determined by flow cytometry. The serum levels of IFNγ and IL-4 in mice injected with cultured cells 7 days earlier were analyzed at 0, 4, and 10 h after i.p. injection of α-GalCer (2 μg).

3. Results

3.1. Expansion of mouse Vα14i NKT cells in vitro

Consistent with previous results [25,26], we observed that 1.2% of freshly isolated spleen cells comprised Vα14i NKT cells (Fig. 1A). When spleen cells were cultured in the presence of 50 ng/ml α-GalCer, the percentage and absolute number of Vα14i NKT cells increased to 6.7% and 8-fold, respectively, after 4 days culture (Fig. 1). α-GalCer-induced human $Vα24^+Vβ11^+$ NKT cell expansion can be potentiated by IL-2, IL-7, or IL-15 [13–18]. To examine whether IL-2 augments mouse Vα14i NKT cell expansion by α-GalCer, spleen cells were cultured in the presence of α-GalCer and 100 U/ml IL-2. Vα14i NKT cells were more vigorously expanded when cultured with α-GalCer and IL-2 as compared with α-GalCer alone. IL-15 also enhanced α-GalCer-induced Vα14i NKT cell expansion (data not shown). Vα14i NKT cell expansion peaked after 4 days of culture with α-GalCer alone, whereas IL-2 prolonged Vα14i NKT cell expansion by α-GalCer past 4 days in culture (Fig. 1B). Furthermore, Vα14i NKT cells in thymus, liver, and bone marrow could be expanded in the presence of α-GalCer and IL-2 (data not shown). However, Vα14i NKT cells were not expanded in the presence of IL-2 alone (Fig. 1). It should be noted that in vitro expansion of Vα14i NKT cells is dependent on α-GalCer.

$NK1.1^+$ T cells, which are classical NKT cells, increased to around 5-fold after 4 days culture in the presence of IL-2 with or without α-GalCer (Fig. 1). Furthermore, they increased to around 20-fold after 6 days culture. Like $NK1.1^+$ T cells, NK cells expanded in the presence of IL-2. Thus, the expansion of $NK1.1^+$ T cells and NK cells is dependent on IL-2 but not α-GalCer.

3.2. Phenotypes of in vitro-expanded Vα14i NKT cells

Because the expansion of Vα14i NKT cells and $NK1.1^+$ T cells was dependent on α-GalCer and IL-2, respectively, we next examined whether the Vα14i NKT cells were identical to $NK1.1^+$ NKT cells after culture with α-GalCer and IL-2. Consistent with previous studies [25,26], half of $NK1.1^+$ T cells were CD1d/α-GalCer $tetramer^+$ cells and around 70% of Vα14i NKT cells expressed NK1.1 in the spleen (Fig. 2A). However, after culture, almost all Vα14i NKT cells lost expression of NK1.1. Our results indicate that the expanded Vα14i NKT cells are distinct from the expanded $NK1.1^+$ T cells following in vitro culture.

We analyzed the phenotype of the expanded Vα14i NKT cells as compared with fresh Vα14i NKT cells. As shown in Fig. 2B, the expanded Vα14i NKT cells maintained memory

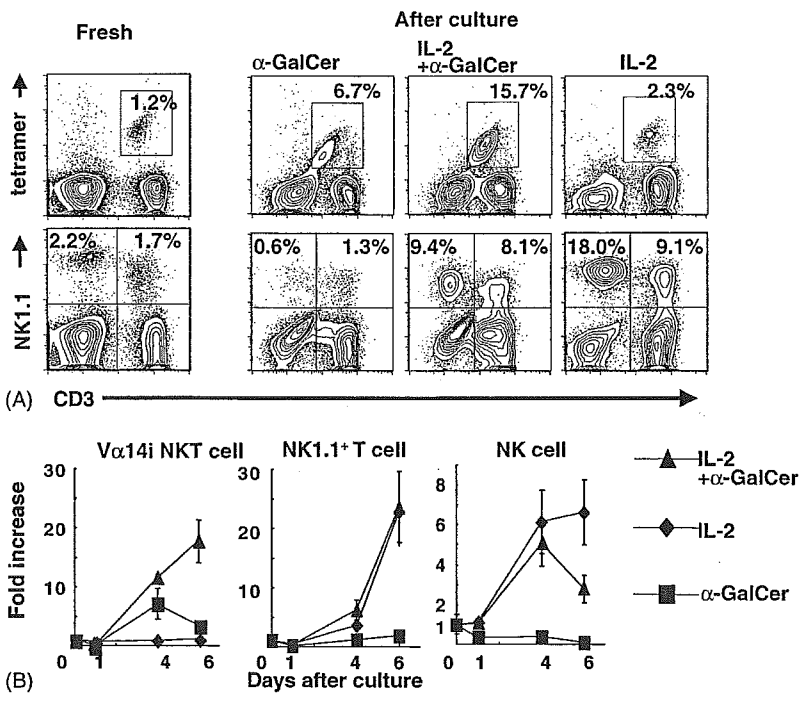

Fig. 1. In vitro expansion of Vα14i NKT cells. (A) C57BL/6 mice spleen cells (7×10^6) were cultured with 50 ng/ml α-GalCer, α-GalCer plus 100 U/ml IL-2, or IL-2 for 4 days. The percentages of Vα14i NKT cells, NK cells, and $NK1.1^+$ T cells were determined. Fresh and cultured cells were stained with mAbs and CD1d/α-GalCer tetramer and analyzed by flow cytometry. The fluorescence profiles are representative of at least five independent experiments. (B) The fold increase in Vα14i NKT cells after culture with IL-2 plus α-GalCer (▲), IL-2 alone (♦), or α-GalCer alone (■) was calculated from cell counts and flow cytometric data. Data are means obtained from three mice per point.

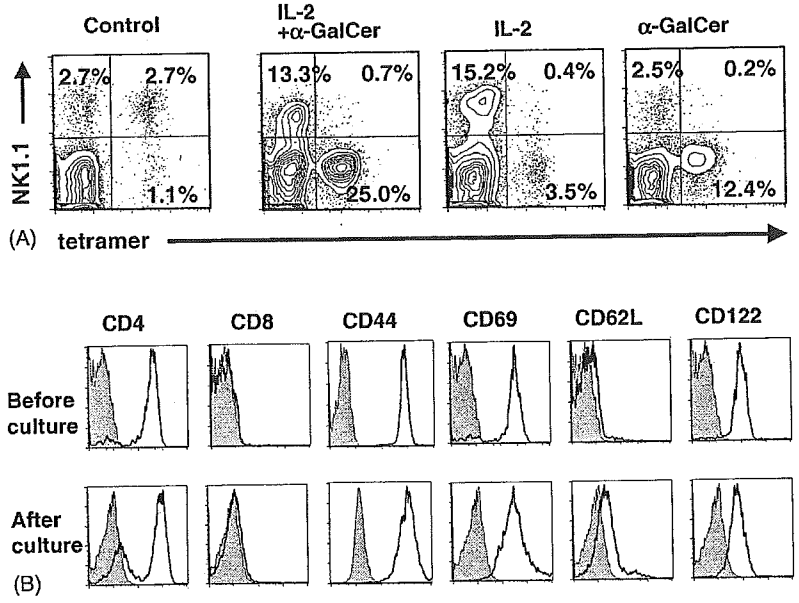

Fig. 2. Phenotype of in vitro-expanded Vα14i NKT cells. (A) NK1.1 expression in in vitro-expanded Vα14i NKT cells was examined after 4 days culture. Fresh and cultured cells were stained with CD1d/α-GalCer tetramer, anti-CD3, and anti-NK1.1 mAb. The cells were analyzed by flow cytometry and gated on CD3-positive cells. The fluorescence profiles are representative of at least five independent experiments. (B) The surface marker expression of fresh and expanded Vα14i NKT cells was compared by flow cytometry. Histogram panels are gated on CD1d/α-GalCer tetramer$^+$ CD3$^+$ cells. Shadow histograms indicate non-stained controls. The fluorescence profiles are representative of at least three independent experiments.

or activated phenotypes (CD44^{high}CD62L$^-$CD69$^+$) whereas the expression of IL-2Rβ was down-regulated. Similar to fresh Vα14i NKT cells, the expanded Vα14i NKT cells consisted of CD4$^+$ and CD4$^-$CD8$^-$ double negative subsets (Fig. 2B). In addition, some expanded Vα14i NKT cells maintained CD94 expression and down-regulated NKG2D, although some fresh Vα14i NKT cells express NK receptors, such as CD94 and NKG2D (data not shown). These results indicate that expanded Vα14i NKT cells maintain the memory or activated phenotypes but modulate the expression of NK cell-related molecules.

3.3. In vitro-expanded Vα14i NKT cells retain the ability to produce cytokines

It has been reported that α-GalCer induces rapid activation of Vα14i NKT cells and a burst of IL-4 and IFNγ secretion in vivo and in vitro [8–10]. Therefore, we examined the ability of expanded Vα14i NKT cells to secrete IL-4 and IFNγ. First, we analyzed IL-4 and IFNγ levels in the supernatants of spleen cells cultured with α-GalCer and/or IL-2 (Fig. 3A). We detected a larger amount of IL-4 and IFNγ in the supernatant when cultured in the presence of α-GalCer. Furthermore, the addition of IL-2 to the culture with α-GalCer slightly enhanced the production of IL-4 and IFNγ. No IL-4 and a low amount of IFNγ were detected when spleen cells were cultured with IL-2 alone.

Next, we used intracellular cytokine staining to determine if expanded Vα14i NKT cells directly secrete IL-4 and IFNγ in vitro (Fig. 3B). IL-4 producing cells comprised mainly expanded Vα14i NKT cells. Seventy percent of expanded Vα14i NKT cells contained intracellular IFNγ. These results suggest that expanded Vα14i NKT cells retain the ability to produce cytokines in vitro. In addition to expanded Vα14i NKT cells, 70% of CD1d/α-GalCer tetramer$^-$ T cells and 40% of CD1d/α-GalCer tetramer$^-$ CD3$^-$ cells were also positive for intracellular IFNγ. The IFNγ producing CD1d/α-GalCer tetramer$^-$ CD3$^-$ cells were mainly NK cells but not B cells (data not shown). It should be noted that NK cells and some T cells acquired the ability to produce IFNγ when cultured with α-GalCer and IL-2 but not IL-2 alone.

3.4. In vivo survival and cytokine production of expanded Vα14i NKT cells after adoptive transfer

We examined the ability of expanded Vα14i NKT cells to survive and migrate to peripheral tissues after adoptive transfer. Spleen cells cultured with α-GalCer and IL-2 were transferred into lymphopenic SCID mice. Seven days after transfer, the mice were killed and the CD1d/α-GalCer tetramer$^+$ cells in the liver and spleen were analyzed. As shown in Fig. 4, around 7% of hepatic mononuclear cells were CD1d/α-GalCer tetramer$^+$ T cells. The percentage of CD1d/α-GalCer tetramer$^+$ cells in the spleen was equal to that observed in normal mice (Figs. 1A and 4). Although it has been known that Vα14i NKT cells are abundant in bone marrow in normal mice, we observed very few CD1d/α-GalCer tetramer$^+$ cells in bone marrow (data not shown). The expanded Vα14i NKT cells were detected at least 3 weeks after transfer (data not shown). There were no differences in the phenotype of in vitro-expanded Vα14i NKT cells before and after transfer into SCID mice.

