Supplementary material for

Murine chronic graft-versus-host disease proteome profiling discovers CCL15 as a novel biomarker in patients and potential therapeutic target

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Supplementary methods

Mice

C57Bl/6 (B6; H2b) mice, purchased from the National Cancer Institute, were used at 8-12 weeks old. B10.BR (H2k) mice, purchased from the Jackson Laboratory, were used at 10-14 weeks old. Mice homozygous for both floxed Crk and Crkl genes were crossed with B6 CD4-Cre transgenic mice (Taconic) to generate Crk/Crkl$^{-/-}$ T cells.[1,2] Animal protocols were approved by Institutional Animal Care and Use Committee at the University of Minnesota and Children's Hospital of Philadelphia.

Induction of cGVHD in vivo

B10.BR mice were conditioned with intraperitoneal Cytoxan (Sigma, 120mg/kg/day, i.p., day-3&-2) and total body irradiation (8.3 Gray, day-1), followed by infusion of 10^7 B6 T cell depleted bone marrow (TCD-BM) only as non-cGVHD control, or plus 70,000 purified splenic T cells to induce cGVHD (day 0), purified as described.[3,4] Organs were taken at day 56 post-T cell injection (established cGVHD), and day 28 post- T cell injection (prior cGVHD onset).

Pulmonary Function Tests

cGVHD was assessed by pulmonary function tests on day 56 ± 4 as described.[3,4] Nembutal anesthetized mice were intubated and ventilated using the Flexivent system (Scieq). Airway resistance, elastance and compliance were recorded and analyzed using the Flexivent software version 5.1.

Serum collection for murine proteomics

Mice were first anesthetized using a ketamine/xylazine mix. An intracardiac puncture was performed using a 27G needle and 1 mL syringe from the right ventricle. Blood was directly transferred into a dry Eppendorf tube on ice for 30 min then spun at 2000g for 10-15 min. Serum was collected and frozen at -80°C until day of use (only one freeze-thaw cycle was permitted).

Proteomics

Sample preparation and protein fractionation

Each pool contained 100 µl of serum. The two pooled serum samples were then individually immunodepleted of the 3 common hyper-abundant proteins (albumin, IgG, and transferrin) with a Multi Affinity Removal Column (MARS) 4.6 x 50 mm (Agilent) according to manufacturer's protocol. After measuring protein concentrations of depleted serum with a Micro BCA protein assay reagent kit (ThemroFisher Scientific), the volume of 100 µg aliquots were reduced to approximately 1/10 using Vivaspin® 500 (Vivaproducts) and precipitated using acetone at -20°C overnight. After centrifugation for 25 minutes at 15,000×g, acetone was decanted, and the air-dried protein pellets were dissolved in 100 µL of 8 M urea. Each sample was reduced by adding 50 µL of 10 mM dithiothreitol (DTT) in 100mM ammonium bicarbonate and incubated for 45 min at 56°C. The cysteine residues were alkylated by adding 30 µL of 50 mM iodoacetamide in 100mM ammonium bicarbonate to each vial and incubating for 30 minutes in darkness. Additional 30 µL of 10 mM DTT in 100mM ammonium biarbonate was added to neutralize iodoacetamide, and all solutions were diluted with 50 mM ammonium bicarbonate to 1M urea concentration. All samples were trypsinized by adding 2 µg of trypsin (Promega) in 20 µL of 50 mM ammonium bicarbonate to each and incubating them overnight at 37°C. Each sample was diluted further with

0.1% TFA to 800 µL and cleaned using Oasis HLB 10mg cartridge (Waters). The cartridges were conditioned with 1 mL acetonitrile, 2 × 1 mL 65% acetonitrile 0.1% TFA in water, and 2 × 1 mL 0.1% TFA. After sample loading and washing 2 × 1 mL 0.1% TFA, peptides were eluted using 1 mL of 65% acetonitrile and 0.1% TFA in water and dried in a speed vac. Each sample was then labeled with a unique tag allowing for differential quantification. The dried peptides were dissolved in 100 µL of 200mM HEPES, pH8.5. 41 µL of acetonitrile was added to 0.8 mg each of TMT reagent, 126 and 127 (Thermo Scientific). The samples were labeled in the following order: 1) control (bone marrow (BM) only) with label 126, and 2) cGVHD (BM and T cells) with label 127. 40 µL out of 41 µL TMT solution was then added to each sample vial, and the vials were incubated at room temperature for 1 hour. The labeling reaction was quenched by adding 8 µL hydroxlamine in water. All vials were dried in a speed vac separately, re-desolved in 0.1% TFA, combined, and cleaned with Oasis HLB 10mg cartridge (Waters) as before. The dried TMT labeled peptide sample was stored at -20°C until fractionation by strong cation-exchange (SCX) chromatography.

