

Dendritic cells

Blood dendritic cells are decreased in acute graft-versus-host disease

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Summary:

The recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT) often develop acute graft-versus-host disease (aGVHD), which is closely related to morbidity and mortality. However, the essential part of the immune responses elicited in aGVHD remains largely unknown. We attempt to determine if peripheral blood dendritic cells (PBDCs) are altered in aGVHD, and find that the number of PBDCs (both myeloid and lymphoid DCs) is significantly decreased. Immunohistochemical staining of the biopsied skin from patients with aGVHD demonstrates that a number of fascin⁺ cells with dendritic projections infiltrate the dermis of the skin. Based on these findings, we hypothesize that the PBDCs are recruited to the affected tissues and may thus play important roles in immune responses elicited in aGVHD.

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quently attack the recipient organs.³ It has been suggested that dysregulation of cytokines plays an important role in the development of aGVHD.^{4–6} However, the essential mechanism of the immune responses elicited in aGVHD remains largely unknown.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that have a unique capacity to prime naive T cells. Recent progress in DCs biology has highlighted the central roles of DCs in inducing various immune responses.^{7–9} In the human peripheral blood, two major subsets of DCs – CD11c⁺ and CD11c[–] subsets – have been identified.^{10,11} The CD11c⁺ subset belongs to the myeloid lineage and is considered to consist of very immature DCs, whereas the CD11c[–] subset, recognized as plasmacytoid cells, consists of the direct precursors of lymphoid DCs. These peripheral blood DC (PBDC) subsets may migrate to the tissues of various organs and experience multi-step maturation processes during their migration. DCs in the fully mature stage act as potent APCs, and thus dictate the quality and the magnitude of T-cell responses.^{12,13} Therefore, both PBDC subsets are regarded as circulating pools for the DCs in the peripheral tissues. Emerging evidence has suggested that DCs are involved in various disease conditions.^{14–16} Indeed, we have recently reported that, in patients with primary Sjögren's syndrome, which is an organ-specific autoimmune disease, a subset of PBDCs was recruited to the salivary glands and thus induced a type 1T helper (Th1) response, indicating a novel mechanism of this disease.¹⁴ Thus, PBDCs may provide an essential viewpoint and a useful tool in dissecting the pathophysiology of various diseases.

In this study, we examine the number of PBDCs before and after the occurrence of aGVHD in patients with hematological malignancies who received allo-HSCT, and find that the number of PBDCs and their fractions is decreased in parallel with the occurrence of aGVHD.

Patients and methods

Patients

A total of 11 patients who received allo-HSCT (five men and six women, mean age 34.7 years, range 17–57 years), at our hospital, between June 2000 and September 2001, were enrolled in this study with their consents; three had acute myelogenous leukemia (AML), four had acute lymphoblastic leukemia (ALL), three had chronic myelogenous leukemia (CML) and one had refractory anemia with

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been established to be a beneficial therapeutic strategy for hematological malignancies. The primary idea underlying allo-HSCT is to reconstitute hematopoietic cells that originate from the transplanted donor stem cells after abrogation of recipient bone marrow cells induced by myeloablative preconditioning.¹ During or after the reconstitution of the hematopoietic cells, the recipients often develop acute graft-versus-host disease (aGVHD), which currently constitutes a major cause of treatment-related mortality (TRM).² Acute GVHD is a complicated post transplant condition, and a myriad of factors are suggested to be involved in this process. The essential basis of aGVHD is recognized to be primarily the immune responses that are evoked by the existence of allogeneic disparities between donor and recipient, and that subse-

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excess of blasts in transformation (RAEB-t), as shown in Table 1. Eight patients received allo-BMT, two received allo-PBSCT and one received CBSCT. For the treatment of residual disease, two patients (patients 8 and 10) received donor lymphocyte infusion (DLI) (1×10^7 T cells/kg) after allo-HSCT. Six patients received allo-HSCT from human leukocyte antigen (HLA)-matched donors. As a conditioning regimen, nine patients received total body irradiation (TBI total 12Gy) and various combinations of high-dose chemotherapy. The remaining two patients received busulphan (BU) and cyclophosphamide (CY). For GVHD prophylaxis, six patients received a combination of cyclosporin A (CyA) and methotrexate (MTX), and five patients received tacrolimus (FK-506) and MTX. The diagnosis of aGVHD was made based on the histological findings of the specimens obtained by biopsy. Grading of aGVHD was performed according to the criteria described by Przepiorka *et al.*¹⁷ All patients developed aGVHD after allo-HSCT. In all, two patients received DLI after allo-HSCT, as shown in Table 1. Immunosuppressive agents such as corticosteroids or antithymocyte globulin (ATG) were used to treat severe aGVHD. Six patients had an infection caused by a bacterium or fungus and five patients had cytomegalovirus (CMV) antigenemia after allo-HSCT. However, none of the patients had CMV antigenemia or other major infections during the periods of examination. Informed consent was obtained for this study in accordance with the provisions of the Declaration of Helsinki.

