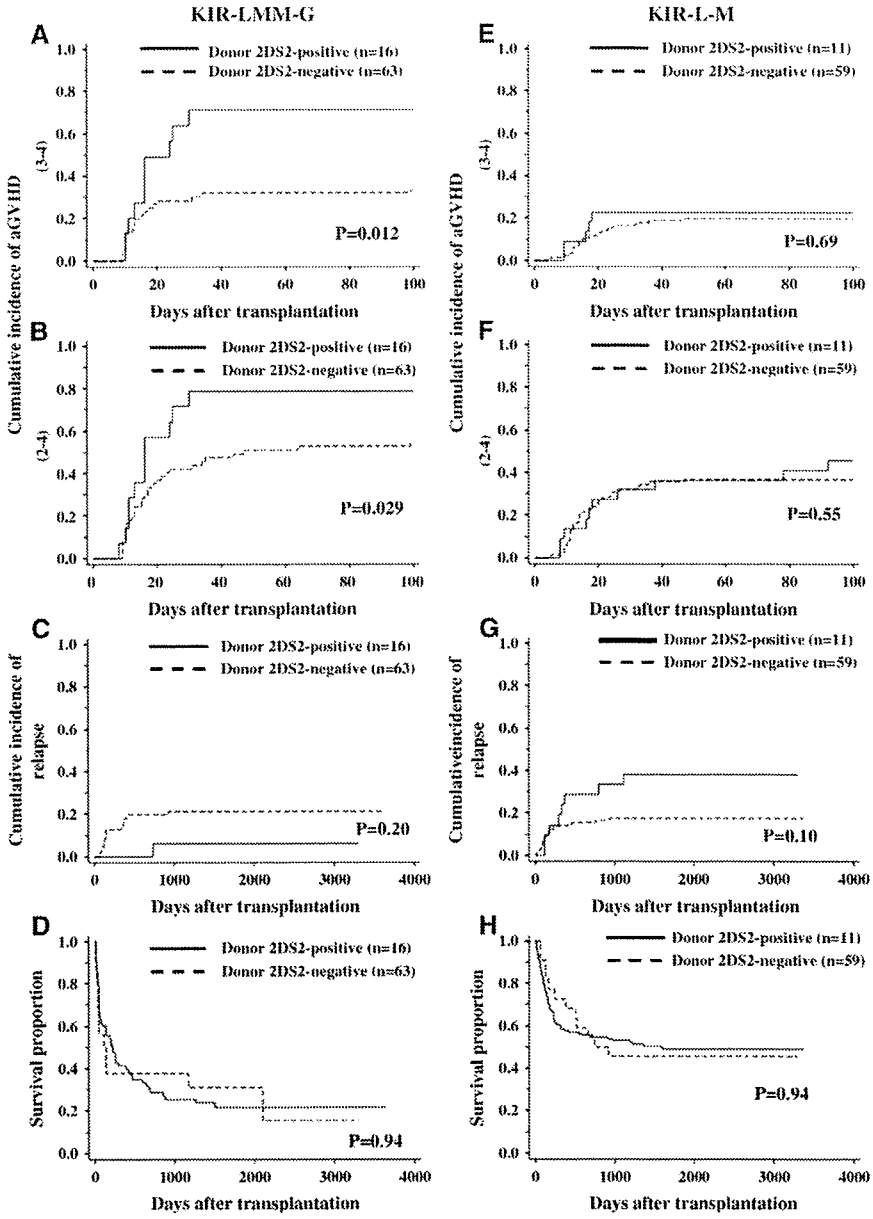

Figure 2. Effects of donor *KIR2DS2* in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival with presence or absence of donor *KIR2DS2* gene in HLA-C–mismatched patients. Grade III-IV GVHD (A and E), grade 2-4 GVHD (B and F), relapse (C and G), and overall survival (D and H) with KIR-L-MM-G (A-D) or KIR-L-M (E-H) cases were analyzed. The solid line represents donor *KIR2DS2*-positive; the dotted line, donor *KIR2DS2*-negative.

KIR genotype and profile between patient and donor [receptor–receptor analysis] [17,19,25], compatibility score [24], ligand homozygosity in patients [21,27,38,39], and "missing ligand" effect [3,11,13,40,41]), and found no significant associations in this cohort (data not shown).

ATG Preadministration Ameliorates the Adverse Effects of KIR-L-MM-G on aGVHD and OS

In our previous study [5], the incidence of aGVHD was high in KIR-L-MM-G, where all cases did not involve ATG administration in the conditioning regimen, which is common in the JMDP cases. In the present study, we included rare ATG-administered cases (n = 94) in the analysis and evaluated the effects of ATG administration on KIR-L-MM-G. We found no significant differences in most of the parameters between the ATG-administered and non–ATG-administered groups, except for patient average age (18 years vs 27 years). Multivariate analysis (Table 2; group A [n = 1489]) indicated that ATG administration was a risk-reducing factor for severe aGVHD (grade III-IV

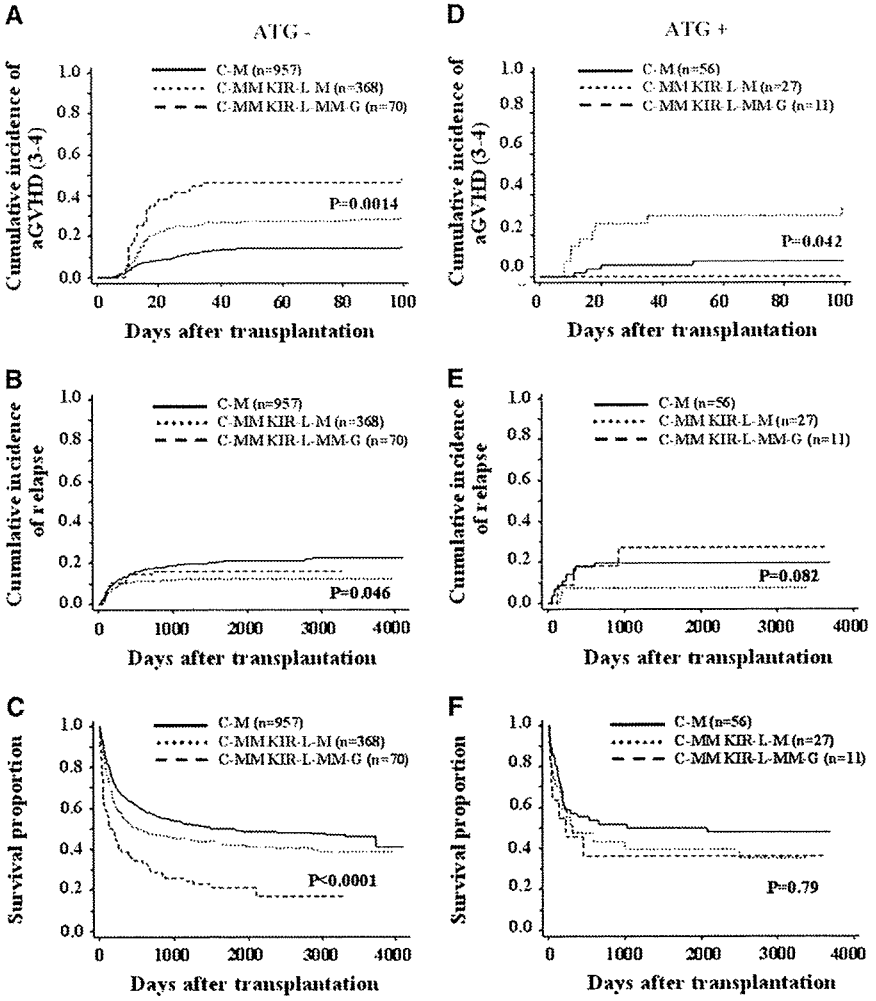

Figure 3. Effects of ATG preadministration in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival of patients not receiving ATG (A-C) and those receiving ATG (D-F). The solid line represents HLA-C match (C-M), the thick dotted line represents HLA-C mismatch KIR ligand match in the GVHD direction (C-MM KIR-L-M), and the thin dotted line represents HLA-C mismatch KIR ligand mismatch in the GVH direction (C-MM KIR-L-MM-G). The log-rank test was applied between CMM KIR-L-MM-G and CMM KIR-L-M.

GVHD: HR = 0.56; P = .047; grade II-IV GVHD: HR = 0.63, P = .019), whereas no significant effects on relapse or OS could be seen.

The cumulative incidence of aGVHD was assessed separately in the non–ATG-administered and ATG-administered groups (Figures 3A and 3D, respectively). In the non–ATG-administered group, the incidence of grade III-IV GVHD was significantly higher in KIR-L-MM-G than in KIR-L-M (47.7% [95% CI = 35.2%-59.2%] vs 29.4% [95% CI=24.8%-34.1%]; P = .0014), as found in our previous study [5]. In contrast, no grade III-IV aGVHD was observed in KIR-L-MM-G cases in the ATG-administered group (2 cases of grade 2, 2 cases of grade 1, and 7 cases of grade 0), and the preventive effects of KIR-L-MM-G on severe aGVHD were significant (P = .042) although only a small number were analyzed (n = 38). We analyzed the effects of *2DS2* in the non–ATG-administered cases. In KIR-L-MM-G, the incidence of grade III-IV aGVHD was significantly higher in the donor *2DS2*-positive cases (n = 15) than in the donor *2DS2*-negative cases (n = 54) (76.4% [95% CI = 43.5-91.7%] vs 40.1% [95% CI = 26.5%-53.2%]; P = .048), suggesting that the adverse effects of donor *2DS2* are independent of ATG administration. In ATG-administered cases, no grade III-IV aGVHD was observed in donor *2DS2*-negative KIR-L-MM-G (n = 15); in 1 donor *2DS2*-positive KIR-L-MM-G case, the patient failed engraftment but showed no aGVHD, and died on day 35. Therefore, we could not statistically evaluate the effect of ATG on the *2DS2*-positive cases.

As shown in Figure 3B, in non–ATG-administered cases, the cumulative incidence of relapse was higher in KIR-L-MM-G than in KIR-L-M (16.1% [95% CI = 8.6%-25.8%] vs 11.9% [95% CI = 8.9%-15.3%];

P = .046), which was seen mainly in ALL (data not shown), as was found in our previous study [5]. In contrast, no significant increase in relapse was obtained in ATG-administered cases (P = .082) (Figure 3E). As in our previous study [5], in non–ATG-administered cases, overall survival rate was significantly lower in KIR-L-MM-G than in KIR-L-M (21.0% [95% CI = 12.2%-31.3%] vs 42.0% [95% CI =36.8%-47.0%]; P < .0001) (Figure 3C). On the other hand, in ATG-administered cases, no significant difference was observed between KIR-L-MM-G and KIR-L-M (36.4% [95% CI = 11.2%-62.7%] vs 39.5% [95% CI = 21.2%-57.3%]; P = .79) (Figure 3F), suggesting that ATG preadministration in the conditioning regimen abolished the adverse effect of KIR-L-MM-G on survival.

DISCUSSION

In the present study, we identified donor *KIR* genotype–patient KIR ligand combination and no ATG preadministration as critical factors for the adverse effects of KIR-L-MM-G on transplantation outcomes in the JMDP. The cases analyzed in this study were all HLA-A, -B, and -DR serologically matched; thus, we were able to evaluate the HLA-C ligand compatibility effects, because the HLA-Bw4 and HLA-A3 and -A11 KIR ligands were all matched. Other groups included mostly Bw4 ligand mismatch cases in KIR-L-MM-G analysis [2,6,7,10-12,14,21,42]. The Bw4 (patient) -*3DL1* (or -*3DS1*) (donor) combinatory effect also may affect transplantation outcome.

