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Brief report

Predominant infiltration of monocytes in chronic graft-versus-host disease

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Abstract

Pathogenesis of chronic graft versus host disease (cGVHD) is largely unknown. It is important to determine the responsible cell types and the factors that play roles to recruit these cells into sites of disease. We examined whether monocytes and chemokine fractalkine/receptor CX3CR1 axis might be involved. We found that the absolute number of CX3CR1+ monocytes in the blood was significantly decreased in patients with severe cGVHD. Immunohistochemical staining revealed the extensive infiltration of CD14+ cells as well as strong expression of fractalkine in the cGVHD skin. The number of infiltrated CD14+ cells on the margin of fractalkine+ epidermis was larger in cGVHD skin compared to that of acute GVHD, whereas no difference was observed in CD3+ T cells. These results suggest that CX3CR1+ monocytes may be recruited from the circulation to the fractalkine+ epidermis in cGVHD, and highlight these cells and this chemokine/receptor axis as additional targets for cGVHD therapy.

(word count 150)

Introduction

Chronic graft versus host disease (cGVHD) is the most common late complication of allogeneic stem cell transplantation (alloSCT) (*1*). Clinical management of cGVHD depends on immunosuppressive agents to target lymphocytes, but therapy is complicated by untoward side effects, infections and frequent failure to control the underlying process. More than 20% of primary causes of death is cGVHD among patients who are disease free two years after alloSCT (*2*). To establish a better therapeutic strategy, it is important to determine the cell types and the mediators that are responsible for cGVHD.

The role of chemokines in acute GVHD (aGVHD) has been actively studied (*3*). However, few reports have examined their role in cGVHD (*4-6*). Fractalkine/CX3CL1 is an unique chemokine that exists not only as a chemo-attracting soluble factor but also as a membrane-anchored form that mediates adhesion of leukocytes expressing its cognitive receptor CX3CR1 to the endothelium (*7, 8*). Monocyte is one of the major cell types which express CX3CR1 (*8, 9*). The immune responses of fractalkine-deficient mice to a variety of T cell-mediated inflammatory stimuli, such as delayed-type hypersensitivity, were not impaired (*10*). However, fractalkine- or CX3CR1-deficient mice exhibited reduced atherogenesis due to impaired recruitment of monocytes (*11, 12*). The strong expression of fractalkine or predominant infiltration of monocytes has been reported in some chronic autoimmune disorders

(*13-15*). Because autoimmune disorders and cGVHD share many clinical manifestations, we focused on monocytes as a candidate of responsible cell type other than lymphocytes. We examined their distribution in the blood and skin of cGVHD patients in association with chemokine fractalkine/receptor CX3CR1 axis.

Materials and Methods

Blood and tissue samples

Peripheral blood samples for flow cytometric analyses were obtained from 19 patients who had undergone alloSCT (11 men, 8 women; age: range 20-71, median 41; days after transplantation: range 97-3523, median 681, conditioning: 11 received myeloablative, 8 non-myeloablative regimen, 8 containing total body irradiation (TBI), stem cell source: 11 related peripheral blood stem cell, 5 unrelated bone marrow, 2 related bone marrow, 1 cord blood. The profile of each patient is listed in Supplemental Table 1). Skin biopsy specimens were obtained from alloSCT patients (7 with active rash due to aGVHD, 7 with active rash due to cGVHD and 7 without skin cGVHD. Each group consists of 4 patients who received myeloablative transplantation with TBI and 3 non-myeloablative transplantation with fludarabine containing regimen. The profile of each patient is listed in Supplemental Table 2). All patients studied had no evidence of infection. The protocol was approved by the Institutional Review Board of

Okayama University and informed consent was obtained from patients.

Clinical assessment

The clinical severity of cGVHD was assessed according to the report of National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in cGVHD (*16*). The sum of the each score was used as cGVHD score. The severity of cGVHD was categorized in cGVHD(-)~mild, moderate and severe groups as described (*16*).

