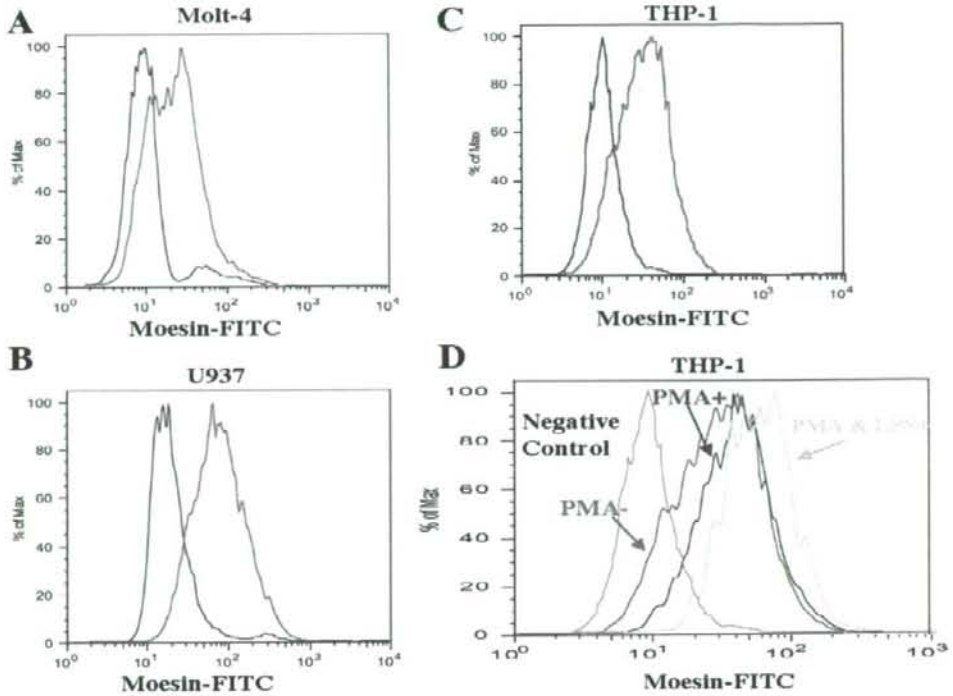

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1×10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

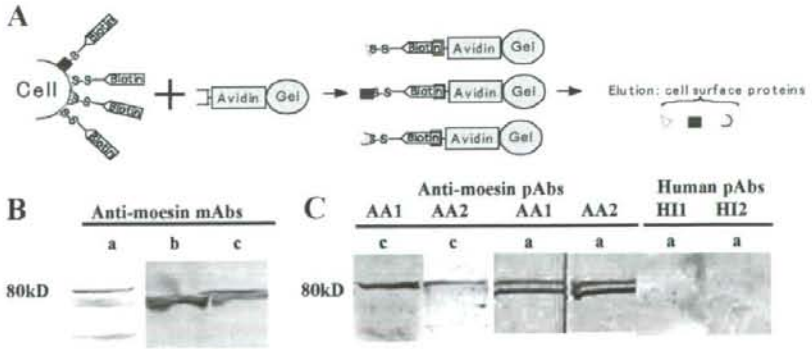

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean ± SD. Comparisons were made using the paired *t* test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM $CD34^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 ± 2.2 and 6.6 ± 2.1, respectively, and the difference was significant (mean fluorescence intensity ± SD, $p < 0.05$, unpaired *t* test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

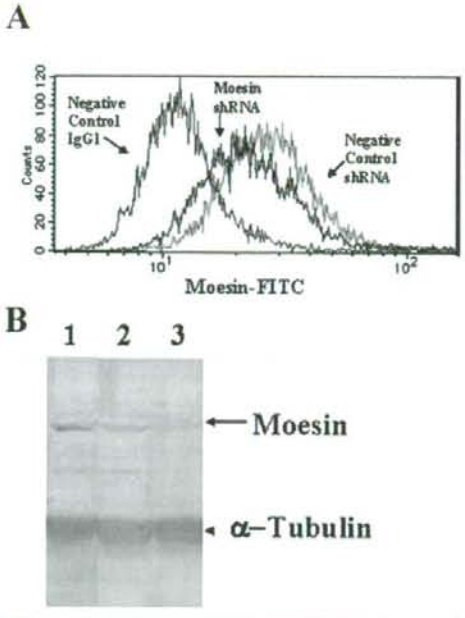

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

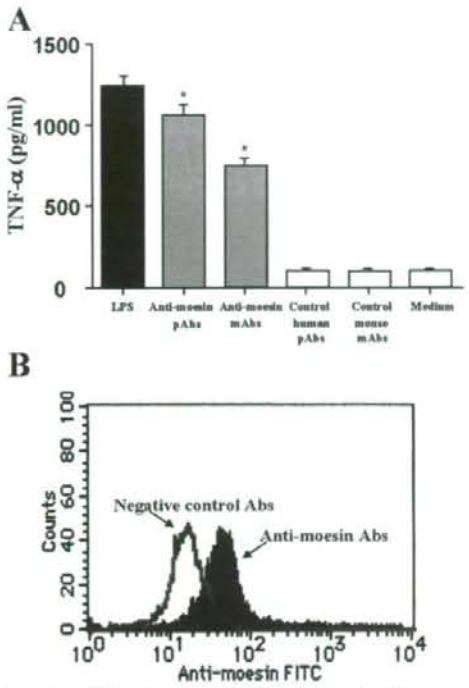

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration ± SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

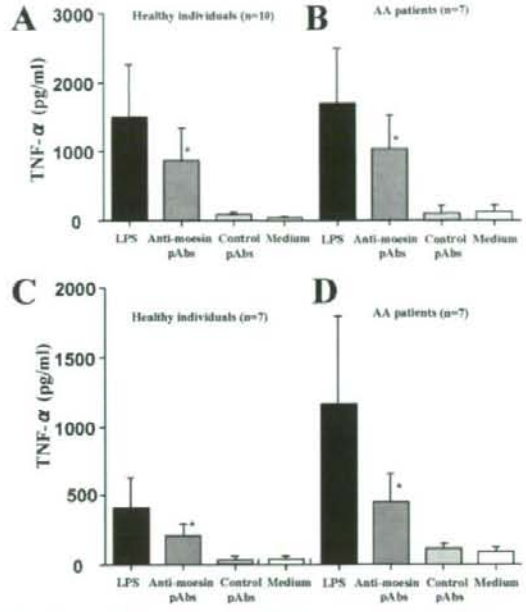

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration ± SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 ± 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 ± 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 ± 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 ± 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