In the KIR-L-MM-G combination, the patient lacks the donor's KIR ligand. In this situation, donor NK cells may react with the patient cells according to the "missing self" model [43]. Previous KIR ligand compatibility data, together with the present data, confirm that the KIR-L-MM-G has potent adverse effects on UR-HSCT. In most KIR-L-MM-G cases in the JMDP, the donor and patient ligand types are C1C2 and C1C1, respectively, suggesting that C1C2 donor NK cells (and/or some T cells) respond to C1C1 patient cells. In this case, donor NK cells lack the inhibitory KIR for C1 (2DL2 and 2DL3) in terms of genotype or phenotype, or both. As shown in the present results, almost all JMDP donors examined posessed an inhibitory *KIR* gene for C1 (*2DL3*). The subpopulation of donor NK cells thus appears to lack cell surface expression of the C1-inhibitory KIR molecule, despite the presence of the genes. This is explained by the "at least one inhibitory receptor expression" model [44], in which each NK cell must express 1 inhibitory receptor for the self–major histocompatibility complex (MHC) class I to avoid autoreactivity, but expression of other receptors is "stochastic." Consequently, NK cell subpopulations lacking the C1-inhibitory *KIR* (*2DL2* and *2DL3*) but having the C2-inhibitory *KIR* (*2DL1*) would react with C1C1 (C2-lacking) patient cells. Therefore, the donor inhibitory KIR repertoire at the expression level, not at the genomic level, appears to influence outcome in the JMDP. The importance of the inhibitory KIR expression repertoire and functional analysis of donor NK cells has been discussed previously [3,45].

With a lack of inhibitory KIR signals, NK cells respond to target cells through activation signals from activating receptors. 2DS1 and 2DS2 are assigned to recognize C2 and C1, respectively, but other activating KIR ligand specificities (2DS3-5 and 3DS1) are unidentified [35]. Therefore, we were able to evaluate only these 2 KIRs for combinatory effects with their ligands. As described in Results, a higher incidence of severe aGVHD was observed in the *2DS2*-positive donors in the KIR-L-MM-G cases, but not in the KIR-L-M cases. This suggests that *2DS2*-positive lymphocytes (NK cells and/or some T cells) react with cognate ligand (C1)-positive cells and exacerbate aGVHD. Recently, La Nasa et al.[27] reported that the patient KIR ligand homozygosity, but not donor KIR genotype, is predictive for the outcome of HLA-matched UR-HCT in patients with beta-thalassemia. Their cases were all KIR ligand-matched transplantation and the donor-activating *KIR*–patient cognate ligand combination had no significant effect on the outcomes. Their results are consistent with our findings indicating that the donor *2DS2*–patient C1 combination of ligand-matched pairs has no effect on any outcomes (Figure 2E-H). This is in accordance with the notion that an activating *KIR* works only when the patient has the cognate ligand and that the donor inhibitory KIR does not function (Fig. 4). Chewning et al [36] reported that *KIR 2DS1*-positive NK cells recognized C2-expressing target cells and showed alloreactivity in vitro supporting the concept of this model.

Although adverse impacts of donor *2DS2* on transplantation outcome have been documented previously [15,18,23], the present study is the first report on the adverse effects of the *2DS2*–cognate ligand C1 combination on aGVHD incidence. Because we had an a priori hypothesis, we did not apply adjustment of P-value in our analysis; however, our results must be interpreted with caution. KIR-L-MM-G is infrequent in the JMDP (only 81 of 1489 cases in the present study), and the frequency of *2DS2* is low in Japan [32,33], and confirmation in other independent cohorts from different populations will support our findings. Combinatory effects of *2DS2* and cognate ligand C1 also have been reported in disease susceptibility studies, including studies of type I diabetes mellitus [46], ulcerative colitis [47], rheumatoid vasculitis [48], and tuberculosis [49]. Furthermore, extensive genetic analysis of *KIR* and HLA genotypes of various ethnic populations have demonstrated a strong negative correlation of activating *KIR* and its putative ligand

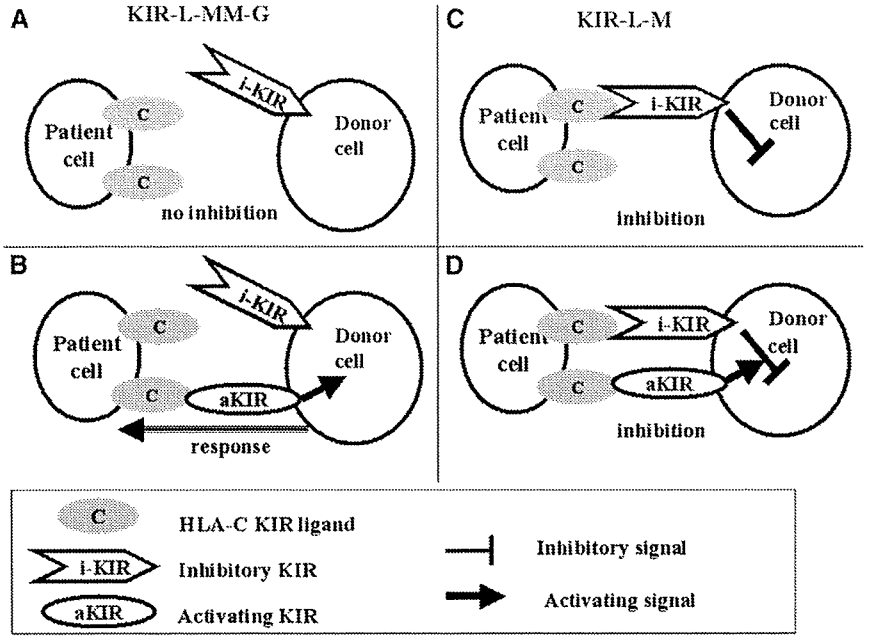

Figure 4. Model of interaction between activating KIR and cognate KIR ligand. Donor-activating KIR transduces an activating signal on recognition of the cognate KIR ligand of the patient cell in KIR-L-MM-G case (B). The activating signal is canceled by an inhibitory signal from inhibitory KIR, which recognizes the KIR ligand of the patient cell in KIR-L-M case (D).

combination including *2DS2*–C1, suggesting coevolution of the activating receptor–ligand loci [50]. Taken together with our data, these clinical and population genetic studies suggest a direct receptor–ligand interaction between 2DS2 and C1; however, binding studies using soluble 2DS2 molecules have shown no or a very weak binding to C1 molecules or C1-transfected cells, challenging the notion of C1 as a 2DS2 ligand [51-53]. Recombinant 2DS1 also showed very low or no affinity to C2 [54]. This disparity may be linked to differences in the nature of ligand binding between inhibiting and activating receptors. One possible factor is class I-binding peptides. The peptide-dependent binding with class I-binding receptors is recognized in most of the inhibitory receptors [53,55-58] and also has been suggested in activating KIR [53,58,59]. The peptide repertoire that allows strong KIR binding might be more restricted in activating KIR cases than in inhibitory ones. Alternatively, activating KIR–ligand binding may be somehow strengthened under stress conditions, such as transplantation or viral infection. Epstein Barr virus–transformed C1-positive cells were found to be stained slightly by recombinant 2DS2 tetramers [53]. A mutation study found that only 1 amino acid substitution in 2DS2 increased its level of binding to C1 to that of inhibitory 2DL2, suggesting that a very fine conformational microstructure change controls KIR binding specificity [60].

Inhibitory *2DL2* also showed a significant association with the incidence of severe aGVHD. This may be secondary to the *2DS2*–C1 association [50]; alternatively, donor 2DL2-positive NK cells might have a different effect than 2DL3-positive NK cells on acute GVHD incidence, because the binding affinity to C1 is higher in 2DL2 than in 2DL3 [60]. Other groups have analyzed activating *KIR* gene number and outcome and have reported both beneficial and adverse associations [10,16,26,37]. We did not find such quantitative *KIR* loci effects in this JMDP cohort (data not shown); *KIR* genotype variation among various ethnic groups may be responsible for these differences.

Preadministration of ATG to a patient is also a critical factor in attenuating the adverse effects of KIR-L-MM-G on transplantation outcome. Our findings demonstrate that KIR-L-MM-G had potent adverse effects (higher aGVHD incidence and lower OS) without ATG administration, and that ATG administration in the conditioning regimen ameliorated most of these adverse effects. Although the average patient age in the ATG-administered group was about 10 years younger than that in the non–ATG-administered group in this study, multivariate analysis including age as a confounder also identified the ATG effect as an independent factor for incidence of aGVHD (see Table 2). To the best of our knowledge, this is the first direct comparison UR-HSCT study on the effects of ATG preadministration under the same transplantation regimen with similar genetic backgrounds. Because far fewer ATG-administrated cases than

non–ATG-administered cases are included in the JMDP (an imbalance that could bias statistical results), further evaluation of large numbers of ATG-preadministered cases in different ethnic populations are needed.

Administration of ATG extensively depletes patient and donor T cells, thus strongly inhibiting the responses of alloreactive T cells. Because the JMDP cases are all unmanipulated T cell–replete marrow, donor alloreactive T cell response may be very strong, which would obscure some of the NK cell beneficial effects [61]. In KIR-L-MM-G without ATG preadministration, alloreactive NK cells were activated by 2DS2–C1 interaction without inhibitory KIR signals and may have augmented alloreactive donor T cell responses, resulting in increased aGVHD incidence and mortality. Alternatively, KIR-positive T cells may have been responsible for inducing aGVHD. In contrast, with ATG preadministration, donor T cells are largely depleted, and the beneficial effects of NK cell alloreactivity on aGVHD incidence may become prominent. Too few ATG-treated cases were analyzed (n = 11) to allow confirmation of the preventive effects of KIR-L-MM-G on acute GVHD, but the results are consistent with those for the HLA haplo-mismatched, ATG-preadministered R-HSCT [2]. In mouse GVHD models, alloreactive NK cells prevented donor alloreactive T cell stimulation and suppressed aGHVD by lysing donor antigen-presenting cells [2]. These mechanisms might explain the preventive effects of KIR-L-MM-G on the incidence of aGVHD. NK cell reconstitution after transplantation might be influenced by ATG treatment as well as by KIR ligand and KIR genotype variability [39,62,63]. Our data suggest that the KIR-L-MM-G combination must be avoided in JMDP transplantation unless ATG is used in the conditioning regimen.

Another possible factor is mismatch combination dissimilarity resulting from genetic variability in *HLA* and *KIR* in populations with different ethnic backgrounds. There are allele frequency differences in *HLA-C* among human populations in terms of the *HLA-C* KIR ligand [50]. Because the C1 ligand type is dominant in the Japanese population (allele frequency 0.92), KIR-L-MM-G is relatively rare (5%) compared with the incidence in White populations. Furthermore, in the KIR-L-MM-G, the C1C1 (patient)–C1C2 (donor) combination is common (95%) [5]. Therefore, we could focus on the KIR ligand incompatibility and the *2DS2* effects on the C1-homozygous patients in this study. In contrast, the White population more frequently exhibits the C2 type [50]. Consequently, the KIR-L-MM-G frequency is higher in Caucasian than Japanese and might include C2C2 (patient)–C1C2 (donor), C2C2–C1C1, and C1C1–C2C2 combinations, in addition to the C1C1–C1C2 combination. Therefore, not only the C1C1 (patient)–2DS2 (donor) combination, but also the C2C2 (patient)–2DS1 (donor) combination, might contribute considerably to the effects of KIR-L-MM-G in White [36]. The inhibitory capacity of C1 is reportedly weaker than that of C2 [64], and the binding strength of inhibitory KIR to the ligand HLA-C is different as well (2DL1 > 2DL2 > 2DL3) [60]. There may be more variability in inhibitory pathways in White populations; indeed, several groups have reported that the transplantation outcomes vary between C1-homozyous and C2-homozygous patients [15,19,38,39]. *KIR* genotype also shows ethnic variability [33,50]; Japanese have a markedly high frequency of the A haplotype and a very low frequency of *2DS2* (16% in the JMDP, compared with a frequency of > 40% in most Caucasian and African populations). One potential factor not examined in the present study is *KIR* allelic polymorphism. Yawata et al. [34] have shown that allelic polymorphism modulates the level and frequency of KIR3D expression, as well as its inhibitory capacity. These allelic differences might influence outcomes even though HLA-A- and B-KIR ligand specificities were the same in donors and recipients in the present study.

