Title: Plasma CXCL9 elevations correlate with chronic GVHD diagnosis.
Short Running Title: Plasma CXCL9 is elevated at diagnosis of chronic GVHD

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Scientific Category: Transplantation

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**Key Points:**

1. Plasma concentrations of CXCL9 are elevated at the onset of cGVHD diagnosis, but not in patients with cGVHD for more than 3 months
2. Plasma concentrations of CXCL9 are impacted by immunosuppressive therapy
Abstract: There are no validated biomarkers for chronic GVHD (cGVHD). We used a protein microarray and subsequent sequential ELISA to compare 17 patients with treatment-refractory de novo onset cGVHD and 18 time-matched control patients without acute or chronic GVHD to identify five candidate proteins that distinguished cGVHD from no cGVHD: CXCL9, IL2Rα, Elafin, CD13 and BAFF. We then assessed the discriminatory value of each protein individually and in composite panels in a validation cohort (n=109). CXCL9 was found to have the highest discriminatory value with an area under the receiver operating characteristic curve of 0.83 (95% confidence interval, 0.74-0.91). CXCL9 plasma concentrations above the median were associated with a higher frequency of cGVHD even after adjustment for other factors related to developing cGVHD including age, diagnosis, donor source and degree of HLA matching (71% vs. 20%, p<0.001). A separate validation cohort from a different transplant center (n=211) confirmed that CXCL9 plasma concentrations above the median were associated with more frequent newly diagnosed cGVHD after adjusting for the aforementioned factors (84% vs. 60%; p=0.001). Our results confirm that CXCL9 is elevated in patients with newly diagnosed cGVHD.
Introduction

Improvements in survival following allogeneic hematopoietic cell transplantation (HCT) have been achieved by decreasing early post-HCT toxicities through better human leukocyte antigen (HLA) matching, improved supportive care and less toxic conditioning regimens. Despite multiple clinical trials investigating innovative treatments for chronic graft-versus-host disease (cGVHD), standard treatment has not changed in the past 30 years, and cGVHD remains the leading cause of morbidity and mortality for long-term transplant survivors. The reasons for this lack of improvement are multifactorial and include an incomplete understanding of the pathophysiology as well as inconsistent definitions for diagnostic and response criteria. In 2005, the National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in cGVHD published a series of articles to help standardize the clinical approach to these patients, and promoted new interest in this important post-transplant complication.

Acute GVHD (aGVHD) biomarkers have been identified that predict disease occurrence, distinguish new onset GVHD from non-GVHD, have organ specificity and can predict treatment response. There is increasing interest in identifying cGVHD biomarkers that could also provide clinically meaningful information. Several publications have reported discovery of cGVHD biomarkers, but validation studies of biomarkers in independent populations are currently lacking. Furthermore, newly diagnosed and established cGVHD cases are often studied together, although the pathologic processes culminating in a new diagnosis may be different than those present in established disease. Therefore, we focused on identifying biomarkers for newly diagnosed cGVHD. We interrogated patient samples with a microarray approach to identify candidate proteins elevated in the plasma of patients with newly diagnosed cGVHD. The leading five protein candidates were tested in two independent populations to validate the findings using high throughput assays.
Of the five proteins, chemokine (C-X-C motif) ligand 9 (CXCL9) had the most significant association with cGVHD. CXCL9 is an interferon gamma (IFN-γ) inducible ligand for chemokine (C-X-C motif) receptor 3 (CXCR3), which is expressed on effector CD4+ Th1 cells and CD8+ CTL. CXCL9 has been shown to influence the interactions and migration patterns of effector T cells to inflamed tissue.\textsuperscript{13} We found that CXCL9 was elevated in the plasma of all three cohorts studied, and emerged as the best potential cGVHD biomarker.

### Methods

#### Patients

This study was approved by the Institutional Review Boards (IRB) of both the University of Michigan and the Fred Hutchinson Cancer Research Center (FHCRC). Informed consent was obtained from all patients or their legal guardians in accordance with the Declaration of Helsinki. Patient characteristics are summarized in Table 1. The University of Michigan discovery cohort consisted of 17 patients with treatment refractory de novo onset cGVHD (defined as rapidly progressive in severity or refractory to initial therapy) and 18 patients without a history of either aGVHD or cGVHD in order to identify two groups most likely to show differences in protein concentrations and to remove biomarkers only associated with aGVHD. The University of Michigan validation set was made up of a separate group of 109 patients. There were 45 patients with de novo onset cGVHD who had prospectively collected plasma samples obtained within 50 days of the onset of cGVHD. There were an additional 64 patients who had plasma samples collected at matched time points to the 45 cGVHD patients, had not developed cGVHD at the time of sample acquisition and any aGVHD had resolved (22%). Both the University of Michigan discovery and validation patients provided plasma samples for an IRB approved biorepository from 2002 to 2008. Chronic GVHD specific data was retrospectively reviewed by two clinicians (C.L.K.
and D.R.C.) with expertise in cGVHD who confirmed that patients met the NIH consensus criteria for diagnosis of the disease and assigned individual organ involvement and global score according to the 2005 National Institutes of Health Consensus Criteria. Details of cGVHD characteristics are provided in Table S1.