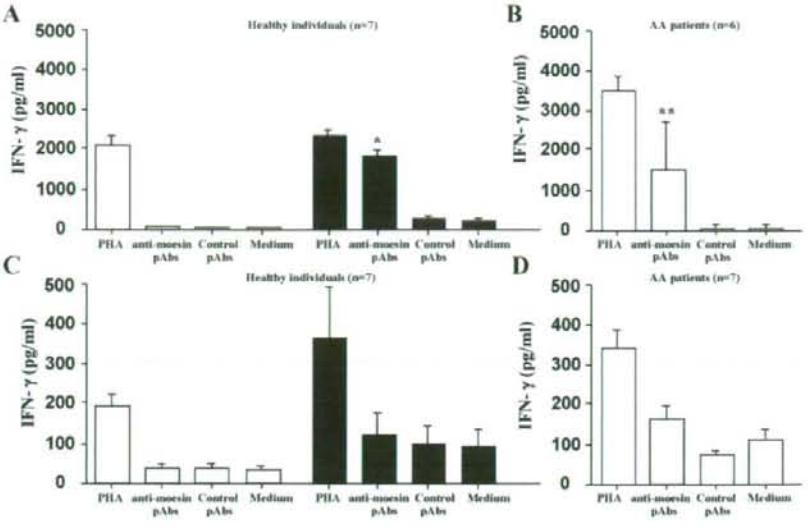

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

Acknowledgments

We gratefully acknowledge Prof. A. Yachie of Kanazawa University for suggestions and helpful discussion. We also thank M. Yoshii, A. Hamano, R. Oumi, and T. Tanaka of Cellular Transplantation Biology of Kanazawa University for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Young, N. S. 2002. Acquired aplastic anemia. *Ann. Intern. Med.* 136: 534–546.
2. Bacigalupo, A., G. Broccia, G. Corda, W. Arcese, M. Carotenuto, A. Gallamini, F. Locatelli, P. G. Mori, P. Saracco, G. Todeschini, et al. 1995. Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. *Blood* 85: 1348–1353.
3. Rosenfeld, S. J., J. Kimball, D. Vining, and N. S. Young. 1995. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood* 85: 3058–3065.
4. Hoffman, R., E. D. Zanjani, J. D. Lutton, R. Zalusky, and L. R. Wasserman. 1977. Suppression of erythroid-colony formation by lymphocytes from patients with aplastic anemia. *N. Engl. J. Med.* 296: 10–13.
5. Nissen, C., P. Cornu, A. Gratwohl, and B. Speck. 1980. Peripheral blood cells from patients with aplastic anemia in partial remission suppress growth of their own bone marrow precursors in culture. *Br. J. Haematol.* 45: 233–243.
6. Nakao, S., A. Takami, H. Takamatsu, W. Zeng, N. Sugimori, H. Yamazaki, Y. Miura, M. Ueda, S. Shiobara, T. Yoshioka, et al. 1997. Isolation of a T-cell clone showing HLA-DRB1*0405-restricted cytotoxicity for hematopoietic cells in a patient with aplastic anemia. *Blood* 89: 3691–3699.
7. Zeng, W., J. P. Maciejewski, G. Chen, and N. S. Young. 2001. Limited heterogeneity of T cell receptor BV usage in aplastic anemia. *J. Clin. Invest.* 108: 765–773.
8. Hirano, N., M. O. Butler, M. S. Von Bergwelt-Baildon, B. Maecker, J. L. Schultze, K. C. O'Connor, P. H. Schur, S. Kojima, E. C. Guinan, and L. M. Nadler. 2003. Autoantibodies frequently detected in patients with aplastic anemia. *Blood* 102: 4567–4575.
9. Feng, X., T. Chuhjo, C. Sugimori, T. Kotani, X. Lu, A. Takami, H. Takamatsu, H. Yamazaki, and S. Nakao. 2004. Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria-type cells. *Blood* 104: 2425–2431.
10. Hirano, N., M. O. Butler, E. C. Guinan, L. M. Nadler, and S. Kojima. 2005. Presence of anti-kinectin and anti-PMS1 antibodies in Japanese aplastic anaemia patients. *Br. J. Haematol.* 128: 221–223.
11. Takamatsu, H., X. Feng, T. Chuhjo, X. Lu, C. Sugimori, K. Okawa, M. Yamamoto, S. Iseki, and S. Nakao. 2007. Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood* 109: 2514–2520.
12. Weetman, A. P. 2003. Grave's disease 1835–2002. *Horm. Res.* 59 (*Suppl.* 1): 114–118.
13. Kitajima, Y., and Y. Aoyama. 2007. A perspective of pemphigus from bedside and laboratory-bench. *Clin. Rev. Allergy Immunol.* 33: 57–66.
14. Baroni, S. S., M. Santillo, F. Bevilacqua, M. Luchetti, T. Spadoni, M. Mancini, P. Fraticelli, P. Sambo, A. Funaro, A. Kazlauskas, et al. 2006. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N. Engl. J. Med.* 354: 2667–2676.
15. Svegliati, S., A. Olivieri, N. Campelli, M. Luchetti, A. Poloni, S. Trappolini, G. Moroncini, A. Bacigalupo, P. Leoni, E. V. Avvedimento, and A. Gabrielli. 2007. Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* 110: 237–241.
16. Ralston, D. R., C. B. Marsh, M. P. Lowe, and M. D. Wewers. 1997. Antineutrophil cytoplasmic antibodies induce monocyte IL-8 release: role of surface proteinase-3, α1-antitrypsin, and Fcγ receptors. *J. Clin. Invest.* 100: 1416–1424.
17. Tsukita, S., and S. Yonemura. 1999. Cortical actin organization: lessons from ERM (ezrin/radixin/moesin) proteins. *J. Biol. Chem.* 274: 34507–34510.
18. Ariel, A., R. Hershkoviz, I. Altbaum-Weiss, S. Ganor, and O. Lider. 2001. Cell surface-expressed moesin-like receptor regulates T cell interactions with tissue components and binds an adhesion-modulating IL-2 peptide generated by elastase. *J. Immunol.* 166: 3052–3060.
19. Matsuyama, A., N. Sakai, H. Hiraoka, K. Hirano, and S. Yamashita. 2006. Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages. *J. Lipid Res.* 47: 78–86.
20. Elkord, E., P. E. Williams, H. Kynaston, and A. W. Rowbottom. 2005. Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells. *Immunology* 114: 204–212.
21. Jensen, O. N., A. Podtelejnikov, and M. Mann. 1996. Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. *Rapid Commun. Mass Spectrom.* 10: 1371–1378.
22. Krawetz, R., M. J. MacKenzie, Q. Sun, P. A. Walton, and G. M. Kelly. 2006. Gα13 activation rescues moesin-depletion induced apoptosis in F9 teratocarcinoma cells. *Exp. Cell. Res.* 312: 3224–3240.
23. Masumoto, J., J. Sagara, M. Hayama, E. Hidaka, T. Katsuyama, and S. Taniguchi. 1998. Differential expression of moesin in cells of hematopoietic lineage and lymphatic systems. *Histochem. Cell Biol.* 110: 33–41.
24. Amar, S., K. Oyaisu, L. Li, and T. Van Dyke. 2001. Moesin: a potential LPS receptor on human monocytes. *J. Endotoxin Res.* 7: 281–286.
25. Tohme, Z. N., S. Amar, and T. E. Van Dyke. 1999. Moesin functions as a lipopolysaccharide receptor on human monocytes. *Infect. Immun.* 67: 3215–3220.
26. Solomou, E. E., K. Keyvanfar, and N. S. Young. 2006. T-bet, a Th1 transcription factor, is up-regulated in T cells from patients with aplastic anemia. *Blood* 107: 3983–3991.
27. Debets, J. M., C. J. van der Linden, I. E. Spronken, and W. A. Buurman. 1988. T cell-mediated production of tumour necrosis factor-α by monocytes. *Scand. J. Immunol.* 27: 601–608.
28. Tsukaguchi, K., B. de Lange, and W. H. Boom. 1999. Differential regulation of IFN-γ, TNF-α, and IL-10 production by CD4$^+$ αβTCR$^+$ T cells and Vδ2$^+$ γδ T cells in response to monocytes infected with *Mycobacterium tuberculosis*-H37Ra. *Cell Immunol.* 194: 12–20.
29. Herold, K. C., J. B. Burton, F. Francois, E. Poumian-Ruiz, M. Glandt, and J. A. Bluestone. 2003. Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3γ1(Ala-Ala). *J. Clin. Invest.* 111: 409–418.
30. Gaston, R. S., M. H. Deierhoi, T. Patterson, E. Prasthofer, B. A. Julian, W. H. Barber, D. A. Laskow, A. G. Diethelm, and J. J. Curtis. 1991. OKT3 first-dose reaction: association with T cell subsets and cytokine release. *Kidney Int.* 39: 141–148.
31. Wagatsuma, M., M. Kimura, R. Suzuki, F. Takeuchi, K. Matsuta, and H. Watanabe. 1996. Ezrin, radixin and moesin are possible auto-immune antigens in rheumatoid arthritis. *Mol. Immunol.* 33: 1171–1176.
32. Kaufman, D. W., J. P. Kelly, M. Levy, and S. Shapiro. 1991. *The Drug Etiology of Agranulocytosis and Aplastic Anemia.* Oxford University Press, New York.
33. Lipsky, P. E., D. M. van der Heijde, E. W. St. Clair, D. E. Furst, F. C. Breedveld, J. R. Kalden, J. S. Smolen, M. Weisman, P. Emery, M. Feldmann, et al. 2000. Infliximab and methotrexate in the treatment of rheumatoid arthritis: anti-tumor necrosis factor trial in rheumatoid arthritis with Concomitant Therapy Study Group. *N. Engl. J. Med.* 343: 1594–1602.
34. Klareskog, L., D. van der Heijde, J. P. de Jager, A. Gough, J. Kalden, M. Malaise, E. Martin Mola, K. Pavelka, J. Sany, L. Settas, et al. 2004. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomized controlled trial. *Lancet* 363: 675–681.
35. Maini, R., E. W. St. Clair, F. Breedveld, D. Furst, J. Kalden, M. Weisman, J. Smolen, P. Emery, G. Harriman, M. Feldmann, and P. Lipsky. 1999. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomized phase III trial. ATTRACT Study Group. *Lancet* 354: 1932–1939.
36. Deeg, H. J., J. Gotlib, C. Beckham, K. Dugan, L. Holmberg, M. Schubert, F. Appelbaum, and P. Greenberg. 2002. Soluble TNF receptor fusion protein (etanercept) for the treatment of myelodysplastic syndrome: a pilot study. *Leukemia* 16: 162–164.
37. Raza, A., A. Candoni, U. Khan, L. Lisak, S. Tahir, F. Silvestri, J. Billmeier, M. I. Alvi, M. Mumtaz, S. Gezer, P. Venugopal, P. Reddy, and N. Galili. 2004. Remicade as TNF suppressor in patients with myelodysplastic syndromes. *Leuk. Lymphoma* 45: 2099–2104.
38. Hansen, P. B., and A. M. Lauritzen. 2005. Aplastic anemia successfully treated with rituximab. *Am. J. Hematol.* 80: 292–294.
39. Castiglioni, M. G., P. Scatena, C. Pandolfo, S. Mechelli, and M. Bianchi. 2006. Rituximab therapy of severe aplastic anemia induced by fludarabine and cyclophosphamide in a patient affected by B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* 47: 1985–1986.

Biology of Blood and Marrow Transplantation 14:510-517 (2008)
© 2008 American Society for Blood and Marrow Transplantation
1083-8791/08/1405-0001$32.00/0
doi:10.1016/j.bbmt.2008.02.008

Preengraftment Serum C-Reactive Protein (CRP) Value May Predict Acute Graft-versus-Host Disease and Nonrelapse Mortality after Allogeneic Hematopoietic Stem Cell Transplantation

Shigeo Fuji, Sung-Won Kim, Takahiro Fukuda, Shin-ichiro Mori, Satoshi Yamasaki, Yuriko Morita-Hoshi, Fusako Ohara-Waki, Yuji Heike, Kensei Tobinai, Ryuji Tanosaki, Yoichi Takaue

Department of Hematology and Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan

Correspondence and reprint requests: Yoichi Takaue, MD, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan (e-mail: ytakaue@ncc.go.jp).

