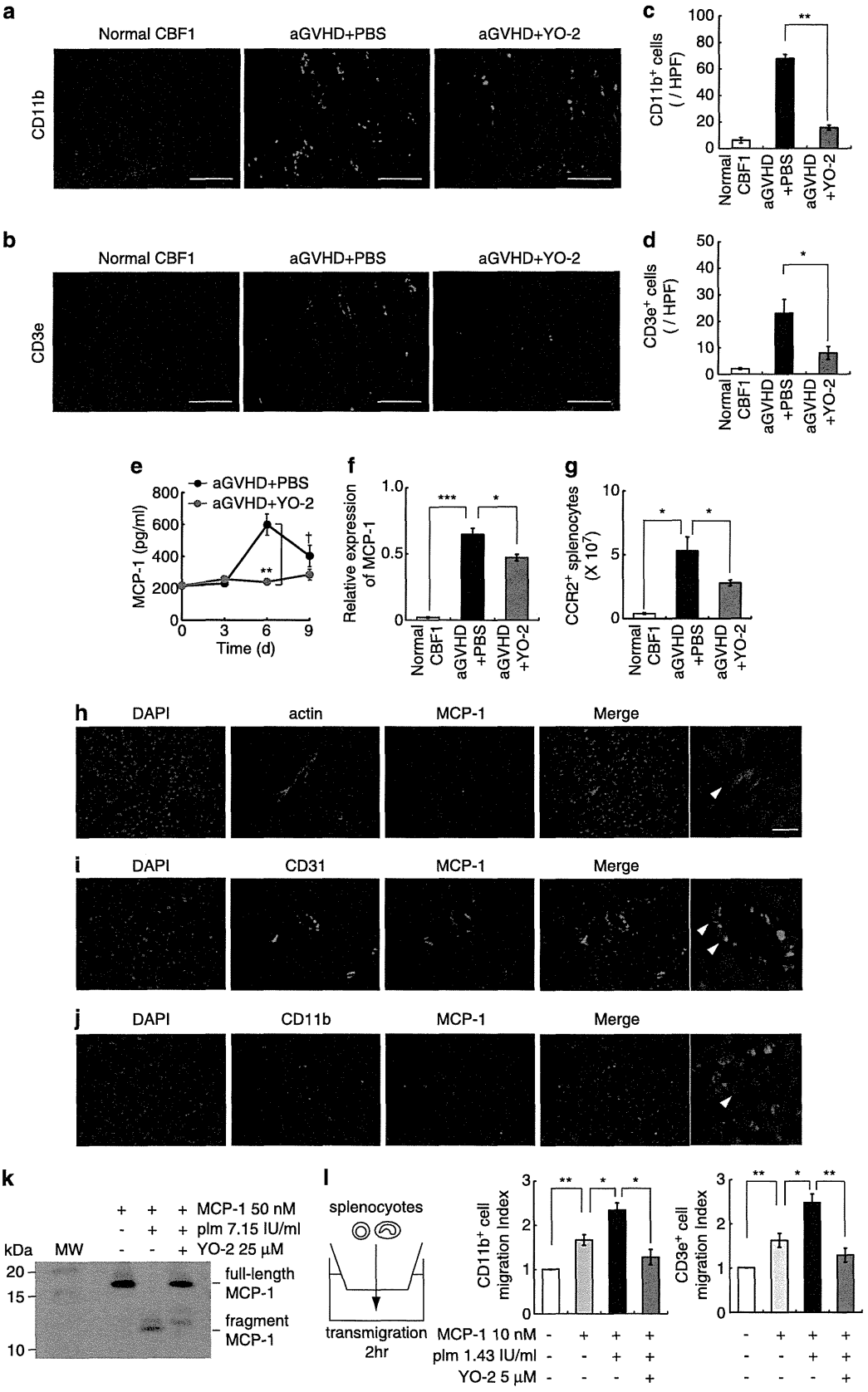
aGVHD did not occur in CBF1 recipients adoptively cotransferred with syngenic BM cells plus SP (CBF1:BM + SP) or allogenic C57Bl/6 (B6) BM cells (B6:BM), whereas aGVHD developed in CBF1 recipients transplanted with B6 BM cells and SP (B6:BM + SP). Even though all mice had received total body irradiation, plm activation as determined by the increase in circulating mouse PAP was low in



CBF1 recipients transplanted with CBF1:BM + SP or B6:BM cells (Figure 1f), indicating that total body irradiation alone was not sufficient to activate plm. In contrast, activation of plm was found in CBF1 mice receiving B6:BM + SP peaking on day 7 (Figure 1f).

T_H1 cytokines are released during aGVHD under MHC-mismatch conditions. T_H1 cytokines like TNF-α and IFN-γ were not upregulated in syngenic BM cells plus SP (CBF1:BM + SP) or allogenic C57Bl/6 (B6) BM cells (B6:BM) (Figures 1g and h). TNF-α production after aGVHD induction was suppressed in YO-2-treated aGVHD mice. These data suggest that plm occurred under MHC-mismatch conditions in the early phase of aGVHD and that plm and T_H1 cytokines peaked around day 7.

Plm inhibition protects against aGVHD-associated lethality by controlling proinflammatory cytokine/chemokine production

Because T-cell alloreactivity rather than irradiation seems to be important for plm activation, we next used another MHC-mismatched mouse model, a so-called 'parent-to-F1' model of lethal aGVHD (Figure 2a). Mixed chimerism was found in SPs of day 28 (data not shown). Because plm is activated mainly during the early phase of aGVHD, YO-2 was only injected from day 0 to 8 in this MHC-mismatched mouse aGVHD model. YO-2 treatment improved the survival and prevented aGVHD-associated BW loss in SPs transfer-induced aGVHD mice (Figures 2b and c). Even though YO-2 had been reported to induce thymocyte apoptosis *in vitro*, thymocytes isolated from YO-2-treated aGVHD animals did not show increased T-cell apoptosis (Supplementary Figure S2b). Next, we injected another plm inhibitor (YO-57), which had been reported to not affect thymocyte apoptosis. Similarly to YO-2 treatment, YO-57 treatment improved the survival and prevented aGVHD-associated BW loss (Figures 2b and c). These data indicate that plm inhibition rather than T-cell apoptosis seemed to be important to improve clinical symptoms of aGVHD *in vivo*. As signs of the activation of fibrinolysis during aGVHD progression, murine PAP, uPA and tPA plasma levels increased after transplantation (Figures 2d–f). PAP levels were low in YO-2-treated aGVHD mice. As the function of plm is to dissolve fibrin clots, and plm inhibition might cause fibrin deposition/clot formation, accelerate coagulation, d14 liver tissues from aGVHD mice were stained for fibrin(ogen). No difference in fibrin(ogen) staining pattern was observed in YO-2- and vehicle-treated aGVHD-derived tissues (Supplementary Figures S3a and b). Fibrin deposition was only found in tissues from $Plg^{-/-}$ mice. Similarly, no abnormal plasma levels of thrombin–antithrombin (Supplementary Figure S3c) were found after YO-2 treatment.

Increased serum levels of matrix metalloproteinase-9 (MMP-9) have been reported in aGVHD patients after allo-HSCT.[31] Plm inhibition partially prevented the rise in total plasma MMP-9 levels in circulation, with a decrease in both the pro-MMP and active form of MMP-9 as determined by zymography (Figures 2g and h). MMP-9 and MMP-2 proteolytic activity was high in $CD11b^+$, but not $CD3e^+$ FACS-sorted SPs of aGVHD mice (Figure 2i).

Because MMP-9 protein increased and active MMPs/MMP-9 can convert cytokines/chemokines into more active or inactive immune signals,[32] or can process membrane-bound proteins,[30,33] we hypothesized that plm inhibitor treatment can control cytokine processing. Circulating TNF-α, FasL, IL-1β and IL-6, but not IFN-γ levels were reduced in YO-2-treated aGVHD mice (Figures 2j–n). Plm addition increased gene transcription of *TNF-α, IL-1β, IL-6* and *MCP-1* in monocytes in a dose-dependent manner, a process that could be blocked when YO-2 was added to the cultures (Supplementary Figure S4a). These data indicate that blockade of the naturally occurring activation of plm during the early phase of aGVHD development impaired the production of cytokines/chemokines.

Plm inhibition reduces aGVHD-associated inflammatory changes

On day 10 after transplantation, skin from YO-2-treated aGVHD mice showed less cell infiltration, hyperkeratosis and loss of hair follicles, the liver showed less bile duct damage and portal cell infiltrates and the large intestines showed less cell infiltration, lamina propria inflammation, crypt destruction and mucosal atrophy when compared with PBS-treated aGVHD mice (Figures 3a–c). YO-2-treated mice compared with controls showed a lower disease score by histopathological evaluation (Figure 3d). We observed SP expansion 8 days after transplantation, followed by lymphoid hypoplasia 14 days after transplantation (Figure 3e). The numbers of T cells, $CD11b^+Ly6G^{low}Ly6C^{hi}$ inflammatory monocytes, $CD11b^+F4/80^+$ macrophages and $CD11c^+$ dendritic cells were decreased in YO-2-treated aGVHD mice compared with the PBS-treated aGVHD mice (Figure 3f). These findings demonstrate that pharmacological inhibition of plm delays aGVHD-associated skin, liver and intestine damage, and that it suppresses the infiltration of effector cells in aGVHD tissues.

Plm inhibition reduces lymphoid hypoplasia

GVHD-associated lymphoid hypoplasia and B-cell dysfunction has been shown to be dependent upon donor T cell-mediated function.[34] On day 14 after transplantation, when compared with PBS-treated aGVHD mice, the spleen from YO-2 treated aGVHD mice showed less lymphoid atrophy with decreased cellularity and structural disorganization, the BM showed less atrophy and a paucity of hematopoietic cells, and the thymus showed less disorganized demarcation between the thymic cortex and medulla (Figures 3g–i). In the SPs from YO-2-treated GVHD mice, $B220^+$ B cells of host or donor origin were preserved as compared with PBS-treated aGVHD mice (Figure 3j). These findings suggest that plm inhibition reduced lymphoid atrophy and immunosuppression associated with aGVHD.

Plasmin inhibition impairs MCP-1-mediated cell migration *in vitro*

During aGVHD reaction, donor T cells initially migrate to the spleen and peripheral lymphoid tissues within hours.[35] YO-2 treatment reduced the number of $CD11b^+$ cells and $CD3e^+$ T effector cells as determined by immunohistochemistry on spleen sections after 8 days of aGVHD initiation (Figures 4a–d). Because T cell and inflammatory cell trafficking into parenchymal organs requires specific selectin–ligand, integrin–ligand[36] and chemokine–receptor interactions, for example, MCP-1 with its receptor CCR2, we next examined whether plm could alter MCP-1/CCR2 signaling. YO-2-treated aGVHD mice showed lower

Figure 4. Plm inhibition influences both the initial T-cell expansion and its concomitant effector cell migration to aGVHD target organs. (**a–d**) Immunofluorescent staining of (**a**) CD11b and (**b**) CD3e of intestinal tissue derived from CBF1 mice and PBS of YO-2-treated aGVHD mice 8 days after SP transfer (scale bar, 100 μm). Quantification of (**c**) $CD11b^+$ and (**d**) $CD3e^+$ cells in intestinal tissues ($n = 3$/group). (**e**) ELISA of MCP-1 in pooled plasma samples of three SP transfer model mice obtained at indicated times. (**f**) *MCP-1* gene expression in spleen on day 8 was determined by qPCR ($n = 3$) (**g**) $CCR2^+$ cells within SPs harvested from mice after SP transfer on day 8 ($n = 3$–4) were determined by FACS. (**h–j**) MCP-1 was costained with (**h**) actin, (**i**) CD31 and (**j**) CD11b on spleen sections derived from PBS-treated aGVHD mice 8 days after SP transfer (scale bar, 10 μm; $n = 3$). (**k**) Western blot analysis of MCP-1 after incubation of recombinant (rec.) murine MCP-1with/without plasmin in the presence/absence of YO-2 ($n = 3$). (**l**) Transmigration assay of $CD11b^+$ or $CD3e^+$ SPs migrating towards medium in the lower chamber containing MCP-1 in the presence or absence of rec. plasmin with or without YO-2 (right; $n = 3$). Each experimental condition was assayed in triplicate. Data represent mean ± s.e.m from three independent experiments. *P*-values: PBS versus YO-2: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

MCP-1 plasma levels (Figure 4e), MCP-1 mRNA expression in the spleen (Figure 4f) and a lower number of CCR2$^+$ cells in SPs when compared with PBS controls (Figure 4g). MCP-1 has been shown to be important for monocyte/macrophage, memory T cells and dendritic cells recruitment in several inflammatory models.[37,38] Immunohistochemical analysis identified actin$^+$, most likely smooth muscle cells/pericytes and CD31$^+$ endothelial cells as the major cellular source for MCP-1 in spleen sections of aGVHD mice (Figures 4h–j). Plm can enhance MCP-1 signaling by releasing a MCP-1 fragment with improved chemoattractive ability.[39,40] YO-2 prevented the release of the MCP-1 fragment as determined by western blot analysis, and blocked the improved chemoattractive ability of plm-processed MCP-1 using CD11b$^+$ or CD3e$^+$ cells, derived from the spleens of aGVHD mice *in vitro* (Figures 4k and l).

