Results and discussion

Patient characteristics

The characteristics of the 34 allo-SCT recipients are presented in Table 1. Of these, 22 had cGVHD at the sampling, including 7 and 4 with moderate and severe cGVHD respectively. Sampling was performed at a median 189 days after SCT (range: 177-434 days). Their median age was 38.5 years (range: 16-65). Their median weight loss after SCT and median BMI at sampling were 4.1 kg (range: -16.9-26.5 kg) and 20.3 (15.3-34.6), respectively. The 26 normal healthy subjects included 11 males and 15 females. Their median age and BMI were 40 years (range: 28-68) and 21.71 (range: 17.31-29.47), respectively.

Comparison of the concentrations of HMW-adiponectin

Gender and BMI are known to be associated with the concentration of adiponectin [13]. Therefore, HMW-adiponectin levels were compared among groups stratified according to gender and BMI (Figure 1-A and 1-B). The concentrations of HMW-adiponectin were significantly higher in recipients with cGVHD than those in subjects without cGVHD (21.7±11.0 vs 9.1±6.1 μg/ml in females ($P<0.001$), and 10.1±6.8 vs 4.3±2.9 μg/ml in males ($P=0.003$), respectively, Figure 1-A). The concentration of HMW-adiponectin was inversely correlated with BMI ($R^2=0.20$, $P=0.0003$). Furthermore, the HMW-adiponectin levels in recipients with cGVHD were higher than those in normal subjects and recipients without cGVHD in each stratified BMI group (Figure 1-B).

When we analyzed only allo-SCT recipients, a univariate analysis revealed that a high HMW-adiponectin level was associated with female gender, low BMI, the presence and the severity of cGVHD, gut involvement and steroid administrations. Multiple regression analysis revealed that a high HMW-adiponectin level was associated with female gender, steroid administrations, and the severity of cGVHD among allo-SCT recipients (Table 1). When the 26 normal subjects were included, it was further confirmed that a high

HMW-adiponectin level was associated with female gender (β-coefficient 8.2 (95% confidential interval: 4.7-11.8), P<0.0001) and the severity of cGVHD (β-coefficient 6.6 (1.8-11.4), 12.7 (7.0-18.3), and 15.6 (8.4-22.9), P <0.01, each for mild, moderate, and severe cGVHD, respectively), while there was no difference between recipients without cGVHD and normal subjects (P=0.28) (Figure1-C).

Impact of clinical changes in cGVHD on HMW-adiponectin

To assess the impact of clinical changes in cGVHD on serum HMW-adiponectin levels, we analyzed 58 serial sera with a median interval of 84 days (range: 24-196) from 19 patients. Worsening, improving and stable cGVHD were observed in the 13, 7, and 19 sample pairs, respectively. The ratios of the later-to-prior HMW-adiponectin levels between adjacent samples were associated with clinical changes in cGVHD; the HMW-adiponectin level increased as cGVHD progressed, decreased as cGVHD improved, and did not change with stable cGVHD (Figure 1-D, P<0.01 each for comparison between worsening and stable / improving pairs).

Adiponectin is a type of adipokine, which are peptides secreted mainly from adipose tissues. It exists in multimers, and HMW-adiponectin is thought to have the greatest effect on immunity and inflammation [7]. However, adiponectin has been shown to have both pro-inflammatory and anti-inflammatory functions and thus it is difficult to interpret the relationship between a high serum adiponectin level and the development of cGVHD.

First, adiponectin itself might induce cGVHD via its pro-inflammatory effect. In rheumatoid arthritis models, adiponectin stimulated the secretion of pro-inflammatory cytokines including IL-6, IL-8, matrix metalloproteinase, and monocyte chemoattractant protein-1 [6,8,14]. In addition, adiponectin has been reported to stimulate the production of extracellular matrix (ECM) by dermal fibroblasts [15]. Therefore, adiponectin may directly induce excessive ECM and fibrosis as a symptom of cGVHD.

On the other hand, the high adiponectin level in patients with cGVHD might be a response to inflammation in cGVHD, since adiponectin has been shown to have an anti-inflammatory function. *In vitro,* adiponectin suppressed pro-inflammatory cytokines including TNF-α, IL1-β, and adhesion molecule activities in human endothelial and cardiac cells [16-18]. In fact, several clinical observations have suggested that a high adiponectin level may help protect against vascular inflammation in obesity-related diseases such as type 2 diabetes mellitus, cardiovascular disease, and metabolic syndrome [7,19-22]. The adiponectin level may increase to suppress the systemic inflammation of cGVHD as observed in Sjogren syndrome, in which increasing adiponectin rescues salivary gland epithelial cells from apoptosis [23].

To date, an Italian group has evaluated the serum adiponectin levels in the context of metabolic syndrome in long-term allo-SCT survivors [24]. The mean adiponectin level was 15.8 and 22.6 μg/ml in recipients with and without metabolic syndrome, respectively. The values seemed higher than those in normal subjects. Although they did not analyze the relationship between the serum adiponectin level and cGVHD, a high adiponectin level might also be associated with cGVHD.

To our knowledge, the current study is the first to suggest an association between the serum adiponectin level and the severity of cGVHD. However, there might be a bias because of the retrospective nature of the study and the small population. Therefore, a further, large prospective study is warranted to assess the association between adiponectin and the severity of cGVHD. In addition, a further basic investigation is also needed to elucidate whether a high adiponectin level in cGVHD is a primary or secondary event and how adiponectin influences the pathophysiology of cGVHD.

Author contribution

H.N. designed the study, collected and analyzed data, and wrote the manuscript, P.N.T.B, R.Y., Y.T., K.S., M.A., M.S., K.T., S.K., M.K., S.K., S.O., K.O., A.T., J.N. and Y.A. collected data, and Y.K. designed the study, analyzed data and wrote the manuscript.