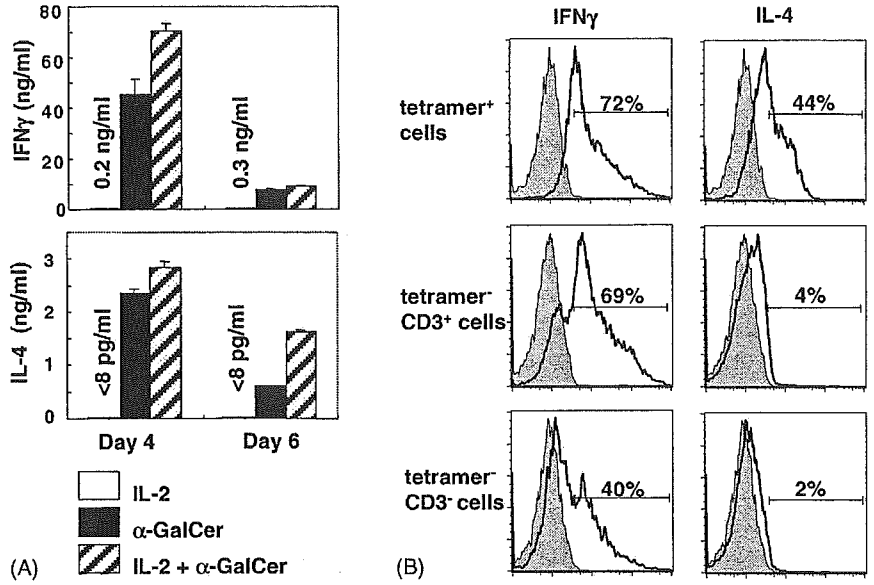

Fig. 3. Cytokine production profile of Vα14i NKT cells, NK cells, and NK1.1$^+$ T cells after culture. (A) Spleen cells (7×10^6) from C57BL/6 mice were cultured with 50 ng/ml α-GalCer, α-GalCer plus 100 U/ml IL-2, or IL-2 for 4 and 6 days. IFNγ and IL-4 in the supernatants were measured by ELISA. Data are representative of three independent experiments. (B) Intracellular cytokine staining for IFNγ and IL-4 in spleen cells cultured with α-GalCer and IL-2 for 4 days. The cultured cells were stimulated with PMA and ionomycine for 2 h. Then, the cells were stained with CD1d/α-GalCer tetramer, anti-CD3 mAb, and anti-IL-4, IFNγ, or isotype control mAb and analyzed by flow cytometry. Histogram panels are on CD1d/α-GalCer tetramer$^+$ CD3$^+$ cells (Vα14i NKT cells), CD1d/α-GalCer tetramer$^-$ CD3$^+$ cells (including NK1.1$^+$ T cells), or CD1d/α-GalCer tetramer$^-$ CD3$^-$ cells (including NK cells). Closed histograms indicate isotype controls. The fluorescence profiles are representative of three independent experiments.

Next, we examined the ability of the adoptively transferred, in vitro-expanded Vα14i NKT cells, to secrete IL-4 and IFNγ after administration of α-GalCer. Seven days after cell transfer, the mice were injected with 2 μg of α-GalCer.

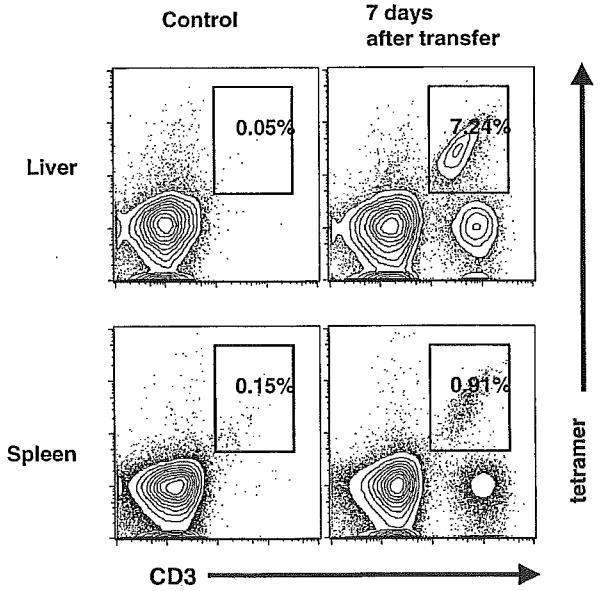

Fig. 4. Migration of in vitro-expanded Vα14i NKT cells after adoptive transfer. Spleen cells from BALB/c mice were cultured with α-GalCer plus 100 U/ml IL-2 for 4 days. The cultured cells (2×10^7) were injected into C.B-17/Icr SCID mice. Recipient mice were killed after 7 days, and the presence of transferred Vα14i NKT cells in the liver and spleen was determined by flow cytometry. The fluorescence profiles are representative of three independent experiments.

The serum levels of IL-4 and IFNγ were analyzed by ELISA. Four hours after the α-GalCer injection, IL-4 and IFNγ were detected in the serum of mice that had received cultured cells (Fig. 5A). One hour after α-GalCer administration, intracellular cytokine staining for CD1d/α-GalCer tetramer$^+$ T cells in the spleen revealed that intracellular IL-4 and IFNγ were detected in 50 and 30% of CD1d/α-GalCer tetramer$^+$ T cells, respectively (Fig. 5B). These results indicate that the expanded Vα14i NKT cells re-exposed to α-GalCer retain the ability to produce IL-4 and IFNγ after adoptive transfer.

It has been reported that increased IFNγ levels in the serum of normal mice 10–16 h after α-GalCer injection were due to IFNγ production by NK cells [11]. However, 10 h after the α-GalCer injection, the IFNγ level was decreased in the mice that had previously received the cultured cells. Therefore, the in vitro-expanded Vα14i NKT cells could not induce NK cell IFNγ production in vivo. Previous reports have demonstrated that diminished IFNγ levels in the serum of α-GalCer-primed mice were caused by a failure of NK cell IFNγ production after α-GalCer re-injection [27]. Thus, the in vitro-expanded Vα14i NKT cells might be similar to primed Vα14i NKT cells.

4. Discussion

NKT cells play an important role in various immune responses, including autoimmunity and tumor immunity [1–3]. The administration of α-GalCer, a specific ligand

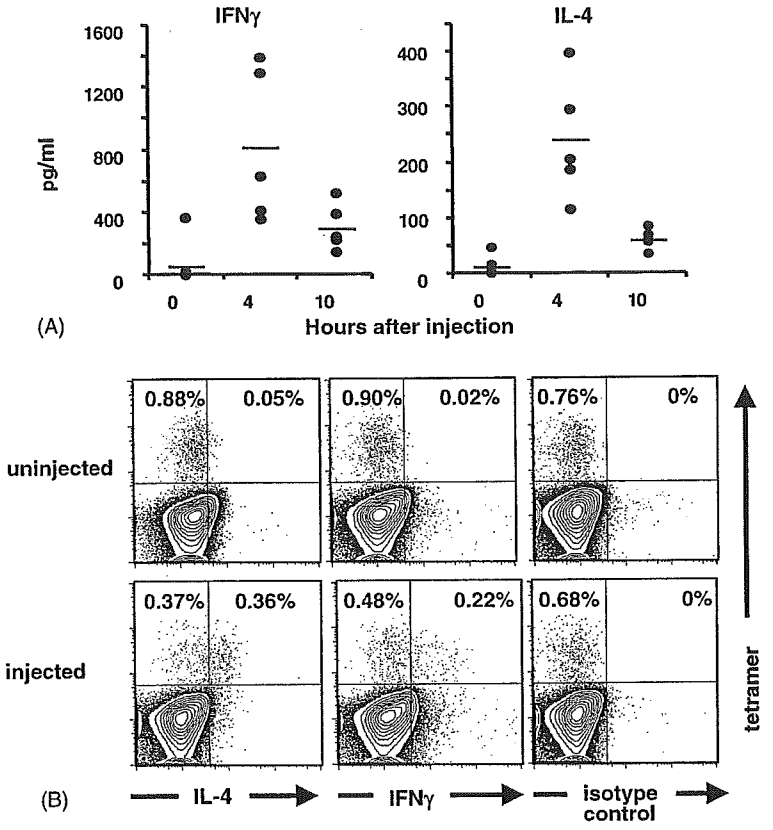

Fig. 5. IFNγ and IL-4 production of in vitro-expanded Vα14i NKT cells after adoptive transfer. Spleen cells from BALB/c mice were cultured with α-GalCer plus 100 U/ml IL-2 for 4 days. The cultured cells (2×10^7) were injected into C.B-17/Icr SCID mice. (A) Serum IFNγ and IL-4 levels. Seven days after the cells were injected, the serum cytokine levels were analyzed 0, 4, and 10 h after i.p. injection of α-GalCer. Data were obtained from 5 to 7 mice. (B) Intracellular cytokine staining of splenocytes 2 h after i.p. injection of α-GalCer (2 μg) in mice injected with cultured cells (2×10^7) 7 days earlier. Cells were stained with CD1d/α-GalCer tetramer and anti-IL-4, IFNγ, or isotype control mAb. Stained cells were analyzed by flow cytometry. The fluorescence profiles are representative of three independent experiments.

for Vα14i NKT cells, prevents tumor metastasis [9,10] and autoimmune disease [28–30]. Moreover, the adoptive transfer of NKT cells in mice prevents type I autoimmune diabetes [31] and tumor metastasis [12,32]. These studies suggest several possible therapeutic applications for adoptive immune therapy with NKT cells. However, it is apparent that the frequency of NKT cells is very low in human blood. Therefore, in vitro NKT cell expansion is required for adoptive immunotherapy with these cells. In this study, we found that in vitro-expanded Vα14i NKT cells are able to migrate into liver and spleen, and produce cytokines after adoptive transfer.

Human $V\alpha24^+V\beta11^+$ T cells in peripheral blood mononuclear cells expand in vitro using α-GalCer and IL-2, IL-7, or IL-15 [13–19], and mouse Vα14i NKT cells also proliferate in the presence of α-GalCer in vitro [1–3]. However, the function and phenotype of in vitro-expanded Vα14i NKT cells have not been well characterized because there is no appropriate marker to identify these cells. In previous studies, NKT cells have been identified as $NK1.1^+$ T cells. However, some Vα14i NKT cells do not express NK1.1 [25,26], and Vα14i NKT cells lose or down-regulate the expression of NK1.1 in vivo after stimulation [33,34]. Therefore, the NK1.1 marker is not expressed on Vα14i NKT cells after stimulation. We could detect in vitro-expanded Vα14i NKT cells by CD1d/α-GalCer tetramer. However, an issue with CD1d/α-GalCer tetramer staining is that the surface expression of Vα14i NKT cells is also down-regulated at 8–12 h after α-GalCer-stimulation [33,34]. Although their TCR expression was recovered to normal levels at 24–48 h [33,34], it is not an issue is whether the numbers of in vitro-expanded Vα14i NKT cells (at 4 and 6 days after culture) is an underestimate of the actual number. These in vitro-expanded Vα14i NKT cells in the presence of α-GalCer do not express NK1.1. It has been reported that $NK1.1^-$ CD1d/α-GalCer $tetramer^+$ T cells exist in normal mice and that some of these cells are immature NKT cells that have recently emigrated from the thymus [35,36]. However, recent studies have shown that expanded $NK1.1^-$ Vα14i NKT cells originate from $NK1.1^+$ Vα14i NKT cells that down-regulate their surface NK1.1 expression [33,34]. We considered two possibilities for the origin of in vitro-expanded Vα14i NKT cells: expansion of NK1.1 down-regulated NKT cells and/or expansion of $NK1.1^-$ precursor NKT cells. We observed that some Vα14i NKT cells expanded when $NK1.1^-$ spleen cells were cultured (data not shown). Therefore, we concluded that both

NK1.1$^+$ and NK1.1$^-$ NKT cells expand after in vitro α-GalCer-stimulation.

Previous studies demonstrated that mouse and human invariant NKT cells could produce both Th1 and Th2 cytokines [1–3]. Furthermore, it was reported that adult Vα24$^+$ Vβ11$^+$ NKT cells did not polarize into Th1 or Th2 after expansion [37]. However, NKT cells display polarization induced by type 1 or 2 dendritic cells [19]. Th1 or Th2 polarization of NKT cells is believed to be influenced by culture environment, such as the type of dendritic cell. We showed that in vitro-expanded Vα14i NKT cells continuously produced both IL-4 and IFNγ and did not polarize into Th1- or Th2-type. By contrast, a previous study has demonstrated that the robust expanded Vα14i NKT cells (after α-GalCer administration) continue to produce IFNγ in vivo [34]. This suggests that in vivo-expanded Vα14i NKT cells favor Th1 polarization. In contrast to in vivo-expanded Vα14i NKT cells, in vitro-expanded Vα14i NKT cells might remain continually activated and produce both IFNγ and IL-4 because they are continually exposed to α-GalCer in the culture conditions. Indeed, in the expansion phase of Vα14i NKT cells (after α-GalCer injection), these Vα14i NKT cells had the ability to secrete large amounts of both cytokines when re-injected with α-GalCer (Ikarashi et al., unpublished data).

In addition to the IFNγ production by in vitro-expanded Vα14i NKT cells, we showed that NK cells and NK1.1$^+$ T cells acquired the ability to produce IFNγ when cultured with α-GalCer and IL-2. IL-2 alone could induce the proliferation, but not IFNγ production, by NK cells and NK1.1$^+$ T cells in vitro. Our results indicate that α-GalCer-induced Vα14i NKT cell activation leads to IFNγ production of NK and NK1.1$^+$ T cells in vitro. Previous in vivo studies have demonstrated rapid cytokine production by Vα14i NKT cells in response to α-GalCer triggered activation and IFNγ production by NK cells [7,8], and bystander activation by conventional T cells and B cells [38,39]. Taken together, the mechanisms of NK cell activation by Vα14i NKT cells in vitro might be similar to the in vivo mechanisms.