Plm inhibition protects against lethal endotoxin shock and reduced MMP-9 and TNF-α production *in vivo*

We next examined whether plm inhibition can control TNF-α production through MMPs. Administration of GalN/LPS is a model of endotoxin shock that is governed by monocyte/macrophage-released TNF-α. YO-2-treated B6 mice and *Plg*$^{-/-}$

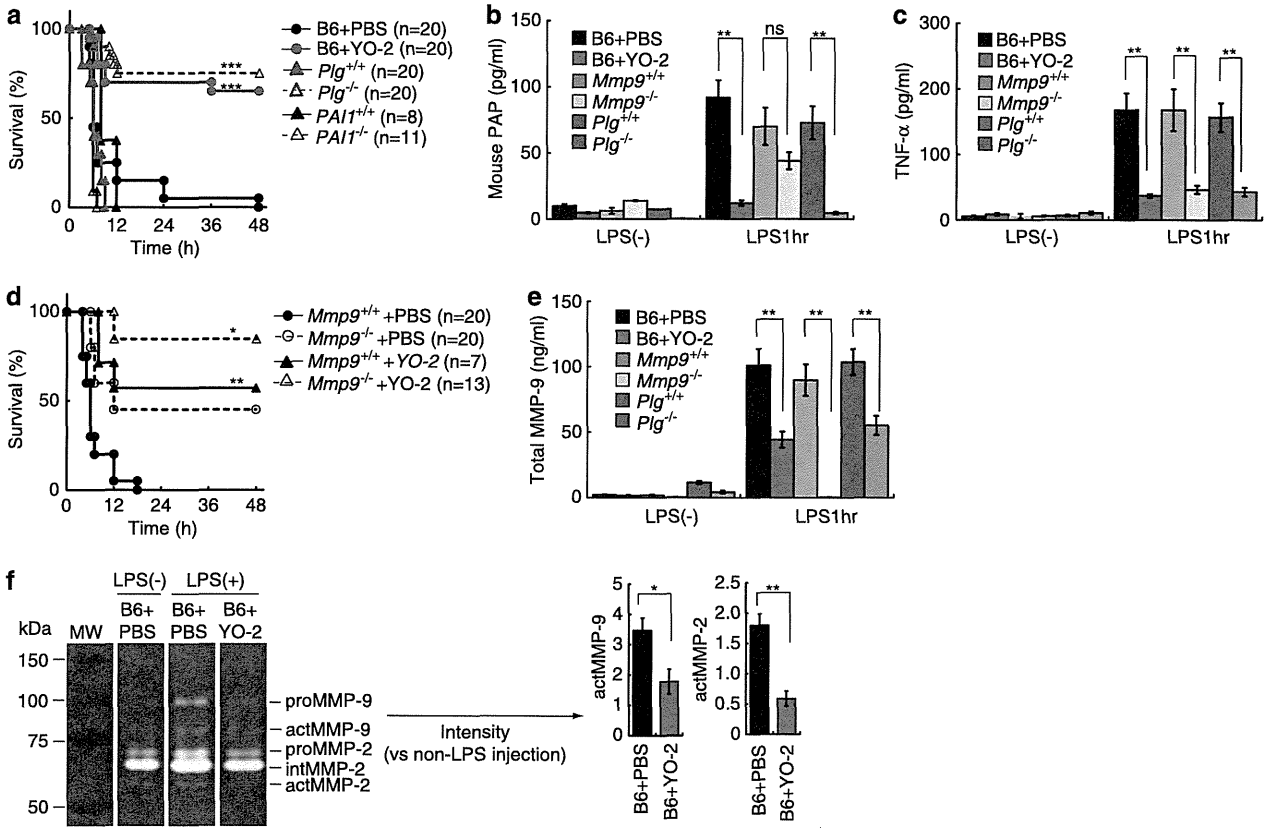
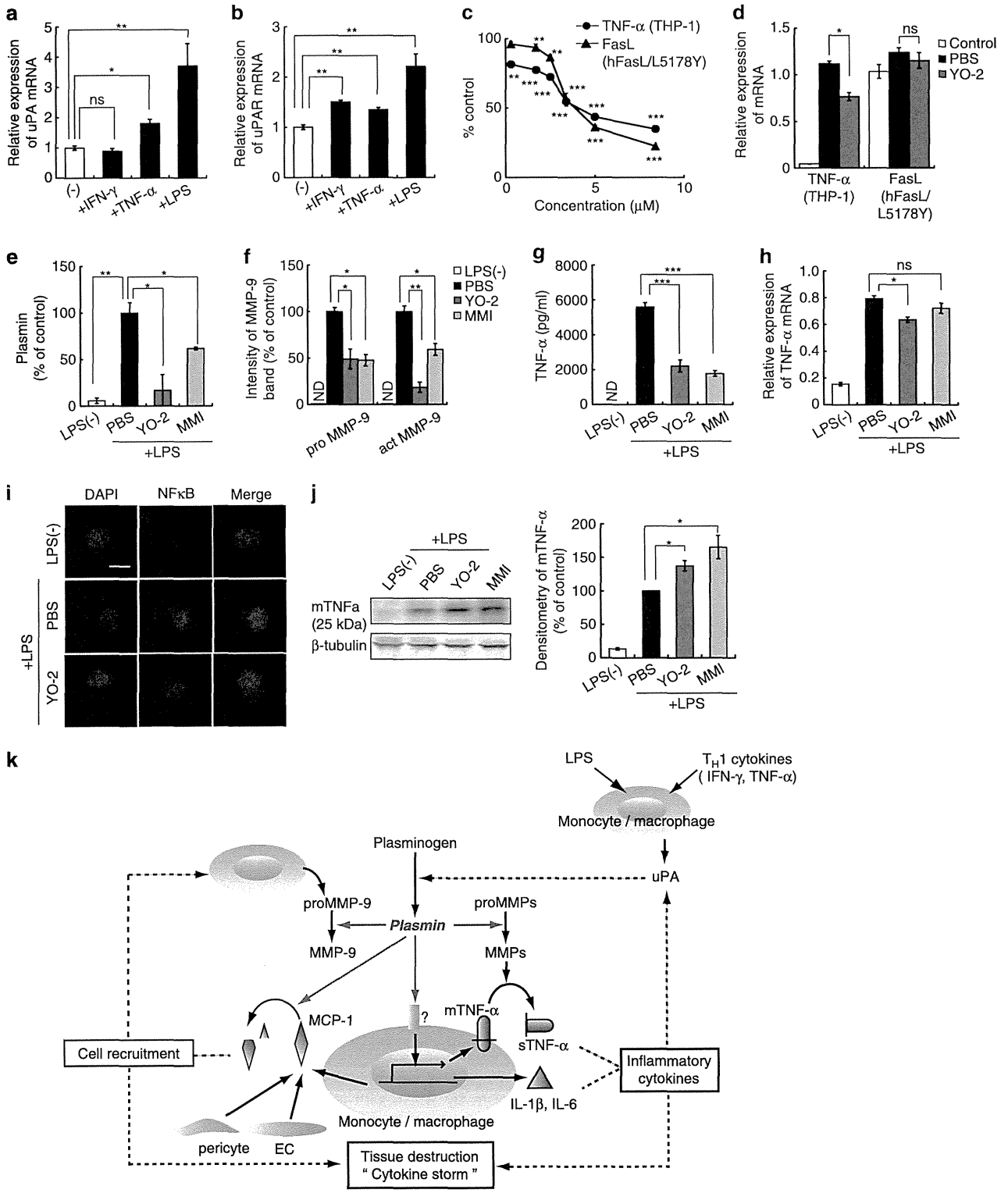


Figure 5. Plm inhibition protects against lethal endotoxin shock. (**a**) Kaplan–Meier curves showing survival of WT B6 mice treated with PBS or YO-2, *Plg*$^{+/+}$, *Plg*$^{-/-}$, *PAI1*$^{-/-}$ and *PAI1*$^{-/-}$ mice ($n=8$–20/group). Significant differences were found between B6 mice treated with PBS and YO-2: ***$P<0.001$; between *Plg*$^{+/+}$ and *Plg*$^{-/-}$ mice: ***$P<0.001$. (**b**, **c**) Sera and plasma were collected at 1 h after GalN/LPS injection. (**b**) PAP ($n=6$–10) and (**c**) TNF-α ($n=6$–15) were analyzed in plasma derived from GalN/LPS-injected mice by ELISA. (**d**) Kaplan–Meier curve showing survival of *Mmp9*$^{+/+}$, *Mmp9*$^{-/-}$ mice treated with or without YO-2 ($n=13$–20/group). Significant differences were found between *Mmp9*$^{+/+}$ and *Mmp9*$^{-/-}$ mice with PBS: **$P<0.01$; between *Mmp9*$^{-/-}$ treated with PBS and YO-2: *$P<0.05$. (**e**, **f**) Plasma samples were collected at 1 h after GalN/LPS injection. (**e**) Total MMP-9 ($n=5$) in plasma was measured by ELISA. (**f**) Blood samples were analyzed by gelatin zymography (left). Quantification of the intensity of actMMP-9 and actMMP-2 bands (right; $n=3$). Data represent the mean ± s.e.m. from two independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 6. Plm inhibition prevents TNF-α shedding and FasL production. (**a**, **b**) THP-1 monocytes were stimulated by 5 ng rhTNF-α, 1 μg/ml LPS and 20 ng rIFN-γ for 2 h. (**a**) *uPA* and (**b**) *uPAR* gene expressions in cultured cells were determined by qPCR ($n=3$). (**b**, **c**) Dose-responsive inhibition of cytokine release from LPS-stimulated THP-1 and hFasL/L5178Y cells by YO-2. TNF-α and FasL levels were determined in culture supernatants by ELISA ($n=3$). (**d**) Gene expression of cytokines in cultured cells as determined by qPCR ($n=3$). (**e**–**i**) WEHI-274.1 cells were preincubated with 0.5 μM mouse plasminogen and 5 μM YO-2, 5 μM MMI270 or PBS, followed by 1 μg/ml LPS stimulation in the absence of serum. (**e**) Plm in culture supernatants was detected using a plm chromogenic substrate ($n=3$). (**f**) Quantitation of relative intensity of proMMP-9 and actMMP-9 bands in supernatants as determined by zymography ($n=3$). (**g**) Secreted TNF-α were determined by ELISA ($n=3$). (**h**) *TNF-α* gene expression in cultured cells as determined by qPCR ($n=3$). (**i**) Confocal immunofluorescence staining of NF-κB (p65) in LPS-stimulated WEHI-274.1 cells (scale bar, 10 μm). (**j**) Left: western blot of membrane-TNF-α (mTNF-α) in cell lysates ($n=3$). Right: quantification of mTNF-α relative to β-tubulin. (**k**) Proposed mechanism by which plm exacerbates the cytokine storm in inflammatory diseases. Inflammatory cells like monocytes, by releasing the plasminogen activator uPA catalyze the generation of plm. Plm by activating other MMPs can generate a proteolytic environment resulting in the shedding of TNF-α and in an increased production of other cytokines, thereby fueling the so-called 'cytokine storm'. As cytokines in turn can promote the production of inflammatory cells, a vicious cycle is initiated. In addition, plm promotes the recruitment of inflammatory cells, such as CCR2$^+$ by generating a MCP-1 fragment with improved chemoattractive properties. Data represent the mean ± s.e.m. from three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

mice, but not *PAI1*$^{-/-}$ mice, were protected against lethal endotoxin shock (Figure 5a). We found high PAP plasma levels in PBS-treated B6 mice 2 h after LPS injection, indicating that plm is activated in the early phase after LPS injection. PAP plasma levels were decreased in YO-2-treated B6 mice and *Plg*$^{-/-}$ mice, but not *Mmp9*$^{-/-}$ mice (Figure 5b). A reduction in circulating PAP levels was observed, but this did not reach significance in *Mmp9*$^{-/-}$ mice. We determined whether TNF-α levels were reduced after GalN/LPS injection. Indeed, YO-2-treated B6 mice and *Plg*$^{-/-}$ and *Mmp9*$^{-/-}$ mice showed decreased TNF-α plasma levels (Figure 5c). YO-2 treatment could not prevent death in mice that were intravenously injected with recombinant mouse TNF-α (data not shown). These results indicate that plm is activated during the early phase after the onset of endotoxin shock and that plm inhibition blocks the release of TNF-α *in vivo*.



