A Distinct Evolution of the T Cell Repertoire Categorizes Treatment Refractory Gastrointestinal Acute Graft-Versus-Host Disease

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Running Title: T cell repertoire in GVHD patients.
Key Points:

1. T cell clones identified in the GI tract of patients with steroid-refractory acute GI GVHD expand in the blood with disease progression.

2. The T cell repertoire in the GI tract of steroid-refractory patients at the time of diagnosis is more similar than for responsive patients.

Abstract:
Steroid refractory gastrointestinal (GI) acute graft versus host disease (aGVHD) is a major cause of mortality in hematopoietic stem cell transplantation (HCT) without immune markers to establish a diagnosis or guide therapy. We found that T cell receptor β (TCRβ) CDR3 repertoire sequencing reveals patterns that could eventually serve as a disease biomarker of T cell alloreactivity in aGVHD. We identified T cell clones in GI biopsies in a heterogeneous group of 15 allogeneic HCT patients with GI aGVHD symptoms. Seven steroid-refractory aGVHD patients showed a more conserved TCRβ clonal structure between different biopsy sites in the GI tract than eight primary-therapy responsive patients. Tracking GI clones identified at endoscopy longitudinally in the blood also revealed an increased clonal expansion in patients with steroid-refractory disease. Immune repertoire sequencing-based methods could enable a novel personalized way to guide diagnosis and therapy in diseases where T cell activity is a major determinant.
Introduction

Hematopoietic cell transplantation (HCT) is an effective therapy for a broad range of hematologic malignancies\textsuperscript{1-3}. The benefits of HCT are in large part related to the development of effective immune responses against the underlying malignancy. However, immune reactivity against the recipient in the form of acute graft-versus-host disease (aGVHD) is a leading cause of morbidity and mortality that limits the use of HCT. Acute GVHD typically manifests within 100 days of transplant, during which donor T cells attack host skin, gut, or liver in a process fueled by inflammatory signals from host antigen-presenting cells (APCs) and tissues\textsuperscript{2,4}. For the 30-45\% of patients that develop aGVHD, just over half develop gastrointestinal (GI) tract manifestations, and these acute GVHD patients with significant GI involvement account for most of the mortality\textsuperscript{1,3}.

Symptoms of GI aGVHD include nausea, anorexia, diarrhea, abdominal pain, and hemorrhage\textsuperscript{5}. Severity of these symptoms is a poor predictor of survival, as they also frequently occur in the post-transplant setting secondary to conditioning regimen toxicity, infection, or medications. Endoscopic biopsy confirmation of aGVHD is standard, but histologic grade at diagnosis does not reliably predict disease severity or survival and cannot be used to guide therapy\textsuperscript{6}.

Front-line therapy for GI aGVHD involves increased immunosuppression using corticosteroid and other medications. This initial therapy effectively treats 24-41\% of patients\textsuperscript{6}. There is no consensus on treatment of steroid-refractory GI aGVHD, a subgroup with high mortality. A fundamental obstacle to identifying and treating aGVHD, and thus improving HCT outcomes, is a lack of objective disease biomarkers to guide therapy and standardize patients in studies. Some groups have proposed panels of blood biomarkers of tissue damage to predict aGVHD treatment outcomes\textsuperscript{7,8}. Though promising, these approaches do not monitor alloreactive T cells
known to be causally related to disease activity. A minimally invasive, reliable blood measure of T cell alloreactivity could significantly aid the management of GI aGVHD.

Despite decades of research, the identity and activity of the alloreactive donor T cells that cause aGVHD have remained inaccessible to clinical practitioners, largely due to the highly personalized and complex repertoire of T cell receptor hypervariable regions that arises when unique donor T cells are transplanted into recipients with unique histocompatibility antigens. Several groups have found an association between oligoclonal T cell populations and poor outcomes in HCT recipients. Quantitative studies of patient-specific T cell repertoires post-HCT can now be scaled, due to the use of next-generation sequencing to enumerate the full repertoire of recombinated TCR complementarity-determining region 3 (CDR3) repertoire sequences.

