



Quantitative Assessment of T Cell Clonotypes in Human Acute Graft-versus-Host Disease Tissues

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Owing to the 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, TCR- β deep sequencing of various GVHD tissue samples and concurrent peripheral blood obtained from transplant recipients 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 in 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 alloreactive 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.

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INTRODUCTION

Acute graft-versus-host disease (GVHD) is a major complication that is largely 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 caused mainly by donor-derived alloreactive T cells that infiltrate target tissues [2]. However, owing to difficulty in isolating T cells from human biopsy samples, the characteristics of human acute GVHD remain largely uninvestigated.

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 and 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 semiquantitative methods and enable assessment of only a small fraction of the entire population of T cells in tissues. The development of deep sequencing technology over the last decade has 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 analyzing 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 patients with GVHD. 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, primarily, whether the dominant T cell clones in GVHD tissue actually have an alloreactive cytotoxicity.

Unfortunately, TCR repertoire analysis provides no information about the function of T cells, such as cytotoxicity and cytokine production. The functional analysis of T cells, or

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preferably T cell clones that are isolated from GVHD tissue, will contribute to elucidating the pathogenesis of acute GVHD. However, it is extremely difficult to isolate T cell clones from quite small biopsy samples 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 patients post-transplantation was performed to evaluate the T cell repertoire in GVHD tissues. The successful isolation of T cell clones from GVHD tissue enabled evaluation of alloreactivity 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.

METHODS

Patients

Patients who had undergone allogeneic HSCT at Nagoya University Hospital and developed acute GVHD were enrolled in this study. Acute GVHD was diagnosed and graded using 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 tissues were performed when a patient was considered to have developed acute GVHD and before first-line treatment. The biopsy samples were divided, with one piece used for pathological diagnosis and the other piece used for this study. Peripheral blood was obtained from patients around the same time that biopsies specimens were obtained. As controls, 3 blood samples were obtained from 3 healthy volunteers.

RNA Extraction

Tissue samples were homogenized by a bead-based homogenizer (Multi-Beads Shocker; Yasui Kikai, Kyoto, Japan) after being frozen with liquid nitrogen, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). CD3⁺ 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⁺ cells or, if there were fewer than 5×10^5 cells, all available CD3⁺ cells.

TCR- β Deep Sequencing

TCR- β deep sequencing was performed as described previously [14,15], with some modifications, using an Illumina MiSeq sequencer (Illumina, San Diego, CA) instead of a Roche 454 sequencer (Roche, Basel, Switzerland). In brief, 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 a universal forward primer (Clontech, Mountain View, CA) and a reverse primer compatible with both *TRBC* genes (5'-GCACACAGTGTGGCCTTTGGG-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.

TCR- β Repertoire Analysis

Sequencing data were analyzed using MiTCR software with default settings [16]. Nonfunctional CDR3 sequences and clonotypes with only 1 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 diversity index (*1/Ds*) was calculated using the following formula:

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

where *S* is the total number of clonotypes, *N* is the total number of reads, and *n* is 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 ranged widely, 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 IL-2 (R&D Systems, Minneapolis, MN). 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 described previously [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 ⁵¹Cr-labeled EBV-LCLs and PBMCs was performed as described previously [19]. In brief, EBV-LCLs and PBMCs were labeled for 1 hour with ⁵¹Cr and then incubated for 4 hours with cytotoxic T lymphocyte (CTL) clones at various E:T ratios.

Determination of TCR V β 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 a Clontech nested forward primer and a nested *TRBC* primer (5'-CTTTGGGTGTGGAGATCTCTG-3'). The purified amplicons were directly sequenced [20]. The *TRBV* gene and CDR3 were analyzed using the international ImMunoGeneTics information system software, IMG/ 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 8 blood samples were obtained from 8 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 antithymocyte globulin, mesenchymal stem cells, or mycophenolate mofetil.

The T Cell Repertoire Is More Skewed in GVHD Tissues Than 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 2 clones accounted for approximately 98% of T cells infiltrating the skin GVHD lesion of patient 1.

The mean *1/Ds* of 8 peripheral blood samples obtained from 8 patients with GVHD was 249 (range, 18.9 to 898), whereas that of 12 GVHD tissues, including 7 skin, 3 stomach, and 2 colon, was 7.9 (range, 1.0 to 21.2) ($P < .0001$) (Figure 1B). In addition, the mean frequency of the most frequent clonotypes in each peripheral blood sample was 9.4% (range, 1.4% to 16.4%; $n = 8$), whereas that in each GVHD tissue was 41.2% (range, 9.0% to 99.8%; $n = 12$) ($P < .01$). These data indicate that the T cell repertoire is more skewed in GVHD tissues than in the peripheral blood.

