of splenic Foxp3$^+$CD4$^+$ Treg phenotype cells in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells was higher than in recipients of WT CD4$^+$ T cells, but the total percentage was still only ~1% (Fig. 6*B*, *left panel*). The absolute number was ~4-fold higher, but the actual number was only ~4 × 10^5 of the total number of splenocytes (~4 × 10^7) (Fig. 6*B*, *right panel*). In contrast to posttransplantation, pretransplantation splenocytes from IL-21R$^{-/-}$ mice did not show an increase in Foxp3$^+$CD4$^+$ T cells compared with cells from WT mice (Supplemental Fig. 3), suggesting that the increased Treg cell after transplantation was an induced Treg cell during GVHD reaction. For that reason, we did not deplete CD25$^+$ cells prior to transplantation.

CD25 depletion did not restore the suppressed alloreaction in vitro and did not exacerbate the ameliorated GVHD

To investigate the importance of Treg cells in diminishing GVHD, we performed an MLR, which corresponds to alloreaction in vitro, with or without CD25$^+$CD4$^+$ T cells. Because Foxp3 is an intracellular protein, and Foxp3 staining cannot be used to purify or deplete Treg cells, anti-CD25 Ab is widely used for this purpose (9, 29–32). The impaired MLR of IL-21R$^{-/-}$ CD4$^+$ T cells after transplantation was not restored by CD25 depletion (Fig. 7*A*), nor was the impaired IFN-γ production by IL-21R$^{-/-}$CD4$^+$ T cells in an MLR (Fig. 7*B*). Moreover, analogous to cytokine production by anti-CD3/CD28 stimulation (Fig. 3), IL-21R$^{-/-}$ CD4$^+$ T cells before transplantation were not defective for alloreaction (Fig. 7*C*).

Consistent with the in vitro experiments above, CD25$^+$ depletion in vivo did not alter the severity of GVHD in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells, without altering the body weight loss and survival (Fig. 8*A*, 8*B*). In contrast, the severity of GVHD in recipients of WT CD4$^+$ T cells seemed to be slightly diminished by CD25$^+$ depletion (Fig. 8*A*, 8*B*). In this condition, as previously reported (30), the depletion efficacy of CD25$^+$CD4$^+$ T cells was >95% and that of Foxp3$^+$CD4$^+$ T cells was ≥50% (Fig. 8*C*, *upper* and *lower panels*). Interestingly, Foxp3 expression was higher in CD25$^-$CD4$^+$ T cells from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than from recipients of WT CD4$^+$ T cells (Fig. 8*D*). Together with the results in vitro (Fig. 7), this suggests a relationship between the unresponsiveness of CD25$^-$CD4$^+$ T cells and greater expression of Foxp3.

Discussion

In this article, we reported evidence indicating that IL-21 is critical for the pathogenesis of CD4$^+$ T cell-mediated GVHD, at least in part because of its effects on CD4 differentiation. In this study, we focused on CD4$^+$ T cell-mediated GVHD; a role for IL-21 in CD8$^+$ T cell-mediated GVHD remains to be investigated.

We found a profound defect in T cell effector function only after transplantation, although serum cytokine concentrations showed no obvious difference. According to these results, T cell differentiation into Th1 and Th2 cells seemed to be altered in the absence of IL-21 during GVHD. Cytokines are believed to have positive and negative roles in GVHD. For example, although T cells from IFN-γ–deficient mice resulted in more severe GVHD (33–35), T cells from Stat4 (Th1)-deficient mice resulted in less severe GVHD than did T cells from WT mice with less severe colitis (36). In contrast to IFN-γ$^{-/-}$ T cells, T cells from IL-4–deficient mice induced less severe GVHD (34); analogously, T cells from Stat6 (Th2)-deficient mice induced less severe GVHD than did those from WT mice (36). T cells from TNF-α–deficient mice developed less severe GVHD, with less severe colitis (37). Our data suggest a strong correlation between the defect in effector function in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells and the attenuated phenotype of GVHD, indicating a role for IL-21 in this process.