Conflict of interest

The authors report no potential competing conflicts of interest.

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Table 1. Patient characteristics and adiponectin concentrations among subgroups

	Number	HMW-adiponectin (Mean±SD μg/ml)	Univariate P-value	Multivariate β-coefficient (95%CI)	Multivariate P-value
Gender					
Male	18	7.9±6.0	0.0008	-	-
Female	16	18.9±10.8		9.1 (3.9-14.3)	0.0013
Age					
Median (years)	38.5	# -0.0002±0.11	NS	-	
(range)	(16-65)				
Weight loss from SCT					
Median	4.1kg	# -0.14±0.22	NS	-	
(range)	(-16.9-26.5)				
Body mass index at the sampling					
Median	19.1	# -1.0±0.41	0.016	NS	
(range)	(15.3-34.6)				
Conditioning regimen					
Cy/TBI	19	10.1±8.5	NS	-	
Cytarabine/TBI	1	18.6			
RIC	15	16.4±11.4			
Donor gender					
Male	16	10.8±9.7	NS	-	
Female	18	15.2±10.3			
Donor source					
Matched related	10	11.8±12.2	NS	-	
Matched unrelated	13	13.5±9.5			
Mismatched related	6	12.9±8.3			
Mismatched unrelated	5	15.0±11.9			
Bone marrow or Peripheral blood					
Bone marrow	19	14.1±9.7	NS	-	
Peripheral blood	15	11.9±10.9			
Acute GVHD					
Grade 0-1	20	11.3±9.3	NS	-	
Grade 2-4	14	15.7±11.0			
Type of GVHD at sampling					
None	10	7.0±5.0	0.031	NA	
Late onset aGVHD	2	7.2±3.0			
cGVHD (classic/overlapped)	22(19/3)	6.4±10.9			
Severity of cGVHD at sampling					
None+late aGVHD	12	7.1±4.6	0.031	-	-
Mild	11	13.6±12.0		5.3 (-0.93-11.5)	0.09
Moderate	7	19.1±8.4		9.0 (2.3-15.7)	0.011
Severe	4	19.4±12.1		15.4 (7.2-23.6)	0.0006
Organ involvements of cGVHD at sampling					
Skin	11	14.9±9.7	NS	-	
Mouth	16	16.3±9.7	NS	-	
Eye	3	17.9±9.0	NS	-	
Gut	3	26.0±14.1	0.022	NS	
Liver	10	15.3±7.7	NS	-	
Other	4	17.6±7.9	NS	-	
Lung (BO / BOOP)	0 / 1				
Muscle/joint	1				
Genital tract	1				
Serositis	1				
Steroid use at sampling day					
No administration	17	9.2±6.4	0.021	-	-
On administration	17	17.0±11.8		6.9 (1.7-12.1)	0.012

Abbreviation:

Cy, cyclophosphamide; TBI, total body irradiation; RIC, reduced intensity conditioning; GVHD, graft-versus-host disease; BO, bronchiolitis obliterans; BOOP, bronchiolitis obliterans organizing pneumonia; NS, not significant; NA, not assessed; CI, confidential interval; P-value <0.05 was considered significant. # Changes in adiponectin are shown with a 1-unit increase in continuous variables.

Figure legends

Figure 1-A

Concentrations of high-molecular-weight adiponectin by gender among normal healthy subjects and recipients without GVHD, with late acute GVHD, and with NIH cGVHD

Figure 1-B

Concentrations of high-molecular-weight adiponectin among normal health subjects and recipients without GVHD, with late acute GVHD, and with NIH cGVHD according to BMI <18, 18-22, and >22 groups

Figure 1-C

Concentrations of high-molecular-weight adiponectin by gender and the NIH severity of cGVHD

Figure 1-D

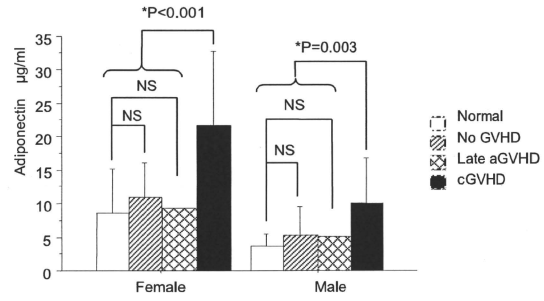
Comparison of the ratios of the later-to-prior high-molecular-weight adiponectin between adjacent samples according to clinical changes in cGVHD; worsening, improving and stable cGVHD groups

Ratio >1 means that adiponectin levels increased over time.

Ratio=1 means that adiponectin levels did not change over time.

Ratio<1 means that adiponectin levels decreased over time.

Figure1-A



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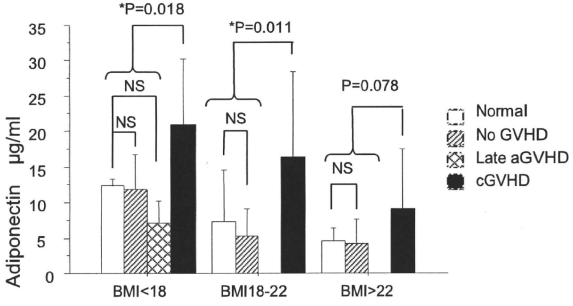

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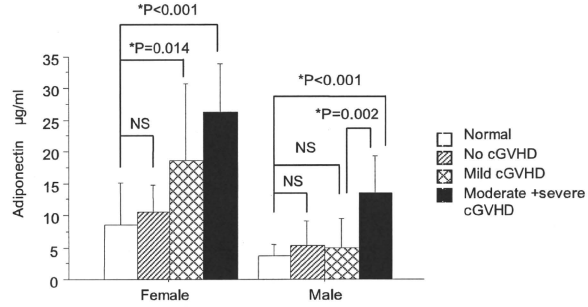
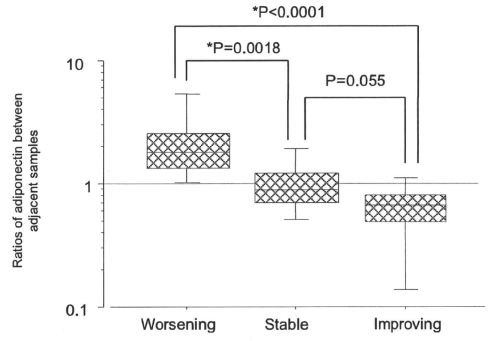