Here we found that the combination of donor-activating *KIR* genotype–patient cognate KIR ligand type and ATG administration in the conditioning regimen were critical factors in the adverse effects of KIR-L-MM-G on transplantation outcome. Alloreactivity of NK cells may be either beneficial or adverse depending on the above factors. However, other important parameters also may contribute to transplantation outcome. Further large-scale international collaborative studies, including a variety of ethnic populations and statistical comparisons under uniform regimens, are needed to gain further insight into the effects of NK cell alloreactivity on transplantation and to guide the development of cell therapy using alloreactive NK cells for leukemia and other diseases.

ACKNOWLEDGMENTS

The authors thank the staff members of the transplantation center, the donor centers, and the JMDP office. They also thank Drs Kenji Tadokoro, Hiroshi Furukawa, and Nobuyo and Makoto Yawata for useful suggestions and discussion, and Ms. Mutsuko Minemoto and Mr. Takahiro Ichihara for technical assistance. This work was supported in part by a Health and Labor Science Research Grant from the Ministry of Health, Labor and Welfare of Japan (Research on Human Genome, Tissue Engineering), a grant from Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Grant-in Aid B (15390309) from the Japan Society for the Promotion of Science, and a Grant of Third-Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan. The following institutions participated in and

registered patients in the present study: Hokkaido University Hospital, Sapporo University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Asahikawa Medical College Hospital, Hirosaki University Hospital, Iwate Medical University Hospital, Tohoku University Hospital, Yamagata University Hospital, Akita University Hospital, Fukushima Medical College, Toranomon Hospital, National Cancer Center Central Hospital, National Center for Child Health and Development, Institute of Medical Science at the University of Tokyo, Toho University Hospital, Omori Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Hospital, Itabashi Hospital, Jikei University Hospital, Keio University Hospital, Tokyo Medical College Hospital, Tokyo Medical and Dental University Hospital, Tokyo University Hospital, Yokohama City University Hospital, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, St. Marianna University Hospital, Chiba University Hospital, Chiba Children's Hospital, Kameda General Hospital, Saitama Children's Medical Center, Saitama Cancer Center Hospital, Saitama Medical School Hospital, Ibaraki Children's Hospital, Jichi Medical School Hospital, Tsukuba University Hospital, Dokkyo University Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata University Hospital, Niigata Cancer Center Hospital, Shinshu University Hospital, Hamamatsu University Hospital, Hamamatsu Medical Center, Shizuoka General Hospital, Shizuoka Children's Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Nagoya Ekisaikai Hospital, Nagoya Medical Center, Aichi Cancer Center Hospital, Aichi Medical University Hospital, Nagoya City University Hospital, Showa Hospital, Anjo Kousei Hospital, Fujita Health University Hospital, Mie University Hospital, Yamada Red Cross Hospital, Kanazawa University Hospital, Kanazawa Medical University Hospital, Toyama Prefectural Central Hospital, Fukui Medical School Hospital, Shiga University of Medical Science, Center for Adult Disease in Osaka, Kinki University Hospital, Osaka University Hospital, Osaka City University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kobe City General Hospital, Kobe University Hospital, Kyoto University Hospital, Kyoto Prefectural University of Medicine Hospital, Kyoto City Hospital, Kansai Medical University Hospital, Tenri Hospital, Nara Medical University Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Yamaguchi University Hospital, Ehime Prefectural Central Hospital, Okayama Medical Center, Kurashiki Central Hospital, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St. Mary's Hospital, Kokura Memorial Hospital, Nagasaki University Hospital, Kumamoto Medical Center, Oita Medical University Hospital, Imamura Hospital, and Kagoshima University Hospital.

REFERENCES

1. Ruggeri L, Aversa F, Martelli MF, et al. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol Rev.* 2006;214:202-218.
2. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002;295:2097-2100.
3. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol.* 2004;172:644-650.
4. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang.* 2006;90:10-20.
5. Morishima Y, Yabe T, Matsuo K, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell–replete marrow from an unrelated donor. *Biol Blood Marrow Transplant.* 2007;13:315-328.
6. Davies SM, Ruggieri L, DeFor T, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. *Blood.* 2002;100:3825-3827.
7. Giebel S, Locatelli F, Lamparelli T, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood.* 2003;102:814-819.
8. Bornhauser M, Schwerdtfeger R, Martin H, et al. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood.* 2004;103:2860-2862.
9. Schaffer M, Malmberg KJ, Ringden O, et al. Increased infection-related mortality in KIR ligand–mismatched unrelated allogeneic hematopoietic stem-cell transplantation. *Transplantation.* 2004;78:1081-1085.
10. De Santis D, Bishara A, Witt CS, et al. Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens.* 2005;65:519-528.
11. Hsu KC, Gooley T, Malkki M, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant.* 2006;12:828-836.
12. Farag SS, Bacigalupo A, Eapen M, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry. *Biol Blood Marrow Transplant.* 2006;12:876-884.
13. Sun JY, Dagis A, Gaidulis L, et al. Detrimental effect of natural killer cell alloreactivity in T cell–replete hematopoietic cell transplantation (HCT) for leukemia patients. *Biol Blood Marrow Transplant.* 2007;13:197-205.
14. Sivula J, Volin L, Porkka K, et al. Killer-cell immunoglobulin-like receptor ligand compatibility in the outcome of Finnish unrelated donor hematopoietic stem cell transplantation. *Transpl Immunol.* 2007;18:62-66.

15. Cook MA, Milligan DW, Fegan CD, et al. The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood.* 2004;103:1521-1526.
16. Cook M, Briggs D, Craddock C, et al. Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell–replete stem cell transplantation. *Blood.* 2006;107: 1230-1232.
17. Chen C, Busson M, Rocha V, et al. Activating KIR genes are associated with CMV reactivation and survival after non–T-cell–depleted HLA-identical sibling bone marrow transplantation for malignant disorders. *Bone Marrow Transplant.* 2006; 38:437-444.
18. Verheyden S, Schots R, Duquet W, et al. A defined donor-activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia.* 2005;19:1446-1451.
19. McQueen KL, Dorighi KM, Guethlein LA, et al. Donor–recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol.* 2007;68: 309-323.
20. Clausen J, Wolf D, Petzer AL, et al. Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human leucocyte antigen-identical haematopoietic stem cell transplantation. *Clin Exp Immunol.* 2007;148:520-528.
21. Sobecks RM, Ball EJ, Maciejewski JP, et al. Survival of AML patients receiving HLA-matched sibling donor allogeneic bone marrow transplantation correlates with HLA-Cw ligand groups for killer immunoglobulin-like receptors. *Bone Marrow Transplant.* 2007;39:417-424.
22. Zhao XY, Huang XJ, Liu KY, et al. Prognosis after unmanipulated HLA-haploidentical blood and marrow transplantation is correlated to the numbers of KIR ligands in recipients. *Eur J Haematol.* 2007;78:338-346.
23. Giebel S, Nowak I, Wojnar J, et al. Impact of activating killer immunoglobulin-like receptor genotype on outcome of unrelated donor-hematopoietic cell transplantation. *Transplant Proc.* 2006;38:287-291.
24. Sun JY, Gaidulis L, Dagis A, et al. Killer Ig-like receptor (KIR) compatibility plays a role in the prevalence of acute GVHD in unrelated hematopoietic cell transplants for AML. *Bone Marrow Transplant.* 2005;36:525-530.
25. Gagne K, Brizard G, Gueglio B, et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol.* 2002;63:271-280.
26. Kroger N, Binder T, Zabelina T, et al. Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukemia patients after in vivo T-cell–depleted unrelated stem cell transplantation. *Transplantation.* 2006;82:1024-1030.
27. La Nasa G, Littera R, Locatelli F, et al. Status of donor–recipient HLA class I ligands and not the KIR genotype is predictive for the outcome of unrelated hematopoietic stem cell transplantation in beta-thalassemia patients. *Biol Blood Marrow Transplant.* 2007; in press.
28. Bacigalupo A. Antilymphocyte/thymocyte globulin for graft-versus-host disease prophylaxis: efficacy and side effects. *Bone Marrow Transplant.* 2005;35:225-231.
29. Sasazuki T, Juji T, Morishima Y, et al. 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. *N Engl J Med.* 1998;339:1177-1185.
30. Morishima Y, Sasazuki T, Inoko H, et al. 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.* 2002;99:4200-4206.
31. Gomez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: an update. *Tissue Antigens.* 2002;59:184-193.
32. Miyashita R, Tsuchiya N, Yabe T, et al. Association of killer cell immunoglobulin-like receptor genotypes with microscopic polyangiitis. *Arthritis Rheum.* 2006;54:992-997.
33. Yawata M, Yawata N, McQueen KL, et al. Predominance of group A KIR haplotypes in Japanese associated with diverse NK cell repertoires of KIR expression. *Immunogenetics.* 2002; 54:543-550.
34. Yawata M, Yawata N, Draghi M, et al. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med.* 2006;203:633-645.
35. Moretta A, Sivori S, Ponte M, et al. Stimulatory receptors in NK and T cells. *Curr Top Microbiol Immunol.* 1998;230:15-23.
36. Chewning JH, Gudme CN, Hsu KC, et al. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J Immunol.* 2007;179:854-868.
37. Bishara A, De Santis D, Witt CC, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens.* 2004;63:204-211.
38. Giebel S, Locatelli F, Wojnar J, et al. Homozygosity for human leucocyte antigen-C ligands of KIR2DL1 is associated with increased risk of relapse after human leucocyte antigen C–matched unrelated donor haematopoietic stem cell transplantation. *Br J Haematol.* 2005;131:483-486.
39. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C–specific inhibitory killer Ig-like receptor. *J Immunol.* 2007;178:3918-3923.
40. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood.* 2005;105:4878-4884.
41. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood.* 2007;109:5058-5061.
42. Beelen DW, Ottinger HD, Ferencik S, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood.* 2005;105:2594-2600.
43. Ljunggren HG, Karre K. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol Today.* 1990;11: 237-244.
44. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity.* 1997;7:739-751.

45. Han M, Fallena M, Guo Y, et al. Natural killer cell cross-match: functional analysis of inhibitory killer immunoglobulin-like receptors and their HLA ligands. *Hum Immunol.* 2007;68:507-513.
46. van der Slik AR, Koeleman BP, Verduijn W, et al. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes.* 2003;52:2639-2642.
47. Jones DC, Edgar RS, Ahmad T, et al. Killer Ig-like receptor (KIR) genotype and HLA ligand combinations in ulcerative colitis susceptibility. *Genes Immun.* 2006;7:576-582.
48. Yen JH, Moore BE, Nakajima T, et al. Major histocompatibility complex class I–recognizing receptors are disease risk genes in rheumatoid arthritis. *J Exp Med.* 2001;193:1159-1167.
49. Mendez A, Granda H, Meenagh A, et al. Study of KIR genes in tuberculosis patients. *Tissue Antigens.* 2006;68:386-389.
50. Single RM, Martin MP, Gao X, et al. Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet.* 2007;39: 1114-1119.
51. Vales-Gomez M, Erskine RA, Deacon MP, et al. The role of zinc in the binding of killer cell Ig-like receptors to class I MHC proteins. *Proc Natl Acad Sci U S A.* 2001;98:1734-1739.
52. Saulquin X, Gastinel LN, Vivier E. Crystal structure of the human natural killer cell– activating receptor KIR2DS2 (CD158j). *J Exp Med.* 2003;197:933-938.
53. Stewart CA, Laugier-Anfossi F, Vely F, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A.* 2005;102: 13224-13229.
54. Biassoni R, Pessino A, Malaspina A, et al. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur J Immunol.* 1997;27:3095-3099.
55. Maenaka K, Juji T, Nakayama T, et al. Killer cell immunoglobulin receptors and T cell receptors bind peptide major histocompatibility complex class I with distinct thermodynamic and kinetic properties. *J Biol Chem.* 1999;274:28329-28334.
56. Thananchai H, Gillespie G, Martin MP, et al. Cutting edge: allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *J Immunol.* 2007;178:33-37.
57. Hansasuta P, Dong T, Thananchai H, et al. Recognition of HLA-A3 and HLA-A11 by *KIR3DL2* is peptide-specific. *Eur J Immunol.* 2004;34:1673-1679.
58. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* 1999;18:4250-4260.
59. Carr WH, Rosen DB, Arase H, et al. Cutting edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a *DAP12*-associated receptor expressed on NK cells that triggers NK cell activation. *J Immunol.* 2007;178:647-651.
60. Winter CC, Gumperz JE, Parham P, et al. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol.* 1998;161: 571-577.
61. Lowe EJ, Turner V, Handgretinger R, et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell–depleted HLA-nonidentical paediatric bone marrow transplantation. *Br J Haematol.* 2003;123:323-326.
62. Savani BN, Mielke S, Adams S, et al. Rapid natural killer cell recovery determines outcome after T-cell–depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia.* 2007;21: 2145-2152.
63. Zhao XY, Huang XJ, Liu KY, et al. Reconstitution of natural killer cell receptor repertoires after unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation: analyses of CD94:NKG2A and killer immunoglobulin-like receptor expression and their associations with clinical outcome. *Biol Blood Marrow Transplant.* 2007;13:734-744.
64. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol.* 2005;5:201-214.