Controls

For normal controls, we collected blood from healthy adult volunteers ($n = 20$, nine men and 11 women, mean age 35.2 years, range 19–59 years). No patients or volunteers showed evidence of infection at the time of study.

Media

RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ng/ml streptomycin and 10% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA) was used throughout the experiments.

Enrichment and analyses of PBDCs

PBDCs were enriched using a previously described protocol.¹⁸ Briefly, peripheral blood mononuclear cells (PBMCs) from normal donors and patients after allo-HSCT were prepared by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation of heparinized venous blood. PBMCs were incubated with anti-CD3, anti-CD14 and anti-CD19 monoclonal antibodies (mAbs) (PharMingen, San Diego, CA, USA) for 30 min on ice, and cells binding these mAbs were removed using sheep anti-mouse IgG-coated magnetic beads (M-450; Dynal, Oslo, Norway). Using this method, the percentage of PBDCs (less than 1% of total PBMCs) was increased to 2–5% (DC-enriched population). Three fractions of PBDCs were identified:¹⁸

Table 1 Patient characteristics

Case	Diagnosis	Disease status	Sex	Age	Graft	HLA correspondence	Pretransplant conditioning	GVHD prophylaxis	aGVHD grade	Outcome	Day post HSCT of PBDC sampling		Day post HSCT of donor lymphocyte	Day post HSCT of immunohistochemical stainings
											Before aGVHD	After aGVHD		
1	MDS	RAEB-t	F	50	BM	6/6	BU/CY	CyA/MTX	I	Alive	19	33	ND	30
2	ALL (L2)	Ist CR	M	29	BM	6/6	CY/TBI	CyA/MTX	II	Alive	17	33, 49, 70	ND	31
3	AML (M2)	Non CR	M	27	BM	5/6	CY/TBI	CyA/MTX	II	Dead	N.T.	45	ND	NT
4	CML	CP	F	32	BM	5/6	CY/TBI	FK/MTX	II	Dead	N.T.	36	ND	NT
5	CML	BC	F	38	BM	5/6	CY/TBI	FK/MTX	IV	Dead	N.T.	80	ND	32
6	AML (M5a)	Ist CR	M	50	BM	5/6	CY/TBI	FK/MTX	I	Dead	N.T.	14	ND	NT
7	CML	CP	M	30	BM	6/6	CY/TBI	FK/MTX	IV	Dead	N.T.	20	ND	NT
8	ALL (L2)	Non-CR	F	17	BM	6/6	CA/CY/TBI	CyA/MTX	II	Alive	85	97	87	95
9	ALL (L2)	Non-CR	M	57	PB	6/6	BU/CY	FK/MTX	III	Dead	24, 31	17, 43, 53, 63	ND	17
10	AML (M1)	Non-CR	F	29	PB	6/6	CY/TBI	CyA/MTX	III	Alive	17	70	58	65
11	ALL (L2)	Non-CR	F	53	CB	3/6	CA/CY/TBI	CyA/MTX	II	Dead	27	83	ND	NT

MDS = myelodysplastic syndrome; ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; RAEB-t = refractory anemia with excess of blasts in transformation; CR = complete remission; CP = chronic phase; BC = blastic crisis; BM = bone marrow; PB = peripheral blood; CB = cord blood; BU = busulfan; CY = cyclophosphamide; TBI = total body irradiation; CyA = cyclosporin A; MTX = methotrexate; FK = tacrolimus; NT = not tested; ND = not done.