The sample was dissolved in buffer A (7mM potassium phosphate, 30% acetonitrile, pH 2.65) before fractionation with a SCX column (polySULFOETHYL A, 5 µm, 2.1 × 100 mm, PolyLC). Fractions were collected at 1 minute intervals at a flow rate of 200 µL/min from 1% solvent B (7 mM potassium phosphate, 500 mM KCl, 30% acetonitrile, pH 2.65) to 60% over 40 min (1% B for 7 minutes, 6–15% B for 23 min, 15–34% B for 15 min, and 34–60% B for 10 min) as well as during column washing with 98% solvent B for 10 min. The chromatographic elution was monitored using a UV detector at λ=220 nm. These fractions were consolidated into 12 fractions using the UV trace to distribute the peptide quantities similarly. After drying them in a speed vac,

peptides were desalted with Oasis HLB 10mg cartridge (Waters) as before and dried in a speed vac.

LC-MS/MS analysis

LC-MS/MS analysis was performed with an Easy-nLC 1000 (Thermo Scientific) coupled to an Orbitrap Fusion mass spectrometer (Thermo Scientific). The LC system configured in a vented format format[5] consisted of a fused-silica nanospray needle (PicoTip™ emitter, 75 μm ID, New Objective) packed in-house with 25 cm of Magic C18 AQ 100Å reverse-phase media (Michrom Bioresources Inc.), and a trap (IntegraFrit™ Capillary, 100 μm ID, New Objective) containing 2 cm Magic C18 AQ 200Å. The peptide sample was diluted in 30 μL of 2% acetonitrile and 0.1% formic acid in water, and injection volumes ranging between 1-4 uL were loaded onto the column in triplicate and separated using a two-mobile-phase system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A 90-min gradient from 7% to 30% B at a flow rate of 400 nL/min was used for chromatographic separation. The mass spectrometer was operated in a data-dependent MS/MS mode over the m/z range of 400–1500. The precursor scan mass resolution was set to 120,000. The cycle time was set to 3 seconds, and the most abundant ions from the precursor scan were isolated for MS/MS analysis using a quadrupole with 1.6 (*m/z*) mass window, dissociated with 37% normalized HCD collision energy and analyzed with an orbitrap with the resolution set to 60,000. Selected ions were dynamically excluded for 30 seconds.

Peptide and protein identification from mass spectra of digested fragments

Proteome Discoverer™ version 2.1 was used for data analysis. The acquired LC-MS/MS data were data were searched against the Swiss-Prot mouse proteome database (August 4, 2015) using SEQUEST[6] with the following parameters: trypsin was set as the digestion protease, with 2 maximum missed cleavages; precursor and fragment error tolerance were 10 part per million (ppm)

and 0.6 Dalton, respectively; Tandem Mass Tag™ (TMT) modification of N-termini was a fixed modification; and TMT modification of lysine residues, carbamidomethyl on cysteine residues, and oxidation of methionine residues as variable modifications. Identified peptides were filtered according to a 1% peptide-level false discovery rate (FDR) using Percolator.[7] Proteins with at least one identified peptide were reported. Ratios were normalized to the median of all PSMs (Peptide Spectrum Match).

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium[8] via the PRIDE partner repository with the dataset identifier PXD006278.

Candidate biomarker selection

This study was designed to discover biomarkers by profiling the proteome of murine cGVHD sera then validating the cause-and-effect relationship for those preferentially elevated in cGVHD mice. Candidates were selected for tested based upon available, relevant knockout donor mice or infusing neutralizing antibody to reverse established cGVHD. Four candidates (CCL8, CXCL7, CCL9, CRKL) were chosen for further in vivo analysis. Based upon in vivo murine cGVHD treatment data, three proteins (CCL15, the human equivalent of CCL9; CXCL7; CRKL) were selected to be measured in the plasma of a cohort of 211 allo-HCT recipients previously reported.[9]

Chemokine neutralizing antibody treatment

Monoclonal rat anti mouse CCL9 (MAB463), CCL8 (MAB790) and CXCL7 (MAB1091) were purchased from R&D Systems. To determine whether chemokine blockade could reverse cGVHD, cGVHD mice were intraperitoneally injected with 50-200 μg of each antibody, as indicated, from day 28-56, 3 doses/week. For CCL9 and CCL8, we used the previously published doses.[10,11] For

CXCL7, we used a classical 100 mcg/injection based on the 50% neutralizing dose (ND_{50}) of 2-8 µg/mL provided by the manufacturer. To evaluate the effect of anti-CCL9 on the GC reaction induced by sheep red blood cells (SRBCs), mice were immunized using SRBCs as described.[12] A cohort of mice was treated with anti-CCL9 antibody on days -1, 1, and 3 after immunization. On day 6 post-immunization, germinal center (GC) reactions were evaluated.