We used repertoire sequencing to identify dominant personal T cell clones in the GI tracts of patients with aGVHD at the time of diagnosis. Here we report the first analysis of the clonal TCRβ repertoire in patients with gastrointestinal GVHD and find that the T cell repertoire was more similar at different sites in the GI tracts of patients with severe treatment refractory GI GVHD when compared to patients with mild, treatment responsive aGVHD or with aGVHD-like symptoms without an identified cause. Importantly, each patient had a unique repertoire with little overlap amongst patients. Lastly, longitudinal tracking of GI-identified clones in peripheral blood at time of diagnosis and at 30 days after diagnosis revealed statistically significant clonal expansion in patients with steroid-refractory disease versus primary-responsive patients.
Methods

Patients and aGVHD classification

Between July 2011 and July 2012, 15 patients provided informed consent in accordance with the Declaration of Helsinki and were enrolled in a research and biobanking protocol approved by the Stanford University Institutional Review Board. Patients were clinically scored for acute GVHD using the Beardsman criteria by treating BMT attending physician\textsuperscript{17}. Patients with known CMV colitis were excluded.

Sample collection and DNA extraction

For identified patients undergoing HCT with suspected GI aGVHD, we requested that our gastroenterology colleagues collect 1-2 upper and 1-2 lower GI samples in areas of disease involvement. If no areas showed involvement, samples were to be collected in the right and left colon and the duodenum and stomach. GI biopsy specimens and placed them in saline at 4°C for 30-120 minutes before removal from saline and freezing at -80°C.

TCR\textsubscript{\beta} repertoire sequencing

Genomic DNA was isolated using standard methods (Qiagen). TCR\textsubscript{\beta} repertoire sequencing was performed using GigaMune\textsuperscript{®} Rep-Seq\textsuperscript{™} molecular kits and ClonoByte\textsuperscript{™} repertoire analysis software (GigaGen). Briefly, 1.6µg genomic DNA was amplified by PCR with a set of 45 primers targeting the TRBV genes paired with 13 primers targeting the TRBJ genes. This set of 58 primers amplifies the CDR3 region of TCR\textsubscript{\beta} and also introduces universal priming sites to the amplicons. A second round of PCR was performed on the resulting amplicons using universal primers. Each sample was indexed with a unique 6-nucleotide tag, allowing
demultiplexing of samples after sequencing. Samples were then sequenced on a Genome Analyzer IIx or MiSeq (Illumina).

The analysis of the sequencing results were performed blinded as to if the patient has steroid-refractory or primary-responsive GVHD. The sequencing reads for each sample were analyzed using ClonoByte™ TCRβ repertoire analysis software (GigaGen). We aligned each sequence to the set of TRBV and TRBJ genes by identifying the conserved cysteine and phenylalanine which form the boundaries of the CDR3. For quality purposes we discard reads that do not have a uniquely identifiable V gene, are out of frame as defined by the conserved cysteine and phenylalanine, contain a stop codon or a sequencing error in the form of an uncalled base. All other nucleotide sequences are translated into their amino acid equivalent.

Errors can accrue during the pre-sequencing PCR and during the sequencing process, distorting the real diversity of the TCRβ CDR3 repertoire by creating a tail of low-abundance clones. We removed the background of spurious low-abundance clones: First, we ran a no-template negative control (NTC) alongside each batch of 12 samples at every stage. In the rare cases where the NTC showed a strong spurious CDR3 signal we removed those clones from all corresponding samples. Second, for each sample we removed all reads mapping to clones whose frequency is less than 0.1% of the most abundant clone's frequency, and then normalized the remaining clones' frequencies to the total number of remaining reads.