Received September 29, 2007; accepted February 10, 2008

ABSTRACT

In a mouse model, inflammatory cytokines play a primary role in the development of acute graft-versus-host disease (aGVHD). Here, we retrospectively evaluated whether the preengraftment C-reactive protein (CRP) value, which is used as a surrogate marker of inflammation, could predict posttransplant complications including GVHD. Two hundred twenty-four adult patients (median age, 47 years; range: 18-68 years) underwent conventional stem cell transplantation (CST, n = 105) or reduced-intensity stem cell transplantation (RIST, n = 119). Patients were categorized according to the maximum CRP value during neutropenia: the "low-CRP" group (CRP < 15 mg/dL, n = 157) and the "high-CRP" group (CRP ≥ 15 mg/dL, n = 67). The incidence of documented infections during neutropenia was higher in the high-CRP group (34% versus 17%, $P = .004$). When patients with proven infections were excluded, the CRP value was significantly lower after RIST than after CST ($P = .017$) or after related than after unrelated transplantation ($P < .001$). A multivariate analysis showed that male sex, unrelated donor, and HLA-mismatched donor were associated with high CRP values. The high-CRP group developed significantly more grade II-IV aGVHD ($P = .01$) and nonrelapse mortality (NRM) ($P < .001$), but less relapse ($P = .02$). The present findings suggest that the CRP value may reflect the net degree of tissue damage because of the conditioning regimen, infection, and allogeneic immune reactions, all of which lead to subsequent aGVHD and NRM.
© 2008 American Society for Blood and Marrow Transplantation

KEY WORDS

C-reactive protein • Allogeneic transplantation • Acute graft-versus-host disease • Nonrelapse mortality

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with high treatment-related mortality (TRM) because of acute graft-versus-host disease (aGVHD) and infections [1,2]. Inflammatory cytokines, for example, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 [3-11], are produced following conditioning and play a primary role in activating T cells, leading to GVHD and resultant target tissue destruction [12,13]. An acute-phase protein, C-reactive protein (CRP), is produced by hepatocytes downstream of IL-6 [14] and is widely used as a reliable surrogate marker of infectious diseases [15-19]. This process is further stimulated by other cytokines including TNF-α [12,13]. After allogeneic HSCT, the elevation of CRP was observed with infectious complications, but not in uncomplicated aGVHD [8,20]. On the other hand, elevation of CRP has been shown to be associated with TRM [21-24]. Nevertheless, these previous studies adopted the sporadic measurement of CRP and mostly focused on patients undergoing conventional HSCT (CST) with a myeloablative regimen. It has been hypothesized that recently developed reduced-intensity HSCT (RIST) decreases regimen-related toxicities and, hence, may reduce inflammation

that augments the subsequent allogeneic immune reaction to induce GVHD and nonrelapse mortality (NRM).

In this study, the correlation between the preengraftment CRP value and subsequent clinical events was analyzed to test whether high CRP reflected the degree of tissue damage because of the conditioning regimen, infections, and allogeneic immune reactions and/or inflammation, all of which could contribute to subsequent aGVHD and NRM.

MATERIALS AND METHODS

Patient Characteristics

The data from a cohort of 224 consecutive adult patients with hematologic malignancies, who were treated between January 2002 and July 2006 at the National Cancer Center Hospital (NCCH, Tokyo, Japan), were reviewed retrospectively. Patients who developed graft failure or who had previous allogeneic transplantation were excluded. Their characteristics are listed in Table 1. The median age of the patients was 47 years (range: 18-68 years), and their diagnosis included acute myeloid leukemia (AML, n = 94), acute lymphoblastic leukemia (ALL, n = 23), non-Hodgkin lymphoma (NHL, n = 62), myelodysplastic syndrome (MDS, n = 27) and chronic myeloid leukemia (CML, n = 12). Standard risk included acute leukemia in first complete remission, chronic leukemia in the first chronic phase, MDS in refractory anemia, and NHL in complete remission, with the rest of the patients categorized as a high-risk group. Stem cell sources used for transplantation included bone marrow (BM, n = 108), peripheral blood stem cells (PBSC, n = 98) and cord blood cells (CB, n = 18). One-hundred five patients received a CST regimen including total-body irridiation (TBI)-based (n = 50) and non-TBI-based busulfan-containing regimens (n = 55), whereas 119 patients received a RIST regimen including fludarabine or cladribine plus busulfan or melphalan (Table 1). CMV serostatus was positive in 157 patients and negative in 67 patients. The median age of the patients was 49 years in the high-CRP group (range: 19-67) and 47 years in the low-CRP group (range: 18- 68). Written informed consent was obtained according to the Declaration of Helsinki.

Transplantation Procedures

GVHD prophylaxis included cyclosporine- (n = 174) and tacrolimus-based regimens (n = 50), with an additional short course of methotrexate (MTX) in 165 patients. Granulocyte colony-stimulating factor (G-CSF) was administered in all patients from day +6 of transplantation until engraftment was confirmed. Most patients received ciprofloxacin (200 mg orally 3 times daily) for bacterial prophylaxis until neutrophil engraftment. Fluconazole (100 mg once daily) was administered for fungal prophylaxis. Low-dose acyclovir was given for prophylaxis against herpes simplex virus and varicella zoster virus until the cessation of immunosuppressive agents. Prophylaxis against *Pneumocystis jiroveci* infection was provided with trimethoprim-sulfamethoxazole (400 mg of sulfamethoxazole once daily) from the first day of conditioning to day −3 of transplantation, and from day +28 until day +180 or the discontinuation of immunosuppressive agents. Patients with fever during the neutropenic period were treated with cefepime, and additional agents including vancomycin and aminoglycosides, and amphotericin B were given as clinically indicated. Neutrophil engraftment was defined as the first of 3 consecutive days after transplantation that the absolute neutrophil count exceeded 0.5×10^9/L. In our institute, the CRP level was serially measured as part of our routine checkup at least 3 times a week. Hence, all serially admitted patients were subjected to this analysis. Every patient had started CRP measurement

Table 1. *Patients' Characteristics*

	N (%)/ Median		
Variable	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	*P* Value
Age (year)	47 (18-68)	49 (19-67)	.85
<40	53 (34)	26 (39)	
≥40	104 (66)	41 (61)	.47
Patient sex			
Male	84 (54)	48 (72)	
Female	73 (46)	19 (28)	.01
Donor sex			
Male	81 (52)	30 (45)	
Female	76 (48)	37 (55)	.35
CMV serostatus			
Positive	140 (89)	64 (96)	
Negative	17 (11)	3 (4)	.20
Disease risk			
Standard	35 (22)	17 (25)	
High	122 (78)	50 (75)	.62
Conditioning			
CST	72 (47)	33 (50)	
RIST	85 (53)	34 (50)	.64
GVHD prophylaxis			
Cyclosporin-based	122 (78)	52 (78)	
Tacrolimus-based	35 (22)	15 (22)	.99
Short term MTX (+)	107 (68)	58 (87)	.004
Relation to donor			
Related	94 (60)	13 (19)	
Unrelated	63 (40)	54 (81)	<.001
Stem cell source			
Bone marrow	63 (40)	45 (67)	
PBSC	87 (55)	11 (16)	
Cord blood	7 (5)	11 (16)	<.001

CRP indicates C-reactive protein; CMV, cytomegalovirus; CST, conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation; GVHD, graft-versus-host disease; MTX, methotrexate; PBSC, peripheral blood stem cells; HLA, human leukocyte antigen.

Table 2. *Comparison of Preengraftment CRP Value Stratified According to the Conditioning Regimen (CST versus RIST) and the Relation to Donor (Related versus Unrelated)*

Patients' Characteristics	CRP Value Median (Range)
All patients	8.9 (0.1-42.7)
CST	10.5 (0.3-31.3)*
Related	9.4 (0.6-30.0)†
Unrelated	10.6 (0.3-31.3)†
RIST	6.2 (0.1-42.7)*
Related	1.6 (0.1-9.7)‡
Unrelated	16.2 (0.5-42.7)‡

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation.
*P = .017.
†P = .33.
‡P < .001.

before the initiation of the conditioning regimen, and the median pretransplant CRP level was 0.3 mg/dL (range: 0.0-20.5 mg/dL). The median maximum CRP value during neutropenia was 8.9 mg/dL (0.1-42.7, Table 2).

The "maximum CRP level" was determined by measuring both the CRP level and the neutrophil count, as shown in the example in Figure 1A. The average number of levels assessed for each patient was 8 (range: 1-30). The median day of the maximum CRP level was day 10 of HSCT (range: 0-25), with 79% of patients developing this in later days (≥8 days). The patients were categorized according to the maximum CRP level after the threshold CRP level was determined following a preliminary analysis of the maximum CRP level after CST using an ROC curve analysis (data not shown). The "low-CRP" group (CRP <15 mg/dL) included 157 patients and the "high-CRP" group (CRP ≥15 mg/dL) included 67 patients.

Statistical Analyses

The primary endpoint of this study was the occurrence of grade II-IV and grade III-IV aGVHD, according to the Consensus Criteria [25]. The secondary endpoints were overall survival (OS) and nonrelapse mortality (NRM). Standard descriptive

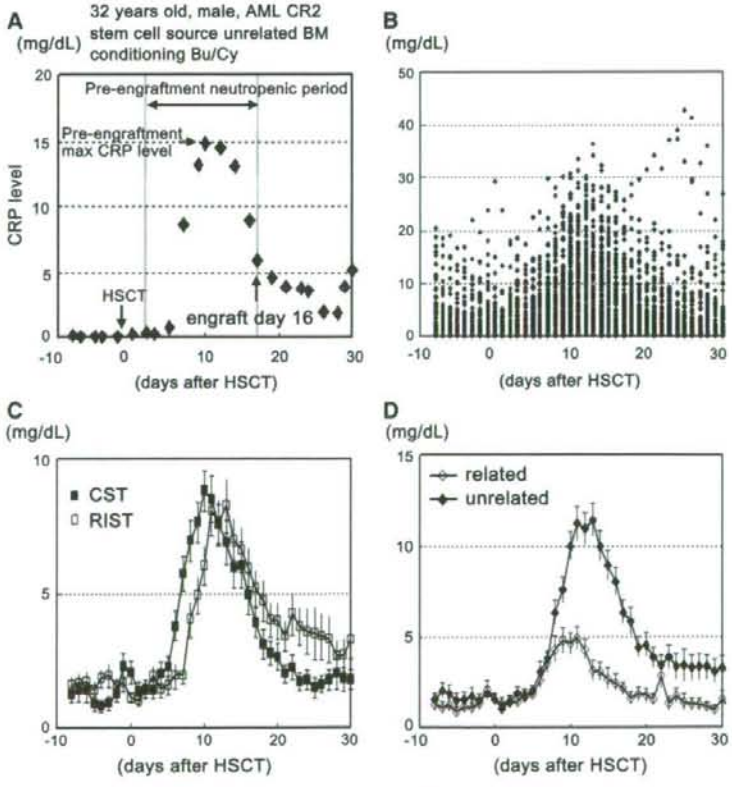

Figure 1. An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's exact test, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. To analyze the pretransplant risk factors for a high CRP level, logistic analysis was used. OS was estimated using Kaplan-Meier curves. The cumulative incidence of aGVHD and NRM was estimated based on a Cox regression model for cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for the multivariate analysis of variables in aGVHD, NRM, and OS after HSCT. Clinical factors that were assessed for their association with aGVHD included patient age, patient sex, donor sex, CMV serostatus, conditioning regimen (CST versus RIST), donor (human leukocyte antigen [HLA]-matched versus HLA-mismatched, related versus unrelated), GVHD prophylaxis (cyclosporine-based versus tacrolimus-based, short-term MTX versus no MTX) and disease risk (standard versus high risk). NRM and OS were also assessed for their association with these factors. Factors with $P < .10$ in the univariate analyses were subjected to a multivariate analysis using a multiple logistic analysis and Cox proportional hazard modeling. In Japan, only BM and CB are allowed for unrelated transplantation, and most transplantations with a related donor use PBSC as a stem cell source. Therefore, the stem cell source was not included as a factor in the multivariate analysis. A level of $P < .05$ was defined as statistically significant. All P values are 2-sided. All analyses were made with SPSS ver 10.0 statistical software (Chicago, IL). This analysis was approved by the institutional review board.