Available online at www.sciencedirect.com
ScienceDirect
Cancer Letters 260 (2008) 137–145
CANCER Letters
www.elsevier.com/locate/canlet

Effective induction of anti-tumor immune responses with oligomannose-coated liposome targeting to intraperitoneal phagocytic cells

Yuzuru Ikehara [a,b,*], Nobumitsu Shiuchi [c], Sanae Kabata-Ikehara [a,b], Hayao Nakanishi [b], Naoaki Yokoyama [d], Hideaki Takagi [c], Toshi Nagata [e], Yukio Koide [e], Kiyotaka Kuzushima [f], Toshitada Takahashi [g], Kunio Tsujimura [e], Naoya Kojima [c]

[a] *Molecular Medicine Team of Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Centeral 2-12, Room 211, 1-1-1 Umezono, Tsukuba, Japan*
[b] *Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan*
[c] *Department of Applied Biochemistry, The Institute of Glycotechnology, Tokai University, Hiratsuka, Japan*
[d] *Research Unit for Molecular Diagnosis, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan*
[e] *Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan*
[f] *Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan*
[g] *Aichi Comprehensive Health Science Center of the Aichi Health Promotion Foundation, Aichi, Japan*

Received 2 July 2007; received in revised form 22 October 2007; accepted 29 October 2007

Abstract

We recently established a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) which are probably taken up by macrophages (Mϕ) to carry anti-cancer drugs to milky spots known as preferential metastatic sites of gastric cancers [Y. Ikehara, T. Niwa, L. Biao, S.K. Ikehara, N. Ohashi, T. Kobayashi, Y. Shimizu, N. Kojima, H. Nakanishi, A carbohydrate recognition-based drug delivery and controlled release system using intraperitoneal macrophages as a cellular vehicle, Cancer Res. 66 (2006) 8740–8748]. In the present study, we applied this intraperitoneal DDS for systemic cancer immunotherapy employing ovalbumin (OVA) as a model antigen. The cells taking up the OMLs containing FITC-OVA injected into the peritoneal cavity were predominantly Mϕ, as they showed adhesive characteristics and expressed F4/80 and CD11b almost exclusively. The phagocytic cells also took up bare OVA directly to the same extent as OML-enclosed OVA (OML-OVA), as it is a highly mannosilated protein. The phagocytic cells taking up OML-OVA, however, could activate OVA-specific $CD8^+$ (from OT-I: H-$2K^b$/$OVA_{257-264}$-specific) and $CD4^+$ (from OT-II: H-$2A^b$/$OVA_{323-339}$-specific) T cells much more effectively *in vitro* than those taking up bare OVA. Furthermore, only the mice pre-immunized with OML-OVA rejected E.G7-OVA (OVA-transfected EL4) but not EL4. These results indicate that

* Corresponding author. Address: Molecular Medicine Team of Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan. Tel.: +81 29 861 5853; Fax: +81 29 861 3462.
E-mail address: yuzuru-ikehara@aist.go.jp (Y. Ikehara).

0304-3835/$ - see front matter
doi:10.1016/j.canlet.2007.10.038

the OMLs can also be used as an effective antigen delivery system for cancer immunotherapy activating both CTL and Th subsets.

Keywords: Drug delivery system; Cancer vaccine; Immune responses to cancer; Oligomannose liposome

1. Introduction

While recent advances in tumor immunology enable us to identify tumor antigens recognized by T cells and understand the molecular and cellular bases of T cell-mediated anti-tumor responses, the clinical realization of effective immunotherapy for solid tumors has not yet been convincingly achieved [1,2]. Many $CD8^+$ and $CD4^+$ T cells recognizing tumor antigen in the context of MHC class I and II, respectively, have been reported, and the former are known to be a major effector of the adaptive anti-tumor immune responses [3–5]. $CD4^+$ T cells play an important role for the expansion and persistence of $CD8^+$ T cells, while some of them are known to function as regulatory cells [5–7]. Optimal anti-tumor immune responses are therefore considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the selective activation of $CD4^+$ T cells with helper but not regulatory functions [8]. Endogenous and exogenous antigens are presented as peptides preferentially by MHC class I and II, respectively, and most tumor antigen peptides are derived from the proteins expressed endogenously. Novel methods to make tumor antigens presented simultaneously by both MHC class I and II molecules are therefore needed for the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells, and many attempts have been made for this purpose [2,3,8].

We recently developed a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) [9,10] which are effectively taken up by $F4/80^+$ intraperitoneal cells to carry anti-cancer drugs to milky spots known as a preferential metastatic site of gastric and ovarian cancers [9,11,12]. We demonstrated that this system could control the formation of overt metastasis of seeded gastric cancer cells at the extra-nodal lymphoid tissues such as the omentum [10].

In the present study, we applied this OML-based intraperitoneal DDS for cancer immunotherapy using ovalbumin (OVA) as a model antigen, aiming at the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells. Peritoneal phagocytic cells took up OML containing OVA and then migrated into milky spots as previously reported. In addition, they activated both OVA-specific $CD8^+$ [13,14] and $CD4^+$ [15] T cells effectively *in vitro*. Spleen cells from OML-enclosed OVA (OML-OVA)-injected mice showed an effective killing activity against E.G7-OVA (OVA-transfected EL4) [16] but not EL4 [17] *in vitro*, and only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 *in vivo*. In light of these results obtained *in vitro* and *in vivo*, the potential of our novel OML-based immunization method for the prevention of tumor metastasis is discussed.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice ($H\text{-}2^b$) at 8–12 weeks of age were obtained from Charles River Japan Inc. (Yokohama, Japan) and kept under standard housing conditions. T cell receptor transgenic mice OT-I (specific for $H\text{-}2K^b/OVA_{257\text{-}264}$) [13,14] and OT-II ($H\text{-}2A^b/OVA_{323\text{-}339}$) [15] were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. All animal experiments were performed under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Aichi Cancer Center.

2.2. Cell lines

EL4 [17], a B6-derived thymoma cell line, was maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum, 0.2% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (complete RPMI). EG.7-OVA (EL4 transfected with *OVA* gene) [16] was obtained from ATCC (Manassas, VA) and maintained in complete RPMI supplemented with 400 μg/ml G418 (Wako, Osaka, Japan) in a humidified 5% CO_2 incubator at 37 °C.

2.3. Man3-DPPE and liposome preparation

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma–Aldrich (St. Louis, MO). Mannotriose (Man3: Manα1-6(Manα1-3)Man) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Man3-DPPE was prepared by conjugation of the mannotriose with DPPE by reductive amination as described in previous papers [10,18]. The purity of Man3-DPPE was confirmed by high-performance thin-layer chromatography (Silica gel 60 HPTLC plate, MERCK, Darmstadt, Germany) and time-of-flight mass spectrometry (Auto FLEX, Bruker Daltonics, Bremen, Germany). The purified Man3-DPPE was quantified by determination of the phosphate contained.

Liposomes were prepared as described previously [10]. Briefly, a chloroform–methanol (2:1, v/v) solution containing 1.5 μmol of DPPC and 1.5 μmol of cholesterol was placed in a conical flask and dried by rotary evaporation. Subsequently, 2 ml ethanol containing 0.15 μmol of Man3-DPPE was added to the flask and evaporated to prepare a lipid film containing neoglycolipids. Procedures for protein-encasing of oligomannose-coated liposomes (OMLs) were performed as described previously [10]. The multilamellar vesicles were generated with either 200 μl of FITC-labelled or non-labelled OVA (5.0 mg/ml, Sigma–Aldrich), Alexa Fluor 680 (Molecular Probes, Eugene, OR)-labelled bovine serum albumin (BSA, 5 mg/ml, Sigma–Aldrich), or PBS in the dried lipid film by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through polycarbonate membranes of 1 μm pore (Nucleopore, Pleasanton, CA). Liposomes entrapping proteins were separated from free untrapped proteins by four successive rounds of washing in PBS with centrifugation (20,000*g*, 30 min) at 4 °C. The amounts of entrapped proteins were measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL) in the presence of 0.3% (w/v) sodium dodecyl sulfate using BSA as the standard.

2.4. Flow cytometry

One hour after intraperitoneal injection, peritoneal exudate cells (PEC) were recovered from B6 mice with 5 ml ice cold PBS. PEC were incubated on ice for 30 min with fluorescein-labelled antibodies against mouse hematopoietic cell lineage markers after blocking with mouse Fc Blocker (BD Biosciences, San Jose, CA) and then analysed on a FACS Calibur (BD Biosciences). The following monoclonal antibodies used in this study were purchased or kindly provided: anti-F4/80 (A3-1, Serotec Ltd., Oxford, UK), anti-MHC class II (M5/114.15.2, e-Bioscience, Boston, MA), anti-CD11b (M1/70.15, Caltag Laboratories, Burlingame, CA), anti-CD3ε (145-2C11, BD Biosciences), anti-CD19 (1D3, BD Biosciences), and anti-H-2K^{b}D^b (20-8-4S, Dr. E. Nakayama, Okayama University).

2.5. Macrophage depletion by plastic adhesion

PEC suspension (2×10^7 cells in 10 ml of complete RPMI) was poured into a 75 cm^2 tissue culture flask and incubated at 37 °C for 2 h in a humidified 5% CO_2 incubator. Non-adherent cells were collected with serum-free DMEM and subjected to FACS analysis.

2.6. In vitro activation of OVA-specific T cells

One hour after injection of either OML-encased OVA or bare OVA into the peritoneal cavity of B6 mice, PEC were recovered with 5 ml ice cold PBS. The PEC suspended in complete RPMI were seeded into a 96-well culture plate (5×10^5 cells in each well) and incubated at 37 °C for overnight in a humidified 5% CO_2 incubator. On the next day, non-adherent cells were washed out with complete RPMI, and co-cultured with 5×10^5 cells of either CD8$^+$ or CD4$^+$ T cells from the spleen of OT-I and OT-II mice, respectively. CD8$^+$ and CD4$^+$ T cells were prepared with the isolation kits for corresponding subsets (Miltenyi Biotec Inc., Auburn, CA). The supernatants were collected at 24, 48, and 72 h and assayed for IFN-γ production with Mouse IFN-γ ELISA kit (Pierce Biotechnology, Inc., Rockford, IL).

2.7. CTLs assay

B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA in liposome/mouse with or without oligomannose coating. Spleen cells were isolated from the mice 1 week after the last challenge, and 1×10^6 spleen cells were stimulated with 10 μg OVA in 1 ml for 72 h. The effector cells thus prepared were co-cultured with target cells (E.G7-OVA or EL4) at various effector/target ratios for 8 h at 37 °C, and the cytotoxicity was measured with CytoTox96 Non-Radioactive Cytotoxicity assay kit (Promega, Madison, WI).

2.8. Tumor assay

Tumor cells (in 0.2 ml) were injected intradermally into the backs of mice with a 27 gauge needle. The diameter of the tumors was measured with Vernier calipers twice at right angles to calculate the mean diameter, and the survival time after tumor challenges was followed.