Vα14i NKT cells have been known to regulate immune responses [1–3]. In fact, previous studies have shown that adoptive transfer of thymic NKT cells prevented type I diabetes in NOD mice in an IL-4- and IL-10-dependent manner (T helper 2) [31]. Furthermore, hepatic metastasis of B16 melanoma was prevented by adoptive transfer of IL-12-activated Vα14i NKT cells from Vα14 TCR transgenic mice [32]. These observations indicate that adoptive Vα14i NKT cell immunotherapy is useful for autoimmune diabetes and cancer. However, important questions remain as to whether in vitro-expanded Vα14i NKT cells can survive in the recipients and maintain the ability to produce IFNγ and IL-4 after transfer, similar to resident Vα14i NKT cells. A previous study demonstrated that fresh mouse Vα14i thymocytes proliferated and survived in an IL-15-dependent manner after adoptive transfer into lymphopenic mice [40]. We found that in vitro-expanded Vα14i NKT cells survived and dominantly migrated into the liver of lymphopenic mice. Furthermore, we revealed that in vitro-expanded Vα14i NKT cells 7 days after transfer could respond to α-GalCer and secrete IL-4 and IFNγ after administration of α-GalCer. However, IFNγ production patterns of lymphopenic mice transferred with in vitro-expanded Vα14i NKT cells after administration of α-GalCer were similar to those of α-GalCer-primed mice, as reported previously [27].

Previous studies have demonstrated that the antitumor effect of α-GalCer is mediated by Vα14i NKT cells [9–12] and that the IFNγ production by Vα14i NKT cells and the subsequent IFNγ production by NK cells are critical for α-GalCer to mediate antitumor activity [11,12]. Although it appears that α-GalCer-based immunotherapy is useful for cancer, treatment with α-GalCer has shown little therapeutic effect in patients with solid tumors [41]. For these reasons, it has been proposed that human Vα24i NKT cells from cancer patients have impaired proliferative responses to α-GalCer and have lost the ability to produce IFNγ [22,23]. We believe that adoptive Vα24i NKT cell therapy may be beneficial for cancer patients. Further studies are needed to clarify the mechanism and clinical applicability of in vitro-expanded Vα24i NKT cell therapy in cancer.

Acknowledgments

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References

[1] Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today 2000;21:573–83.
[2] Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. Nat Rev Immunol 2002;2:557–68.
[3] Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Vα14 NKT cells in innate and acquired immune response. Annu Rev Immunol 2003;21:483–513.
[4] Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. Science 1997;278:1623–6.
[5] Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med 2000;191:661–8.
[6] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, et al. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14 NKT cells. Proc Natl Acad Sci USA 1998;95:5690–3.
[7] Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. J Immunol 1999;163:4647–50.

[8] Eberl G, MacDonald HR. Selective induction of NK cell proliferation and cytotoxicity by activated NKT cells. Eur J Immunol 2000;30:985–92.
[9] Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y. KRN7000 a novel immunomodulator, and its antitumor activities. Oncol Res 1995;7:529–34.
[10] Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an α-galactosylceramide, KRN7000. Cancer Res 1998;58:1202–7.
[11] Hayakawa Y, Takeda K, Yagita H, Kakuta S, Iwakura Y, Van Kaer L, et al. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. Eur J Immunol 2001;31:1720–7.
[12] Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, et al. Sequential production of interferon-gamma by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. Blood 2002;99: 1259–66.
[13] Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. J Exp Med 1998;188:1521–8.
[14] Nishi N, van der Vliet HJ, Koezuka Y, von Blomberg BM, Scheper RJ, Pinedo HM, et al. Synergistic effect of KRN7000 with interleukin-15, -7, and -2 on the expansion of human Vα 24$^+$V β11$^+$ T cells in vitro. Hum Immunol 2000;60:357–65.
[15] van der Vliet HJ, Nishi N, Koezuka Y, von Blomberg BM, van den Eertwegh AJ, Porcelli SA, et al. Potent expansion of human natural killer T cells using α-galactosylceramide (KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. J Immunol Methods 2001;247:61–72.
[16] Asada-Mikami R, Heike Y, Harada Y, Kanai S, Ikarashi Y, Kato K, et al. Increased expansion of Vα24$^+$ T cells derived from G-CSF-mobilized peripheral blood stem cells as compared to peripheral blood mononuclear cells following α-galactosylceramide stimulation. Cancer Sci 2003;94:383–8.
[17] Rogers PR, Matsumoto A, Naidenko O, Kronenberg M, Mikayama T, Kato S. Expansion of human Vα24$^+$ NKT cells by repeated stimulation with KRN7000. J Immunol Methods 2004;285:197–214.
[18] Nieda M, Nicol A, Koezuka Y, Kikuchi A, Takahashi T, Nakamura H, et al. Activation of human Vα24NKT cells by α-glycosylceramide in a CD1d-restricted and Vα24TCR-mediated manner. Hum Immunol 1999;160:10–9.
[19] van der Vliet HJ, Molling JW, Nishi N, Masterson AJ, Kolgen W, Porcelli SA, et al. Polarization of Vα24$^+$ Vβ11$^+$ natural killer T cells of healthy volunteers and cancer patients using α-galactosylceramide-loaded and environmentally instructed dendritic cells. Cancer Res 2003;63:4101–6.
[20] Kawano T, Nakayam T, Kamada N, Kaneko Y, Harada M, Ogura N, et al. Antitumor cytotoxicity mediated by ligand-activated human Vα24 NKT cells. Cancer Res 1999;59:5102–5.
[21] Takahashi T, Nieda M, Koezuka Y, Nicol A, Porcelli SA, Ishikawa Y, et al. Analysis of human Vα24$^+$ CD4$^+$ NKT cells activated by α-glycosylceramide-pulsed monocyte-derived dendritic cells. J Immunol 2000;164:4458–64.
[22] Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-γ production by invariant NK T cells in advanced cancer. J Immunol 2001;167:4046–50.
[23] Yanagisawa K, Seino K, Ishikawa Y, Nozue M, Todoroki T, Fukao K. Impaired proliferative response of Vα24 NKT cells from cancer patients against α-galactosylceramide. J Immunol 2002;168: 6494–9.
[24] Ikarashi Y, Mikami R, Bendelac A, Terme M, Chaput N, Terada M, et al. Dendritic cell maturation overrules H-2D-mediated natural killer T (NKT) cell inhibition: critical role for B7 in CD1d-dependent NKT cell interferon γ production. J Exp Med 2001;194:1179–86.
[25] Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to α glycolipid antigen using CD1d tetramers. J Exp Med 2000;192:741–54.
[26] Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. J Exp Med 2000;191:1895–903.
[27] Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, et al. Mouse Vα14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci USA 2003;100:8395–400.
[28] Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature 2001;413:531–4.
[29] Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, et al. Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med 2001;7:1057–62.
[30] Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, et al. The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat Med 2001;7:1052–6.
[31] Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. αI/β-T cell receptor (TCR)$^+$CD4$^-$CD8$^-$ (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. J Exp Med 1998;187:1047–56.
[32] Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Vα14 NKT cells. Int J Cancer 2001;91:523–8.
[33] Wilson MT, Johansson C, Olivares-Villagomez D, Singh AK, Stanic AK, Wang CR, et al. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. Proc Natl Acad Sci USA 2003;100:10913–8.
[34] Crowe NY, Uldrich AP, Kyparissoudis K, Hammond KJ, Hayakawa Y, Sidobre S, et al. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. J Immunol 2003;171:4020–7.
[35] Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. Science 2002;296:553–5.
[36] Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1$^-$CD4$^+$ CD1d-dependent precursor stage. J Exp Med 2002;195:835–44.
[37] Kadowaki N, Antonenko S, Ho S, Rissoan MC, Soumelis V, Porcelli SA, et al. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. J Exp Med 2001;193:1221–6.
[38] Singh N, Hong S, Scherer DC, Serizawa I, Burdin N, Kronenberg M, et al. Cutting edge: activation of NK T cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. J Immunol 1999;163:2373–7.
[39] Kitamura H, Ohta A, Sekimoto M, Sato M, Iwakabe K, Nakui M, et al. α-Galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. Cell Immunol 2000;199:37–42.
[40] Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, et al. Homeostasis of Vα14i NKT cells. Nat Immunol 2002;3:966–74.
[41] Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M. A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res 2002;8:3702–9.

bjh research paper

Impact of human leucocyte antigen mismatch on graft-*versus*-host disease and graft failure after reduced intensity conditioning allogeneic haematopoietic stem cell transplantation from related donors

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Summary

The impact of human leucocyte antigen (HLA) incompatibility between donor and recipient on graft-*versus*-host disease (GVHD) and graft failure after reduced-intensity conditioning stem cell transplantation (RICT) remains to be elucidated. We retrospectively analysed outcome in 341 patients who underwent RICT from related donors for haematological malignancies. The overall cumulative incidence of grade II–IV acute GVHD (aGVHD) was 40% for all subjects; 39% in recipients with HLA-matched donors, 44% in those with one-locus-mismatched donors, and 50% in those with two- to three-loci-mismatched donors. In a Cox regression model adjusted for potential confounders, the tendency for grade II–IV aGVHD ($P = 0{\cdot}01$), chronic GVHD (cGVHD) ($P = 0{\cdot}05$) and graft failure ($P = 0{\cdot}033$) increased with HLA disparity. Use of peripheral blood grafts instead of marrow was a risk factor for cGVHD. Use of antithymocyte globulin was associated with reduced aGVHD and cGVHD. Overall survival (OS) in recipients of two- to three-loci-mismatched RICT at 2 years (18%) was significantly worse than that in patients who received one-locus-mismatched RICT (51%) and HLA-matched RICT (48%) ($P < 0{\cdot}0001$). A two- to three-loci mismatch was identified as an independent risk factor for OS ($P < 0{\cdot}001$), but there was no significant difference in OS between HLA-matched and one-locus-mismatched RICT. HLA incompatibility between the donor and recipient is an important risk factor for graft failure, aGVHD, cGVHD and OS after RICT. RICT from a one-locus-mismatched donor may represent an effective alternative approach in patients with high-risk malignancies who lack HLA-matched related donors.

Keywords: human leucocyte antigen, graft-*versus*-host disease, rejection, reduced intensity conditioning, antithymocyte globulin.

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Allogeneic haematopoietic stem cell transplantation (SCT) is a potentially curative treatment for haematologic malignancies. A growing body of evidence suggests that allogeneic SCT is also useful for the treatment of bone marrow failure, congenital metabolic disorders, non-haematologic disorders including solid tumours and autoimmune diseases (Burt *et al*, 2003; Slavin *et al*, 2004). However, high transplant-related mortality (TRM) precludes the wider application of allogeneic SCT for these diseases. Recently, several investigators have reported encouraging results with allogeneic SCT using a reduced-intensity conditioning (RIC) transplant regimen (RICT) (Bacigalupo, 2004). These regimens have been designed to reduce TRM and provide a platform for durable donor cell engraftment to exploit a graft-*versus*-tumour effect.

Graft-*versus*-host disease (GVHD) is still a major obstacle to allogeneic SCT. Human leucocyte antigen (HLA) disparity between the SCT donor and recipient is the most critical factor that governs the severity of GVHD after conventional allogeneic SCT. Studies in patients who have been transplanted from a related donor other than an HLA-identical sibling after myeloablative conditioning have shown that HLA incompatibility increases the incidence and severity of acute GVHD (aGVHD), as well as the incidence of graft failure (Beatty *et al*, 1985). Although it was initially assumed that RIC may reduce the incidence of GVHD, GVHD appears to be a significant clinical problem following RICT (Khouri *et al*, 1998; Slavin *et al*, 1998; Nagler *et al*, 2000; Schetelig *et al*, 2002; Mielcarek *et al*, 2003; Bacigalupo, 2004; Diaconescu *et al*, 2004). While most studies on RICT have been performed in an HLA-matched related setting, alternative donor grafts are increasingly used in RICT (Kottaridis *et al*, 2000; Giralt *et al*, 2001; Maris *et al*, 2003; Niederwieser *et al*, 2003; Wong *et al*, 2003; Bacigalupo, 2004; Goggins & Rizzieri, 2004). Our current knowledge regarding the association of HLA incompatibility with GVHD, graft failure and survival is based primarily on results obtained in the setting of conventional and myeloablative allogeneic SCT. However, the risk factors that affect the transplant outcome after RICT, including engraftment, GVHD and survival, are still poorly defined. The present study was performed to analyse the impact of HLA incompatibility on graft failure, aGVHD, chronic GVHD (cGVHD) and survival in patients with haematological malignancies who received RICT from a related donor.