**Whole-repertoire statistics**

The Bhattacharyya coefficient measures the amount of overlap between two statistical samples, and was applied to unique clonotypes and their frequencies\(^1\). It is calculated as

\[
\sum_{j=1}^{n} \sqrt{f_{j,1} \times f_{j,2}}
\]

where \(f_{j,1}\) and \(f_{j,2}\) are the frequencies of clonotype \(j\) in samples 1 and 2, respectively, and \(n\) is the
number of unique clonotypes present across samples 1 and 2. A Bhattacharyya coefficient of 1 between two Rep-Seq samples would mean that the samples are identical in number, sequence, and frequency of unique clonotypes. A value of 0 would mean that no clonotypes are present in both samples.

The Gini coefficient is a metric of inequality in a frequency distribution. It is commonly used to measure economic distributions such as income inequality, but has been applied in biology as a measure of oligoclonality of HTLV-1–infected T cell clones. The Gini coefficient is defined as the ratio of the area between the Lorenz curve of the distribution and the line of equality to the area under the line of equality. Thus, a Gini coefficient of 1 corresponds to a completely monoclonal sample containing a single clone, while a Gini coefficient of 0 corresponds to a sample with all clones present at equal frequencies. For a Rep-Seq data set containing unique clonotypes ranked from least frequent \((k = 1)\) to most frequent \((k = n)\), the Gini coefficient can be approximated with the trapezoidal rule as

\[
1 - \sum_{k=1}^{n} \left( X_k - X_{k-1} \right) \left( Y_k + Y_{k-1} \right)
\]

where \(X_k\) is the cumulative proportion of unique clonotypes \(1, \ldots, k\) and \(Y_k\) is the cumulative frequency of clonotypes \(1, \ldots, k\).

For patient-level comparisons, statistical evaluation was performed on the mean of any repeated measures (e.g., multiple biopsy tissue sites within one patient), except as specified. All statistical significance testing was performed using Prism 6 (GraphPad).

**Indicator Clone Index at day 30 (ICI-30)**

The ICI-30 is derived from the average fold change of indicator clone frequencies in peripheral blood over time, weighted by each indicator clone’s frequency in the tissue of discovery. An
ICI-30 value of 100 indicates that on average, indicator clone frequency in blood at day 30 post-Dx remained unchanged from time of diagnosis, while a value of 500 indicates that the weighted average fold change was five-fold. Performing repertoire sequencing on a patient’s GI tissue, blood at time of diagnosis, and blood at day 30 post-Dx yields three sets of unique CDR3 sequences and their frequencies. From these data, the ICI-30 is calculated as

$$100 \times \left( \frac{\sum_{i=1}^{n} f_{i,t} \left[ \frac{f_{i,b30} / (1-f_{i,b30})}{f_{i,bDx} / (1-f_{i,bDx})} \right]}{\sum_{i=1}^{n} f_{i,t}} \right)$$

where $n$ is the number of tissue indicator clones in tissue $t$; and $f_{i,t}$, $f_{i,bDx}$, and $f_{i,b30}$ are the frequencies of clonotype $i$ in tissue, blood at time of diagnosis, and blood at day 30 post-Dx, respectively. Indicator clones that were not detected above background in either blood sample were assigned a fold change of 1 and included in the weighted average. If an indicator clone was detected above background in one blood sample but not the other, we assigned the clone a frequency equal to the background cutoff of the sample from which the clone was absent, i.e. 0.1\% of the frequency of the most abundant clone.
Results

Patient enrollment and sample collection.

We sequenced the TCRβ repertoires of biopsy tissue obtained from a heterogeneous group of 15 consecutive allogeneic HCT patients who underwent endoscopy for suspected GI aGVHD (Table 1). We also obtained blood from each patient at the time of aGVHD diagnosis and at 25-35 days after diagnosis (day 30 post-Dx; Fig. 1a). We compared seven HCT patients with aGVHD requiring secondary therapy (steroid-refractory) and eight HCT patients who recovered from symptoms seen in aGVHD with or without primary therapy (primary-responsive). All but one of the 15 patients received steroids as primary GVHD therapy (Supplementary Table S1).