Table 1
Patient Characteristics and Collected Samples

Patient	Sex	Age, yr	Donor Type	Graft	Conditioning Regimen	GVHD Prophylaxis	Site (Day) of Biopsy	GVHD Grade (Skin, Gut, and Liver Stage) at Biopsy	First-Line Treatment (Day)	Steroid Response	Second-Line Treatment (Day)
1	Male	51	MMUD	BM	Cy + TBI	Tac + sMTX	Skin (12)	III (3, 0, 2)	mPSL (12)	Refractory	ATC (43)
2	Male	68	MUD	BM	Flu + ivBU	Tac + sMTX	Skin (39) Stomach (42)	II (2, 1, 0) II (2, 1, 0)	mPSL (54)	Sensitive	–
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) Stomach (77) Colon (80)	III (3, 3, 0) III (3, 3, 0) III (3, 3, 0)	mPSL (81)	Sensitive Refractory	MSC (90)
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) Colon (42)	III (2, 2, 0) III (2, 2, 0)	mPSL (42)	Sensitive	–
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; ATC, antithymocyte globulin; MSC, mesenchymal stem cells; MMF, mycophenolate mofetil.

Only a Few Different Alloreactive Cytotoxic T Cell Clones Might Have Caused the Skin GVHD in 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 established from 4 biopsy tissues: 2 skin, 1 stomach, and 1 colon. Eventually 2 T cell clones, designated clones 1 and 2, were successfully isolated from skin specimens of patient 1 and expanded up to a sufficient number for cell functional analyses. TCR V β subfamilies of clones 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 alloreactive CTL clones.

To analyze the frequencies of these 2 alloreactive CTL clones in the original GVHD skin, the sequences of CDR3 region within TCR V β were determined. The CDR3 of clone 1, CASSPDLGQVYNQPQHF, was identical to the most frequent (75.6%) clonotype in the skin of patient 1, and the CDR3 of clone 2, CASSRAVEKLFF, was identical to the second most frequent (23.0%) clonotype (arrows in Figure 1A). These data suggest that only a few different alloreactive CTL clones might have caused the skin GVHD of patient 1.

Frequency of Clonotypes Differs Among Tissues

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. As in patient 1's skin specimen, only the top 1 or 2 clone(s) in each GVHD tissue (arrows) accounted for approximately 99% of the T cells in the corresponding tissue. Interestingly, the most frequent (92.8%) clonotype in skin accounted for only .029% in stomach and .0092% in colon, and the second most frequent (7.1%) clonotype in skin was not detected in either stomach or colon. The most frequent (64.4%) clonotype in stomach accounted for only .033% in colon, and the second most frequent (35.5%) clonotype accounted for only .0032% in skin and .012% in colon. The most frequent (99.8%) clonotype in colon was not detected in either skin or 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. Thirty-two clonotypes accounted for $\geq 5\%$ in at least 1 GVHD tissue sample (Table 2). In patient 2, 5 clonotypes that accounted for $\geq 5\%$ in skin were detected in stomach at a frequency of $\leq 5\%$, and conversely, 7 clonotypes that accounted for $\geq 5\%$ in stomach were detected in skin at a frequency of $\leq 3.4\%$ or not detected. In patient 4, clonotypes that accounted for $\geq 5\%$ in skin (n = 2), stomach (n = 2), and colon (n = 1) were detected in other tissues at an extremely low frequency ($\leq 0.33\%$) or not detected. In patient 6, only 1 clonotype, CASSLTGPN SPLHF, was detected in both stomach and colon at a frequency of $\geq 5\%$ (7.5% in stomach and 19.1% in colon); all other clonotypes were detected in other tissues at a low frequency ($\leq 3.3\%$) or not detected. These data suggest that frequent clonotypes differ among tissues in the same patient with GVHD.

