In the present study, we established a new detection method for alloreactive T cells by intracellular staining for interferon (IFN)-γ, using CD40-activated B cells that have an excellent antigen-presenting capacity[10,11] as stimulator cells. Using this system, we assessed the temporal changes of alloreactive T-cell frequencies in relation to rejection episodes in patients who underwent living donor liver transplantation.

MATERIALS AND METHODS

Patients

The study design and purpose were approved by the institutional review board of Mie University Graduate School of Medicine and fully explained to all the donors and the recipients according to the Declaration of Helsinki. The characteristics of the 19 recipients of the liver allografts from living donors between June 2003 and July 2004 and the outcomes of the analyses are shown in Table 1. Human leukocyte antigen (HLA) class I and II typings were performed by serotyping and polymerase chain reaction (PCR), respectively. The immunosuppressive regimen consisted of tacrolimus and low-dose steroids. The target trough levels for tacrolimus in whole blood were 10–15 ng/mL during the first 2 weeks, approximately 10 ng/mL during the following 2 weeks, and 5–10 ng/mL thereafter. Methylprednisolone (10 mg/kg body weight) was administered intravenously immediately before perfusion of the graft portal vein. Methylprednisolone at 1 mg/kg per day was given intravenously on postoperative days (PODs) 1–3, followed by 0.5 mg/kg per day on PODs 4–6. The steroid was then switched to oral prednisolone at 5 mg/kg per day on POD 7, and the dosage was reduced to 1 mg/kg per day at 1 month. If the liver function stabilized, patients were weaned off of the steroids at 3–6 months postoperatively. During the follow-up period, 12 patients maintained normal levels of liver enzymes and bilirubin and normal clotting times, and had no evidence of vascular or biliary abnormalities. In addition, no signs of rejection were observed in the protocol biopsy at 1 year posttransplantation; these patients were categorized as "nonrejection." In the case of clinical suspicion of rejection based on elevated liver enzymes without an obvious alternative cause, a liver biopsy was performed and the degree of rejection was graded according to the Banff schema.[12] Acute rejection was observed in 5 patients and resolved upon 3 boluses of 1-g methylprednisolone. Two patients developed chronic transplant dysfunction as a consequence of chronic rejection of the transplant.

Responder Cells

Blood samples were obtained from the allograft recipients at 0 month (preoperative) and 1, 3, and 6 months after transplantation. Blood was drawn at each time point before the intake of immunosuppressants. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on a Ficoll-Paque gradient (GE Healthcare, Little Chalfont, UK) and cryopreserved until use. PBMCs were also obtained from healthy volunteers and used in some experiments.

Stimulator Cells

PBMCs were isolated from donor blood obtained at the time of organ harvest and cryopreserved until use. CD40-activated B cells were generated from the PBMCs by using an NIH/3T3 cell line with the expression of the human CD40 ligand (kindly provided by Dr G. Freeman, Dana-Farber Cancer Institute, Boston, Mass) as previously described[11,13] and used as stimulator cells. Mixed CD40-activated B cells derived from 4 healthy donors were also prepared as the third-party control. Unmixed CD40-activated B cells were also used in some experiments.

Table 1. Patients' Characteristics and Results of the Analyses

Patient No.	Gender	Age	Primary Disease	Donor	ABO Mismatch	HLA Mismatch†		Rejection Episode
						Class I	Class II	
1	M	54	HCC/HCV	Low-Son	Identical	5	2	Acute
2	M	43	LC	Wife	Identical	4	3	Acute
3	F	13	BA	Parent	Identical	2	2	Acute
4	M	60	LC	Wife	Identical	0	2	Acute
5	F	70	HCC	Nephew	Identical	3	2	Acute
6	M	57	HCC/HCV	Child	Identical	3	3	Chronic
7	M	60	HCC/HCV	Wife	Identical	5	3	Chronic
8	M	53	LC/HBV	Wife	Identical	6	2	None
9	M	66	LC	Child	Identical	3	2	None
10	M	50	LC	Wife	Identical	3	3	None
11	F	63	PSC	Child	Identical	2	2	None
12	F	66	HCC/HCV	Child	Identical	3	2	None
13	F	67	HCC/HCV	Child	Identical	3	2	None
14	M	25	BA	Sibling	Incompatible	4	3	None
15	M	55	LC/HBV	Child	Identical	2	1	None
16	F	64	LC	Child	Identical	3	2	None
17	F	46	LC	Child	Identical	3	2	None
18	M	37	LC/HBV	Cousin	Identical	3	2	None
19	M	60	LC/HCV	Cousin	Incompatible	4	3	None

BA, biliary atresia; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PSC, primary sclerosing cholangitis.

†HLA-A/B/C (class I) and HLA-DRA/DRB (class II) were examined.

Quantification of Alloreactive T Cells by Flow Cytometry

Primary mixed lymphocyte cultures (MLCs) were performed by culturing responder PBMCs (1×10^6 per well) with irradiated (33 Gy) stimulator cells (1×10^6 per well) for 7 days in AIM-V medium using 24-well plates. Before intracellular staining for IFN-γ, harvested viable responder cells (1×10^6 per tube) were restimulated with irradiated stimulator cells (1×10^6 per tube) for 5 hours in AIM-V medium, using round-bottom polypropylene tubes. Brefeldin A (10 μg/mL; Sigma-Aldrich, St Louis, Mo) was added to the culture as a blocker of cytokine secretion. After restimulation, the cells were treated with 2 mmol/L EDTA for 10 minutes and then fixed in prewarmed 4% paraformaldehyde in phosphate-buffered saline for 10 minutes at room temperature. The cells were then permeabilized with IC PERM (BioSource/Invitrogen, Carlsbad, Calif) and stained for 30 minutes at room temperature in the dark using appropriate concentrations of phycoerythrin (PE)-labeled anti-CD69 monoclonal antibody (mAb; Coulter, Buffalo, NY) and fluorescein isothiocyanate (FITC)-labeled anti–IFN-γ mAb (BD Biosciences Pharmingen, San Diego, Calif), together with PE-Cy5 (PC5)-labeled anti-CD4 or CD8 mAb (BD Biosciences Pharmingen). After staining, the cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, Calif). At least 10,000 $CD4^+$ or $CD8^+$ T cells were gated and analyzed by the CellQuest software (BD Biosciences Immunocytometry Systems). In some experiments, the frequencies of alloreactive T cells were analyzed in the same way without primary MLCs.

Normalization of the Frequencies of Donor-Specific T Cells: Calculation of Donor–Third-Party and Postoperative–Preoperative Ratios

To avoid the effect of the primary frequencies of the alloreactive T cells and/or the immunosuppressive reagents, the donor–third-party ratio was used for the evaluation of the donor-specific T-cell responses, dividing the frequency of the donor-specific precursors by that of the third-party–specific precursors.[14] The postoperative–preoperative ratio was also calculated by defining the donor–third-party ratio of each individual at preoperative period as 1.

Statistical Analysis

The differences in frequencies were analyzed by the Wilcoxon matched pairs test. Differences were considered statistically significant when $P < .05$.

RESULTS

Establishment of a New Detection System for Alloreactive T Cells

We first attempted to establish a new detection system for alloreactive T cells by intracellular staining for IFN-γ. For this purpose, we used PBMCs as responders and CD40-activated B cells as stimulators, both derived from healthy volunteers. The PBMCs were cultured for 5 hours with autologous CD40-activated B cells or allogeneic CD40-activated B cells from 3 healthy volunteers at a responder-stimulator ratio of 1:1. As shown in the upper part of Table 2, the IFN-γ–producing $CD4^+$ but not $CD8^+$ T cells were better detected in the responder cells from the 3 donors cultured with allogeneic stimulators than in those with the autologous stimulators ($P < .05$).

To detect $CD8^+$ T-cell responses better, we then conducted the same assay using the prestimulated responder cells from the MLCs (Fig 1). Alloreactive $CD4^+$ and $CD8^+$ T cells were detected only when the cells were cultured with allogeneic CD40-activated B cells in primary and secondary stimulations. Similar results were obtained using the responder cells from the 3 volunteers (Table 2, lower part), and the frequencies of alloreactive T cells are significantly higher than those of autoreactive T cells in the $CD4^+$ and $CD8^+$ subsets ($P < .01$). Although $CD8^+$ T-cell responses were much more enhanced than the $CD4^+$ T-cell responses by primary MLCs, we concluded that this detection system is applicable for the assessment of alloreactive responses of $CD4^+$ and $CD8^+$ T cells.

Temporal Changes in the Frequencies of Alloreactive $CD4^+$ and $CD8^+$ T Cells After Liver Transplantation

We then assessed the temporal changes in the frequencies of the alloreactive $CD4^+$ and $CD8^+$ T cells after liver transplantation, using the newly established method (Table 3 and Fig 2). As shown in Fig 2, the frequencies of $CD4^+$ and $CD8^+$ T cells against the donor but not the third party gradually decreased in the nonrejection group after transplantation, whereas no significant changes were observed in the rejection group.

Table 2. Alloreactivity of $CD4^+$ and $CD8^+$ T Cells From Healthy Volunteers

Primary MLC	CD4/CD8	Responder	Stimulator			
			Auto	Allo 1	Allo 2	Allo 3
(−)	CD4	1	0.02*	0.40	0.21	0.26
		2	0.07	0.22	0.22	0.21
		3	0.02	0.20	0.08	0.08
	CD8	1	0.04	0.09	0.11	0.08
		2	0.06	0.04	0.06	0.10
		3	0.13	0.03	0.05	0.04
(+)	CD4	1	0.20	1.19	1.25	0.82
		2	0.35	4.20	5.79	5.13
		3	0.47	2.40	9.86	8.57
	CD8	1	1.05	6.93	9.15	25.67
		2	2.49	29.93	17.09	16.96
		3	1.99	19.15	22.92	20.26

*Percentage of IFN-γ–producing cells in activated ($CD69^+$) $CD4^+$ or $CD8^+$ T cells detected by intracellular staining.