RESULTS

Infections

The median duration of follow-up in surviving patients was 965 days (61 to 1432 days) in the high-CRP group and 915 days (76 to 1803 days) in the low-CRP group, and the incidence of total documented infections during neutropenia was, respectively, 23 cases in the high-CRP group (34%) and 27 cases in the low-CRP group (17%, $P = .004$). The incidence of bacteremia was, respectively, 20 cases (30%) and 20 cases (13%, $P = .002$), and the incidence of pneumonia was 7 cases (10%) and 4 cases (3%, $P = .01$). The incidence of central venous catheter infection was, respectively, 4 cases (6%) and 7 cases (4%, $P = .63$).

Serial changes in the CRP level are shown in Figure 1B; in most cases, the CRP level was elevated within 2 weeks of HSCT. Stratified data according to conditioning regimen (CST versus RIST) or relation to donor (related versus unrelated) are shown in Figure 1C and D, respectively.

To clarify the pretransplant risk factors for high CRP values during neutropenia, we performed a logistic regression analysis, which showed that male, unrelated donor, stem cell source with BM or CB transplantation (versus PBSCT), HLA-mismatched donor, and immunosuppression with MTX were associated with high CRP values during neutropenia (Table 1). Factors that showed significant associations ($P < .1$) were subjected to a multiple logistic regression analysis, and the results showed that unrelated donor, HLA mismatch and male sex were associated with high CRP ($P < .001$, $P = .005$, $P = .028$, respectively), as shown in Table 3. The median CRP levels after CST and RIST were 10.5 (0.3-31.3) and 6.2 (0.1-42.7), respectively, with a significant difference ($P = .017$) (Table 2). Notably, within the RIST group, the median CRP level was significantly lower in related than in unrelated transplantation (1.6 mg/dL [0.1-9.7] veruss 16.2 mg/dL [0.5-42.7]: $P < .001$). However, the logistic analysis failed to disclose any overall significant difference between CST and RIST.

Primary Outcomes

The cumulative incidences of aGVHD grade II-IV and grade III-IV are shown, respectively, in Figure 2A and B. Grade II-IV and grade III-IV aGVHD were both more frequent in the high-CRP group than in the low-CRP group ($P = .001$ and $P = .04$, respectively). A Cox proportional hazard model showed that a high CRP level and CMV serostatus were associated with an increased risk of grade II-IV aGVHD (Table 4). Similar results were obtained when we included only the patients who received a myeloablative conditioning regimen (grade II-IV aGVHD 25% in the low-CRP group and 58% in the high-CRP group, $P < .001$, grade III-IV aGVHD 7% in the low-CRP group and 21% in the high-CRP group, $P = .047$).

Secondary Outcomes

OS and NRM are shown, respectively, in Figure 3A and B. OS was significantly worse in the

Table 3. *Multiple Logistic Regression Analysis of Risk Factors for High CRP during Neutropenia*
*Factors with P < .10 in a Multivariate Analysis Was Shown**

Outcomes and Variables	Multiple Logistic Regression Analysis		
	Odds	95% CI	*P* Value
Unrelated donor	4.6	2.2-9.6	<.001
HLA mismatch	2.6	1.3-5.0	.005
Patient sex (male)	2.1	1.1-4.2	.0028

CRP indicates C-reactive protein; CI, confidence interval; HLA, human leukocyte antigen; CMV, cytomegalovirus.
*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source.

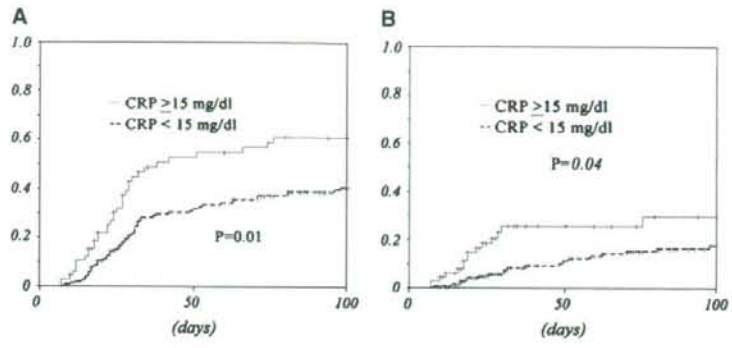

Figure 2. Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

high-CRP group than in the low-CRP group (1-year OS 47% versus 75%, $P = .001$). NRM was significantly higher in the high-CRP group than in the low-CRP group (1-year NRM 47% versus 13%, $P < .001$). Similar results were obtained when we included only patients who received a myeloablative conditioning regimen (1-year NRM 8% in the low-CRP group and 38% in the high-CRP group, $P = .007$). A Cox proportional hazard model showed that the risk factors for poor OS were high CRP ($P = .002$, hazard ratio [HR] 2.0, 95% confidence interval [CI] 1.3-3.1) and high-risk disease ($P = .015$, HR 2.2, 95% CI 1.2-4.0), whereas those for high NRM were high CRP ($P < .001$, HR 4.0, 95% CI 2.0-8.0) and high-risk disease ($P = .029$, HR 2.6, 95% CI 1.1-6.2), as shown in Table 4. When the threshold was set at 15 mg/dL, the sensitivity and specificity of the CRP level for prediction of grade II-IV aGVHD, NRM, or OS were 37% and 75%, 59% and 79%, and 40% and 78%, respectively. The relapse rate was significantly lower in the high-CRP group than in the low-CRP group (1-year relapse 21% versus 33%, $P = .02$).

Causes of death are summarized in Table 5. A total of 57 patients (36%) in the low-CRP group and 39 patients (58%) in the high-CRP group died ($P = .002$, OR 2.4 [1.4-4.4]). Six patients (4%) in the low- and 5 (7%) in the high-CRP group died because of aGVHD, for example, death because of infectious diseases associated with aGVHD and its treatment. Seven patients (4%) in the low- and 11 (16%) in the high-CRP group ($P = .003$, OR 4.2 [1.6-11.4]) died because of chronic GVHD (cGVHD), including death because of infectious diseases associated with cGVHD and its treatment. No patient (0%) in the low- and 5 (7%) in the high-CRP group ($P = .002$) died because of infectious diseases excluding infectious disease concomitant with GVHD. No patient in the low-CRP group and 4 (6%) in the high-CRP group ($P = .008$) died because of multiple-organ failure (MOF) excluding MOF because of GVHD and infectious disease.

DISCUSSION

The results of this retrospective study suggested that higher CRP values during the neutropenic period may reflect net inflammation secondary to tissue damage because of the conditioning regimen, infection, and subsequent allogeneic immune reactions, all of which lead to aGVHD/cGVHD and ultimate NRM. In a mouse model, the concept that the production of inflammatory cytokines plays an important role in the development of aGVHD, by affecting the afferent and effector phase [12,13], has been accepted. Cooke et al. [26] showed that LPS antagonism reduced aGVHD in a mouse model, as indicated by Ferrara et al. [4]. However, in human studies, the value of determining individual levels of cytokines to monitor aGVHD has not been fully explored, because this approach is very costly and requires sophisticated techniques, which impedes its universal applicability. On the other hand, CRP is already being widely used

Table 4. *Multiple Variate Analysis for aGVHD, NRM, and OS**

Outcomes and Variables	Hazard Ratio	95% CI	P value
Grade II-IV aGVHD			
High CRP	1.7	1.1-2.6	.02
CMV positivity	3.1	1.0-9.8	.5
Disease risk (high)	1.6	0.9-2.7	.10
NRM			
High CRP	4.0	2.0-8.0	<.001
Age (≥40 years old)	1.9	0.9-3.9	.07
Disease risk (high)	2.6	1.1-6.2	.03
OS			
High CRP	2.0	1.3-3.1	.002
Disease risk (high)	2.2	1.2-4.0	.02

CRP indicates C-reactive protein; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-versus -host disease; TBI, total body irradiation; NRM, nonrelapse mortality; OS, overall

*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source

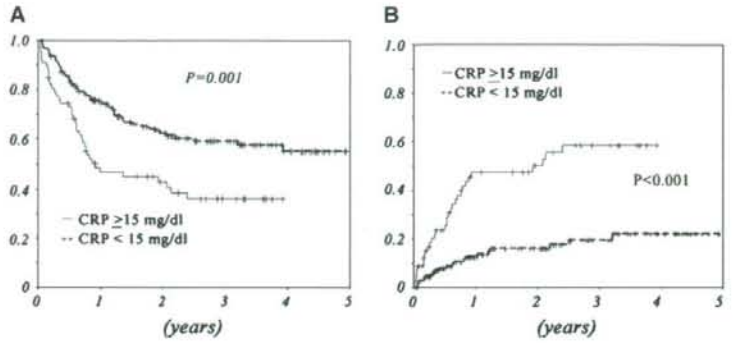

Figure 3. OS stratified according to the maximal CRP level during neutropenia (A). Cumulative incidence of TRM stratified according to the maximal CRP level during neutropenia (B).

worldwide, especially in Japan, to distinguish bacterial infections from other causes of fever [15-19]. Based on this practice, we reviewed the value of the CRP level after HSCT, and our data suggest that it might be useful to monitor the CRP value as a net surrogate marker for produced cytokines, and for predicting the subsequent development of aGVHD and NRM.