Next, we tested whether plm-mediated lethality and TNF-α production required endogenous MMP-9 in a model of endotoxin shock. *Mmp9*$^{-/-}$ mice were partially resistant to endotoxin shock-associated lethality (Figure 5d). These data suggest that plm inhibition prevents TNF-α production in part through MMP-9.

YO-2 treatment restored survival in GalN/LPS-injected *Mmp9*$^{-/-}$ mice (Figure 5d). YO-2 treatment reduced the total amount of MMP-9 and active MMP-9 and MMP-2 protein in circulation, as determined by ELISA and zymography (Figures 5e and f). As plm inhibition inactivates not only MMP-9, but also other MMPs (like for example, MMP-2 as shown here), the survival-enhancing effects of plm inhibition after LPS administration is both MMP-9-dependent and MMP-9-independent.

Plm inhibition prevents TNF-α shedding and FasL production *in vitro*

We next examined the influence of T_H1 cytokines on the expression of fibrinolytic factors in monocytes/macrophages *in vitro*. The addition of LPS and TNF-α increased the expression of uPA (Figure 6a), whereas LPS, TNF-α and IFN-γ augmented uPAR expression in THP-1 cells (Figure 6b). These data indicate that inflammatory T_H1 cytokines enhance the gene expression of fibrinolytic factors in monocytes/macrophages. YO-2 inhibited the release of TNF-α and FasL in monocyte culture supernatants in a dose-dependent manner (Figure 6c). The reduction of FasL release was not the result of impaired gene expression, as shown by qPCR (Figure 6d), but rather seems to be due to impaired protein release of the cytokine.

When WEHI-274.1 cells were maintained in serum-free medium, plm and MMP-9 activity increased in culture supernatant after LPS stimulation, but was inhibited in cultures treated with YO-2 or the MMP inhibitor MMI270 (Figures 6e and f). TNF-α secretion from WEHI-274.1 cells was blocked by either YO-2 or MMI270 (Figure 6g). *TNF-α* mRNA accumulation in LPS-stimulated WEHI-274.1 cells was reduced by YO-2, but was not affected by MMI270 (Figure 6h). Plm-mediated upregulation of cytokines in human monocytes has been reported to involve NF-κB activation.[8] After LPS stimulation, p65 subunit of the transcription factor NF-κB immunofluorescence staining was localized in the nucleus in control cultures, but was detected in the cytosolic compartment in YO-2-treated cells (Figure 6i). These data suggest that YO-2 blocked plm-mediated NF-κB translocation to the nucleus, thereby blocking gene expression. Next we investigated whether plm induces TNF-α shedding from its 25-kDa membrane-associated form (mTNF-α) to its 17-kDa secretory form. Both YO-2 and MMI270 treatment enhanced the expression of 25-kDa mTNF-α as determined by western blot analysis (Figure 6j). These data indicate that plm accelerates the ectodomain shedding of mTNF-α *in vitro*, and can regulate its transcription.

We show that pharmacological plm inhibition prevents inflammation-associated lethality and tissue destruction in models of endotoxin shock and aGVHD (Figure 6k) by impairing the release of inflammatory cytokines/chemokines, which will further attract inflammatory cells or fuel the influx of inflammatory cells.

DISCUSSION

Here, we show that plm is activated during the early phase of endotoxin shock and aGVHD in mice and humans. Plm inhibition protects against the proinflammatory cytokine storm in these inflammatory diseases by blocking cytokine/chemokine production and inflammatory cell infiltration.

Donor T cells recognize MHC and their associated peptides on host APCs, which results in T-cell activation and T_H1 cytokine production. T_H1 cytokines increase due to a reaction between MHC-mismatch donor T cells and recipient APC, or LPS-activated APC including monocyte/macrophage. We observed that plm peak levels coincided with a peak in IFN-γ levels, a cytokine, mainly released by activated T cells. We therefore suspected that there is a link between T_H1 cytokines and plm activation. Monocytes exposed to IFN-γ and TNF-α upregulated the expression of the fibrinolytic factors uPA and uPAR. uPA binding to its receptor uPAR will convert more plg into plm. The newly produced plm will further increase the production of proinflammatory cytokines, establishing a vicious cycle of plm activation and cytokine production.

We provide evidence that plm inhibition blocks shedding of the membrane-associated proapoptotic cytokine TNF-α after LPS stimulation *in vitro* and promotes the release of FasL. Plm has been reported to cleave FasL, releasing a soluble proapoptotic FasL fragment from the surface of endothelial cells. The Fas/FasL pathway is particularly important in hepatic GVHD. Plm inhibitor treatment improved the pathological score in liver tissues of aGVHD mice. Similar to reports by others,[8,9] we show that plm inhibition inhibited the transcription of TNF-α,IL-1β, IL-6 and MCP-1 in monocytes by activating the NF-κB pathway. These data indicate that plm inhibition controls cytokine/chemokine production by blocking the processing and transcription.

Plm inhibition decreased the total amount of circulating MMP-9 in murine models of septic shock and aGVHD, and in a partial MMP-9-dependent manner improved survival in a murine septic shock model. Plasmin, via activation of MMP-3 is a potent activator of pro-MMP-9 *in vitro*.[41] In an experimental model of the autoimmune disease bullous pemphigoid, it was reported that plm, in concert with other unidentified mechanism(s) caused MMP-9 activation.[42] How can we explain the impaired rise in circulating MMP-9 after plm inhibition? One possibility is that plm as shown by others and us induces IL-1β, TNF-α production in monocytes.[8] These cytokines, like reported for IL-1β,[43] in turn can induce MMP-9. Plm inhibition prevented the cytokine increase.

Another scenario is that plm regulates the infiltration of MMP-9 producing cells in inflamed tissues during aGVHD. We identified $CD11b^+$ cells as major supplier of MMP-9 during aGVHD. The increase in total numbers of $CD11b^+$ cells, rather than changes on a cellular basis, most likely accounts for the net MMP-9 activation.

We show that plm improves MCP-1-mediated $CD11b^+$ and $CD3e^+$ cell migration *in vitro*. We found that MCP-1 gene transcription and protein release are reduced in YO-2-treated aGVHD mice *in vivo*. Furthermore, we confirmed and extended a report demonstrating that plm proteolytically removes the C terminus of MCP-1 thereby enhancing the chemotactic potency of MCP-1.[39,40] Even though we focused on MCP-1, other chemotactic molecules or alterations in the proteolytic environment by plm might contribute to the observed impaired myeloid cell recruitment into inflamed tissues. Coagulation is highlighted during septic shock and aGVHD. The serine proteinase activated protein C reduces mortality in animal models of sepsis,[44] and inhibits coagulation by blocking PAI-1 activity.[45,46] Often inflammatory diseases are associated with an increase in PAI-1 plasma levels, indicating that fibrinolysis is blocked during disease progression. On the other hand, increased tPA levels have been reported during sepsis in mice and the early phase of HSCT in humans.[5,47–49] We found that plm is activated during the early phase of aGVHD and endotoxin shock, causing severe tissue destruction. YO-2 can block excessive circulating plm thereby blocking fibrinolysis, without the requirement of binding to the lysine-binding site of the plg molecule-binding fibrin as other plm inhibitors. Clinically, patients often show signs of excessive fibrinolysis and coagulation at the same time. Especially in these cases, YO-2 treatment seems to be a good choice in cases with known risk of bleeding while using fibrinolysis-activating agents.

During sepsis, patients die from bacteremia or sepsis-induced hyperinflammation due to an uncontrolled 'cytokine storm'. During the late phase of immunosuppression, patients often die due to secondary infection.[50,51] These observations gave the rationale to conduct clinical studies to block proinflammatory

cytokines during sepsis and aGVHD. But the results have been disappointing due to immunosuppressive side effects.[51,52] We propose that pharmacological plm inhibition in the early profibrinolytic phase regulates the fatal cytokine storm without immunosuppressive side effects. Blockade of the activation of plm during the early phase of aGVHD development delays the onset of aGVHD, but is followed by a phase of the activation of the coagulation system demonstrating that there is an important therapeutic window to benefit from plm inhibition in the treatment of aGVHD.

The matrix-degrading PA/plm system is a general proteolytic enzyme system which mediates tissue destruction in, for example, sepsis,[53] group A streptococcus infection,[54] influenza virus infection[55] and in autoimmune inflammatory diseases. We propose that plm is a novel therapeutic target and biomarker for these diseases, where tissue destruction impairs the life quality of patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Changes in the Clinical Impact of High-Risk Human Leukocyte Antigen Allele Mismatch Combinations on the Outcome of Unrelated Bone Marrow Transplantation

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A B S T R A C T

Several high-risk HLA allele mismatch combinations (HR-MMs) for severe acute graft-versus-host disease (GVHD) have been identified by analyzing transplantation outcomes in Japanese unrelated hematopoietic stem cell transplant recipients. In this study, we analyzed the effects of HR-MMs in 3 transplantation time periods. We confirmed that the incidence of grade III to IV acute GVHD in the HR-MM group was significantly higher than that in the low-risk (LR) MM group (hazard ratio [HR], 2.74; $P < .0001$) in the early time period (1993 to 2001). However, the difference in the incidence of grade III to IV acute GVHD between the HR-MM and LR-MM groups was not statistically significant (HR, 1.06; $P = .85$ and HR, .40; $P = .21$, respectively) in the mid (2002 to 2007) and late (2008 to 2011) time periods. Similarly, survival in the HR-MM group was significantly inferior to that in the LR-MM group (HR, 1.46; $P = .019$) in the early time period, whereas the difference in survival between the 2 groups was not statistically significant in the mid and late time periods (HR, 1.06; $P = .75$ and HR, .82; $P = .58$, respectively). In conclusion, the adverse impact of HR-MM has become less significant over time. Unrelated transplantation with a single HR-MM could be a viable option in the absence of a matched unrelated donor or an unrelated donor with a single LR-MM.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) from an unrelated donor has been established as an effective treatment option for patients with hematological diseases who lack a human leukocyte antigen (HLA)–matched related donor. However, an HLA mismatch at the genetic level (allele mismatch) may be observed even in HSCT from a serologically HLA-matched donor (antigen match), and the presence of an allele mismatch adversely affects the incidence of severe acute graft-versus-host disease (GVHD) and survival [1-4]. We recently showed that the presence of single HLA allele mismatches at the HLA-A, -B, -C, or -DRB1 loci equivalently affect the outcome of HSCT, although a previous study from Japan reported that an HLA-A or -B allele mismatch impairs overall survival more strongly than an HLA-C or -DRB1 allele mismatch [4,5]. These findings suggest that the

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Table 1
Patient Characteristics