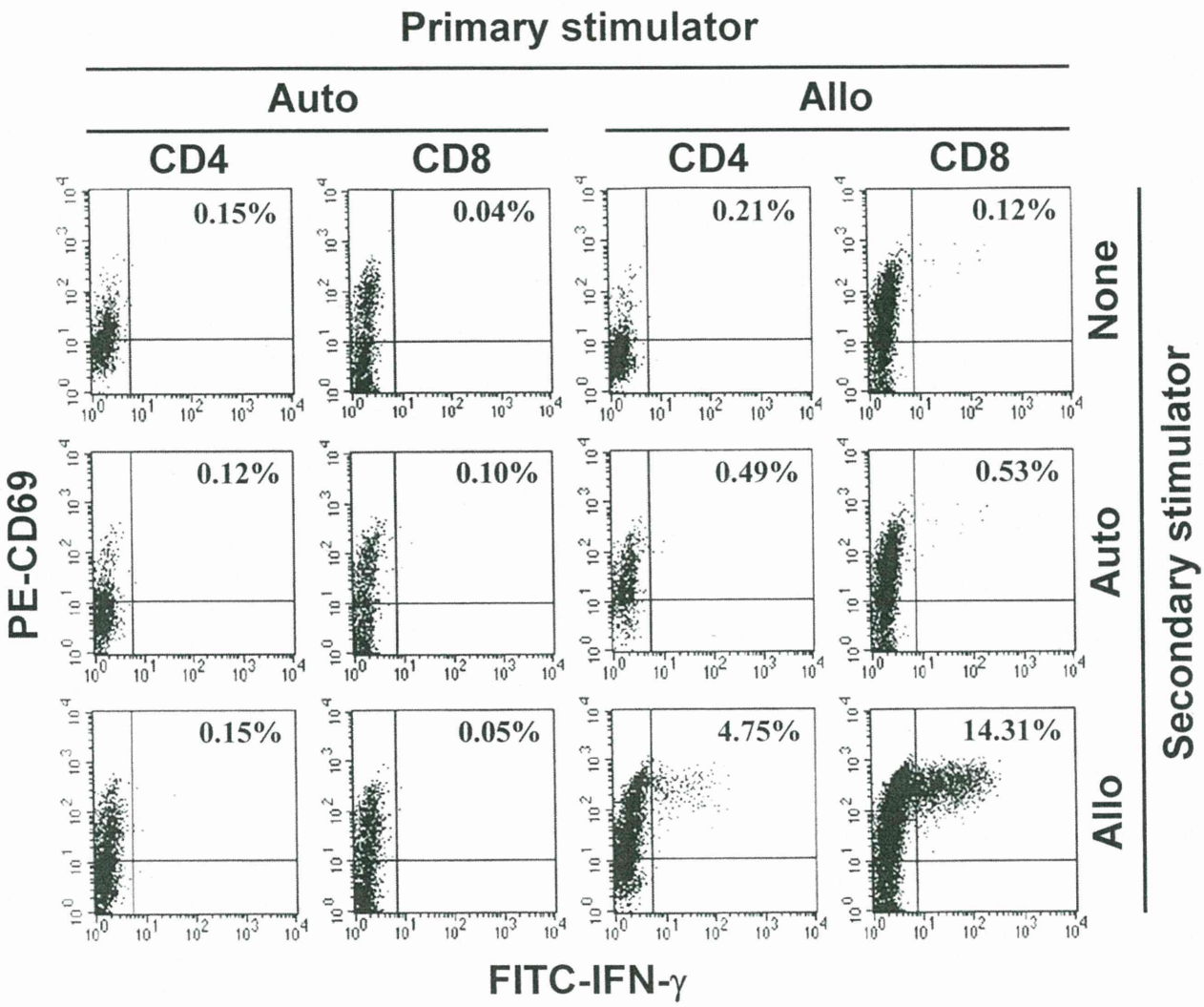

Fig 1. IFN-γ production of $CD4^+$ and $CD8^+$ T cells stimulated with CD40-activated B cells. PBMCs were stimulated with autologous or allogeneic CD40-activated B cells for 7 days, and each cell population was then restimulated with autologous or allogeneic CD40-activated B cells for 5 hours in a crisscross manner. After the secondary stimulation, intracellular IFN-γ was analyzed flow cytometrically using FITC—anti–IFN-γ, PE–anti-CD69, and PC5–anti-CD4 or CD8. The frequencies of the IFN-γ–producing cells in activated ($CD69^+$) T-cell fractions gated with CD4 or CD8 are shown as percentages.

Chronic immunosuppression, viral infections, and lymphopenia reduce whole T-cell responses in an antigen-nonspecific manner and may affect the results on the antigen-specific T-cell responses. Because the changes in T-cell responses against third-party stimulators are assumed to reflect the influences of such antigen-nonspecific immunosuppression, the ratio between the donor-specific and third-party–specific T-cell responses (donor–third-party ratio) was calculated as previously described[14] to further analyze the temporal changes of the allospecific T-cell responses. The mean donor–third-party ratio of the $CD8^+$ but not the $CD4^+$ T cells in the nonrejection group was significantly lower than that in the rejection group throughout the postoperative periods (data not shown). As shown in the upper panels of Fig 3, when the relative postoperative–preoperative ratio was calculated by defining the donor–third-party ratios at the preoperative period as 1, the means of the ratio of $CD8^+$ T cells in the nonrejection group were significantly lower than those in the rejection group. In addition, the ratio of $CD8^+$ T cells in most individuals in the nonrejection group was lower than 1 during the postoperative periods (Fig 3, right upper panel), indicating the induction of donor-specific suppression of $CD8^+$ T-cell responses. In contrast, the relative postoperative–preoperative ratios of the $CD8^+$ T cells of the rejection group in the postoperative periods were generally higher than those in the preoperative periods (Fig 3, left upper panel). For the $CD4^+$ T-cell subsets, no differences were found in the relative postoperative–preoperative (Fig 3, lower panels) and donor–third-party (data not shown) ratios between the rejection and nonrejection groups.

Table 3. Alloreactivity of $CD4^+$ and $CD8^+$ T Cells Derived From the Recipients

	CD4				CD8			
Patient no.	0 Mo (Pre)	1 Mo	3 Mos	6 Mos	0 Mo (Pre)	1 Mo	3 Mos	6 Mos
1	0.40* (1.54)	ND†	0.04 (0.84)	0.48 (0.86)	1.18 (7.31)	4.52 (4.29)	0.50 (1.20)	1.58 (1.36)
2	2.41 (10.80)	2.29 (8.80)	0.40 (5.85)	3.66 (14.58)	1.92 (9.58)	1.78 (8.44)	2.68 (8.34)	2.19 (25.14)
3	13.64 (5.79)	4.43 (4.01)	ND	6.03 (8.44)	15.38 (10.87)	5.47 (9.32)	ND	3.64 (16.72)
4	2.45 (3.28)	2.84 (4.05)	5.04 (5.44)	2.68 (1.18)	1.23 (3.66)	1.12 (1.44)	8.16 (4.86)	2.39 (3.86)
5	0.30 (1.64)	0.55 (0.71)	0.45 (0.64)	0.34 (0.85)	1.07 (1.71)	3.41 (2.54)	1.90 (2.31)	2.63 (2.70)
6	0.74 (9.61)	0.33 (2.07)	1.96 (0.79)	2.48 (2.56)	7.19 (36.24)	0.88 (11.67)	1.16 (3.80)	15.06 (17.31)
7	0.80 (2.16)	0.76 (1.51)	0.98 (1.84)	0.96 (1.12)	4.27 (9.77)	1.47 (0.60)	1.40 (2.54)	0.59 (2.47)
8	11.11 (6.46)	0.43 (1.86)	0.30 (2.20)	0.38 (2.25)	21.05 (6.52)	1.30 (6.58)	0.48 (6.74)	0.81 (4.71)
9	1.30 (3.80)	3.20 (2.90)	ND	2.20 (3.70)	4.80 (5.20)	7.90 (3.70)	ND	3.10 (6.60)
10	1.13 (1.90)	1.14 (0.47)	0.27 (0.45)	0.22 (0.53)	4.93 (1.56)	1.69 (0.90)	1.59 (1.29)	1.08 (1.38)
11	1.45 (3.74)	5.88 (2.05)	3.50 (5.20)	ND	6.15 (34.67)	1.45 (4.70)	1.91 (11.83)	ND
12	1.64 (4.63)	1.43 (3.86)	1.60 (5.09)	0.78 (1.03)	2.87 (9.49)	0.72 (11.91)	2.47 (13.34)	2.51 (5.29)
13	0.16 (1.91)	0.19 (0.40)	0.48 (0.75)	0.27 (0.53)	0.51 (8.05)	0.22 (2.26)	0.43 (4.52)	0.31 (3.40)
14	0.36 (0.37)	0.29 (0.30)	0.63 (1.02)	0.29 (0.86)	3.35 (1.12)	0.96 (0.35)	0.44 (0.72)	0.49 (0.66)
15	0.39 (2.41)	0.51 (1.09)	0.50 (0.59)	0.21 (0.77)	1.28 (5.21)	0.20 (6.99)	0.51 (5.60)	0.50 (2.52)
16	1.04 (3.44)	3.49 (5.08)	2.63 (11.64)	0.84 (4.52)	2.21 (10.40)	6.33 (11.80)	1.37 (11.03)	1.29 (12.04)
17	0.68 (3.74)	0.34 (1.87)	1.45 (2.71)	0.51 (4.73)	3.42 (16.55)	1.17 (7.19)	5.26 (7.28)	0.83 (14.28)
18	4.40 (8.24)	0.78 (8.10)	0.53 (12.79)	3.33 (13.10)	11.95 (11.99)	1.75 (11.89)	3.08 (23.57)	5.42 (15.11)
19	9.81 (3.09)	0.68 (3.89)	0.37 (6.33)	0.87 (5.34)	3.49 (2.03)	1.91 (2.50)	0.22 (7.58)	0.29 (9.84)

*Percentage of IFN-γ–producing cells stimulated with CD40-activated B cells from the donors (results with those from the third party are shown in parenthesis).
†Not determined.

These observations together indicate that donor-specific responses of $CD8^+$ T cells correlate with rejection episodes much more than those of $CD4^+$ T cells.

DISCUSSION

In the present study, we established a new flow cytometric method for the detection of alloreactive T cells by intracellular staining for IFN-γ, using CD40-activated B cells as stimulator cells. Using this system, we found that the relative postoperative–preoperative ratio of the donor-specific $CD8^+$ T cells is a possible evaluative indicator of the risk for graft rejection.