When compared to primary-responsive patients, steroid-refractory patients showed no significant difference in stool output, aGVHD grade (Mann-Whitney, P = 0.577), maximum GI histology grade (Mann-Whitney, P = 0.27), or white blood cell count at endoscopy (data not shown; t-test, P = 0.5). All patients received blood-mobilized peripheral stem cells, and the two groups showed rough equivalency between numbers of matched related, unrelated, and mismatched donors, with the exception of one patient in the steroid-refractory group who had a haploidentical donor.

Notable differences between groups include two patients in the primary-responsive group who received non-myeloablative conditioning, but had full donor chimerism at the time of endoscopy. Two patients in the steroid-refractory group underwent prophylactic treatment with experimental T regulatory cell therapy. Two patients had CMV PCR positivity at less than 150 copies with negative shell vial and CMV culture results from the GI biopsy; they were among four patients treated for CMV viremia with gancyclovir without evidence of end-organ involvement (Table 1). Some patients had skin aGVHD, most prior to onset of GI symptoms (Supplementary Table S1).
**TCRβ repertoires in endoscopic tissue samples have similar characteristics in steroid-refractory and primary-responsive patients**

We performed repertoire sequencing of TCRβ CDR3 regions from genomic DNA to analyze 2-4 GI biopsies from each patient at the time of diagnosis of suspected GI aGVHD. In each sample, between 6,739–1,141,229 total productive translated amino acid sequences were obtained after a conservative background subtraction with no statistically significant difference in sequencing depth between groups (Supplementary Table S2; Mann-Whitney, P = 0.2785). Normal healthy controls run in triplicate showed pearson correlation coefficients between 0.90-0.95 with Bhattacharya coefficient values between 0.7-0.88.

Between groups, GI tissue repertoires did not show a statistically significant difference in oligoclonality, as measured using the Gini coefficient19,20 (Fig. 1b, Mann-Whitney, P = 0.6). We also found that within patients, areas of higher-grade histology did not show any difference in oligoclonality of TCRβ repertoires compared to areas of lower-grade histology (Fig. 1d, Kruskall-Wallis, P = 0.3). These data suggest that oligoclonality of tissue-infiltrating TCRβ repertoires is not an indicator of aGVHD outcomes.

**aGVHD tissue samples do not share public T cell clonotypes**

We performed comparisons between tissue samples across all patients and found that there were not unique CDR3 sequences present in all tissues. Nor were public clones found in all tissues of either the steroid-refractory or primary-responsive groups. Patients with shared HLA alleles did not appear to show a tendency towards more shared T cell clones (data not shown). This suggests that tissue-infiltrating TCRβ repertoires are highly patient-specific.
The T cell repertoire is more similar across different tissue sites in patients with steroid-refractory versus primary-responsive GVHD

For all same-patient tissue-tissue pairs, we calculated the Bhattacharyya coefficient (BC) to measure pairwise similarity of TCR repertoires\textsuperscript{21} (Fig. 2a). When averaging tissue-tissue BC values by patient, the intra-patient similarities between colon and upper GI were higher in steroid-refractory patients than in primary-responsive patients (Fig. 2b; Mann-Whitney, P = 0.045;). We saw no statistically significant differences in intra-patient colon-colon or upper GI-upper GI similarities (Fig. 2b), although these results are for few comparisons.

To comprehensively examine factors that might influence intra-patient tissue-tissue similarity, we fit all intra-patient tissue-tissue BC data to several linear mixed-effects models by maximum likelihood. The models included combinations of the following fixed effects: patient group (SR vs. PR), tissue site (upper GI vs. colon), and disparity in tissue histology (same histology vs. different; 0–5, given six possible histology grades: normal, apoptotic bodies, or GVHD grades 1–4).