Characteristic	Match n = 2504			Low-Risk Mismatch n = 1057			High-Risk Mismatch n = 157		
	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
	802	814	888	412	351	294	64	71	22
Age (recipient)									
Median	32	38	43	31	38	43	33	39	41
Age (donor)									
Median	34	34	36	33	34	37	35	36	37
Sex (recipient)									
Female	292	305	378	162	165	123	27	27	9
Male	510	509	510	250	186	171	37	44	13
Sex (donor)									
Female	286	262	266	164	158	107	20	28	5
Male	512	548	622	247	190	187	43	43	17
N.A.	4	4	0	1	3	0	1	0	0
Sex mismatch									
Match	507	537	512	238	209	166	35	40	14
Male to female	148	158	244	85	72	72	17	15	6
Female to male	143	115	132	88	67	56	11	16	2
N.A.	4	4	0	1	3	0	1	0	0
ABO blood type									
Match	454	462	500	167	151	121	33	31	9
Minor mismatch	154	162	175	112	84	81	15	18	3
Major mismatch	125	114	142	82	67	61	9	18	4
Bidirectional mismatch	58	70	71	45	46	31	7	4	6
N.A.	11	6	0	6	3	0	0	0	0
Disease									
AML	269	415	495	134	168	170	15	29	12
ALL	229	229	249	116	96	76	11	23	8
CML	237	84	29	125	42	14	30	3	0
MDS	67	86	115	37	45	34	8	16	2
Disease risk									
Low	552	533	607	265	219	181	40	38	12
High	230	239	280	135	116	113	21	28	10
Others	20	42	1	12	16	0	3	5	0
Cell dose (cells/kg)									
Median	3.0	2.7	2.7	3.0	2.6	2.6	3.1	2.8	2.6
GVHD prophylaxis									
CSA-based	545	306	185	267	114	47	45	21	2
TAC-based	240	499	689	135	227	240	19	50	20
N.A.	17	9	14	10	10	7	0	0	0
Conditioning regimen									
TBI regimen	760	639	560	394	272	194	59	53	15
Non-TBI regimen	30	114	328	17	52	100	3	11	7
N.A.	12	61	0	1	27	0	2	7	0

N.A. indicates not available; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus; TBI, total body irradiation.

clinical impact of an HLA mismatch may have changed over time periods.

Some investigators have tried to identify specific donor-recipient allele combinations that may be associated with a higher risk of severe acute GVHD [6,7]. Kawase et al. found 16 high-risk HLA allele mismatch combinations (HR-MMs) for severe acute GVHD [7]. They also showed that the number of HR-MMs was associated with severe GVHD and poor survival, whereas the presence of mismatch combinations other than HR-MMs (low-risk mismatch combinations, LR-MMs) did not affect the outcome of HSCT. However, their study included a variety of benign and malignant hematological diseases. In addition, they included donor-recipient pairs with more than 1 HLA mismatch. The impact of each specific mismatch combination was evaluated after adjusting for the number of HLA mismatches in other loci in a multivariate model, but the possible presence of HR-MMs in other loci or the interaction between HLA mismatch combinations could not be appropriately treated in their model. At that time, the study design was inevitable, because the number of each HLA mismatch combination was limited. However, several years have passed and the amount of unrelated HSCT data in the Transplant Registry Unified Management Program (TRUMP) has increased to more than 13,500 donor-recipient pairs. Therefore, in this study, we reanalyzed the impact of HR-MMs, excluding HSCT with multiple HLA mismatches in patients with relatively homogeneous background diseases. In addition, we evaluated the impact of HLA mismatch on transplantation outcomes considering the period effect, because the impact of HR-MM mismatch might have changed over time periods, as we previously reported in an analysis of single HLA allele mismatches at the HLA-A, -B, -C, and -DRB1 loci [5].

METHODS

Patients

Patients aged at least 16 years with acute myeloblastic leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, or chronic myelogenous leukemia (CML) who underwent a first HSCT from a serologically HLA-A, -B, and -DR matched unrelated donors between 1993 and 2011, and who had full HLA-A, -B, -C, and -DRB1 allele data, were included in this study. Bone marrow was exclusively used as a stem cell source. Clinical data for

Table 2
Multivariate Analysis to Evaluate the Impact of Single HLA Allele Mismatches on the Incidence of Grade III to IV Acute GVHD Stratified according to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Donor age		1.02 (1.00-1.03)	.082
	Donor sex	Female	1.00	
		Male	1.65 (1.05-2.60)	.031
	Female to male transplantation	No	1.00	
		Yes	1.52 (.91-2.55)	.11
	Disease	AML	1.00	
		ALL	1.15 (.79-1.68)	.47
		CML	1.62 (1.11-2.36)	.012
		MDS	.65 (.32-1.35)	.25
	Disease risk	Low	1.00	
		High	1.30 (.93-1.83)	.13
		Others	.80 (.23-2.85)	.74
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.83 (.61-1.14)	.25
	HLA	Low-risk mismatch	1.00	
		Match	.89 (.65-1.21)	.44
		High-risk mismatch	2.74 (1.73-4.32)	<.0001
2002-2007				
	Donor age		1.03 (1.01-1.05)	.0028
	Donor sex	Female	1.00	
		Male	1.50 (.96-2.33)	.076
	Female to male transplantation	No	1.00	
		Yes	1.53 (.89-2.64)	.13
	Disease	AML	1.00	
		ALL	1.36 (.95-1.96)	.094
		CML	1.27 (.74-2.20)	.38
		MDS	1.25 (.77-2.02)	.37
	Disease risk	Low	1.00	
		High	1.76 (1.25-2.48)	.0011
		Others	1.65 (.82-3.34)	.16
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.86 (.63-1.19)	.37
	HLA	Low-risk mismatch	1.00	
		Match	.64 (.46-.89)	.008
		High-risk mismatch	1.06 (.58-1.93)	.85
2008-2011				
	Donor age		1.03 (1.01-1.06)	.0016
	Donor sex	Female	1.00	
		Male	1.28 (.78-2.12)	.33
	Female to male transplantation	No	1.00	
		Yes	.98 (.52-1.88)	.96
	Disease	AML	1.00	
		ALL	1.18 (.80-1.74)	.42
		CML	1.53 (.69-3.37)	.3
		MDS	.66 (.36-1.20)	.17
	Disease risk	Low	1.00	
		High	1.53 (1.08-2.17)	.018
		Others	NA (NA-NA)	NA
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.82 (.55-1.24)	.34
	HLA	Low-risk mismatch	1.00	
		Match	.56 (.39-.80)	.0014
		High-risk mismatch	.40 (.10-1.64)	.21

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

these patients were obtained from the TRUMP [8]. We excluded patients who lacked data on survival status, those with more than 1 allele or antigen mismatch, those who received a reduced-intensity conditioning regimen, and those who received ex vivo or in vivo T cell depletion, such as antithymocyte globulin or alemtuzumab. Finally, 3718 patients were included in the main part of this study. As a post hoc analysis, 415 patients with 2 LR-MMs and 66 patients with 2 allele mismatches including at least 1 HR-MM were added to compare the impact of 1 HR-MM and 2 LR-MMs and to analyze the statistical interaction between HR-MM and the presence of an additional allele mismatch. The study was approved by the data management committee of TRUMP and by the institutional review board of Saitama Medical Center, Jichi Medical University.

Histocompatibility

Histocompatibility data for serological and genetic typing for the HLA-A, HLA-B, HLA-C, and HLA-DR loci were obtained from the TRUMP database, which includes HLA allele data determined retrospectively by the Japan Marrow Donor Program using frozen samples [7,9]. In this study, the following donor-recipient HLA-mismatch combinations were regarded as HR-MMs: A*02:06-A*02:01, A*02:06-A*02:07, A*26:02-A*26:01, A*26:03-A*26:01, B*15:01-B*15:07, C*03:03-C*15:02, C*03:04-C*08:01, C*04:01-C*03:03, C*08:01-C*03:03, C*14:02-C*03:04, C*15:02-C*03:04, C*15:02-C*14:02, DR*04:05-DR*04:03, and DR*14:03-DR*-DR1401, as we did not have enough data on HLA-DP and -DQ [7]. In HR-MM pairs, the donor and the recipient must have the HLA allele as shown above, and at the same time, these donor and recipient HLA alleles should not be shared by the recipient and the donor, respectively. For example, if the donor has HLA-A*02:06/02:06 and the recipient has HLA-A*02:01/02:06, this pair was not regarded as HR-MM pair, as the donor's HLA-A*02:06 was shared by the recipient. Other HLA mismatch pairs were regarded as LR-MM pairs. Only the HLA-C mismatch group included HLA mismatch at a serological (antigen) level.

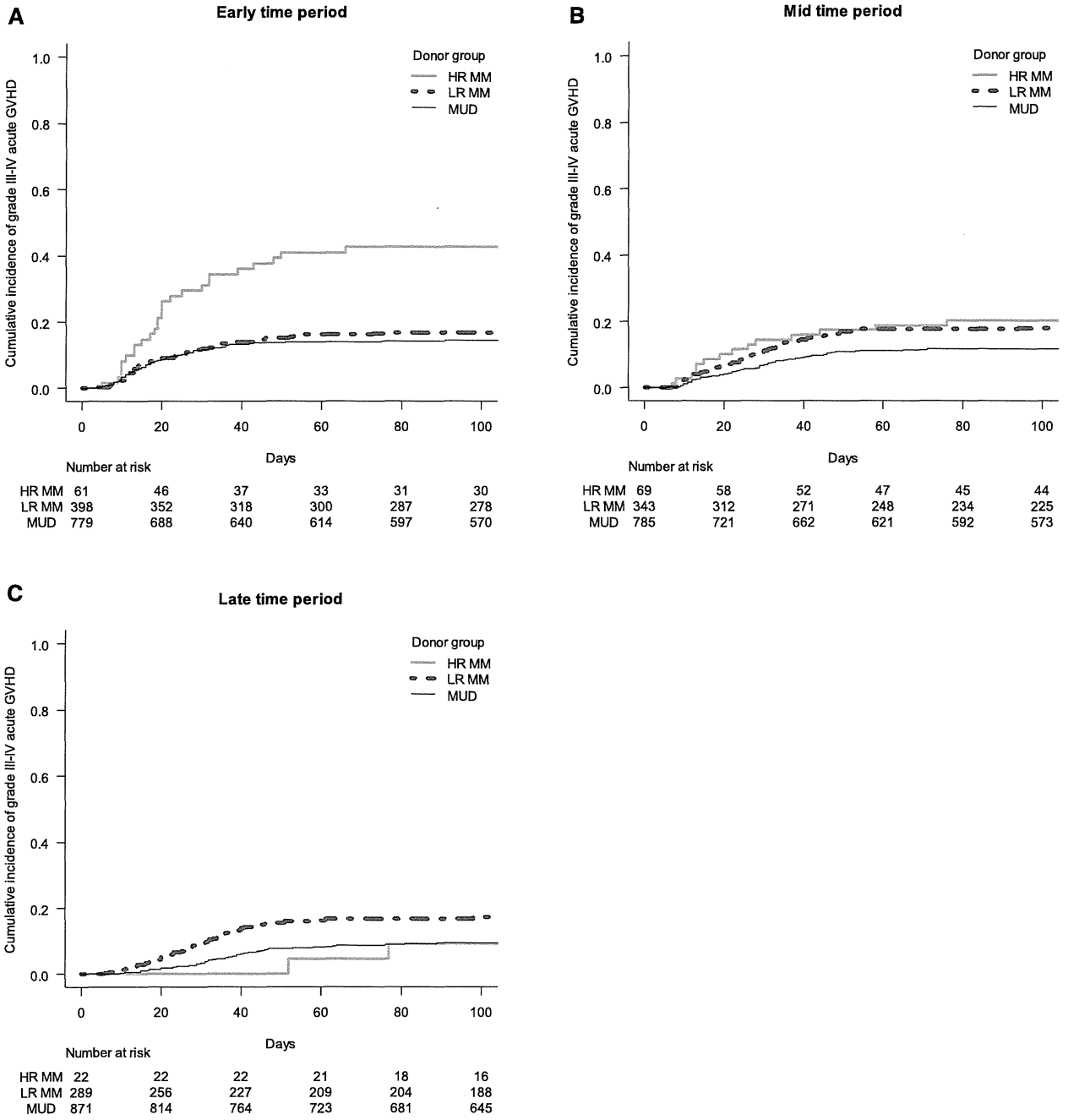

Figure 1. The cumulative incidence of grade III to IV acute GVHD grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). HR-MM indicates high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

Statistical Analyses

We divided the patients into 3 groups according to the time period when HSCT was performed to evaluate whether the impact of HR-MM changed over time periods: the early, mid, and late groups included HSCT performed from 1993 through 2001, 2002 through 2007, and 2008 through 2011, respectively. The break points among groups were determined to make the number of patients in each group equivalent (n = 1278, 1236, and 1204, respectively). To avoid making misleading conclusions by arbitrary grouping, we confirmed that there was a statistically significant interaction between the presence of HR-MMs and transplantation year as a continuous variable, both for overall survival ($P = .0098$) and the incidence of grade III to IV acute GVHD ($P < .001$). The following analyses were performed separately in each group. However, in post hoc analyses to evaluate the impact of HR-MMs at each locus and to compare 1 HR-MM and 2 LR-MMs, the mid and late groups were combined to increase the statistical power, after confirming that similar results were obtained in the 2 groups.