Patients and methods

Patients

We retrospectively analysed data from patients with haematological malignancies who underwent RICT from a related donor at 21 transplant centres in Japan. This study was approved by Institutional Review Board of each individual centre. All patients were treated with RIC regimens before allogeneic SCT because of high-risk clinical features that made them ineligible for conventional myeloablative allogeneic SCT. The stem cell source was either bone marrow or granulocyte-colony stimulating factor (G-CSF)-mobilised peripheral blood stem cells (PBSC) from related donors. Patients who received a manipulated graft and those who received cord blood were excluded from the analyses. Patients who received a graft from an HLA-matched non-sibling donor were also excluded because the numerous secondary factors and minor histocompatibility antigens present were significantly different to those in full-sibling matches. A total of 341 patients who underwent allogeneic RICT from related donors for haematological malignancies between 1998 and 2004 were evaluated in this study.

Transplantation procedure

Serologic typing for HLA-A, -B and -DR antigens of the donor and recipient was performed with a standard two-stage complement-dependent test of microcytotoxicity. Serologically HLA-matched sibling pairs were considered to be genotypically HLA-identical based on the results of family analysis. In pairs other than serologically identical sibling pairs, alleles at the HLA-A, -B and -DRB1 loci were identified by middle-resolution DNA typing as described previously (Sasazuki *et al*, 1998). HLA-mismatch in the graft-*versus*-host (GVH) vector was defined when the recipient's antigens or alleles were not shared by the donor, while mismatch in the host *versus* donor (HVG) vector was defined as when the donor's antigens or alleles were not shared by the recipients. The conditioning regimen and GVHD prophylaxis were conducted according to the guidelines of each institution. RIC regimens were defined as reported previously (Bacigalupo, 2002, 2004; Champlin *et al*, 2000). The most frequently used RIC regimens were fludarabine-based (fludarabine 150–180 mg/m^2 with either cyclophosphamide 60 mg/kg, busulphan 8 mg/kg or melphalan 80–140 mg/m^2) with or without either total body irradiation (TBI) 2–4 Gy or antithymocyte globulin (ATG) 5–10 mg/kg. Patients conditioned with >6 Gy TBI and those conditioned with >8 mg/kg of busulphan were excluded from the study. The most frequently used prophylaxis regimens for GVHD were ciclosporin (CSP) alone or CSP plus methotrexate (MTX).

Definitions

Risk status at transplantation was categorised as either standard risk or high risk. Standard-risk diseases included acute leukaemia in first complete remission, chronic myeloid leukaemia in first chronic phase and refractory anaemia of myelodysplastic syndrome. Other diseases were categorised as high-risk disease. Graft failure was analysed in patients who survived more than 28 d post-transplant according to the criteria reported previously (Petersdorf *et al*, 2001); graft failure was defined as failure of the absolute neutrophil count (ANC) to surpass $0{\cdot}5 \times 10^9$/l before relapse, death or second

transplantation, as well as a decrease in the ANC to $<0{\cdot}1 \times 10^9$/l on at least three consecutive determinations with a finding of severe hypoplastic marrow. The aGVHD, graded according to the standard criteria (Przepiorka *et al*, 1995), was defined as moderate to severe (grade II–IV) disease. All patients who had no evidence of graft failure were considered to be evaluable for aGVHD. GVHD persisting beyond day +100 or *de novo* GVHD occurring after day +100 was classified as cGVHD. Biopsy-proven cGVHD occurring between days 80 and 100 was also included. The incidence of cGVHD was calculated in patients followed for at least 100 d and was classified as none, limited or extensive as well as none, *de novo*, quiescent or progressive (Sullivan *et al*, 1991). Overall survival (OS) was defined as the duration of survival between transplant and either death or the last follow-up.

Statistical analysis

The primary endpoint of this study was the incidence of grade II–IV aGVHD and graft failure. The secondary endpoint was the incidence of cGVHD and OS among the patients. The cumulative incidence of aGVHD was calculated using a method described by Gooley *et al* (1999) to eliminate the effect of competing risks. The competing event for aGVHD was defined as death without aGVHD II–IV. For each endpoint, a Cox proportional hazard model was used for uni- and multivariate analyses. The factors included in the analysis were HLA disparity (one-locus mismatch, two- to three-loci mismatch *versus* identical), type of graft (bone marrow *versus* PBSC), previous history of SCT (yes *versus* no), type of donor (family *versus* sibling), recipient age (age 60 years or more *versus* less than 60 years), use of TBI (yes *versus* no), use of ATG (yes *versus* no), GVHD prophylaxis (CSP with MTX, tacrolimus with MTX, and others *versus* CSP alone), and risk status (standard *versus* high). To evaluate the association between CD34 cell counts and the development of aGVHD, subjects were categorised into three groups by tertile and linearity was assessed by score test in a proportional hazard model. We defined statistical significance as a P-value $<0{\cdot}05$. All the statistical analyses were performed using STATA version 8 (STATA Corp., College Station, TX, USA).

Results

Patient characteristics

The numbers of patients who received a graft from an HLA-matched, one-locus-mismatched and two- to three-loci-mismatched donor were 250, 57 and 34 respectively (Table I). The respective median age of these patients were 54, 50·5 and 46·5 years. Among 341 patients, 286 received a graft from a sibling donor and 55 received a graft from a family member other than a sibling. Family donors included 22 sons, 17 daughters, three fathers, 10 mothers, one uncle and two unknown. A total of 110 patients had malignant lymphoma, 106 had acute leukaemia, 74 had mylodysplastic syndrome, 30 multiple myeloma and 21 had chronic myeloid leukaemia. A total of 323 patients received PBSC, whereas the remaining 18 were given bone marrow. The HLA-matched group included significantly higher proportions of patients who did not receive ATG ($P < 0{\cdot}001$) and those who were given CSP alone for GVHD prophylaxis ($P < 0{\cdot}001$) compared with the HLA-mismatched group. Gender, disease, risk status at transplant, previous history of SCT, stem cell source, use of a TBI-containing conditioning regimen and year of transplant were evenly distributed between the groups (Table I).

Acute GVHD

The cumulative incidence of grade II–IV aGVHD in this study population was 40% (95% CI, 35–46%) (Fig 1A). It was 39% (95% CI, 33–45%) in recipients with HLA-matched donors, 44% (95% CI, 30–57%) in those with one-locus-mismatched donors, and 50% (95% CI, 29–68%) in those with two- to three-loci-mismatched donors (Fig 1B); there was a marginally significant difference between two- to three-loci-mismatched RICT and HLA-matched RICT [hazard ratio (HR), 1·72; 95% CI, 0·94–3·14; $P = 0{\cdot}079$]. Similar results were obtained when the incidence of grade III–IV severe aGVHD was analysed (data not shown). A relationship between multiple incompatibility for HLA and a risk of aGVHD was further supported by a Cox regression model adjusted for potential confounders (Table II). Patients who received a graft from a one-locus-mismatched donor and a two- to three-loci-mismatched donor had a HR for aGVHD of 1·83 (95% CI, 1·04–3·22; $P = 0{\cdot}035$) and 2·44 (95% CI, $1{\cdot}14–5{\cdot}21_3$; $P = 0{\cdot}021$), respectively, when compared with those from an HLA-matched donor. A greater incidence of grade II–IV aGVHD was observed with increased HLA disparity ($P = 0{\cdot}010$). Thus, the number of mismatched HLA loci between the donor and recipient was thus a continuous variable with respect to the incidence of grade II–IV aGVHD. No other variables significantly influenced the development of aGVHD after RICT. In patients receiving PBSC grafts, there was no association between the numbers of $CD34^+$ cells and the development of aGVHD ($P = 0{\cdot}904$).

Of note, the development of aGVHD did not reach a plateau within 3 months after RICT, with a median onset on day 30 (Fig 1). The onset of aGVHD was earlier after two- to three-loci-mismatched RICT compared with HLA-matched RICT, and reached a plateau within 40 d post-transplant. The median number of days before the onset of aGVHD after HLA-matched RICT, one-locus-mismatched RICT and two-to three-loci-mismatched RICT was 39, 18 and 24 respectively.

Human leucocyte antigen-C typing is not routinely performed in haemopoietic stem cell transplantation (HSCT) from a related donor in Japan. In this study, HLA-C typing data were available in 75 donor–recipient pairs. Acute GVHD developed in 32% (95% CI, 21–44%) of patients who received a graft from an HLA-C matched donor, and in 56% (95% CI,

Table I. Characteristics of subjects according to human leucocyte antigen-matching status.

	Identical ($n = 250$)	One-mismatched ($n = 57$)	Two or more mismatched ($n = 34$)	*P*-value
Recipient age (range, median)	16–70, 54	25–61, 50·5	21–60, 46·5	0·03
Recipient sex, female:male (unknown)	109:141	43:14	20:14	0·007
Previous history of HCT, no:yes	205:45	27:9	36:19	0·64
Disease				
Acute leukaemia	73	17	16	
Chronic myeloid leukaemia	16	3	2	
Myelodysplastic syndrome	48	18	8	
Malignant lymphoma	90	15	5	
Multiple myeloma	23	4	3	0·163
Risk status				
Standard	52	8	3	
High	198	49	31	0·154
HCT type				
PBSC	236	55	32	
BM	14	2	2	0·805
Donor				
Sibling	250	28	8	
Family	0	29	26	
Year of transplant				
<2000	5	1	1	
2000+	245	56	33	0·922
TBI				
No	213	44	25	
Yes	37	13	9	0·117
ATG				
No	236	36	20	
Yes	14	21	14	<0·001
GVHD prophylaxis				
CSP alone	99	8	10	
CSP + MTX	122	30	10	
FK + MTX	5	13	12	
Other	24	6	2	<0·001

HCT, haematopoietic cell transplantation; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; ATG, antithymocyte globulin; CSP, ciclosporin; MTX, methotrexate; FK, tacrolimus.

21–86%) of those who received a graft from an HLA-C mismatched donor. Although this difference was not statistically significant ($P = 0·261$), the impact of HLA-C mismatch on the incidence of aGVHD remains to be elucidated because of limited numbers of subjects for evaluation.

CGVHD

Recipients of HLA-matched RICT ($n = 156$) and 42 recipients of HLA-mismatched RICT (one-locus mismatch, 32; two- to three-loci mismatch, 10) were evaluable for cGVHD. The cumulative incidence of cGVHD was 61% (95% CI, 52–67%), 69% (95% CI, 44–77%) and 67% (95% CI, 28–88%) after HLA-matched RICT, HLA one-locus-mismatched RICT and two- to three-loci-mismatched RICT respectively (Fig 2). The cumulative incidence of extensive cGVHD was 38%, 34% and 60% after HLA-matched RICT, HLA one-locus and two- to three-loci-mismatched RICT respectively. There was no significant difference between the three groups regarding the incidence of cGVHD or extensive cGVHD. Similarly, there was no significant difference between the groups in the incidence of *de novo*, quiescent and progressive cGVHD. As shown in Table III, a tendency for cGVHD was observed with increased HLA disparity in multivariate analysis ($P = 0·05$). In addition, use of PBSC grafts, no use of ATG, and high-risk disease were identified as independent risk factors for cGVHD. In patients receiving PBSC grafts, there was no association between the numbers of $CD34^+$ cells and the development of cGVHD ($P = 0·613$; HR, 0·95; 95% CI, 0·76–118).

Graft failure

The incidence of graft failure was 3·7% (95% CI, 1·7–6·9%) in recipients with an HLA-matched donor, 5·7% (95% CI,

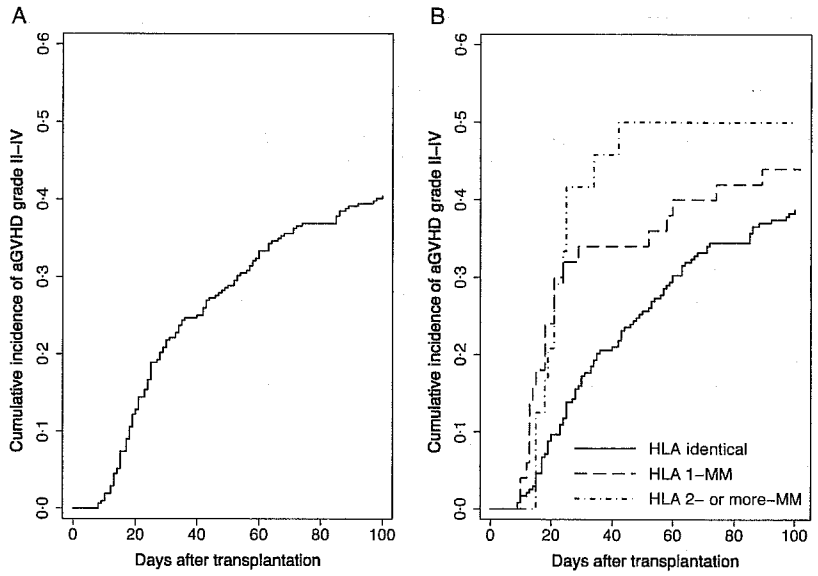

Fig 1. Incidence of grade II–IV acute graft-*versus*-host disease (aGVHD). The curves represent the cumulative incidence of grade II–IV aGVHD in patients with haematological malignancies following reduced-intensity conditioning transplant regimen from a related donor as a function of time after transplantation (A) for all available subjects ($n = 312$) and (B) in relation to the extent of human leucocyte antigen mismatch (identical, $n = 238$; one-locus mismatch, $n = 22$ and two to three-loci mismatch, $n = 7$).