The development of calcineurin inhibitors, such as tacrolimus, has reduced the rate of acute rejection and improved short-term but not long-term graft survival. However, long-term use of immunosuppressive drugs leads not

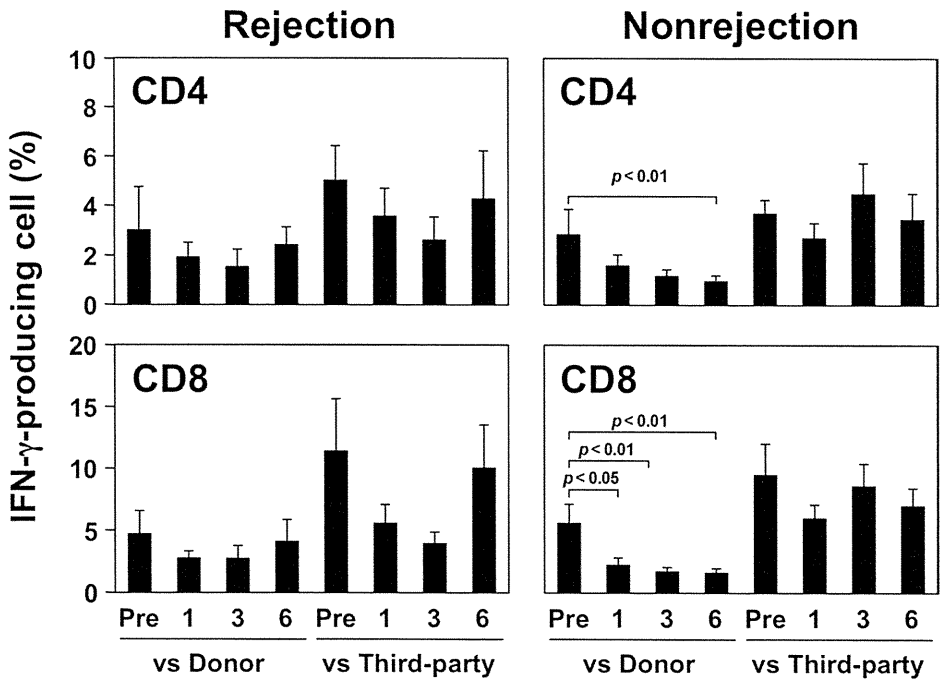

Fig 2. Temporal change in the frequencies of donor-specific T cells. PBMCs from the recipients were stimulated with CD40-activated B cells from the corresponding donors for 7 days, and each cell population was then restimulated for 5 hours with CD40-activated B cells from the donor or third party. After secondary stimulation, intracellular IFN-γ was analyzed flow cytometrically using FITC—anti–IFN-γ, PE–anti-CD69, and PC5–anti-CD4 or CD8. The average frequencies of IFN-γ–producing cells in the $CD4^+CD69^+$ or $CD8^+CD69^+$ cells at the indicated time points are shown. The bars represent the standard errors.

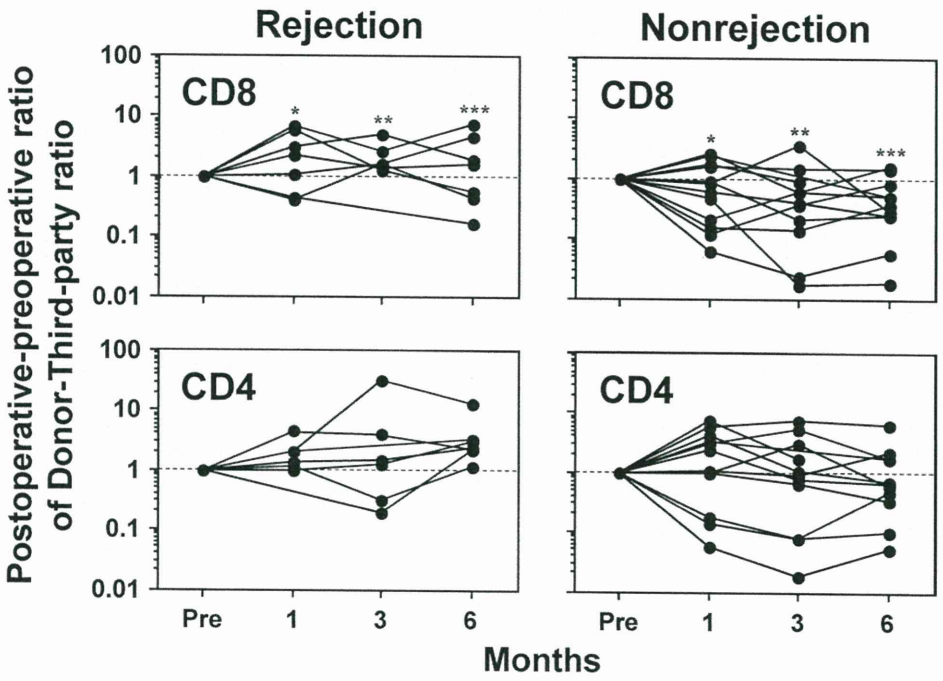

Fig 3. Temporal change in the relative postoperative–preoperative ratios. Ratios between the frequencies of the donor– and third-party–specific T cells (donor–third-party ratio) were first calculated. The relative postoperative-preoperative ratio was then calculated, defining the donor–third-party ratio of each individual at preoperative period as 1. All *P* values were <.05.

only to renal toxicity and metabolic disorders, but also to adverse effects of immunosuppression, such as opportunistic infections and cancers.[1, 2] Maintenance of a normal allograft function despite withdrawal of all immunosuppressive drugs was observed in a certain fraction of liver transplant recipients.[4] Various attempts have been made to define the differences in phenotype and gene expression between PBMCs obtained from operationally tolerant liver recipients and those obtained from patients requiring continuous immunosuppressive therapies.[4,5] Although these studies have provided valuable information on the cellular and molecular bases of the maintenance of tolerance, translation of these observations into clinical practice has been difficult. Biomarkers are thus still needed to better evaluate the immune status of transplant recipients, diagnose graft rejection noninvasively, and individualize immunosuppressive therapy.[4–6]

Beside classic measurement analyses using MLCs,[15] several approaches have been established to more accurately assess the alloreactivity of recipient T cells.[16–19] Until recently, it was believed that activated helper T (Th) cells terminally differentiated into either Th1 (producing IFN-γ) or Th2 (producing interleukin [IL]-4, IL-5, and IL-10), which had cytopathic or cytoprotective effects, respectively.[20–22] Therefore, most of the newly established methods were based on the production of these cytokines. However, more recent studies indicate that Th1 and Th2 cells can stimulate graft rejection,[21, 22] whereas regulatory T cells, rather than Th2, are the crucial inhibitors of allospecific immune responses.[23] Together with the discovery of Th17-producing IL-17,[24] the current paradigm is that the relative balance of cytopathic Th1 and Th17 cells versus cytoprotective regulatory T cells determines whether the graft is accepted or rejected.[4,25] Because these T cells are distinguished by the expression profiles of their cytokines, surface markers, and transcription factors, flow cytometric analysis has a huge advantage in quantifying these factors simultaneously.[6–9] In the present study, we therefore attempted to establish a new detection system of alloreactive T cells by intracellular staining for IFN-γ as the first step for the multiparameter analysis of alloreactive T cells.

Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCLs) have been widely used as stimulator cells to assess allospecific T-cell responses[18, 19] because they can be repetitively used after their establishment. However, the antigenicity of EBV itself is known to be high, and EBV-specific T cells therefore exist in high frequencies in the peripheral blood of EBV carriers.[26] In addition, EBV can be reactivated during immunosuppressive conditions, such as those in posttransplant periods.[27] Considering that EBV is a ubiquitous latent gamma-herpes virus infecting >90% of the world's population,[28] the use of LCLs to detect alloreactive T cells might affect the interpretation of the results in some cases. As shown in Table 2 and Fig 1, our detection system had a fine antigen specificity, indicating that CD40-activated B cell is a very good tool for allostimulation, avoiding EBV-specific T-cell responses.

Animal studies show the importance of regulatory T cells for graft acceptance,[29, 30] and some clinical studies suggest the involvement of $CD4^+CD25^+$ regulatory T cells in graft survival.[31,32] We checked temporal changes in the expression of *Foxp3,* a master regulator of $CD4^+CD25^+$ regulatory T cells,[7] by quantitative PCR but did not find any difference in peripheral $CD4^+$ and $CD8^+$ T cells between

the rejection and nonrejection groups, although the difference in *Foxp3* expression was observed by using T cells within the grafts.[32] One explanation is that the number and/or the ratio of alloreactive Foxp3$^+$ regulatory T cells might be smaller in the blood than in the grafts.

Recent observations clearly indicate that the relative balance of various T-cell subsets determines the fate of allografts.[4,25] Our present study paved the way for the establishment of multiparameter flow cytometric analyses of alloreactive T cells that would bring us useful information to define the biomarkers for detrimental immune responses to the grafts.

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Isolation of human mAbs that directly modulate FMS-related tyrosine kinase 3 signaling

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FMS-related tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase that plays important roles in hematopoiesis, including early progenitors and dendritic cell development. FLT3 is expressed at high levels in 70–100% of cases of AML and in virtually all cases of B-lineage acute lymphoblastic leukemia. FLT3 is regarded as a molecular target in the development of novel therapies for acute leukemia patients. Currently, many small-molecule FLT3 inhibitors have been developed, but clinical trials have resulted in limited antileukemia effects because of off-target toxicities and drug resistance. The development of anti-FLT3 Abs might overcome these difficulties and enhance the antileukemia efficacy of FLT3 inhibitors. In the present study, we demonstrate the isolation of novel human mAbs against FLT3 with antagonistic or agonistic activities. An antagonistic Ab, designated A2, continuously inhibits FLT3 ligand (FL)-induced phosphorylation of FLT3 and MAPK. A2 cooperatively induces apoptosis with daunorubicin, even in the presence of FL. An agonistic Ab, designated 3E6, surprisingly induces the phosphorylation of FLT3 and MAPK, and supports the growth of a factor-dependent cell line independently of FL addition. In addition, A2 showed complement-dependent cytotoxicity activity, but was devoid of Ab-dependent cell mediated cytotoxicity. Finally, we evaluated Ab internalization in a cell line. Immunofluorescence and flow cytometry analyses revealed that A2 is efficiently internalized. Collectively, these data demonstrate that A2 is a potent human Ab that might be capable of delivering cytotoxic reagents and that has antagonistic effects on FLT3 signaling. In addition, 3E6 might be a potential scaffold for novel dendritic cell-based immunotherapies. (*Cancer Sci* 2012; 103: 350–359)

FMS-related tyrosine kinase 3 (FLT3) is a class III receptor tyrosine kinase expressed on early hematopoietic progenitor cells that plays important roles in hematopoiesis.[1–4] The FLT3 ligand (FL) is active in both soluble and membrane-bound forms, and is produced by bone marrow stromal cells, T cells, and endothelial cells. The FLT3 ligand acts in synergy with other cytokines in promoting hematopoietic expansion.[1,5] Upon stimulation with FL, FLT3 dimerizes and undergoes autophosphorylation, which results in an upregulation of its tyrosine kinase activity. This increase in activity triggers signaling through an array of downstream pathways, including the phosphatidylinositol-3 kinase and MAPK cascades, thereby promoting cell proliferation and inhibiting apoptosis.[2,3] FLT3 is expressed at high levels in 70–100% of cases of AML and in virtually all cases of B-lineage acute lymphoblastic leukemia (ALL).[2,3,6,7] Therefore, FLT3 is expected to be a potent molecular target in the development of efficient AML therapies.[8] Several inhibitors are now in preclinical investigation stages. Available clinical trial data show that there is a strong decline in peripheral blood blasts in response to current FLT3 inhibition-based therapies, but bone marrow responses are less common. Thus, molecular-targeting therapies against FLT3 are urgently sought as combination therapies that could potentially overcome drug resistance to FLT3 inhibitors. One of reasons for the drug resistance has been considered as the contribution of the leukemia microenvironment, harboring an abundance of FL.[9,10]

In the present study, we aimed to isolate human mAbs against FLT3 that could be useful in clinical use. Recently, our group established a procedure for the comprehensive identification of tumor-associated antigens through the extensive isolation of human mAbs that might be therapeutically advantageous.[11,12] Using this strategy, several mAbs with agonist and antagonist activities were isolated. Two of the identified mAbs are antagonists and continuously inhibit the FL-induced phosphorylation of FLT3 and MAPK. In addition, the A2 mAb induces complement-dependent cytotoxicity (CDC) and was shown to be efficiently internalized. Therefore, A2 is a potent human Ab able to deliver cytotoxic reagents and has an antagonistic effect on FLT3 signaling.