Flow cytometry, Immunohistochemistry, Statistics

Methods can be found in supplemental online materials.

Results and Discussion

CX3CR1+ monocytes were decreased in the blood of severe cGVHD patients

As shown in Figure 1A, CD14+ monocytes in patients with no cGVHD symptom displayed high CX3CR1 expression level (range: 52-83%). Non-transplanted normal individuals showed similar level of expression (not shown). We noticed that this expression was low in patients with severe cGVHD (Figure 1A). Correlation between percentage of CX3CR1 positive cells in CD14+ fraction and cGVHD severity score is shown in Figure 1B. There was a strong inverse correlation between cGVHD score and CX3CR1 expression ($n=19$, $p<0.01$). Although the numbers of monocytes in the blood were similar in each category (Figure 1C), the absolute number of CX3CR1 positive monocytes was drastically decreased in patients with severe cGVHD (Figure 1D). Although it is still possible that some minor lymphocyte subsets might show a correlation similar to that observed in monocytes, the analysis of total lymphocytes characterized by low forward and side scatter from same 19 samples did not display such a trend (Supplemental Figure 1). These data suggest that the reduction of CX3CR1 positive cells in severe cGVHD patients may be specific for monocytes.

Fractalkine expression and monocyte infiltration in GVHD skin tissues

We first examined skin tissues at the sites of normal appearance, pigmentation and active rash from a patient with skin cGVHD (Figure 2A). Fractalkine was faintly

expressed in epidermis at the sites of normal appearance and pigmentation (Figure 2B) similar to that in skin from non-transplanted healthy donor (not shown). In contrast, it was strongly expressed in epidermis with hyperplasia in the skin with active rash (Figure 2B). Very few CD14+ cells were observed at the sites of normal appearance and pigmentation (Figure 2B) as well as in the skin from non-transplanted healthy donor (not shown). In sharp contrast, dramatic infiltration of CD14+ cells was observed within the superficial dermis in active rash (Figure 2B). We observed strong epidermal fractalkine expression in all cGVHD samples with some variety of its level. The number of infiltrated CD14+ cells was significantly higher in skin with rash compared to those with normal appearance from transplanted patients (Figure 2C, $n=7$, $p<0.01$). These data suggest that the strong expression of fractalkine and the infiltration of CD14+ cells are not due to the preparative regimen for alloSCT but likely specific phenomenon in active skin cGVHD.

Epidermal fractalkine expression was also observed in aGVHD skin, with a wide variety in its level (from no to relatively strong expression). Remarkably, the fractalkine positive area was greatly wider in cGVHD (Supplemental Figure 2), which is consistent with the feature that aGVHD does not show epidermal hyperplasia (*17*). Epidermal hyperplasia is due to the hyperproliferation of keratinocytes, known as a main fractalkine producer in the skin (*15*). The total amount of fractalkine production from epidermis in cGVHD skin may be much higher than that in aGVHD.

During the study of CD14+ cells in cGVHD skin tissues, we noticed that the infiltration of CD14+ cells was strongly observed at the site along the edge of the dermis bordering the epidermis (Figure 2D), as if these cells were attracted to the epidermis. The number of CD3+ T cells in this specific area showed no difference between acute and chronic GVHD (Figure 2E). Interestingly, there was a trend toward higher number of total cells in cGVHD, and the number of CD14+ cells in cGVHD was significantly higher than that in aGVHD skin in this bordering area (Figure 2E). These results suggest that, in comparison with aGVHD, cGVHD skin is characterized by the strong expression of epidermal fractalkine accompanied by the extensive infiltration of CD14+ cells near this area. However, this may not necessarily apply to all cGVHD patients because cGVHD affects various organs and clinical presentation of skin lesions varies.

It has been reported that, among CD14+ monocytes, $CX3CR1^{high}$ cells are preferentially attracted by fractalkine compared to other monocytes and that these cells are the precursor of antigen presenting cells (APCs) (*9*). Shlomchik et al. have reported that, in contrast to acute GVHD which requires host APCs, donor APCs are required to initiate cGVHD in their mouse model (*18*). It would be important to determine whether the CD14+ cells in the skin are definitely monocytes or could be APCs such as dendritic cells, and whether these cells are host or donor origin.