The primary endpoint was the incidence of grade III to IV acute GVHD. Overall survival was evaluated as a secondary endpoint. The chi-square test or Fisher exact test was used to compare categorical variables and Student *t*-test or an analysis of variance test was used for continuous variables to evaluate the homogeneity of background characteristics of the HR-MM, LR-MM, and HLA-matched (MUD) groups. *P* values were adjusted using the Bonferroni's method and Tukey's method for multiple comparisons between each pair. Overall survival was estimated according to the Kaplan-Meier method, and compared among groups with the log-rank test. The incidence of acute GVHD was calculated treating death without GVHD as a competing event, and it was compared using Gray's test [10].

The impact of HR-MMs was evaluated using multivariate models: the Cox proportional hazards model was used for overall survival and Fine and Gray's proportional hazards model was used for acute GVHD [11]. The LR-MM group was regarded as the reference group. Potential confounding factors that were considered in these analyses included recipient/donor age, recipient/donor sex, sex mismatch, ABO major/minor mismatch, the use of

Table 3
Multivariate Analysis to Evaluate the Impact of Single High-Risk Allele Mismatches on Overall Survival Stratified According to the Transplantation Time Period

Year	Factor		Hazard Ratio	*P* Value
1993-2001				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.06 (.90-1.23)	.51
	Disease	AML	1.00	
		ALL	1.20 (.99-1.45)	.065
		CML	.89 (.72-1.10)	.29
		MDS	.61 (.45-.83)	.0015
	Disease risk	Low	1.00	
		High	2.72 (2.30-3.23)	<.0001
		Others	2.03 (1.27-3.23)	.0029
	ABO major mismatch	Absent	1.00	
		Present	1.25 (1.06-1.47)	.0092
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.85 (.72-1.00)	.049
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.73-1.01)	.063
		High-risk mismatch	1.46 (1.06-2.01)	.019
2002-2007				
	Age		1.01 (1.00-1.02)	.0025
	Sex	Female	1.00	
		Male	1.20 (1.02-1.41)	.0027
	Disease	AML	1.00	
		ALL	1.16 (.96-1.39)	.13
		CML	.84 (.62-1.12)	.23
		MDS	.56 (.43-.73)	<.0001
	Disease risk	Low	1.00	
		High	2.87 (2.41-3.40)	<.0001
		Others	2.23 (1.58-3.15)	<.0001
	ABO major mismatch	Absent	1.00	
		Present	.97 (.81-1.16)	.77
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.97 (.83-1.15)	.76
	HLA	Low-risk mismatch	1.00	
		Match	.83 (.69-.98)	.032
		High-risk mismatch	1.06 (.75-1.48)	.75
2008-2011				
	Age		1.02 (1.01-1.03)	<.0001
	Sex	Female	1.00	
		Male	1.08 (.89-1.31)	.42
	Disease	AML	1.00	
		ALL	.97 (.76-1.25)	.83
		CML	.97 (.57-1.64)	.9
		MDS	.65 (.48-.87)	.004
	Disease risk	Low	1.00	
		High	2.73 (2.23-3.35)	<.0001
		Others	NA (NA-NA)	NA
	ABO major mismatch	Absent	1.00	
		Present	1.14 (.92-1.41)	.22
	GVHD prophylaxis	CSA-based	1.00	
		TAC-based	.95 (.75-1.21)	.69
	HLA	Low-risk mismatch	1.00	
		Match	.86 (.69-1.06)	.15
		High-risk mismatch	.82 (.42-1.62)	.58

AML indicates acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; GVHD, graft-versus-host disease; CSA, cyclosporine; TAC, tacrolimus.

total body irradiation in the conditioning regimen, cell dose in the bone marrow graft, the use of cyclosporine or tacrolimus as GVHD prophylaxis, background disease, and disease risk. Acute leukemia in first or second remission, CML in first or second chronic phase, CML in accelerated phase, and myelodysplastic syndrome of refractory anemia or refractory anemia with excess blasts were considered low-risk diseases, and other conditions were considered high-risk diseases. All of these potential confounding factors were included in the multivariate analyses and then deleted in a stepwise fashion from the model to exclude factors with a *P* value of .05 or higher. Finally, HLA mismatch was added to the model. Different multivariate models were compared using the likelihood ratio test. The quantity of interest was the deviance difference between the 2 models, under the null hypothesis that 2 models fit the data equally well and the deviance difference has an approximate chi-square distribution with degrees of freedom equal to the difference in the number of independent variables between the compared models.

All *P* values were 2 sided and *P* values of .05 or less were considered statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University) [12], which is a graphical user interface for R (The R Foundation for Statistical Computing). More precisely, it is a modified version of R commander that was designed to add statistical functions frequently used in biostatistics.

RESULTS

Patients

The patient characteristics are summarized in Table 1. HR-MMs were observed in 64 of 1278, 71 of 1236, and 22 of 1204 donor-recipient pairs in the early, mid, and late time periods, respectively. On the other hand, 412, 351, and 294 pairs had LR-MMs, respectively. With regard to the

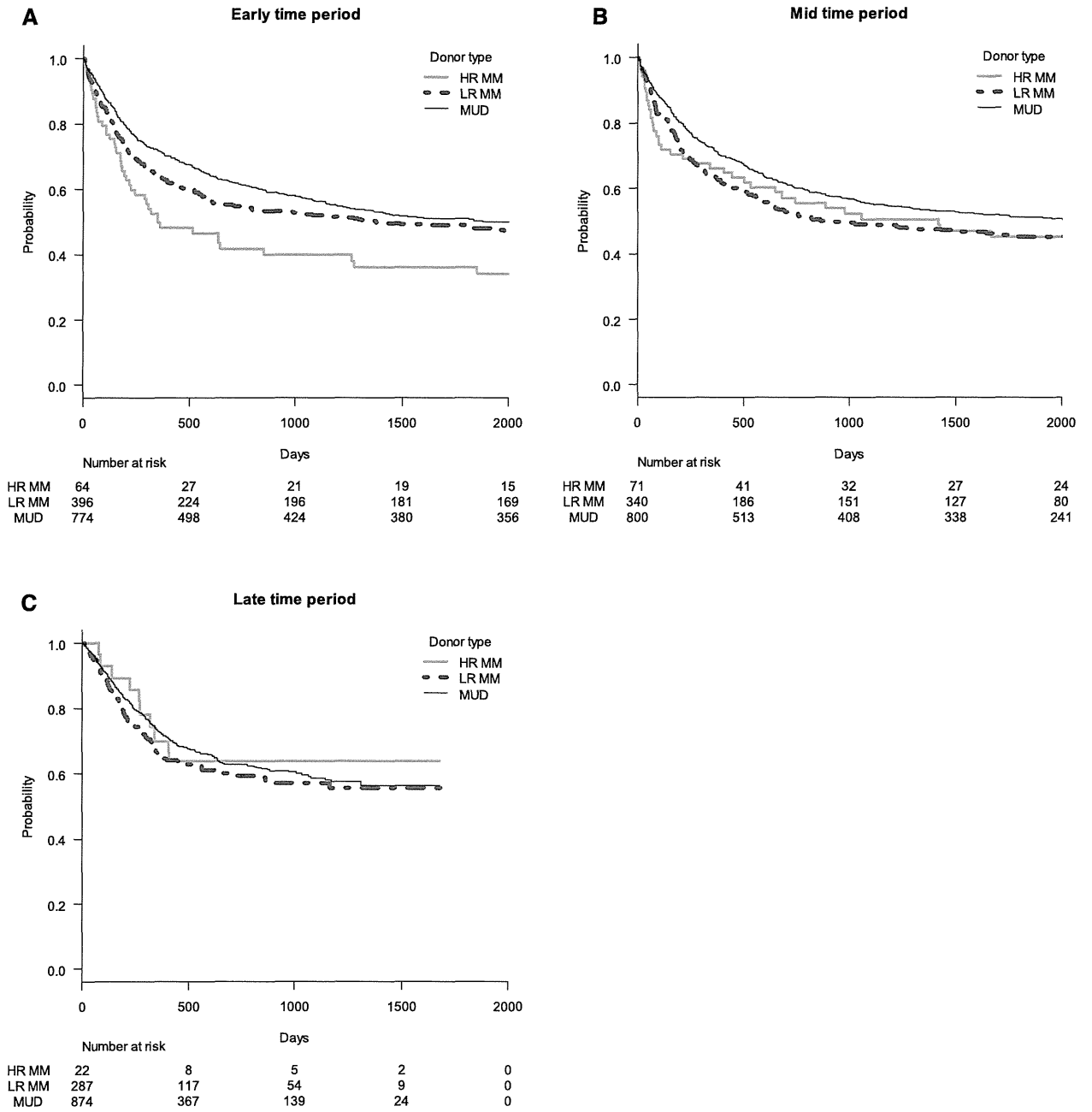

Figure 2. Overall survival grouped according to the HLA mismatch between the donor and recipient in the early (A), mid (B), and late time periods (C). The survival curves were adjusted for other significant factors by the mean of covariates method, in which average values of covariates are entered into the Cox proportional hazards model. HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

differences among transplantation time periods, the numbers of LR-MMs and HR-MMs decreased in the late time periods, ie, after the introduction of routine typing for HLA-C and the publication of a paper about HR-MMs [7]. The proportion of HSCTs for CML also dramatically decreased over time periods (30.7%, 10.4%, and 3.6% in the early, mid, and late periods, respectively). With regard to the difference among HLA mismatch groups, the proportion of patients with high-risk underlying disease in the MUD group (29.9%) was significantly lower than those in the HR-MM (37.6%) and LR-MM groups (34.4%). In addition, the proportion of HSCTs for CML was significantly higher in the HR-MM group in the early time period (29.6%, 30.3%, and 46.9% in the MUD, LR-MM, and HR-MM groups, respectively).