(i) CD1b/c⁺/CD11c⁺ (fraction 1) PBDCs: The PBDC-enriched population was stained with FITC-labeled anti-CD1b/c mAbs (B-B5: Biosource, Camarillo, CA, USA), a mixture of PE-labeled mAbs against lineage markers (CD3, CD14, CD16 and CD19; Exalpa, Boston, MA, USA; CD15, Coulter, Hialeah, FL, USA; CD56, Becton-Dickinson, San Jose, CA, USA) and phycoerythrin-cyanin5-1 (PC5)-conjugated anti-HLA-DR mAbs (Immu-357: Immuno Tech, Marseille, France), and the CD1b/c⁺/DR⁺/lin⁻ population was identified as CD1b/c⁺/CD11c⁺ PBDCs. Note that we confirmed that the identified CD1b/c⁺/DR⁺/lin⁻ PBDCs are CD11c⁺ by four-color staining. (ii) CD1b/c⁻/CD11c⁺ (fraction 2) and CD1b/c⁻/CD11c⁻ (fraction 3) PBDCs: The PBDC-enriched population was stained with FITC-labeled mAbs against lineage markers (CD3, CD14 and CD16; Exalpa, CD15, CD19 and CD56; Becton Dickinson) and CD1b/c, PE-labeled anti-CD11c (Becton Dickinson) and PC5-conjugated anti-HLA-DR mAbs. CD1b/c⁻/lin⁻/CD11c⁺/DR⁺ PBDCs and CD1b/c⁻/lin⁻/CD11c⁻/DR⁺ PBDCs were identified as fraction 2 and fraction 3 PBDCs, respectively. Before staining, the cells were incubated with polyclonal mouse immunoglobulin (Cappel, Aurora, OH, USA) to block nonspecific binding of labeled mAbs. The absolute numbers (per ml) of PBDCs were calculated by multiplying the percentage of the lin⁻/DR⁺ population among the DC-enriched population by the count (per ml) of the DCs-enriched population. The absolute number (per ml) of each fraction of PBDCs was calculated by multiplying the percentage of each fraction within the lin⁻/DR⁺ population by the total number of the lin⁻/DR⁺ population.

Histopathological analyses

Formalin-fixed, paraffin-embedded sections were prepared from biopsied specimens of skins of patients with aGVHD. Hematoxylin and eosin (HE) staining was performed in all patients with aGVHD. The immunohistochemical analyses of paraffin-embedded sections was performed on six patients with aGVHD, using the monoclonal antibodies against CD1a (Novocastra, Newcastle, UK), fascin (Dako, Carpinteria, CA, USA) and HLA-DR (Nichirei, Tokyo, Japan), known to react with DCs.¹⁹⁻²² Site-matched specimens (skin from anterior chest) obtained from four age-matched volunteers were examined as normal controls. Four random fields containing the epidermis and the dermis were selected from each specimen and the cells immunoreactive to anti-CD1a Ab or anti-fascin Ab or anti-HLA-DR Ab in each field were counted under a microscope at a magnification of ×200. We obtained the mean numbers of immunoreactive cells per field for each specimen. Statistical analysis of the number of immunoreactive cells was performed between aGVHD and normal control groups.

Statistical analysis

The Mann-Whitney *U*-test was used for statistical analysis with a StatView statistical program (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant when tied *P*-values were less than 0.05.

Results

Absolute numbers of PBDCs after allo-HSCT

We examined the number of PBDCs and their fractions by flow cytometry in 11 patients who received allo-HSCT for hematological malignancies and 20 normal control subjects. There was no significant difference in the total numbers of white blood cells among patients with and without aGVHD and control subjects (data not shown). Indeed, the total numbers of white blood cells in the patients with and without aGVHD were within normal levels (3000–8500/ μ l) at all time points examined. The normal control subjects had a mean absolute PBDC count of 19 173/ml (range 13 566–31 762/ml). Six patients without aGVHD had a mean absolute PBDC count of 22 762/ml (range 9735–42 727/ml). There was no significant difference between the two groups. On the other hand, 11 patients with aGVHD had a significantly lower PBDC count (mean 4849/ml, range 349–19 078/ml) than patients without aGVHD (*P* = 0.0048) (Figure 1a).