3. Results

3.1. OMLs are taken up preferentially by intraperitoneal macrophages

We showed that neoglycolipid-coated liposomes are ingested by intraperitoneal cells much more effectively than non-coated liposomes [10]. Of those, OMLs are incorporated most effectively, and the cells ingesting

OMLs are preferentially F4/80$^+$ and migrate into extranodal lymphoid tissues in the omentum after the uptake. We have also shown that the OML-ingesting cells are very useful drug delivery vehicles for cancer chemotherapy in the previous study [9,10]. To verify whether the OMLs are applicable also for cancer immunotherapy, we first analyzed in detail the peritoneal cells incorporating OMLs. Bovine serum albumin (BSA, Sigma–Aldrich) was labelled with Alexa Fluor 680, encased in OML and then injected into the peritoneal cavity of B6 mice. One hour after the injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and analyzed. As shown in Fig. 1A, PEC were divided into three groups based on the incorporation of OMLs. When adherent cells were removed by plastic dish adherence, only the population with higher OML uptake (R1) disappeared (Fig. 1B). In addition, most cells of R1 express F4/80 and CD11b but not CD3 and CD19 (Fig. 1C), suggesting that R1 population preferentially consists of macrophages (Mϕ). The PEC with lower OML uptake (R2) did not express F4/80, and nearly 2/3 of them were considered to be B cells because of their CD19 expression. These results together confirmed that OMLs injected into the peritoneal cavity were ingested preferentially by Mϕ, and also indicate that OML is a good vehicle for the phagocytosis of non-glycosylated proteins.

3.2. *Phagocytic cells ingesting OMLs activate both CD8 and CD4 T cells in vitro in an antigen-specific manner*

We next analyzed the antigen-presenting capacity of the phagocytic cells ingesting OMLs containing ovalbumin (OVA) as an antigen. CD8$^+$ T cells from OT-I (a transgenic strain of T cell receptor (TCR) recognizing $OVA_{257-264}$ peptide presented by H-2K^b) and CD4$^+$ T cells from OT-II (a transgenic strain of TCR recognizing $OVA_{323-339}$ peptide presented by H-2A^b) were used as responder cells. When these T cells were co-cultured with adherent cells enriched from PEC of the mouse intraperitoneally injected with OML-encased OVA (OML-OVA), both CD8$^+$ and CD4$^+$ T cells produced large amounts of IFN-γ (Fig. 2). Though adherent cells from the mice injected with soluble OVA also stimulated both CD8$^+$ and CD4$^+$ T cells, much higher amounts of OVA were needed compared to those from the mice injected with OML-OVA. Mϕ ingesting OML-OVA are supposed to

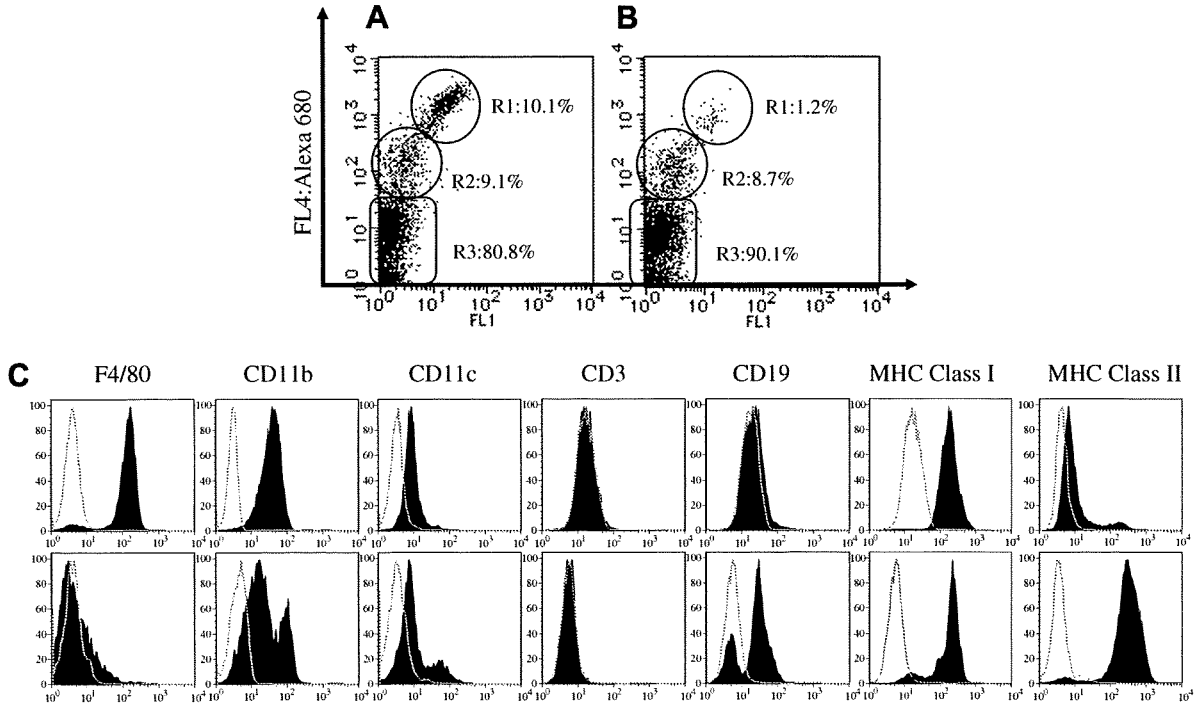

Fig. 1. OMLs injected into the peritoneal cavity were ingested preferentially by adhesive cells. One hour after injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and their fluorescence was analyzed by flow cytometry (A). Non-adherent PEC were further isolated by plastic adherence for 2 h and analyzed (B). (C) Phenotypic analysis of PEC derived from OML-injected mice. One hour after injection of OMLs containing Alexa Fluor 680-labelled OVA, PEC were collected and stained with mAbs indicated. As shown in (A) and (B), PEC were divided into three groups based on their fluorescence intensity of Alexa Fluor 680, and the surface phenotypes of R1 and R2 were further analyzed.

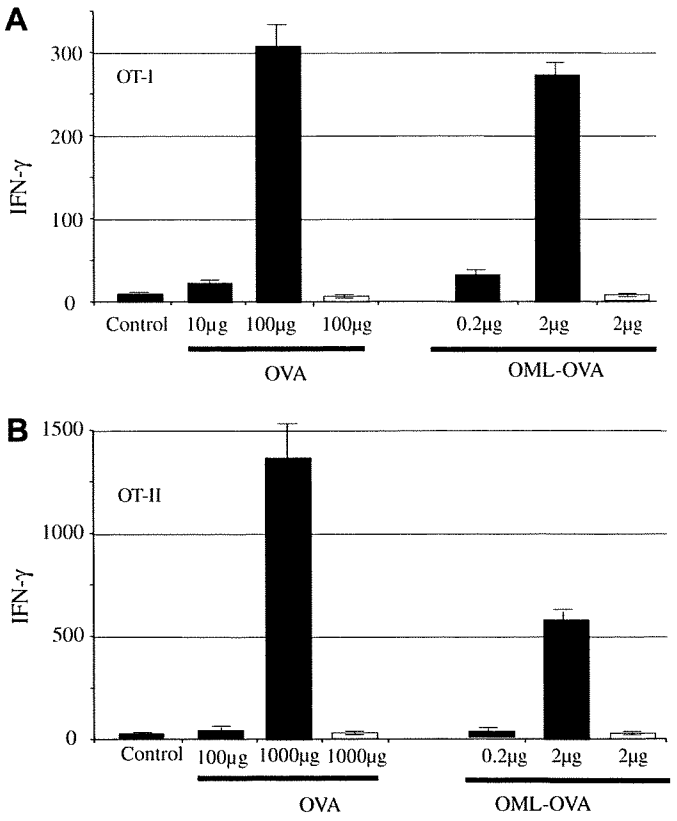

Fig. 2. Mϕ ingesting OVA encased in OML activate OVA-specific $CD8^+$ and $CD4^+$ T cells much more effectively than those ingesting soluble OVA. One hour after intraperitoneal injection of antigens, PEC were prepared from mice, and adherent cells were enriched by plastic adherence. $CD8^+$ and $CD4^+$ T cells were purified from spleen of OT-I (A) and OT-II (B), respectively, co-cultured (closed bar) with adherent PEC or cultured adherent PEC alone (open bar) for 24 h, and then production of IFN-γ in supernatants was tested by ELISA. PEC recovered from mice without any treatment was used as control. OML-OVA, OVA encased in OML; OVA, OVA; control, OML containing PBS. The graph shows the average and standard error from three independent experiments.

present antigen effectively also *in vivo*, as they effectively induced proliferation responses of OVA-specific $CD8^+$ T cells in the spleens of OT-I mice (Supplement Figure 1).

We next analyzed the uptake efficiency of OML-encased and soluble OVA and found that peritoneal phagocytic cells effectively uptake OVA irrespective of sugar encapsulation (Fig. 3). The uptake of soluble OVA is probably mediated by mannose receptors, as it is known as a highly mannosylated protein [19]. These results together indicate that OML-mediated ingestion promotes the presentation of OVA peptides by both MHC class I and II molecules by enhancing the antigen processing but not the uptake efficiency.

3.3. Induction of antigen-specific cytotoxic T lymphocytes (CTL) in vitro by OML-mediated immunization

We next performed CTL assay to detect OVA-specific T cells in the spleen. Only the spleen cells from mice immunized with OML-OVA but not bare liposome (BL)-encased OVA showed cytotoxicity against E.G7-OVA. The spleen cells from neither group showed cytotoxicity against EL4, confirming that OVA-specific CTL can be effectively induced *in vivo* by OML-OVA immunization (Fig. 4).

3.4. OML-mediated immunization induces antigen-specific anti-tumor immunity in vivo

We finally examined whether intraperitoneal immunization with OMLs also induces antigen-specific anti-tumor immunity *in vivo*. Mice were immunized intraperitoneally with OVA with or without OML encasing and then challenged with E.G7-OVA or EL4. As shown in Fig. 5, only the mice immunized with OML-OVA survived for more than 70 days when challenged with E.G7-OVA, while naïve and bare OVA-immunized mice died within 55 days. All the mice including those immunized with OML-OVA died within 30 days when challenged with EL4, indicating that the rejection of

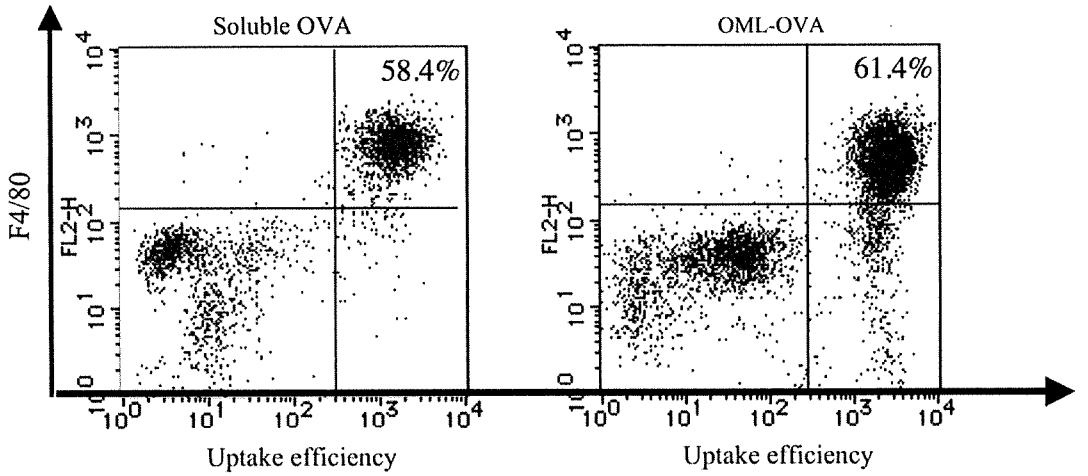

Fig. 3. Peritoneal F4/80$^+$ cells uptake OVA effectively irrespective of carbohydrate encapsulation. One hour after injection of either soluble FITC-OVA (20 μg) or OML-encapsulated FITC-OVA (20 μg) into the peritoneal cavity of B6 mice, uptake efficiency of FITC-OVA by peritoneal cells was analyzed by flow cytometry together with F4/80 expression.