Our patients had various interacting backgrounds, and it is still difficult to predict whether a patient with a high CRP level is destined to suffer from GVHD or major infectious complications. Infectious diseases were previously reported to be a primary cause of elevated CRP [8,20], which might, in turn, affect the severity of aGVHD. In this study, we made every effort, including intense culture studies, to exclude infection as a primary cause of increased CRP, and showed that there were significantly more documented infections in the high-CRP group than in the low-CRP group. Current practice for the prevention of infection mostly focuses on the effective control of Gram-negative bacteria, considering the potent immediate pathologic effect of the organisms. However, if the hypothesis that decreasing the net production of cytokines is important for the prevention of subsequent GVHD is correct, more effort should be paid to broadly cover other types of organisms or even clinically less significant infection, that is, stomatitis, at least during the early period of neutropenia, particularly in patients carrying risk factors for high CRP, which included unrelated donor, HLA mismatch, BM, and CB transplantation in this study. The addition of other markers, such as procalcitonin, may be useful for identifying the risk of major infectious complications [24].

Table 5. *Causes of Death Stratified According to CRP Value during Neutropenia*

Causes of death	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	*P* Value
Total	57 (36%)	39 (58%)	.002
Relapse/progressive disease	34 (22%)	8 (12%)	.09
acute GVHD (total)	6 (4%)	5 (7%)	.25
acute GVHD	5 (3%)	3 (5%)	.63
acute GVHD + infection	1 (1%)	2 (3%)	.16
chronic GVHD (total)	7 (4%)	11 (16%)	.003
chronic GVHD	3 (2%)	7 (10%)	.005
chronic GVHD + infection	4 (3%)	4 (6%)	.21
Infection*	0 (0%)	5 (7%)	.002
MOF†	0 (0%)	4 (6%)	.008
Respiratory failure‡	3 (2%)	4 (6%)	.11
Others	Stroke 2	VOD 1	
	VOD 2	Myocardial infarction 1	
	Secondary cancer 1		
	Unknown 2		

CRP indicates C-reactive protein; GVHD, graft-versus-host disease; TBI, total-body irradiation; MOF, multiple organ failure; VOD, veno-occlusive disease.

*Excluding infection during GVHD or GVHD treatment.

†Excluding MOF due to GVHD, infection.

‡Excluding respiratory failure because of GVHD, infection, and MOF.

Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

ACKNOWLEDGMENTS

This work was presented in part as a poster presentation at the annual Meeting of EBMT, Lyon, March 2007. This study was supported in part by grants from the Ministry of Health, Labor and Welfare, and Advanced Clinical Research Organization, Japan. There is no potential conflict of interest to declare.

REFERENCES

1. Wojnar J, Giebel S, Krawczyk-Kulis M, et al. Acute graft-versus-host disease. The incidence and risk factors. *Ann Transplant.* 2006;11:16-23.
2. Weisdorf D, Hakke R, Blazar B, et al. Risk factors for acute graft-versus-host disease in histocompatible donor bone marrow transplantation. *Transplantation.* 1991;51:1197-1203.
3. Krenger W, Hill GR, Ferrara JL. Cytokine cascades in acute graft-versus-host disease. *Transplantation.* 1997;64:553-558.
4. Ferrara JL. The cytokine modulation of acute graft-versus-host disease. *Bone Marrow Transplant.* 1998;21(Suppl 3):S13-S15.
5. Cooke KR, Olkiewicz K, Erickson N, Ferrara JL. The role of endotoxin and the innate immune response in the pathophysiology of acute graft versus host disease. *J Endotoxin Res.* 2002;8:441-448.
6. Toren A, Novick D, Or R, Ackerstein A, Slavin S, Nagler A. Soluble interleukin-6 receptors in hematology patients undergoing bone marrow transplantation. *Transplantation.* 1996;62:138-142.
7. Liem LM, van Houwelingen HC, Goulmy E. Serum cytokine levels after HLA-identical bone marrow transplantation. *Transplantation.* 1998;66:863-871.
8. Schwaighofer H, Herold M, Schwarz T, et al. Serum levels of interleukin 6, interleukin 8, and C-reactive protein after human allogeneic bone marrow transplantation. *Transplantation.* 1994;58:430-436.
9. Chasty RC, Lamb WR, Gallati H, Roberts TE, Brenchley PE, Yin JA. Serum cytokine levels in patients undergoing bone marrow transplantation. *Bone Marrow Transplant.* 1993;12:331-336.
10. Lange A, Karabon L, Klimczak A, et al. Serum interferon-gamma and C-reactive protein levels as predictors of acute graft-vs-host disease in allogeneic hematopoietic precursor cell (marrow or peripheral blood progenitor cells) recipients. *Transplant Proc.* 1996;28:3522-3525.
11. Symington FW, Symington BE, Liu PY, Viguet H, Santhanam U, Sehgal PB. The relationship of serum IL-6 levels

to acute graft-versus-host disease and hepatorenal disease after human bone marrow transplantation. *Transplantation*. 1992;54: 457-462.
12. Fowler DH, Foley J, Whit-Shan Hou J, et al. Clinical "cytokine storm" as revealed by monocyte intracellular flow cytometry: correlation of tumor necrosis factor alpha with severe gut graft-versus-host disease. *Clin Gastroenterol Hepatol*. 2004;2: 237-245.
13. Antin JH, Ferrara JL. Cytokine dysregulation and acute graft-versus-host disease. *Blood*. 1992;80:2964-2968.
14. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J*. 1990;265:621-636.
15. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999;340:448-454.
16. Santolaya ME, Cofre J, Beresi V. C-reactive protein: a valuable aid for the management of febrile children with cancer and neutropenia. *Clin Infect Dis*. 1994;18:589-595.
17. Manian FA. A prospective study of daily measurement of C-reactive protein in serum of adults with neutropenia. *Clin Infect Dis*. 1995;21:114-121.
18. Persson L, Engervall P, Magnuson A, et al. Use of inflammatory markers for early detection of bacteraemia in patients with febrile neutropenia. *Scand J Infect Dis*. 2004;36:365-371.
19. von Lilienfeld-Toal M, Dietrich MP, Glasmacher A, et al. Markers of bacteremia in febrile neutropenic patients with hematological malignancies: procalcitonin and IL-6 are more reliable than C-reactive protein. *Eur J Clin Microbiol Infect Dis*. 2004; 23:539-544.
20. Rintala E, Remes K, Salmi TT, Koskinen P, Nikoskelainen J. The effects of pretransplant conditioning, graft-versus-host disease and sepsis on the CRP levels in bone marrow transplantation. *Infection*. 1997;25:335-338.
21. Schots R, Kaufman L, Van Riet I, et al. Monitoring of C-reactive protein after allogeneic bone marrow transplantation identifies patients at risk of severe transplant-related complications and mortality. *Bone Marrow Transplant*. 1998;22:79-85.
22. Schots R, Van Riet I, Ben Othman T, et al. An early increase in serum levels of C-reactive protein is an independent risk factor for the occurrence of major complications and 100-day transplant-related mortality after allogeneic bone marrow transplantation. *Bone Marrow Transplant*. 2002;30:441-446.
23. Min CK, Kim SY, Eom KS, et al. Patterns of C-reactive protein release following allogeneic stem cell transplantation are correlated with leukemic relapse. *Bone Marrow Transplant*. 2006;37: 493-498.
24. Pihusch M, Pihusch R, Fraunberger P, et al. Evaluation of C-reactive protein, interleukin-6, and procalcitonin levels in allogeneic hematopoietic stem cell recipients. *Eur J Haematol*. 2006; 76:93-101.
25. Przepiorka D, Weisdorf D, Martin P, et al. 1994 consensus conference on acute GVHD grading. *Bone Marrow Transplant*. 1995;15:825-828.
26. Cooke KR, Gerbitz A, Crawford JM, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J Clin Invest*. 2001;107:1581-1589.

Bone Marrow Transplantation (2008) 41, 515–521
© 2008 Nature Publishing Group All rights reserved 0268-3369/08 $30.00
www.nature.com/bmt

ORIGINAL ARTICLE

Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation

Y Morita-Hoshi[1,2], Y Heike[1], M Kawakami[1], T Sugita[3], O Miura[2], S-W Kim[1], S-I Mori[1], T Fukuda[1], R Tanosaki[1], K Tobinai[1] and Y Takaue[1]

[1]*Department of Medical Oncology, National Cancer Center Hospital, Tokyo, Japan;* [2]*Department of Hematology and Oncology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan and* [3]*Cellular Immunology Section, SRL Inc., Hachioji-city, Tokyo, Japan*

In order to evaluate whether we could predict reactivation of CMV by monitoring the number of CMV-specific cytotoxic T-lymphocytes (CTL), tetramer analysis was performed in 37 patients who underwent hematopoietic stem cell transplantation (HSCT). The results disclosed that the mean number of CMV-specific CTL at day 30 did not differ among patients who developed CMV antigenemia (22/μl) and those who did not (12/μl). Serial tetramer analysis showed that 21% of the patients had >10/μl CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/μl CMV-specific CTL at the onset of CMV disease. Intracellular staining upon stimulation by CMV lysates and peptide in patients with CMV colitis revealed that both IFN-γ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and 8/μl), which increased with recovery of the disease (19 and 47/μl). These data suggest that it is difficult to predict CMV reactivation solely by the number of CMV-specific CTL. We suggest that additional functional analysis by intracellular cytokine assay may be useful for immunomonitoring against CMV.