Figure1-D



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Mycophenolic Acid Inhibits Natural Killer Cell Proliferation and Cytotoxic Function: A Possible Disadvantage of Including Mycophenolate Mofetil in the Graft-Versus-Host Disease Prophylaxis Regimen

Kinya Ohata, J. Luis Espinoza, Xuzhang Lu, Yukio Kondo, Shinji Nakao

To determine how immunosuppressant agents used for graft-versus-host disease (GVHD) prophylaxis affect natural killer (NK) cells, we examined the effects of cyclosporine (CSP), tacrolimus (TAC), mycophenolic acid (MPA, an active form of mycophenolate mofetil), and methotrexate (MTX) on the proliferation and cytotoxicity of NK cells. The proliferation of NK cells from healthy individuals in the presence of interleukin (IL)-2 and IL-15 was suppressed to 51% ± 16% of that of the controls with CSP, to 31% ± 19% with TAC, to 14% ± 6% with MPA, and to 87% ± 18% with MTX. Both CSP and TAC increased the proportion of $CD16^{-}CD56^{bright}$ cells, a NK cell subset capable of secreting high amount of cytokines, and also enhanced NKp30 expression, whereas MPA markedly decreased the proportion of $CD16^{-}CD56^{bright}$ cells and reduced the expression of all activating NK cell receptors, including NKG2D, NKp30, NKp44, and NKp46. MPA also reduced the cytotoxicity against K562 cells from 61% ± 15% to 17% ± 7% and that against Daudi cells from 44% ± 4% to 4% ± 4%, whereas the other 3 drugs did not diminish these cytotoxicities. The inhibition of NK cell proliferation and cytotoxicity against leukemic cell lines by MPA was partially abolished by the inclusion of guanosine in the culture. Similar to the effect of MPA on T cells, MPA inhibited the down-regulation of p27 on NK cells induced by the incubation of NK cells in the presence of IL-2. These results suggest that MPA is a potent inhibitor of NK cells, and that its inclusion in the GVHD prophylaxis regimen might diminish the graft-versus-leukemia effect of NK cells.

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KEY WORDS: Allogeneic stem cell transplantation, Graft-versus-leukemia effect, Cyclosporine, Tacrolimus, Methotrexate, p27

INTRODUCTION

Graft-versus-host disease (GVHD) and disease relapse are major causes of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT) for hematologic disease. Both murine and human studies show that natural killer (NK) cells mediate a number of potentially beneficial effects after allo-HSCT, including the elimination of residual malignant cells and virally infected cells [1,2]. The Perugia group demonstrated that NK cells play a critical role in the development of the graft-versus-leukemia (GVL) effect [2,3]. Patients with acute myelogenous leukemia (AML) who received a haploidentical transplant from a killer immune globulin–like receptor (KIR) ligand–mismatched donor in the GVHD direction showed a marked reduction in the relapse rate compared with an otherwise similar group of AML patients without this mismatch (3% vs 47%) [4].

In contrast to several subsequent reports supporting that original report, a large retrospective study of T cell–replete transplantation from the National Marrow Donor Program showed no decrease in the relapse rate of AML following KIR-mismatched transplantation [5]. Similarly negative results have been reported by other groups as well [6,7]. Those studies differed in various respects, including donor source (related vs unrelated), HLA parity of donors (haploidentical vs matched unrelated), T cell content in the graft (strictly depleted vs T cell replete), and the use of posttransplantation immunosuppression (absent vs present). The GVHD prophylaxis regimen may particularly affect the potency of the GVL effect by

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NK cells. Wang et al. [8] recently reported that cyclosporine (CSP) augments NK cell cytotoxicity against leukemia cell lines.

In addition to CSP and methotrexate (MTX), new immunosuppressants, including tacrolimus (TAC) and the inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolate mofetil (MMF), are being successfully used for GVHD prophylaxis. Although sparing NK cells from the inhibitory effect of immunosuppressants is potentially important to retain the GVL effect after allo-HSCT, precisely how these new drugs influence NK cells remains to be clarified. In the present study, we compared the effects of 4 different immunosuppressants on NK cells and found a potent inhibitory effect of mycophenolic acid (MPA), an active form of MMF, on NK cells.

MATERIALS AND METHODS

$CD3^-CD56^+$ NK Cell Enrichment

Peripheral blood mononuclear cells (PBMCs) were isolated from 7 healthy donors using lymphocyte separation media. After washing with phosphate-buffered saline, PBMCs were subjected to NK cell enrichment using the negative selection with immunomagnetic beads (Dynal NK cell isolation kit; Invitrogen Dynal AS, Smestad, Norway) according to the manufacturer's recommendations. Enriched NK cells contained more than 85%-90% $CD56^+CD3^-$ (data not shown).

NK Cell Culture

Enriched NK cell populations were cultured in Dulbecco's Modified Eagle's Medium/Hams F12 (2:1) with 10% human AB^- sera, 2-β mercaptoethanol (24 μM), L-ascorbic acid (24 mg/L), sodium selenite (50 μg/L), ethanolamine (50 μM), and penicillin-streptomycin (100 U/mL of each). At the start of each culture, interleukin (IL)-2 (100 U/mL) and IL-15 (10 ng/mL) were added to the culture medium [8]. Then 4 different immunosuppressive drugs were added to the culture at the following concentrations: CSP, 1000 ng/mL; TAC, 20 ng/mL; MPA, 10 μg/mL; and MTX, 100 ng/mL. Each drug was diluted with ethanol and included in the culture at the indicated concentration. Vehicle control cultures were set up in parallel using ethanol (EtOH) at a final concentration of 0.1%. In some experiments, guanosine was included in the culture at a concentration of 100 μM with MPA.

