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Increased Treg cells and eosinophils characterize atopic dermatitis-like graft-versus-host disease compared to lichen planus-like graft-versus host disease

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37

Abstract

Background: Graft-versus-host disease (GVHD) has various cutaneous manifestations. Little is known about the mechanisms of cutaneous GVHD with different clinical features.

Objective: To characterize the immunological features and skin barrier functions of cutaneous GVHD.

Methods: Nineteen patients with atopic dermatitis (AD)-like GVHD, eight patients with lichen planus (LP)-like GVHD, twenty-four patients with AD and fifteen healthy controls were included in this study. T cell subpopulation in peripheral blood and skin lesions were measured by flow cytometry and immunofluorescence respectively. Filaggrin expression in skin lesions was measured by Western blot and immunohistochemistry. Trans-epidermal water loss (TEWL) was also measured using Tewameter® TM 300.

Results: The peripheral blood eosinophils in AD-like GVHD were significantly higher than that in LP-like GVHD. Th2 cells in peripheral blood and skin lesions were increased in AD-like GVHD and LP-like GVHD. Treg cells in peripheral blood and skin lesions were increased in AD-like GVHD. Filaggrin expression and TEWL were increased in skin lesions of AD-like GVHD and LP-like GVHD.

Limitations: The number of patients is limited.

Conclusions: Although AD-like GVHD and LP-like GVHD both had elevated Th2 cells and impaired skin barrier, increased eosinophils and Treg cells were found only in AD-like GVHD.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an important therapeutic option for treatment of hematological malignancies, solid tumors and severe immunodeficiency disorders. Graft-versus-host disease (GVHD) is a common complication of HSCT, occurring in 50% of patients.¹ GVHD has been traditionally divided into acute GVHD and chronic GVHD based on clinical presentations and time of onset. Skin is the most affected organ. Chronic cutaneous GVHD can mimic various skin diseases such as poikiloderma, lichen planus (LP), scleroderma, psoriasis, dermatomyositis, atopic dermatitis (AD) and other skin diseases.²⁻⁵

T cells are considered to play crucial roles in cutaneous GVHD.⁶ AD-like GVHD and LP-like GVHD both have erythematous lesions and scales and the T cell infiltrations are mainly in upper portion of dermis. However, LP-like GVHD is often associated with oral and nail involvements while AD-like GVHD is characterized by dry skin, eczematoid dermatitis and itching. In this study, we attempted to characterize the immunological features and skin barrier functions in patients with AD-like GVHD and LP-like GVHD.

Patients and methods

Study subjects

Nineteen patients with AD-like GVHD, eight patients with LP-like GVHD, twenty-four patients with AD and fifteen healthy controls were enrolled in this study. Peripheral blood samples were obtained from all subjects for measurement of eosinophils and T cell subpopulation. Sera were obtained after centrifugation and stored at -80°C before use. Skin biopsies were performed in five patients with AD-like GVHD, five patients with LP-like GVHD, six patients with AD and eight healthy controls. This study was approved by the Ethics Committee of Peking University People's Hospital. All subjects gave their written informed consent before they participated in this study.

Enzyme-linked immunosorbent assay

Total serum IgE level was measured by enzyme-linked immunosorbent assay (ELISA) using human Ready-Set-Go! ELISA Kits (eBioscience, CA, USA) and performed according to the manufacturer's protocol.

PBMCs culture and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare, Sweden). Cell suspensions were adjusted to a concentration of $1 \times 10^6/\text{ml}$ and then incubated for 5 hours in the 37°C, 5% CO₂ incubator with or without PMA (50ng/ml), Ionomycin Calcium (1μg/ml) and BFA/Monensin Mixture

(3 μ g/ml BFA, 1.4 μ g/ml Monensin, MultiSciences Biotech Co. Ltd, China) dissolved in RPMI-1640 supplemented with 10% FBS (Gibco, Life Technologies, USA).

T cell subpopulation staining

Treg cells were stained with surface markers (anti-CD4-FITC, anti-CD25-PE) and nuclear marker (anti-Foxp3-APC) according to the instruction of the Human Regulatory T Cell Staining Kit (eBioscience, CA, USA). Th1, Th2, Th17 and Th22 cells were stained with surface marker (anti-CD4-FITC) and intracellular markers (anti-IFN- γ -PerCP-Cyanine5.5, anti-IL-4-PE, anti-IL-17A-APC, and anti-IL-22-PE, respectively) according to manufacturer's protocols (eBioscience, CA, USA). Cells were acquired on flow cytometry and data were analyzed using analysis software (BD Biosciences). All stainings were adapted to isotype-matched controls and gated accordingly.