Incidence of Grade III to IV Acute GVHD

To adjust the impact of HLA mismatch for possible confounding factors, we identified the following independently significant factors for the incidence of grade III to IV acute GVHD: donor age, donor sex, sex mismatch, disease, disease risk, and GVHD prophylaxis. After we adjusted for these factors, we confirmed that the incidence of grade III to IV acute GVHD in the HR-MM group was significantly higher than that in the LR-MM group (hazard ratio [HR], 2.74; 95% confidence interval [CI], 1.73 to 4.32; $P < .0001$) in the early time period, whereas the difference between the MUD and LR-MM groups was not significant (HR, .89; 95% CI, .65 to 1.21; $P = .44$) (Table 2, Figure 1). On the other hand, in the mid and late time periods, the difference in the incidence of

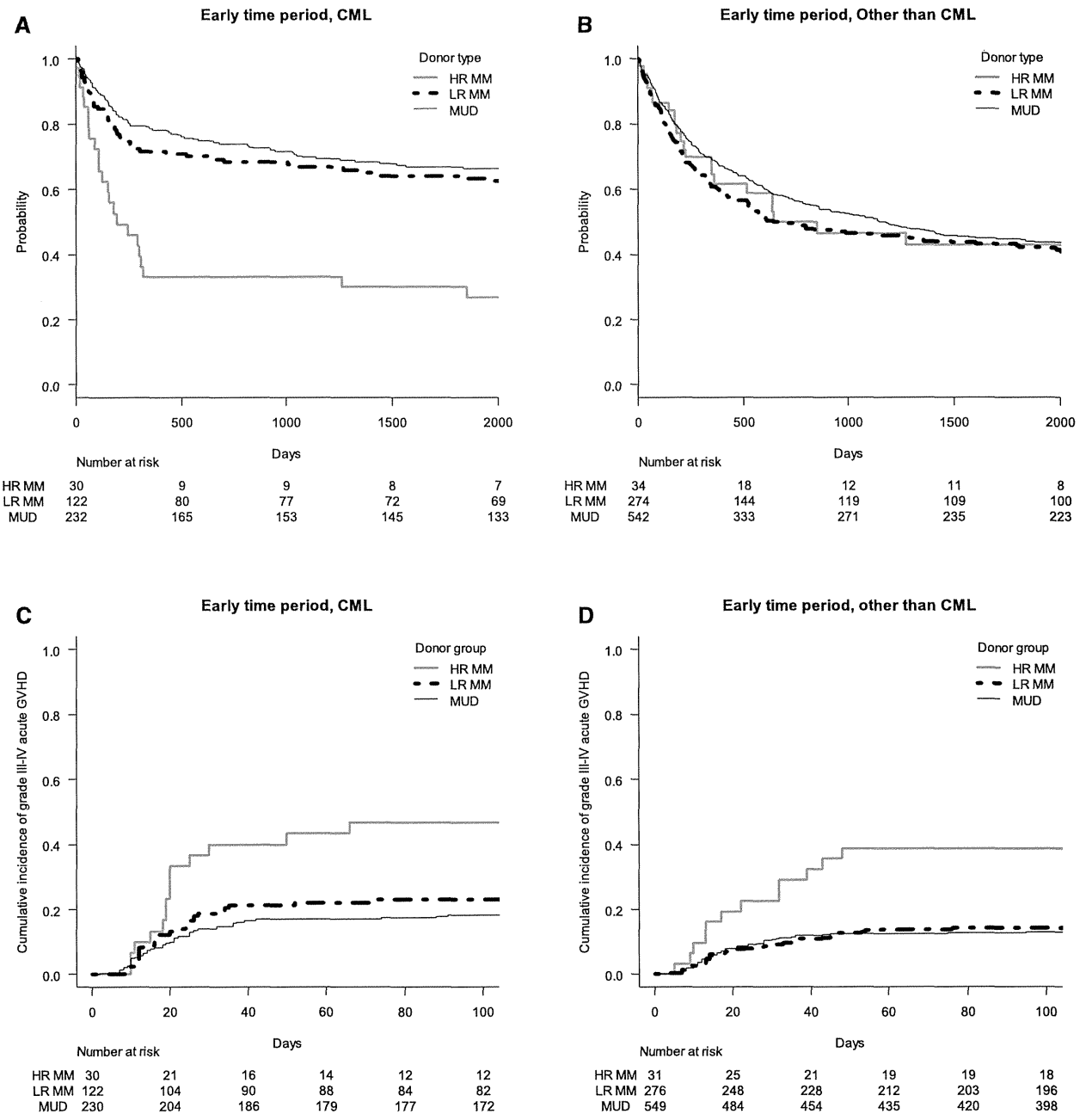

Figure 3. Adjusted overall survival (A,B) and the cumulative incidence of grade III to IV acute GVHD (C,D) grouped according to the underlying disease in the early time period. CML, chronic myelogenous leukemia; HR-MM, high-risk mismatch; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

grade III to IV acute GVHD between the HR-MM and LR-MM groups was not statistically significant (HR, 1.06; 95% CI, .58 to 1.93; $P = .85$ and HR, .40; 95% CI; .10 to 1.64; $P = .21$, respectively). The presence of LR-MM significantly adversely affected the incidence of grade III to IV acute GVHD in the mid and late periods (HR, .64; 95% CI, .46 to .89; $P = .008$ and HR, .56; 95% CI, .39 to .80; $P = .0014$, respectively, for the MUD group).

Similarly, the presence of HR-MM significantly affected the incidence of grade II to IV acute GVHD compared with LR-MM only in the early time period (HR, 1.53; 95% CI, 1.05 to 2.24; $P = .028$), and not in the mid and late periods (HR, .92; 95% CI, .61 to 1.37; $P = .67$ and HR, .79; 95% CI, .40 to 1.58; $P = .51$, respectively).

Overall Survival

After adjusting for recipient age, recipient sex, presence of ABO-major mismatch, disease, disease risk, and GVHD prophylaxis, we again confirmed that survival in the HR-MM group was significantly inferior to that in the LR-MM group (HR, 1.46; 95% CI, 1.06 to 2.01; $P = .019$) in the early time period, whereas there was no significant difference between the MUD and LR-MM groups (HR, .86; 95% CI, .73 to 1.01; $P = .063$) (Table 3). On the other hand, the difference in survival between the HR-MM and LR-MM groups was not statistically significant in the mid and late time periods (HR, 1.06; 95% CI, .75 to 1.48; $P = .75$ and HR, .82; 95% CI, .42 to 1.62; $P = .58$, respectively). The difference in survival between the MUD and LR-MM groups was consistent among

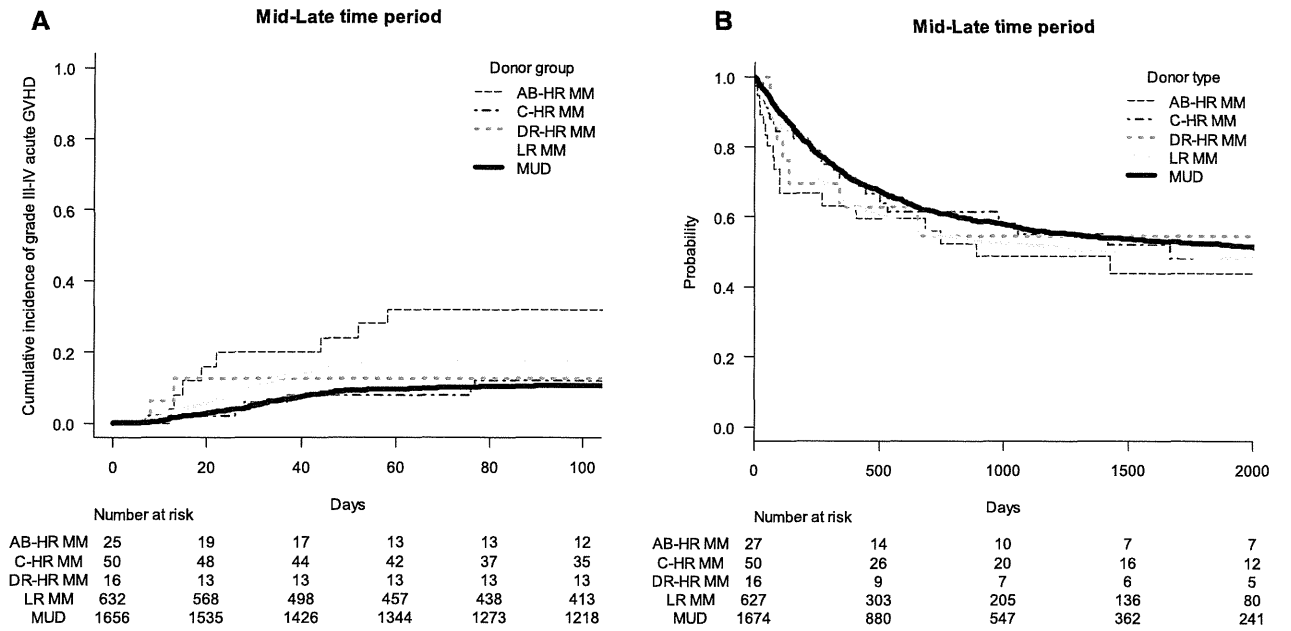

Figure 4. The cumulative incidence of grade III to IV acute GVHD (A) and adjusted overall survival (B) grouped according to the HLA mismatch loci between the donor and recipient in the mid or late time period. AB-HR MM, high-risk mismatch at the HLA-A or -B locus; C-HR MM, high-risk mismatch at the HLA-C locus; DR-HR MM, high-risk mismatch at the DRB1 locus; LR-MM, low-risk mismatch; MUD, matched unrelated donor.

the 3 time periods but statistically significant only in the mid period (HR, .83; 95% CI, .69 to .98; $P = .032$). Figure 2 shows the overall survival curves grouped according to the HLA-mismatch groups in each time period, adjusted for other significant factors by the mean of covariates method.

Disease-specific Effects of HR-MM in the Early Period

The number of patients with CML was significantly higher in the early period than in the mid and late periods. Therefore, we evaluated the disease-specific impact of HR-MM in the early period. As shown in Figures 3A and B, the presence of HR-MM had an adverse impact on overall survival only in patients with CML, although HR-MM showed a similar adverse impact on the incidence of grade III to IV acute GVHD regardless of the underlying disease (Figure 3C, D). Of the 24 CML patients who died after HSCT with HR-MM, 23 died without relapse of CML, and 10 of these patients died without grade III to IV acute GVHD.

Impact of HR-MM at Each Locus

To evaluate the impact of HR-MM at each locus in the mid and early periods, we combined the 2 periods together to

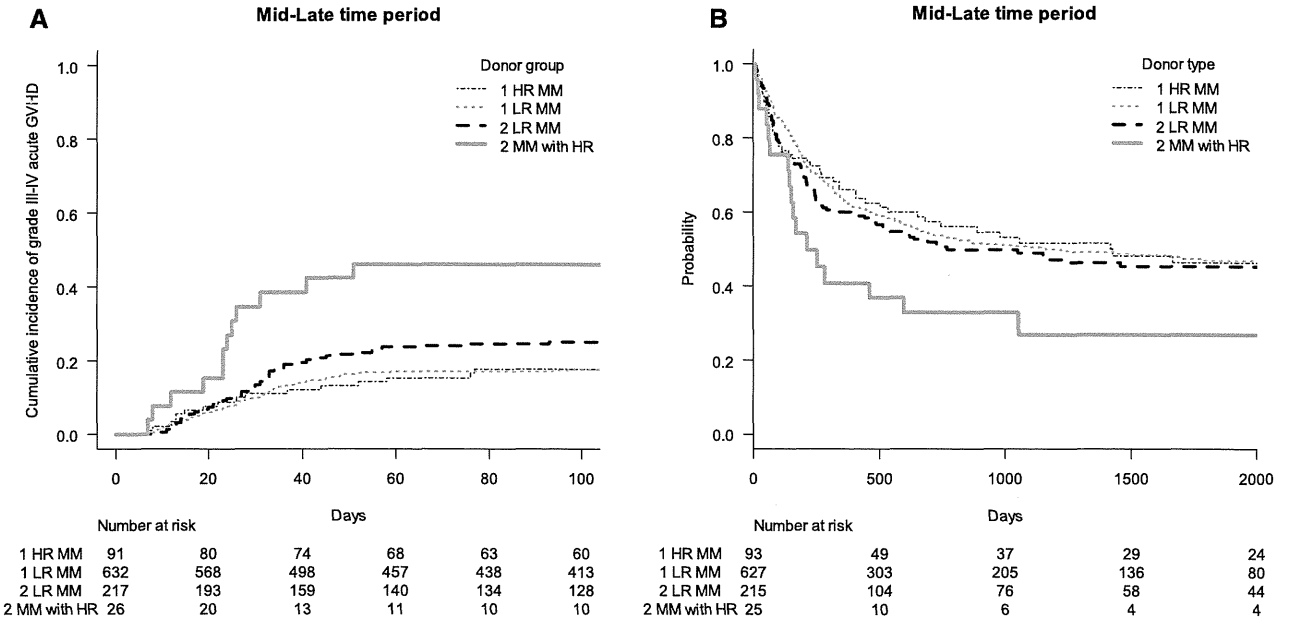

Figure 5. The cumulative incidence of grade III to IV acute GVHD (A) and adjusted overall survival (B) grouped according to the HLA mismatch between the donor and recipient in the mid or late time period. 1HR-MM, 1 high-risk mismatch; 1LR-MM, 1 low-risk mismatch; 2LR-MM, 2 low-risk mismatches; 2MM with HR, 2 allele mismatches including at least 1 HR-MM.

increase statistical power because the impact of HR-MM on acute GVHD and survival tended to be similar in these 2 time periods. The presence of HR-MMs at the HLA-A/B (HLA-A or -B), HLA-C, and HLA-DRB1 loci was not associated with significantly different survival compared with the LR-MM group (HR, 1.23; 95% CI, .76 to 1.98; $P = .41$; HR, .96; 95% CI, .65 to 1.44; $P = .86$; and HR, .95; 95% CI, .45 to 2.02; $P = .89$, respectively. Figure 4A). However, the incidence of grade III to IV acute GVHD was higher in patients who had HR-MM at the HLA-A/B locus than in those with LR-MM, although this difference was not statistically significant (HR, 1.78; 95% CI, .86 to 3.66; $P = .12$; HR, .63; 95% CI, .28 to 1.41; $P = .26$; and HR, .69; 95% CI, .15 to 3.12; $P = .63$ for HLA-A/B, HLA-C, and HLA-DRB1, respectively.) (Figure 4B).

