Highlights:

Frequent clonotypes differ from tissue to tissue in a certain GVHD patient.

Frequent clonotypes in a certain GVHD tissue differ from patient to patient.

A few T cell clones account for almost all of human GVHD tissue-infiltrating T cells.

Quantitative assessment of T cell clonotypes in human acute graft-versus-host disease tissues

Daisuke Koyama, MD[1], Makoto Murata, MD, PhD[1], Ryo Hanajiri, MD, PhD[1], Tomohiro Akashi, PhD[2], Shingo Okuno, MD[1], Sonoko Kamoshita, MD[1], Jakrawadee Julamanee, MD[1], Erina Takagi, MD[1], Kotaro Miyao, MD, PhD[1], Reona Sakemura, MD, PhD[1], Tatsunori Goto, MD, PhD[1], Seitaro Terakura, MD, PhD[1], Tetsuya Nishida, MD, PhD[1], and Hitoshi Kiyoi, MD, PhD[1]

[1] Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan

[2] Division of Omics Analysis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan

Corresponding author

Makoto Mutara, MD, PhD

Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa, Nagoya, Aichi 466-8550, Japan.

Phone: +81-52-744-2141

Fax: +81-52-744-2157

E-mail: mmurata@med.nagoya-u.ac.jp

Category of manuscript

Regular manuscript

Short title

T cell clonotypes in human acute GVHD tissues

Key words

Transplantation; graft-versus-host disease; T cell repertoire; cytotoxic T cell

Abstract

Due to difficulty in isolating T cells from human biopsy samples, the characteristics of T cells that are infiltrating human acute graft-versus-host disease (GVHD) tissues remain largely uninvestigated. In the present study, T cell receptor-β deep sequencing of various GVHD tissue samples and concurrent peripheral blood obtained from post-transplant patients was performed in combination with functional assays of tissue-infiltrating T cell clones. The T cell repertoire was more skewed in GVHD tissues than in the peripheral blood. The frequent clonotypes differed from tissue to tissue of the same patient, and the frequent clonotypes in the same tissue differed from patient to patient. Two T cell clones were successfully isolated from GVHD skin of a patient. In a cytotoxicity assay, both T cell clones lysed patient peripheral blood mononuclear cells, but not donor-derived Epstein Barr virus-transformed lymphoblastoid cells. Their clonotypes were identical to the most and second most frequent T cell clonotypes in the original GVHD skin and accounted for almost all of the skin-infiltrating T cells. These results suggest that human acute GVHD may result from only a few different allo-reactive cytotoxic T cell clones, which differ from tissue to tissue and from patient to patient. The characterization of T cells infiltrating human GVHD tissues should be further investigated.

Introduction

Acute graft-versus-host disease (GVHD) is a major complication and in large part responsible for early mortality after allogeneic hematopoietic stem cell transplantation (HSCT) (1). Based on the findings in mouse experimental models, the pathogenesis of acute GVHD is understood to be the tissue damage mainly caused by donor-derived, allo-reactive T cells that are infiltrating target tissues (2). However, due to difficulty in isolating T cells from human biopsy samples, the characteristics of human acute GVHD remain largely uninvestigated.

T cell receptor (TCR) repertoire analysis is an attractive approach to understanding the whole context of T cells in GVHD tissues without isolating tissue-infiltrating T cells. Previous studies using TCR spectratyping or PCR subcloning techniques have shown that the TCR repertoire in GVHD tissues is skewed (3-6), suggesting an oligoclonal expansion of T cells in GVHD tissues. However, these techniques are only semi-quantitative methods and enable an assessment of only a small population of the whole T cells in the tissues. The development of deep sequencing technology over the last decade made it possible to perform massively parallel sequencing of highly diverse TCR genes with utmost depth, resolution, and accuracy (7, 8). There is a single report showing the T cell clonotypes of human GVHD tissue samples (9). In that analysis, patients with steroid-refractory GVHD

had a more consistent TCR-β clonal structure between different biopsy sites in the gastrointestinal tract than patients with steroid-sensitive GVHD, and no TCR-β complementarity-determining region 3 (CDR3) sequences were shared among GVHD patients. Nonetheless, questions left unresolved were whether the T cell repertoire overlaps between organs, such as between GVHD gut and skin, whether the diversity of the T cell repertoire in GVHD tissue is associated with the response to steroid treatment, and in the first place, whether the dominant T cell clones in GVHD tissue actually have an allo-reactive cytotoxicity.

Unfortunately, TCR repertoire analysis gives us no information about the function of T cells, such as cytotoxicity and cytokine production. The functional analysis of T cells or preferably T cell clones that are isolated from GVHD tissue contributes to elucidating the pathogenesis of acute GVHD. However, it is extremely difficult to isolate T cell clones from quite small biopsy samples that are obtained from patients under immunosuppressive conditions by GVHD prophylaxis in the early period after transplantation. In fact, to the best of our knowledge, all reported human T cell clones that are specific for GVHD-associated minor histocompatibility antigens were isolated from peripheral blood or bone marrow, but not from tissue (10-12).

In the present study, TCR-β deep sequencing of various tissue samples obtained from

post-transplant patients was performed to evaluate the T cell repertoire in GVHD tissues. The successful isolation of T cell clones from GVHD tissue enabled evaluation of allo-reactivity of dominant T cell clones in GVHD tissues. This study is the first to analyze comprehensive TCR sequences in GVHD tissues in combination with functional assays of tissue-infiltrating T cells at the clonal level.