Frequency of Clonotypes in GVHD Tissues Differ Among Patients

Acquisition of skin samples from the 7 patients enabled the comparison of the most frequent clonotypes in skin GVHD tissues among these patients. Forty-one clonotypes accounted for $\geq 5\%$ in at least 1 skin sample (Table 3). For example, the most frequent and second most frequent clonotypes in the skin of patient 1, CASSPDLGQVYNQPQHF (75.6%) and CASSRAVEKLFF (23.0%), were not detected in the skin of the other 6 patients. The most

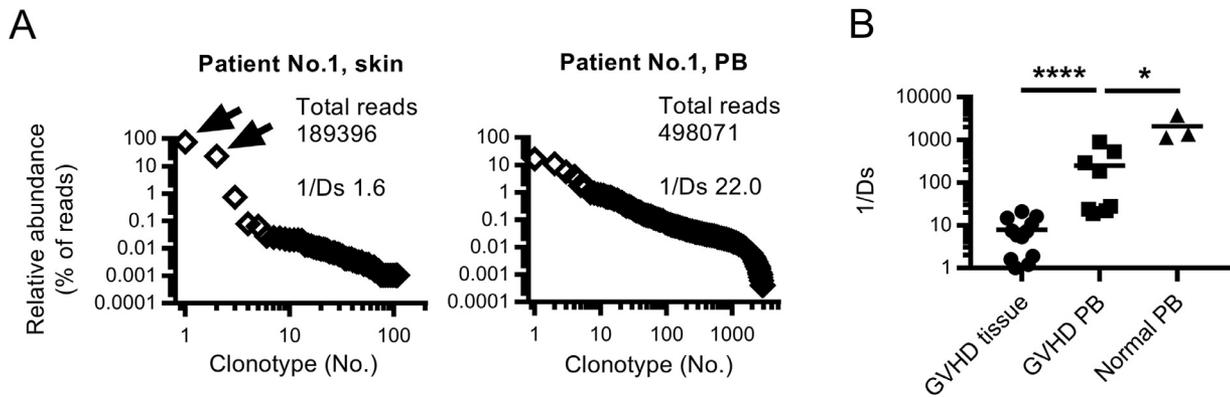


Figure 1. Comparison of clonotype distributions among GVHD tissues and peripheral blood (PB). (A) Clonotype distribution plots of T cells in the skin and concurrent PB obtained from patient 1. Each dot represents a distinct CDR3 amino acid sequence. Arrows indicate the most frequent and second most frequent 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 ($n = 3$). * $P < .05$; **** $P < .0001$.

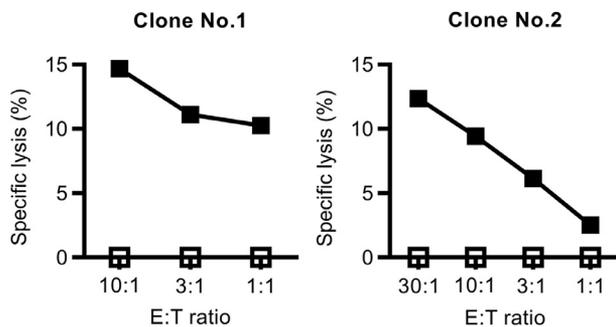


Figure 2. Allogeneic responses of isolated T cell clones. Cytotoxicities of T cell clones 1 and 2 against patient PBMCs (solid squares) and donor EBV-LCLs (open squares) were determined by a chromium release assay. Specific lysis is shown as the mean of triplicate cultures at various E:T ratios.

frequent clonotype in the skin of patient 4, CASRSLYGYTF (92.8%), was not detected in the skin of any other patient except patient 5 (.00094%). Taken together, of the 41 clonotypes, 30 were detected only in 1 patient (ie, unique to an individual patient) and 11 were detected in other patients' skin at an extremely low frequency ($\leq .066\%$). These data suggest that the most frequent clonotypes in certain GVHD tissues differ from patient to patient.

Frequent Clonotypes in GVHD Tissues Are Not Always Detected in Concurrent Peripheral Blood

Sixty-seven clonotypes that accounted for $\geq 5\%$ in a tissue sample and their frequencies in the concurrent

peripheral blood of the corresponding patient were analyzed (**Supplementary Table 1**). Although 1 clonotype, CASSVNTAEFF, was detected in both the skin and the corresponding peripheral blood of patient 7 at a frequency of $\geq 5\%$ (20.8% in skin and 13.4% in peripheral blood), all other frequent clonotypes detected in a GVHD tissue were detected in the corresponding peripheral blood at a frequency of $< 5\%$ or not detected.