The best-fit model according to the Akaike information criterion (AIC) included two fixed covariates (patient group and disparity in histology) and fit the data significantly better than a null model containing the random effect but no fixed covariates (P = 0.032). The combined model (three fixed covariates) did not fit the data significantly better than the two fixed covariate model. This model predicts a BC of 0.34 for a pair of tissues from a PR patient that have the same histology; pairs from a SR patient are predicted to be more similar (BC +0.21) and those with different histology to be less similar (BC -0.097). These findings may indicate that patients with severe steroid-refractory GI aGVHD show a more restricted reconstitution of the entire GI tract, with the possibility that a common suite of aGVHD-causing T cell clones affect multiple tissue sites within a patient.
**Whole TCRβ repertoires in blood do not distinguish steroid-refractory from primary-responsive patients at time of endoscopic evaluation**

Since repeated endoscopic biopsies are prohibitive, we cannot directly monitor alloreactive clones in the GI tract, which likely expand as aGVHD worsens. We therefore investigated whether characteristics of TCRβ repertoires in peripheral blood alone distinguish the two groups of patients. T cell repertoire sequencing of blood samples yielding 9,193–2,196,016 total sequence reads per sample, with no statistically significant difference between groups (Supplementary Table S2; Mann-Whitney, $P = 0.37$). Oligoclonality in blood at time of diagnosis as measured by the Gini coefficient was not statistically different between patient groups (Fig. 1c; Mann-Whitney, $P = 0.19$). Neither group showed significant changes in oligoclonality between time of diagnosis and day 30 post-Dx (Fig. 1e, Wilcoxon, primary-responsive $P > 0.99$, steroid-refractory $P = 0.22$). We were also unable to distinguish between the groups by difference in Gini coefficient from time of diagnosis to day 30 post-Dx (Not shown, Mann-Whitney, $P = 0.39$).

**Relative frequency at diagnosis and longitudinal expansion of aGVHD indicator clones in blood correlates with steroid-refractory disease**

We hypothesized that a blood-derived signature that follows patient-specific, dominant “indicator clones” identified in GI tissue samples could distinguish between steroid-refractory and primary-responsive patients. We define “indicator clones” as the 100 most frequent clones in a GI tissue sample. We developed a novel “fence plot”, to depict indicator clone frequency and rank in the tissue and blood samples of a patient with severe steroid-refractory aGVHD (Fig. 3a) and another patient with primary-responsive aGVHD (Fig. 3b). The number of tissue indicator clones detected in blood at time of diagnosis is shown in Figure 3c. At time of diagnosis, the number of indicator clones detected in blood was indistinguishable between
groups (Mann-Whitney, p = 0.22). Similarly, no statistically significant difference in mean or median indicator clone frequency in blood at the time of diagnosis could be detected between groups (not shown; Mann-Whitney, mean P = 0.33, median P = 0.65).

However, at day 30 post-Dx, fewer indicator clones were detected in the blood of primary-responsive patients than in steroid-refractory patients (Fig. 3d; Mann-Whitney, p < 0.0001). We hypothesized that indicator clones in the peripheral blood of steroid-refractory patients would increase in frequency over time, but remain the same or decrease in frequency in primary-responsive patients.

We next examined the behavior of GI indicator clones for each patient and plotted the ratio fold change of indicator clone frequencies at day 30 post-Dx versus time of diagnosis (Figure 4a). We observed that a number of indicator clones in steroid-refractory subjects expanded 10-100 fold, but none did in the primary-responsive group. To evaluate this further, we devised the Indicator Clone Index at day 30 (ICI-30) as a metric of expansion or contraction of tissue-identified indicator clones in peripheral blood at day 30 post-Dx compared to at diagnosis.

We found statistically significant expansion of indicator clones in patients with steroid-refractory GI aGVHD compared to primary-responsive patients, as measured by higher ICI-30 tissue values (Fig. 4b, Mann-Whitney, P < 0.0001). Averaging all tissue ICI-30 values for a patient also showed a statistically significant difference between groups (Fig. 4c; Mann-Whitney, P = 0.007). Although day 30 after diagnosis is late in disease progression and intervention, we performed a ROC analysis as an early evaluation of eventual clinical feasibility (AUC = 0.910, p < 0.008; Supplementary Figure S1). As a control, we calculated ICI-30 values for each patient, defining indicator clones as the top 100 clones by frequency in blood at time of diagnosis (Fig. 4d). Control ICI-30 values for steroid-refractory and primary-responsive groups were statistically indistinguishable (Mann-Whitney, P = 0.094).
We broadened our definition of indicator clone to include any clone above background in GI tissue biopsy and there was still a statistically significant difference (Supplementary Fig. S2; Mann-Whitney, P = 0.021). Lastly, we found that steroid dose does not correlate with ICI-30 values for either patient group (not shown; linear regression, primary-responsive $R^2 = 0.031$, P = 0.68, steroid-refractory $R^2 = 0.23$, P = 0.28).