IL-21, as well as IL-6, induces Th17 differentiation in the presence of TGF-β, suggesting a possible involvement of IL-17 in

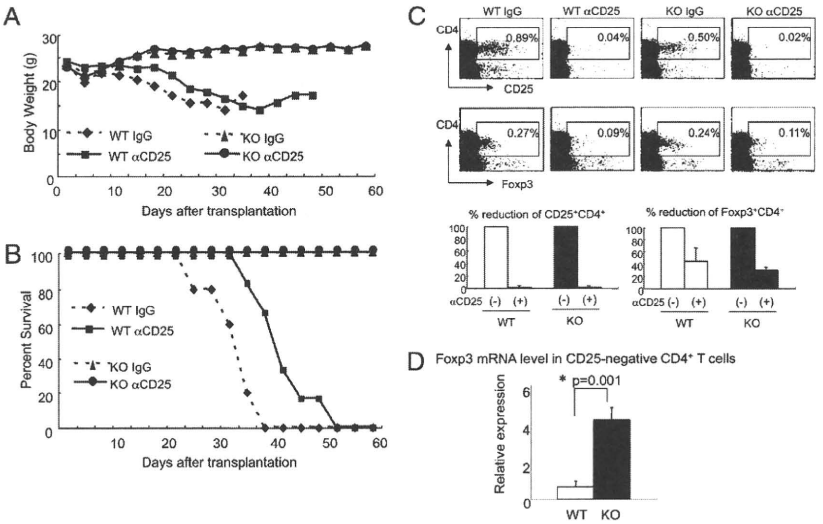

FIGURE 8. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells is not dependent on CD25$^+$CD4$^+$ T cells. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells was not exacerbated by depletion of CD25$^+$CD4$^+$ T cells. Body weight (*A*) and survival (*B*) of recipients are shown. Comparisons of recipients of WT CD4$^+$ T cells and IL-21R$^{-/-}$ CD4$^+$ T cells and additional comparisons with and without anti-CD25 Ab treatment were performed. Nonspecific rat IgG was used as the control Ab. *C*, Splenic CD25$^+$CD4$^+$ T cells and splenic Foxp3$^+$CD4$^+$ T cells at day 14 after transplantation with or without anti-CD25 Ab treatment were analyzed by flow cytometry (*upper two rows*). The lower panels indicate the mean reduction in the percentage of CD25$^+$CD4$^+$ and Foxp3$^+$CD4$^+$ cells from three similar results. *D*, Foxp3 mRNA level in CD25$^-$CD4$^+$ T cells at day 21 after transplantation. Cell sorter-purified CD25$^-$CD4$^+$ T cells were subjected to mRNA purification, reverse-transcriptase treatment, and TaqMan quantitative PCR. Relative value to β-actin is denoted.

the phenotype we observed. However, our results with IL-17$^{-/-}$ CD4$^+$ T cells demonstrated that IL-17 was dispensable for CD4$^+$ T cell-mediated GVHD, indicating that the attenuated GVHD in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells was not due to an IL-17–related defect. During the preparation of this manuscript, a role for IL-17 in GVHD was reported (38–40). These reports varied, but one suggested that the lack of IL-17 promotes GVHD (38). Another report suggested that IL-17$^{-/-}$ CD4$^+$ T cells can ameliorate GVHD only at the early stages, which suggested a promoting effect for IL-17 at an early stage of GVHD (39). The third report suggested that ex vivo-differentiated Th17 cells induced skin and lung GVHD (40). Thus, the role of IL-17 may be complex and dependent on the specific experimental conditions.

Because there are reciprocal relationships between Th1/Th2 and Treg cell differentiation (41–43) and between IL-21 and Treg cell differentiation (8), we investigated the level of Treg cells in the spleens of recipients. Foxp3$^+$CD4$^+$ T cells were increased in percentage and absolute number but still represented only ~1% of splenocytes. Regarding the relationship between the defective effector T cell function and the increased number of Treg cells, it is possible that increased Treg cells suppress functional effector T cells. Alternatively, it is possible that effector differentiation itself is defective, and the resulting effector T cells cannot respond to alloantigen, analogous to the situation in T cell anergy, and that the increased Treg cell number is also a result of a dysregulated differentiation. Our results might be more consistent with the latter possibility, given that Treg cell depletion by anti-CD25 treatment did not alter the results in vitro and in vivo, although the efficiency of depletion of Foxp3$^+$CD4$^+$ T cells in vivo was incomplete. It is also conceivable that the upregulation of Foxp3 in CD25$^-$CD4$^+$ T cells (which would not be removed by CD25$^+$ depletion) in the absence of IL-21 signaling might result in unresponsiveness or poor responsiveness of effector T cells and that more than one mechanism can contribute to the attenuated GVHD.