Three fractions of PBDCs were identified within the lin⁻, HLA-DR⁺, population, based on the differential expression of CD1b/c and CD11c. As shown in Figure 1b and d, the absolute numbers of PBDCs from fractions 1 and 3

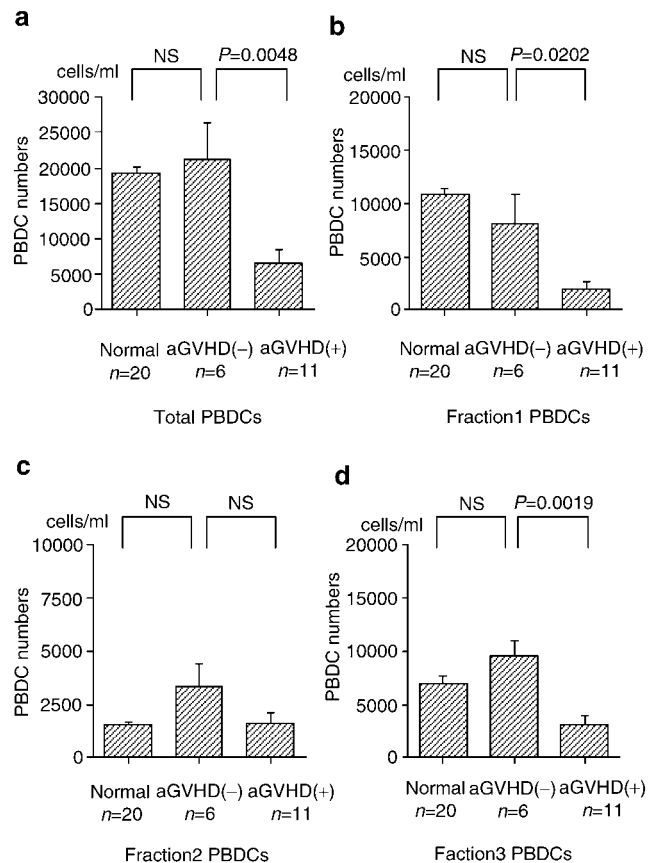


Figure 1 Absolute numbers of the total and each fraction of PBDCs. (a) Total PBDCs, (b) fraction 1 PBDCs, (c) fraction 2 PBDCs, (d) fraction 3 PBDCs, NS = not significant.

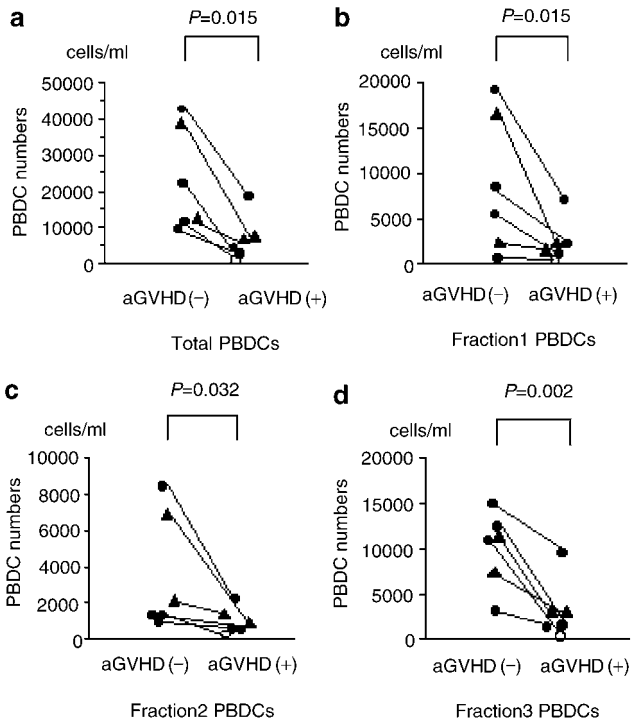


Figure 2 Reduction of the number of PBDCs in aGVHD. Six patients were sequentially examined for the number of total PBDCs and three PBDC fractions before and after the occurrence of aGVHD. Of the six patients, five (black circles and triangles) were free from corticosteroids at the time of examination. The remaining one patient (white circles) had already been treated with corticosteroids at the time of examination after the occurrence of aGVHD. Of the six patients, two (triangles) received DLI after allo-HSCT. (a) Total PBDCs, (b) fraction 1 PBDCs, (c) fraction 2 PBDCs, (d) fraction 3 PBDCs.

were significantly lower in patients with aGVHD than in patients without aGVHD ($P=0.0202$ and 0.0019). However, the DC count of fraction 2 was not significantly different between the two groups ($P=0.0782$) (Figure 1c).

