary recipients. We then evaluated the re-enugraftment of AML cells in secondary recipients 8 weeks after transplantation. All seven mice transplanted with bone marrow cells from primary recipients treated with control IgG developed AML, whereas none of 10 mice transplanted with cells from ATIK2a-treated primary recipients developed AML. Representative data in patient 27 are shown in Figure 7C. These data again suggest that functional LSCs were effectively eliminated by ATIK2a treatment in primary recipients.

DISCUSSION

To selectively kill AML LSCs sparing normal HSCs, one of the most practical approaches is to target the AML LSC-specific surface or functionally indispensable molecules. To achieve specificity for LSCs, the target molecule should be expressed

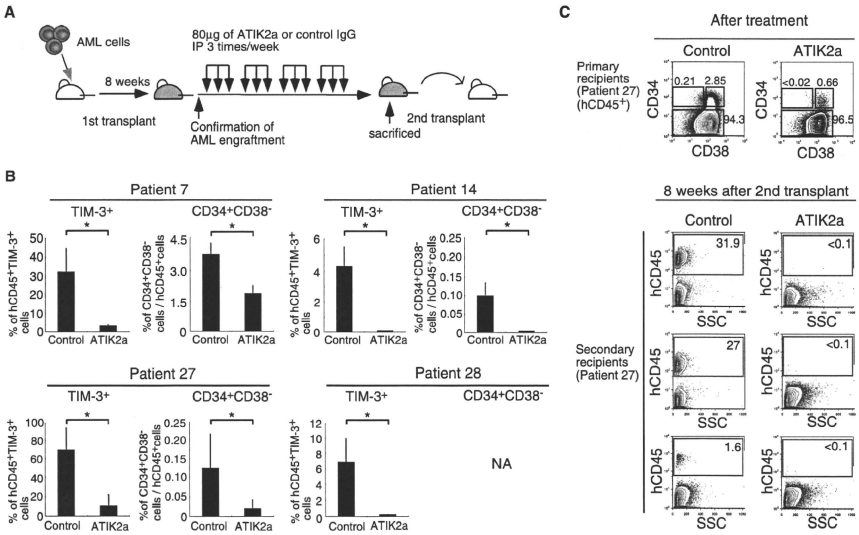


Figure 7. ATIK2a Antibodies Reduced the AML Burden at Least Targeting Functional LSCs

(A) Schedule of ATIK2a administration to test the effect on established human AML in NRG mouse experiments. ATIK2a treatment was started 8 weeks after transplantation.

(B) Summary of four independent experiments to assess the effect of ATIK2a on established human AML cells in vivo (patients 7, 14, 27, and 28). In all experiments, ATIK2a treatment significantly reduced hCD45⁺ AML burden. Within the hCD45⁺ population, the TIM-3⁺ AML fraction that should contain AML LSCs (see Figure 3) was also reduced by this treatment. The percentages of CD34⁺CD38⁺ cells, in which LSCs were concentrated, were also reduced. Three to six mice in each group were analyzed.

(C) The phenotype of grafted hCD45⁺ cells in primary recipients (top). 10⁶ hCD45⁺ AML cells were then harvested from primary recipients treated with ATIK2a or control IgG, and then retransplanted into the secondary NRG recipients. ATIK2a efficiently blocked reconstitution of AML cells (bottom). Representative results of patient 27 are shown.

on LSCs at a high level but not on normal HSCs. In addition, when the molecule is expressed also in leukemic progenitors or blasts, it will help mass reduction of AML clones. It should not matter whether the molecule is expressed in normal mature blood cells or progenitor cells, because if normal HSCs are spared, they should be able to replenish all mature blood cells after treatment.

TIM-3 is expressed in the CD34⁺CD38⁻ AML LSC fraction as well as the majority of their downstream CD38⁺ leukemic progenitors in most AML types except for M3. TIM-3⁺ but not TIM-3⁻ AML population engrafted and reconstituted human AML in NRG mice, suggesting that functional LSCs almost exclusively reside in TIM-3⁺ cells. In contrast, normal HSCs do not express TIM-3. Thus, TIM-3 should be useful molecules to target AML LSCs without seriously affecting normal hematopoiesis. In steady-state human hematopoiesis, TIM-3 is not expressed in HSCs or myeloid progenitor populations. TIM-3 expression begins at the GMP stage, in parallel with monocyte lineage commitment (Figure 4). Furthermore, in addition to TIM-3, the expression profiling data show that the CD34⁺

CD38⁻ LSC fraction expressed many monocyte lineage-related molecules such as CD86 and CSF1R at a high level (Figure 1). In this context, LSCs in most AML types, except for M3 that might be of granulocytic lineage leukemia, may activate some monocyte lineage-related programs.