1·2–15·7%) in those with a one-locus-mismatched donor, and 10·3% (95% CI, 2·2–27·4%) in those with a two- to three-loci-mismatched donor. Multivariate analysis revealed a significant increase of graft failure in patients who received a graft from a two- to three-loci-mismatched donor (HR, 8·58; 95% CI, 1·37–53·9; $P = 0·022$, Table IV), and the extent of HLA mismatch between the donor and recipient was a continuous variable with respect to the incidence of graft failure ($P = 0·033$). Use of ATG did not significantly influence the incidence of rejection after RICT ($P = 0·166$).

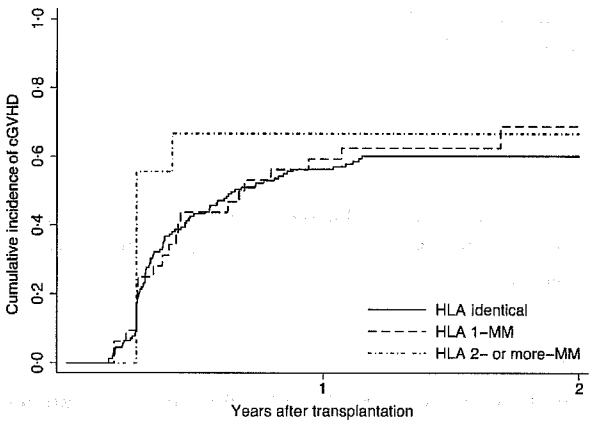

Fig 2. Incidence of chronic graft-*versus*-host disease (cGVHD). The curves represent the cumulative incidence of cGVHD in patients with haematological malignancies following reduced-intensity conditioning transplant regimen from a related donor as a function of time after transplantation in relation to the extent of human leucocyte antigen mismatch (identical, $n = 156$; one-locus mismatch, $n = 32$ and two-to three-loci mismatch, $n = 10$).

Survival

To elucidate the impact of HLA mismatch on transplant outcome, OS was analysed. With a median follow-up of 347 d, OS in patients who received a graft from an HLA-matched donor, a one-locus-mismatched donor and a two-to three-loci-mismatched donor was 48% (95% CI, 42–54%), 51% (95% CI, 39–61%) and 18% (95% CI, 7–32%), respectively, at 2 years after RICT. OS after HLA one-locus-mismatched RICT was comparable with that after HLA-matched RICT (Fig 3). However, OS after two- to three-loci-mismatched RICT was significantly worse post-transplant compared with that after HLA-matched RICT. Multivariate analysis identified two- to three-loci HLA mismatch (HR, 3·41; 95% CI, 2·03–5·73; $P < 0·001$), previous history of haematopoietic cell transplantation (HR, 1·42; 95% CI, 1·00–2·02; $P = 0·052$), and high-risk disease (HR, 2·06; 95% CI, 1·33–3·30; $P = 0·002$) as independent risk factors for shorter survival (Table V). As only three patients with standard-risk disease received RICT from a two- to three-loci-mismatched donor, the relationship between multiple HLA incompatibility and OS was further evaluated in patients with high-risk diseases and those with standard diseases separately. In high-risk patients, the 2-year OS was 15% (95% CI, 5–30%), 52% (95% CI, 40–63%) and 43% (95% CI, 36–49%) in recipients with a two- to three-loci-mismatched donor, one-locus-mismatched donor and matched donor respectively. HLA

Table II. Uni- and multivariate analyses for possible risk factors for acute GVHD of grade II or more.

	Evaluable (n = 313)					
	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
HLA						
Identical	1·00			1·00		
1-MM	1·29	0·81–2·05	0·282	1·83	1·04–3·22	0·035
2 or more MM	1·72	0·94–3·14	0·079	2·44	1·14–5·21	0·021
		Trend	0·068		Trend	0·010
HCT type						
PBSC	1·00			1·00		
BM	0·65	0·26–1·59	0·342	0·70	0·28–1·73	0·441
Previous history of HCT						
No	1·00			1·00		
Yes	1·15	0·75–1·77	0·52	0·90	0·57–1·44	0·663
Recipient age (years)						
<60	1·00			1·00		
≥60	0·90	0·57–1·42	0·656	0·94	0·59–1·50	0·808
Recipient sex						
Female	1·00			1·00		
Male	1·16	0·82–1·66	0·401	1·28	0·88–1·84	0·196
TBI						
No	1·00			1·00		
Yes	0·96	0·60–1·55	0·881	0·77	0·43–1·38	0·386
ATG						
No	1·00			1·00		
Yes	0·85	0·50–1·44	0·543	0·55	0·29–1·02	0·057
GVHD prophylaxis						
CSP alone	1·00			1·00		
CSP + MTX	0·74	0·50–1·09	0·124	0·70	0·46–1·04	0·079
FK + MTX	1·07	0·57–2·02	0·826	0·70	0·32–1·50	0·355
Other	1·12	0·61–2·06	0·724	1·37	0·65–2·90	0·410
Risk						
Standard	1·00			1·00		
High	1·52	0·93–2·47	0·095	1·43	0·87–2·36	0·159

GVHD, graft-*versus*-host disease; HLA, human leucocyte antigen; MM, mismatched; HCT, haematopoietic cell transplantation; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; ATG, antithymocyte globulin; CSP, ciclosporin; MTX, methotrexate; FK, tacrolimus.
Multivariable adjusted for all variables listed.

two- to three-loci mismatch, but not one-locus mismatch, was again a risk factor for shorter survival ($P < 0·0001$, Fig 4A). In standard-risk patients, the 2-year OS was 73% (95% CI, 58–84%), 40% (95% CI, 12–67%) and 38% (95% CI, 1–81%) in recipients with a matched, one-locus-mismatched donor and two- to three-loci-mismatched donor respectively (Fig 4B). In contrast to high-risk disease, one-locus mismatch was a risk factor for shorter survival in patients with standard-risk disease ($P = 0·079$).

Of the 178 patients who died following RICT, 90 deaths were directly attributed to disease progression or relapse. Non-relapse mortality was 49%, including 60 deaths (34%) because of infection and/or GVHD, 22 deaths (12%) from other transplant-related toxicities and six deaths (3%) from other diseases. There was no difference in the cause of death between HLA-matched RICT and HLA-mismatched RICT (data not shown).

Discussion

In this study, we found that HLA disparity was a continuous and independent risk factor for grade II–IV aGVHD and cGVHD as well as graft failure following RICT. Furthermore, two- to three-loci HLA mismatch was a risk factor for survival, and one-locus mismatch was a risk factor for survival in patients with standard-risk disease, but not in those with high-risk disease. Our finding, that HLA disparity impacts on grade II–IV aGVHD in patients receiving RICT, was quite consistent

Table III. Uni- and multivariate analyses for possible risk factors for chronic GVHD.

	Evaluable (n = 198)					
	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
HLA						
Identical	1·00			1·00		
1-MM	1·09	0·68–1·76	0·709	1·57	0·89–2·75	0·116
2 or more MM	1·25	0·55–2·85	0·6	2·20	0·84–5·75	0·108
		Trend	0·543		Trend	0·050
HCT type						
PBSC	1·00			1·00		
BM	0·31	0·10–0·97	0·044	0·28	0·88–0·90	0·032
Previous history of HCT						
No	1·00			1·00		
Yes	0·90	0·56–1·42	0·641	0·75	0·45–1·26	0·274
Recipient age (years)						
<60	1·00			1·00		
≥60	1·31	0·86–1·99	0·214	1·16	0·74–1·81	0·512
Recipient sex						
Female	1·00			1·00		
Male	1·18	0·82–1·69	0·375	1·27	0·87–1·87	0·221
TBI						
No	1·00			1·00		
Yes	0·76	0·46–1·24	0·265	0·61	0·33–1·11	0·104
ATG						
No	1·00			1·00		
Yes	0·66	0·36–1·23	0·194	0·42	0·20–0·85	0·016
GVHD prophylaxis						
CSP alone	1·00			1·00		
CSP + MTX	1·10	0·72–1·68	0·67	1·00	0·63–1·56	0·966
FK + MTX	0·76	0·37–1·55	0·444	0·53	0·23–1·24	0·143
Other	0·98	0·51–1·88	0·961	1·36	0·63–2·96	0·435
Risk						
Standard	1·00			1·00		
High	1·67	1·06–2·64	0·027	1·74	1·09–2·79	0·021

GVHD, graft-*versus*-host disease; HLA, human leucocyte antigen; MM, mismatch; HCT, haematopoietic cell transplantation; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; ATG, antithymocyte globulin; CSP, ciclosporin; MTX, methotrexate; FK, tacrolimus.
Multivariable adjusted for all variables listed.

Table IV. Uni- and multivariate analyses for host-*versus*-graft human leucocyte antigen mismatching on graft failure risk.

	Evaluable (n = 325)							
			Univariate			Multivariate		
	Rejected	Not-rejected	HR	95% CI	P-value	HR	95% CI	P-value
Identical	9	234	1·00	Reference		1·00	Reference	
1-MM	3	50	1·6	0·42–6·12	0·337	1·18	0·19–7·26	0·855
2 or more MM	3	26	3·01	0·77–11·8	0·114	8·58	1·37–53·9	0·022
				Trend	0·035		Trend	0·033

HR, hazard ratio; MM, mismatch.
Multivariate analysis adjusted for age, sex, donor type, previous history of haematopoietic cell transplantation, total body irradiation conditioning, antithymocyte globulin conditioning, graft-*versus*-host disease prophylaxis and risk status.

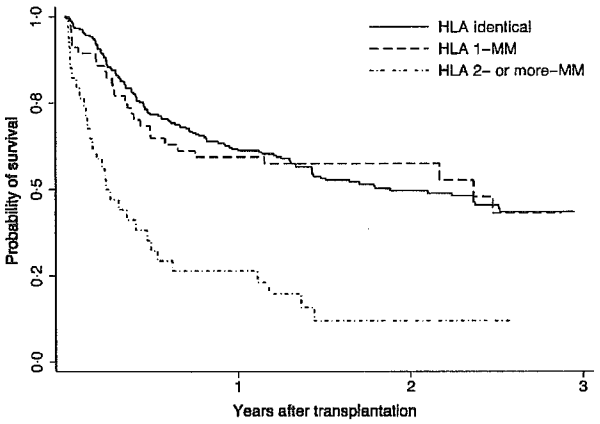

Fig 3. Overall survival (OS) based on the extent of human leucocyte antigen (HLA) mismatch. The curves represent OS in patients with haematological malignancies following reduced-intensity conditioning transplant regimen from a related donor as a function of time after transplantation in relation to the extent of HLA mismatch (identical, $n = 250$; one-locus mismatch, $n = 56$ and two to three-loci mismatch, $n = 34$).

with earlier findings in conventional myeloablative SCT (Beatty *et al*, 1985; Ringden & Nilsson, 1985; Anasetti *et al*, 1990; Anasetti & Hansen, 1994; Sasazuki *et al*, 1998; Morishima *et al*, 2002; Kanda *et al*, 2003), but has not been well described in the setting of RICT.

The cumulative incidence of grade II–IV aGVHD after HLA-matched RICT was 39% in this study population, which is similar to that in recent reports from other groups following RICT (Levine *et al*, 2003; Martino *et al*, 2003; Wong *et al*, 2003; Bacigalupo, 2004; Diaconescu *et al*, 2004; Goggins & Rizzieri, 2004), although a recent retrospective comparison of myeloablative SCT with RICT conditioned with 2 Gy TBI and fludarabine showed less aGVHD after RICT in matched unrelated donor transplants (Sorror *et al*, 2004).