Hematoxylin-eosin staining and immunohistochemistry

The formalin-fixed skin tissues were embedded in paraffin and cut into 5 μ m sections, stained with hematoxylin and eosin. For immunohistochemistry, the section slices were incubated in 3% hydrogen peroxide and then washed in phosphate-buffered saline (PBS), followed by antigen retrieval using 0.01 mM citrate buffer (PH=6.0) and 5% bovine serum albumin blockade. The sections were incubated with the mouse anti-filaggrin antibody (1:50, Santa Cruz, CA, USA) overnight at 4 $^{\circ}$ C. The sections were then incubated with the secondary antibody,

121 stained with DAB and counterstained using hematoxylin. The results were recorded
122 using the digital Leica Application Suite imaging system (Leica, Solms, Germany).

124 **Immunofluorescence**

125 Dual immunofluorescence was carried out to co-localize IFN- γ , IL-4, IL-17A,
126 IL-22 and Foxp3 to CD4. Briefly, OCT-embedded skin tissues were cut into 5 μ m
127 sections, boiled in EDTA buffer (PH=9.0) for antigen retrieval. The sections were
128 incubated with the primary antibody overnight at 4 $^{\circ}$ C. Then the sections were
129 incubated with corresponding fluorescence secondary antibody and counterstained
130 using DAPI. The sections were observed under inverted fluorescent microscope
131 (Nikon, Tokyo, Japan) at $\times 200$ magnification under appropriate absorption/emission
132 wavelengths (Alexa Fluor 594nm or 488nm). The images were captured using Nikon
133 Digital Still Camera and analyzed by two independent investigators. Immuno-stained
134 cells in the entire epidermis and dermis were counted and results were expressed as
135 the number of positive cells/mm². Inter-observer variations for the counts were less
136 than 10%.

138 **Western Blotting**

139 The skin tissues were lysed with RIPA Lysis Buffer (Solarbio, Beijing, China)
140 using TissueLyserII (Qiagen, CA, USA), followed by centrifugation at 12,000 $\times g$ for
141 15min. Protein concentrations were determined using the BCA assay. Subsequently,
142 an equal amount of protein sample was loaded in each well and subjected to sodium

dodecyl sulfate-polyacrylamide gel for electrophoresis. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the PVDF membrane was incubated with the mouse anti-filaggrin (1:1000, Santa Cruz) overnight at 4°C. Mouse anti- β -actin (1:1000, Proteintech) was used as a control. Then the membranes were incubated with corresponding secondary antibodies. The specific bands were observed by ImageQuant 350 (GE, MA, USA) with standard chemiluminescence (Thermo, IL, USA). The band intensity was calculated by ImageJ software compared with β -actin.

Trans-epidermal water loss

Trans-epidermal water loss (TEWL) was measured using Tewameter® TM 300, which is an open chamber device (Courage & Khazaka electronic GmbH, Cologne, Germany). The value of TEWL was expressed in g/m²/h. The Tewameter® TM 300 has accuracy of ± 0.5 g/m²/h under normal room condition (10°C-30°C) and humidity 40-60% according to the manufacturer.

Statistical analysis

All data were analyzed by SPSS version 21.0 (IBM Corp., NY, USA). The comparisons between two groups were analyzed using Mann-Whitney U test or the Fisher exact test. One-way analysis of variance was used to multiple comparisons. A value of $p < 0.05$ was considered statistically significant. GraphPad Prism software

164 (GraphPad, CA, USA) was used to plot the graphs.

165

Results

Patient characteristics

The characteristics of patients with cutaneous GVHD were summarized in Table I . Increased peripheral blood eosinophils were found in 36.8% patients with AD-like GVHD, significantly higher than that of LP-like GVHD ($p=0.011$, Figure 1). Eosinophils were also found in the infiltration of the skin lesions of AD and AD-like GVHD (Figure 1). Elevated total serum IgE was found in 63.2% patients with AD-like GVHD and 50.0% patients with LP-like GVHD (data not shown) .

T helper cells in cutaneous GVHD

Th1 cells were increased in patients with AD-like GVHD both in peripheral blood ($p=0.001$, Figure 2) and skin lesions ($p=0.002$) as compared with healthy control. Th2 cells were increased in peripheral blood of patients with AD-like GVHD and LP-like GVHD ($p=0.002$, $p=0.014$, vs. healthy control respectively, Figure 2). The infiltration of Th2 cells was also increased in skin lesions of AD-like GVHD and LP-like GVHD ($p=0.003$, $p<0.001$, vs. healthy control respectively). Th22 cells were increased in peripheral blood of LP-like GVHD ($p=0.033$, vs. healthy control, Figure 2). The infiltration of Th22 cells was increased in lesions of AD-like GVHD and LP-like GVHD as compared with healthy control ($p<0.001$, $p<0.001$, respectively). The infiltration of Th17 cells was elevated in lesions of AD-like GVHD ($p=0.002$, vs. healthy control).