In conclusion, our data suggest the potential involvement of monocytes in

cGVHD via the fractalkine-CX3CR1 pathway, and highlight these previously unappreciated cells and chemokine-receptor axis as additional targets for cGVHD therapy.

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Figure legends

Figure 1. CX3CR1 positive monocyte in the blood is decreased in patients with severe cGVHD. (A) Representative histograms of CX3CR1 expression in CD14+ fraction are shown. Gray: isotype matched control. (B) CX3CR1 expression level inversely correlates with severity of clinical symptoms in cGVHD patients. Spearman's rank correlation coefficient, n=19, $p<0.01$, $R=-0.73$. (C) The numbers of peripheral blood total monocytes and (D) CX3CR1 positive monocytes. n=8 for cGVHD(-)~mild, 5 for moderate, and 6 for severe group. *$p<0.05$, **$p<0.01$.

Figure 2. Immunohistochemical staining of fractalkine and CD14 in GVHD skin tissues. (A) A picture of a patient with active cGVHD skin rash. (B) Fractalkine and CD14 staining in skin samples from this patient as indicated in (A). Violet color shows positive staining of target antigens visualized by VIP. Methylgreen was used for the counterstain. No non-specific staining with control antibody was observed in all experiments. Bar: 200 μ m. (C) The number of infiltrated CD14 positive cells in skin tissues from transplanted patients with or without skin cGVHD. Box-and whisker plots. n=7 for each group. **$p<0.01$. (D) A representative picture of CD3 (visualized by DAB, brown)/CD14 (VIP, violet) dual color staining of cGVHD skin. Red dotted line indicates the margin of epidermis (200μm). (E) The number of cells located on the

margin of epidermis in acute and chronic GVHD skin tissues (/ 1 mm). Box-and whisker plots. n=7 for each group. *$p<0.05$.