Disclosures

K. Ozaki and W.J.L. are inventors on patents and patent applications related to IL-21.

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High incidence of false-positive *Aspergillus* galactomannan test in multiple myeloma

Yasuo Mori,[1,2] Yoji Nagasaki,[1,2] Kenjiro Kamezaki,[1] Katsuto Takenaka,[1] Hiromi Iwasaki,[2] Naoki Harada,[1] Toshihiro Miyamoto,[1] Yasunobu Abe,[3] Nobuyuki Shimono,[1] Koichi Akashi,[1,2] and Takanori Teshima[2]

[1]Medicine and Biosystemic Science, [2]Center for Cellular and Molecular Medicine, and [3]Medicine and Bioregulatory Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Corresponding author: Takanori Teshima, MD, PhD
Center for Cellular and Molecular Medicine, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Phone: +81-92-642-5947, Fax: +81-92-642-5951
E-mail: tteshima@cancer.med.kyushu-u.ac.jp

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Key Words; galactomannan, false-positive, myeloma, aspergillus

Invasive aspergillosis (IA) remains one of the most significant causes of morbidity and mortality in patients with hematological malignancies undergoing chemotherapy and hematopoietic stem cell transplantation (HSCT), mainly due to the difficulty in its early diagnosis. Monitoring of galactomannan (GM) antigen, an exoantigen of Aspergillus, in the blood by sandwich ELISA is a useful and non-invasive method for early diagnosis of IA. The GM test has a sensitivity of 67-100% with a specificity of 81-99% in neutropenic patients and allogeneic transplant recipients (1-3). Although it has been widely used as a diagnostic criterion for IA (4, 5), one of the major limitations of this assay is false-positivity, particularly in pediatric patients (1), patients with graft-versus-host disease (GVHD) (6, 7), and those taking dietary GM (8, 9) or fungus-derived antibiotics, such as piperacillin-tazobactam (PIPC/TAZ) (10-12).

Multiple myeloma results from malignant proliferation of a single clone of plasma cells, which produces a monoclonal immunoglobulin. Opportunistic infection is a major cause of death in patients with myeloma (13, 14). The risk for infection primarily resides during periods of chemotherapy-induced neutropenia or in the terminal stages of the disease. Therefore, monitoring of Aspergillus is recommended during chemotherapy-induced neutropenia.

124 patients with hematological disorders hospitalized in our institution from April 2007 to September 2009 were analyzed retrospectively. The clinical characteristics of these patients are summarized in supplementary materials (Table 1) Twenty-seven patients had plasma cell associated disorders (IgG type

myeloma: 14, IgA type myeloma: 1, IgD type myeloma: 1, Bence-Jones type myeloma: 3, lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia: 3, plasma cell leukemia: 1, primary AL amyloidosis: 1, and POEMS syndrome: 2). The remaining 97 patients were diagnosed as acute leukemia (MDS/AML: 32, ALL: 15) or malignant lymphoma (n = 50). Out of the 124 patients, those receiving cytotoxic chemotherapy, autologous peripheral blood stem cell transplantation (PBSCT), and allogeneic HSCT were 81, 10, and 28, respectively. 111 patients received antifungal prophylaxis, mostly with FLCZ or ITCZ. Eight patients were administered PIPC/TAZ at sampling time points. Seventy patients were low-risk for the development of IA, while the remaining 54 patients were high-risk.

In 21 of the 124 (16.9%) patients, GM antigenemia was positive at least 2 consecutive times and their characteristics are shown in Table 1. However, only 7 of the 21 patients showed clinical features of IA and were diagnosed with probable IA in the lung (cases 1-7). Clinical features were relieved with treatment with VRCZ in these patients, confirming a diagnosis of IA. All of the 7 patients had received antifungal prophylaxis, but not antibiotics known to cause false-positive results, such as PIPC/TAZ (10-12), amoxicillin/clavulanic acid, or amoxicillin (17). Four of the 7 patients were high-risk for the developing IA and 3 were low-risk. Possible IA was diagnosed in 4 patients in the absence of positive GM results. On the other hand, no proven or probable IA was detected in 103 patients with negative GM antigenemia.