Reagents and Cell Lines

The immunosuppressants were kindly provided by the producers: CSP, Novartis Pharma, Tokyo, Japan; TAC, Astellas Pharma, Tokyo, Japan; MTX, Wyeth, Tokyo, Japan; and MPA, Roche Bioscience, Tokyo, Japan. Guanosine was purchased from Sigma-Aldrich (St Louis, MO). K562 and Daudi were purchased from the Health Science Research Resources Bank (Osaka, Japan) and RIKEN BRC (Ibaraki, Japan), respectively.

Phenotypic Analysis of NK Cells

The NK cell surface phenotype was determined by 3-color flow cytometry. Fresh or cultured NK cells were stained with various monoclonal antibodies specific to cell surface proteins, including CD3, CD56, and CD16 (BD Pharmingen, Franklin Lakes, NJ) and NKG2D, NKp30, NKp44, and NKp46 (Beckman Coulter, Marseille, France), and then analyzed with a FACScan flow cytometer (BD Bioscience, Mountain View, CA).

Carboxy Fluoroscein Succinimidyl Ester Staining

NK cells were stained with carboxy fluoroscein succinimidyl ester (CFSE) using the Cell Trace CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA). After a 10-minute incubation with CFSE, NK cells were cultured in the presence or absence of each drug. The CFSE content of the NK cells was measured using the FACScan flow cytometer at various time points during the culture.

Cytotoxicity Assay of NK Cells against Leukemia Cell Lines

NK cell cytotoxicity against K562 and Daudi cells was assessed using a standard chromium-release assay [9]. The percentage of specific lysis was calculated using the following formula: 100 × (count per minute [cpm] released from test sample − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release).

Western Blot Analysis

The preparations of activated T cells, as well as their MPA and IL-2 treatments, were as described by Quemeneur et al. [10]. Freshly isolated NK cells and activated T cells were cultured for the indicated times in the presence or absence of IL-2 with and without MPA. Lysates of NK cells and T cells were subjected to Western blot analysis as described previously [11], with minor modifications. In brief, the cultured cells were sonicated using a Sonifier B-12 cell disrupter (Branson, Danbury, CT). Equal amounts of the lysate proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). The membranes were incubated with the primary antibodies (rabbit anti-human p27 pAb; Ana Spec, San Jose, CA) and mouse anti-human α-tubulin antibody (Sigma-Aldrich), and then incubated with the appropriate horseradish

peroxide–labeled secondary antibody. Specific bands were visualized using a peroxidase chemiluminescent substrate (Pierce, Rockford, IL) and analyzed using an LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Statistical Analyses

The significance of differences in cell number, percentage of $CD16^{+}CD56^{-}$ cells, and cytotoxicity among NK cells cultured in the presence of different immunosuppressants was assessed by the Student *t* test. The significance of differences in the expression levels of NKG2D, NKp30, NKp44, and NKp46 among NK cells cultured with different immunosuppressants was assessed by the Mann-Whitney *U* test. A *P* value <.05 was considered significant.

RESULTS

Effects of Immunosuppressants on NK Cell Proliferation

NK cells isolated from 7 healthy donors were cultured in the presence of interleukin (IL)-2 (100 U/mL) and IL-15 (10 ng/mL) with CSP 1000 ng/mL [12], TAC 20 ng/mL [13], MTX 100 ng/mL [14], MPA 10 μg/mL [15], or vehicle control (EtOH 0.1%), and the number of NK cells in each culture was measured after 7 days (Figure 1A). The concentration of each immunosuppressant in the culture medium was similar to the peak level measured in the blood after the administration of standard doses. CSP, TAC, and MPA significantly inhibited the proliferation of NK cells, with MPA having the strongest inhibitory effect, whereas MTX did not influence NK cell proliferation.

Next, freshly isolated NK cells were labeled with the membrane dye CFSE and cultured under the same conditions as described above. The CFSE levels in NK cells harvested at days 1, 5, and 9 of culture were determined by flow cytometry. Although all 4 immunosuppressants inhibited the decline of CFSE levels associated with NK cell proliferation, the inhibitory effect of MPA was most prominent (Figure 1B). MPA blocked the CFSE decline almost completely, even in cells harvested after 9 days of culture. The inhibitory effect of MPA on NK cell proliferation was dose-dependent, as shown in Figure 1C. MPA inhibited NK cell proliferation at a much lower concentration (1 μg/mL) than that achieved after the administration of standard-dose MMF. These results indicate that MPA inhibits NK cell proliferation more potently than the other immunosuppressants at a concentration