Figure 1

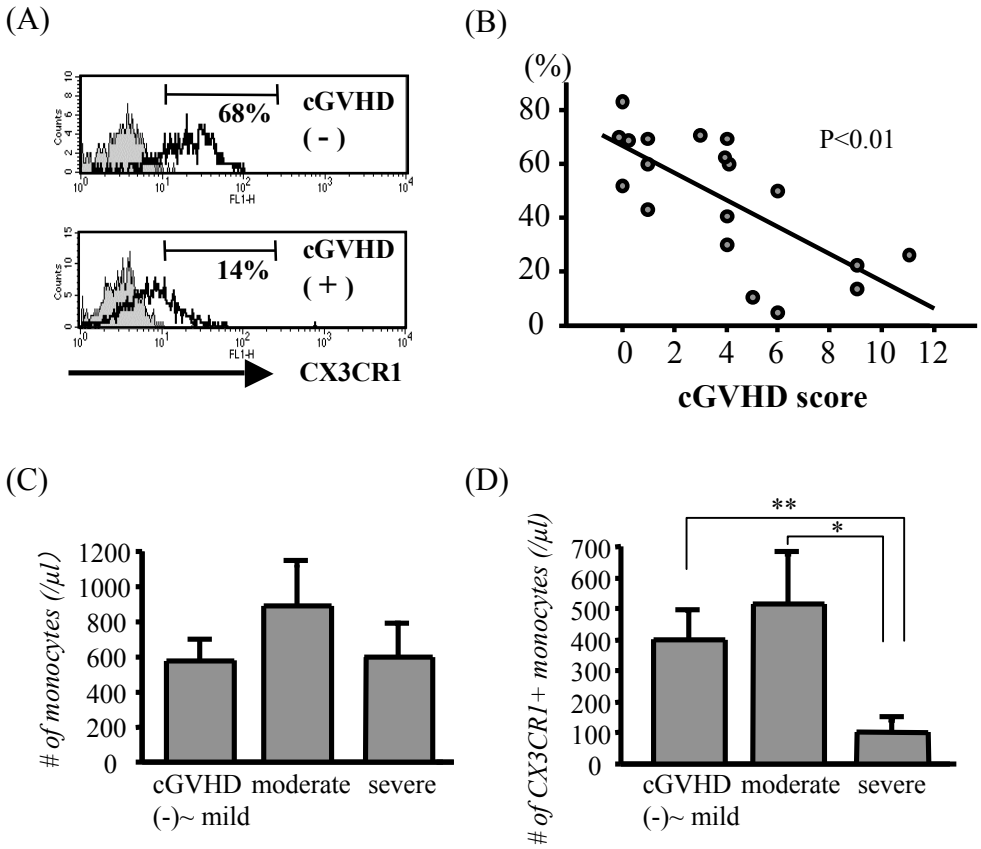
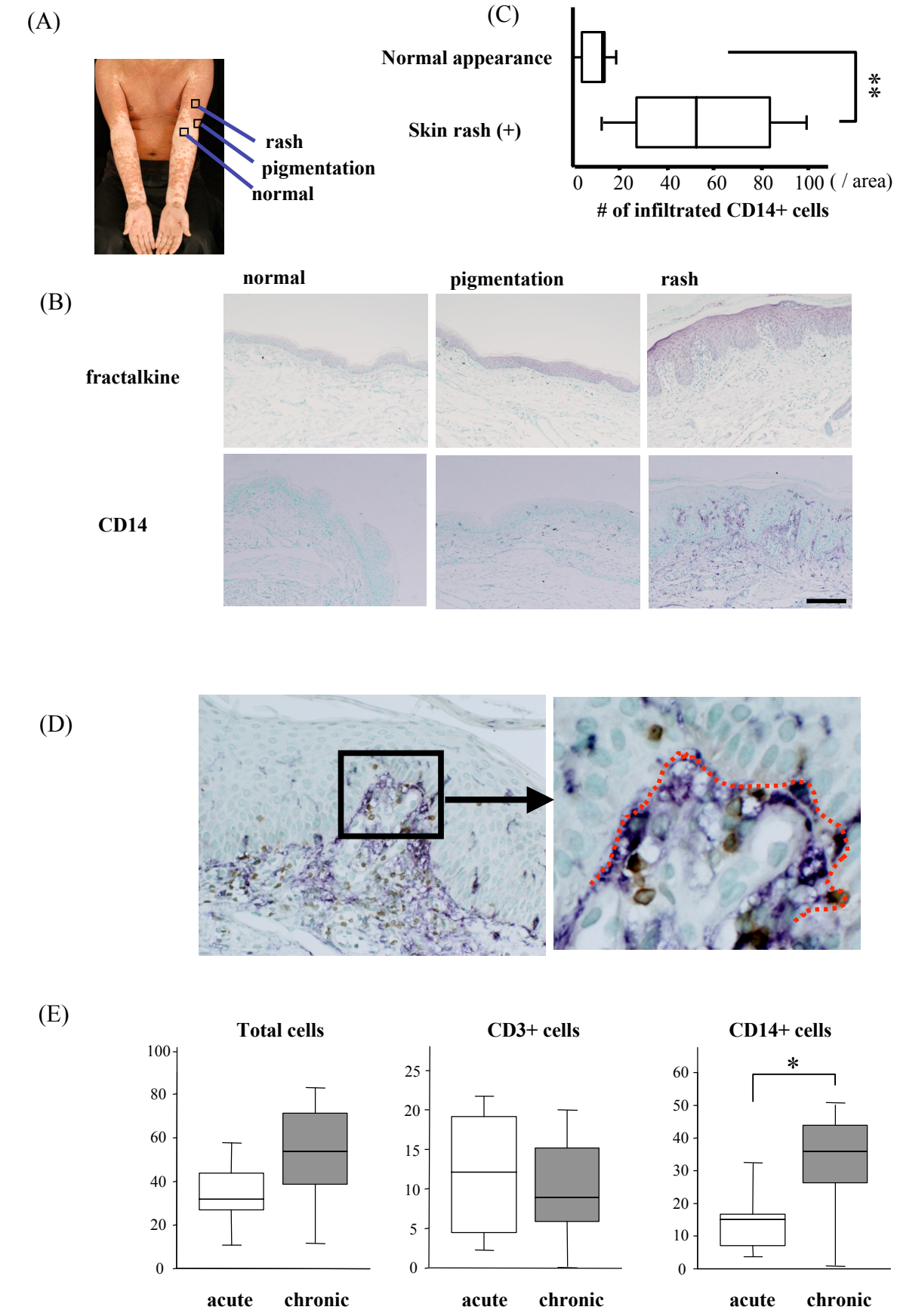