Bone Marrow Transplantation (2008) **41,** 515–521; doi:10.1038/sj.bmt.1705932; published online 19 November 2007
Keywords: CMV; intracellular IFN-γ; CTL; HSCT; HLA-A02

Introduction

Reactivation of CMV is one of the major complications in patients undergoing hematopoietic stem cell transplantation (HSCT) and is significantly related to morbidity and mortality despite the recent development of potent antiviral medications.[1,2] The decision to administer antiviral therapy is currently based on the clinical risk and the detection of viremia by various methods including PCR for CMV-derived DNA or CMV antigenemia assay. However, treatment with antiviral drugs such as ganciclovir and foscarnet increases the risk for secondary graft failure and other infectious complications due to myelotoxicity. To optimize the therapy with minimum drug exposure, it is important to monitor the recovery of CMV-specific immunity accurately. For this purpose, tetramer-based monitoring of CMV-specific cytotoxic T-cells (CTL) has been widely performed in patients with an HLA-A02 or HLA-B07 serotype.[3–11] Some of the results have demonstrated that the reconstitution of CMV-specific CTL as evaluated by quantitative tetramer to levels >10–20/μl is adequate for protection against CMV infection.[5–7] However, some patients with CMV-specific CTL above this level still experience CMV reactivation.[9] It has also been reported that the cellular response to CMV in immunosuppressed patients reflects functional impairment,[10] and CMV reactivation following HSCT has been shown to be associated with the presence of dysfunctional CMV-specific T-cells.[11] Therefore, by itself, the quantification of CMV-specific CTL seems to be insufficient and a simultaneous qualitative analysis of CMV-specific lymphocytes is needed. Furthermore, it is essential that we should develop a universal monitoring method, which is not limited to HLA to cover larger populations, since an epitope that is potent enough for immunomonitoring is not obtained in some HLA types such as HLA-A24.[12] In this study, simultaneous functional analysis of CMV-specific lymphocytes by intracellular cytokine assay upon stimulation with CMV lysate and antigen peptide were performed with tetramer-based CTL quantification in patients who underwent HSCT to identify an optimal monitoring system.

Correspondence: Dr Y Takaue, Director, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: ytakaue@ncc.go.jp
Received 7 August 2007; revised 10 October 2007; accepted 15 October 2007; published online 19 November 2007

Materials and methods

Study patients

CMV seropositive patients with an HLA-A*0201 or HLA-A*0206 genotype who had undergone allogeneic non-T-cell

depleted-HSCT between February 2002 and May 2005 were included in this study. Patients were eligible with the availability for 160 days of follow-up. The study was approved by the Ethics Committee and a written informed consent was given by all patients. Peripheral blood samples were obtained at days 30 ± 7 and 60 ± 7 after transplantation. When patients agreed to additional sampling, additional samples were obtained every 2–3 weeks. The median age of studied patients was 52 (21–68). The genotype for HLA-A02 in 37 eligible patients was HLA-A*0201 in 20 patients, HLA-A*0206 in 16 patients and both the HLA-A*0201 and HLA-A*0206 genotypes in one patient. Nine patients received BMT from an unrelated donor, two received BMT from a related donor and the remaining 26 received peripheral blood HSCT from a related donor. With regard to the conditioning regimen, 11 patients received a conventional regimen that included 120 mg/kg CY plus 16 mg/kg BU or 120 mg/kg CY plus 12 Gy of TBI, whereas 26 received a reduced-intensity regimen with 0.66 mg/kg cladribine (2-chlorodeoxyadenosine) plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU. For patients who received a graft from an unrelated donor or DNA-mismatched donor, 4 Gy of TBI or 5 mg/kg of rabbit antithymocyte globulin (ATG) were added to reduced-intensity conditioning.

Diagnostic tests for CMV infection and CMV disease

CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. All patients and 31 donors (84%) were seropositive for CMV. CMV antigenemia was monitored weekly after engraftment to day 60, and at longer intervals thereafter, by using the immunocytochemical detection of pp65 antigen in leukocytes. Test results were considered to be positive when more than one cell per 50 000 leukocytes was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ. Pre-emptive antiviral therapy was given with an antigenemia of more than 10 positive cells per 50 000 leukocytes, which we defined as high antigenemia. The initial therapy was ganciclovir 5 mg/kg once per day, which was adjusted according to the follow-up CMV antigenemia value.

Peptide and CMV antigen

A >80% pure HLA-A02-binding peptide NLVPMVATV (AA 495–503, referred to as NLV peptide) from the CMV pp65 phosphoprotein was obtained using high-performance liquid chromatography (Qiagen, Tokyo, Japan).

Tetramer staining

Tetramer staining was performed as recently described.[13] Briefly, 5 μl CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 μl PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 μl heparinized blood and incubated for 30 min. After RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). More than 20 000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ CMV-tetramer-positive fraction of the lymphocyte gate was defined as CMV-specific CTL.

Intracellular cytokine assay

Intracellular cytokine staining was performed as recently described[14] with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 μg/ml NLV peptide or 1 μg/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 μg/ml each). Breferdin A (Sigma, St Louis, MO, USA; 10 μg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 μg/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 μl CD69-FITC, 20 μl IFN-γ-PE, 0.6 μl CD3-APC and 10 μl CD8− or CD4− PerCP. More than 10 000 cells in the lymphocyte gate were acquired and analyzed using an FACS Calibur. The cells were gated on the CD3+ fraction of the lymphocyte gate and the proportion of IFN-γ and CD8 or CD4 was analyzed. CD69 was used as a marker for activated T-cells.

Statistical analysis

The difference between groups was compared with the Wilcoxon–Mann–Whitney *U*-test and the probabilities of $P<0.05$ were defined as statistically significant.

Results

Tetramer staining

CMV antigenemia was observed in 27 patients (73%) between day 23 and day 56 (median, day 34) after transplantation; 13 (35%) of them had a peak antigenemia level of >10/50 000 leukocytes (high antigenemia) which required ganciclovir therapy and four (11%) subsequently developed CMV disease. The median number of leukocytes and lymphocytes were 3500 (1300–17 200)/μl and 576 (228–3333)/μl at day 30 and 3900 (1400–9700)/μl and 1018 (192–6790)/μl at day 60, respectively. The median percentages of CD4+ and CD8+/lymphocytes were 35% (7–64%) and 38% (20–83%) at day 30 and 25% (6–37%) and 52% (27–83%) at day 60, respectively.

The tetramer analysis showed that the mean and median number of CMV-specific CTL at day 30 was, respectively, 11 and 1.9/μl for patients without CMV antigenemia, 23 and 7.8/μl for those with antigenemia, 33 and 15/μl for those with peak antigenemia <10/50 000, 12 and 3.7/μl for those with high antigenemia, and 21 and 2.4/μl for those who developed CMV disease. There was no significant correlation between the number of CMV-specific CTL and the incidence or severity of CMV antigenemia ($P>0.05$) (Figure 1).

To further evaluate the accurate number of CMV-specific CTL at the onset of CMV antigenemia, serial analysis of CMV-specific CTL was performed weekly in 14 patients (Figures 2 and 3). Patient's characteristics are shown in Table 1. CMV antigenemia was observed in 12 patients, and five of them (UPN1-5) developed high

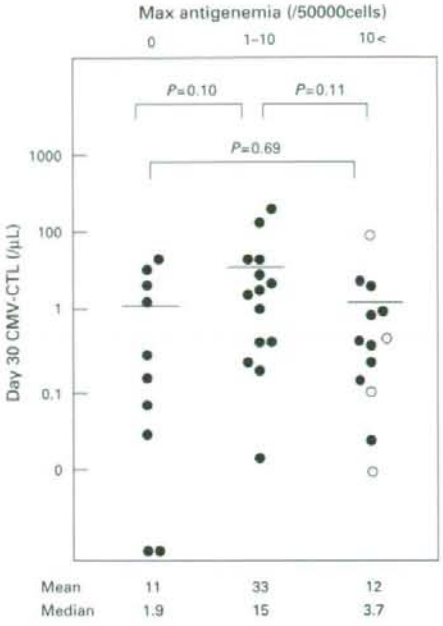

Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50 000, who had antigenemia of >10/50 000. The outlined circle ○ indicates patients who developed CMV colitis.

antigenemia, including three (UPN1-3) with CMV colitis. The mean and median number of CMV-specific CTL at the first detection of CMV antigenemia was 21/μl and 4.7 (0–100)/μl in the 12 patients, and three (UPN2, 13, 14) showed >10/μl. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/μl throughout the observation time, the maximum CTL count was >200/μl for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/μl, which was 14 and 80/μl when diarrhea occurred, and 88 and 63/μl, respectively at the time of colon biopsy which proved CMV colitis.

It has been demonstrated that in patients coexpressing HLA-A02 and HLA-B07, CMV-specific cellular immune responses restricted by HLA-B07 dominate those restricted by HLA-A02, possibly because CD8+ T cells specific for dominant epitopes are able to suppress immune responses to less favored epitopes.[3] The allele frequency of HLA-B07 is low (5.2%) among Japanese[15] and only one patient coexpressed HLA-B07 in this study. We did not exclude this patient (UPN14) from the analysis because the number of HLA-A02-restricted CMV-specific CTL in this patient was 9.5/μl on day 30 and the maximum value reached 243/μl on day 128 suggesting that the coexpression of HLA-B07 seems not to have affected the immunoresponse of HLA-A2 in this patient.