ATIK2a, a TIM-3 antibody with ADCC and CDC activities, selectively blocked the human AML engraftment and/or development in NOD-SCID mice, whereas it did not disturb normal HSC engraftment. Furthermore, in NRG mice transplanted with human AML cells where percentage of engrafted human cells reached 5%–60% (Figure 7B), ATIK2a treatment reduced or eliminated CD34⁺CD38⁻ and TIM-3⁺ LSC-containing fractions within the bone marrow of primary recipients, resulting in failure of re-enugraftment of primary recipients' bone marrow cells into secondary recipients (Figure 7C). Collectively, it is likely that ATIK2a eradicated functional AML LSCs in vivo, sparing normal HSCs.

To use surface markers for targeting AML LSCs, specificity as well as sensitivity should be critical. TIM-3 has an advantage against other candidate markers in several aspects: Detectable levels of TIM-3 protein is not expressed in normal HSCs or other

progenitors except for only a fraction of GMPs. Furthermore, TIM-3 is expressed in LSCs at a high level, and its expression was found in the vast majority of CD34⁺CD38⁻ cells of M0, M1, M2, and M4 AMLs in all cases tested. As shown in Figure 1, the mRNA expression level of CD25, CD32, CD44, and CD47 in LSCs was only 2- to 3-fold higher as compared to normal HSCs, and in some AML cases, LSCs did not express these molecules. CD33 and CD123 proteins were detectable in normal HSCs (Figure 1B) as well as most myeloid progenitors including CMPs and GMPs (Taussig et al., 2005). In fact, prolonged cytopenias have been observed in AML patients treated with gemtuzumab, a recombinant humanized CD33 monoclonal antibody conjugated with the cytotoxic antibiotic calicheamicin, and this side effect could be due to CD33 expression in normal HSCs (Taussig et al., 2005). CLL-1, CSF1R, TIM-3, and CD96 are the group of molecules that are specifically expressed in LSCs. Among all, the sensitivity of TIM-3 is likely to be the highest at least for AML M0, M1, M2, and M4 (Figures 1B and 2). Thus, TIM-3 might be one of the most useful therapeutic targets at least for these AML types.

It may also be important to understand function of these molecules in maintenance or reconstitution capability of LSCs. For example, it was shown that CD44 monoclonal antibodies reduced the leukemic burden and blocked secondary engraftment in a NOD-SCID model (Jin et al., 2006). This effect on LSCs was mediated in part by the disruption of LSC-niche interactions (Jin et al., 2006). CD47 antibodies can block LSC reconstitution and inhibited the growth of engrafted human AML in a NOD-SCID model (Majeti et al., 2009). However, the interpretation of this result is difficult because the anti-LSC effect of CD47 antibody treatment in this xenograft model could be due to induction of xenogeneic rejection by blocking the ligation of human CD47 expressed on LSCs with mouse SIRPA: NOD-type SIRPA expressed on host macrophage is agonistic for human CD47 to block phagocytotic signals, resulting in the induction of tolerance for human cells in this model (Takenaka et al., 2007). The effect of TIM-3 antibodies in our study might be due to killing activity for their target cells that should include LSCs. It is, however, still important to understand the role of TIM-3 signaling in LSC functions by, for example, testing the effect of activation or suppression of TIM-3 signaling on LSC fate decision.

In summary, TIM-3 is a promising surface molecule to target AML LSCs of most FAB types. Our *in vivo* experiments strongly suggest that targeting this molecule by monoclonal antibody treatment is a practical approach to eradicate human AML.

EXPERIMENTAL PROCEDURES

Clinical Samples

The bone marrow samples of 34 adult AML cases diagnosed according to French-American-British (FAB) and WHO criteria were enrolled. Human adult bone marrow and peripheral blood cells were obtained from healthy donors. Cord blood cells were obtained from full-term deliveries. Informed consent was obtained from all patients and controls in accordance with the Helsinki Declaration of 1975 that was revised in 1983. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects.

Antibodies, Cell Staining, and Sorting

For the analyses and sorting of human HSCs and progenitors, cells were stained and sorted by FACS Aria (BD Biosciences) as we have previously reported (Kikushige et al., 2008; Yoshimoto et al., 2009). In brief, for the analyses

and sorting of HSCs and myeloid progenitors, cells were stained with a Cy5-PE- or PC5-conjugated lineage cocktail, including anti-CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD10 (HI10a), CD19 (HIB19), CD20 (FAC7), CD11b (ICRF44), CD14 (RMO52), CD56 (NKH-1), and GPA (GA-R2); FITC-conjugated anti-CD34 (8G12), anti-CD90 (5E10), or anti-CD45RA (HI100); PE-conjugated anti-TIM-3 (344823), CD33 (HIM3-4), CD96 (NK92.39), or anti-CD123 (6H6); APC-conjugated anti-CD34 (8G12) or anti-CD38 (HIT2); and Pacific Blue conjugated anti-CD45RA (HI100), and biotinylated anti-CD38 (HIT2), or anti-CD123 (9F5). For analysis and sorting of human cells in the immunodeficient mice, FITC-conjugated anti-CD33 (HIM3-4), PE-conjugated anti-CD19 (HIB19), PE-Cy7-conjugated anti-CD38 (HIT2), and APC-conjugated anti-CD45 (J.33) monoclonal antibodies were used in addition to the antibodies described above. Streptavidin-conjugated APC-Cy7 or PE-Cy7 was used for visualization of the biotinylated antibodies (BD Pharmingen, San Jose, CA). Nonviable cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control monoclonal antibodies were used to determine the level of background staining. The cells were sorted and analyzed by FACS Aria (BD Biosciences, San Jose, CA). The sorted cells were subjected to an additional round of sorting within the same gate to eliminate contaminating cells and doublets. For single-cell assays, an automatic cell-deposition unit system (BD Biosciences, San Jose, CA) was used.