An analysis of correlations between the frequencies of clonotypes that accounted for $\geq 5\%$ in any of the skin ($n = 41$), stomach ($n = 19$), and colon ($n = 7$) samples (**Supplementary Table 1**) and their frequencies in the concurrent peripheral blood of the corresponding patient revealed no significant correlations (skin, $P = .73$; stomach, $P = .71$; colon, $P = .67$) (**Figure 4**). 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 finding is compatible with previous studies showing an oligoclonal expansion pattern of T cells by TCR spectratyping or PCR subcloning techniques [3–6], as well as a recent study using the deep sequencing technique [9]. The present study 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 2 T cell clones accounted for almost all ($\sim 98\%$) of the T cells

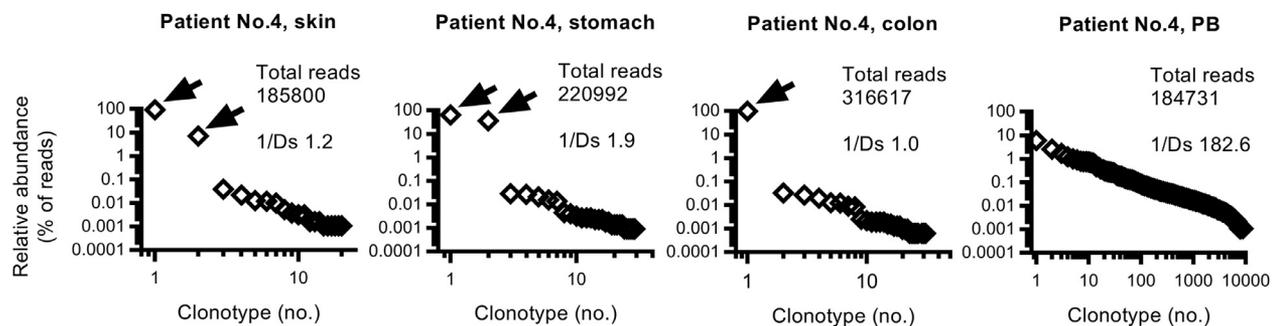


Figure 3. Clonotype distribution plots of T cells in the skin, stomach, colon, and concurrent PB obtained from patient 4. Each dot represents a distinct CDR3 amino acid sequence. Arrows indicate the most frequent and second most frequent clonotypes in the skin and stomach and the most frequent clonotype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

Table 2
Comparison of the Frequent T Cell Clonotypes Between GVHD Tissues of the Same Patient

Patient	CDR3 of the Frequent Clonotypes Accounting for $\geq 5\%$ in at Least 1 GVHD Tissue	Relative Abundance in Each GVHD Tissue, %		
		Skin	Stomach	Colon
2	CASSLSQGAANEQFF	16.8*	.011	–
2	CASSRSNGGRGVPLHF	7.3	.0084	–
2	CSAEGTVGAGELFF	6.9	.5	–
2	CASSLEGRTGAKNIQYF	6.1	.0019	–
2	CASTRGGSTDTQYF	5.7	.0046	–
2	CASSFMTRTETSPYEQYF	.0061	35.0	–
2	CASQGPNTAEFF	ND	12.8	–
2	CASSVVAGGPADTQYF	3.4	9.9	–
2	CASSSQGDEAFF	.0016	8.7	–
2	CASGGFGTSYEQYF	ND	8.5	–
2	CASSSTGGGTAEFF	.026	5.2	–
2	CASSLAGVASEQYF	.0022	5.0	–
4	CASRSLYGYF	92.8	.029	.0092
4	CASSLQLREASWTGELFF	7.1	ND	ND
4	CASSQVYGGSGDTQYF	ND	64.4	.033
4	CASSPWGLLEQFF	.0032	35.5	.012
4	CASSQGRANEQFF	ND	ND	99.8
6	CSLRGRPMASEQYF	–	13.4	.0033
6	CASSLTGPNSPLHF	–	7.5	19.1
6	CASNIGLLYSTDTQYF	–	7.3	.0016
6	CASSLQGQGGNQPHF	–	7.1	.0021
6	CASSDGTNQPQHF	–	7.0	.0015
6	CASSLDGRNQPHF	–	6.8	.0028
6	CASTSEGLSPNEQFF	–	6.8	.00066
6	CASLGAEPYEYF	–	6.6	.0023
6	CASSSRLRGGWETQYF	–	6.6	ND
6	CASSLVGDRRPTQYF	–	5.1	.0049
6	CAIKNPGAGQPQHF	–	.0055	28.8
6	CASSLETPFDRVRETQYF	–	.57	17.4
6	CASSSGTPSTDTQYF	–	.0023	12.9
6	CASSPGLAGTRSYEQYF	–	ND	10.1
6	CASSLFGDKAAEQYF	–	3.3	6.9

– indicates not done; ND, not detected.