Materials and Methods

Cell lines. The human leukemia cell lines, EOL-1, was purchased from RIKEN (Tsukuba, Japan). KOCL58 and KOPB26 were kindly provided by Dr Kanji Sugita (University of Yamanashi, Yamanashi, Japan).[9] These cell lines, BaF3/FLT3–internal tandem duplication (ITD), and BaF3/FLT3–D835Y[7] were maintained in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% FBS (Equitech-Bio, Kerrville, TX, USA) at 37°C in a humidified atmosphere of 5% CO_2. OCI-AML5 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and maintained in alpha-minimum essential medium (MEM) with 20% FBS and 10% (v/v) conditioned medium from cell line 5637 (DSMZ).

Screening of the phage Ab library with the FLT3 antigen. The AIMS5 phage Ab library was used as a source of human mAbs.[11,12] The antigen used in the screenings of the Ab library was prepared as follows: cDNA encoding an myc-tagged extracellular portion of human FLT3 (myc-rEC-FLT3) was inserted into a pcDNA3 expression vector.[13] The resultant plasmid DNA was transfected into 293T cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the transformants were grown. The myc-rEC-FLT3 protein was purified from the supernatant of the cell culture with an anti-myc tag Ab-conjugated column. The myc-rEC-FLT3 protein was mixed with protein G Dynabeads (Veritas, Tokyo, Japan) and anti-myc tag Ab (MBL,

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Nagoya, Japan), and the mixture was incubated for 2 h at 4°C. Phages (1×10^{13} c.f.u.) from the AIMS5 library were then added to the mixture and incubated for 2 h at 4°C. Following several PBS washes using a magnetic device, phages that bound to the beads were directly infected into *Escherichia coli* (*E. coli*) DH12S cells (Invitrogen). Following phage preparation, this panning procedure was performed three times. The phages prepared after three pannings were subjected to isolation of antigen/antibody complexes through organic solvent (ICOS) screening.[11] The EOL-1 cells expressing FLT3 were used as antigens. ICOS screenings were performed twice.

Selection of clones that bound to FLT3. Following the final selection rounds, *E. coli* infected with the recovered phages were spread on plates, and several hundred colonies were picked. When *E. coli* with phagemid was grown without helper phages, the scFv-C_L molecule fused with cp3 was secreted into the medium.[14] The supernatant of the culture was subjected to ELISA with anti-cp3 Abs (MBL) and with the myc-rEC-FLT3 protein. Clones that reacted with both the anti-cp3 Ab and myc-rEC-FLT3 protein were selected. The DNA sequence was determined using an ABI Prism 3100 genetic analyzer and a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Preparation of Ab forms. The scFv-C_L-cp3 levels were determined with ELISA assay. After the scFv-C_L-cp3 molecules were converted into the scFv-C_L-PP (P denotes a single Fc-binding domain of protein A) form of the Ab, they were purified with an IgG-conjugated column. IgG_1 was prepared using a high-expression vector, and purified with a protein G-conjugated column.

Flow cytometry. Cells (5×10^5) were incubated with 10 μg scFv-pp or IgG in 100 μL PBS for 45 min on ice. After the cells were washed with PBS, ALEXA488-conjugated antihuman IgG (Invitrogen) was incubated with the cells for 45 min on ice. After two washes, the cells were subjected to flow cytometry (FCM) analysis with a FACSCalibur system (Becton Dickinson, San Jose, CA, USA).

Immunoprecipitation and immunoblotting analysis. Cells were first cultured in serum-free X-VIVO 10 medium (LONZA, Basel, Switzerland) overnight, and then treated with FL and/or in serum-free medium under various conditions. Following treatments, cells were lysed in lysis buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5 mM EDTA, 10 mM NaF, 1 mM PMSF, 0.5 mM $NaVO_4$, and 0.5 mM PhosSTOP (Roche, Basel, Switzerland). Equal amounts of cell lysate from each sample were mixed with anti-FLT3 Ab (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), agitated for 1 h at 4°C, and incubated with protein G agarose (Santa Cruz Biotechnology) for 2 h at 4°C. Protein mixtures were separated by SDS-PAGE, blotted onto a PVDF membrane, and probed with an anti-phosphotyrosine Ab (4G10; Upstate Biotechnology, Lake Placid, NY, USA) or anti-FLT3 Ab.[7] Proteins were visualized with chemiluminescence (Santa Cruz Biotechnology). To detect phospho-MAPK or the signal transducer and activator of transcription 5 (STAT5) protein, equal amounts of cell lysate were separated by SDS-PAGE, transferred onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA) and immunoblotted with anti-phospho-MAPK or STAT5 Ab (Cell Signaling, Danvers, MA, USA), respectively. To detect total MAPK or the STAT5 protein, stripped membranes were reprobed with anti-MAPK or STAT5 Ab (Cell Signaling), respectively.[15]

Cell proliferation assay. OCI-AML5 cells were first grown in the presence of FL (1.7 nM),[16] and were then split into a 96-well plate at a concentration of 2×10^4/100 μL. IgG Abs (67 nM) were added to the medium. Cells were then cultured with or without FL for 7 days. Cell growth was measured using CellTiter96 (Promega, Madison, WI, USA). Optical absorbance was determined using an ARVO X spectrophotometer (Perkin Elmer, Waltham, MA, USA). Experiments were performed in triplicate.

Dye-exclusion assay. KOCL58 (2×10^4/mL) cells were precultured in the presence or absence of FL (1.7 nM) and the IgG Abs (67 nM) overnight. Cells were treated with daunorubicin (30 ng/mL; Meiji Seika, Tokyo, Japan) for 48 h at 37°C. Living and dead cells were counted in triplicate using a dye-exclusion assay.[9] The percentage of viability was calculated as (living cell number)/(total cell number) × 100.

Immunofluorescence analysis and microscopy. The EOL-1 or BaF3 cells were incubated with the IgG Abs (67 nM) under various conditions. Cells were fixed for 30 min in methanol at −20°C. The slides were then incubated with 200 μL Image-iT FX signal enhancer (Molecular Probes, Carlsbad, CA, USA) for 30 min at room temperature. After washing three times with PBS, the cells were incubated with the primary Ab at a 1:50 dilution for 1 h at room temperature. Following the three PBS washes, cells were incubated for 1 h at room temperature with antihuman IgG–Alexa Fluor 488 Ab (Molecular Probes) at a 1:200 dilution. After three PBS washes, the slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Confocal laser images were captured with an Olympus BX51 microscope (Olympus, Tokyo, Japan). Original magnification was ×400 for all panels.[17]

Complement-dependent cytotoxicity and Ab-dependent, cell-mediated cytotoxicity. Complement-dependent cytotoxicity and Ab-dependent, cell-mediated cytotoxicity (ADCC) were measured with a cytotoxicity detection kit (Roche). The assay measures lactate dehydrogenase (LDH) activity released from damaged cells. MOLM14 and KOCL58 were used as the target cells. Peripheral blood monocytes and, complement were isolated from human whole blood from healthy donors. Target cells, prepared as 1×10^6 cells/well, and containing several concentration of anti-FLT3 Abs, were incubated with effector cells (effector-to-target ratio: 50:1) or complement. To determine the percentage of cell-mediated cytotoxity, we calculated the average absorbance of the triplicates and substracted the background. These values were substituted as follows: Percentage of cytotoxity = ([effector − target cell mix − effector cell control] − low control)/(high control − low control) × 100. ''High control'' provides information about detergent lysed target cells; ''low control'' provides information about the untreated target cell.

Colony-forming cell assay. Methocult (H4435; STEMCELL Technologies, Vancouver, Canada) was used for the colony-forming cell (CFC) assay according to the manufacturer's instructions. CD34-positive bone marrow cells from healthy donors under an institutional protocol were isolated using the CD34-MicroBeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Colony-forming cells were counted at day 14.

Results

Isolation of human mAbs specifically bound to FLT3. The AIMS5 phage Ab library was used as a source of human mAbs. To isolate mAbs able to bind native FLT3 on the cell surface, a two-step screening technique was used, as described in the Materials and Methods. Of the several hundred clones picked at the final screening stage, 265 positively reacted with both anti-cp3 Ab and myc-rEC-FLT3 in the ELISA assays. These clones were classified by sequencing. Five unique clones were isolated and designated as A2, G3, 1B8, 1E3, and 3E6. The total numbers obtained for each clone were 239, 13, 10, 1, and 2 for A2, G3, 1B8, 1E3, and 3E6, respectively.

The binding ability of these clones to FLT3 on the cell surface was directly examined using FCM. As shown in Figure 1(a), these clones bound to the molecule on FLT3-expressing 293T