A second independent validation set was comprised of 211 patients treated at FH CRC from 2008 to 2011. The FHCRC validation cohort included samples obtained at the time of enrollment on an IRB-approved long-term follow-up study. Patients entered this study from 3 months to 66 months post-transplant, thus, there was greater heterogeneity in timing of sample acquisition relative to cGVHD onset. Therefore, we divided the FHCRC cohort into three groups: controls without cGVHD, newly diagnosed cGVHD (sample obtained within 90 days of diagnosis) and those with established cGVHD (sample obtained 3-36 months post-cGVHD diagnosis). Time to sample acquisition relative to HCT and diagnosis of cGVHD for both cohorts are provided in Table 2. In contrast to the University of Michigan patients, the FHCRC cGVHD cohort included all types of cGVHD presentation (de novo, quiescent and progressive). In both the University of Michigan and FHCRC cohorts, the onset of cGVHD was defined as the first time the NIH consensus criteria for diagnosis of cGVHD occurred, which was not necessarily when a patient first received systemic therapy.

**Antibody Array and ELISA**

Plasma samples in the discovery set were analyzed using a customized quantitative microarray dotted with 130 antibodies that targeted a diverse group of proteins detailed in Table S2 (Raybiotech, Norcross, GA). Briefly, we used an array of matched pair antibodies for detection of each target protein. Samples (50 μl) were incubated with the arrays, nonspecific proteins were washed off, and detection was carried out using a cocktail of biotinylated antibodies, followed by a streptavidin-conjugated fluor. Signals were
visualized using a fluorescence laser scanner, and quantified by comparison to array-specific protein
standard curves. Proteins that could distinguish between the cGVHD positive and negative groups with
a p value of 0.1 or less met the threshold for validation with enzyme-linked immunosorbent assay
(ELISA).

Validation of the proteins of interest from the microarray was performed with a sequential ELISA
protocol to maximize the number of measured analytes per sample by reusing the same aliquot
consecutively in individual ELISA plates. Commercial antibody pairs were available for: CXCL9
(RayBiotech, Norcross, GA), elastin, interleukin 2 receptor alpha (IL2Rα) and soluble B-cell activating
factor (BAFF) (R&D systems, Minneapolis, MN). The specificity of the capture and detection antibodies
for CXCL9 from Raybiotech is as follows: **Capture Antibody**: Host: Mouse, Isotype: Mouse IgG1, κ, Immunogen: baculovirus-expressed full-length recombinant human CXCL9 protein Clonality:
  Monoclonal; **Detection Antibody**: Host: Mouse, Isotype: Mouse IgG1, κ, Immunogen: baculovirus-
  expressed full-length recombinant human CXCL9 protein, Clonality: Monoclonal. These antibodies have
  shown less than 0.1% cross-reactivity with many human CXC chemokines (CXCL1, CXCL2, CXCL3,
  CXCL4/PF4, CXCL7, CXCL10) as well as a variety of other immunologic proteins. Samples and standards
  were analyzed in duplicate according to a previously described protocol.\(^{14}\)

In addition, because CD13 has been reported to be elevated in patients at onset of cGVHD,\(^ {11}\) we
developed a novel sandwich ELISA using two mouse anti-human CD13 monoclonal antibodies directed at
distinct epitopes of CD13 to analyze CD13 plasma concentrations in the discovery set. Briefly, plates
were coated with anti-CD13 antibody WM15\(^ {15}\) in carbonate buffer, then blocked with a blocking solution
devoid of animal protein (Vector Laboratories, Burlingame, CA). Test samples were applied and CD13
was detected using a biotinylated anti-CD13 antibody termed 591.1D7.34 that was generated in the Fox
laboratory, followed by streptavidin-HRP and TMB substrate. We used the same technique for measuring CD13 concentrations in the validation cohort as CD13 met our \textit{a priori} criteria for a candidate biomarker. Plasma samples were run by a technician blinded to clinical factors or case/control status.