It was initially assumed that the incidence and severity of aGVHD might decrease after RICT compared with conventional SCT. Pretransplant conditioning can activate host tissues to secrete inflammatory cytokines and amplify GVHD (Xun *et al*, 1994). The relationship between conditioning intensity, inflammatory cytokine and GVHD severity was further supported by animal models (Hill *et al*, 1997) and clinical observation (Gale *et al*, 1987; Clift *et al*, 1990; Deeg *et al*, 1991). In experimental models, the development of mixed donor–host chimaerism may facilitate the establishment of anti-host tolerance (Colson *et al*, 1996; Manilay *et al*, 1998). In contrast, minimally cytotoxic conditioning may enable the persistence of host antigen-presenting cells, which would enhance presentation of host alloantigens to donor T cells (Shlomchik *et al*, 1999; Teshima *et al*, 2002; Duffner *et al*, 2004).

There are several possible explanations that can account for this unexpectedly high incidence of aGVHD after RICT. First, the median age of patients who underwent RICT in the current study was 53 years, which was much higher than that among patients who received conventional SCT. A greater age has been associated with an increased risk for GVHD after conventional SCT (Ringden & Nilsson, 1985; Gale *et al*, 1987; Anasetti *et al*, 1990; Weisdorf *et al*, 1991; Nash *et al*, 1992). Secondly, CSP alone was administered for GVHD prophylaxis in one-third of the patients in this study, whereas a combination of two agents was exclusively used in conventional SCT. Thirdly, the RIC regimens used in our study were more intensive than the non-myeloablative conditioning regimens used by the Seattle group (Sorror *et al*, 2004). These differences may counterbalance the potential beneficial aspects of reducing the intensity of conditioning. Nonetheless, the onset of GVHD was delayed in RICT; 15% of aGVHD developed between days 60 and 100 in our study, as previously reported (Mielcarek *et al*, 2003). Following conventional SCT, most of aGVHD develops within 50 d post-transplant (Snover, 1984; Beatty *et al*, 1985; Sasazuki *et al*, 1998; Morishima *et al*, 2002; Kanda *et al*, 2003).

The incidence of aGVHD rose with increasing HLA mismatch, from match through multi-loci mismatch, following RICT. In addition, the onset of aGVHD was earlier with increasing HLA mismatch. These findings are consistent with data from the myeloablative setting (Beatty *et al*, 1985; Ringden & Nilsson, 1985; Anasetti *et al*, 1990; Anasetti & Hansen, 1994; Petersdorf *et al*, 1998; Sasazuki *et al*, 1998; Morishima *et al*, 2002; Kanda *et al*, 2003). Studies from the Japan Marrow Donor Programme and others have demonstrated the importance of HLA-C mismatching in rejection, GVHD and mortality in myeloablative SCT from unrelated donors (Petersdorf *et al*, 2001, 2004; Morishima *et al*, 2002; Flomenberg *et al*, 2004; Sasazuki *et al*, 1998). We did not find a significant association between HLA-C mismatch and the development of aGVHD, but this association needs to be further investigated in a larger prospective study because only 75 donor–recipient pairs were available for analysis in this study.

We found that the use of ATG was associated with a reduction in both acute and chronic GVHD without an increased risk of graft failure, as previously shown in studies using alemtuzumab or ATG (Kottaridis *et al*, 2000; Khouri *et al*, 2001; Mohty *et al*, 2003; Nakai *et al*, 2003; Faulkner *et al*, 2004). Initial clinical trials of matched unrelated RICT or haploidentical RICT appear to be encouraging with the use of T-cell depletion (Sykes *et al*, 1999; Kottaridis *et al*, 2000; Giralt *et al*, 2001; Nagler *et al*, 2001; Chakraverty *et al*, 2002; Maris *et al*, 2003; Niederwieser *et al*, 2003; Wong *et al*, 2003; Goggins & Rizzieri, 2004). Mohty *et al* (2003) reported that a high $CD34^+$ cell dose was associated with an increased incidence of chronic, but not acute, GVHD following RICT with G-CSF-mobilised PBSC. We did not find an association between acute or chronic GVHD and the number of $CD34^+$ cells infused. Interestingly, in contrast to data from a myeloablative setting (Ringden & Nilsson, 1985; Gale *et al*, 1987; Anasetti *et al*, 1990; Weisdorf *et al*, 1991; Nash *et al*, 1992), there was no

Table V. Uni- and multivariate analyses for possible risk factors for overall survival.

	Evaluable (n = 286)					
	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
HLA						
Identical	1·00			1·00		
1-MM	1·01	0·67–1·53	0·966	1·03	0·64–1·66	0·899
2 or more MM	3·21	2·14–4·82	<0·001	3·41	2·03–5·73	<0·001
		Trend	<0·001		Trend	<0·001
HCT type						
PBSCT	1·00			1·00		
BMT	1·19	0·63–2·25	0·598	1·53	0·79–2·94	0·205
Previous history of HCT						
No	1·00			1·00		
Yes	1·64	1·18–2·28	0·003	1·42	1·00–2·02	0·052
Recipient age (years)						
<60	1·00			1·00		
≥60	1·05	0·73–1·51	0·808	1·09	0·75–1·60	0·65
Recipient sex						
Female	1·00			1·00		
Male	1·29	0·96–1·74	0·095	1·19	0·88–1·61	0·264
TBI						
No	1·00			1·00		
Yes	1·25	0·86–1·82	0·239	1·28	0·84–1·94	0·25
ATG						
No	1·00			1·00		
Yes	1·15	0·77–1·72	0·482	0·71	0·44–1·16	0·177
GVHD prophylaxis						
CSP alone	1·00			1·00		
CSP + MTX	0·92	0·66–1·27	0·614	0·89	0·64–1·24	0·488
FK + MTX	1·17	0·70–1·98	0·546	0·87	0·48–1·57	0·64
Other	0·89	0·52–1·51	0·66	0·69	0·38–1·25	0·223
Risk						
Standard	1·00			1·00		
High	2·21	1·42–3·43	<0·001	2·06	1·31–3·25	0·002

HLA, human leucocyte antigen; MM, mis-match; HCT, haematopoietic cell transplantation; PBSC, peripheral blood stem cells; BM, bone marrow; TBI, total body irradiation; ATG, anti-thymocyte globulin; CSP, ciclosporin; MTX, methotrexate; FK, tacrolimus.
Multivariable adjusted for all variables listed.

increase in the incidence of aGVHD in elderly patients, as previously reported (Mohty *et al*, 2002; Wong *et al*, 2003). It has been shown in experimental models that donor T-cell responses are enhanced under stimulation with antigen-presenting cells from older mice in the context of proinflammatory milieu (Ordemann *et al*, 2002). The absence of excessive inflammation in RICT may be associated with a similar incidence of GVHD in aged recipients following RICT.

The incidence of cGVHD was similar to that following myeloablative HSCT in Japan (Kanda *et al*, 2003). A greater incidence of cGVHD was observed with increased HLA disparity, although the difference was marginal. Chronic GVHD was influenced by the use of ATG, disease status and the stem cell source, with PBSC grafts having a fourfold risk of cGVHD. It has been shown that the use of PBSC grafts instead of marrow increases the frequency of cGVHD following myeloablative HSCT (Blaise *et al*, 2000; Bensinger *et al*, 2001; Cutler *et al*, 2001). Our study suggest that the use of PBSC grafts is also a risk factor for cGVHD following RICT, although there was a large difference between the number of recipients of a PBSC graft and those who received a bone marrow (BM) graft.

In a conventional allogeneic transplant setting, the incidence of graft failure has been shown to be correlated with the degree of HLA mismatch between donors and recipients (Beatty *et al*, 1985; Anasetti *et al*, 1989; Petersdorf *et al*, 1998, 2001; Petersdorf *et al*, 1997). We found that HLA mismatch was also a risk factor for graft failure following RICT. The incidence of graft failure after HLA-matched RICT was comparable with that after conventional SCT in Japan

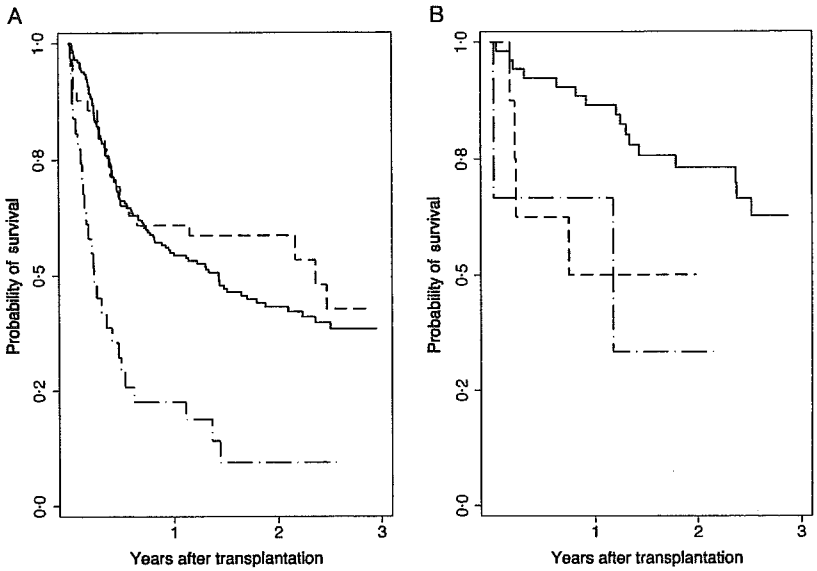

Fig 4. Overall survival (OS) according to the extent of human leucocyte antigen (HLA) mismatch and disease status. The curves represent OS as a function of time after transplantation in relation to the extent of HLA mismatch in (A) patients with a high-risk disease (total, $n = 277$; identical, $n = 198$; one-locus mismatch, $n = 48$ and two to three-loci mismatch, $n = 31$); (B) those with standard-risk disease (total, $n = 63$; identical, $n = 52$; one-locus mismatch, $n = 3$ and two- to three-loci mismatch, $n = 8$).

(Morishima *et al*, 2002; Kanda *et al*, 2003). Thus, the RIC regimens used in this study appear to have been sufficient for achieving donor cell engraftment in these patients. However, we found that the risk of rejection was extremely high (10·8%) in patients who received a graft from a two- to three-loci-mismatched donor in the HVG vector and myeloablative conditioning should be considered in this setting. Previous reports have demonstrated that the incorporation of low-dose TBI in the conditioning or the use of PBSC could reduce the incidence of graft failure (Deeg *et al*, 2001; Maris *et al*, 2003). These associations were not observed in our study probably because rate of graft failure was too low to detect a significant decrease.

The most important factor that affected OS after RICT was multiple HLA mismatch in patients with haematological malignancies. RICT from a two- to three-loci-mismatched donor resulted in a poor outcome, as has been shown in conventional SCT (Beatty *et al*, 1985; Hows *et al*, 1993; Szydlo *et al*, 1997). However, the 2-year OS after one-locus-mismatched RICT was comparable with that after HLA-matched RICT. When stratified according to disease status, one-locus mismatch was a risk factor for survival in patients with standard-risk disease, but not in those with high-risk disease. These results suggest that RICT from a one-locus-mismatched related donor may be warranted in patients with high-risk haematological malignancies when an HLA-matched sibling donor is not available. In previous studies, a younger age of patients (Faulkner *et al*, 2004) and the use of PBSC (Maris *et al*, 2003) were associated with a superior outcome after RICT, but these factors did not influence OS in the present study. However, the present study has several limitations. First, there was a large difference between the number of recipients with an HLA-matched donor and those with an HLA-mismatched donor. Secondly, since the follow-up period was short following RICT, it is too early to determine long-term outcome to these treatment regimens. Thirdly, HLA-C typing and high resolution DNA typing was not routinely performed in HSCT from a related donor.

Nonetheless, the large cohort of patients in the current study allowed us to make several important observations. First, HLA mismatch between the donor and recipient is an important risk factor for graft failure, aGVHD, cGVHD and OS after RICT. Secondly, RICT from a one-locus-mismatched related donor may represent an alternative approach in patients with high-risk haematological malignancies who lacked an HLA-matched sibling donor. These findings should be carefully confirmed in a prospective study.

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References

Anasetti, C. & Hansen, J.A. (1994) Effect of HLA incompatibility in marrow transplantation from unrelated and HLA-mismatched related donors. *Transfusion Science*, **15**, 221–230.

Anasetti, C., Amos, D., Beatty, P.G., Appelbaum, F.R., Bensinger, W., Buckner, C.D., Clift, R., Doney, K., Martin, P.J., Mickelson, E., Nisperos, B., O'quigley, J., Ramberg, R., Sanders, J.E., Stewart, P., Storb, R., Sullivan, K.M., Witherspoon, R.P., Thomas, E.D. & Hansen, J.A. (1989) Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *New England Journal of Medicine*, **320**, 197–204.