Six of the 11 patients could be sequentially examined for the number of PBDCs before and after the occurrence of aGVHD (Figure 2). Of the six patients, five (patient no. 1, 2, 8, 9 and 10) were free from corticosteroids at the time of examination. On the other hand, the remaining patient (patient no. 11) had already been treated with corticosteroids at the time of examination after the occurrence of aGVHD. Patients 8 and 10 received DLI after allo-HSCT because of persisting residual diseases, and were examined for the PBDC count before and after the occurrence of DLI-induced GVHD (Table 1). These two patients received neither corticosteroids nor immunosuppressants such as cyclosporin A at the time of examinations. As shown in Figure 2a, the number of total PBDCs was significantly decreased after the occurrence of aGVHD in all the six patients ($P=0.015$), although there was a wide variation in total PBDC counts among the patients, especially before the onset of aGVHD. We also examined the numbers in each of the three PBDC fractions

at various time points in the same six patients (Figure 2b–d), and found all to be significantly decreased when aGVHD occurred in all of the six patients ($P=0.015$, 0.032 and 0.002).

Temporal profile of the number of PBDCs in aGVHD

We could examine the absolute PBDC counts in two patients (patients 2 and 9) at multiple time points after allo-HSCT. In patient no. 2, we observed a reconstitution of PBDCs as early as day 17 post allo-BMT. However, the number of PBDCs gradually decreased after the occurrence of aGVHD. The reduced number of PBDCs was restored to the pre-aGVHD level after aGVHD had been ameliorated (Figure 3a). On the other hand, patient no. 9 developed skin GVHD on day 14 post allo-PBSCT. After the skin rash resolved, the number of PBDCs was restored to the normal level. However, the patient experienced skin and liver GVHD (grade 3) again on day 40 post allo-PBSCT, when the total PBDC count was dramatically lower than the pre-GVHD level (Figure 4b).

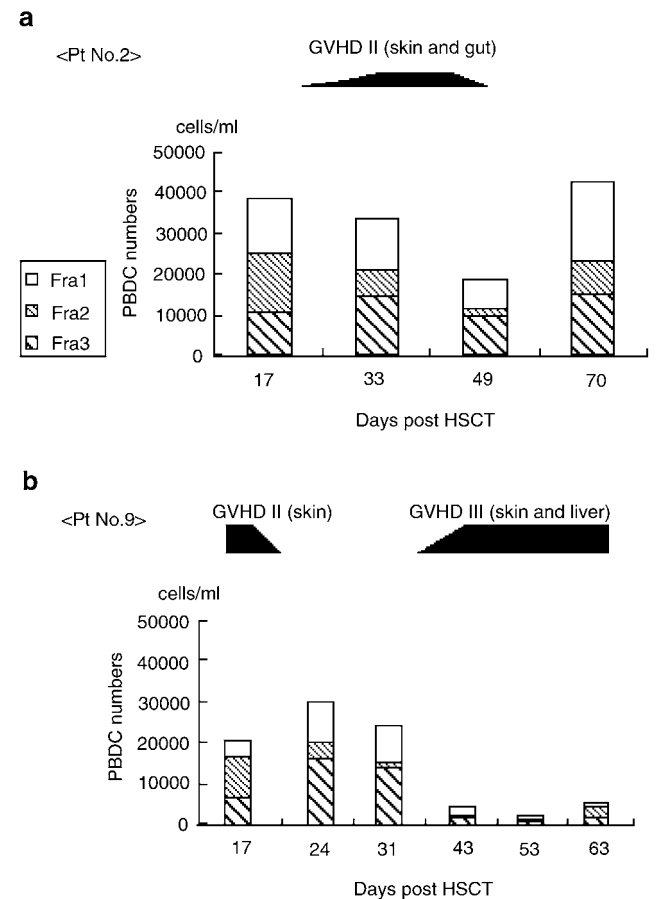


Figure 3 Correlation between absolute numbers of PBDCs and clinical manifestation of aGVHD. Two patients were examined for the total number of PBDCs and three PBDC fractions at multiple time points after allo-HSCT. The clinical manifestation of aGVHD is represented. (a) Pt No. 2, (b) Pt No. 9; Fra 1, Fraction 1 PBDCs; Fra 2, Fraction 2 PBDCs; Fra 3, Fraction 3 PBDCs.