**Statistical Methods**

Differences in the groups with and without cGVHD were compared with Student’s \textit{t} tests for continuous variables, and with Fisher’s exact tests for categorical variables. Differences in patient characteristics between training and validation sets were assessed with a Breslow-Day test for homogeneity of the odds ratios. Median protein concentrations were compared using the Wilcoxon-Mann-Whitney test. The chi-square test was used for unadjusted comparison of proportions. Logistic regression with adjustment for clinical factors known to be related to cGVHD in the two cohorts was used to compare proportions of patients with cGVHD in the high vs. low CXCL9 groups, classified by division at the median. A probability level of $<0.05$ was considered to be statistically significant. P-values were not corrected for multiple comparisons in \textit{a priori} analyses. Receiver operating characteristic (ROC) area under the curves (AUC) were estimated nonparametrically.

**Results**

We hypothesized that samples at onset of \textit{de novo} cGVHD from patients who ultimately developed treatment-refractory disease would be most likely to contain cGVHD-specific biomarkers. Using the protein microarray (Table S2) and subsequent ELISA workflow outlined, we identified five proteins (out of the 131 tested; 130 from the microarray + CD13 which was measured separately) that distinguished refractory cGVHD patients at disease onset from patients who never had aGVHD or cGVHD: CXCL9, IL2Rα, Elafin, CD13 and BAFF (Figure 1A-E).
We then measured concentrations of these five proteins in samples from the validation cohort of University of Michigan patients. Of note, patients in the cGVHD group were older and more likely to have received a transplant for a malignant condition than the no cGVHD controls. Otherwise, there were no statistically significant differences between the patients with cGVHD and without cGVHD based on donor type, graft source, HLA match or conditioning intensity. Likewise, samples were collected at similar times for both the cGVHD cases and controls. Samples were obtained at a median of 154 days after HCT in the cGVHD group compared to 135 days after HCT in the no cGVHD group (p=0.25). As in the discovery set, all five candidate proteins were significantly elevated in patients with newly diagnosed de novo onset cGVHD compared to those without cGVHD (Figure 1F-J), validating our initial findings. As others have also reported, we found an association of higher CD13 concentrations in patients whose chronic GVHD included liver involvement compared to cGVHD patients without liver involvement (median 1382 vs 725 ng/ml; p<0.0001)\(^9\).

To better define the potential clinical utility of these proteins elevated at the onset of cGVHD we performed area under the ROC curve analyses for each protein comparing no chronic GVHD to de novo onset cGVHD. The AUCs were similar for IL2R\(\alpha\), Elafin, CD13 and BAFF and ranged from 0.62–0.67 while the AUC for CXCL9 was 0.83 (Figure S1A). Given the similar AUCs for 4 of the proteins, we combined them into a composite panel (without CXCL9), which provided an improved AUC of 0.74. When CXCL9 was added to the composite panel, the AUC improved further to 0.83, but was not better than CXCL9 alone (Figure S1B). Since there was no additional diagnostic value to using the composite panels, we determined that CXCL9 had the best correlation with de novo onset cGVHD, and further analyses were confined to CXCL9.
Next we determined that the median CXCL9 plasma concentration provide an 87% sensitivity and a 77% specificity for identifying de novo cGVHD (Table S3). We then assessed the correlation of CXCL9 plasma concentrations and diagnosis of cGVHD by chi-square analysis. CXCL9 plasma concentrations above the median (6.5 pg/ml) were strongly associated with the presence of newly diagnosed cGVHD (71% vs 20%; p<0.001), a finding that remained statistically significant (p<0.001) after adjusting for potential confounding factors associated with the development of cGVHD [patient age, graft source (bone marrow/cord blood vs peripheral blood HCT), HLA match (matched sibling vs other) and diagnosis (malignant vs non-malignant)] (Table 3).

Finally, we assessed if CXCL9 concentrations were associated with other factors. Since changes in CXCL9 concentrations may reflect differences in immune recovery, we first analyzed for an association of CXCL9 concentrations and absolute lymphocyte count, and found none. We then examined whether CXCL9 concentrations were higher as time post-HCT increased, an alternative way to look for an association with CXCL9 and immune recovery. In the cGVHD patients, we did not detect an association of CXCL9 concentration and time post-HCT. Therefore, we concluded that CXCL9 elevated concentrations at the time of de novo cGVHD were due to the presence of the disease. We then sought to further validate CXCL9 as a marker of cGVHD activity in a second, more heterogeneous, independent cohort.