Materials and Methods

Patients

Patients who had received allogeneic HSCT at Nagoya University Hospital and developed acute GVHD were enrolled in this study. Acute GVHD was diagnosed and graded by established criteria (13). Pathological diagnoses of all tissue samples were compatible with GVHD. Patients who failed a first-line treatment and required second-line treatment were defined as steroid-refractory cases. This study was approved by the institutional review board of Nagoya University Hospital, and written, informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Sample collection

Biopsies of skin, stomach, and colon were performed when the patients were thought to have developed acute GVHD and prior to first-line treatment. The obtained biopsy sample was divided, and one piece was used for pathological diagnosis, while the other portion was used for this study. Peripheral blood was obtained from patients around the same time that biopsies were performed. As controls, three blood samples were obtained from three healthy volunteers.

RNA extraction

Tissue samples were homogenized by a bead-based homogenizer (Multi-Beads Shocker, Yasui Kikai Co., Kyoto, Japan) after being frozen with liquid nitrogen, and total RNA was extracted from them using the RNeasy mini kit (Qiagen, Manchester, UK). CD3-positive cells were isolated from peripheral blood mononuclear cells (PBMCs) with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and total RNA was extracted from 5×10^5 CD3-positive cells, or if there were fewer than 5×10^5 cells, all available CD3-positive cells.

TCR-β deep sequencing

TCR-β deep sequencing was performed as previously described with some modifications because an Illumina Miseq sequencer (Illumina, San Diego, CA, USA) was used instead of a Roche 454 sequencer (Roche, Basel, Switzerland) (14, 15). Briefly, cDNA was generated from total RNA using a SMARTer RACE 5'/3' kit (Takara Bio, Kusatsu, Japan). *TRB* gene products were amplified through 5' rapid amplification of cDNA ends (RACE) PCR with Clontech's universal forward primer and a reverse primer compatible with both TRBC genes (5'-GCACACCAGTGTGGCCTTTTGGG-3'). Barcode and adaptor sequences were incorporated into each sample with a 2-step PCR method using the Nextera XT Index kit

V2 (Illumina) according to the manufacturer's instructions. Samples were pooled and sequenced on the Illumina MiSeq sequencer (Illumina).

TCR-β repertoire analysis

Sequencing data were analyzed using MiTCR software with default settings (16). Non-functional CDR3 sequences and clonotypes with only one read were discarded using VDJtools software (17). The relative abundance curve was constructed for each sample. To estimate the TCR repertoire diversity in each sample, the inverse Simpson's diversity index (*1/Ds*) were calculated using the following formula.

$Ds = \sum_{i=1}^{S} n_i(n_i-1)/N(N-1)$

S = total number of clonotypes

N = total number of reads

n = the number of reads belonging to a clonotype

The target read count for each sample was set to be 100,000; however, actual read counts for productive clonotypes were wide-ranging, from 98,423 to 706,550 (median, 314,936). To avoid the impact of read depth on the statistical analysis, downsampling to 98,423 reads (the minimum reads within the samples) was performed using VDJtools software. The *1/Ds* was calculated with the downsampled datasets.

Isolation of skin-infiltrating T cell clones

A portion of biopsied skin was minced with a scalpel and digested at 37°C with collagenase D (Roche Applied Science, Mannheim, Germany). The skin extract was cultured for nonspecific expansion in 96-well round-bottom plates containing unrelated allogeneic irradiated (75 Gy) Epstein Barr virus-transformed lymphoblastoid cells (EBV-LCLs) and irradiated (25 Gy) PBMCs as feeder cells with 50 IU/ml recombinant human interleukin-2 (R & D Systems, Minneapolis, MN, USA). After 2 weeks, established T cell lines were cloned by limiting dilution, and the cloned T cells were expanded using a rapid expansion protocol, as previously described (18). Human T cells were cultured in RPMI-1640 supplemented with 10% pooled, heat-inactivated human serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin.

Chromium release assay

A cytotoxicity assay with ^{51}Cr-labeled EBV-LCLs and PBMCs was performed as previously described (19). Briefly, EBV-LCLs and PBMCs were labeled for 1 h with ^{51}Cr and incubated for 4 h with cytotoxic T cell (CTL) clones at various E:T ratios.

Determination of TCR Vβ gene and CDR3 of skin-infiltrating T cell clones

Total RNA was extracted from individual T cell clones, and *TRB* gene products were amplified through 5' RACE PCR, as described above. The nested PCR reaction was performed using Clontech's nested forward primer and a nested TRBC primer (5'-CTTTGGGTGTGGGAGATCTCTG-3'). The purified amplicons were directly sequenced (20). *TRBV* gene and CDR3 were determined by the international ImMunoGeneTics information system software, IMGT/V-QUEST (http://www.imgt.org/).

Statistics

Spearman's ρ was calculated to determine the correlations between the relative abundances of the clonotypes in GVHD tissues and those in peripheral blood of the corresponding patients.

Results

Patient characteristics and collected samples

The clinical characteristics and the treatment profiles of the patients are summarized in **Table 1**. Twelve tissue samples and eight blood samples were obtained from eight adult patients, who had received transplantation of bone marrow, peripheral blood stem cells, or umbilical cord blood, after GVHD development and before first-line GVHD treatment. Three patients failed a first-line steroid treatment and received a second-line treatment with anti-thymocyte globulin, mesenchymal stem cells, or mycophenolate mofetil.