* Frequencies $\geq 5\%$ are indicated in bold type.

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 alloreactive CTL clones may cause acute GVHD after allogeneic HSCT.

A recent study assessed TCR- β sequences in peripheral blood and tissue samples obtained from 6 HLA-matched bone marrow transplant recipients using post-transplantation cyclophosphamide [21]. In a skin sample obtained on day +369 from a male patient who had a female donor and experienced acute GVHD, 3 clonotypes were identical to the TCR- β sequences of minor histocompatibility antigen H-Y-specific cytotoxic T cell clones that had been established in the laboratory. These 3 clonotypes accounted for 36% of the sequences in the corresponding skin biopsy specimen. Although the authors did not report whether those T cell clones actually existed in the skin tissue during acute GVHD, or whether there were more frequent clones than H-Y-specific T cell clones, their findings support the hypothesis that human acute GVHD in a specific organ may result from only a few different alloreactive T cell clones.

An unresolved question is 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 development of GVHD in the corresponding organ. This hypothesis is compatible with our clinical experience with

acute GVHD, including the difference in severity among organs, difference in onset day among organs, and mixed response to steroid treatment. Proving this hypothesis will require the identification of minor histocompatibility antigens recognized by frequent T cell clones in each tissue.

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 did not identify shared T cell clonotypes among 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 TCRs, because HLA types vary from patient to patient. Further analysis of more patients who share HLA alleles may provide more insight into 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 biopsy specimens 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]

Table 3
Comparison of the Frequent T Cell Clonotypes in Skin GVHD Tissues Between Patients

CDR3 of the Frequent Clonotypes Accounting for $\geq 5\%$ in at Least 1 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*	ND	ND	ND	ND	ND	ND
CASSRAVEKLF	23.0	ND	ND	ND	ND	ND	ND
CASSLSQGAANEQFF	ND	16.8	ND	ND	.0020	ND	ND
CASSRSNGRGRVPLHF	ND	7.3	ND	ND	ND	ND	ND
CSAEGTVGAGELFF	ND	6.9	.0020	ND	.0048	ND	.027
CASSLEGRTGAKNIQYF	ND	6.1	ND	ND	ND	ND	ND
CASTRGGSTDTQYF	ND	5.7	ND	ND	ND	ND	ND
CASSFNQPQHF	ND	ND	9.0	ND	ND	ND	ND
CASSLYTGQETQYF	ND	ND	8.2	.0016	ND	ND	ND
CASSLGSGRITETQYF	ND	ND	7.2	ND	ND	ND	ND
CSVDTLRNGYTF	ND	ND	6.8	ND	.0011	ND	ND
CASSFSRGGQGPLHF	ND	ND	6.5	.0032	ND	ND	ND
CASSFLGGRADTQYF	ND	ND	5.5	.0011	ND	ND	ND
CASSPTGQGNVGYTF	ND	ND	5.2	ND	ND	ND	ND
CASRSLYGYTF	ND	ND	ND	92.8	.00094	ND	ND
CASSLQREASWTGELFF	ND	ND	ND	7.1	ND	ND	ND
CASSVGGTGANVLT	ND	ND	ND	ND	15.7	ND	ND
CATSDLGTSAGETQYF	ND	ND	ND	ND	12.8	ND	ND
CSARDQGADGYTF	ND	ND	ND	ND	10.5	ND	ND
CASSLWQLGETQYF	ND	ND	ND	ND	9.3	ND	ND
CATSDRRGGGNEQFF	ND	ND	ND	ND	9.1	ND	ND
CSARDWASGVDEQFF	ND	ND	ND	ND	7.5	ND	ND
CASTTHRGGGANVLT	ND	ND	ND	ND	7.1	ND	ND
CASSPQADTQYF	ND	.017	ND	ND	6.6	ND	ND
CASSLATKNQPQHF	ND	.0013	ND	ND	6.0	ND	ND
CASSSTGGGGTEAFF	ND	.027	ND	ND	5.9	ND	ND
CASSLLGVAGGEETQYF	ND	ND	ND	ND	ND	21.8	ND
CASSVNTEAFF	ND	ND	ND	ND	ND	20.8	ND
CASSLRPGLGQETQYF	ND	ND	ND	ND	ND	14.1	ND
CASSPLNQETQYF	ND	ND	ND	ND	ND	9.5	ND
CASSFGTGTGANVLT	ND	ND	ND	ND	ND	8.4	ND
CASISGAGGPLSLQFF	ND	ND	ND	ND	ND	7.3	ND
CASRSQGANSSELFF	ND	ND	ND	ND	ND	5.6	ND
CAISDTQGPKETQYF	ND	ND	ND	ND	ND	ND	20.1
CASSLVPQQYF	ND	ND	ND	ND	ND	ND	16.5
CSASRDQDEKLF	ND	ND	ND	ND	ND	.066	16.3
CASSYSNLNRDRIGTGANVLT	ND	ND	ND	ND	ND	ND	12.7
CASSPSTGVPYNEQFF	ND	ND	ND	ND	ND	ND	7.4
CSARQGGAGELFF	ND	ND	ND	ND	ND	ND	7.0
CASSLGTTYEQYF	ND	ND	ND	ND	ND	ND	6.3
CASSKVEGASGSENEQYF	ND	ND	ND	ND	ND	ND	5.8