Anasetti, C., Beatty, P.G., Storb, R., Martin, P.J., Mori, M., Sanders, J.E., Thomas, E.D. & Hansen, J.A. (1990) Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. *Human Immunology*, **29**, 79–91.

Bacigalupo, A. (2002) Second EBMT workshop on reduced intensity allogeneic hemopoietic stem cell transplants (RI-HSCT). *Bone Marrow Transplantation*, **29**, 191–195.

Bacigalupo, A. (2004) Third EBMT/AMGEN workshop on reduced-intensity conditioning allogeneic haemopoietic stem cell transplants (RIC-HSCT), and panel consensus. *Bone Marrow Transplantation*, **33**, 691–696.

Beatty, P.G., Clift, R.A., Mickelson, E.M., Nisperos, B.B., Flournoy, N., Martin, P.J., Sanders, J.E., Stewart, P., Buckner, C.D., Storb, R., Thomas, E.D. & Hansen, J.A. (1985) Marrow transplantation from related donors other than HLA-identical siblings. *New England Journal of Medicine*, **313**, 765–771.

Bensinger, W.I., Martin, P.J., Storer, B., Clift, R., Forman, S.J., Negrin, R., Kashyap, A., Flowers, M.E., Lilleby, K., Chauncey, T.R., Storb, R. & Appelbaum, F.R. (2001) Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *New England Journal of Medicine*, **344**, 175–181.

Blaise, D., Kuentz, M., Fortanier, C., Bourhis, J.H., Milpied, N., Sutton, L., Jouet, J.P., Attal, M., Bordigoni, P., Cahn, J.Y., Boiron, J.M., Schuller, M.P., Moatti, J.P. & Michallet, M. (2000) Randomized trial of bone marrow versus lenograstim-primed blood cell allogeneic transplantation in patients with early-stage leukemia: a report from the Societe Francaise de Greffe de Moelle. *Journal of Clinical Oncology*, **18**, 537–546.

Burt, R.K., Arnold, R., Emmons, R., Oyama, Y. & Marmont, A. (2003) Stem cell therapy for autoimmune disease: overview of concepts from the Snowbird 2002 tolerance and tissue regeneration meeting. *Bone Marrow Transplantation*, **32**(Suppl. 1), S3–S5.

Chakraverty, R., Peggs, K., Chopra, R., Milligan, D.W., Kottaridis, P.D., Verfuerth, S., Geary, J., Thuraisundaram, D., Branson, K., Chakrabarti, S., Mahendra, P., Craddock, C., Parker, A., Hunter, A., Hale, G., Waldmann, H., Williams, C.D., Yong, K., Linch, D.C., Goldstone, A.H. & Mackinnon, S. (2002) Limiting transplantation-related mortality following unrelated donor stem cell transplantation by using a nonmyeloablative conditioning regimen. *Blood*, **99**, 1071–1078.

Champlin, R., Khouri, I., Shimoni, A., Gajewski, J., Kornblau, S., Molldrem, J., Ueno, N., Giralt, S. & Anderlini, P. (2000) Harnessing graft-versus-malignancy: non-myeloablative preparative regimens for allogeneic haematopoietic transplantation, an evolving strategy for adoptive immunotherapy. *British Journal of Haematology*, **111**, 18–29.

Clift, R.A., Buckner, C.D., Appelbaum, F.R., Bearman, S.I., Petersen, F.B., Fisher, L.B., Anasetti, C., Beatty, P., Bensigner, W.I., Doney, K., Hill, R.S., McDonald, G.B., Martin, P., Sanders, J., Singer, J., Stewart, P., Sullivan, K.M., Witherspoon, R., Storb, R., Hansen, J.A. & Thomas, E.D. (1990) Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. *Blood*, **76**, 1867–1871.

Colson, Y.L., Lange, J., Fowler, K. & Ildstad, S.T. (1996) Mechanism for cotolerance in nonlethally conditioned mixed chimeras: negative selection of the Vbeta T-cell receptor repertoire by both host and donor bone marrow-derived cells. *Blood*, **88**, 4601–4610.

Cutler, C., Giri, S., Jeyapalan, S., Paniagua, D., Viswanathan, A. & Antin, J.H. (2001) Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. *Journal of Clinical Oncology*, **19**, 3685–3691.

Deeg, H.J., Spitzer, T.R., Cottler-Fox, M., Cahill, R. & Pickle, L.W. (1991) Conditioning-related toxicity and acute graft-versus-host disease in patients given methotrexate/cyclosporine prophylaxis. *Bone Marrow Transplantation*, **7**, 193–198.

Deeg, H.J., Amylon, I.D., Harris, R.E., Collins, R., Beatty, P.G., Feig, S., Ramsay, N., Territo, M., Khan, S.P., Pamphilon, D., Leis, J.F., Burdach, S., Anasetti, C., Hackman, R., Storer, B. & Mueller, B. (2001) Marrow transplants from unrelated donors for patients with aplastic anemia: minimum effective dose of total body irradiation. *Biology of Blood and Marrow Transplantation*, **7**, 208–215.

Diaconescu, R., Flowers, C.R., Storer, B., Sorror, M.L., Maris, M.B., Maloney, D.G., Sandmaier, B.M. & Storb, R. (2004) Morbidity and mortality with nonmyeloablative compared to myeloablative conditioning before hematopoietic cell transplantation from HLA matched related donors. *Blood*, **104**, 1550–1558.

Duffner, U.A., Maeda, Y., Cooke, K.R., Reddy, P., Ordemann, R., Liu, C., Ferrara, J.L. & Teshima, T. (2004) Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. *Journal of Immunology*, **172**, 7393–7398.

Faulkner, R.D., Craddock, C., Byrne, J.L., Mahendra, P., Haynes, A.P., Prentice, H.G., Potter, M., Pagliuca, A., Ho, A., Devereux, S., McQuaker, G., Mufti, G., Yin, J.L. & Russell, N.H. (2004) BEAM-alemtuzumab reduced-intensity allogeneic stem cell transplantation for lymphoproliferative diseases: GVHD, toxicity, and survival in 65 patients. *Blood*, **103**, 428–434.

Flomenberg, N., Baxter-Lowe, L.A., Confer, D., Fernandez-Vina, M., Filipovich, A., Horowitz, M., Hurley, C., Kollman, C., Anasetti, C., Noreen, H., Begovich, A., Hildebrand, W., Petersdorf, E., Schmeckpeper, B., Setterholm, M., Trachtenberg, E., Williams, T., Yunis, E. & Weisdorf, D. (2004) Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood*, **104**, 1923–1930.

Gale, R.P., Bortin, M.M., van Bekkum, D.W., Biggs, J.C., Dicke, K.A., Gluckman, E., Good, R.A., Hoffman, R.G., Kay, H.E.M., Kersey, J.H., Marmont, A., Masaoka, T., Rimm, A.A., van Rood, J.J. & Zwaan, F.E. (1987) Risk factors for acute graft-versus-host disease. *British Journal of Haematology*, **67**, 397–406.

Giralt, S., Thall, P.F., Khouri, I., Wang, X., Braunschweig, I., Ippolitti, C., Claxton, D., Donato, M., Bruton, J., Cohen, A., Davis, M., Andersson, B.S., Anderlini, P., Gajewski, J., Kornblau, S., Andreeff, M., Przepiorka, D., Ueno, N.T., Molldrem, J. & Champlin, R. (2001)

Melphalan and purine analog-containing preparative regimens: reduced-intensity conditioning for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. *Blood*, **97**, 631–637.

Goggins, T.F. & Rizzieri, D.R. (2004) Nonmyeloablative allogeneic stem cell transplantation using alternative donors. *Cancer Control*, **11**, 86–96.

Gooley, T.A., Leisenring, W., Crowley, J. & Storer, B.E. (1999) Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Statistics in Medicine*, **18**, 695–706.

Hill, G.R., Crawford, J.M., Cooke, K.J., Brinson, Y.S., Pan, L. & Ferrara, J.L.M. (1997) Total body irradiation and acute graft versus host disease. The role of gastrointestinal damage and inflammatory cytokines. *Blood*, **90**, 3204–3213.

Hows, J., Bradley, B.A., Gore, S., Downie, T., Howard, M. & Gluckman, E. (1993) Prospective evaluation of unrelated donor bone marrow transplantation. The International Marrow Unrelated Search and Transplant (IMUST) Study. *Bone Marrow Transplantation*, **12**, 371–380.

Kanda, Y., Chiba, S., Hirai, H., Sakamaki, H., Iseki, T., Kodera, Y., Karasuno, T., Okamoto, S., Hirabayashi, N., Iwato, K., Maruta, A., Fujimori, Y., Furukawa, T., Mineishi, S., Matsuo, K., Hamajima, N. & Imamura, M. (2003) Allogeneic hematopoietic stem cell transplantation from family members other than HLA-identical siblings over the last decade (1991–2000). *Blood*, **102**, 1541–1547.

Khouri, I.F., Keating, M., Korbling, M., Przepiorka, D., Anderlini, P., O'Brien, S., Giralt, S., Ippoliti, C., von Wolff, B., Gajewski, J., Donato, M., Claxton, D., Ueno, N., Andersson, B., Gee, A. & Champlin, R. (1998) Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *Journal of Clinical Oncology*, **16**, 2817–2824.

Khouri, I.F., Saliba, R.M., Giralt, S.A., Lee, M.S., Okoroji, G.J., Hagemeister, F.B., Korbling, M., Younes, A., Ippoliti, C., Gajewski, J.L., McLaughlin, P., Anderlini, P., Donato, M.L., Cabanillas, F.F. & Champlin, R.E. (2001) Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. *Blood*, **98**, 3595–3599.

Kottaridis, P.D., Milligan, D.W., Chopra, R., Chakraverty, R.K., Chakrabarti, S., Robinson, S., Peggs, K., Verfuerth, S., Pettengell, R., Marsh, J.C., Schey, S., Mahendra, P., Morgan, G.J., Hale, G., Waldmann, H., de Elvira, M.C., Williams, C.D., Devereux, S., Linch, D.C., Goldstone, A.H. & Mackinnon, S. (2000) In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation. *Blood*, **96**, 2419–2425.

Levine, J.E., Uberti, J.P., Ayash, L., Reynolds, C., Ferrara, J.L., Silver, S.M., Braun, T., Yanik, G., Hutchinson, R. & Ratanatharathorn, V. (2003) Lowered-intensity preparative regimen for allogeneic stem cell transplantation delays acute graft-versus-host disease but does not improve outcome for advanced hematologic malignancy. *Biology of Blood and Marrow Transplantation*, **9**, 189–197.

Manilay, J.O., Pearson, D.A., Sergio, J.J., Swenson, K.G. & Sykes, M. (1998) Intrathymic deletion of alloreactive T cells in mixed bone marrow chimeras prepared with a nonmyeloablative conditioning regimen. *Transplantation*, **66**, 96–102.

Maris, M.B., Niederwieser, D., Sandmaier, B.M., Storer, B., Stuart, M., Maloney, D., Petersdorf, E., McSweeney, P., Pulsipher, M., Woolfrey, A., Chauncey, T., Agura, E., Heimfeld, S., Slattery, J., Hegenbart, U., Anasetti, C., Blume, K. & Storb, R. (2003) HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. *Blood*, **102**, 2021–2030.

Martino, R., Giralt, S., Caballero, M.D., Mackinnon, S., Corradini, P., Fernandez-Aviles, F., San Miguel, J. & Sierra, J. (2003) Allogeneic hematopoietic stem cell transplantation with reduced-intensity conditioning in acute lymphoblastic leukemia: a feasibility study. *Haematologica*, **88**, 555–560.

Mielcarek, M., Martin, P.J., Leisenring, W., Flowers, M.E., Maloney, D.G., Sandmaier, B.M., Maris, M.B. & Storb, R. (2003) Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood*, **102**, 756–762.

Mohty, M., Kuentz, M., Michallet, M., Bourhis, J.H., Milpied, N., Sutton, L., Jouet, J.P., Attal, M., Bordigoni, P., Cahn, J.Y., Boiron, J.M. & Blaise, D. (2002) Chronic graft-versus-host disease after allogeneic blood stem cell transplantation: long-term results of a randomized study. *Blood*, **100**, 3128–3134.

Mohty, M., Bay, J.O., Faucher, C., Choufi, B., Bilger, K., Tournilhac, O., Vey, N., Stoppa, A.M., Coso, D., Chabannon, C., Viens, P., Maraninchi, D. & Blaise, D. (2003) Graft-versus-host disease following allogeneic transplantation from HLA-identical sibling with antithymocyte globulin-based reduced-intensity preparative regimen. *Blood*, **102**, 470–476.