(A)
68%
cGVHD
(-)
14%
cGVHD
(+)
CX3CR1
(B)
(%)
P<0.01
cGVHD score
(C)
# of monocytes (/μl)
cGVHD moderate severe
(-)~ mild
(D)
**
*
# of CX3CR1+ monocytes (/μl)
cGVHD moderate severe
(-)~ mild

Figure 2


(A)
rash
pigmentation
normal
(C)
Normal appearance
Skin rash (+)
**
0 20 40 60 80 100 (/ area)
# of infiltrated CD14+ cells
(B)
normal
pigmentation
rash
fractalkine
CD14
(D)
(E)
Total cells
CD3+ cells
CD14+ cells
*
acute
chronic

Supplemental Materials

Supplemental Methods

Flow cytometry

Blood samples were stained with PE-conjugated anti-human CD14 antibody (CALTAG, Burlingame, CA) together with FITC-conjugated anti-human CX3CR1 antibody (MBL, Nagoya, Japan) or FITC-conjugated isotype-matched control (MBL), followed by the lysis of red blood cells with lysing solution (BD Pharmingen, San Jose, CA). Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Immunohistochemistry

Formalin-fixed and paraffin-embedded specimens were sliced at 5 μ m thickness and deparaffinized followed by antigen retrieval with microwave in citrate buffer. Samples were dipped in 3% H_2O_2 to quench endogenous peroxidase, then blocked with phosphate-buffered saline (PBS)+5% horse serum, incubated with polyclonal goat anti-human Fractalkine antibody (R&D systems, Minneapolis, MN) or control goat IgG (Sigma, St. Louis, MO), followed by the incubation with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA). Avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories) was used to enhance the staining signals.

Antigens were visualized by VIP (Vector Laboratories). For CD14 staining, samples were blocked with PBS+5% sheep serum, incubated with biotinylated sheep anti-human CD14 antibody (R&D systems) or control biotinylated sheep IgG (Vector Laboratories). ABC kit (Vector Laboratories) and VIP (Vector Laboratories) were used to visualize the staining. For dual color staining with CD3, samples were then washed twice in PBS, blocked with PBS+5% horse serum, and incubated with mouse anti-human CD3 antibody (Novocastra, Newcastle upon Tyne, UK) or control mouse IgG (Sigma) followed by biotinylated-horse anti-mouse IgG (Vector Laboratories). ABC kit (Vector Laboratories) and DAB (Vector Laboratories) were used to visualize the staining. Methylgreen (Vector Laboratories) was used for the counterstain. Stainings with control antibodies were performed in all experiments and showed no significant non-specific staining (not shown). The number of infiltrated CD14+ cells in cGVHD and transplanted normal skin tissues was counted in randomly selected three rectangles (200 x 400 μm) in dermis near epidermis, and was represented by the average (Figure 2C). For the comparison between acute and chronic GVHD, both CD14+ and CD3+ cells in dual color stained-skin tissues were counted on the edge of the dermis bordering the epidermis (Figure 2D, red dotted line).