Comparison of One HR-MM and Two LR-MMs

To evaluate whether a donor with 1 HR-MM or a donor with 2 LR-MMs should be preferred, we added patients with 2 LR-MMs and those with 2 allele mismatches including at least 1 HR-MM to the dataset, and we compared the outcome of HSCT from these donors with that of HSCT from a donor with 1 LR-MM as a reference in the combined mid and late periods.

The presence of 2 LR-MMs was associated with a significantly higher incidence of grade III to IV acute GVHD (HR, 1.44; 95% CI, 1.04 to 2.00; $P = .030$), but the impact of 1 HR-MM was not statistically significant (HR, .94; 95% CI, .56 to 1.59; $P = .83$) (Figure 5A). However, the impact of 2 LR-MMs was not associated with inferior survival. The HR for survival of 1 HR-MM and 2 LR-MMs were 1.05 (95% CI, .78 to 1.42; $P = .75$) and 1.12 (95% CI, .90 to 1.39; $P = .33$), respectively (Figure 5B).

On the other hand, the presence of 2 allele mismatches including at least 1 HR-MM was associated with an extremely poor outcome; HR, 3.61 (95% CI, 1.96 to 6.66; $P < .001$) for grade III to IV acute GVHD and HR, 2.02 (95% CI, 1.25 to 3.26; $P = .0040$) for overall survival. These results suggested that the impact of HR-MM may change according to the presence or absence of an additional allele mismatch. In fact, there was a statistically significant interaction between the presence of HR-MM and the presence of an additional allele mismatch ($P = .020$). The likelihood ratio test revealed that the prognostic value of Fine and Gray's proportional hazards model for acute GVHD was significantly improved by adding the interaction term to the model ($P = .024$).

DISCUSSION

In this study, we reevaluated the clinical impact of HR-MMs in unrelated HSCT. We confirmed that the presence of HR-MMs was associated with a significantly higher incidence of grade III to IV acute GVHD and significantly inferior survival in the early transplantation time period. However, in the mid and late periods, ie, after 2002, there was no statistically significant difference in overall survival or the incidence of grade III to IV acute GVHD between patients with HR-MMs and those with LR-MMs. The methods used for the statistical analyses were somewhat different than those in a previous study, but this is not the major reason for the different results, as the significant impact of HR-MMs on survival and acute GVHD was reproduced in the early time period. Another possible explanation is a bias caused by the availability of information about HR-MMs. After the publication of a paper that reported the importance of HR-MM, physicians may have tended to intensify prophylaxis against GVHD in unrelated HSCT with HR-MMs, and, thereby, the impact of HR-MMs might have become less significant. However, this is not the case because the impact of HR-MMs was already not apparent in the mid time period, before the paper was published. We also considered that the difference in the underlying disease might have influenced the effect of HR-MMs. The proportion of patients with CML decreased from 30.7% in the early period to 10.4% and 3.6% in the mid and late periods, respectively. Therefore, we analyzed the impact of HR-MMs grouped according to the underlying disease in the early period. The effect of HR-MMs on survival was observed only in patients with CML (Figure 3A,B). However, HR-MMs had an adverse effect on the incidence of grade III to IV acute GVHD regardless of the underlying disease (Figure 3C,D). Therefore, the different effects of HR-MMs on the incidence of grade III to IV acute GVHD among the time periods could not be explained solely by the underlying diseases. We could not clarify the reason for this different effect, but the changes in the transplantation procedure, including prophylaxis against GVHD, might have reduced the clinical impact of HR-MM. In fact, the incidence of grade III to IV acute GVHD decreased from 42.6%, 16.8%, and 14.5% in the HR-MM, LR-MM, and MUD groups, respectively, in the early time period to 17.6%, 17.7%, and 10.6% in the mid or late period. Improved survival in patients who developed severe acute GVHD might also reduce the effect of HR-MMS on survival. The 1-year survival in patients who developed grade III to IV acute GVHD improved from 32.1% in the early period to 44.4% in the mid and late time periods. This change may have resulted from the progress in supportive care, including strategies against fungal or viral infections.

Another important finding is that the impact of HR-MM was significantly enhanced by the presence of an additional allele mismatch in the mid and late time periods. This fact may be explained by a hypothesis that the HR-MM biologically increases the graft-versus-host (GVH) reaction, but the recent improvement in GVHD prophylaxis has masked its effect, if HR-MM exists as a single allele mismatch, whereas the adverse impact of HR-MM is not suppressed even by recent methods of GVHD prophylaxis when an additional allele mismatch is present. Based on these findings, interaction terms should be incorporated into the statistical model when the impact of HR-MMs is analyzed in datasets that include HSCT with multiple allele mismatches.

A major limitation of this study is the small number of patients with HR-MMs, especially in the late time period. We cannot deny the possibility that an important effect of HR-MMs might be overlooked because of the poor statistical power. The lack of a significant difference in the incidence of grade III to IV acute GVHD between unrelated HSCT with HR-MMs at the HLA-A/B locus and HSCT with LR-MM should be interpreted with caution, because of the small number of patients. Furthermore, it was impossible to evaluate the effect of each mismatch combination, as the number of patients with each mismatch combination was most often fewer than 10. HR-MMs associated with at least a 20% incidence of grade III to IV acute GVHD in the mid and late periods included A*0206-A*0201 (4 of 14), A*0206-A*0207 (3 of 4), B*1501-B*1507 (1 of 1), C*0801-C*0303 (4 of 15), and C*1402-C*0304 (1 of 5), but the number of patients in each pair was too small to draw any definitive conclusions.

When we consider the impact of HR-MMs, especially at the HLA-C locus, we should also consider the effect of a killer immunoglobulin-like receptor ligand (KIR) mismatch [13,14]. Among the 50 patients with HR-MMs at the HLA-C locus in the mid and late periods, 20 had a KIR mismatch in the GVH direction, whereas 30 did not. The incidence of grade III to IV acute GVHD was 5% and 16.7%, respectively, but this

difference was not statistically significant ($P = .24$). The incidence of grade III to IV acute GVHD in the 21 patients who had LR-MMs and a KIR mismatch in the GVH direction was 15.0%. We could not conclude that a KIR mismatch had an impact in this study because of the small number of patients with a KIR mismatch in the GVH direction.

We should note that the results of the current study are applicable to patients who receive bone marrow graft after a myeloablative conditioning regimen. The impact of HR-MMs may change according to the stem cell source or the conditioning regimen. Therefore, further analyses are required to evaluate the impact of HR-MMs in peripheral blood stem cell transplantation and reduced-intensity conditioning transplantation.

In conclusion, this retrospective study revealed that the clinical impact of HR-MMs became less significant after 2002. Although HR-MMs may have a biological impact, their effect may be controlled by recent methods for GVHD prophylaxis when they exist as a single allele mismatch. It may still be prudent to avoid a donor with HR-MMs, especially at the HLA-A or -B locus, if a donor with the other mismatch combination is available. However, in the absence of MUD or an unrelated donor with a LR-MM, a donor with a single HR-MM could be a viable option for unrelated HSCT, and it is preferred over a donor with 2 LR-MMs. In addition, we should be aware that the clinical impact of risk factors may change over time periods, and therefore, we should repeatedly confirm the validity of risk factors.

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Comparison of Cord Blood Transplantation with Unrelated Bone Marrow Transplantation in Patients Older than Fifty Years

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A B S T R A C T

We retrospectively compared the transplantation outcomes for patients 50 years or older who received umbilical cord blood transplantation (UCBT) with those who received unrelated bone marrow transplantation (UBMT) for hematologic malignancies. A total of 1377 patients who underwent transplantation between 2000 and 2009 were included: 516 received 8/8 HLA allele-matched UBMT, 295 received 7/8 HLA allele-matched UBMT, and 566 received 4/6 to 6/6 HLA-matched UCBT. Adjusted overall survival (OS) was significantly lower in those who underwent UCBT than those who underwent 8/8 HLA–matched UBMT but was similar to that of 7/8 HLA–matched UBMT (the 2-year OS after 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and UCBT were 49% [95% confidence interval (CI), 45% to 55%], 38% [95% CI, 32% to 45%], and 39% [95% CI, 34% to 43%], respectively). However, adjusted OS was similar between 8/8 HLA–matched UBMT and UCBT receiving $\geq .84 \times 10^5$ $CD34^+$ cells/kg among those with acute myeloid leukemia and those with acute lymphoblastic leukemia (the 2-year OS was 49% [95% CI, 43% to 55%], and 49% [95% CI, 41% to 58%], respectively). These data suggest that UCB is a reasonable alternative donor/stem cell source for elderly patients with similar outcomes compared with UBM from 8/8 HLA–matched unrelated donors when the graft containing $\geq .84 \times 10^5$ $CD34^+$ cells/kg is available.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for patients with high-risk hematologic malignancies. The frequency of adverse cytogenetic abnormalities is higher in elderly patients with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) than in younger patients, and overall survival (OS) after intensive chemotherapy in elderly patients is shorter than that in younger patients [1,2]. Inductions of reduced-intensity and nonmyeloablative stem cell transplantations allow elderly patients to receive allogeneic HSCT [3,4], and these patients have increasingly received this type of transplantation [5]. Only approximately 30% of patients

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have an HLA-identical sibling, and some elderly patients have siblings who cannot serve as a donor because of their age or underlying comorbidities; in such cases, an alternative donor is needed.

HLA-matched unrelated bone marrow or peripheral blood stem cells have been used as an alternative to an HLA-identical sibling donor. Umbilical cord blood has been used more frequently over the past decade, and several studies and meta-analyses have compared the outcomes of umbilical cord blood transplantation (UCBT) with that of unrelated bone marrow transplantation (UBMT) or unrelated peripheral blood stem cell transplantation (UPBSCT) [6-15]. However, the findings of those reports varied, and most of those studies included a small number of elderly patients. To the best of our knowledge, there has been no report that compared the outcomes of elderly patients who received UCBT with those who received UBMT or UPBSCT. Therefore, the main objective of this study was to compare the outcomes of patients 50 years or older who received UCBT with those who received UBMT using the Japanese nationwide registry data.

METHODS

Data Collection

Data regarding transplantations were extracted from the Transplant Registry Unified Management Program system of the Japan Society for Hematopoietic Cell Transplantation [16]. A total of 171 transplantation centers performed unrelated HSCT for adults and reported transplantation data to Japan Society for Hematopoietic Cell Transplantation between 2000 and 2009. All patients gave written informed consent at each transplantation center. The trial was conducted in accordance with the Declaration of Helsinki.

Patients with acute leukemia or myelodysplastic syndrome (MDS) who were 50 years or older and who received unrelated HSCT between 2000 and 2009 were included. Because the bone marrow was exclusively harvested from volunteer unrelated donors in Japan, cases of peripheral blood stem cell transplantation were not included in this analysis. Only 7 patients received double UCBT; therefore, these patients were also excluded. For the bone marrow recipients, recipients whose HLA matched 8/8 or 7/8 with their donor at the allelic level for HLA-A, HLA-B, HLA-C, and HLA-DRB1 were included. For UCBT, recipients whose HLA matched 4/6 to 6/6 with their donor at the antigen level for HLA-A and HLA-B and at the allelic level for HLA-DRB1, and who received a single unit of umbilical cord blood containing 2.0×10^7 or more total nucleated cells per kilogram of recipient's body weight at cryopreservation were included. Patients who had previously received autologous or allogeneic transplantation were excluded.