Morishima, Y., Sasazuki, T., Inoko, H., Juji, T., Akaza, T., Yamamoto, K., Ishikawa, Y., Kato, S., Sao, H., Sakamaki, H., Kawa, K., Hamajima, N., Asano, S. & Kodera, Y. (2002) The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood*, **99**, 4200–4206.

Nagler, A., Slavin, S., Varadi, G., Naparstek, E., Samuel, S. & Or, R. (2000) Allogeneic peripheral blood stem cell transplantation using a fludarabine-based low intensity conditioning regimen for malignant lymphoma. *Bone Marrow Transplantation*, **25**, 1021–1028.

Nagler, A., Aker, M., Or, R., Naparstek, E., Varadi, G., Brautbar, C. & Slavin, S. (2001) Low-intensity conditioning is sufficient to ensure engraftment in matched unrelated bone marrow transplantation. *Experimental Hematology*, **29**, 362–370.

Nakai, K., Mineishi, S., Kami, M., Saito, T., Hori, A., Kojima, R., Imataki, O., Hamaki, T., Yoshihara, S., Ohnishi, M., Kim, S.W., Ando, T., Fumitoh, A., Kanda, Y., Makimoto, A., Tanosaki, R., Kanai, S., Heike, Y., Ohnishi, T., Kawano, Y., Wakasugi, H. & Takaue, Y. (2003) Antithymocyte globulin affects the occurrence of acute and chronic graft-versus-host disease after a reduced-intensity conditioning regimen by modulating mixed chimerism induction and immune reconstitution. *Transplantation*, **75**, 2135–2143.

Nash, A., Pepe, M.S., Storb, R., Longton, G., Pettinger, M., Anasetti, C., Appelbaum, F.R., Bowden, R.A., Deeg, H.J., Doney, K., Martin, P.J., Sullivan, K.M., Sanders, J. & Witherspoon, R. (1992) Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood*, **80**, 1838–1845.

Niederwieser, D., Maris, M., Shizuru, J.A., Petersdorf, E., Hegenbart, U., Sandmaier, B.M., Maloney, D.G., Storer, B., Lange, T., Chauncey, T., Deininger, M., Ponisch, W., Anasetti, C., Woolfrey, A., Little, M.T., Blume, K.G., McSweeney, P.A. & Storb, R.F. (2003) Low-dose total body irradiation (TBI) and fludarabine

followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. *Blood*, **101**, 1620–1629.

Ordemann, R., Hutchinson, R., Friedman, J., Burakoff, S.J., Reddy, P., Duffner, U., Braun, T.M., Liu, C., Teshima, T. & Ferrara, J.L. (2002) Enhanced allostimulatory activity of host antigen-presenting cells in old mice intensifies acute graft-versus-host disease. *Journal of Clinical Investigation*, **109**, 1249–1256.

Petersdorf, E.W., Longton, G.M., Anasetti, C., Mickelson, E.M. & McKinney, S.K. (1997) Association of HLA-C disparity with great failure after marrow transplantation from unrelated donors. *Blood*, **89**, 1818–1823.

Petersdorf, E.W., Gooley, T.A., Anasetti, C., Martin, P.J., Smith, A.G., Mickelson, E.M., Woolfrey, A.E. & Hansen, J.A. (1998) Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood*, **92**, 3515–3520.

Petersdorf, E.W., Hansen, J.A., Martin, P.J., Woolfrey, A., Malkki, M., Gooley, T., Storer, B., Mickelson, E., Smith, A. & Anasetti, C. (2001) Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. *New England Journal of Medicine*, **345**, 1794–1800.

Petersdorf, E.W., Anasetti, C., Martin, P.J., Gooley, T., Radich, J., Malkki, M., Woolfrey, A., Smith, A., Mickelson, E. & Hansen, J.A. (2004) Limits of HLA mismatching in unrelated hematopoietic cell transplantation. *Blood*, **104**, 2976–2980.

Przepiorka, D., Weisdorf, D., Martin, P., Klingemann, H.G., Beatty, P., Hows, J. & Thomas, E.D. (1995) 1994 Consensus conference on acute GVHD grading. *Bone Marrow Transplantation*, **15**, 825–828.

Ringden, O. & Nilsson, B. (1985) Death by graft-versus-host disease associated with HLA mismatch, high recipient age, low marrow cell dose, and splenectomy. *Transplantation*, **40**, 39–44.

Sasazuki, T., Juji, T., Morishima, Y., Kinukawa, N., Kashiwabara, H., Inoko, H., Yoshida, T., Kimura, A., Akaza, T., Kamikawaji, N., Kodera, Y. & Takaku, F. (1998) Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *New England Journal of Medicine*, **339**, 1177–1185.

Schetelig, J., Kroger, N., Held, T.K., Thiede, C., Krusch, A., Zabelina, T., Dubiel, M., Rick, O., Bornhauser, M., Ehninger, G., Zander, A.R. & Siegert, W. (2002) Allogeneic transplantation after reduced conditioning in high risk patients is complicated by a high incidence of acute and chronic graft-versus-host disease. *Haematologica*, **87**, 299–305.

Shlomchik, W.D., Couzens, M.S., Tang, C.B., McNiff, J., Robert, M.E., Liu, J., Shlomchik, M.J. & Emerson, S.G. (1999) Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science*, **285**, 412–415.

Slavin, S., Nagler, A., Naparstek, E., Kapelushnik, Y., Aker, M., Cividalli, G., Varadi, G., Kirschbaum, M., Ackerstein, A., Samuel, S., Amar, A., Brautbar, C., Ben-Tal, O., Eldor, A. & Or, R. (1998) Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood*, **91**, 756–763.

Slavin, S., Morecki, S., Weiss, L., Shapira, M.Y., Resnick, I. & Or, R. (2004) Nonmyeloablative stem cell transplantation: reduced-intensity conditioning for cancer immunotherapy – from bench to patient bedside. *Seminars in Oncology*, **31**, 4–21.

Snover, D.C. (1984) Acute and chronic graft versus host disease: histopathological evidence for two distinct pathogenetic mechanisms. *Human Pathology*, **15**, 202–205.

Sorror, M.L., Maris, M.B., Storer, B., Sandmaier, B.M., Diaconescu, R., Flowers, C., Maloney, D.G. & Storb, R. (2004) Comparing morbidity and mortality of HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative and myeloablative conditioning: influence of pretransplantation comorbidities. *Blood*, **104**, 961–968.

Sullivan, K.M., Agura, E., Anasetti, C., Appelbaum, F., Badger, C., Bearman, S., Erickson, K., Flowers, M., Hansen, J., Loughran, T., Martin, P., Matthews, D., Petersdorf, E., Radich, J., Riddell, S., Rovira, D., Sanders, J., Schuening, F., Siadak, M., Storb, R. & Witherspoon, R.P. (1991) Chronic graft-versus-host disease and other late complications of bone marrow transplantation. *Seminars in Hematology*, **28**, 250–259.

Sykes, M., Preffer, F., McAfee, S., Saidman, S.L., Weymouth, D., Andrews, D.M., Colby, C., Sackstein, R., Sachs, D.H. & Spitzer, T.R. (1999) Mixed lymphohaemopoietic chimerism and graft-versus-lymphoma effects after non-myeloablative therapy and HLA-mismatched bone-marrow transplantation. *Lancet*, **353**, 1755–1759.

Szydlo, R., Goldman, J.M., Klein, J.P., Gale, R.P., Ash, R.C., Bach, F.H., Bradley, B.A., Casper, J.T., Flomenberg, N., Gajewski, J.L., Gluckman, E., Henslee-Downey, P.J., Hows, J.M., Jacobsen, N., Kolb, H.J., Lowenberg, B., Masaoka, T., Rowlings, P.A., Sondel, P.M., van Bekkum, D.W., van Rood, J.J., Vowels, M.R., Zhang, M.J. & Horowitz, M.M. (1997) Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. *Journal of Clinical Oncology*, **15**, 1767–1777.

Teshima, T., Ordemann, R., Reddy, P., Gagin, S., Liu, C., Cooke, K.R. & Ferrara, J.L. (2002) Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nature Medicine*, **8**, 575–581.

Weisdorf, D., Hakke, R., Blazar, B., Miller, W., McGlave, P., Ramsay, N., Kersey, J. & Filipovich, A. (1991) Risk factors for acute graft-versus-host reactions. *Transplantation*, **51**, 1197.

Wong, R., Giralt, S.A., Martin, T., Couriel, D.R., Anagnostopoulos, A., Hosing, C., Andersson, B.S., Cano, P., Shahjahan, M., Ippoliti, C., Estey, E.H., McMannis, J., Gajewski, J.L., Champlin, R.E. & de Lima, M. (2003) Reduced-intensity conditioning for unrelated donor hematopoietic stem cell transplantation as treatment for myeloid malignancies in patients older than 55 years. *Blood*, **102**, 3052–3059.

Xun, C.Q., Thompson, J.S., Jennings, C.D., Brown, S.A. & Widmer, M.B. (1994) Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. *Blood*, **83**, 2360–2367.

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Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A*02 or HLA-A*24 phenotype undergoing hematopoietic stem cell transplantation

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Summary:

Cytomegalovirus-specific cytotoxic T-lymphocytes (CMV-CTL) are essential for the control of CMV reactivation. To monitor the quantity and function of CMV-CTL after hematopoietic stem cell transplantation (HSCT), two CMV epitopes that bind to HLA-A*0201 NLVPMVATV (A*02NLV) and HLA-A*2402 QYDPVAALF (A*24QYD) were evaluated for their immunological potential. Samples from patients with the HLA-A*02 or HLA-A*24 serotype were analyzed by tetramer, intracellular cytokine staining and enzyme-linked immunospot (ELISPOT) assay. There were significantly more A*02NLV-specific CMV-CTL than A*24QYD (23×10^6 *vs* 0.4×10^6/l). The frequency of IFN-γ-producing cells was also higher upon stimulation with A*02NLV than with A*24QYD (2.5 *vs* 0.1%/CD8). Furthermore, the magnitude of CMV-CTL expansion was two- to 50-fold when cells were cultured with A*02NLV, while only an insignificant increase was observed in culture with A*24QYD. Although the number of A*24QYD-specific CMV-CTL was very low in most of the HLA-A*24 patients, the incidence of CMV reactivation did not differ between those with HLA-A*02 and HLA-A*24 serotype alone. These results suggest that an epitope other than A*24QYD plays a major role in patients with HLA-A*24. Our study also showed that A*02NLV may be a useful epitope for monitoring CMV-CTL not only in patients with HLA-A*0201 but also in those with the A*0206 genotype.

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Patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) are at high risk for cytomegalovirus (CMV)-associated disease as a consequence of prolonged T-cell immunodeficiency.[1] Although prophylactic administration of gancyclovir or foscarnet reduces the risk of severe CMV disease, these prophylactic strategies are complicated by significant myelosuppression, nephrotoxicity and even secondary engraftment failure. The development of a reliable method to evaluate CMV-specific immunity will be of critical importance for the prompt initiation of treatment. To address this need, several studies have been conducted with monitoring of tetramer-based CMV-specific cytotoxic-T-lymphocytes (CMV-CTL) in patients with HLA-A*0201 and HLA-B*0702.[2 9] However, the allele frequency of A*0201 is about 26%, with lower frequencies among African-American (16%) and Asian populations (15%).[10] This frequency is much lower in Japanese (11%).[11] Furthermore, the allele frequency of B*0702 is only 5% among Japanese.[11] To make the approach realistic, practical and more widely applicable, the further development of CMV-CTL monitoring among other HLA types is required. HLA-A*2402 is a major alternate candidate, since it is highly represented in Japanese (58%) and other ethnic groups including Caucasian (17%), Hispanic (27%) and Chinese (33%).[12] HLA-A*0206 is also attractive, since its frequency is as high as that of HLA-A*0201 in the Asian population, and is also significant in a certain population of Caucasians in North America.[10,11]

HLA-A*2402-restricted CMV-specific epitope was introduced by Kuzushima *et al* in 2001,[13] and is now widely recognized as the major epitope for HLA-A*2402.[14 17] In this study, we compared the immunological potential of two major CMV pp65 epitope peptides for HLA-A*0201 and HLA-A*2402 for use in monitoring of CMV-CTL by tetramer assay, intracellular cytokine assay, enzyme-linked immunospot (ELISPOT) assay and CTL culture. We also evaluated whether the HLA-A*0201-restricted CMV pp65 epitope could also be used for the A*0206 genotype.

Materials and methods

Subjects

After we obtained written informed consent, peripheral blood samples were obtained from patients with an HLA-

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