The T cell repertoire in GVHD tissues is more skewed than that in concurrent peripheral blood

The frequencies of each TCR-β clonotype of T cells in the skin and concurrent peripheral blood obtained from patient 1 are shown in **Figure 1A**. The *1/Ds* of peripheral blood was 22.0, whereas that of the skin was 1.6, indicating that the T cell repertoire was more skewed in the GVHD skin than in peripheral blood. Interestingly, the frequencies of the most and second most frequent clonotypes in the skin (arrows in **Figure 1A**) were 75.6% and 23.0%, respectively, suggesting that only two clones accounted for approximately 98% of T cells infiltrating the skin GVHD lesion of patient 1.

The mean *1/Ds* of eight peripheral blood samples obtained from eight GVHD patients was 249 (range, 18.9-898), whereas that of 12 GVHD tissues including seven skin, three stomach, and two colon was 7.9 (range, 1.0-21.2) ($P < 0.0001$) (**Figure 1B**). In addition, the mean frequency of the most frequent clonotypes in each peripheral blood sample was 9.4% (range, 1.4-16.4%; n = 8), whereas that in each GVHD tissue was 41.2% (range, 9.0-99.8%; n = 12) ($P < 0.01$). These data indicate that the T cell repertoire is more skewed in GVHD tissues than in the peripheral blood.

Only a few different allo-reactive cytotoxic T cell clones might cause skin GVHD of patient 1

To analyze the functional characteristics of GVHD tissue-infiltrating T cells, an attempt was made to isolate T cell clones from 10 biopsy tissues. However, 18 T cell lines were actually established from four biopsy tissues: skin (n = 2), stomach (n = 1), and colon (n = 1). Eventually, two T cell clones, termed clone nos. 1 and 2, were successfully isolated from patient 1's skin and expanded up to a sufficient number to be analyzed for cell functional analyses. TCR Vβ subfamilies of clone nos. 1 and 2 were TRBV2-1 and TRBV28-1, respectively, supporting the idea that these were independent T cell clones. In a cytotoxicity assay, both T cell clones lysed patient PBMCs, but not donor-derived

EBV-LCLs (**Figure 2**), indicating that they were allo-reactive CTL clones.

To analyze the frequencies of these two allo-reactive CTL clones in the original GVHD skin, the sequences of CDR3 region within TCR Vβ were determined. CDR3 of clone no. 1, CASSPDLLGQVYNQPQHF, was identical to the most frequent (75.6%) clonotype, and CDR3 of clone no. 2, CASSSRAVEKLFF, was identical to the second most frequent (23.0%) clonotype in the skin of patient 1 (arrows in **Figure 1A**). These data suggest that only a few different allo-reactive CTL clones might cause the skin GVHD of patient 1.

Frequent clonotypes differ from tissue to tissue

The frequencies of each TCR-β clonotype of T cells in GVHD tissues and concurrent peripheral blood obtained from patient 4 are shown in **Figure 3**. Like patient 1's skin, only the top one or two clone(s) in each GVHD tissue (arrows) accounted for approximately 99% of T cells in the corresponding tissue. Interestingly, the most frequent (92.8%) clonotype in skin accounted for only 0.029% in stomach and 0.0092% in colon, and the second most frequent (7.1%) clonotype in skin was detected in neither stomach nor colon. The most frequent (64.4%) clonotype in stomach accounted for only 0.033% in colon, and the second most frequent (35.5%) clonotype accounted for only 0.0032% in skin and 0.012% in colon. The most frequent (99.8%) clonotype in colon was detected in neither

skin nor stomach.

Acquisition of tissue samples from multiple organs in patients 2, 4, and 6 enabled the comparison of the frequent T cell clonotypes between tissues of the same patient. There were 32 clonotypes that accounted for 5% or more in at least one GVHD tissue sample (**Table 2**). In patient 2, five clonotypes that accounted for ≥ 5% in skin were detected in stomach of the same patient at a frequency of 0.5% or less. Conversely, seven clonotypes that accounted for ≥ 5% in stomach were detected in skin of the same patient at a frequency of ≤ 3.4% or not detected. In patient 4, clonotypes that accounted for ≥ 5% in skin (n = 2), stomach (n = 2), and colon (n = 1) were detected in other tissues at an extremely low frequency (≤ 0.033%) or not detected. In patient 6, only one clonotype, CASSLTGPNSPLHF, was detected in both stomach and colon at a frequency of ≥ 5% (7.5% in stomach and 19.1% in colon). However, all other clonotypes were detected in the other tissue at a low frequency (≤ 3.3%) or not detected. These data suggest that frequent clonotypes differ from tissue to tissue of the same GVHD patient.

Frequent clonotypes in a GVHD tissue differ from patient to patient

Acquisition of skin samples from seven patients enabled the comparison of the frequent clonotypes in skin GVHD tissues between patients. There were 41 clonotypes that

accounted for ≥ 5% in at least one skin sample (**Table 3**). For example, the most and second most frequent clonotypes in the skin of patient 1, CASSPDLLGQVYNQPQHF (75.6%) and CASSSRAVEKLFF (23.0%), were not detected in the skin of the other six patients. The most frequent clonotype in the skin of patient 4, CASRSLYGYTF (92.8%), was not detected in the skin of other patients, except patient 5 (0.00094%). Taken together, of 41 clonotypes, 30 were detected only in one patient, that is, unique to a certain patient, and 11 were detected in other patients' skin at an extremely low frequency (≤ 0.066%). These data suggest that the frequent clonotypes in a certain GVHD tissue differ from patient to patient.