Taken together, our data suggest that longitudinal expansion of tissue-infiltrating clones in blood is a biomarker of aGVHD disease activity that can be quantified and used to discriminate outcomes.
Discussion
This study is the first to use comprehensive T cell receptor sequencing to evaluate the T cell repertoire of patients with acute GVHD following allogeneic HCT for treatment of hematological malignancies. GI aGVHD carries a high mortality and it cannot yet be anticipated or well stratified by symptomology or histology at the time of diagnosis. An additional challenge in treatment of GI aGVHD is that no objective immune system metrics exist that can be used to guide therapy. We hypothesized that TCRβ repertoire sequencing might identify patterns that could eventually be used to predict severity and/or track the progress of GI aGVHD.

Since T cells are known to drive the pathophysiology of GVHD, assessment of T cell repertoires is likely to directly reflect disease activity. Sophisticated immune monitoring of this type may allow greater clarity into the pathophysiology of aGVHD, as well as provide much-needed objective measures for guiding therapy and standardizing patients in clinical studies. Further, it may eventually be possible to deduce the identity of the target antigens through analysis of the TCR repertoire. We used repertoire sequencing to identify dominant personal T cell clones in the GI tracts of patients with GI aGVHD symptoms at the time of diagnosis, and to track their behavior in blood over time. Importantly, we have demonstrated that this technology is applicable to the study of a complex disease such as GVHD and analysis of personalized TCR repertoire is readily achievable.

Repertoire sequencing is subject to the introduction of errors and artifacts due to primer dimerization, contamination, polymerase errors, and PCR amplification biases. We controlled for some potential technical errors by using conservative background subtraction to minimize the contribution of artifacts. We also designed our analysis to minimize any effect of PCR amplification bias, specifically by following the relative change in frequency of indicator clones in samples from the same patient.
Our study is the first to demonstrate a number of key points. First, despite the fact that primary-responsive and steroid-refractory patients showed the same degree of oligoclonality in their tissue samples at the time of diagnosis (Fig. 1b), we found that patients with steroid-refractory GVHD appeared to have a more consistent TCRβ clonal structure between different biopsy sites in the GI tract than primary-responsive counterparts (Fig. 2). High similarity of T cell repertoires between upper and lower GI samples could be used as a way to predict and stratify steroid-refractory versus steroid-responsive disease at the time of diagnosis. This finding could also suggest that steroid-refractory patients may have a unique pathology. For example, antigens targeted by common T cell clones within patients with severe, steroid-refractory disease might not necessarily be unique to specific areas of the GI tract, as may be the case for primary-responsive patients. These potential common, allogeneic antigens may be present and/or targeted throughout the GI tract and at other common aGVHD sites, such as skin and liver. Future studies will require standardized collection of skin or liver sites, in addition to multiple sites throughout the GI tract.

A second major finding in our study is that longitudinal tracking of these GI-identified clones in peripheral blood revealed an increased clonal expansion in patients with steroid-refractory disease versus primary-responsive patients. When we tracked GI-identified aGVHD indicator clones in blood from time of diagnosis, we were able to detect a sizable number of these clones, but at 30 days after diagnosis, we detected fewer indicator clones in the blood of primary-responsive patients. We expect that in successfully treated cases aGVHD-associated clones that presumably decrease in frequency would be less likely to be detected in the blood.