Treg cells in cutaneous GVHD

The number of Treg cells was increased in patients with AD-like GVHD both in peripheral blood ($p=0.024$, vs. healthy control, Figure 2) and skin lesions ($p=0.023$, vs. healthy control). No increase of Treg cells was found in LP-like GVHD. Data about the infiltrations of T cell subpopulation in skin lesions were not shown in this manuscript.

TEWL, filaggrin expression in cutaneous GVHD

TEWL was significantly elevated in patients with AD-like GVHD and LP-like GVHD ($p<0.001$, $p<0.001$, vs. healthy control respectively, Figure 3). The filaggrin expression was increased in patients with AD-like GVHD and LP-like GVHD ($p<0.001$, $p<0.001$, vs. healthy control respectively, Figure 4), whereas it was decreased in patients with AD ($p<0.001$, vs. healthy control, Figure 4).

Discussion

Chronic cutaneous GVHD represents a wide range of clinical manifestations reflecting a spectrum of superficial (lichenoid) and deep (scleroderma-like) cutaneous inflammations. We had reported AD-like GVHD.⁵ The clinical features of patients with AD-like GVHD include eczematous lesions, dry skin, itching. The histopathologic changes of AD-like GVHD include epidermal spongiosis, scattered keratinocyte necrosis and a sparse perivascular infiltration of lymphocytes and eosinophils. Perifollicular infiltrations are found in some patients. These patients usually have an observed favorable outcome. The clinical features and laboratory findings are quite similar to atopic dermatitis.⁷ These patients are different from the eczematoid GVHD reported by *Creamer et al.*⁸ Patients with eczematoid GVHD have features of weeping lesions, erythroderma and are associated with the poor outcome, suggesting that they represent different forms of cutaneous GVHD. Moreover, drug eruption was ruled out by careful history taking. In fact, most patients developed cutaneous GVHD when corticosteroid or immunosuppressive agents were tapered to lower doses.

None of the patients with AD-like GVHD had history of AD or other allergic diseases. However, five AD-like GVHD patients received HSCT from donors with history of AD or other allergic diseases, suggesting that atopy could be transmitted by HSCT. This has been reported by *Ozdemir*.⁹

Increased peripheral blood eosinophils were found in patients with AD-like GVHD, which is often a biologic marker in allergic syndromes including asthma and AD.

Eosinophilia after allogeneic HSCT had been reported as a favorable prognostic marker.¹⁰⁻¹² This was confirmed by our AD-like GVHD patients, all of whom had an observed favorable outcome.

GVHD has a complicated aetiology but ultimately is the result of donor T cells identifying disparate histocompatibility antigens of the recipient and causing injuries to host tissues.¹³ T cells play a crucial role in development of GVHD. CD4⁺ T cells are able to differentiate into Th1, Th2, Th17, Th22, and Treg cells.^{14,15} A balanced interaction among CD4⁺ T cell subpopulation is important in maintaining immune homeostasis. AD is regarded as a Th2-driven disease. However, Th1 cells also play roles in chronic phase of AD.^{16,17} The Th1/Th2 paradigm also occurs in GVHD. Acute GVHD is thought to be a Th1-biased immune response¹⁸ and chronic GVHD is considered as a Th2-predominant paradigm.¹⁹ In this study, we found that Th2 cells were increased in AD-like GVHD and LP-like GVHD. Although Th1 cells were also increased in patients with AD-like GVHD, the ratio of Th2/Th1 was still increased. The results suggest that AD-like GVHD and LP-like GVHD might have the similar pathogenesis - a Th2-predominant paradigm.

Th17 cells have been implicated in many inflammatory conditions.²⁰ Reinhardt *et al* reported that Th17 were increased in cutaneous GVHD.²¹ We found that Th17 cells were increased in the skin infiltration of patients with AD-like GVHD but not LP-like GVHD.

Nogralles *et al*²² has reported that increased Th22 cells infiltration was found in AD. However, the reports about Th22 cells in patients with GVHD were controversial.^{23, 24}

In this study, we found that Th22 cells were increased in lesions of AD-like GVHD and LP-like GVHD.

We found that Treg cells were increased in peripheral blood and skin lesions of AD-like GVHD. Treg cells are committed to suppressive functions and play a crucial role in maintaining immune homeostasis and self-tolerance. Adoptive transfer of Treg cells is a treatment option for patients with various autoimmune diseases or receiving transplantation.²⁵ High target Treg dose had been reported to decrease the incidence of GVHD.²⁶ Our findings might suggest that Treg cells infiltration was associated with the favorable outcome of AD-like GVHD.