Fourteen of the 21 (66.7%) patients with positive GM antigenemia did not satisfy the diagnostic criteria of proven or probable IA (cases 8-21 in Table 2); thus their episodes were considered to be false-positive. None of the 14 patients were treated with antibiotics potentially causing false-positivity of GM test. Antifungal prophylaxis had been given in all 14 episodes (FLCZ in 5, ITCZ in 5, and MCFG in 4). These patients did not show any clinical features suggestive of IA. Chest CT scans did not show any abnormal findings in 10 patients, while 4 patients showed abnormalities in the lung which were not suspicious of IA. Diagnosis of these lung lesions were history of pneumoconiosis in one patient, proven bacterial pneumonia in one, and idiopathic interstitial pneumonia in the remaining 2 patients. These lesions were not deteriorated without antifungal treatment. (1→3)-β-D-glucan was negative in all patients showing false-positive GM test. With a median follow-up of 10 months (range: 1-19 months), these patients did not develop fungal infection without treatment. On the other hand, false negative GM results were obtained in 4 patients (3.2 %). In the current study, the sensitivity, specificity, PPV, and NPV of the GM ELISA test were 63.6% (7/11), 87.6% (99/113), 33.3% (7/21), and 96.1% (99/103), respectively.

Surprisingly, 11 out of the 14 patients showing the false-positive results had diagnosis of multiple myeloma. The false-positivity of GM antigenemia was significantly higher in myeloma patients (11/22, 50%) than those with other hematological malignancies (3/102, 2.9%) ($p < 0.001$). Moreover, in myeloma patients false-positive results were exclusively detected in those with IgG subtype. Thus, rate of false-positivity was extremely high in patients with IgG

myeloma (11/14, 78.6%). We could not find any difference in the characteristics of IgG myeloma patients with or without GM-false positivity, including serum levels of IgG (5083 ± 2077 mg/dl versus 4713 ± 3729 mg/dl). In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in 8 of the 11 patients. We also evaluated GM antigenemia in frozen serum samples collected prior to chemotherapy in 3 myeloma patients who showed false-positivity after chemotherapy to rule out the possibility that administration of myeloma-specific chemotherapy is associated with the false-positivity, and confirmed GM positivity prior to chemotherapy in these samples.

In a univariate logistic regression analysis, IgG myeloma and low risk category were strongly associated with false-positive GM antigenemia. Sex, type of treatment, antibiotics, corticosteroid usage, and serum levels of immunoglobulins were also significant or marginally associated with false-positivity. Multivariate analysis confirmed diagnosis of IgG myeloma as the only independent risk factor for false-positivity (odds ratio, 59.41; 95% confidence interval, 8.19 – 431.0; $p < 0.001$) (see supplementary materials, Table 2). In patients with other diseases, the GM assay showed a high sensitivity (7/11, 63.6%), specificity (96/99, 97.0%), PPV (7/10, 70%), and NPV (96/100, 96%). In contrast, for patients with IgG myeloma, specificity and PPV of the assay were very low (3/14, 21.4%, and 0/11, 0%), while NPV was 100% even in this cohort.

A recent meta-analysis addressing the accuracy of a GM assay for diagnosing IA confirmed the clinical usefulness of this test with a sensitivity of 71% and a specificity of 89% (18). Although our study demonstrated similar sensitivity (7/11:64%) and specificity (103/117:88%) of the GM test, PPV (33%) was lower, compared to previous studies that demonstrate 40-60% PPV (7, 19-21). This difference is due to an unexpectedly high incidence of GM antigen false-positivity (11.3%) in our study. It should be noted, however, that screening of GM antigen was performed less frequently in this study compared to previous studies, where GM antigenemia was evaluated 2 to 3 times per week (1, 2, 22), and such a frequent monitoring is ideal to assure the optimal PPV and NPV.

Diagnosis of multiple myeloma is a major risk factor for GM false-positivity. In particular, the false-positivity was exclusively observed in patients with IgG myeloma and was not observed in patients with other types of plasma cell disorders. These observations should be confirmed in a larger study because some studies previously reported the usefulness of GM antigen assay as a diagnostic tool for IA among patients with hematological malignancies including IgG myeloma (23, 24), and only small numbers of patients with plasma cell disorders other than IgG myeloma were included in this study. Low risk category of developing IA was a risk for false positive results in a univariate, but not multivariate analysis. IgG myeloma remained a strong risk for false positivity even after the compensation by the risk categorization.