Statistics

Spearman's rank correlation coefficient was used to determine the correlations

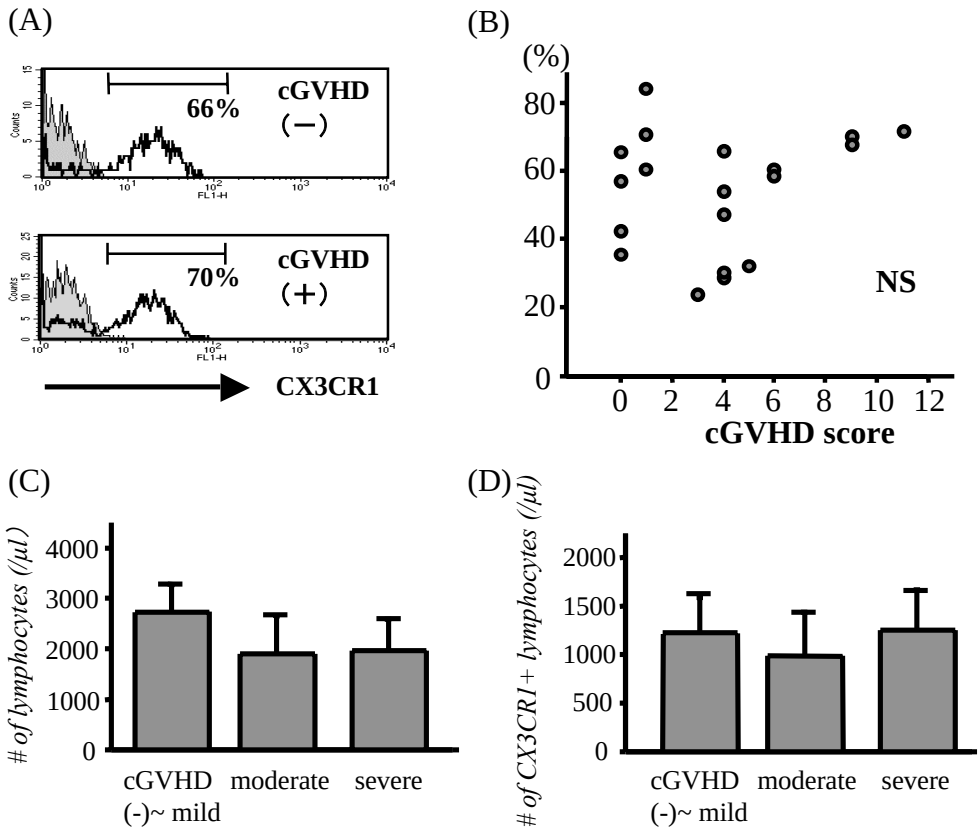
(Figure 1B and Supplemental Figure 1B). Mann-Whitney's U-test was used for non-parametric comparison. $P<0.05$ was considered statistically significant.