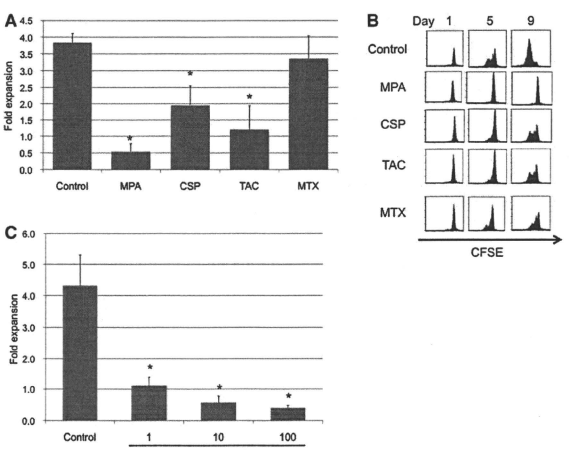

Figure 1. Effect of immunosuppressants on the proliferation of NK cells induced by IL-2 and IL-15. (A) NK cells isolated from healthy individuals were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with a vehicle control (EtOH 0.1%, MPA 10 μg/mL, CSP 1000 ng/mL, TAC 20 ng/mL, or MTX 100 ng/mL) for 7 days. The vertical axis represents the fold expansion (mean ± SD; n = 6) of NK cells calculated by dividing the number of cells after culture by that before culture. An asterisk indicates values showing significantly less degree of proliferation ($P < .05$) than the vehicle controls. (B) CFSE contents in $CD3^{-}CD56^{+}$ cells were determined using flow cytometry at days 1, 5, and 9 of culture. The figure shows a representative set of histograms from a healthy individual. The results from 5 other individuals showed similar results. (C) NK cells were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with MPA (1-100 μg/mL) for 7 days. The vertical axis represents the fold expansion (mean ± SD; n = 4) of NK cells calculated by dividing the number of cells after culture by that before culture. An asterisk indicates values showing significantly less proliferation ($P < .05$) compared with the vehicle controls.

similar to that measured in blood after administration of standard doses.

Changes in NK Cell Phenotype Associated with Immunosuppressants

NK cells can be separated into 2 subsets on the basis of CD56 density and CD16 expression [16], namely $CD56^{dim}CD16^{+}$ cells, characterized by potent cytotoxicity, and $CD56^{bright}$ $CD16^{-}$ cells, featuring high cytokine secretion activity. The 1-week culture of NK cells with EtOH alone resulted in an increase in $CD56^{bright}CD16^{-}$ cells, with a reciprocal decrease in $CD56^{dim}CD16^{+}$ cells. The addition of MPA markedly reduced the proportion of $CD56^{bright}CD16^{-}$ cells and left $CD56^{dim}CD16^{+}$ cells unchanged. CSP and TAC reduced the proportion of $CD56^{dim}CD16^{+}$ cells and reciprocally increased that of $CD56^{bright}CD16^{-}$ cells, whereas MTX did not affect the proportion of either subset (Figure 2A). Figure 2B shows the proportion of the $CD56^{bright}CD16^{-}$ NK cell subpopulation after 7 days of culture with immunosuppressants or EtOH alone. The median proportion of this NK cell subset was significantly lower in the culture with MPA (4.0%) than in the vehicle control (15.5%). In contrast, the proportion was significantly higher in the culture with CSP (36.5%) and TAC (33.0%) than in the control, whereas the proportion of the culture with MTX (17.5%) was comparable to that in the control.

Effect of Immunosuppressants on NK Cell Receptor Expression

Activating NK cell receptor expression after a 1-week culture was compared among the NK cells treated with the different immunosuppressants using the relative geometric mean fluorescent intensity (MFI) of NKG2D, NKp30, NKp44, and NKp46 to that of the control culture. NKG2D expression was decreased significantly after the treatment with TAC and MPA. In contrast, NKp30 expression was augmented significantly by CSP and TAC (Figure 3; $P < .05$). Of note, the expression of all NK-activating receptors decreased significantly after MPA treatment (Figure 3; $P < .05$), suggesting poor cytotoxic function of MPA-treated NK cells.

Cytotoxicity of NK Cells Treated with Immunosuppressants

Figure 4A shows specific cytotoxicities against K562 and Daudi cells at an effector:target ratio of 1:1. MPA-treated NK cells showed significantly lower cytotoxicity against K562 cells (61.3% ± 14.5% vs 16.7% ± 6.7%; $P < .01$) and Daudi cells (44.0% ± 3.6% vs 4.3% ± 4.0%; $P < .01$) compared with the control. There was a trend toward a higher cytotoxicity against Daudi cells in the CSP- or TAC-treated NK cells, but this enhancing effect was not observed against K562 cells. MTX did not affect NK cell cytotoxicity against either cell line. The reduced cytotoxicity against K562 and Daudi cells by NK cells treated with MPA was dependent on the MPA dose (Figure 4B).

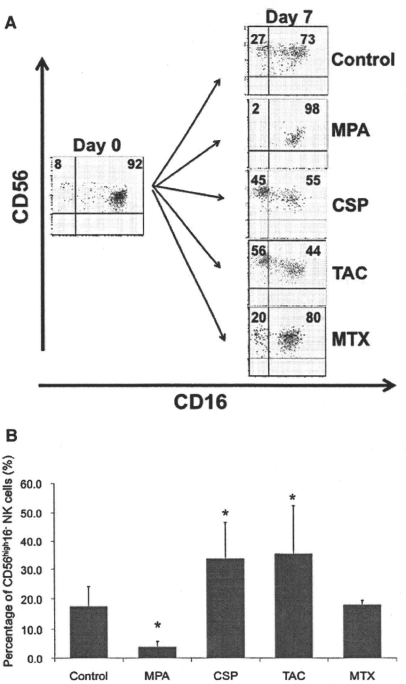

Figure 2. Changes in the NK cell phenotype after a 1-week culture with different immunosuppressants. NK cells were cultured in the presence of IL-2 (100 U/mL) and IL-15 (10 ng/mL) with different immunosuppressants or a vehicle for 7 days, and the percentages of $CD56^{dim}CD16^{+}$ and $CD56^{hig}CD16^{-}$ cells before and after culture were determined. (A) Scattergrams of a representative donor from 4 different ones. The analyses of other individuals produced similar results. (B) The percentages of $CD56^{high}CD16^{-}$ NK cells in the total $CD3^{-}CD56^{+}$ cells after culture in the presence of various immunosuppressants. The *y*-axis represents the mean percentage ± SD of $CD56^{high}CD16^{-}$ NK cells calculated from 4 different experiments. An asterisk indicates a value showing a significant difference in the $CD56^{high}CD16^{-}$ NK cell percentage compared with the vehicle control.