Frequent clonotypes in GVHD tissues are not always detected in the concurrent peripheral blood

Sixty-seven clonotypes that accounted for ≥ 5% in a tissue sample and their frequencies in the concurrent peripheral blood of the corresponding patient were analyzed (**Supplemental Table 1**). Although one clonotype, CASSSVNTEAFF, was detected in both the skin and the corresponding peripheral blood of patient 7 at a frequency of ≥ 5% (20.8% in skin and 13.4% in peripheral blood), all other frequent clonotypes in a GVHD tissue were detected in the corresponding peripheral blood at a frequency of < 5% or not detected.

Whether there was a correlation between the frequencies of clonotypes that accounted

for ≥ 5% in any of the skin (n = 41), stomach (n = 19) and colon (n = 7) samples (**Supplemental Table 1**) and their frequencies in the concurrent peripheral blood of the corresponding patient was next determined (**Figure 4**). There was no correlation between the frequencies of clonotypes in GVHD tissues and the corresponding peripheral blood (skin, $P = 0.73$; stomach, $P = 0.71$; colon, $P = 0.67$). These data suggest that the predominance of the frequent clonotypes in GVHD tissues is not always replicated in peripheral blood.

Discussion

In the present study, TCR-β deep sequencing analysis clearly demonstrated a highly skewed T cell repertoire in GVHD tissues. This is compatible with the previous studies showing an oligoclonal expansion pattern of T cells by TCR spectratyping or PCR subcloning techniques (3-6), or a recent study by deep sequencing technique (9). One of novelties of the present study is that it is the first to analyze comprehensive TCR sequences in various GVHD tissues in combination with functional assay of tissue-infiltrating T cells at the clonal level. Only two T cell clones accounted for almost all (~98%) of the T cells infiltrating the GVHD skin of patient 1, and both clones lysed patient PBMCs, but not donor-derived EBV-LCLs. These findings suggest that only a few allo-reactive CTL clones may cause acute GVHD after allogeneic HSCT.

In a recent study (21), TCR-β sequences in peripheral blood and tissue samples obtained from six recipients of HLA-matched bone marrow transplantation using post-transplantation cyclophosphamide were assessed. In a skin sample obtained on day 369 from a male patient who had a female donor and experienced acute GVHD, three clonotypes were identical to the TCR-β sequences of minor histocompatibility antigen H-Y-specific cytotoxic T cell clones that had been established in their laboratory. These three clonotypes accounted for 36% of the sequences in the corresponding skin biopsy.

Although it was neither shown whether those T cell clones actually existed in the skin tissue during acute GVHD, nor whether there were more frequent clones than H-Y-specific T cell clones, their findings support the hypothesis that human acute GVHD in a certain organ may result from only a few different allo-reactive T cell clones.

A question left unresolved was whether tissue-infiltrating T cell clonotypes are different among GVHD tissues of the same patient. In the present comprehensive TCR-β sequencing analysis, the frequent T cell clonotypes differed from tissue to tissue of the same patient (patients 2, 4, and 6). These patients received HSCT from HLA-matched donors, suggesting that different CTL clones specific for minor histocompatibility antigens preferentially expressed in each tissue may be involved in the GVHD development of the corresponding organ. This hypothesis is compatible with our clinical experience with acute GVHD, such as the difference in severity between organs, difference in onset day between organs, and mixed response to steroid treatment. To prove the hypothesis, the identification of minor histocompatibility antigens recognized by frequent T cell clones in each tissue is required.

Frequent clonotypes in the skin of a certain patient were detected in other patients' skin at an extremely low frequency or not detected. The previous TCR-β sequencing analysis for gastrointestinal GVHD samples also could not find shared T cell clonotypes between

different patients (9). These results suggest that the clonality of tissue-infiltrating T cells is highly patient-specific, even in the same GVHD organ. Nonetheless, this does not exclude the possibility that different T cell clones recognize the same antigen using different TCR, because HLA types vary from patient to patient. Further analysis of more patients who share HLA alleles may answer this question.

Nearly all frequent clonotypes in GVHD tissue samples were detected in the concurrent peripheral blood of the corresponding patient at an extremely low frequency or not detected. In addition, there was no correlation between the frequencies of clonotypes in tissue samples and peripheral blood of the corresponding patient. Kanakry et al. (21) demonstrated that frequent clonotypes in peripheral blood were infrequent in gastrointestinal tissues and almost never observed in skin biopsies obtained from recipients with acute GVHD. Hirokawa et al. (6) also demonstrated that dominant clonotypes differed between tissue and peripheral blood using the PCR cloning technique of TCR. On the other hand, Beck et al. (5) demonstrated that some clonotypes were shared by tissue and peripheral blood. Although it is difficult to interpret the difference in these observations, a possible explanation is that the T cell clones recognizing minor histocompatibility antigens that are preferentially expressed in tissues are detected only in tissues, whereas those recognizing the antigens that are expressed both in tissue and peripheral blood are detected

in both. Nonetheless, it may not be very surprising that the frequencies of tissue-infiltrating T cells were low in peripheral blood, since peripheral blood is essential for trafficking of all T cells. Thus, it is natural that the T cell repertoire in peripheral blood is not likely to be present in the organs. This emphasizes the importance of investigating the human T cell responses in GVHD tissues to elucidate the mechanism of human acute GVHD development.