Intracellular cytokine assay

Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high

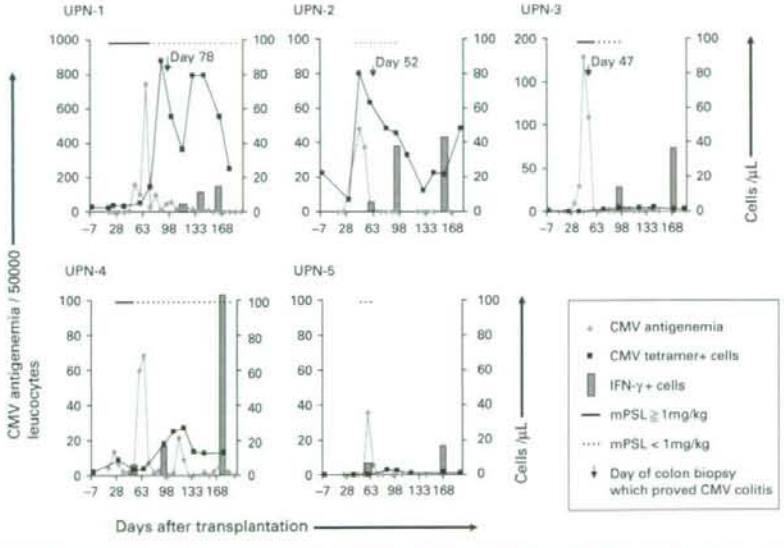

Figure 2 Serial analysis of patients who had high antigenemia of >10/50 000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ♦ indicates CMV antigenemia, gray bar indicates the number of IFN-γ+cells/μl peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone administration of 1 mg/kg/day or more, the dashed line indicates corticosteroid administration less than 1 mg/kg/day and ↓ indicates the day of colon biopsy which CMV disease was diagnosed. UPN1, 2, 3 developed CMV disease. Intracellular IFN-γ was undetectable on day 60 and day 90 for UPN1 and on day 60 for UPN3.

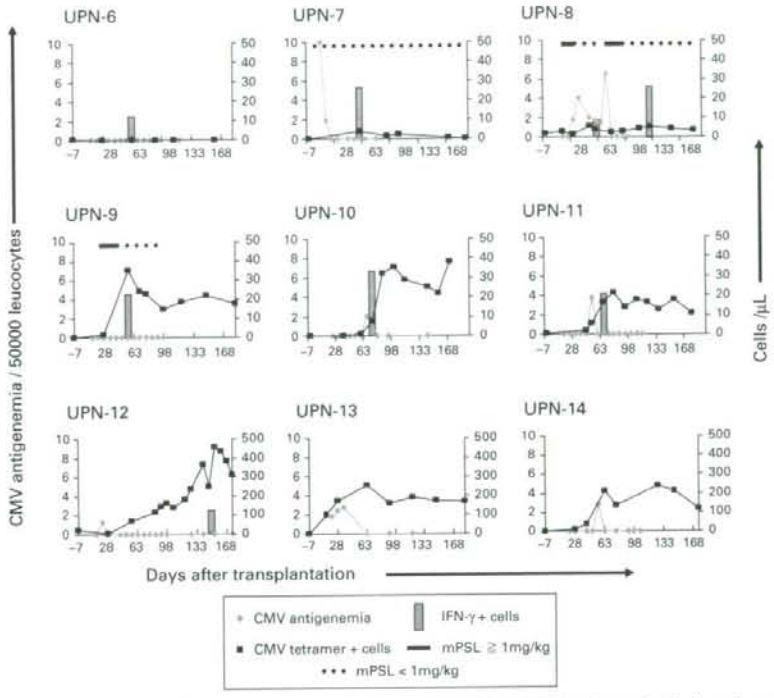

Figure 3 Serial analysis in patients with CMV antigenemia of <10/50000 or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

Table 1 Patients' characteristics

ID	*Age*	*HLA-A locus*	*Primary disease*	*Conditioning regimen*	*GVHD prophylaxis*	*Stem cell source*	*CMV serology*		*Max CMV-Ag*	*CMV disease*
							Recipient	*Donor*		
UPN-01	63	0201, 0206	CML (AP)	CdA/BU	CSP→TAC	PB	+	+	740	+
UPN-02	57	0201	NHL (DLBCL)	CdA/BU	CSP	PB	+	+	48	+
UPN-03	49	0201	NHL (low grade)	CdA/BU	CSP→TAC	PB	+	+	178	+
UPN-04	54	0206	MCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	68	−
UPN-05	59	0206	AML	CdA/BU/TBI	CSP+sMTX	UBM	+	+	35	−
UPN-06	66	0206	MDS (RA)	Flu/BU	CSP+sMTX	PB	+	−	0	−
UPN-07	61	0201	NHL (low grade)	Flu/BU/ATG	CSP	UBM	+	+	10	−
UPN-08	62	0201	AML	CdA/BU	TAC	PB	+	+	6.5	−
UPN-09	43	0201	MDS (RA)	BU/CY	CSP+sMTX	UBM	+	−	0	−
UPN-10	41	0206	AML	BU/CY	CSP+sMTX	RBM	+	+	2.1	−
UPN-11	54	0201	NHL (low grade)	Flu/BU	CSP+sMTX	PB	+	+	3.7	−
UPN-12	32	0206	RCC	CdA/BU	CSP	PB	+	+	2.8	−
UPN-13	42	0206	PCL	CdA/BU/ ATG	CSP+sMTX	PB	+	+	2.8	−
UPN-14	43	0206	RCC	CdA/BU/ ATG	CSP	PB	+	+	1.3	−

Abbreviations: ATG = antithymocyte globulin; CdA = cladribine; CML (AP) = CML (accelerated phase); CSP = cyclosporine; DLBCL = diffuse large B-cell lymphoma; Flu = fludarabine; MCL = mantle cell lymphoma; MDS (RA) = myelodysplastic syndrome (refractory anemia); NHL = non-Hodgkin lymphoma; PB = peripheral blood; PCL = plasma cell leukemia; RBM = related bone marrow; RCC = renal cell carcinoma; sMTX = short term methotrexate; TAC = tacrolimus; UBM = unrelated bone marrow.

antigenemia and required antiviral therapy showed that the mean number of IFN-γ-producing cells was 3.6 (0–6.7)/μl at day 60, which subsequently increased to 72 (15–250)/μl at day 160. As for three patients with CMV colitis (UPN1–3), only one patient (UPN2) had detectable level of IFN-γ-producing cells (4.8/μl) at the time of disease

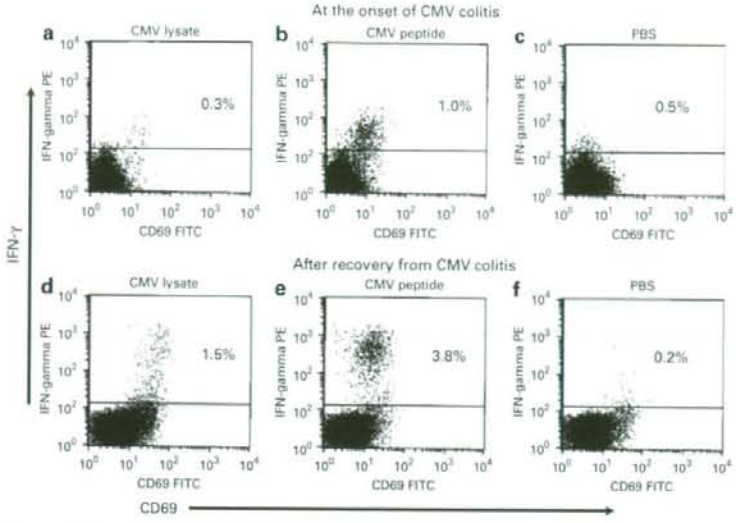

Figure 4 Intracellular cytokine assay in a patient with CMV colitis (UPN2). The samples were taken at the onset of CMV colitis (**a**–**c**) and after recovery from CMV colitis (**d**–**f**). The numbers of IFN-γ-producing cells on lysate stimulation (**a**, **d**) and peptide stimulation (**b**, **e**) both increased after recovery from CMV colitis. (**c**) and (**f**) are negative controls.

onset and were undetectable for the other two patients, which remained negative until day 90 for UPN1. The mean number of IFN-γ+ cells subsequently increased to 19 (5–38)/μl after recovery from CMV disease (Figures 2, 4a and d). Among the patients who did not require antiviral therapy, the IFN-γ-producing cells were all >10/μl at day 60.

When stimulated with CMV peptide, IFN-γ-producing cells numbered 8 (0–16)/μl at the time of disease onset with a subsequent increase to 47 (15–95)/μl after recovery from CMV disease (Figures 4b and e).

Regarding the phenotype of IFN-γ-producing cells, median of 81% (76–100) were CD4+ and <20% were CD8+ upon stimulation by CMV lysate. The staining of IFN-γ was brighter in CD4+ than in CD8+ cells and CD69 was positive for both CD4+ and CD8+ fraction. IFN-γ-producing cells were CD69 low positive and median of 42% (25–68) were CD8+, while the rest were CD8−/CD4− phenotype upon CMV peptide stimulation.

Discussion

Our results showed that it is difficult to predict CMV infection by the number of CMV-specific CTL alone as this did not correlate with the incidence and severity of CMV infection. While UPN1 and UPN2 developed CMV colitis after the recovery of sufficient number of CTL, UPN6, UPN7 and UPN8 did not require antiviral therapy despite low CMV-specific CTL. These results showed that CMV disease could occur after HSCT even in patients with >10/μl CMV-specific CTL as evaluated by tetramer assay, which has been considered to be sufficient to protect against CMV infection.[5–7]

CMV-specific CTL emerged immediately following the detection of antigenemia in most patients, suggesting that CMV infection can be a trigger for the recovery of CMV-specific immunity. However, UPN9 had recovery of CMV-specific CTL at day 60 even though his CMV antigenemia and CMV DNA as evaluated by PCR were negative throughout the course.