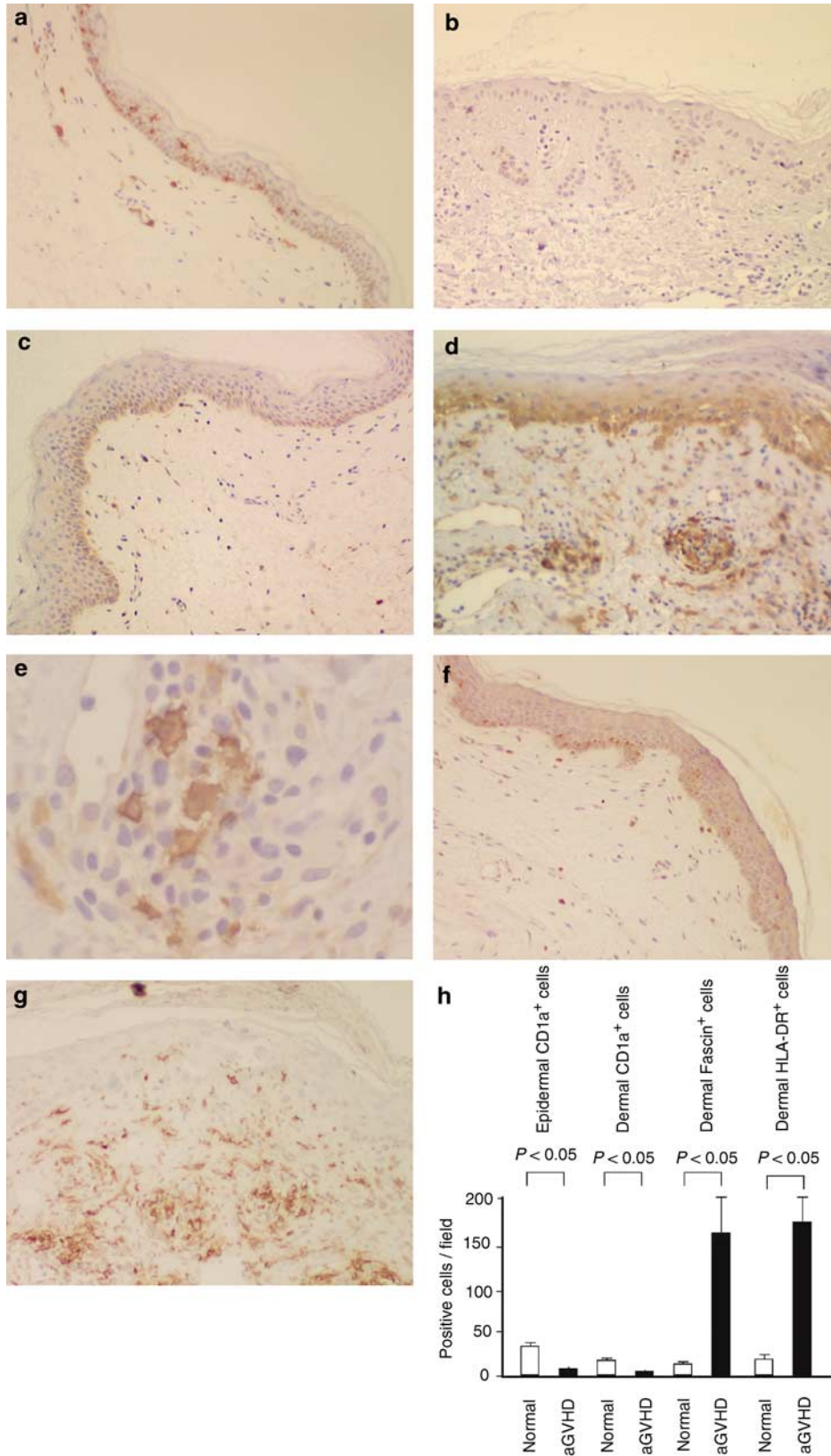


Figure 4 Immunohistochemical staining of skin with anti-CD1a Ab, anti-fascin Ab and anti-HLA-DR Ab. (a–g) Representative immunohistochemical staining of aGVHD and normal control skin. (a, b) Staining with anti-CD1a Ab at a magnification of $\times 200$. (c, d) Staining with anti-fascin Ab at a magnification of $\times 200$. (e) Staining with anti-fascin Ab at a magnification of $\times 1000$. (f, g) Staining with anti-HLA-DR at a magnification of $\times 200$. (a, c, f) Control skin; (b, d, e, f) aGVHD skin (patient no. 9); (h) frequency of epidermal and dermal CD1a⁺ cells, dermal fascin⁺ cells and dermal HLA-DR⁺ cells.