We obtained 211 samples from the FHCRC for validation. Unlike the University of Michigan cohort, the FHCRC validation cohort included patients with any type of cGVHD presentation (de novo, quiescent or progressive). In order to create more homogenous subsets within the FHCRC cohort, we divided the cGVHD patients into a newly diagnosed group (within 90 days of diagnosis; n=86) and an established cGVHD group (diagnosed 3-36 months prior to sample acquisition; n=92). Control patients (n=33) did
not have cGVHD, but prior treated aGVHD was allowed (73%). The median plasma concentration of CXCL9 was significantly higher in the FHCRC cohort than in the University of Michigan cohort (26 vs 6.5 pg/ml; p<0.0001), presumably reflecting the differences in the two populations described above. For our initial analysis of this independent cohort, we limited comparisons to no cGVHD controls versus newly diagnosed patients since they were most similar to the University of Michigan cohort. Despite differences in absolute values of CXCL9, as in the University of Michigan results, CXCL9 plasma concentrations were significantly higher in patients with newly diagnosed cGVHD compared to the no cGVHD patients (p=0.003; Figure 2). Area under the ROC curve analyses for CXCL9 comparing no chronic GVHD controls to newly diagnosed cGVHD revealed an AUC of 0.68 with a sensitivity and specificity at the median of 59% and 70% respectively (Table S3). Given the similarity of these results to those seen in the University of Michigan validation set, we performed an identical adjusted chi-square analysis for the FHCRC newly diagnosed cGVHD patients. As in the University of Michigan analysis, CXCL9 plasma concentrations above the median were strongly associated with the presence of cGVHD (84% vs. 60%; p=0.001; Table 3).

Given the strong correlation between CXCL9 plasma concentrations above the median and the presence of newly diagnosed cGVHD, we evaluated whether CXCL9 plasma concentrations were also associated with cGVHD severity at diagnosis. Very few patients in either the University of Michigan cohort (n=4) or the newly diagnosed FHCRC cohort (n=3) had mild cGVHD, so those patients were combined with patients who presented with moderate cGVHD. In both the University of Michigan cohort and FHCRC cohorts, CXCL9 plasma concentrations were significantly higher in patients who presented with severe cGVHD compared to no cGVHD group (p<0.0001 and p=0.0009 respectively; Figure 3A and B). Although University of Michigan patients who presented with mild/moderate cGVHD had significantly higher
CXCL9 plasma concentrations compared to no cGVHD controls (p<0.001; Figure 3A), we were unable to reproduce this finding in the FHCRC patients with mild/moderate cGVHD (p=0.17; Figure 3B).

Finally, because previously reported biomarkers for both acute and chronic GVHD have been shown to decrease following initiation of immunosuppressive therapy (IST)\textsuperscript{10, 16}, we analyzed the effect of treatment with IST on CXCL9 concentrations. In the University of Michigan cohort, where samples were obtained closer to the time of onset and possible initiation of therapy, we found that median CXCL9 concentrations were higher in patients not on IST (n=19) compared to patients on IST (n=43; 39 vs 15 ng/ml; p=0.009), furthermore, both groups had higher concentrations than the no cGVHD controls (n=82; 4 ng/ml, p<0.001 for both comparisons; Figure 4A). We performed the same analysis in the newly diagnosed FHCRC cohort. As in the University of Michigan cohort, patients not on IST at the time of sample acquisition (n=43) had higher CXCL9 concentrations than patients on IST (n=43; 77 vs 23 ng/ml; p<0.0001; Figure 4B) and the no cGVHD controls (n=33; 20 ng/ml; p<0.0001). Unlike the University of Michigan cohort however, concentrations of CXCL9 in patients on IST was not higher than the no cGVHD controls (p=0.51). This result might be explained by differences in the intensity and duration of IST between the cohorts. University of Michigan patients on IST were generally not on systemic steroids at the time of sample acquisition (84%), while only 2% of FHCRC patients were not treated with steroids when samples were acquired. Taken together, this finding suggests that intensity and duration of cGVHD treatment lowers CXCL9 concentrations. Lastly, because both cohorts consisted entirely of patients with multi-organ involvement, we could not validate CXCL9 as a biomarker with target organ specificity (data not shown).
We also were able to study CXCL9 concentrations in the FHCRC patients with established cGVHD (n=92; sample obtained 3-36 months after cGVHD diagnosis). CXCL9 plasma concentrations in this group of patients with long-standing and treated cGVHD were not statistically different compared to the no cGVHD controls (p=0.18). Likewise, there was no correlation between CXCL9 plasma concentrations above the median and the presence of cGVHD (Table 3), nor by disease severity (data not shown).