Effect of Guanosine on the Inhibition of NK Cell Proliferation and Cytotoxicity by MPA

MPA has been shown to inhibit T cell proliferation by depleting pools of guanosine triphosphate (GTP) in activated T cells through IMPDH inhibition, whereas guanosine has been shown to be able to restore the proliferation of MPA-treated T cells by increasing the GTP concentration in T cells [17,18]. To

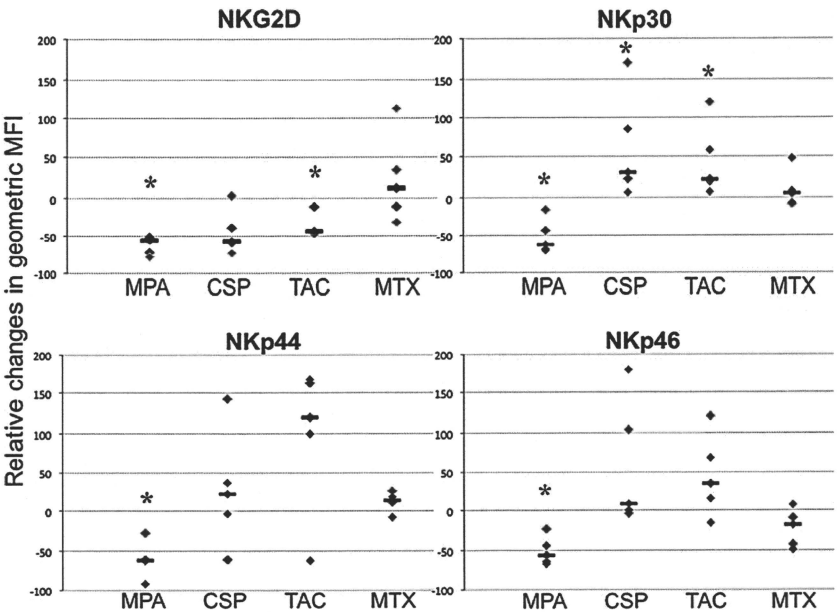

Figure 3. Changes in the expression of NK cell–activating receptors after culture with immunosuppressants. The changes in the expression levels of NKp30, NKp44, NKp46, and NKG2D in NK cells from 5 different individuals after a 1-week culture with immunosuppressants are shown. The vertical axis represents relative changes in the geometric MFI (gMFI) that is calculated by (gMFI of the receptor in each immunosuppressant -gMFI of the receptor in control)/gMFI of the receptor in control) × 100. An asterisk indicates a significant difference compared with the vehicle control. "–" denotes the mean of the relative change.

determine whether the same mechanism is involved in the NK cell inhibition by MPA, we examined the effects of guanosine on NK cell proliferation and cytotoxicity. As shown in Figure 5A, the addition of guanosine to the culture partially restored the NK cell proliferation that had been markedly inhibited by MPA; NK cell expansion increased from 0.7 ± 0.2-fold to 2.6 ± 0.9-fold by guanosine. Guanosine significantly increased the cytotoxicity against K562 by MPA-treated NK cells (from 16.0% ± 7.0% to 36.7% ± 3.2%) (Figure 5B), indicating that IMPDH inhibition is a major mechanism of NK cell inhibition by MPA.

MPA Inhibits Down-Regulation of CDK Inhibitor p27

The down-regulation of the CDK inhibitor $p27^{Kip1}$ is a critical event in the control of G1- to S-phase transition of T cells [19] and is dependent on IL-2 signaling [20], possibly through the $p21^{ras}$/Raf/mitogen-activated or extracellular signal–regulated protein kinase/extracellular signal–regulated kinase pathway [21]. MPA has been shown to inhibit down-regulation of $p27^{Kip1}$ and also to interfere with the cytokine-dependent signals that control the cell cycle, thereby inhibiting T cells from entering the mid-G1 phase of the cell cycle [10,22].

To determine whether a similar cell cycle inhibition is induced in NK cells by MPA, we examined expression of the $p27^{Kip1}$ in cultured NK cells and T cells in the presence or absence of MPA. As shown in Figure 6, the degradation of $p27^{Kip1}$ was induced in both T and NK cells within 24 hours in the culture with IL-2 (100 U/mL). Degradation of $p27^{Kip1}$ did not occur when the T and NK cells were stimulated with IL-2 in the presence of 10 μg/mL of MPA.

DISCUSSION

MMF is being increasingly used in GVHD prophylaxis after allo-HSCT because its use in combination with calcineurin inhibitors (CIs) produces comparable neutrophil recovery to that seen with the MTX + CI regimen [23]. The MMF + CI regimen is commonly

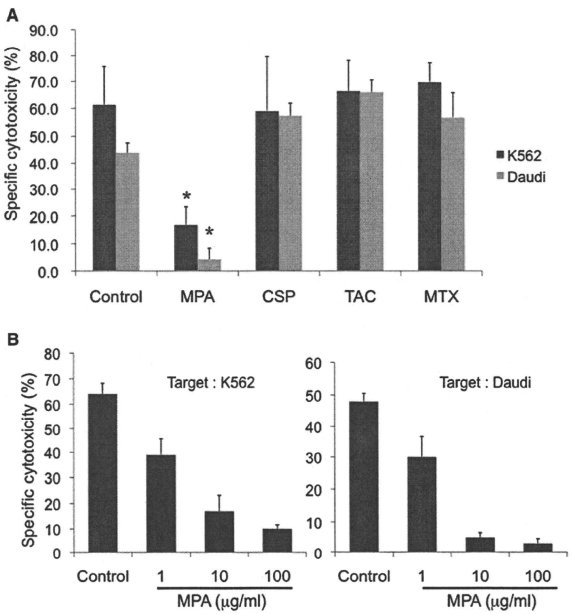

Figure 4. Cytotoxicity of cultured NK cells against leukemia cell lines. (A) Cytotoxicities by NK cells against K562 and Daudi cells at a 1:1 E:T ratio after the 1-week culture in the presence of different immunosuppressants were compared. The mean ± SD cytotoxicity from experiments using 3 different donors is shown. An asterisk indicates a significant difference compared with the control. (B) Cytotoxicities by NK cells against K562 and Daudi cells at a 1:1 E:T ratio after the 1-week culture in the presence of different concentration of MPA were compared. The mean ± SD cytotoxicities of experiments using NK cells from 3 different donors are shown.