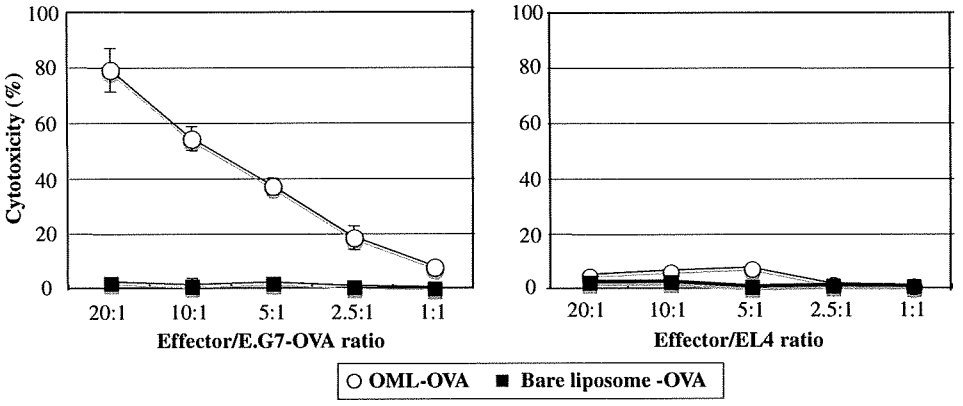

Fig. 4. OML-OVA-generated OVA-specific cytotoxicity. B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA encased in oligomannose-coated (OML-OVA, open symbols) or bare liposomes (bare liposome-OVA, closed symbols). Spleen cells were isolated from mice one week after the last challenge, and 1×10^6 cells were stimulated with 10 μg OVA in 1 ml for 72 h. The graph shows the average and standard error from three independent experiments.

E.G7-OVA is OVA-specific. These results together showed that OML-mediated immunization can induce systemic immune response robust enough to protect mice from tumor challenge in an antigen-specific manner.

4. Discussion

In this study, we demonstrated that our novel OML-based drug delivery system (DDS) targeted to intraperitoneal phagocytic cells can also be used for the induction of systemic immune responses. After ingesting OML-encased OVA (OML-OVA), intraperitoneal phagocytic cells to extra-nodal lymphoid tissues in abdominal cavity and presented OVA-derived peptides in the context of both MHC class I and II molecules. Only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 challenged subcutaneously. These results together indicate that the OMLs can be used as an effective antigen delivery system for immunotherapy activating both CTL and Th subsets. Fig. 6 shows the plausible induction process of anti-tumor immunity starting from phagocytic cells triggered by OML injection.

OMLs are very useful not only for the promotion of non-glycosylated protein uptake by antigen-presenting cells but also for the enhancement of antigen-processing of encased antigens. Endogenous

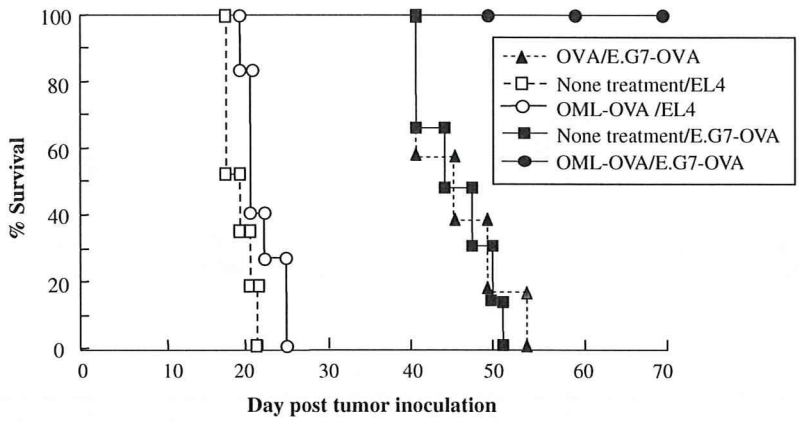

Fig. 5. OML-mediated immunization induces antigen-specific anti-tumor immunity *in vivo*. OML-OVA-immunized (circles) and naïve (squares) mice were challenged with E.G7-OVA (closed circles and squares) or EL4 cells (open circles and squares). As a control, mice were immunized with soluble OVA and challenged with E.G7-OVA (closed triangles).

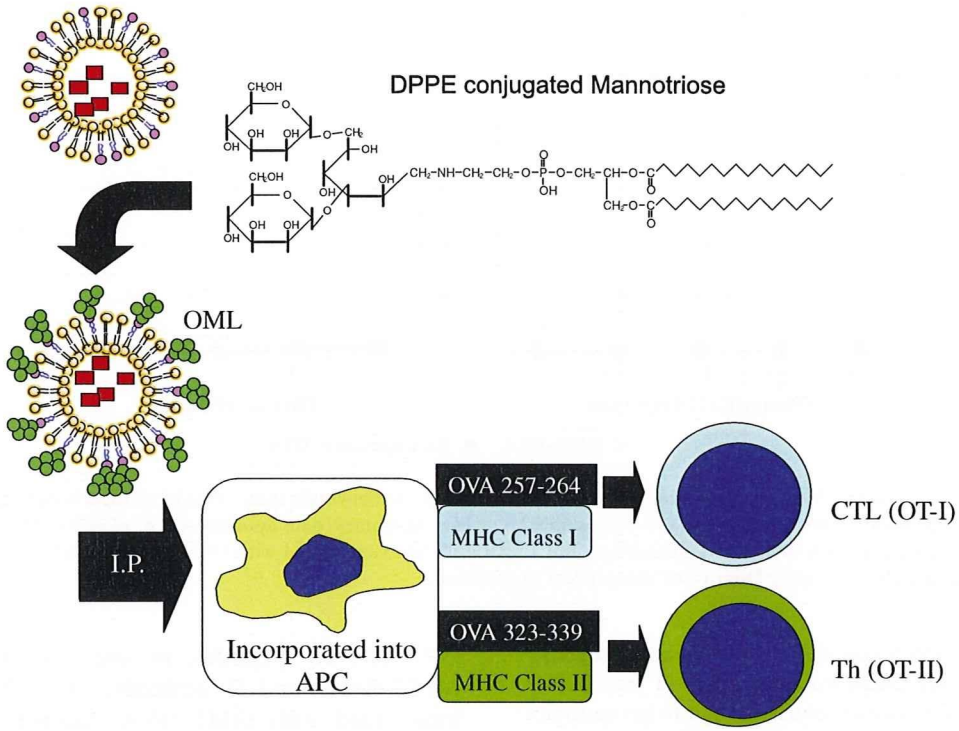

Fig. 6. Possible mechanism of OML-based vaccine delivery. Coating of bare liposomes with DPPE-conjugated mannotriose facilitates functions of intraperitoneal macrophages, resulting in antigen-specific activation of both CTL and Th populations.

and exogenous antigens are presented preferentially by MHC class I and II, respectively. OML-OVA, however, were effectively directed to both pathways, even when added exogenously. This advantage of OML-mediated immunization will hopefully facilitate the simultaneous activation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells as shown here with OVA. It is also very interesting to study the mechanism by which OML-mediated ingestion of antigens enhances the antigen presentation by both MHC molecules [20–22]. So far, we observed the up-regulation of CD80 and CD86 on OML-ingesting cells (in press on Cytokine, H. Takagi et al.), but it seems very important to know the additional signals to

make antigen presenting cells more immunogenic [23]. Additional adjuvant effects of various cytokines and/or toll-like receptor ligands on OML-mediated immunization are now being investigated.

Another advantage of OML-mediated immunization is Th1-skewing of the cytokine profiles. Indeed, OT-I and OT-II T cells stimulated with antigen-presenting cells ingesting OML-OVA produced IFN-γ but not IL-4 or IL-10 (unpublished observation). Moreover, our previous study demonstrated that the OML-mediated immunization protects BALB/c mice against *Leishmania major* infection, possibly due to the Th1-skewing of immune responses [24]. We observed that phagocytic cells ingesting OML preferentially produce IL-12 (unpublished observation), suggesting this cytokine is a key of Th1-skewing as reported previously [25]. Further investigation of the mechanism of this Th1-skewing of immune responses induced by OML-mediated immunization is currently underway.

Cells belonging to the monocyte-Mϕ lineage have been known to be heterogeneous, reflecting the plasticity and versatility of these cells in response to various microenvironmental signals [26]. Mϕ are now roughly categorized into M1 and M2 based on their functional properties, and several studies revealed that M1 and M2 promote type I and type II Th responses, respectively [27–29]. It is also reported that M1 and M2 are prone to induce inflammatory and immunoregulatory responses, respectively [29]. A possible concern of our DDS system is therefore the protumoral effects by M2 with antigen-encased OMLs, as they are supposed to express macrophage mannose receptors induced by IL-4 [30]. Although at least our *in vitro* study clearly showed OML-mediated skewing to type I immune responses, more precise investigation including the conditions for M1 polarization should be done especially in tumor-bearing mice. In addition, characteristics of the small population of non-Mϕ cells ingesting OMLs should be investigated as well.

In order to use our DDS in clinical study, the best administration routes should be determined to pursue repetitive vaccination while avoiding possible side effects. As generally acknowledged, intraperitoneal administration is accompanied with a high risk of side effects such as catheter-related complications, and abdominal pain [31]. In this connection, we have already obtained anti-tumor effects by subcutaneous injection of OML-OVA similar to those by intraperitoneal injection. However, side effects induced by subcutaneous injection of OMLs should be further investigated to assure their safe clinical application.

In the previous study, we reported that the formation of intraperitoneal metastasis of seeded gastric cancer cells in milky spots can be controlled with OMLs containing anti-cancer drugs [10]. In the present study, we have further extended the possibility of OMLs for the immunotherapy of systemic metastasis and existing tumor cells aside from milky spots. Oligomannose coating of liposomes showed the best uptake efficiency by intraperitoneal Mϕ among the neoglycolipids so far tested, and the encased antigen was effectively presented by both MHC class I and II molecules. However, the additional effects for immune responses by other neoglicolipids (shown here) have not been studied at all so far. We have a great interest in their effects on immune responses and are seeking sugar materials with immunoregulatory properties. If such materials are found, we believe that further study of our sugar-coated liposome technology will find it also to be applicable for antigen-specific regulation of autoimmune diseases and allergy.

Acknowledgments

We thank Ms. Y. Matsudaira and Ms. K. Nishida for their excellent technical assistance. This work was supported by the Industrial Technology Research Grant Program in 04' from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and in part by a grant for Hi-tech research program from Tokai University and by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2007.10.038.

References

[1] L. Gattinoni, D.J. Powell Jr., S.A. Rosenberg, N.P. Restifo, Adoptive immunotherapy for cancer: building on success, Nat. Rev. Immunol. 6 (2006) 383–393.