Supplemental Figure legends

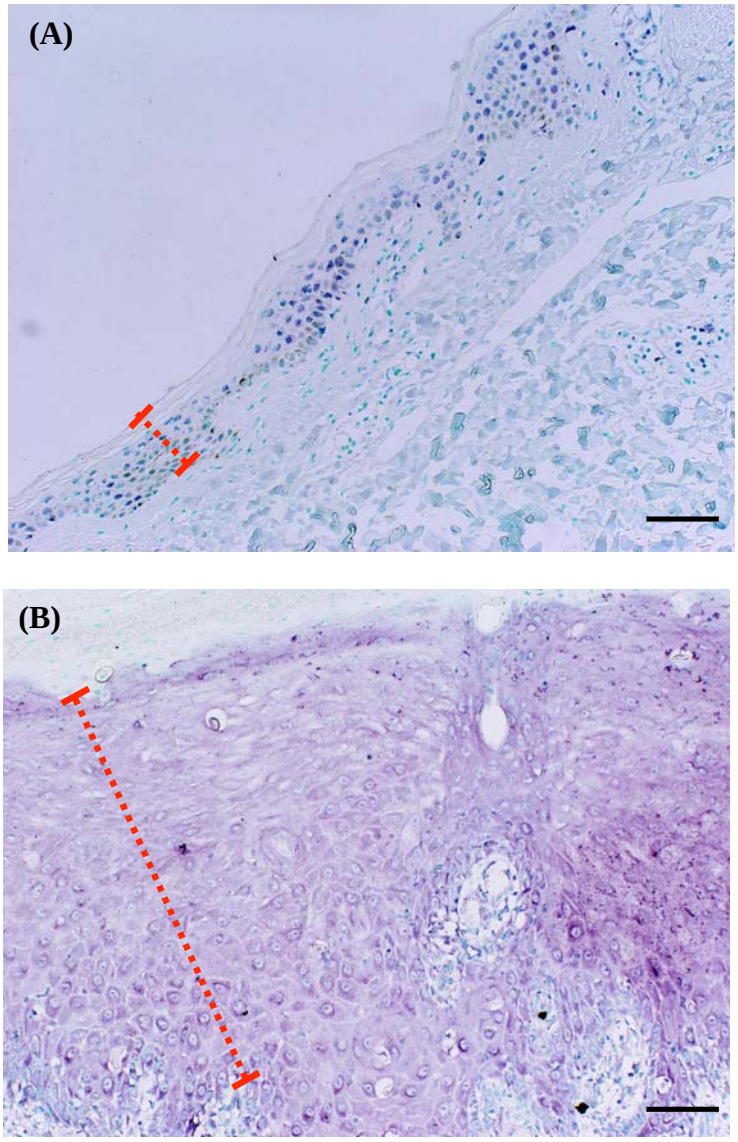
Supplemental Figure 1. CX3CR1 expression on peripheral blood lymphocytes in cGVHD patients. (A) Representative histograms of CX3CR1 expression in lymphocyte fraction (low forward and side scatter) are shown. Gray: isotype control. (B) CX3CR1 expression level shows no correlation with severity of clinical symptoms in cGVHD patients (Spearman's rank correlation coefficient). NS: not significant. (C) The numbers of peripheral blood total lymphocytes and (D) CX3CR1 positive lymphocytes. n=8 for cGVHD(-)~mild, 5 for moderate, and 6 for severe group.

Supplemental Figure 2. Immunohistochemical staining of fractalkine (VIP, violet) in (A) acute and (B) chronic GVHD skin tissues. Red dotted line: the width of epidermis. Black bar: 100μm.

Supplemental Figure 1



Supplemental Figure 2



Grade	cGVHD score	Sex (recepient/donor)	Age	Disease	Stem cell source	Conditioning	Day after trasplatation	Immunosuppressant/day	cGVHD status	Duration of cGVHD(day)	% CX3CR1+ in CD14+ cells
-	0	M/F	56	MDS	CB	Flu+CY+TBI	206	CsA 100mg	-	-	83
-	0	M/F	57	AML	R-BM	Flu+BU	461	-	-	-	69
-	0	F/F	47	CML	R-PBSC	BU+CY	2195	-	-	-	70
-	0	F/M	41	CML	R-PBSC	BU+CY	2292	-	-	-	52
mild	1	M/F	35	AML	R-PBSC	AraC+TBI	855	FK506 0.5/ 1mg alternative	stable	300	69
mild	1	F/F	20	AML	UR-BM	CY+TBI	122	FK506 2.5mg	onset	0	40
mild	1	F/F	61	NHL	R-PBSC	Flu+CY	97	-	onset	0	60
mild	3	M/M	30	AML	R-PBSC	AraC+TBI	3523	PSL 5mg/2 days	stable	2947	71
moderate	4	F/M	40	NHL	UR-BM	Flu+BU	337	PSL 10mg, FK506 2mg	stable	238	41
moderate	4	M/F	71	NHL	R-PBSC	Flu+CY+Rit	1531	CsA 25mg(twice/week)	stable	973	63
moderate	4	M/F	55	MDS	R-PBSC	Flu+CY	231	CsA 250mg	worsening	7	69
moderate	4	M/F	64	NHL	UR-BM	Flu+BU	308	CsA 100mg , PSL 5mg	onset	0	60
moderate	4	M/F	45	MM	R-PBSC	Flu+L-PAM	314	CsA 100mg , PSL 20mg	stable	151	32
severe	5	F/F	50	CML	R-PBSC	BU+CY	1747	CsA 25mg , PSL 20mg	stable	1357	11
severe	6	F/M	29	ALL	UR-BM	CY+TBI	706	CsA 150mg , PSL 50mg	stable	147	5
severe	6	M/F	34	ALL	R-PBSC	CY+TBI	681	CsA 100mg	stable	39	52
severe	9	M/F	37	CML	R-BM	BU+CY	2786	PSL 15mg, FK506 4mg	stable	2786	22
severe	9	M/M	31	NHL	UR-BM	L-PAM+TBI	142	CsA 50mg , PSL 5mg	stable	30	14
severe	11	F/F	33	AML	R-PBSC	CY+TBI	692	PSL 15mg	stable	907	27