In conclusion, human acute GVHD can be caused by a few different allo-reactive CTL clones, which differ from tissue to tissue and from patient to patient. The characterization of human T cells infiltrating GVHD tissues should be further investigated.

Grant support

This study was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) (15K09498 and 18K08321) from the Japan Society for the Promotion of Science (JSPS) to M. Murata, a Practical Research Project for Allergic Diseases and Immunology (18ek0510022h0002) from the Japan Agency for Medical Research and Development (AMED) to M. Murata, and a grants-in-aid from the 24th General Assembly of the Japanese Association of Medical Sciences to M. Murata.

Acknowledgments

The authors would like to thank Chika Wakamatsu, Yoko Matsuyama, and Yasutomo Itoh for their technical assistance. The authors also express their sincere appreciation for experimental and administrative support from the Radioisotope Research Center, Nagoya University Graduate School of Medicine, and the Division of Medical Research Engineering, Nagoya University Graduate School of Medicine.

H. Kiyoi received research funding from Chugai Pharmaceutical Co., Ltd., Bristol-Myers Squibb, Kyowa Hakko Kirin Co., Ltd., Zenyaku Kogyo Co., Ltd., FUJIFILM Corporation, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc. and Celgene Corporation, consulting fees from Astellas Pharma Inc. and Daiichi Sankyo Co.,

Ltd., and honoraria from Bristol-Myers Squibb and Pfizer Japan Inc. These companies were not directly involved in any part of this study. The remaining authors have declared there are no financial conflicts of interest with regard to this study.

Author contributions

D. Koyama and M. Murata designed and performed experiments, analyzed the data, and wrote this manuscript. R. Hanajiri and T. Akashi performed experiments and assisted with data analysis. S. Okuno, S. Kamoshita, J. Julamanee, E. Takagi, K. Miyao, R. Sakemura, T. Goto, S. Terakura, and T. Nishida gave advice regarding the study design and data collection. H. Kiyoi supervised the project. All authors read and approved the final manuscript.

References

1. Ferrara JL, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet*. 2009;373:1550-1561.

2. Beilhack A, Schulz S, Baker J, et al. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. *Blood*. 2005;106:1113-1122.

3. Kubo K, Yamanaka K, Kiyoi H, et al. Different T-cell receptor repertoires between lesions and peripheral blood in acute graft-versus-host disease after allogeneic bone marrow transplantation. *Blood*. 1996;87:3019-3026.

4. Margolis DA, Casper JT, Segura AD, et al. Infiltrating T cells during liver graft-versus-host disease show a restricted T-cell repertoire. *Biol Blood Marrow Transplant*. 2000;6:408-415.

5. Beck RC, Wlodarski M, Gondek L, et al. Efficient identification of T-cell clones associated with graft-versus-host disease in target tissue allows for subsequent detection in peripheral blood. *Br J Haematol*. 2005;129:411-419.

6. Hirokawa M, Matsutani T, Saitoh H, et al. Distinct TCRAV and TCRBV repertoire and CDR3 sequence of T lymphocytes clonally expanded in blood and GVHD lesions after human allogeneic bone marrow transplantation. *Bone Marrow Transplant*.

2002;30:915-923.

7. Freeman JD, Warren RL, Webb JR, Nelson BH, Holt RA. Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing. *Genome Res*. 2009;19:1817-1824.

8. Mamedov IZ, Britanova OV, Zvyagin IV, et al. Preparing unbiased T-cell receptor and antibody cDNA libraries for the deep next generation sequencing profiling. *Front Immunol*. 2013;4:456.

9. Meyer EH, Hsu AR, Liliental J, et al. A distinct evolution of the T-cell repertoire categorizes treatment refractory gastrointestinal acute graft-versus-host disease. *Blood*. 2013;121:4955-4962.

10. Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. *J Exp Med*. 2001;193:195-206.

11. Murata M, Warren EH, Riddell SR. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. *J Exp Med*. 2003;197:1279-1289.

12. Griffioen M, van Bergen CA, Falkenburg JH. Autosomal minor histocompatibility antigens: how genetic variants create diversity in immune targets. *Front Immunol*. 2016;7:100.

13. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15:825-828.

14. van Heijst JW, Ceberio I, Lipuma LB, et al. Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation. *Nat Med*. 2013;19:372-377.

15. Hanajiri R, Murata M, Sugimoto K, et al. Integration of humoral and cellular HLA-specific immune responses in cord blood allograft rejection. *Bone Marrow Transplant*. 2015;50:1187-1194.

16. Bolotin DA, Shugay M, Mamedov IZ, et al. MiTCR: software for T-cell receptor sequencing data analysis. *Nat Methods*. 2013;10:813-814.

17. Shugay M, Bagaev DV, Turchaninova MA, et al. VDJtools: unifying post-analysis of T cell receptor repertoires. *PLoS Comput Biol*. 2015;11:e1004503.

18. Terakura S, Murata M, Warren EH, et al. A single minor histocompatibility antigen encoded by UGT2B17 and presented by human leukocyte antigen-A*2902 and -B*4403. *Transplantation*. 2007;83:1242-1248.

19. Sugimoto K, Murata M, Terakura S, Naoe T. CTL clones isolated from an HLA-Cw-mismatched bone marrow transplant recipient with acute graft-versus-host disease. *J Immunol*. 2009;183:5991-5998.