Discussion

Discovery of valid and reproducible biomarkers for cGVHD remains a significant challenge. Compared to aGVHD, cGVHD is clinically more heterogeneous, and can involve many more target organs, often simultaneously. Additionally, the timing of sample acquisition for biomarker assessment is also critical. Once immunosuppression has been initiated, the biomarker pattern may change, as has been previously been observed with BAFF plasma concentrations after patients are treated with corticosteroids\(^{10}\) and was observed in our study as well. Therefore, one of the strengths of our study design was the inclusion of only de novo cGVHD in the first validation cohort, when the length of prior therapy was minimized. Another strength of our study was that we were then able to reproduce the strong correlation of CXCL9 with cGVHD in a second more heterogeneous cohort. Taken together, these findings provide convincing evidence that elevated CXCL9 concentrations are a marker for newly diagnosed cGVHD.

CXCL9 is an IFN-γ inducible chemokine that binds to CXCR3, its only known receptor. CXCR3 expression can be rapidly induced in both CD4+ Type 1 helper cells, as well as CD8+ cytotoxic following dendritic cell activation of naïve lymphocytes.\(^{13}\) In both human and mouse autoimmune disease studies, the binding of CXCL9 to CXCR3 promotes lymphocytes to migrate to inflamed tissues.\(^{17,18}\) CXCR3 has also been shown to be critical for the recruitment of alloreactive T-cells in acute GVHD, while\(^ {19,20}\) CXCL9 has been shown to be elevated in tissue samples from patients with oral,\(^ {21}\) ocular\(^ {22}\) and cutaneous\(^ {23}\) cGVHD. We found that the plasma of patients with newly diagnosed cGVHD, but not established cGVHD, contains
higher concentrations of CXCL9 than patients without cGVHD. These results suggest that this T-cell chemoattractant is involved in the initiating steps of the cGVHD disease process, particularly around the time that clinical manifestations are first noted. The role of CXCL9 in the pathophysiology of cGVHD after the disease is well established, and systemic therapy has been given, is not as clear. Given the well described relationship of CXCL9-CXCR3 in Th1 mediated disease states, it is intriguing to speculate that the Th1 pathways may be important during the early stages of cGVHD.

One other group reported that in a study of 28 patients with cGVHD, CXCL9 serum concentrations were associated with cGVHD involving the skin, but not other phenotypes. Our cohort did not include patients with isolated skin involvement, which precluded us from performing the same analysis. However, as noted, we did not find that CXCL9 correlated with any particular organ involvement (data not shown). Given our large sample size and reproducibility of our results in independent validation cohorts, we believe that CXCL9 may be useful as a marker of cGVHD that presents with a variety of clinical phenotypes. Of note, the same group also reported an association of CXCL10 and CXCL11 and cGVHD. Though CXCL10 was in the discovery array, we did not find a difference in CXCL10 levels in our discovery experiments and CXCL11 was not included in our discovery array and therefore, neither marker was pursued further.

Several limitations should be noted. First, although we included a large number of candidate biomarkers in our discovery array, our approach was not unbiased in that we pre-selected the candidates for study. Thus, proteins not included in our array but associated with cGVHD were missed. In addition, a previous study demonstrated a strong correlation of BAFF/B cell ratios with active cGVHD. Our study did not include analyses such as B cell enumeration, so we cannot confirm the BAFF/B cell ration correlation. Using plasma protein concentrations alone, we found that CXCL9 had the highest AUC and best
sensitivity and specificity for diagnosis of cGVHD of the 5 proteins tested. A direct comparison of the
diagnostic utility of CXCL9 compared to BAFF/B cell ratios could be useful. Next, this study does not
address whether CXCL9 can predict the development of cGVHD as our focus was on samples obtained at
the time of diagnosis. Furthermore, although two independent validation cohorts were included, the
similarities in HCT practices, such as the paucity of haploidentical donors and bone marrow and cord
blood stem cell sources may limit the generalizability of the findings to patients who develop cGVHD
subsequent to transplants where these factors are present, if they impact cGVHD. Finally, as noted in
the results, the median concentrations of CXCL9 around the time of diagnosis of cGVHD were
significantly different in the two validation cohorts. We suspect these differences can be explained by
differences in the timing of sample acquisition relative to the onset of cGVHD (there was a wider
window for the FHCRC patients), the inclusion in the FHCRC cohort of patients with cGVHD presentation
other than de novo, the impact of immunosuppressive therapy, including intensity or duration
treatment, or center effects due to other differences between the cohorts that we have not yet been
able to define. We believe that the difference in median values highlights the importance of internally
controlling each experiment and testing samples blinded to clinical characteristics, which maximizes the
interpretability of validation studies.