In Vitro Assays to Determine the Differentiation Potential of Myeloid Progenitors

Clonogenic colony-forming unit (CFU) assays were performed with a methylcellulose culture system that was set up to detect all possible outcomes of myeloid differentiation as reported previously (Kikushige et al., 2008; Manz et al., 2002). Colony numbers were enumerated on day 14 of culture. All of the cultures were incubated at 37°C in a humidified chamber under 5% CO₂.

Microarray Analysis

Twelve AML samples and five normal adult HSCs samples were investigated with Sentrix Bead Chip Assay For Gene Expression, Human-6 V2 (Illumina). In brief, total RNA was extracted with Trizol (Invitrogen) from FACS-sorted AML CD34⁺CD38⁻ cells and normal CD34⁺CD38⁻Lin⁻ HSCs, and biotinylated complementary RNA was synthesized with two round amplification steps via MessageAmpII aRNA Amplification Kit and Illumina TotalPrep RNA Amplification Kit (Applied Biosystems). 1.5 µg of cRNA from each sample was hybridized to the Bead Chip. After staining and washing, Bead Chip was scanned with an Illumina Bead Array reader. Microarray data were analyzed with Gene Spring GX11.01 software (Agilent Technologies). According to the guided workflow for Illumina single color experiment, normalization algorithm of 75-percentile shift was used, and the preprocessing baseline was adjusted to median of all samples.

Production of Recombinant Anti-Human TIM-3 Mouse Monoclonal Antibody

Human TIM-3 cDNA were cloned from normal pancreas cDNA (Clontech). Female Balb/C mouse (7-week-old, Purchased from Charles River) was immunized with L929 cells stably expressing TIM-3 four times and soluble human TIM-3 protein one. Four days after the final injection, spleen cells were fused with SP2/O cells by the PEG method and selected in the HAT-medium. Hybridomas were screened by FACS and clone-sorted. cDNAs encoding the variable regions amplified by SMART RACE cDNA Kit (Clontech) and specific primers (Doenecke et al., 1997) were ligated to mouse IgG2a or Igκ constant region.

Evaluation of ADCC and CDC Activities of ATIK2a Antibodies

ADCC and CDC were determined as previously described with slight modification (Shields et al., 2001; Tawara et al., 2008). For ADCC, target cells and IL-2-cultured peripheral blood mononuclear cells prepared from healthy volunteers were incubated with antibodies (1 µg/mL, Effector/Target ratio = 25). Cytotoxicity was analyzed by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) as follows: specific lysis [%] = $(A_E - A_{AB}) / (A_{MAX} - A_{TS}) \times 100$, where A_E is absorbance of experiment, A_{AB} is allogeneic reaction (no antibody control), A_{MAX} is maximum, A_{TS} is target spontaneous release. For CDC, viability of target cells incubated with rabbit sera was assayed by CellTiterGlo (Promega, no antibody control = 100%). UPC 10 (Sigma) replaced in PBS was used as an isotype control.

Transplantation of AML Cells into Immunodeficient Mice

NOD-SCID and NRG mice (stock#7799) were purchased from The Jackson Laboratory. The mice were housed in a specific-pathogen-free facility in micro-isolator cages at the Kyushu University. Animal experiments were performed in accordance with institutional guidelines approved by the Kyushu University animal care committee. NOD-SCID and NRG mice were irradiated at a sublethal dose (2.4 Gy and 4.8 Gy, respectively). In transplantation of AML cells, NOD-SCID mice additionally received a single intraperitoneal injection of 200 µg purified CD122 antibodies that were generated from TM-β1 hybridoma (Tanaka et al., 1993) before transplantation, based on the expectation that it induces transient reduction of NK cells and helps human cell engraftment. We did not inject CD122 antibodies in transplantation of normal bone marrow or cord blood cells. AML cells or CD34⁺ cells from adult bone marrow and cord blood cells were transplanted via a tail vein.

Statistical Analysis

Data were presented as the mean ± standard deviation. The significance of the differences between groups was determined via Student's t test.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number 24395.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at doi:10.1016/j.stem.2010.11.014.

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