Grade 3 aGVHD persisted despite the treatment with corticosteroids, and in parallel with that the number of PBDCs remained low thereafter. The patient eventually died because of the aGVHD on day 89 post allo-PBSCT.

Infiltration of fascin-positive mononuclear cells (MNCs) in the skin of the patients with aGVHD

The skin from six of eleven patients with aGVHD and skin from four normal individuals was immunohistochemically examined. Hematoxylin and eosin (H-E) staining of the skin from all 11 patients with aGVHD demonstrated infiltration of a number of MNCs in the epidermis and the dermis, while no such infiltration was detected in normal control subjects (data not shown). To examine whether DCs were infiltrating the skin, immunohistochemical staining of the skin was performed using mAb against CD1a, fascin and HLA-DR, a DC-associated marker^{19–22} (Table 1). As shown in Figure 4a, a considerable number of CD1a-positive cells, which might represent Langerhans cells, were detected in the epidermis of normal control skin. In addition, a smaller number of CD1a-positive cells were also found in the superficial dermis of normal control skin (Figure 4a). On the other hand, hardly any CD1a-positive cells were detected in either the epidermis or dermis of aGVHD skin (Figure 4b). Statistical analysis demonstrated that the epidermal and dermal CD1a-positive cells were significantly decreased in aGVHD, compared to normal control ($P=0.023$ and 0.023) (Figure 4h). On the other hand, numerous fascin-positive cells were detected in the epidermis and almost all of the cells in the basal layers appeared to be fascin-positive in both normal control and aGVHD (Figure 4c, d). Although the epidermal fascin-positive cells tended to be increased in aGVHD, the increase was not statistically significant compared to normal control skin ($P=0.0667$) (data not shown). In contrast to the epidermis, the distribution pattern of fascin-positive cells in the dermis was different between normal control and aGVHD. In the dermis of normal skin, only a small number of fascin-positive cells were detected (Figure 4c), whereas, in the dermis of aGVHD, there was a wide distribution of a number of fascin-positive cells (Figure 4d). Furthermore, some fascin-positive cells had formed aggregates with many fascin-negative MNCs and the aggregates were scattered throughout the dermis (Figure 4d). Morphologically, many of the fascin-positive cells in the dermis of aGVHD had dendritic projections (Figure 4e). The dermal fascin-positive cells were significantly increased in aGVHD compared to normal controls ($P=0.023$) (Figure 4h). In addition, many HLA-DR-positive cells were also found in the dermis of aGVHD, whereas almost no such cells were detected in the dermis of normal control (Figure 4f, g). The frequency and the distribution pattern of HLA-DR-positive cells were similar to those of fascin-positive cells in aGVHD (Figure 4f, g). The dermal HLA-DR-positive cells were significantly increased in aGVHD compared to normal controls ($P=0.023$) (Figure 4h).