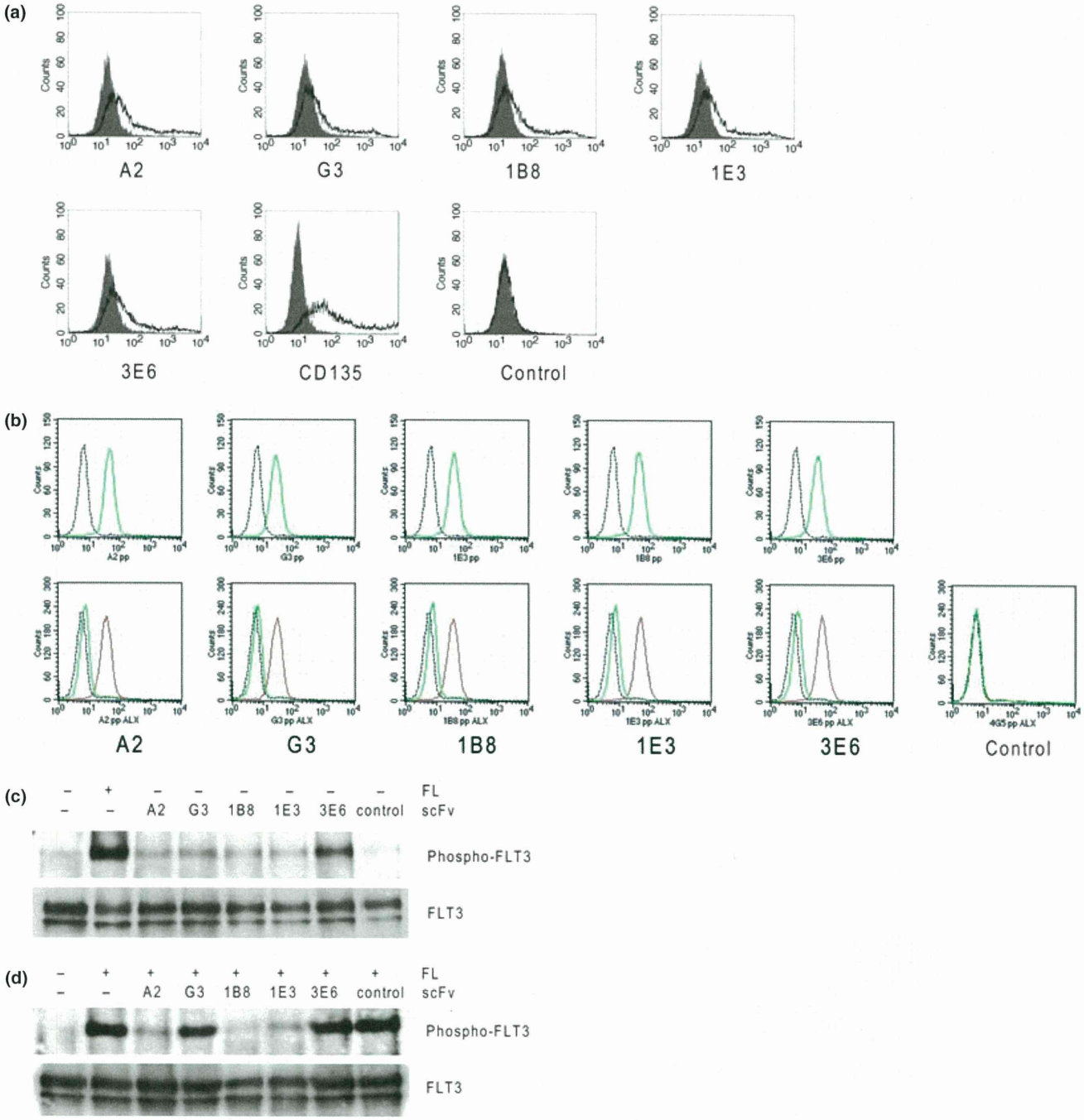

Fig. 1. FMS-related tyrosine kinase 3 (FLT3)-binding assay analyses and effects of isolated Abs on FLT3 phosphorylation. (a) FLT3-binding activity of the scFv form of A2, G3, 1B8, 1E3, and 3E6 Abs were examined using flow cytometry (FCM). CD135 is a polyclonal Ab against FLT3 and was used as a positive control. Open histogram represents the staining of FLT3-transfected 293T cells, and the shaded histogram represents the staining of mock-transfected 293T cells. (b) Effects of the FLT3 ligand (FL) treatment on FLT3–Ab interactions. Upper lane: Following the incubation of EOL-1 cells with the scFv form of the Ab, FL was added at 3.3 nM, and incubated for 10 min at 4°C. Cells were subjected to FCM with the second Ab. Green line represents binding with each scFv Ab, and the broken line represents the negative control with the scFv form of the clone 4G5. Lower lane: After the incubation of EOL-1 cells with 3.3 nM FL for 10 min at 4°C, the scFv form of the Ab was added and incubated. Cells were then subjected to FCM analysis with the second Ab. Red lines represent binding of the Ab without FL treatment. Green lines represent binding of the Ab following the addition of FL. Broken lines represent negative controls. (c) FLT3 ligand and Abs were separately added to the EOL-1 cells. FLT3 phosphorylation was examined as described in the Materials and Methods. (d) After the EOL-1 cells were incubated with mAb, FL was added.

cells, but not on 293T cells not expressing the receptor. We then examined interactions between FLT3, the anti-FLT3 Ab, and FL. In the first experiment, shown in the upper lane of Figure 1(b), FL was added following the incubation of EOL-1 cells with the mAb. In the second experiment, the anti-FLT3 Ab was added following the incubation of EOL-1 cells and FL (Fig. 1b, lower lane, green line). When FL was added following the formation of FLT3/anti-FLT3 Ab complexes, the complexes appeared to remain stable. Alternatively, after FL bound to the FLT3 expressed by EOL-1 cells, the Abs could no longer bind

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to the cell surface. These results suggest that the binding of the Abs to FLT3 on the cell surface clearly competes with that of FL to FLT3, and therefore, the Abs should potently intervene in FLT3 signaling.

Effects of scFv Abs on FLT3 phosphorylation. The binding of FL to FLT3 on the cell surface induces FLT3 dimerization followed by autophosphorylation. The effects of the anti-FLT3 Abs on this process were examined. The Ab was first added to the EOL-1 cells without FL. As shown in Figure 1(c), 3E6 induced the same degree of FLT3 phosphorylation as FL. We then examined the effects of the mAbs on the FL-mediated phosphorylation of FLT3. Three of the isolated mAbs, A2, 1B8, and 1E3, inhibited the FL-mediated phosphorylation of FLT3, but G3 did not show any effect on FLT3 phosphorylation (Fig. 1d). In summary, 3E6 has an agonistic effect on FLT3 signaling, whereas A2, 1B8, and 1E3 have antagonistic effects on FL-induced phosphorylation. The scFv forms of these four mAbs were converted to complete human IgG_1 Abs, and subjected to further analyses.

Agonistic effects of 3E6 on FLT3 signaling. To further analyze the agonistic effects of 3E6 Ab on FLT3 signaling, two leukemia cell lines with abundant FLT3 expression on the cell surface, KOCL58 and KOPB26,[9] were treated with FL; the scFv form and the IgG form of 3E6. FLT3 and MAPK phosphorylation were then examined by Western blotting. As shown in Figure 2(a), the FL, scFv, and IgG forms of 3E6 induced both FLT3 and MAPK phosphorylation. To quantitatively examine the agonistic capability of 3E6, KOPB26 cells were treated with various concentrations of the scFv form of 3E6. As shown in Figure 2(b), MAPK phosphorylation was observed at concentrations >10 nM.

The growth of OCI-AML5 cells requires specific hematopoietic growth factors, including FL, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-3.[16] To further assess the agonistic effects of 3E6, its effects on OCI-AML5 cells were examined (Fig. 2c). The growth of OCI-AML5 cells was supported to a greater degree by both the scFv (200 nM) and the IgG forms of 3E6 (67 nM), rather than by treatment with FL (1.7 nM).

In addition to MAPK signaling, FLT3–ITD also activates cell proliferation through STAT5 signaling.[18] To exclude the possibility that 3E6 transduces aberrant signaling through STAT5, we investigated the STAT5 phosphorylation of OCI-AML5 cells with 3E6 addition (Fig. 2d). As expected, 3E6 did not induce STAT5 phosphorylation.

Antagonistic effects of A2, 1B8, and 1E3 on FLT3 signaling. The scFv forms of A2, 1B8, and 1E3 demonstrated inhibitory effects on FL-induced FLT3 phosphorylation in EOL-1 cells (Fig. 1d). The effects on FLT3 and MAPK phosphorylation were also examined using the IgG forms of the Abs on three acute leukemia cell lines: EOL-1, KOCL58, and KOPB26. Although 1.7 nM FL induced strong FLT3 and MAPK phosphorylation, the IgG forms of A2 and 1B8 (67 nM) clearly inhibited FLT3 phosphorylation, and the IgG form of A2 inhibited MAPK phosphorylation in 1-h incubation (Fig. 3a). Furthermore, KOCL cells were incubated for 48 h in the presence of both 1.7 nM FL and 67 nM IgG mAbs (Fig. 3b). The inhibition of the phosphorylation of MAPK by these two mAbs was observed over this extended culture period. We then quantitatively evaluated the antagonistic capability of A2 on MAPK phosphorylation of the cell line induced with FL. After the KOCL58 cells were incubated with A2 IgG at various concentrations, 1.7 nM FL was added, and MAPK phosphorylation was examined (Fig. 3c). Phosphorylation was clearly inhibited by A2 IgG at concentrations >1.7 nM.

Furthermore, the effect of the IgG forms of the three antagonistic mAbs on the FL-dependent growth of OCI-AML5 was examined. As indicated in Figure 3(d), treatment with A2, 1B8, and

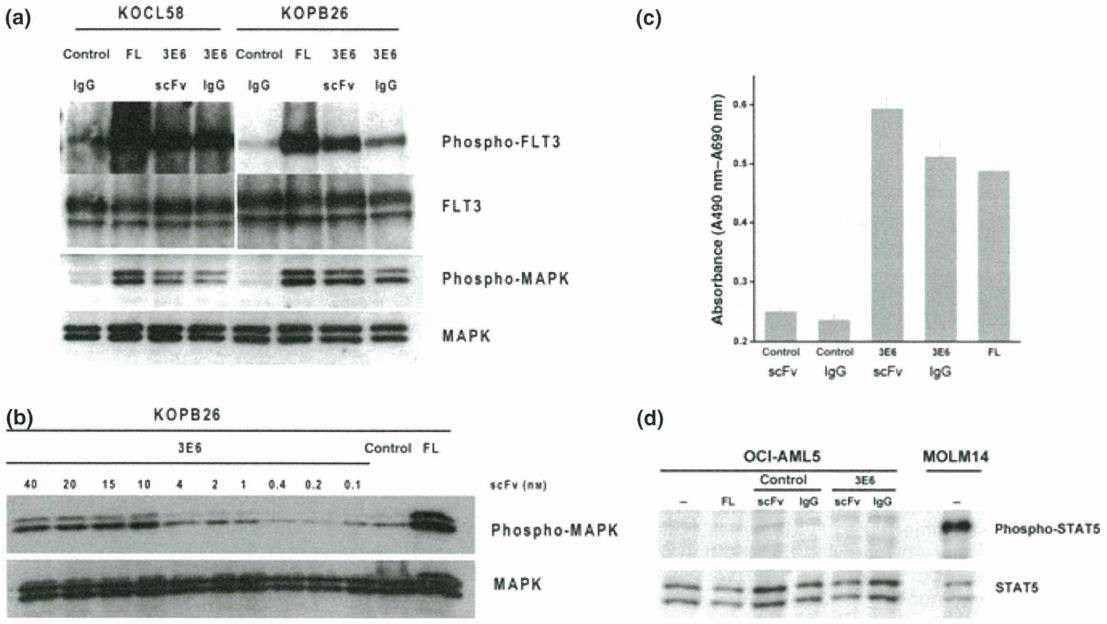

Fig. 2. Agonistic effects of 3E6 on KOCL58, KOPB26, and OCI-AML5 cells. (a) After the KOCL58 and KOPB26 cell lines were incubated with 3E6 IgG (67 nM), 3E6 scFv (200 nM) or the FMS-related tyrosine kinase 3 (FLT3) ligand (FL) (1.7 nM) for 10 min, FLT3 and MAPK phosphorylation were evaluated by immunoblotting. (b) After the KOPB26 cells were incubated with 3E6 scFv at concentrations ranging from 0.1 to 40 nM for 10 min, MAPK phosphorylation was examined by Western blotting. (c) Growth of the OCI-AML5 cells was examined with either FL alone (1.7 nM), 3E6 scFv (200 nM), 3E6 IgG (67 nM), or controls at 7 days' incubation. Cell growth was measured using the MTT assay. (d) Signal transducer and activator of transcription 5 (STAT5) phosphorylation on the OCI-AML5 cells with 3E6. MOLM14 cells, harboring the FLT3–ITD mutation, are the positive control.