On the other hand, intracellular analysis revealed that IFN-γ production in both CD4+ and CD8+ T lymphocytes was depressed in patients with high antigenemia or CMV disease and this had subsequently recovered at disease resolution. Functional analysis methods for CMV-specific immune response by flow cytometry have been established,[16] and it was reported that patients who developed CMV disease after SCT had no detectable IFN-γ production by CD3+/4+ T-cells upon CMV AD-169 antigen stimulation.[17] It has also been demonstrated that levels of IFN-γ-producing CD4+ cells less than one cell/μl and CD8+ less than three cells/μl upon stimulation by CMV-infected autologous dendritic cells are not protective against recurrent infection.[18] As assessed by IFN-γ ELISPOT assay, the threshold level for protection against CMV reactivation was estimated as over one cell/μl peripheral blood upon CMV pp65 peptide stimulation.[19] The number of IFN-γ-producing cells upon CMV lysate stimulation were above ten cells/μl among patients whose antigenemia was <10/50 000 cells in our study, which may be sufficient for protection against CMV reactivation. It is difficult to determine the exact threshold level for protection against CMV since IFN-γ production differs among various stimulating agents. Also the magnitude of response is higher in the cytokine flow cytometry assay while the cytokine flow cytometry assay was less likely than the ELISPOT assay to detect low-level responses.[20]

Several studies on HIV-infected patients have shown the availability of analyzing the phenotype and other cytokine production of virus-specific T-cells such as IL-2, TNF-α.[21–23] It has been demonstrated that virus-specific T-cells, which produce both IFN-γ and IL-2 are important in virus-specific immunity, and that IFN-γ/IL-2 secreting CD8+ T-cells were CD45RA−/CCR7− phenotype and correlated with that of proliferating T-cells, whereas single IFN-γ-secreting cells were either CD45RA−/CCR7− or CD45RA+/CCR7−.[22] Another study has shown that immunorestored patients had increased levels of circulating CMV-specific CD8+ T-cells with 'early' (CD27+/CD28+/CD45RA+, CD27+/CD28+/CD45RA−) and 'intermediate' (CD27−/CD28+/CD45RA−) phenotype.[23] Only IFN-γ production was assessed in our study, however higher-order flow cytometry might have added more discriminatory value. Foster *et al.*[24] demonstrated that CMV-specific CD4+ T-helper cells show the same reconstitution kinetics as CD8+ CTL. Thus, functional analysis of lymphocytes upon lysate stimulation that can be used to assess both CD4+ and CD8+ cells is a useful tool for monitoring T cell immunity against CMV in patients after HSCT. This method is more widely applicable than peptide stimulation or tetramer assay, since it is not restricted to HLA or a single epitope. However, peptide stimulation and tetramer assay may still be a major procedure in the analysis of CD8+ T-cells, since tetramers are widely applied to adoptive immunotherapy of CMV[25] and the dominant population of IFN-γ-producing cells upon lysate stimulation was CD4+. Previous study has demonstrated that flow cytometry following stimulation of PBMC with pp65 and immediate early (IE)-1 peptide pools consisted of 15-aa peptides was highly sensitive and specific in predicting the presence of recognized epitope in the respective proteins.[26] Furthermore, it has been shown that IE-1-specific responses were more important in protective immunity than pp65-specific responses in heart and lung transplant recipients.[27] The stimulation with comprehensive peptide pools might have better assessed both functional CD4+ and CD8+ T-cell responses. Further study is needed to identify whether IE-1 is more important than pp65 in allogeneic HSCT patients, and the significance of IE-1 in Japanese population with low allele frequency of HLA-A1 (1.8%), -B7 (5.2%) or -B8 (<1%),[15] which is known to present IE-1 epitopes.

It is likely that the patients who did not have CMV reactivation despite low CMV-specific CTL had sufficient T-cell immune-recovery against CMV since the number of intracellular IFN-γ positive cells upon CMV lysate stimulation was as high as that in patients who had recovered from CMV reactivation. As for CD8+ T cells in these patients, CTL against other CMV-epitopes besides NLV might have helped to protect against CMV. It is reported that the recovery of CMV specific T-cells is earlier in patients who received reduced-intensity conditioning compared to conventional regimen and this was delayed by the use of ATG.[19,28] Additionally, the graft source and CD3+ T-cell dose significantly influence the recovery of CMV-specific immunity.[28] The difference of immune recovery according to the conditioning regimen and graft source was not demonstrated in this study, probably due to heterogeneous patients and small sample size. Functional depression of the lymphocytes due to corticosteroid for GVHD seems to be the major cause of CMV infection as documented in all patients with high antigenemia. Moreover, 75% of the patients with CMV disease were receiving more than 1 mg/kg/day of methylprednisolone (mPSL), while among those who did not require antiviral therapy, only 13% had received 1 mg/kg/day or more mPSL. The influence of corticosteroid on the number of CMV-specific CTL is controversial. Some studies have reported that a significant reduction of CMV-specific CTL occurred with corticosteroid therapy.[6–8] Others have shown that the frequency and the absolute number of CMV-specific CD8+ T cells were similar in patients receiving corticosteroids and those who didn't, while the CMV-specific CD8+ T cells showed decreased cytokine production.[10,11] Our result was consistent with the latter observation that while the number of CMV-specific CTL does not decrease significantly with corticosteroid therapy, IFN-γ production of CMV-specific CTL is severely suppressed. Therefore, concomitant assessment of T-cell function is essential in patients after HSCT, especially in those who are receiving corticosteroid therapy.

References

1 Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant* 2003; **9**: 543–558.
2 Zaia JA, Sissons JG, Riddell S, Diamond DJ, Wills MR, Carmichael AJ *et al.* Status of Cytomegalovirus Prevention and Treatment in 2000. *Hematology (Am Soc Hematol Educ Program)* 2000, 339–355.
3 Lacey SF, Villacres MC, La Rosa C, Wang Z, Longmate J, Martinez J *et al.* Relative dominance of HLA-B*07 restricted CD8+ T-lymphocyte immune responses to human cytomegalovirus pp65 in persons sharing HLA-A*02 and HLA-B*07 alleles. *Hum Immunol* 2003; **64**: 440–452.
4 Singhal S, Shaw JC, Ainsworth J, Hathaway M, Gillespie GM, Paris H *et al.* Direct visualization and quantitation of cytomegalovirus-specific CD8+ cytotoxic T-lymphocytes in liver transplant patients. *Transplantation* 2000; **69**: 2251–2259.
5 Gratama JW, van Esser JW, Lamers CH, Tournay C, Lowenberg B, Bolhuis RL *et al.* Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood* 2001; **98**: 1358–1364.
6 Aubert G, Hassan-Walker AF, Madrigal JA, Emery VC, Morte C, Grace S *et al.* Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. *J Infect Dis* 2001; **184**: 955–963.
7 Cwynarski K, Ainsworth J, Cobbold M, Wagner S, Mahendra P, Apperley J *et al.* Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood* 2001; **97**: 1232–1240.
8 Engstrand M, Tournay C, Peyrat MA, Eriksson BM, Wadstrom J, Wirgart BZ *et al.* Characterization of CMVpp65-specific CD8+ T lymphocytes using MHC tetramers in kidney transplant patients and healthy participants. *Transplantation* 2000; **69**: 2243–2250.

9 Lacey SF, Gallez-Hawkins G, Crooks M, Martinez J, Senitzer D, Forman SJ *et al*. Characterization of cytotoxic function of CMV-pp65-specific CD8+ T-lymphocytes identified by HLA tetramers in recipients and donors of stem-cell transplants. *Transplantation* 2002; **74**: 722–732.
10 Engstrand M, Lidehall AK, Totterman TH, Herrman B, Eriksson BM, Korsgren O. Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clin Exp Immunol* 2003; **132**: 96–104.
11 Ozdemir E, St John LS, Gillespie G, Rowland-Jones S, Champlin RE, Molldrem JJ *et al*. Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. *Blood* 2002; **100**: 3690–3697.
12 Morita Y, Hosokawa M, Ebisawa M, Sugita T, Miura O, Takaue Y *et al*. Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A*02 or HLA-A*24 phenotype undergoing hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2005; **36**: 803–811.
13 Morita Y, Heike Y, Kawakami M, Miura O, Nakatsuka S, Ebisawa M *et al*. Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation. *Int J Cancer* 2006; **119**: 1360–1367.
14 Rauser G, Einsele H, Sinzger C, Wernet D, Kuntz G, Assenmacher M *et al*. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* 2004; **103**: 3565–3572.
15 Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S *et al*. Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. *Immunogenetics* 1997; **46**: 199–205.
16 Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin Invest* 1997; **99**: 1739–1750.
17 Avetisyan G, Larsson K, Aschan J, Nilsson C, Hassan M, Ljungman P. Impact on the cytomegalovirus (CMV) viral load by CMV-specific T-cell immunity in recipients of allogeneic stem cell transplantation. *Bone Marrow Transplant* 2006; **38**: 687–692.
18 Lilleri D, Gerna G, Fornara C, Lozza L, Maccario R, Locatelli F. Prospective simultaneous quantification of human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. *Blood* 2006; **108**: 1406–1412.
19 Ohnishi M, Sakurai T, Heike Y, Yamazaki R, Kanda Y, Takaue Y *et al*. Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-gamma-enzyme-linked immunospot and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope. *Br J Haematol* 2005; **131**: 472–479.
20 Karlsson AC, Martin JN, Younger SR, Bredt BM, Epling L, Ronquillo R *et al*. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J Immunol Methods* 2003; **283**: 141–153.
21 Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J *et al*. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006; **107**: 4781–4789.
22 Zimmerli SC, Harari A, Cellerai C, Vallelian F, Bart PA, Pantaleo G. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc Natl Acad Sci USA* 2005; **102**: 7239–7244.
23 Sinclair E, Tan QX, Sharp M, Girling V, Poon C, Natta MV *et al*. Protective immunity to cytomegalovirus (CMV) retinitis in AIDS is associated with CMV-specific T cells that express interferon- gamma and interleukin-2 and have a CD8+ cell early maturational phenotype. *J Infect Dis* 2006; **194**: 1537–1546.
24 Foster AE, Gottlieb DJ, Sartor M, Hertzberg MS, Bradstock KF. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar reconstitution pattern after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2002; **8**: 501–511.
25 Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H *et al*. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med* 2005; **202**: 379–386.
26 Kern F, Faulhaber N, Frommel C, Khatamzas E, Prosch S, Schonemann C *et al*. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *Eur J Immunol* 2000; **30**: 1676–1682.
27 Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, Cherepnev G *et al*. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. *J Exp Med* 2005; **201**: 1031–1036.
28 Mohty M, Mohty AM, Blaise D, Faucher C, Bilger K, Isnardon D *et al*. Cytomegalovirus-specific immune recovery following allogeneic HLA-identical sibling transplantation with reduced-intensity preparative regimen. *Bone Marrow Transplant* 2004; **33**: 839–846.