ND indicates not detected.

* Frequencies $\geq 5\%$ are indicated in bold type.

demonstrated that some clonotypes were shared by tissue and peripheral blood. Although interpreting the differences in these observations is difficult, a possible explanation is that the

T cell clones recognizing minor histocompatibility antigens preferentially expressed in tissues are detected only in tissues, whereas those recognizing the antigens expressed in both tissue

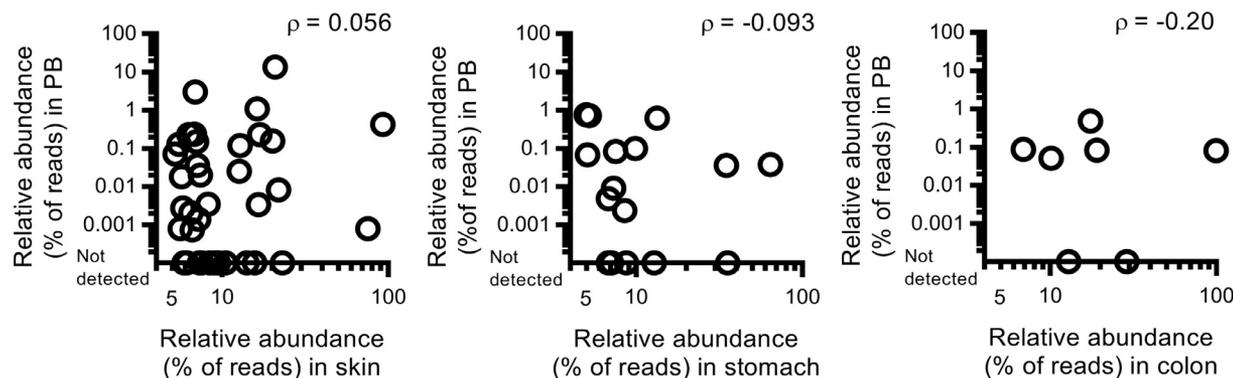


Figure 4. Comparison of frequencies of clonotypes in each GVHD tissue and in PB. The dot plot shows clonotype distributions in T cells that accounted for $\geq 5\%$ in the skin ($n = 41$), stomach ($n = 19$), or colon ($n = 7$) and their frequencies in the concurrent PB of the corresponding patient. The number in the upper right corner is the Spearman correlation coefficient, ρ .

and peripheral blood are detected in both. Nonetheless, the low frequencies of tissue-infiltrating T cells in peripheral blood may be unsurprising, given that peripheral blood is essential for trafficking of all T cells and thus the T cell repertoire in peripheral blood is not likely to be the same as in the organs. This underscores 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 alloreactive CTL clones, which differ from tissue to tissue and from patient to patient. The characterization of human T cells infiltrating GVHD tissues warrants further investigation.

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Authorship statement: D.K. and M.M. designed and performed experiments, analyzed the data, and wrote the manuscript. R.H. and T.A. performed experiments and assisted with data analysis. S.O., S.K., J.J., E.T., K.M., R.S., T.G., S.T., and T.N. contributed to the study design and data collection. H.K. supervised the project. All authors read and approved the final manuscript.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at [doi:10.1016/j.bbmt.2018.10.012](https://doi.org/10.1016/j.bbmt.2018.10.012).

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