20. Narimatsu H, Murata M, Terakura S, Sugimoto K, Naoe T. Potential role of a

mismatched HLA-specific CTL clone developed pre-transplant in graft rejection following cord blood transplantation. *Biol Blood Marrow Transplant*. 2008;14:397-402.

21. Kanakry CG, Coffey DG, Towlerton AM, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight*. 2016;1:e86252.

Figure legends

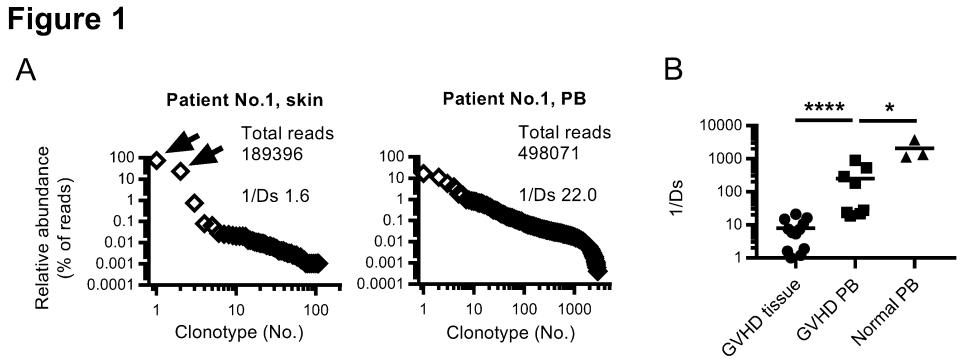

Figure 1. Comparison of clonotype distributions among GVHD tissues and peripheral blood (PB). (A) Clonotype distribution plots of T cells in the skin and concurrent PB obtained from patient 1. Each dot represents a distinct CDR3 amino-acid sequence. Arrows indicate the most and second most frequent clonotypes in the skin. **(B)** Diversity of the TCR repertoire (*1/Ds*) of T cells in the GVHD tissues (n = 12), concurrent PB (n = 8), and normal PB from healthy volunteers (n = 3). * indicates $P < 0.05$, and **** indicates $P < 0.0001$.

Figure 2

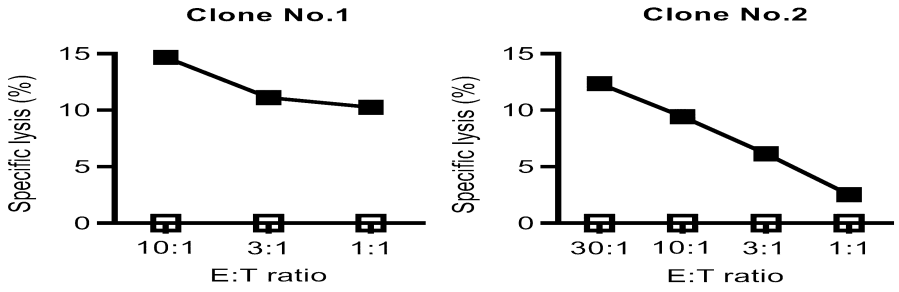

Figure 2. Allogeneic responses of isolated T cell clones. Cytotoxicities of T cell clones nos. 1 and 2 against patient PBMCs (solid squares) and donor EBV-LCLs (open squares) were determined by a chromium release assay. Specific lysis is shown as the mean of triplicate cultures at various E:T ratios.

Figure 3

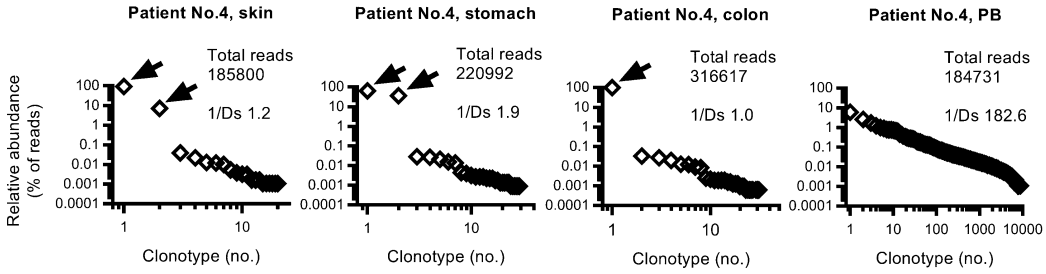

Figure 3. Clonotype distribution plots of T cells in the skin, stomach, colon, and concurrent PB obtained from patient 4. Each dot represents a distinct CDR3 amino-acid sequence. Arrows indicate the most and second most frequent clonotypes in the skin and stomach, and the most frequent clonotype in the colon.

Figure 4

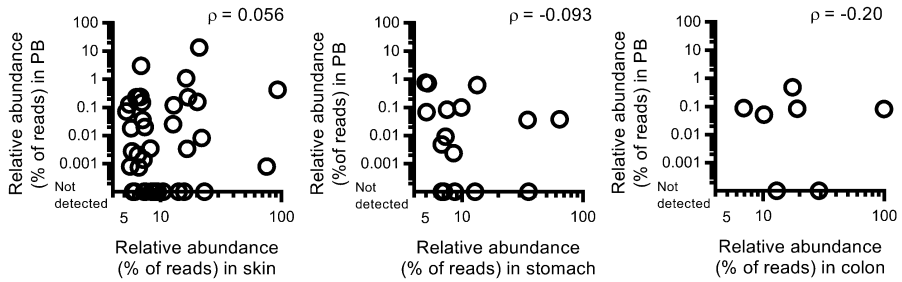

Figure 4. Comparison of frequencies of clonotypes in each GVHD tissue and those in peripheral blood (PB). Dot plot comparing the clonotype distributions between T cells that accounted for ≥ 5% in the skin (n = 41), stomach (n = 19), or colon (n = 7) and their frequencies in the concurrent PB of the corresponding patient are shown. The number in the upper right corner is Spearman's correlation co-efficient (ρ).