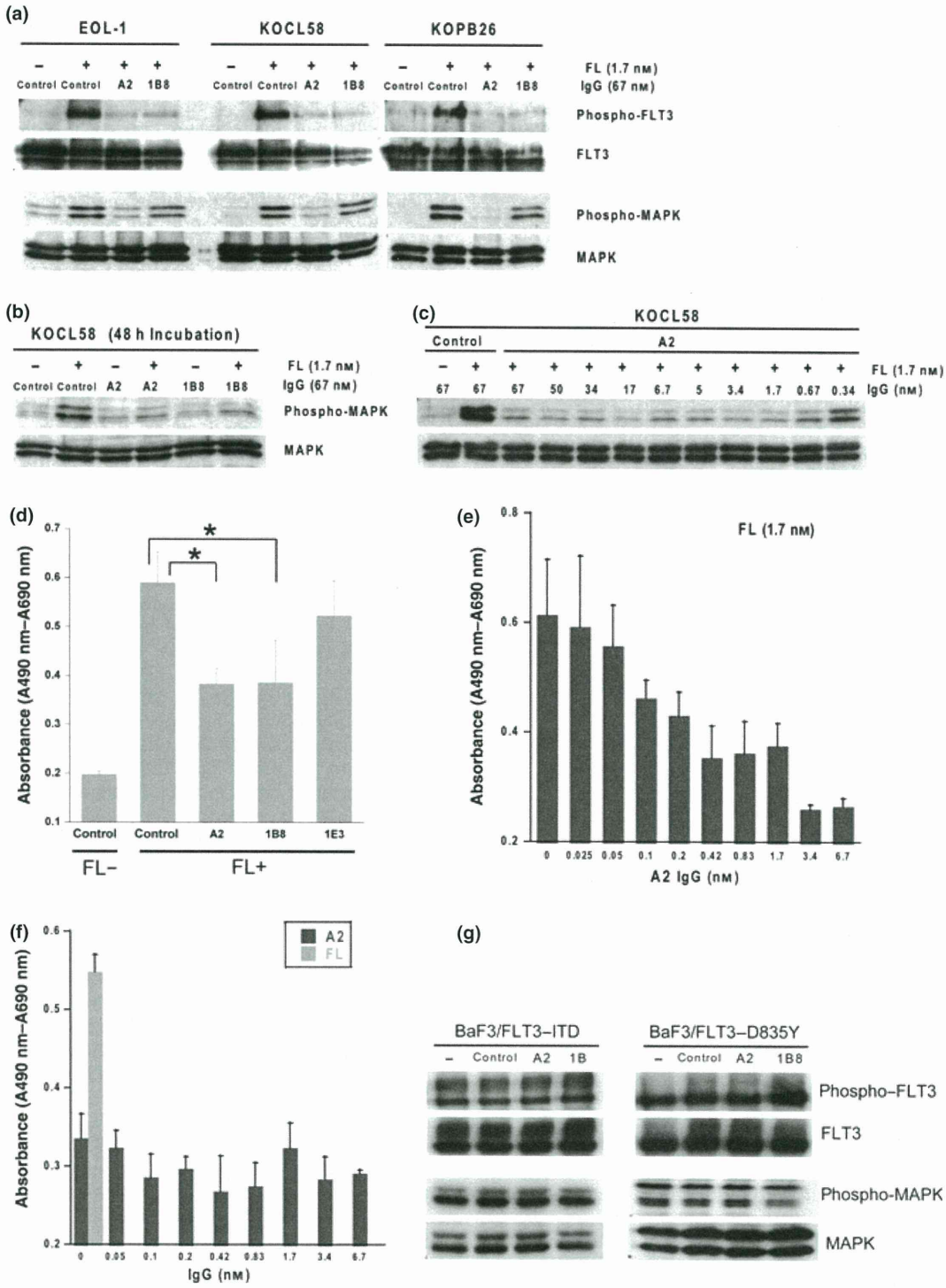

Fig. 3. Antagonistic effects of A2, 1B8, and 1E3 on FMS-related tyrosine kinase 3 (FLT3) signaling. (a) Leukemia cell lines were incubated with IgG Abs against FLT3 (67 nM) for 60 min with or without subsequent incubation with the FLT3 ligand (FL) (1.7 nM). (b) KOCL58 cells were incubated with IgG Abs (67 nM) with or without FL (1.7 nM) for 48 h. (c) KOCL58 cells were incubated with A2 IgG in doses ranging from 0.34 to 67 nM with FL (1.7 nM) for 10 min. (d) Inhibitory effects of the IgG form of A2, 1B8, and 1E3 on the growth of the OCI-AML5 cell line. OCI-AML5 line, which is a factor-dependent leukemia cell line, was grown with the IgG form of the Abs (67 nM) and FL (1.7 nM) for 7 days. Cell growth was measured using the MTT assay. Degree of growth inhibition was calculated by comparing growth with and without FL. Error bar shows the standard deviation. $*P < 0.05$. (e) Dose-dependent inhibition of FL-induced proliferation of OCI-AML5 cells by A2 IgG. Concentration of FL is 1.7 nM. Cell growth was measured using the MTT assay. (f) A2 IgG alone did not induce the proliferation of OCI-AML5 cells. FL (1.7 nM) is the positive control. Cell growth was measured using the MTT assay. (g) Addition of A2 or 1B8 IgG (6.7 nM) did not inhibit the phosphorylation of FLT3 and MAPK in BaF3 cells expressing FLT3–internal tandem duplication (ITD) or FLT3–D835Y.

1E3 resulted in growth inhibitions of 52.8%, 52%, and 17.1% relative to the controls, respectively. These results indicate that A2 and 1B8 possess antagonistic effects on FLT3 signaling. In addition, Figure 3(e,f) shows that A2 has a dose-dependent inhibition of FL-induced proliferation, and A2 alone does not induce the proliferation of OCI-AML5 cells, respectively.

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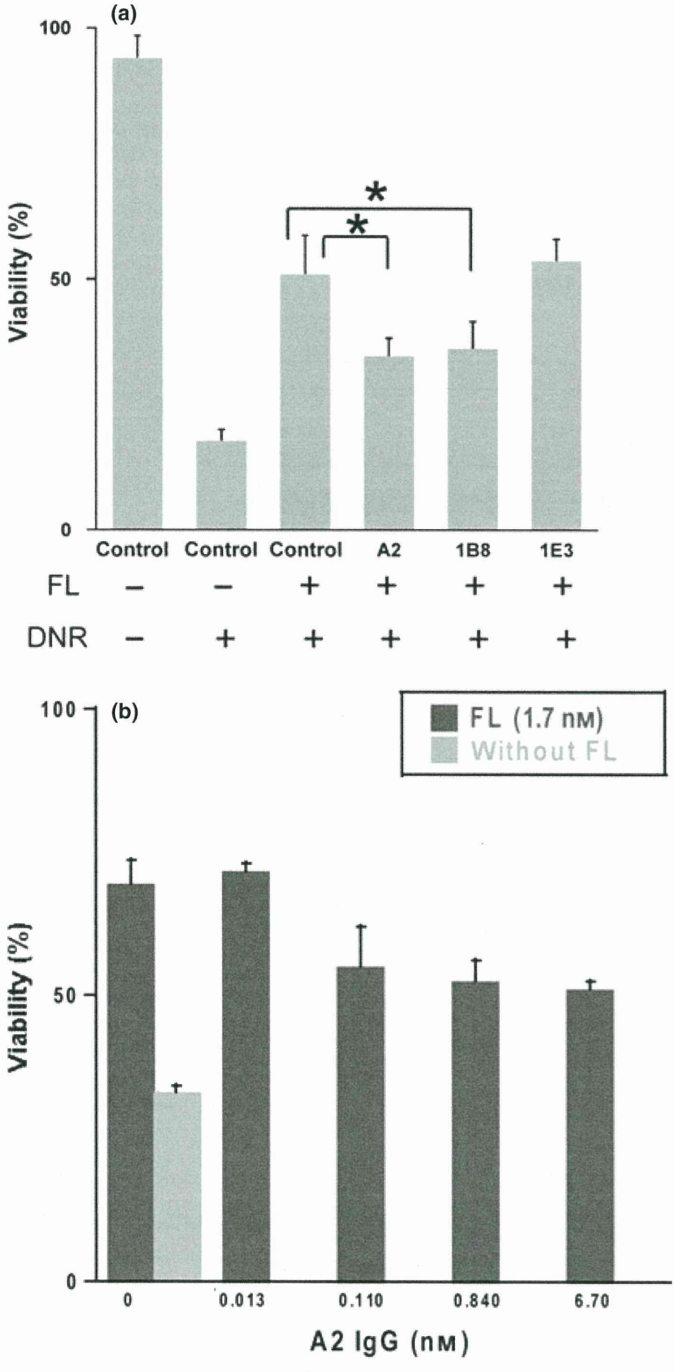

Fig. 4. Effects of the FMS-related tyrosine kinase 3 (FLT3) ligand (FL) and antagonistic anti-FLT3 mAbs on chemotherapy-induced cytotoxicity. (a) KOCL58 cells were precultured in the presence or absence of FL (1.7 nM) and the IgG Abs (67 nM) overnight. Cells were then treated with daunorubicin (DNR) (30 ng/mL) for 48 h at 37°C. In the control experiments, the addition of FL rescued KOCL58 cells from DNR-induced cytotoxicity with FL-treated cells and -untreated cells showing 17.7% and 51% viability, respectively. Incubation with the IgG form of A2 or 1B8 (67 nM) restored DNR-induced cytotoxicity to 34.7% and 37.3% viability, respectively. Cell viability was determined using a dye-exclusion assay. (b) Dose-dependent augmentation of DNR-induced cytotoxicity of KOCL58 cells by A2 IgG. Concentration of FL was 1.7 nM. Error bar shows the standard deviation. *$P < 0.05$.

Finally, we evaluated whether A2 antagonizes mutant FLT3 signaling in BaF3 cells. As shown in Figure 3(g), A2 and 1B8 did not have inhibitory effects in mutant FLT3 phosphorylation and MAPK signaling.