There are a number of potential confounding factors that distinguish the steroid refractory group, including the fact that most patients in the steroid refractory received etanercept and two patients in the group received experimental Tregulatory cells. Neither of these factors are
associated with inducing clonal expansion at the best of our knowledge. Alternatively, patients
with steroid-refractory GVHD and on more intense immunosuppression could have the
proliferation of gut-associated T cells due to infectious causes such as gut translocation of
bacteria. The one steroid-refractory patient with an ICI-30 score similar to those of the eight
primary-responsive patients had CMV PCR positivity. The potential confounding effects of
CMV viremia warrants further investigation.

We do not know how many T cell clones mediate human GI aGVHD. In some patients a few
clones could cause the disease, while in others hundreds might be responsible. Based upon
our tracking of aGVHD indicator clones, a relatively small number of GI identified clones
expanded dramatically (Figure 4a), our data support the use of repertoire sequencing to define
and quantify aGVHD-inducing clones in a personalized fashion.

A third major finding of our study was that no clones were shared between all patients with
aGVHD, indicating that there are no universal TCRβ sequences that cause aGVHD. While one
interpretation of this finding is that there exists no universal aGVHD allogeneic antigen, it is
also possible that multiple TCRβ CDR3 sequences may recognize the same antigen. Our
study is on a small group and evaluating more patients who share HLA alleles could still yield
dominant shared clonotypes and other reproducible patterns.

A fourth major point is that it was absolutely essential to biopsy and study clones identified in
GI tissue affected by GVHD in order to accurately classify patients. Over time, it might be
possible to use chemokine or trafficking receptor selection of T cells to profile clones targeting
specific tissue sites, but until then, studying peripheral blood in isolation from tissue is not likely
to be productive.

Further investigation will be needed to determine whether expansion of GI-identified clones
can be detected 7–14 days after diagnosis, a critical time point at which stratification of steroid-
refractory patients is clinically relevant. The single patient who survived steroid-refractory GI aGVHD had an ICI-30 value at the very lowest end of the steroid-refractory group. This might suggest that our approach could someday be used to titrate therapy. Whether this approach is also applicable to autoimmune disorders warrants investigation.

In summary, our study is the first to use repertoire sequencing to assess the dynamics and complexity of the T cell repertoire in gastrointestinal acute GVHD over time, and offers a new approach to the study of immune function and disease in humans.
Acknowledgments:

This work was supported by the National Institutes of Health, NCI P01 CA049605 NIH/National Cancer Institute. EHM was supported by the ASBMT Young Investigator Award and Stanford Translational and Applied Medicine Pilot Grant (Stanford Department of Medicine).

Authorship Contribution:

EHM designed the experiments and clinical protocol, contributed to analysis and authored the paper. ARH, AL, DBM, DSJ, PL contributed to analysis and writing; EHM, JL, MF, JLZ contributed to sample collection and preparation. RSN, SS contributed to design of protocol and writing.

Disclosure of Conflict of Interest

EHM and AHR have equity in GigaGen, Inc. AL and DSJ have equity and employment with GigaGen, Inc.
References

Table 1. Patient Characteristics.

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<td>URD</td>
<td>FK/MTX</td>
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<td>-/-</td>
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<td>CML Blast crisis</td>
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<td>URD</td>
<td>FK/MTX</td>
<td>2</td>
<td>+/- (*)</td>
<td>CR</td>
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<td>CLL</td>
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<td>URN-1</td>
<td>FK/MTX</td>
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<td>+/-</td>
<td>CR</td>
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*CMV PCR blood positivity. **CMV PCR tissue positivity <150 copies, shell vial and culture negative. Underlined patients are steroid refractory, all others are primary responsive.
Figure 1. Measurement of T cell repertoire oligoclonality in human clinical GVHD samples. (a) Diagram of sample collection for patients. (b) GI tissue samples from primary-responsive (PR; n=8) and steroid-refractory (SR; n=7) patients show the same T cell repertoire oligoclonality as measured by the Gini coefficient (Mann-Whitney, P = 0.5893). Comparison is of patient averages of individual tissue samples. (c) Blood samples from PR and SR patients at time of diagnosis (●) and day +30 after diagnosis (○) show the same T cell repertoire oligoclonality (Mann-Whitney, P = 0.1919). (d) GI tissue samples do not show increased T cell repertoire oligoclonality with increased GVHD histology grade. Comparison is of average Gini coefficients per patient at each histology grade (Kruskal-Wallis, P = 0.2600). (e) No significant change in oligoclonality in the blood between the day of diagnosis and day +30 after diagnosis for either PR or SR patients (Wilcoxon, P = 0.2188).
Figure 2