Table 1. Patient characteristics and collected samples

Pt no.	Sex	Age (y)	Donor type	Graft	Conditioning regimen	GVHD prophylaxis	Site of biopsy (day)	GVHD grade (skin	First-line treatment (day)	Steroid response	Second-line treatment (day)

							, gut, and liver stages) at biopsy				
1	Male	51	MMUD	BM	Cy + TBI	Tac + sMTX	Skin (12)	III (3, 0, 2)	mPSL (12)	Refractory	ATG (43)
2	Male	68	MUD	BM	Flu + ivBU	Tac + sMTX	Skin (39)	II (2, 1, 0)	mPSL (54)	Sensitive	–
							Stomach (42)	II (2, 1, 0)			
3	Male	20	MUD	BM	Cy + TBI	Tac + sMTX	Skin (65)	II (3, 0, 0)	mPSL (73)	Sensitive	–

4	Male	58	MRD	PBSC	Flu + ivBU + Mel	Tac + sMTX	Skin (73)	III (3, 3, 0)				
							Stomach (77)	III (3, 3, 0)	mPSL (81)	Refractory	MSC (90)	
							Colon (80)	III (3, 3, 0)				
5	Male	48	MMUD	UCB	Flu + ivBU + Mel	Tac + sMTX	Skin (18)	II (3, 0, 0)	Topical steroid (18)	Sensitive	–	
6	Male	68	MUD	BM	Flu + ivBU	Tac + sMTX	Stomach (38)	III (2, 2, 0)	mPSL (42)	Sensitive	–	
							Colon (42)	III (2, 2,				

							0)				
7	Female	58	MUD	BM	Flu + Mel	Tac + sMTX	Skin (53)	I (2, 0, 0)	mPSL (56)	Sensitive	–
8	Female	29	MMUD	BM	Flu + Cy	Tac + sMTX	Skin (88)	II (3, 0, 0)	mPSL (88)	Refractory	MMF (106)

MMUD indicates HLA-mismatched unrelated donor; MUD, HLA-matched unrelated donor; MRD, HLA-matched related donor; BM, bone marrow; PBSC, peripheral blood stem cells; UCB, umbilical cord blood; Cy, cyclophosphamide; TBI, total body irradiation; Flu, fludarabine; ivBU, intravenous busulfan; Mel, melphalan; Tac, tacrolimus; sMTX, short-term methotrexate; mPSL, methylprednisolone; ATG, anti-thymocyte globulin; MSC, mesenchymal stem cells; MMF, mycophenolate mofetil.

Table 2. Comparison of the frequent T cell clonotypes between GVHD tissues of the same patient

Patient no.	CDR3 of the frequent clonotypes that accounted for ≥ 5% in at least one GVHD tissue	Relative abundance in each GVHD tissue (%)		
		Skin	Stomach	Colon

2	CASSLSQGAANEQFF	**16.8**[*]	0.011	–
2	CASSRSNGGRGVPLHF	**7.3**	0.0084	–
2	CSAEGTVGAGELFF	**6.9**	0.5	–
2	CASSLEGRTGAKNIQYF	**6.1**	0.0019	–
2	CASTRGGSTDTQYF	**5.7**	0.0046	–
2	CASSFMTRTETSPYEQYF	0.0061	**35.0**	–
2	CASQGPNTEAFF	Not detected	**12.8**	–
2	CASSVVAGGPADTQYF	3.4	**9.9**	–
2	CASSSQGDEAFF	0.0016	**8.7**	–
2	CASGGFGGTSYEQYF	Not detected	**8.5**	–
2	CASSSTGGGGTEAFF	0.026	**5.2**	–
2	CASSLAGVASEQYF	0.0022	**5.0**	–
4	CASRSLYGYTF	**92.8**	0.029	0.0092
4	CASSLQLREASWTGELFF	**7.1**	Not detected	Not detected
4	CASSQVYGGSGDTQYF	Not	**64.4**	0.033

		detected		
4	CASSPWGLLNEQFF	0.0032	**35.5**	0.012
4	CASSQGGRANEQFF	Not detected	Not detected	**99.8**
6	CSLRGRPMASEQYF	–	**13.4**	0.0033
6	CASSLTGPNSPLHF	–	**7.5**	**19.1**
6	CASNIGLLYSTDTQYF	–	**7.3**	0.0016
6	CASSLGQGQGGNQPQHF	–	**7.1**	0.0021
6	CASSDGTNQPQHF	–	**7.0**	0.0015
6	CASSLDGRNQPQHF	–	**6.8**	0.0028
6	CASTSSEGLSPNEQFF	–	**6.8**	0.00066
6	CASLGLAEPYEQYF	–	**6.6**	0.0023
6	CASSSSRLRGGWETQYF	–	**6.6**	Not detected
6	CASSLVGDRRPDTQYF	–	**5.1**	0.0049
6	CAIKNPGAGQPQHF	–	0.0055	**28.8**
6	CASSLETPFDRVRETQYF	–	0.57	**17.4**
6	CASSSGTPSTDTQYF	–	0.0023	**12.9**

6	CASSPGLAGTRSYEQYF	−	Not detected	**10.1**
6	CASSLFGDKAAYEQYF	−	3.3	**6.9**

* Frequencies of ≥ 5% are indicated in bold.