[2] S.A. Rosenberg, J.C. Yang, N.P. Restifo, Cancer immunotherapy: moving beyond current vaccines, Nat. Med. 10 (2004) 909–915.
[3] S.A. Rosenberg, Progress in the development of immunotherapy for the treatment of patients with cancer, J. Intern. Med. 250 (2001) 462–475.
[4] T. Boon, P.G. Coulie, B.J. Van den Eynde, P. van der Bruggen, Human T cell responses against melanoma, Annu. Rev. Immunol. 24 (2006) 175–208.
[5] D.M. Pardoll, S.L. Topalian, The role of CD4+ T cell responses in antitumor immunity, Curr. Opin. Immunol. 10 (1998) 588–594.
[6] R.F. Wang, G. Peng, H.Y. Wang, Regulatory T cells and toll-like receptors in tumor immunity, Semin. Immunol. 18 (2006) 136–142.
[7] S. Sakaguchi, R. Setoguchi, H. Yagi, T. Nomura, Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease, Curr. Top. Microbiol. Immunol. 305 (2006) 51–66.
[8] A.M. Leen, C.M. Rooney, A.E. Foster, Improving T cell therapy for cancer, Annu. Rev. Immunol. 25 (2007) 243–265.
[9] Y. Ikehara, N. Kojima, Development of a novel oligomannose-coated liposome-based anticancer drug-delivery system for intraperitoneal cancer, Curr. Opin. Mol. Ther. 9 (2007) 53–61.
[10] Y. Ikehara, T. Niwa, L. Biao, S.K. Ikehara, N. Ohashi, T. Kobayashi, Y. Shimizu, N. Kojima, H. Nakanishi, A carbohydrate recognition-based drug delivery and controlled release system using intraperitoneal macrophages as a cellular vehicle, Cancer Res. 66 (2006) 8740–8748.
[11] L.F. Krist, M. Kerremans, D.M. Broekhuis-Fluitsma, I.L. Eestermans, S. Meyer, R.H. Beelen, Milky spots in the greater omentum are predominant sites of local tumour cell proliferation and accumulation in the peritoneal cavity, Cancer Immunol. Immunother. 47 (1998) 205–212.
[12] A. Hagiwara, T. Takahashi, K. Sawai, H. Taniguchi, M. Shimotsuma, S. Okano, C. Sakakura, H. Tsujimoto, K. Osaki, S. Sasaki, et al., Milky spots as the implantation site for malignant cells in peritoneal dissemination in mice, Cancer Res. 53 (1993) 687–692.
[13] K.A. Hogquist, S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, F.R. Carbone, T cell receptor antagonist peptides induce positive selection, Cell 76 (1994) 17–27.
[14] S.R. Clarke, M. Barnden, C. Kurts, F.R. Carbone, J.F. Miller, W.R. Heath, Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection, Immunol. Cell Biol. 78 (2000) 110–117.
[15] M.J. Barnden, J. Allison, W.R. Heath, F.R. Carbone, Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements, Immunol. Cell Biol. 76 (1998) 34–40.
[16] M.W. Moore, F.R. Carbone, M.J. Bevan, Introduction of soluble protein into the class I pathway of antigen processing and presentation, Cell 54 (1988) 777–785.
[17] P.A. Gorer, Studies in antibody response of mice to tumour inoculation, Br. J. Cancer 4 (1950) 372–379.
[18] T. Mizuochi, R.W. Loveless, A.M. Lawson, W. Chai, P.J. Lachmann, R.A. Childs, S. Thiel, T. Feizi, A library of oligosaccharide probes (neoglycolipids) from *N*-glycosylated proteins reveals that conglutinin binds to certain complex-type as well as high mannose-type oligosaccharide chains, J. Biol. Chem. 264 (1989) 13834–13839.
[19] T. Tai, K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, A. Kobata, Structural studies of two ovalbumin glycopeptides in relation to the endo-beta-*N*-acetylglucosaminidase specificity, J. Biol. Chem. 250 (1975) 8569–8575.
[20] M.C. Tan, A.M. Mommaas, J.W. Drijfhout, R. Jordens, J.J. Onderwater, D. Verwoerd, A.A. Mulder, A.N. van der Heiden, T.H. Ottenhoff, M. Cella, A. Tulp, J.J. Neefjes, F. Koning, Mannose receptor mediated uptake of antigens strongly enhances HLA-class II restricted antigen presentation by cultured dendritic cells, Adv. Exp. Med. Biol. 417 (1997) 171–174.
[21] A. Lanzavecchia, Mechanisms of antigen uptake for presentation, Curr. Opin. Immunol. 8 (1996) 348–354.
[22] F. Sallusto, M. Cella, C. Danieli, A. Lanzavecchia, Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products, J. Exp. Med. 182 (1995) 389–400.
[23] J.S. Lam, M.K. Mansour, C.A. Specht, S.M. Levitz, A model vaccine exploiting fungal mannosylation to increase antigen immunogenicity, J. Immunol. 175 (2005) 7496–7503.
[24] Y. Shimizu, K. Yamakami, T. Gomi, M. Nakata, H. Asanuma, T. Tadakuma, N. Kojima, Protection against *Leishmania major* infection by oligomannose-coated liposomes, Bioorg. Med. Chem. 11 (2003) 1191–1195.
[25] G. Trinchieri, Interleukin-12 and the regulation of innate resistance and adaptive immunity, Nat. Rev. Immunol. 3 (2003) 133–146.
[26] F.O. Martinez, S. Gordon, M. Locati, A. Mantovani, Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression, J. Immunol. 177 (2006) 7303–7311.
[27] S. Gordon, Alternative activation of macrophages, Nat. Rev. Immunol. 3 (2003) 23–35.
[28] A. Mantovani, A. Sica, M. Locati, Macrophage polarization comes of age, Immunity 23 (2005) 344–346.
[29] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, Trends Immunol. 25 (2004) 677–686.
[30] M. Stein, S. Keshav, N. Harris, S. Gordon, Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation, J. Exp. Med. 176 (1992) 287–292.
[31] S.A. Cannistra, Intraperitoneal chemotherapy comes of age, N. Engl. J. Med. 354 (2006) 77–79.

Alternative splicing due to an intronic SNP in *HMSD* generates a novel minor histocompatibility antigen

Takakazu Kawase,[1,2] Yoshiki Akatsuka,[1] Hiroki Torikai,[1] Satoko Morishima,[1,2] Akira Oka,[3] Akane Tsujimura,[4] Mikinori Miyazaki,[5] Kunio Tsujimura,[1] Koichi Miyamura,[3] Seishi Ogawa,[6,7] Hidetoshi Inoko,[4] Yasuo Morishima,[8] Yoshihisa Kodera,[4] Kiyotaka Kuzushima,[1] and Toshitada Takahashi[1,2]

[1]Division of Immunology, Aichi Cancer Center Research Institute, Aichi; [2]Department of Cancer Genetics, Nagoya University Graduate School of Medicine, Nagoya; [3]Department of Genetic Information, Division of Molecular Life Science, Tokai University School of Medicine, Isehara; [4]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya; [5]Department of Internal Medicine & Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya; [6]Core Research for Evolutional Science and Technology (CREST) of Japan, Science and Technology Corporation (JST), Saitama; [7]Department of Regeneration Medicine for Hematopoiesis, Graduate School of Medicine, University of Tokyo, Tokyo; [8]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan

Here we report the identification of a novel human leukocyte antigen (HLA)-B44–restricted minor histocompatibility antigen (mHA) with expression limited to hematopoietic cells. cDNA expression cloning studies demonstrated that the cytotoxic T lymphocyte (CTL) epitope of interest was encoded by a novel allelic splice variant of *HMSD,* hereafter designated as *HMSD-v.* The immunogenicity of the epitope was generated by differential protein expression due to alternative splicing, which was completely controlled by 1 intronic single-nucleotide polymorphism located in the consensus 5′ splice site adjacent to an exon. Both *HMSD-v* and *HMSD* transcripts were selectively expressed at higher levels in mature dendritic cells and primary leukemia cells, especially those of myeloid lineage. Engraftment of mHA$^+$ myeloid leukemia stem cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID)/γc^{null} mice was completely inhibited by in vitro preincubation with the mHA-specific CTL clone, suggesting that this mHA is expressed on leukemic stem cells. The patient from whom the CTL clone was isolated demonstrated a significant increase of the mHA-specific T cells in posttransplantation peripheral blood, whereas mHA-specific T cells were undetectable in pretransplantation peripheral blood and in peripheral blood from his donor. These findings suggest that the *HMSD-v*–encoded mHA (designated ACC-6) could serve as a target antigen for immunotherapy against hematologic malignancies. (Blood. 2007; 110:1055-1063)

Introduction

Minor histocompatibility antigens (mHAs) are major histocompatibility complex (MHC)-bound peptides derived from cellular proteins encoded by polymorphic genes. Following human leukocyte antigen (HLA)-matched allogeneic hematopoietic cell transplantation (HCT), donor-recipient disparities in mHAs can induce a favorable graft-versus-leukemia (GVL) effect that is often associated with graft-versus-host disease (GVHD).[1-3] Significant efforts have been made to identify mHAs, particularly those specific for hematopoietic cells, since such mHAs are speculated to contribute to the GVL effect. The first report on the identification of a hematopoietic lineage-specific mHA, HA-1, was generated by the Goulmy group in 1998 (den Haan et al[4]) as a result of biochemical analysis of peptides eluted from HLA-A*0201 molecules. The only other mHAs with selective expression in hematopoietic cells described to date are HA-2[5]; ACC-1 and ACC-2[6]; and DRN-7,[7] HB-1,[8,9] and PANE1,[10] the latter 2 of which are B-cell lineage-specific. Thus, identification of more mHAs should facilitate a better understanding of the biology of GVL and the development of effective immunotherapy to induce GVL reactions.

Immunogenicity of most autosomal mHAs identified to date results from single-nucleotide polymorphisms (SNPs) that cause amino-acid substitutions within epitopes, leading to the differential display/recognition of peptides between HCT donor and recipient via several mechanisms: peptide binding to MHC observed in HA-1/A2-,[4] HA-2-,[5] and *CTSH*-encoded mHAs[11]; proteasomal cleavage in HA-3[12]; peptide transport in HA-8[13]; and altered recognition of MHC-peptide complex by cognate T cells in HB-1,[8,9] HA-1/B60,[14] ECGF1/B7,[15] and SP110/A3.[7] Other examples of mechanisms of mHA generation include differential protein expression due to a nonsense mutation in *PANE1*[10] and a frame-shift mutation in *P2X5*.[16] *UGT2B17*[17] is the sole example of differential protein expression due to gene deletion instead of an SNP. Because SNPs are scattered throughout the genome, it has been speculated that mHAs caused by those other than coding SNPs should be present.

In this study, we report the identification of a novel gene encoding an HLA-B44–restricted mHA that is recognized by the 2A12 cytotoxic T lymphocyte (CTL) clone and selectively expressed in primary hematologic malignant cells, especially those of myeloid lineage, multiple myeloma (MM) cells, and normal mature dendritic cells (DCs). The antigenic peptide recognized by 2A12-CTL was encoded by a novel allelic splice variant of *HMSD,*

Submitted February 26, 2007;accepted April 2, 2007. Prepublished online as *Blood* First Edition paper, April 4, 2007; DOI 10.1182/blood-2007-02-075911

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

hereafter designated as *HMSD-v*, due to an intronic SNP located in the consensus 5′ splice site adjacent to an exon. The leukemic stem cell (LSC) engraftment assay using severely immunodeficient mice demonstrated that the engraftment of primary acute myeloid leukemia (AML) cells was completely abolished by coincubation with the CTL clone before injection. These findings suggest that this novel mHA epitope may be an attractive therapeutic target for immunotherapy.

Patients, materials, and methods

Cell isolation and cell cultures

This study was approved by the Institutional Review Board of Aichi Cancer Center. All blood or tissue samples were collected after written informed consent was obtained in accordance with the Declaration of Helsinki. B-lymphoid cell lines (B-LCLs) were derived from donors, recipients, and healthy volunteers. B-LCLs and all cell lines of hematologic malignancy were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (referred to as complete medium). CD40 ligand-activated B (CD40-B) cells were generated as previously described.[18]

Immature DCs were generated by culturing $CD14^+$ cells isolated from peripheral-blood mononuclear cells (PBMCs) with 500 U/mL GM-CSF and 500 U/mL interleukin 4 (IL-4) in AIM-V medium (Invitrogen, Carlsbad, CA) for 2 days, and then DCs were matured by cultivating the immature DCs for 2 additional days with 10 ng/mL IL-1β, 20 ng/mL IL-6, 10 ng/mL tissue necrosis factor α (TNF-α; all cytokines were from R&D Systems, Minneapolis, MN), and 1 μg/mL PGE2 (Cayman Chemical, Ann Arbor, MI). When necessary, cells were retrovirally transduced with restricting HLA cDNA by a method described previously.[18,19]

Generation of CTL lines and clones

CTL lines were generated from PBMCs ($\sim 10^6$) obtained at day 197 after HCT by primary stimulation with irradiated (33 Gy) pre-HCT recipient PBMCs ($\sim 10^6$), thereafter stimulated weekly with irradiated (33 Gy) recipient CD40-B cells (2×10^6) twice in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine (referred to as CTL medium).[11] IL-2 was added on days 1 and 5 after the second and third stimulation. CTL clones were isolated by standard limiting dilution and expanded in CTL medium as previously described.[11,20]

Chromium release assay

Target cells were labeled with 3.7 MBq of ^{51}Cr for 2 hours, and 10^3 target cells/well were mixed with CTLs at the effector-target (E/T) ratio indicated in a standard 4-hour cytotoxicity. All assays were performed at least in duplicate. Some target cells were pretreated with interferon γ (IFN-γ; 500 U/mL) and TNF-α (10 ng/mL; both from R&D Systems) for 48 hours as indicated. Percent specific lysis was calculated as follows: ([experimental cpm − spontaneous cpm]/[maximum cpm − spontaneous cpm]) × 100, where cpm indicates counts per minute.

cDNA library construction

The cDNA library used in the present study was the same one that had been used to identify HLA-A31- and HLA-A33-restricted *cathepsin H*-encoded mHAs (ACC-4 and ACC-5) previously.[11] The cDNA library was constructed from mRNA of a B-LCL derived from an AML patient (UPN-027) using the SuperScript Plasmid System (Invitrogen). The library contained 1.5×10^6 cDNA clones with an average insert size of approximately 2500 bp. cDNA pools, each consisting of approximately 120 and 5 clones for initial and second screens, respectively, were expanded for 24 hours in 96 deep-well plates, and plasmid DNA was extracted with the QIAprep 96 Turbo Miniprep kit (Qiagen, Valencia, CA).