a. Similarity of TCR repertoires between tissue samples within patients. (a) Comparison of TCR repertoire similarity by tissue comparison shown for each patient (blue: colon to colon, pink: upper to colon, green: upper to upper; SR=steroid refractory, PR=primary responsive). (b) Within patients, the TCR repertoire between upper GI and colon tissue shows greater similarity in SR patients than PR patients. Results are reported as the average Bhattacharrya coefficient of upper versus colon comparison per patient with upper and lower samples available (for PR, n=8; for SR, n=5; Mann-Whitney, P=0.045). (c) SR tissue samples are more similar than PR. The best-fit
model according to the Akaike information criterion (AIC) included two fixed covariates (patient group and disparity in histology) and fit the data significantly better than a null model containing no fixed covariates ($P = 0.0323$).

Figure 3. T cell clonal dynamics of GI-identified TCR indicator clones in the blood. (a) Fence plot illustrating change in frequency and rank of GI indicator clones in a representative patient with steroid-refractory GI aGVHD (Patient 5026). Each clone is represented in rank order (most frequent on top) on a sample’s axis by a node with size proportional to the clone’s frequency in the sample. The sample axis represents 100% of the clone counts above background, so the width of a node corresponds to the % frequency of the clone in the sample. Clones are colored based on whether they show an increase (orange) or decrease (blue) in frequency in blood at day 30 post-Dx versus at time of diagnosis. Clones of rare frequency that were detected below background or were undetected are depicted off-axis as $<\text{bkgd}$ or ND, respectively. (b) Fence plot of a representative patient with primary-responsive GI aGVHD (Patient 4998). (c-d) Number of indicator clones detected in blood of patients at time of diagnosis (c) and day 30 post-Dx (d). Points represent indicator clone number for individual tissue biopsies. The same number of GI indicator clones are discovered among PR versus SR patients at the time of diagnosis (c, Mann-Whitney, $P = 0.2174$). More GI-GVHD indicator clones are detected in blood at day 30 post-Dx in SR compared to PR patients (Mann-Whitney, $P < 0.0001$).
Figure 4

(a) Steroid-refractory (SR) patients but not primary-responsive (PR) patients show a longitudinal expansion of GI indicator clones in the blood (top 100 clones in GI tissue). Shown are all GI indicator clones that were detected in blood, reported as ratio fold change of indicator clones at day +30 after diagnosis compared to time of diagnosis. As measured by ICI-30, SR patients show a statistically significant expansion of GI indicator clones as when comparing tissue samples (b) or averaged tissues per patient (c). (d) Control ICI-30 values calculated by defining indicator clones as the 100 most frequent clones in blood at time of diagnosis are not different between PR and SR patients.

Figure 4. Change in frequency of GI indicator clones between diagnosis and day +30 after diagnosis. (a) Steroid-refractory (SR) patients but not primary-responsive (PR) patients show a longitudinal expansion of GI indicator clones in the blood (top 100 clones in GI tissue). Shown are all GI indicator clones that were detected in blood, reported as ratio fold change of indicator clones at day +30 after diagnosis compared to time of diagnosis. As measured by ICI-30, SR patients show a statistically significant expansion of GI indicator clones as when comparing tissue samples (b) or averaged tissues per patient (c). (d) Control ICI-30 values calculated by defining indicator clones as the 100 most frequent clones in blood at time of diagnosis are not different between PR and SR patients.
A distinct evolution of the T cell repertoire categorizes treatment refractory gastrointestinal acute graft-versus-host disease

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