used as a GVHD prophylaxis regimen after cord blood transplantation [24], with the anticipation of rapid hematologic recovery. Although the inhibitory effect of MPA on T cells has been studied extensively, its effect on NK cells remains unclear. The present study is the first to document the potent inhibitory effects of MPA on NK cells. MPA's inhibiting effects on NK cell proliferation and cytotoxic activity are apparently more evident than those of other immunosuppressants, including CSP, TAC, and MTX. The NK-activating receptor expression of NK cells is down-regulated by MPA, and the inhibitory effect is partially abrogated by the addition of guanosine to the NK cell culture.

Wang et al. [8] recently reported that CSP inhibits NK cell proliferation in a dose-dependent manner, while increasing the proportion of $CD56^{bright}CD16^{-}$ NK cells as well as enhancing NK cell cytotoxicity against K562 and the human Burkitt's lymphoma cell line Raji. The present study confirms that the culture of NK cells in the presence of CSP results in enhanced cytotoxicity of NK cells against Daudi cells, as well as an increased proportion of $CD56^{bright}CD16^{-}$ NK cells. Another CI, TAC, exhibited similar effects as CSP on NK cells. In accordance with the previous report, both CSP and TAC significantly augmented the surface expression of NKp30. The NKp30 expression level has been reported to correlate with the cytotoxicitic function of NK cells [25]. Thus, the augmented NKp30 expression might account for the increased cytotoxicity by CI-treated NK cells.

IMPDH is a rate-limiting enzyme in the de novo synthesis of guanosine nucleotides. Mitogenic stimulation of T cells results in a marked increase in IMPDH activity and a 5-fold increase in the guanine nucleotide pool [26,27]. MPA inhibits type II IMPDH, which is expressed in activated lymphocytes more abundantly than the type I IMPDH expressed by most leukocytes [28]. The MPA-induced deprivation of guanosine nucleotides from lymphocytes results in decreased glycosylation and expression of some adhesion molecules, thereby decreasing the recruitment of lymphocytes and monocytes into sites of inflammation and graft rejection [29]. The present study shows that the inhibitory effects of MPA on NK cells are also mediated in

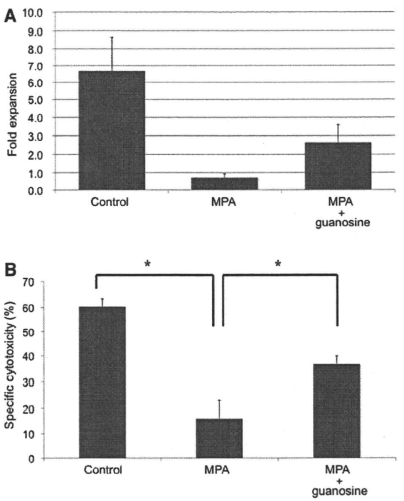

Figure 5. Effect of guanosine on the MPA-induced NK cell inhibition. NK cells were cultured in the presence of 10 μg/mL MPA with or without 100 μM guanosin for 1 week, and the effects of guanosine on MPA-induced NK cell growth inhibition (A) and cytotoxicity inhibition (B) were assessed. The vertical axis in A and B represent the mean ± SD of the fold expansion calculated by dividing the cell number after culture by that before culture and of the cytotoxicity by cultured NK cells against K562 cells at a 1:1 E/T ratio determined from 4 different donors. An asterisk indicates a significant difference ($P < .05$) compared with vehicle controls.

part by the deprivation of guanosine nucleotides. However, given MPA's remarkable inhibitory effect on NK cells, other mechanisms besides inhibition of IMPDH might play a role in this NK cell inhibition.

Laliberte et al. [22] attributed MPA's antiproliferative effect on T cells to the inhibition of cyclin D/cyclin-dependent kinase 6 induction and down-regulation of the CDK inhibitor $p27^{Kip1}$ following phytohemaglutinin stimulation of peripheral blood lymphocytes. $p27^{Kip1}$ is a member of a family of CDK inhibitors that includes $p21^{Cip/Waf1}$ and $p57^{Kip2}$ [20]. It binds to both CDK2 and CDK6 and controls the activity of cyclin D/CDK6 and cyclin E/CDK2 complexes. As a result, guanine nucleotide depletion by MPA significantly retards the degradation of $p27^{Kip1}$ after T cell activation, and $p27^{Kip1}$ gene expression by T cells, which decreases over time after incubation with IL-2, is blocked by MPA [30]. Our findings indicate that MPA also inhibits the IL-2–induced down-regulation of $p27^{Kip1}$ in NK cells, although the expression level of $p27^{Kip1}$ in NK cells was much lower than that in T cells. It is plausible that the diminished degradation of $p27^{Kip1}$ induced by MPA during NK cell activation leads to an increase in the amount of $p27^{Kip1}$ available for inhibiting cyclin E/CDK2 activity. Rapamycin, another potent immunosuppressant, is known to prevent the activation of cyclin E/CDK2 kinase activity and the degradation of $p27^{Kip1}$ associated with T cell activation [31]. Chen et al. [32] recently demonstrated the potent inhibitory effect of rapamycin (sirolimus) on NK cells. Thus, the inhibition of the $p27^{Kip1}$ degradation might be a common mechanism underlying NK cell inhibition by immunosuppressive agents.