An additional challenge for future cGVHD biomarker discovery will be to determine whether specific
biomarkers correlate with clinically relevant outcomes such as clinical phenotypes or treatment
response. Our study was designed to identify a marker of overall cGVHD activity as all of our patients
had at least two target organ involvement at disease onset. Therefore, this study was unable to
determine if a particular organ manifestation may be driving CXCL9 concentrations at disease onset.
Furthermore, the current NIH consensus criteria for treatment response have not been validated, and
publications from the Chronic GVHD Consortium have raised the concern that these measures may in
fact not be valid. The association of CXCL9 concentrations with treatment response would be best analyzed in a controlled context, such as a correlative study in a clinical treatment trial.

The clinical utility of CXCL9 as a biomarker for cGVHD needs to be further defined in future studies. These studies should include serial measurement at important times, such as prior to the typical onset of cGVHD, to determine if elevations precede overt clinical manifestations. If that were the case, CXCL9 levels could be used to guide clinical trials testing pre-emptive strategies. For example, if the CXCL9 level exceeded a certain threshold, planned immunosuppression taper could be delayed. Second, CXCL9 may correlate with clinical outcomes, a possibility suggested by the correlation of high urinary CXCL9 levels and renal allograft rejection. In addition, confirmation of CXCL9 as a cGVHD biomarker opens up the possibility of new therapeutic avenues, if further evidence can be developed implicating the CXCL9-CXCR3 pathway in cGVHD pathogenesis. For example, bortezomib, which has shown promise for acute GVHD prevention in two clinical trials, was recently shown to inhibit T-cell chemotactic movements, decrease expression of CXCR3 and decrease the secretion of CXCL9 by activated T-cells in a mouse study. Other CXCL9 and CXCR3 inhibitors are under development raising the possibility of translating these findings into a future clinical trial.

In conclusion, CXCL9 was the best biomarker for newly diagnosed cGVHD out of 131 candidates we tested. Future directions should include prospective and serial evaluations of CXCL9 in order to further define its clinical utility, potentially as a predictive biomarker prior to the onset of cGVHD. Our findings also implicate the CXCL9-CXCR3 pathway in the pathogenesis of cGVHD, which we believe warrants further exploration. Finally, it may be fruitful to expand the search for cGVHD biomarkers using unbiased large scale proteomic discovery approaches which could detect additional proteins and potentially reveal important pathways in the development of cGVHD that are currently unknown.
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Authorship:

Contribution: C.L.K. conceived and planned the study design, performed clinical data collection and quality assurance, interpreted the data, and wrote the manuscript; D.A.F. and R.M. developed and performed the CD13 ELISAs; T.M.B., B.E.S. and X.C. were the study statisticians and wrote the manuscript; L.C. assisted with the statistical analysis and wrote the manuscript; M.C., B.J.F., P.P. and K.L. performed ELISA experiments, participated in research discussions and wrote the manuscript; D.R.C. performed clinical data quality assurance and wrote the manuscript; J.E.L., J.L.M.F., P.J.M., M.E.F., J.A.H. contributed to patient accrual, clinical data collection and quality assurance, research discussion and
wrote the manuscript; S.J.L. planned the study design, interpreted the data and wrote the manuscript;
S.P. conceived and planned the study design, supervised the experiments, interpreted the data, and
wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