− indicates not done.

Table 3. Comparison of the frequent T cell clonotypes in skin GVHD tissues between patients

CDR3 of the frequent clonotypes that accounted for ≥ 5% in at least one skin GVHD sample (amino acid sequence)	Relative abundance in skin GVHD samples from each patient (%)						
	Patient no.						
	1	2	3	4	5	7	8
CASSPDLLGQVYNQPQHF	**75.6***	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CASSSRAVEKLFF	**23.0**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CASSLSQGAANEQFF	N.D.	**16.8**	N.D.	N.D.	0.0020	N.D.	N.D.
CASSRSNGGRGVPLHF	N.D.	**7.3**	N.D.	N.D.	N.D.	N.D.	N.D.
CSAEGTVGAGELFF	N.D.	**6.9**	0.0020	N.D.	0.0048	N.D.	0.027
CASSLEGRTGAKNIQYF	N.D.	**6.1**	N.D.	N.D.	N.D.	N.D.	N.D.

CASTRGGSTDTQYF	N.D.	**5.7**	N.D.	N.D.	N.D.	N.D.	N.D.
CASSFNQPQHF	N.D.	N.D.	**9.0**	N.D.	N.D.	N.D.	N.D.
CASSLYTGQETQYF	N.D.	N.D.	**8.2**	0.0016	N.D.	N.D.	N.D.
CASSLGSGRTETQYF	N.D.	N.D.	**7.2**	N.D.	N.D.	N.D.	N.D.
CSVDTLRNGYTF	N.D.	N.D.	**6.8**	N.D.	0.0011	N.D.	N.D.
CASSFSRGQGSPLHF	N.D.	N.D.	**6.5**	0.0032	N.D.	N.D.	N.D.
CASSFLGGRADTQYF	N.D.	N.D.	**5.5**	0.0011	N.D.	N.D.	N.D.
CASSPTGQGNYGYTF	N.D.	N.D.	**5.2**	N.D.	N.D.	N.D.	N.D.
CASRSLYGYTF	N.D.	N.D.	N.D.	**92.8**	0.00094	N.D.	N.D.
CASSLQLREASWTGELFF	N.D.	N.D.	N.D.	**7.1**	N.D.	N.D.	N.D.
CASSVGGTGANVLTF	N.D.	N.D.	N.D.	N.D.	**15.7**	N.D.	N.D.
CATSDLGTSAGETQYF	N.D.	N.D.	N.D.	N.D.	**12.8**	N.D.	N.D.
CSARDQGADGYTF	N.D.	N.D.	N.D.	N.D.	**10.5**	N.D.	N.D.
CASSLWQLGETQYF	N.D.	N.D.	N.D.	N.D.	**9.3**	N.D.	N.D.
CATSDRRGGGNEQFF	N.D.	N.D.	N.D.	N.D.	**9.1**	N.D.	N.D.
CSARDWASGVDEQFF	N.D.	N.D.	N.D.	N.D.	**7.5**	N.D.	N.D.
CASTTHRGGGANVLTF	N.D.	N.D.	N.D.	N.D.	**7.1**	N.D.	N.D.
CASSPQADTQYF	N.D.	0.017	N.D.	N.D.	**6.6**	N.D.	N.D.

CASSLATKNQPQHF	N.D.	0.0013	N.D.	N.D.	**6.0**	N.D.	N.D.
CASSSTGGGGTEAFF	N.D.	0.027	N.D.	N.D.	**5.9**	N.D.	N.D.
CASSLLGVAGGEETQYF	N.D.	N.D.	N.D.	N.D.	N.D.	**21.8**	N.D.
CASSSVNTEAFF	N.D.	N.D.	N.D.	N.D.	N.D.	**20.8**	N.D.
CASSLRPGLGQETQYF	N.D.	N.D.	N.D.	N.D.	N.D.	**14.1**	N.D.
CASSPLNQETQYF	N.D.	N.D.	N.D.	N.D.	N.D.	**9.5**	N.D.
CASSFGTGTGANVLTF	N.D.	N.D.	N.D.	N.D.	N.D.	**8.4**	N.D.
CASISGAGGPLSLQFF	N.D.	N.D.	N.D.	N.D.	N.D.	**7.3**	N.D.
CASRSQGANSGELFF	N.D.	N.D.	N.D.	N.D.	N.D.	**5.6**	N.D.
CAISDTQGPKETQYF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**20.1**
CASSLVPGQQYF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**16.5**
CSASRDQDEKLFF	N.D.	N.D.	N.D.	N.D.	N.D.	0.066	**16.3**
CASSYSLNLRDRIGTGANVLTF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**12.7**
CASSPSTGVPYNEQFF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**7.4**
CSARQGGAGELFF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**7.0**
CASSLGTTYEQYF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**6.3**
CASSKVEGASGSENEQYF	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	**5.8**

[*] Frequencies of ≥ 5% are indicated in bold.

N.D. indicates not detected.