Antagonistic IgG enhance daunorubicin-induced cytotoxicity of FLT3-positive leukemia cells in the presence of FL. It is notable that FLT3 is highly expressed in acute leukemias harboring the myeloid/lymphoid or mixed lineage leukemia gene rearrangement,[19] and because of FL stimulation from bone marrow stromal cells, these cells become resistant to chemotherapy.[9,20] In this context, we further examined the chemotherapy-sensitivity of the KOCL58 cell line, which has the *MLL* gene rearrangement,[9] in the presence of FL and antagonistic anti-FLT3 mAbs. Without FL, daunorubicin treatment permitted only 17.7% of KOCL58 cells to survive (Fig. 4a). However, the addition of FL (1.7 nM) interfered with the daunorubicin-induced cytotoxicity of KOCL58 cells up to 51% viability. When 1.7 nM FL was added together with 67 nM A2 and 1B8 IgG, the daunorubicin-induced cytotoxicity of KOCL58 cells recovered again to 34% and 36% viability, respectively. In addition, Figure 4(b) shows the dose-dependent augmentation of daunorubicin-induced cytotoxicity by the A2 Ab. These results suggest that antagonistic IgG against FLT3 enhance the daunorubicin-induced cytotoxicity of FLT3-positive leukemia cell lines, even in the presence of FL, which presumably is supplied from the bone marrow and peripheral blood environments.[20,21]

Internalization of anti-FLT3 mAbs into FLT3-expressing cells. There are many cases where antireceptor tyrosine kinase (RTK) mAbs are internalized following complex formation with the target RTK. Immunofluorescence analyses were performed to examine whether the isolated anti-FLT3 mAbs could internalize into EOL-1 cells. Cells were incubated with the IgG forms of the four isolated Abs (A2, 1B8, 3E6, and 1E3) at 4°C for 15 min. The IgG forms of A2, 1B8, and 3E6 were localized on the cell membrane (Fig. 5a, upper lane). While the scFV form of all four mAbs bound to the cells, the IgG form of 1E3 showed no binding to the cells. The FCM analyses also indicated that the IgG form of IE3 lost FLT3-binding activity (Fig. 5a). Furthermore, we examined the localization of the Abs in differential conditions. After the incubation was prolonged to 4 h, A2 and 1B8 were localized not only on the cell membrane, but also in the cytoplasm (Fig. 5b, upper lane). This tendency was accelerated when the temperature was increased to 37°C for 4 h (Fig. 5b, middle lane). These results were confirmed by FCM analysis, where that the number of cells showing surface binding to A2, 1B8, and 3E6 was attenuated from 69.%, 93.1%, and 95% to 11.8%, 44.7%, and 49.2%, relative to the controls, respectively (Fig. 5b, lower lane). These results indicate that the A2 IgG efficiently internalized into EOL-1 cells. Finally, we examined the internalization of A2 IgG on BaF3/mutant-FLT3 cells. As shown Figure 5(c, lower), A2 internalized into BaF3/FLT3–D835Y cells, as well as EOL-1 cells. However, A2 did not internalize into BaF3/FLT3–ITD cells (Fig. 5c, upper).

A2 and 1B8 IgG induce CDC, but not ADCC. Complement-dependent cytotoxicity and ADCC are major effects for therapeutic Abs. To evaluate them in FLT3-expressing AML cell lines, we used MOLM14 or KOCL58 cells with either A2 or 1B8 IgG. Figure 6 shows that A2 and 1B8 IgG induce CDC mildly, but do not induce any ADCC.

A2 IgG does not have inhibitory effects on normal hematopoietic progenitor cells. FLT3 is a class III receptor tyrosine kinase expressed on early hematopoietic progenitor cells.[1–4] To evaluate the possible inhibitory effects of FLT3 signaling with A2 IgG on normal CD34-positive progenitor cells, we performed standard methylcellulose culture assays. Figure 7 shows that A2 IgG does not have inhibitory effects in CFC assays.

Discussion

FLT3 is a unique target for the development of molecular-targeting therapy for the treatment of leukemia,[8] and is frequently expressed in AML and ALL cells.[2,3] Recently, it was reported

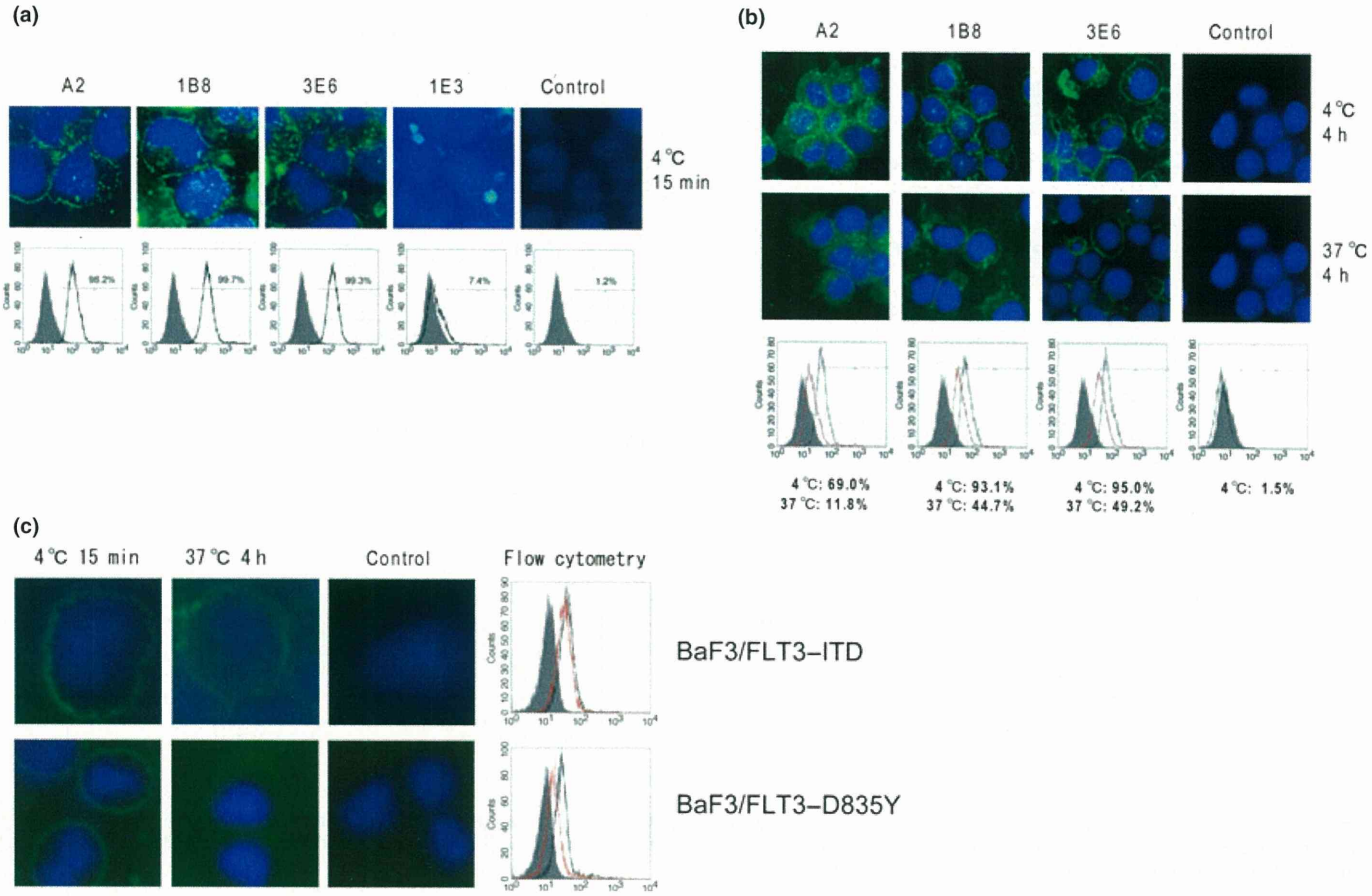

Fig. 5. Localization of anti-FMS-related tyrosine kinase 3 (FLT3) mAbs in FLT3-expressing cells. (a) EOL-1 cells were incubated with the IgG Abs (67 nM) for 15 min at 4°C. Subcellular localization of the IgG Abs was analyzed by confocal laser microscopy (upper lane). DAPI was used for nuclear staining. Surface binding of the IgG Abs was analyzed by flow cytometry (FCM) without any reagents for permeabilization (lower lane). Shaded histograms are negative controls. Positive cell population for FCM was calculated by gating and is indicated at the bottom of the Figure. (b) EOL-1 cells were incubated with the IgG Abs (67 nM) for 4 h. Incubation temperature was 4°C (upper lane) or 37°C (middle lane). Surface binding of the IgG Abs was analyzed by FCM without any reagents for permeabilization (lower lane). (c) Internalization of mutant FLT3 in BaF3 cells by A2 IgG. Concentration of IgG was 67 nM. Black lines represent incubation for 4 h at 4°C. Red lines represent incubation for 4 h at 37°C. Shaded histograms are negative controls.

that differentiation into all hematopoietic lineages involves Flt3-expressing, non-self-renewing progenitors.[4] In contrast, hematopoietic stem cell (HSC) origin and maintenance do not include an Flt3-expressing stage.[4] This finding means that eradication of FLT3-expressing cells does not eliminate normal HSC populations. The FLT3 ligand is expressed at high levels as a soluble or membrane-bound form on bone marrow stromal cells.[22] In bone marrow, the FL/FLT3 interaction from bone marrow stromal cells contributes to persistent minimal residual disease in FLT3-expressing leukemia cells.[9] To inhibit FL/FLT3 interaction in leukemia cells, several small-molecule tyrosine kinase inhibitors against FLT3 are in clinical testing, and some have shown limited clinical activity in patients with relapsed or refractory AML. However, the depth and duration of clinical responses to FLT3 inhibitor monotherapies have been modest.[8,23,24] One of reasons why leukemic blasts acquire resistance to the inhibitors is the contribution of the leukemia microenvironment, harboring an abundance of FL, to the drug resistance of leukemic stem cells.[8,10,23,24]

For developing novel Ab-based FLT3 inhibitors, we isolated several anti-FLT3 mAbs with agonist or antagonist activities by phage-Ab library-based screening. Two of the identified mAb (A2 and 1B8) are antagonists, and 3E6 is an agonist. Because of the functional difference between antagonists and agonists, Verstraete *et al.*[25] recently described the structural base of FL/FLT3 interaction. According to the results, the N-terminal loop of FL binds with the FLT3 D3 domain, in which FL/FLT3 interaction occurs in a single contact site covering <900Å[2] of buried surface area, which is two times less extensive compared with other class III receptor tyrosine kinases. They suggest that FL/FLT3 interaction is reminiscent of a classic lock and key binding mode observed in affinity-matured Ab–antigen interactions. Therefore, we speculate that 3E6 interacts with the FL-binding epitope on the FLT3 D3 domain; A2 and 1B8 interact with the FL/FLT3 binding surface, except the FL-binding epitope; and the G3 Ab interacts with FLT3 outside of the FL/FLT3 binding surface.