Table 1: Patient Characteristics

Table 2: Time to Sample Acquisition

Table 3: Chi square analysis for association of CXCL9 levels above the median and cGVHD
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>UM Discovery Cohort</th>
<th>UM Validation Cohort</th>
<th>FHCRC Validation Cohort</th>
<th>P value for difference between Discovery and Validation</th>
<th>P value for difference between No cGVHD and New Onset cGVHD</th>
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<td>Age, years</td>
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<td>Median</td>
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<td>Range</td>
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<tr>
<td>Malignant*</td>
<td>13 (72%)</td>
<td>17 (100%)</td>
<td>&lt;0.05</td>
<td>11 (17%)</td>
<td>1 (2%)</td>
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<td>Non-malignant†</td>
<td>5 (28%)</td>
<td>0</td>
<td>0.01</td>
<td>44 (98%)</td>
<td>1 (2%)</td>
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<td>Disease Status at HCT‡</td>
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<td>Low</td>
<td>22 (42%)</td>
<td>19 (43%)</td>
<td>0.65</td>
<td>11 (33)</td>
<td>38 (44)</td>
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<td>Intermediate</td>
<td>17 (32%)</td>
<td>11 (25%)</td>
<td>12 (36)</td>
<td>12 (35)</td>
<td>30 (35)</td>
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<td>High</td>
<td>14 (26%)</td>
<td>14 (32%)</td>
<td></td>
<td>14 (36)</td>
<td>18 (21)</td>
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<td>Donor Type</td>
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<td>Matched sibling</td>
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<td>25 (56%)</td>
<td>0.55</td>
<td>18 (55)</td>
<td>30 (35)</td>
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<tr>
<td>Other</td>
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<td>56 (65)</td>
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<td>Source</td>
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<tr>
<td>Bone marrow</td>
<td>18 (28%)</td>
<td>6 (13%)</td>
<td>0.15</td>
<td>7 (21)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Cord Blood</td>
<td>3 (5%)</td>
<td>1 (3%)</td>
<td></td>
<td>0</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>43 (67%)</td>
<td>38 (84%)</td>
<td></td>
<td>26 (79)</td>
<td>78 (91)</td>
</tr>
<tr>
<td>Conditioning Intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>48 (75%)</td>
<td>33 (73%)</td>
<td>1.0</td>
<td>19 (58)</td>
<td>50 (58)</td>
</tr>
<tr>
<td>Reduced</td>
<td>16 (25%)</td>
<td>12 (27%)</td>
<td></td>
<td>14 (42)</td>
<td>36 (42)</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>18 (100%)</td>
<td>17 (100%)</td>
<td>–</td>
<td>–</td>
<td>9 (27)</td>
</tr>
<tr>
<td>I–IV</td>
<td>0</td>
<td>0</td>
<td></td>
<td>24 (73)</td>
<td>70 (81)</td>
</tr>
<tr>
<td>NIH Global Severity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Moderate</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>46 (53)</td>
</tr>
<tr>
<td>Severe</td>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>37 (43)</td>
</tr>
</tbody>
</table>

Abbreviations: cGVHD, chronic graft-vs.-host disease; HCT, hematopoietic cell transplantation; NIH, National Institutes of Health
*Malignant diseases included: Acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myelomonocytic leukemia (CMML), Hodgkin lymphoma (Hod), juvenile myelomonocytic leukemia (JMML), Kostmann’s
syndrome, non-Hodgkin lymphoma (NHL), multiple myeloma (MM), myelodysplastic syndrome (MDS), myeloproliferative disorder (MPD), paroxysmal nocturnal hematuria (PNH), Prolymphocytic leukemia.

† Non-malignant disease included: Malignant infantile osteopetrosis, severe aplastic anemia, sickle cell anemia, thalassemia, severe combined immunodeficiency disorder (SCID), X-linked lymphoproliferative disorder (XLP).

‡Low, Intermediate or High risk disease status according to CIBMTR guidance.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>UM Discovery Cohort</th>
<th></th>
<th>UM Validation Cohort</th>
<th></th>
<th>P value for difference between Discovery and Validation</th>
<th>FHCRC Validation Cohort</th>
<th></th>
<th>P value for difference between No cGVHD and New Onset cGVHD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N=35</td>
<td></td>
<td>N=109</td>
<td></td>
<td></td>
<td>N=211</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>No cGVHD N=18</td>
<td>De Novo cGVHD N=17</td>
<td>P value</td>
<td>No cGVHD N=64</td>
<td>De Novo cGVHD N=45</td>
<td>P value</td>
<td>No cGVHD N=33</td>
<td>New Onset cGVHD N=86</td>
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<tr>
<td>Time post-HCT, days</td>
<td>Median</td>
<td>102</td>
<td>103</td>
<td>0.25</td>
<td>135</td>
<td>154</td>
<td>0.25</td>
<td>0.14</td>
</tr>
<tr>
<td>Time post-cGVHD onset, days</td>
<td>Median</td>
<td>–</td>
<td>0</td>
<td>--</td>
<td>–</td>
<td>0</td>
<td>--</td>
<td>0.45</td>
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<tr>
<td></td>
<td>Range</td>
<td>–</td>
<td>-50 - +12</td>
<td></td>
<td>–</td>
<td>-42 - +35</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: cGVHD, chronic graft-vs.-host disease; HCT, hematopoietic cell transplantation

1 P value for the difference between New Onset vs Established
Table 3: Chi Square Association for CXCL9 Levels above the Median with cGVHD