Mechanisms of high frequency of GM false-positivity in myeloma

patients remain to be investigated. (1→3)-β-D-glucan, which is released from the fungal cell wall, is also widely used to support diagnosis of fungal infections and adopted as one of the microbiological criterion for probable IA in the revised EORTC/MSG definition (5). A previous study reported that high levels of immunoglobulins interfere with the measurement of (1→3)-β-D-glucan by causing precipitation of insolubilized proteins and increase the non-specific optical density levels of reaction fluid (25), although (1→3)-β-D-glucan was negative in patients showing false-positive GM test in this study. This phenomenon has not been reported in the GM assay. However, serum levels of IgG were not directly associated with the false-positivity; IgG levels did not differ between IgG myeloma patients with and without false-positivity. In addition, the GM test remained to be false-positive even after normalization of IgG levels by chemotherapy in 8 of the 11 patients.

Causative role of PIPC/TAZ, amoxicillin/clavulanic acid, and amoxicillin in GM false-positivity has been well documented (10-12, 17), therefore collection of samples prior to infusion of these antibiotics and the use of a relatively higher cut-off level (> 0.7) are recommended in patients receiving these agents (12). In this study, no patients with GM false-positivity received these antibiotics at the time of sampling. It has been hypothesized that dietary contamination by GM causes GM false-positivity by the translocation of dietary GM into the systemic circulation through the disrupted intestinal mucosal barrier, especially in patients with gastrointestinal GVHD after allogeneic HSCT (6, 7, 9). In our cohort, 1 (3.6%) of the 28 patients who underwent allogeneic HSCT showed

false-positivity. This patient with lymphoma had acute GVHD involving in the skin and intestine. A previous study demonstrated that false-positive results were preferentially observed in patients with febrile neutropenic sepsis (26), although subsequent study was unable to replicate this result (22). A recent study revealed that serum GM antigen levels was significantly higher in severely neutropenic patients (< 0.1 x 10^9/L) than in the other patients (27). However, we did not find such an association in this study.

In conclusion, the incidence of false-positive GM antigenemia was high in patients with IgG myeloma. Although the results should be confirmed in a prospective study including larger numbers of patients, positive results of GM antigenemia may be interpreted with caution, and intimate survey including CT scan or other microbiological markers will be recommended in myeloma patients.

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Table 1. Characteristics of 21 patients with positive GM test

Case	Age /Sex	Disease	Treatment	GM (C.O.I)	Radiological / clinical findings	Diagnosis	Times / duration of false positivity	Antibiotics*	Antifungal agents*	Steroids	Neutrophils ($\times 10^9$/L)	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	Risk of IA
1	49/F	MDS/AML	CTx	1.1	nodules with halo	probable IA	-	CFPM	FLCZ	none	0.01	1419	227	50	High
2	63/M	MDS/AML	CTx	1.2	nodules	probable IA	-	MEPM, AMK	FLCZ	none	0	1253	238	62	High
3	50/F	AML	CTx	1.2	nodules	probable IA	-	MEPM, AMK	ITCZ	none	0	1301	293	235	High
4	57/F	MDS	allo-SCT	2.3	nodules	probable IA	-	CAZ, VCM	VRCZ	none	0.009	1198	256	120	High
5	63/M	ML	CTx	1.3	consolidation with pleural pain	probable IA	-	none	MCFG	DEXA	2.034	689	126	92	Low
6	57/F	ML	CTx	1.5	nodules	probable IA	-	CFPM	FLCZ	PSL	0.081	675	117	67	Low
7	59/F	ML	CTx	2.2	consolidation with pleural pain	probable IA	-	CZOP	MCFG	PSL	0.036	719	95	20	Low
8	70/M	ML	CTx	0.8	pneumoconiosis	F-P	7 / 7 months	none	FLCZ	PSL	5.096	1870	315	60	Low
9	52/M	ML	allo-SCT	2.5	bacterial pneumonia	F-P	2 / 1 month	CZOP, CPFX	ITCZ	mPSL	0.806	562	49	19	High
10	54/M	ML	CTx	0.9	negative	F-P	5 / 3 months	CFPM	FLCZ	none	0.395	691	46	10	Low
11	63/F	IgGκ MM	CTx	0.8	negative	F-P	2 / 1 month	none	ITCZ	DEXA	1.497	4770	25	27	Low
12	56/F	IgGκ MM	none	1.2	negative	F-P	3 / 2 months	CAZ	FLCZ	none	3.449	7196	5	38	Low
13	69/M	IgGκ MM	CTx	0.5	negative	F-P	4 / 4 months	none	MCFG	DEXA	0.72	3944	13	<10	Low
14	69/M	IgGκ MM	CTx	1.2	negative	F-P	6 / 3 months	none	FLCZ	DEXA	1.777	2385	6	28	Low
15	64/M	IgGκ MM	CTx	1.6	old inflammatory change	F-P	5 / 2 months	none	MCFG	PSL	1.919	3269	30	<10	Low
16	51/F	IgGκ MM	CTx	2.1	IP	F-P	23 / 15 months	none	ITCZ	PSL	3.791	8006	99	30	Low
17	64/M	IgGκ MM	CTx	1.6	negative	F-P	13 / 8 months	none	ITCZ	DEXA	0.899	3950	6	<10	Low
18	75/M	IgGκ MM	CTx	0.6	negative	F-P	2 / 1 month	MEPM	FLCZ	DEXA	0.823	8986	<5	<10	High
19	49/F	IgGκ MM	CTx	1.1	negative	F-P	8 / 3 months	none	MCFG	DEXA	0.724	4563	37	15	Low
20	74/F	IgGλ MM	CTx	0.7	negative	F-P	6 / 2 months	none	MCFG	none	1.451	3924	25	18	Low
21	56/M	IgGλ MM	CTx	1.1	negative	F-P	10 / 3 months	none	ITCZ	DEXA	1.605	4917	9	10	Low