To overcome the obstacles for developing FLT3-targeting therapy, combination therapy with antagonizing mAbs against FLT3 is considered. In this study, pharmacokinetics of A2 *in vivo* was not yet determined, and the addition of more than 1.7 nM A2 IgG was found to interfere with the addition of 1.7 nM FL *in vitro*. Plasma FL levels in AML patients are reported as being mostly <3 ng/mL (0.1 nM).[10] In addition, membrane-bound FL surrounds leukemia cells in bone marrow.[1,5] Although it is difficult to estimate precisely how many FL molecules interact with leukemia cells in bone marrow, A2 IgG might be enough to overcome the cytoprotective effects of

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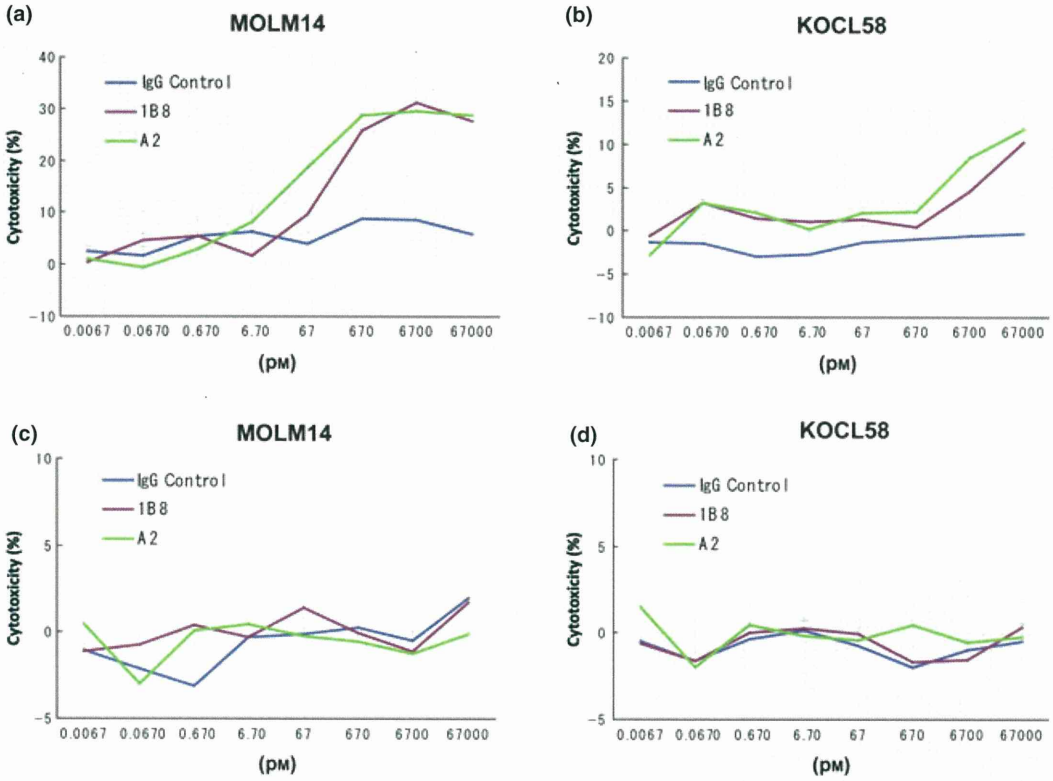

Fig. 6. Effects of A2 and 1B8 IgG on complement-dependent cytotoxicity (CDC) and Ab-dependent, cell-mediated cytotoxicity (ADCC). MOLM14 (a,c) or KOCL58 (b,d) cells were incubated with complement (a,b) or effector cells (c,d) with several concentration of anti-FMS-related tyrosine kinase 3 (FLT3) Abs. Error bar shows the standard deviation.

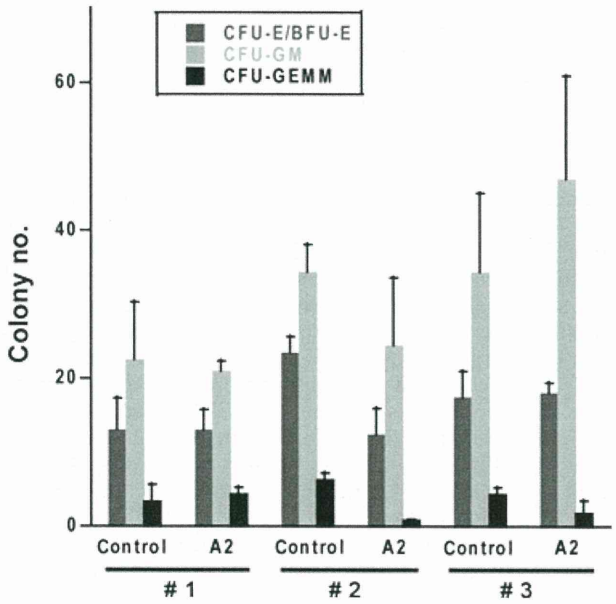

Fig. 7. Effects of A2 IgG on normal hematopoietic progenitors *in vitro*. CD34-positive bone marrow cells were incubated with A2 or control IgG (6.7 nM) in standard methylcellulose culture for 14 days. Each number on the lower lane indicates a sample from a donor. Error bar shows the standard deviation. BFU-E, burst-forming unit–erythroid, CFU-E, colony-forming unit–erythroid, CFU-GEMM, colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte; CFU-GM, colony-forming unit–granulocyte macrophage.

FL in patients because of following reasons. Because the median value of Ab serum levels reported is 554 nM for responders using rituximab pharmacokinetics,[26] it would be possible that A2 IgG antagonizes FL-induced cytoprotective signaling in clinical settings, if the serum levels of A2 IgG achieved are as high as those of rituximab.

Somatic mutations in the *FLT3* gene, including ITD and activation loop mutations, are found in up to 30% of AML cases.[2,3,7] Generally, these FLT3 mutations activate its downstream signaling without FL. We have further examined whether the antagonistic Abs inhibit FLT3 signaling in mutant FLT3-expressing cells. As expected, no significant differences of phospho-FLT3 and phospho-MAPK were observed. Two recent reports indicated that mutated FLT3 is anchored in the perinuclear endoplasmic reticulum and initiates aberrant signaling cascades before translocation of FLT3 to the cell membrane.[27,28] Therefore, it is consistent that the administration of single antagonistic mAbs against FLT3 did not have significant inhibition of mutated FLT3 signaling. Unexpectedly, FLT3–ITD-expressing cells did not internalize A2 IgG. This result might imply that the juxtamembrane domain of FLT3 has important roles for FLT3 internalization, but this requires further investigation.

Complement-dependent cytotoxicity and ADCC are the most important effects of therapeutic Abs for cancer treatment.[29,30] We investigated whether the selected IgG against FLT3 demonstrated CDC an ADCC on FLT3-expressing cell lines. The results show that the IgG forms of A2 and 1B8 induce mild CDC, but not ADCC effects. It is notable that panitumumab does not show ADCC, but is useful in patients.[29]

Gemtuzumab ozogamicin is an Ab-targeted chemotherapy agent for CD33-positive AML, and is conjugated to a derivative of the antitumor compound, calicheamicin.[31] The Ab/antigen complex is internalized into target cells, and the cytotoxic calicheamicin is released intracellularly through hydrolysis. We have shown that A2 IgG efficiently internalize into cells. Therefore, A2 could be utilized as a vehicle of cytotoxic reagents, including antibiotics, radioisotopes, apoptosis-inducing molecules,[32,33] and liposomes[34] carrying siRNA against specific oncogenic fusion transcripts.[35]

FLT3 is expressed at not only hematopoietic progenitors, but also dendritic cells in peripheral lymphoid tissue.[36] The FLT3 ligand treatment dramatically increases the number of functionally-mature dendritic cells in mice.[37] Dendritic cells are powerful antigen-presenting cells and show a remarkable capacity to stimulate antigen-specific T-cell responses.[38] Some reports suggest that FL administration inhibits tumor growth in mice models.[39,40] The agonistic Ab 3E6 is particularly interesting, because virtually a single molecule of the scFv form of 3E6 can induce FLT3 signaling. Although the capability of 3E6 to induce signaling is less than that of FL, the half-life time of therapeutic Abs is more than 14 days.[26] Therefore, it might be possible that 3E6 IgG has long-acting agonistic effects in FLT3 signaling *in vivo*, which lead to novel dendritic cell-based immunotherapies.[38-41]

In summary, A2 is a potent human Ab that has an antagonistic effect, and 3E6 has an agonistic effect on FLT3 signaling. These human mAbs could be potential scaffolds for developing novel FLT3-targeting therapies.

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Disclosure Statement

The authors declare no financial or commercial conflict of interest.

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Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study

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Impact of graft-versus-host disease on outcomes after allogeneic hematopoietic cell transplantation for adult T-cell leukemia: a retrospective cohort study

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Short running head: Impact of GVHD on allografting for ATL
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Abstract

Allogeneic hematopoietic cell transplantation (HCT) is an effective treatment for adult T-cell leukemia (ATL), raising the question about the role of graft-versus-leukemia effect against ATL. In this study, we retrospectively analyzed the effects of acute and chronic graft-versus-host disease (GVHD) on overall survival, disease-associated mortality, and treatment-related mortality among 294 ATL patients who received allogeneic HCT and survived at least 30 days post-transplant with sustained engraftment. Multivariable analyses treating the occurrence of GVHD as a time-varying covariate demonstrated that the development of grade 1-2 acute GVHD was significantly associated with higher overall survival [hazard ratio (HR) for death, 0.65, p 0.018] compared with the absence of acute GVHD. Occurrence of either grade 1-2 or grade 3-4 acute GVHD was associated with lower disease-associated mortality compared with the absence of acute GVHD, while grade 3-4 acute GVHD was associated with a higher risk for treatment-related mortality (HR 3.50, p 0.001). The development of extensive chronic GVHD was associated with higher treatment-related mortality (HR 2.75, p=0.006) compared with the absence of chronic GVHD. Collectively, these results indicate that the development of mild-to-moderate acute GVHD confers a lower risk of disease progression and a beneficial influence on survival of allografted patients with ATL.

Introduction

Adult T-cell leukemia (ATL) is a mature T-cell neoplasm that is causally associated with a retrovirus designated human T-cell leukemia virus type I (HTLV-I).[1-4] HTLV-I is endemic in southwestern Japan, sub-Saharan Africa, the Caribbean Basin, and South America.[3,4] In Japan, more than one million people were estimated to be infected with HTLV-I. Although the majority of HTLV-I-infected individuals remain asymptomatic throughout their lives, about 5% develop ATL at a median age of 40 to 60 years.[4,5]

ATL is categorized into four clinical variants according to its clinical features: smoldering, chronic, acute, and lymphoma types.[6] The acute and lymphoma variants of ATL have an extremely poor prognosis mainly because of resistance to a variety of cytotoxic agents and susceptibility to opportunistic infections; the median survival time is approximately 13 months with conventional chemotherapy,[7,8] although encouraging results have been recently reported with the use of novel agents such as mogamulizumab.[9-11]

Over the past decade, allogeneic hematopoietic cell transplantation (HCT) has been increasingly performed with the aim of improving dismal prognosis of patients who developed ATL.[12-18] Notably, some patients with ATL who relapsed after allogeneic HCT were shown to achieve remission only with the cessation of immunosuppressive agents, raising the question of whether the graft-versus-leukemia effect against ATL can be induced as part of graft-versus-host reaction.[19,20] In one study, among 10 patients who experienced relapse of ATL after transplantation and were withdrawn from immunosuppressive therapy, 8 developed graft-versus-host disease (GVHD)