Supplemental Table 1. Patient profile (blood sampling)

		Sex (recepient/donor)	Age	Disease	Stem cell source	Day after transplantation	Immunosuppressant /day	Conditioning	Skin type	Site
cGVHD	1	M/M	54	AML	UR-BM	615	FK506 2mg	CY/TBI	erythematous plaque	face
	2	F/F	17	ALL	R-BM	391	FK506 4.5mg/ day MTX 2.5mg/week	L-PAM/Thiotepa/CY/TBI	sclerotic	dorsal hand
	3	F/F	44	ALL	R-PBSC	1133	CsA 100mg/PSL 5mg	L-PAM/TBI	lichenoid change	arm
	4	M/F	34	ALL	R-PBSC	768	CsA 100mg	CY/TBI	lichenoid change	palm
	5	M/F	45	MM	R-PBSC	559	CsA 100mg/PSL 20mg	Flu/L-PAM	psoriasiform	arm
	6	M/F	55	MDS	R-PBSC	287	CsA 250mg	Flu/CY	lichenoid change	face
	7	M/F	64	NHL	UR-BM	574	CsA 100mg/PSL 5mg	Flu/BU	erythema	neck
Control	1	M/M	20	AML	UR-BM	393	-	CY/TBI	no eruption	back
	2	M/F	38	CML	R-PBSC	953	PSL 25mg	CY/TBI	no eruption	arm
	3	M/M	47	CML	R-PBSC	1117	FK506 2mg/PSL 5-10mg	AraC/CY/TBI	no eruption	arm
	4	M/F	39	NHL	R-BM	335	CsA 100m/PSL 20mg	L-PAM/TBI	no eruption	abdomen
	5	M/F	45	MM	R-PBSC	559	CsA 100mg/PSL 20mg	Flu/L-PAM	no eruption	arm
	6	M/F	55	MDS	R-PBSC	287	CsA 250mg	Flu/CY	no eruption	back
	7	M/F	64	NHL	UR-BM	574	CsA 100mg/PSL 5mg	Flu/BU	no eruption	back
aGVHD	1	M/F	20	ALL	UR-BM	37	CsA 3mg/kg~, iv	CY/TBI	erythema	dorsal hand
	2	F/M	38	NHL	UR-BM	53	FK506 12.5mg	Flu/BU	erythema	arm
	3	M/M	57	MF	UR-BM	27	CsA 3mg/kg~, iv	Flu/BU	erythema	leg
	4	M/F	63	NHL	UR-BM	54	CsA 3mg/kg~, iv	Flu/BU	erythema, bulla	palm
	5	F/M	35	ALL	UR-BM	14	FK506 12.5mg	CY/TBI	erythema	abdomen
	6	F/F	20	AML	UR-BM	20	FK506 12mg	CY/TBI	erythema, papule	palm
	7	M/F	36	CML	R-PBSC	30	CSA 3mg/kg~, iv	CY/TBI	papule	palm

Supplemental Table 2. Patient profile (skin sampling)