NK cells play an important role in the development of the GVL effect after allo-HSCT, particularly in transplants from HLA-haploidentical donors who have KIR-L mismatches in the GVHD direction. Recently, Dunbar et al. [33] reported an association between high NK cell reconstitution and reduced rates of relapse and death, with no increase in the incidence of GVHD after reduced-intensity conditioning allo-HSCT. Thus, the use of a GVHD prophylaxis regimen that does not impair the reconstitution and function of NK cells early after HSCT is critical. Willemze et al. [34] recently reported a significantly lower relapse rate of AML after cord blood transplantation (CBT) from KIR-L–mismatched donors than after CBT

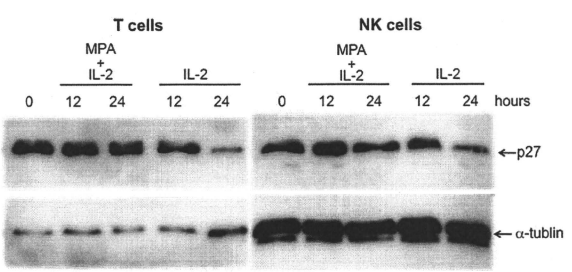

Figure 6. Effect of MPA on $p27^{Kip1}$ expression by T cells and NK cells. T cells and NK cells were harvested at the indicated time points after culture in the presence of IL-2 (100 U/mL) with or without MPA (10 μg/mL), and $p27^{Kip1}$ protein in the T cell and NK cell lysate was detected by Western blot analysis with anti-$p27^{Kip1}$–specific monoclonal antibody. The figure shows representative results from 5 experiments.

from non–KIR-L–mismatched donors. In contrast, another study failed to demonstrate the beneficial effect of KIR-L mismatches in CBT recipients [35]. Only 16% of the 218 patients analyzed in the former study received a GVHD prophylaxis regimen that included MMF [34], whereas 78% of the 257 patients treated in the latter study received MMF [34]. Although a number of factors are involved in the different outcomes between these 2 studies, the use of MMF and the resultant NK cell impairment might be one reason for the difference in the KIR-L–mismatch effect between the 2 studies. Thus, the possible negative effect of MMF on the GVL effect after SCT from KIR-L–mismatched donors merits examination in a prospective randomized study.

In conclusion, our in vitro study shows that MPA has a more potent inhibitory effect on NK cells than other immunosuppresants commonly used for GVHD prophylaxis. Thus, the combination of MTX + CI might be preferred over MMF + CI in terms of the retention of the NK cell–mediated GVL effect as a GVHD prophylaxis regimen.

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ORIGINAL ARTICLE

Long-term outcomes of autologous PBSCT for peripheral T-cell lymphoma: retrospective analysis of the experience of the Fukuoka BMT group

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Peripheral T-cell lymphoma (PTCL) is generally characterized by poor prognosis after conventional chemotherapy compared with aggressive B-cell lymphoma. To elucidate the role of high-dose chemotherapy (HDCT) with auto-SCT, we retrospectively analyzed the outcomes of 39 patients with PTCL who received HDCT and auto-SCT between 1990 and 2005. Eleven patients were histologically typed as angioimmunoblastic, nine as anaplastic large-cell lymphoma, seven as natural killer/T-cell lymphoma and twelve as PTCL unspecified. Clinical conditions at transplantation were complete response (CR) in 27 patients and non-CR in 12 patients. Thirty-two patients received a pre-transplant conditioning regimen (MCEC) comprising ranimustine, carboplatin, etoposide and CY, and seven did other TBI-based regimens. Rapid engraftment was obtained in all cases, and transplant-related death was not seen. An estimated 5-year OS was 62.1% with a median follow-up of 78 months. The 5-year OS was significantly higher in patients transplanted during complete response than in those during other disease status (71.4% vs 27.3%, $P = 0.046$). HDCT supported by auto-SCT may therefore be effective as consolidation in CR for PTCL treatment.

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Keywords: PTCL; autologous PBSCT; HDCT

Introduction

Peripheral T-cell lymphomas (PTCLs) are neoplasms derived from mature T cells and natural killer (NK) cells. They account for <10% of non-Hodgkin's lymphoma (NHL) cases. PTCLs are divided into several subtypes such as PTCL unspecified (PTCL-U), systemic anaplastic large-cell lymphoma (ALCL), angioimmunoblastic T-cell lymphoma and NK/T-cell lymphoma.[1] PTCLs show distinct variations in different geographic regions and races; a higher proportion of NHL is seen in Asia.[2]

Novel and effective agents such as rituximab and yttrium-90 ibritumomab tiuxetan have benefited patients with aggressive B-cell NHL.[3] Several studies have showed the superiority of high-dose chemotherapy (HDCT) with auto-SCT to conventional chemotherapy for patients with aggressive B-cell NHL as consolidation after the initial response.[4,5] Compared with aggressive B-cell NHL, the prognosis of PTCL patients is considerably poorer if they are treated with CY, doxorubicin, vincristine and prednisone (CHOP) or CHOP-like regimens.[6,7] Survival advantage of HDCT with auto-SCT for PTCL patients is contentious because most studies have involved small series and short-term follow-up.[8–10]

Since 1990, in Fukuoka Blood and Marrow Transplantation Group (FBMTG), HDCT with autologous PBSCT (auto-PBSCT) has been evaluated as consolidation for patients in CR1 or partial response (PR) 1, and as salvage for patients with relapsed and refractory disease, to improve PTCL outcomes. In this study, we retrospectively analyzed the results of 39 patients with PTCL who received HDCT with auto-PBSCT to clarify the efficacy of this treatment.

Patients and methods

Patients

Between January 1990 and June 2005, 39 patients who received HDCT with auto-PBSCT for PTCL were enrolled into this study at four institutions of FBMTG in Japan. Histology revealed that nine patients had ALCL, eleven

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