Transfection of 293T cells and ELISA

Twenty thousand 293T cells retrovirally transduced with HLA-B*4403 were plated in each well of 96-well flat-bottomed plates, cultured overnight at 37°C, then transfected with 0.12 μg of plasmid containing a pool of the cDNA library using Trans IT-293 (Mirus, Madison, WI). Ten thousand CTL-2A12 cells were added to each well 20 hours after transfection. After overnight incubation at 37°C, 50 μL of supernatant was collected and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA).

Genotyping of polymorphisms

Genomic DNA was isolated from each B-LCL with a QIAamp DNA blood kit (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized by standard methods. Genomic DNA or cDNA was amplified using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The polymerase chain reaction (PCR) temperature profile was 30 cycles of 94°C for 15 seconds, 58°C for 20 seconds, and 68°C for 40 seconds on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

The primer sequences used to amplify from exon 1 to exon 4 of *HMSD* cDNA were as follows: sense, 5′-CCTCTCCGACCCGGTCTC-3′; antisense, 5′-GGGAAAAGCTAAAGCTAGAGAAAA-3′. Exonic sequence and intronic sequence adjacent to *HMSD* exon 1 and 2 were amplified with primers as follows: exon 1 sense, 5′-GACTGAAAACTCCCGGACAG-3′; exon 1 antisense, 5′-GAAAGGTCTGGAGCAACAGG-3′; exon 2 sense, 5′-GCAGACATTCACTCACAGCA-3′; exon 2 antisense, 5′-AAGCACCCACATGAGTGACC-3′. PCR products were purified and directly sequenced with the same primer.

Construction of minigenes and truncated genes for *HMSD-v*

Mammalian expression plasmids containing the full-length or truncated forms of the *HMSD-v* cDNA were constructed by reverse transcriptase (RT)-PCR using the isolated cDNA clone as a template. The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAA). All products were ligated into *Hin*dIII-*Not*I-cut pEAK10 vector (Edge Bio Systems, Gaithersburg, MD) and verified by sequencing.

Epitope reconstitution assay

The candidate *HMSD*-encoded epitopes were synthesized by standard Fmoc chemistry. ^{51}Cr-labeled donor B-LCLs were incubated for 30 minutes in complete medium containing 10-fold serial dilutions of the peptides and then used as targets in standard cytotoxicity assays.

Real-time PCR assay for *HMSD* and *HMSD-v* expression

cDNAs were prepared from various hematologic malignant cell lines, primary cell cultures, freshly isolated $CD34^+$ bone marrow (BM) and peripheral-blood hematopoietic cells and their subpopulations, immature and mature DCs, activated B and T cells, $CD34^+$ subsets of primary leukemic cells, and $CD138^+$ subsets of primary MM cells. Cell sorting was performed using magnetic-activated cell separation (MACS) immunomagnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany). A panel of cDNA made from different human adult and fetal tissues was purchased (MTC panels human I and II; BD Biosciences, San Diego, CA). Real-time PCR analysis was performed using the TaqMan assay as described previously.[11] Because of uncertainty of which allele(s) were included in each cDNA pool from the MTC panels, quantitative PCR primers and a probe were designed to detect the exon 3-4 boundary, which is shared by both alleles. The following sequences spanning the exon 3-4 boundary were used as primers with TaqMan probe to detect both *HMSD* and *HMSD-v* transcripts simultaneously: sense, 5′-AGAACTGCCAACGGGCTCTT-3′; antisense, 5′-TTGGTAGAATTTGCCACAGGAAT-3′; probe, 5′-(FAM)-CTTATGATTTCCTCACAGGTT-(MGB)-3′. To selectively detect *HMSD-v* transcripts, the following oligonucleotides specific for the exon 1-3 boundary were used: sense, 5′-CTCCGACCCGGTCTCACTT-3′; antisense, 5′-TCTCCATCTTCACCTCCGATTT-3′; probe, 5′-(FAM)-CAAAGTGCCCCAGTTC-(MGB)-3′.

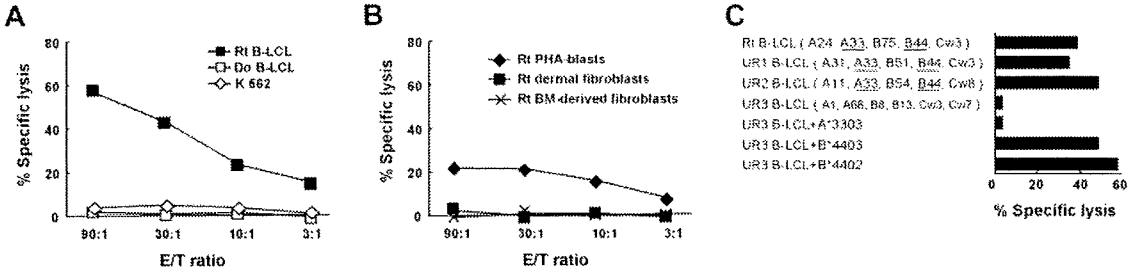

Figure 1. Specificity of the HLA-B44–restricted CTL clone 2A12. The cytolytic activity of CTL-2A12 was evaluated in a standard 4-hour ^{51}Cr release assay at the indicated E/T ratios. (A) CTL-2A12 recognition of target cells derived from recipient (Rt) but not donor (Do) B-LCLs. NK-sensitive K562 cells were used to determine nonspecific lysis. (B) CTL-2A12 recognition of Rt PHA-stimulated T cells (PHA blasts) but not of Rt dermal fibroblasts and bone marrow (BM)-derived fibroblasts pretreated with 500 U/mL IFN-γ and 10 ng/mL TNF-α for 48 hours before ^{51}Cr labeling. (C) CTL-2A12 recognition of an HLA-B*4403- and -B*4402-restricted mHA epitope. The following target cells were tested: Rt B-LCL, B-LCLs of 2 unrelated individuals (UR1 and UR2) sharing an HLA-A33, B44 haplotype with the recipient, and B-LCLs of an HLA class I-mismatched individual (UR3) that were transduced with either HLA-A*3303, B*4403, or B*4402 (E/T ratio, 30:1).

CD45 mRNA expression was detected as described previously.[21] A primer and probe set for human *GAPDH* (Applied Biosystems) was used as an internal control. PCR was performed according to the manufacturer's instructions in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). Samples were quantified using relative standard curves for each experiment. All results were normalized with respect to the internal control and are expressed relative to the levels found in recipient B-LCLs.

LSC engraftment assay of AML cells in immunodeficient NOG mice

BM cells were obtained from patients with AML at diagnosis and then positively selected for $CD34^+$ subsets using MACS immunomagnetic beads (Miltenyi). NOD/Shi-scid, IL-2Rγc^{null} (NOG) mice[22] were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan). All mice were maintained under specific pathogen-free conditions in the Aichi Cancer Center Research Institute. The Ethical Review Committee of the Institute approved the experimental protocol. The ACC-2^D mHA-specific CTL clone $3B5^6$ restricted by the same HLA-B*4403 allele as CTL-2A12 was used as a control CTL clone for this assay. AML cells (7.0×10^6) were preincubated for 16 hours in CTL medium supplemented with 25 units/mL recombinant human IL-2 at 37°C with 5% CO_2 either alone or in the presence of CTL-2A12 or CTL-3B5 at a T-cell/AML cell ratio of 5:1. Thereafter, the cultures were harvested and resuspended in a total volume of 300 μL and were inoculated via the tail vein of 8- to 12-week-old NOG mice (3 mice per group). Five weeks after inoculation, mice were killed, peripheral blood was aspirated from the heart, and BM cells were obtained by flushing the femora with complete medium. Nucleated cells were prepared for flow cytometry by incubation at 4°C for 20 minutes in PBS and 2% FCS with antihuman CD45 and CD34 (all from BD Biosciences) and were analyzed with a FACSCalibur flow cytometer and CellQuest 3.3 software (BD Biosciences). Percentage of engraftment was examined by 1-way analysis of variance (ANOVA) test.

Real-time PCR assay for detecting CTLs specific for ACC-6, a newly identified mHA

Complementary DNAs for a standard curve were prepared from mixtures of ACC-6–specific CTL clone (CTL-2A12) at various ratios with $CD3^+$ cells from healthy donors, and cDNAs of peripheral blood $CD3^+$ cells from the donor and patient before and after HCT were prepared from the AML patient (UPN-027). Real-time PCR analysis was performed using a TaqMan assay as described in "Real-time PCR assay for *HMSD* and *HMSD-v* expression." The primers and fluorogenic probe sequences spanning the CTL-2A12 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-2A12. Samples were quantified with the comparative cycle threshold (C_T) method. The delta C_T value was determined by subtracting the average GAPDH C_T value from the average CTL-2A12 CDR3 C_T value. The standard curve for the proportion of CTL-2A12 among $CD3^+$ cells (Figure 7A) was composed by plotting mean delta C_T values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-2A12A were calculated by using this standard curve.

Results

Characterization of a CTL clone

The $CD8^+$ CTL clone 2A12 (CTL-2A12) was 1 of 24 putative CTL clones isolated from day-197 post-HCT PBMCs of a male with refractory AML with multilineage dysplasia (UPN-027) receiving an HLA-identical HCT from his brother (A*2402, A*3303, B75, B*4403, Cw3, DR4, DR6).[11] The patient developed grade 1 acute GVHD in the first 2 years after transplantation and then suffered from glomerular IgG deposition and mild bronchiolitis obliterans organizing pneumonia. He is alive and in good condition and has been disease free for more than 3 years.

Cytotoxicity assays revealed that CTL-2A12 lysed the recipient B-LCL and less efficiently phytohemagglutinin (PHA)-stimulated T-cell blasts but not donor B-LCL or natural killer (NK)-sensitive K562 cells (Figure 1A,B). No cytotoxicity was observed against the recipient's dermal fibroblasts and BM-derived fibroblasts even after treatment with IFN-γ and TNF-α (Figure 1B). Cytotoxicity against recipient B-LCL was blocked by anti-HLA class I antibody (Ab) but not by anti-HLA-DR Ab, suggesting HLA class I-restricted recognition of mHA (data not shown). Based on the screening results of a panel of B-LCLs derived from individuals partially sharing HLA class I alleles with the recipient (Figure 1C UR1 and UR2; data not shown), those from HLA-mismatched individuals that were transduced with either HLA-A*3303 or -B*4403 were further tested. CTL-2A12 lysed UR3 B-LCLs when transduced with HLA-B*4403. In addition, UR3 B-LCLs transduced with HLA-B*4402 were also recognized, indicating that the mHA peptide can be presented by both HLA-B*4403 and -B*4402 (Figure 1C).

Identification of the gene encoding the mHA and elucidation of the mechanism of antigenicity

cDNA expression cloning using a cDNA library was conducted as described in "Patients, materials, and methods, cDNA library construction." In the first round of screening, 1 of 96 plasmid pools induced IFN-γ production by CTL-2A12. Two-step subclonings (~5 cDNAs and 1 cDNA) of this pool finally resulted in the isolation of a cDNA clone (data not shown).

The cDNA clone was sequenced and a BLAST search[23] revealed that this cDNA clone was previously unreported, but partially identical to XM_209104. XM_209104 was designated histocompatibility (minor) serpin domain containing (*HMSD*) by