*administrated at initial positive sampling, MDS : myelodysplastic syndrome, AML : acute myelogenous leukemia, ML : malignant lymphoma, MM: multiple myeloma, CTx : chemotherapy, allo-SCT : allogeneic stem cell transplantation, C.O.I : cut-off index, IP : interstitial pneumonia, F-P : false-positive, CFPM : cefepime, MEPM : meropenem, AMK : amikacin, CAZ : ceftazidime, VCM : vancomycin, CZOP : cefozopran, PIPC/TAZ : piperacillin/tazobactam, CPFX : ciprofloxacin, PSL : prednisolone, mPSL : methylprednisolone, DEXA : dexamethasone.

Ⅵ. 平成 22 年度研究成果作成資料

JAPAN
MARROW
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PROGRAM

日本骨髄バンク

ドナー適格性判定基準

（BMT／PBSCT）
（初版　2010.10.1）

財団法人　骨髄移植推進財団

ドナー適格性判定基準

【 判定内容および対応 】

A 適　格：骨髄採取や末梢血幹細胞採取および移植の支障となるような疾患(器質的、精神的)が無いと思われるものは、コーディネートを進める。
患者理由で中止となった時のドナー登録は、継続とする。

B 要検討：各ドナーの状況に応じて検討を要するもの。結論が出るまでコーディネートは進めない。
確認検査時に「適格」としてコーディネート進行しても、採取前健康診断時に最終結論として不適格となることもある。
不適格となった場合は内容によって、ドナー登録は保留(原則として１年間)または取消とする。

A 適格 及び B 要検討項目で示してある検査値等はあくまでも参考値であり、最終的な判断は採取施設(採取担当医師及び麻酔科医師)が行う。

C 不適格：当面は全身麻酔下での骨髄採取や末梢血幹細胞採取に支障をきたす可能性があると思われるもの。
該当する場合は原則としてコーディネートは中止とする。
コーディネートを中止としたものは、本人に通知し、一定期間（原則として１年間）ドナー登録を保留とする。内容によっては、取消とする。

D 絶対不適格：将来にわたっても骨髄採取や末梢血幹細胞採取により健康上支障をきたしうる疾患、または患者に移行し得る疾患の既往歴があるものは、ドナー不適格とし、コーディネートを中止とする。ドナー候補者には、ドナー登録取消しの手続きをおこなう。
※※登録取消しのドナー候補者には、敬意をもって対応すること。

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改 訂 履 歴

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改訂No.　　平成　　年　　月　　日改訂	改訂責任者：
改訂理由：	
改訂箇所：	

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