A myeloablative conditioning (MAC) regimen was defined as a total busulfan dose of more than 8 mg/kg, total melphalan dose of more than 140 mg/kg, fractionated total body irradiation (TBI) of 8 Gy or more, or single TBI of 5 Gy or more [17,18]. Other conditioning regimen was defined as reduced-intensity conditioning (RIC). Acute leukemia in the first complete remission (CR), refractory anemia with or without ringed sideroblasts, and refractory cytopenia with multilineage dysplasia for MDS were defined as early phase; acute leukemia in the second or subsequent CR were defined as intermediate phase; and all other statuses were defined as advanced phase. The karyotype at diagnosis for AML, ALL, and MDS were classified as previously reported [2,19,20]. The year of transplantation was divided into 2 groups: 2000 to 2004 was defined as the early period and 2005 to 2009 was defined as the recent period. Neutrophil recovery was defined as the first 3 consecutive days in which absolute neutrophil counts rose to greater than or equal to 500/mm^3. Acute graft-versus-host disease (GVHD) was evaluated based on standard criteria [21]. Chronic GVHD was defined according to the classical classification [22]. Relapse was defined as disease recurrence detected by hematological examination or detected by cytogenetic or molecular examination and requiring any treatment. Patients who did not obtain CR after HSCT were defined as patients who had a relapse the next day after HSCT. Nonrelapse mortality (NRM) was defined as death without relapse. OS was defined as the survival time from the date of transplantation to death from any cause or the last follow-up.

Statistical Analysis

The demographic factors and disease characteristics were compared between patients who underwent transplantation with 8/8 HLA–matched unrelated bone marrow, 7/8 HLA–matched bone marrow, and umbilical cord blood using Fisher's exact test for the categorical data and the Mann-Whitney U test for the continuous variables. OS was calculated from the date of transplantation to death from any cause or last follow-up and was estimated by the Kaplan-Meier method. Cox proportional hazards regression model was used for the multivariate analyses. Adjusted comparison of the stem cell source on OS was performed using the Cox proportional hazards regression model. Gray's test was employed for the comparison of cumulative incidence curves for relapse, NRM, neutrophil and platelet recoveries, and GVHD [23]. NRM and relapse were the competing event for each other. For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD was the competing event. Fine and Gray's proportional hazard regression model was employed for multivariate analyses with competing risks [24]. Multivariate analyses to compare the effect of stem cell source on transplantation outcomes were performed with the consideration of other significant clinical variables in the final models, which were built with the significant variables ($P < .10$) from the univariate analysis, which were then deleted in a stepwise fashion from the model when a variable was not statistically significant ($P > .05$). The stem cell source was added in the final model. The following variables were considered: patient age at transplantation, sex, primary disease (AML versus ALL versus MDS), karyotype at diagnosis (favorable versus intermediate versus adverse), disease status at transplantation (early phase versus intermediate phase versus advanced phase), year of transplantation (early period versus recent period), conditioning regimen (MAC versus RIC), use of TBI, and GVHD prophylaxis (cyclosporine alone versus cyclosporine and other agent versus tacrolimus alone versus tacrolimus and other agent versus other). All tests were 2-sided, and $P < .05$ was considered to indicate statistical significance. Analyses were performed with EZR version 1.20 (Saitama Medical Center, Jichi Medical University) [25], which is a graphical user interface for R version 3.0.2 (R Development Core Team, Vienna, Austria).

RESULTS

Patients and Transplantation Characteristics

Patients and transplantation characteristics are shown in Table 1. A total of 1377 patients were included in this analysis, and of those, 516 patients received 8/8 HLA allele-matched UBMT, 295 patients received 7/8 HLA allelic-matched UBMT, and 566 patients underwent transplantation from 4/6 to 6/6 HLA–matched UCBT. The UCBT recipients were significantly older than the 8/8 or 7/8 HLA–matched UBMT recipients ($P < .001$), and more UCBT recipients underwent RIC or nonmyeloablative transplantation ($P < .001$) and received a TBI-containing conditioning regimen than did the 8/8 or 7/8 HLA–matched UBMT recipients ($P < .001$). More UCBT recipients had advanced phase disease ($P < .001$). Female donor to male recipient transplantation was included in UCBT more than in UBMT ($P < .001$). Compared with those receiving UBMT, more UCBT recipients had AML ($P < .001$) and received GVHD prophylaxis with a single-agent regimen ($P < .001$). The distribution of karyotype at diagnosis was similar (Supplemental Tables 1–3). The distribution of recipients' sex and year of transplantation were similar among the 3 groups. The median duration of follow-up for the surviving patients who underwent transplantation with 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA-matched UCBT was 23.7 months (range, 1.8 to 125.2 months), 18.6 months (range, 1.6 to 94.0 months), and 22.3 months (range, .1 to 107.5 months), respectively.

Hematopoietic Recovery

The median time from transplantation to neutrophil recovery in patients who underwent 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT was 17 days (range, 1 to 100 days), 17 days (range, 4 to 169 days), and 24 days (range, 0 to 95 days), respectively. Neutrophil recovery was faster in recipients with early phase disease or intermediate phase disease than in those with advanced phase disease ($P < .001$). MAC was an independent negative predictor for neutrophil engraftment ($P = .007$). The

Table 1
Patients, Disease, and Transplantation Characteristics

Characteristic	Total	8/8 HLA–Matched Bone Marrow	7/8 HLA–Matched Bone Marrow	Umbilical Cord Blood	*P* Value
Number	1377	516	295	566	
Sex (male)	816 (59%)	310 (60%)	188 (64%)	318 (56%)	.091
Age, median (range), yr	57 (50-82)	56 (50-70)	57 (50-71)	58 (50-82)	<.001
50-59	892 (65%)	376 (73%)	198 (67%)	318 (56%)	
60-69	468 (34%)	138 (27%)	96 (33%)	234 (41%)	
70 or older	17 (1%)	2 (<1%)	1 (<1%)	14 (3%)	
Sex matching					<.001
Female donor to male recipient	1030 (75%)	73 (14%)	67 (23%)	153 (27%)	
Others	293 (21%)	443 (86%)	227 (77%)	360 (64%)	
Unknown	54 (4%)	0 (0%)	1 (<1%)	53 (9%)	
Body weight, median (range), kg	56 (32.0-102.4)	58.5 (32.0-102.4)	58.9 (35.1-92.0)	54.0 (32.0-86.0)	<.001
Disease					<.001
AML	902 (65%)	314 (61%)	180 (61%)	408 (72%)	
ALL	244 (18%)	96 (19%)	47 (16%)	101 (18%)	
MDS	231 (17%)	106 (20%)	68 (23%)	57 (10%)	
Disease status at transplantation					<.001
Early phase	471 (34%)	223 (43%)	94 (32%)	154 (27%)	
Intermediate phase	221 (16%)	82 (16%)	58 (20%)	81 (14%)	
Advanced phase	685 (50%)	211 (41%)	143 (48%)	331 (59%)	
Year of transplantation					
2000-2004	343 (25%)	128 (25%)	74 (25%)	141 (25%)	1
2005-2009	1034 (75%)	388 (75%)	221 (75%)	425 (75%)	
Conditioning regimen					
Myeloablative	653 (47%)	291 (56%)	147 (50%)	215 (38%)	<.001
CY + TBI (≥8 Gy)	174 (12%)	79 (15%)	43 (15%)	52 (9%)	
CY + TBI (≥8 Gy) + other	135 (10%)	46 (9%)	19 (6%)	70 (13%)	
BU + CY	110 (8%)	64 (12%)	33 (12%)	13 (2%)	
FLU + BU (>8 mg/kg)	44 (3%)	34 (7%)	5 (2%)	5 (1%)	
FLU + BU (>8 mg/kg) + TBI (<8 Gy)	40 (3%)	14 (3%)	7 (2%)	19 (3%)	
FLU + MEL (>140 mg/m^2)	57 (4%)	28 (5%)	20 (7%)	9 (2%)	
Other TBI-based regimen	66 (5%)	19 (4%)	13 (4%)	34 (6%)	
Other BU-based regimen	27 (2%)	7 (1%)	7 (2%)	13 (2%)	
RIC/NMA	712 (52%)	217 (42%)	145 (49%)	350 (62%)	
FLU + BU (≤8 mg/kg)	25 (2%)	5 (1%)	5 (2%)	15 (3%)	
FLU + BU (≤8 mg/kg) + TBI (<8 Gy)	206 (15%)	91 (17%)	58 (20%)	57 (10%)	
FLU + BU (≤8 mg/kg) + MEL (≤140 mg/m^2)	26 (2%)	13 (3%)	5 (2%)	8 (1%)	
FLU + BU (≤8 mg/kg) + other	33 (2%)	12 (2%)	16 (5%)	5 (1%)	
FLU + MEL (≤140 mg/m^2)	64 (5%)	33 (6%)	16 (5%)	15 (3%)	
FLU + MEL (≤140 mg/m^2) + TBI (<8 Gy)	219 (16%)	33 (6%)	26 (9%)	160 (28%)	
FLU + MEL (≤140 mg/m^2) + TBI (<8 Gy) + other	20 (2%)	3 (1%)	1 (<1%)	16 (3%)	
FLU + CY + TBI (<8 Gy)	56 (4%)	3 (1%)	2 (1%)	51 (9%)	
Other regimen including TBI (<8 Gy)	33 (2%)	13 (3%)	10 (3%)	10 (2%)	
Other regimen not including TBI (<8 Gy)	30 (2%)	11 (2%)	6 (2%)	13 (2%)	
Unknown	12 (1%)	8 (2%)	3 (1%)	1 (<1%)	
TBI-containing conditioning regimen	962 (70%)	306 (59%)	184 (62%)	472 (83%)	<.001
Addition of ATG to conditioning regimen	46 (3%)	17 (3%)	19 (6%)	10 (2%)	.001
GVHD prophylaxis					
CyA + other	370 (27%)	129 (25%)	52 (18%)	189 (33%)	<.001
CyA alone	68 (5%)	5 (1%)	3 (1%)	60 (11%)	
TAC + other	775 (56%)	359 (70%)	226 (76%)	190 (33%)	
TAC alone	138 (10%)	15 (3%)	11 (4%)	112 (20%)	
Others	13 (1%)	7 (1%)	3 (1%)	3 (1%)	
None	13 (1%)	1 (<1%)	0 (0%)	12 (2%)	
Total cell dose (range, ×10^7/kg)				2.56 (2.00-5.62)	
CD34$^+$ cell dose (range, ×10^5/kg)				.83 (.01-14.02)	
HLA-A, B, DR antigen level					
Matched (6/6)		516 (100%)	295 (100%)	46 (8%)	
One-antigen mismatched (5/6)		0	0	159 (28%)	
Two-antigen mismatched (4/6)		0	0	361 (64%)	

HLA indicates human leukocyte antigen; TBI, total body irradiation; GVHD, graft-versus-host disease; CY, cyclophosphamide; BU, busulfan; FLU, fludarabine; MEL, melphalan; NMA, nonmyeloablative; ATG, antithymocyte globulin; CyA, cyclosporine A; TAC, tacrolimus.

probability of neutrophil recovery by day 50 was significantly lower in recipients of 4/6 to 6/6 HLA–matched UCBT (72% [95% confidence interval (CI), 68% to 75%]) than in those of 8/8 HLA–matched UBMT (95% [95% CI, 92% to 96%]) or 7/8 HLA–matched UBMT (90% [95% CI, 85% to 93%]). On multivariate analysis, the 4/6 to 6/6 HLA–matched UCBT was an independent negative predictor for neutrophil engraftment when compared with the 8/8 HLA–matched UBMT (hazard ratio [HR], .43 [95% CI, .38 to .50]; $P < .001$) and the 7/8 HLA–matched UBMT (HR, .47 [95% CI, .40 to .56]; $P < .001$) (Table 2).

The probability of platelet recovery by day 180 was also significantly lower in the 4/6 to 6/6 HLA–matched UCB recipients (54% [95% CI, 50% to 58%]) than in those who received the 8/8 HLA–matched UBMT (83% [95% CI, 79% to 86%]) or the 7/8 HLA–matched UBMT (75% [95% CI, 70% to 80%]). The median times from transplantation to platelet recovery in the recipients of 8/8 HLA–matched