Skin barrier functions of cutaneous GVHD have not been fully studied. TEWL is a key marker for skin barrier functions. We found that TEWL was increased in patients with AD-like GVHD and LP-like GVHD, illustrating that the skin barrier functions were impaired in cutaneous GVHD. Loss-of-function (null) mutations of filaggrin gene had been found in patients with AD, which may result in skin barrier defect.²⁷⁻²⁹ To our surprise, filaggrin expression was increased in both AD-like GVHD and LP-like GVHD, suggesting that these cutaneous GVHD patients have no filaggrin defect. High expression of filaggrin might be induced by cutaneous inflammation.

Conclusion

AD-like GVHD and LP-like GVHD are two forms of cutaneous GVHD. This study demonstrated that total serum IgE level and Th2 cells were elevated in AD-like GVHD and LP-like GVHD. Although skin barrier functions were impaired in AD-like GVHD and LP-like GVHD, the filaggrin expression was increased in both diseases. Increased eosinophils and Treg cells were found in AD-like GVHD, which might be associated with the favorable outcome of the disease.

273 **Abbreviations**

274 AD, atopic dermatitis; HSCT, allogeneic hematopoietic stem cell transplantation;
275 GVHD, graft-versus-host disease; LP, lichen planus; PBMCs, peripheral blood
276 mononuclear cells; TEWL, trans-epidermal water loss.

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351

352

353 **Table I . Characteristics of the patients**

		AD-like GVHD	LP-like GVHD
Gender			
	Male	10	8
	Female	9	0
Mean age (y)		25.05	28.25
Original diseases			
	AML	7	1
	CML	1	2
	ALL	10	3
	MDS	0	2
	AA	1	0
Donor/Recipient			
	Related	18	8
	Unrelated	1	0
GVHD prophylaxis			
	CSA	13	6
	CSA MMF	5	1
	CSA MTX	1	1
Mean time after HSCT(month)		18.78	28.86
Onset of skin lesion after HSCT(day)		343.0667	699.1
History of allergic disease of donor		5	0

Dry skin		17	6
Itching		16	6
Distribution of lesions			
	Face	0	2
	Palms or soles	0	1
	Trunk and extremities	2	1
	Widespread	17	4
	Nails	2/19	4/8
	Oral mucosa	1/19	5/8
Other system involvements			
	GI	3	2
	Liver	5	4

354 AA, aplastic anemia; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CML,
 355 chronic myeloid leukemia; CSA, cyclosporin A; GI, glucocorticoid; MDS, myelodysplastic
 356 syndrome; MMF, mycophenolate mofetil; MTX, methotrexate
 357

Figure legends

Figure 1. Eosinophils in peripheral blood and skin lesions of cutaneous GVHD

Eosinophils in peripheral blood and representative pictures of hematoxylin and eosin (HE) showing the infiltration of eosinophils in skin lesions. Data were displayed as mean $\pm$ SEM. * p <0.05; *** p <0.001. HC, healthy control; AD, atopic dermatitis; AD-like GVHD, atopic dermatitis-like graft-versus-host disease; LP-like GVHD, lichen planus-like graft-versus-host disease.

Figure 2. T cell subpopulation in cutaneous GVHD.

Representative FACS plots of Th1-, Th2-, Th17-, Th22-, and Treg cells detected in CD4⁺ T cells by flow cytometry were exhibited. The percent of Th1-, Th2-, Th17-, Th22-, and Treg cells in CD4⁺ T cells was analyzed. Data were displayed as mean $\pm$ SEM. * p <0.05. FACS, fluorescence activating cell sorter; HC, healthy control; AD, atopic dermatitis; AD-like GVHD, atopic dermatitis-like graft-versus-host disease; LP-like GVHD, lichen planus-like graft-versus-host disease.

Figure 3. Trans-epidermal water loss (TEWL) in cutaneous GVHD.

Data were displayed as mean $\pm$ SEM. *** p <0.001. HC, healthy control; AD, atopic dermatitis; AD-like GVHD, atopic dermatitis-like graft-versus-host disease; LP-like GVHD, lichen planus-like graft-versus-host disease.

Figure 4. Filaggrin expression in cutaneous GVHD.

Filaggrin expression in skin

lesions was measured by Western blot and immunohistochemistry. The representative images of immunohistochemistry showing filaggrin expression in skin lesions were exhibited (400×). Data were displayed as mean $\pm$ SEM. *** $p < 0.001$. HC, healthy control; AD, atopic dermatitis; AD-like GVHD, atopic dermatitis-like graft-versus-host disease; LP-like GVHD, lichen planus-like graft-versus-host disease.