<table>
<thead>
<tr>
<th></th>
<th>Total n per group (cGVHD %)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ median</td>
<td>&gt; median</td>
</tr>
<tr>
<td>UM Validation</td>
<td>64 (20%)</td>
<td>45 (71%)</td>
</tr>
<tr>
<td>Newly diagnosed</td>
<td>58 (60%)</td>
<td>61 (84%)</td>
</tr>
<tr>
<td>Established FHCRC</td>
<td>71 (68%)</td>
<td>54 (81%)</td>
</tr>
</tbody>
</table>

Abbreviations: CXLC9, chemokine (C-X-C motif) ligand 9; cGVHD, chronic graft-vs.-host disease; UM, University of Michigan; FHCRC, Fred Hutchinson Cancer Research Center; CI, confidence interval
* Adjusted for age, stem cell source (BM/Cord vs PBSC), HLA match (matched sibling vs other) and diagnosis (malignant vs non-malignant)
Figure Legends

**Figure 1: Biomarkers at onset of cGVHD.** (A-E) ELISA results of median plasma concentrations of CXCL9 (A), BAFF (B), CD13 (C), IL2Rα (D) and Elafin (E) in the no GVHD patients (n=18) and refractory de novo cGVHD patients (n=17) from the discovery cohort. (F-J) ELISA results of median plasma concentrations of CXCL9 (F), BAFF (G), CD13 (H), IL2Rα (I) and Elafin (J) in the no cGVHD patients (n=64) and de novo cGVHD patients (n=45) from the validation cohort. Data are illustrated as box and whisker plots with the whiskers indicating the 90th and 10th percentiles.

**Figure 2: CXCL9 is elevated in newly diagnosed cGVHD from an independent cohort.** ELISA results of median plasma concentrations of CXCL9 from no cGVHD patients (n=33) and newly diagnosed cGVHD patients (n=86) in a second validation cohort from the FHCRC. Data are illustrated as box and whisker plots with the whiskers indicating the 90th and 10th percentiles.

**Figure 3: Increased CXCL9 levels are associated with increased cGVHD severity.** (A) ELISA results of median plasma concentration of CXCL9 from no GVHD (n=82), mild/moderate cGVHD (n=35) and severe cGVHD (n=27) from the entire University of Michigan (UM) cohort. (B) ELISA results of median plasma levels of CXCL9 from no GVHD (n=33) and mild/moderate cGVHD (n=49) and severe cGVHD (n=37) from the newly diagnosed FHCRC cohort. Data are illustrated as box and whisker plots with the whiskers indicating the 90th and 10th percentiles.

**Figure 4: CXCL9 is influenced by immunosuppression therapy.** (A) ELISA results from the University of Michigan (UM) cohort of median plasma concentrations of CXCL9 from cGVHD patients on no immunosuppressive therapy at the time of sample acquisition (n=19), cGVHD on any immunosuppressive therapy at the time of sample acquisition (n=43) and patients with no cGVHD (n=82). (B) ELISA results from the FHCRC of median plasma concentrations of CXCL9 from cGVHD patients on no immunosuppressive therapy at the time of sample acquisition (n=43), cGVHD on any immunosuppressive therapy at the time of sample acquisition (n=43) and patients with no cGVHD (n=33). Data are illustrated as box and whisker plots with the whiskers indicating the 90th and 10th percentiles.
Figure 1:

A: Discovery

CXCL9 (ng/ml)

No GVHD | cGVHD

p=0.0001

B: Discovery

BAFF (pg/ml)

No GVHD | cGVHD

p=0.0001

C: Discovery

CD13 (ng/ml)

No GVHD | cGVHD

p=0.003

D: Discovery

IL2Rc (pg/ml)

No GVHD | cGVHD

p=0.04

E: Discovery

Elastin (pg/ml)

No GVHD | cGVHD

p=0.057

F: Validation

CXCL9 (ng/ml)

No GVHD | cGVHD

p<0.0001

G: Validation

BAFF (pg/ml)

No GVHD | cGVHD

p=0.009

H: Validation

CD13 (ng/ml)

No GVHD | cGVHD

p=0.03

I: Validation

IL2Rc (pg/ml)

No GVHD | cGVHD

p=0.007

J: Validation

Elastin (pg/ml)

No GVHD | cGVHD

p=0.002
Figure 2:
Figure 4:

A. UM Cohort

B. FHCRC Cohort

CXCL9 ng/ml

p<0.0001

p=0.009

p<0.0001

p<0.0001

p=0.51

n: 19 43 82

n: 43 43 33
Plasma CXCL9 elevations correlate with chronic GVHD diagnosis