Discussion

In this study, we found a significant reduction in the number of PBDCs and their fractions in the patients with aGVHD who underwent allo-HSCT or DLI post HSCT. A possible basis underlying this phenomenon is the cell death of PBDCs induced by the corticosteroids, which are usually administered for the treatment of aGVHD, since corticosteroids are known to reduce the viability of DCs.^{23,24} However, this does not appear to be the case, since five out of the six patients who could be examined at a series of time points showed a definite decrease in the number of PBDCs on the occurrence of aGVHD, despite no corticosteroids being used for the treatment of aGVHD at the time of examination. In addition, two patients who developed DLI-induced GVHD received neither corticosteroids nor immunosuppressants such as CyA and FK-506 at the time of the examinations. Interestingly, it was demonstrated in two patients that the reduced number of PBDCs were restored to the pre-aGVHD level after aGVHD had been ameliorated. All these findings suggest that the reduction in the number of PBDCs may be directly associated with the development of aGVHD. A more plausible mechanism underlying the decrease in the number of PBDCs might be their active recruitment from the peripheral blood to the affected tissues during the occurrence of aGVHD. In accord with this assumption, we found that many fascin-positive cells had infiltrated the dermal region of the biopsied skin from patients with aGVHD, whereas little such infiltration of DCs was observed in the skin of normal control subjects. Fascin is an actin-bundling protein that is important for the development of dendrites and, among hematopoietic cells, its expression is specific to mature DCs.^{21,22} Indeed, the fascin-positive cells in the dermis of aGVHD showed dendritic projections. We also detected fascin-positive cells in the epidermis of both normal controls and aGVHD. However, the expression of fascin in the epidermis of normal control and aGVHD was manifested in a fashion that most of the cells in the basal layers were fascin-positive. The fascin-positive cells in the epidermis may possibly represent epidermal cells other than DCs. Furthermore, we also detected a number of HLA-DR (an essential marker of DCs)-positive cells in the dermis of aGVHD. The frequency and the distribution pattern of HLA-DR-positive cells were similar to those of fascin-positive cells. All these findings indicate that mature DCs had infiltrated the dermal region in aGVHD. Collectively, the reduction in the number of PBDCs and the concomitant accumulation of mature DCs in the dermis of aGVHD may favorably support, although indirectly, our hypothesis regarding the recruitment of DCs in aGVHD. On the other hand, it has been reported that epidermal and dermal CD1a-positive cells, which may represent epidermal Langerhans cells and dermal DCs, respectively, were significantly decreased in the skin of aGVHD.^{25–27} In this study, we also detected a significant decrease of epidermal and dermal CD1a-positive cells. It was also reported that dermal DCs, determined as factor XIIIa-positive cells, are decreased in the skin in aGVHD.²⁷ Although currently we do not know the basis of the discrepancy in the dynamics of CD1a-, factor XIIIa- and fascin-positive cells in the skin of

aGVHD, it might well be that the expression profile of CD1a, factor XIIIa and fascin in DCs might be different, depending on the maturation state of DCs. In this context, it is interesting to note that epidermal Langerhans cells downregulate CD1a expression under certain circumstances and upregulate fascin expression during maturation.^{21,28} Thus, the reduction in the number of CD1a-positive-cells in aGVHD skin may reflect the maturation of the cells caused by aGVHD.

The recruitment of PBDCs to inflammatory lesions does not seem to be a phenomenon specific to only aGVHD. Indeed, we have recently suggested that the recruitment of PBDCs to inflammatory tissues occurred in Sjögren's syndrome and pulmonary tuberculosis, indicating central roles of DCs in these diseases.^{14,15} Furthermore, in systemic lupus erythematosus (SLE), it is suggested that the blood plasmacytoid cells, the precursors of lymphoid dendritic cells, migrate to the skin.¹⁶ These findings suggest that the recruitment of PBDCs to tissues might be an event that is commonly observed in inflammatory diseases. However, the types of PBDC that decrease and the extent of the decrease seem to be different among inflammatory disease conditions, including aGVHD, suggesting a different level of contribution of DCs in individual diseases.

Acute GVHD is recognized to occur primarily as a consequence of donor lymphocyte reactions in response to the disparities of allo-antigens between the donor and recipient.³ In light of the essential roles of DCs as APCs that trigger immune responses, it is quite possible that DCs play an important role in eliciting and maintaining aGVHD. It has been demonstrated in the murine system that host residual DCs are essential in the pathogenesis of aGVHD after allogeneic bone marrow transplantation.^{29,30} In this context, the current study findings are intriguing in that they implicate donor-derived DCs in the development of aGVHD. Thus, it will be important future work to clarify how donor vs recipient DCs are differentially involved in the development and maintenance of aGVHD.

In summary, we have demonstrated that the PBDCs were significantly decreased and mature DCs had accumulated in the skin in parallel with the occurrence of aGVHD in the patients who underwent allo-HSCT or DLI. The findings obtained in this study have shed light on the possible DC-based mechanism of aGVHD, and thus may be helpful in understanding the nature of aGVHD and also establishing new therapeutic strategies for aGVHD.

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