Enzyme-linked immunosorbent assay (ELISA) of human plasma

Candidate proteins validation was performed with a sequential ELISA protocol.[9,13-15] Commercial antibody pairs were available for 2 proteins (CXCL17 and CCL15, R&D Systems, Minneapolis, MN), and the CRKL kit was customized by Raybiotech, Norcross, GA. ELISAs were run according to manufacturer's recommendations. Samples were diluted as necessary (CRKL undiluted; CXCL7 1/500; and CCL15 1/25). Lower Limit of Quantification (LLOQ), and Upper Limit of Quantification were as followed: CRKL: 1.2, 50 ng/mL; CXCL7: 15, 1000 pg/mL; CCL15: 15, 1000 pg/mL. Samples and standards were analyzed in duplicate as described previously.[9,13-15]

Immunohistochemical and immunofluorescence analysis

Mouse lungs were inflated by 75% OCT and embedded in Optimal Cutting Temperature (OCT) compound, snap frozen in liquid nitrogen, and stored in -80°C. For trichrome staining, 8 µm cryosections were fixed for 15 min in Bouin's solution at 56°C and stained with the Masson's trichrome staining kit (Sigma HT15). For immunofluorescence staining, acetone fixed 8µm cryosections were stained with antibodies against mouse immunoglobulin (Ig) (BD 554001), αSMA (ThermoFisher 53-9760-82), CCL9 (Abcam ab9913) and CD45 (ThermoFisher 11-0451-

82). Splenic GCs were identified using PNA (Vector Laboratories). Confocal images were acquired on an Olympus FluoView500 Confocal Laser Scanning Microscope at 200X, analyzed using FluoView3.2 software (Olympus). The collagen deposition area, CD68 and Ig staining were quantified using the "color segmentation" plugin of Fiji software. The intensity of CCL9 staining was quantified using the "Measure" function of Fiji software.

Flow cytometry analysis of GCs, T follicular helper and T follicular regulatory cells

To analyze GC reaction, single cell suspension of spleens was obtained and stained with fixable viability dye, fluorchrome-labeled anti-CD4 (RM4-5, BD), anti-CXCR5 (SPRCL5, eBioscience), anti-PD-1 (J43, eBioscience), anti-CD19 (eBio1D3, eBioscience), anti-GL7 (GL-7, eBioscience) and anti-Fas (J02, BD). Cells were analyzed on BD LSRFortessa. GC B cells were defined as Fas and GL7 double positive $CD19^+$ B cells. Follicular helper T (Tfh) cells were defined as PD1 and CXCR5hi $CD4^+$ $Foxp3^-$ T cells. Follicular regulatory T (Tfr) cells were defined as PD1 and CXCR5hi $CD4^+$ $Foxp3^+$ T cells.

Patient characteristics and cohorts

This study was approved by the Institutional Review Board (IRB) of the Fred Hutchinson Cancer Research Center (FHCRC), and informed consent was obtained from all patients or their legal guardians. The cohort of patients has previously been described.[9] Patient and GVHD characteristics for the two groups are presented in Table 1. Briefly, the cohort was comprised of 211 patients treated at FHCRC from 2008-2011. Samples were obtained at the time of enrollment on an IRB-approved cGVHD observational study that included enrollment of controls. Patients entered this study from 3-66 months post-transplant. The cohort was divided into 2 groups:

controls without cGVHD (n = 33), and patients diagnosed with cGVHD (n = 178). cGVHD global severity score was evaluated per NIH criteria.[16,17] CCL15 prognostic ability when measured at approximately day +100 after HCT was evaluated in plasma of a cohort of 792 patients (Table 2).

Statistics

For murine experiments:

We assumed that the observations came from a normally distributed population as the recipient mice are from the same genetic background, housed in the same colony, and the BM and T cells they receive are from the same pooled donor graft. Differences between two groups were compared using two-tailed unpaired t test using GraphPad Prism software, version 7.02. Error bars in graphs represent mean ± standard error of the mean (SEM). Values of p less than .05 were considered significant.

For patients:

Clinical differences between groups with and without cGVHD were compared with Student's t-tests for continuous variables and Fisher's exact tests for categorical variables. Differences of CCL15 levels were compared with Wilcoxon two-sample tests. Error bars in graphs represent mean ± SEM. Differences in cGVHD severity between groups were evaluated using the Wilcoxon two-sample tests. The analysis of NRM divided CCL15 levels at the median value among cGVHD cases and compared cases above and below the median. NRM was estimated using cumulative incidence methods, treating relapse as a competing risk, and compared between groups using Cox regression. Severity and NRM analyses were performed using SAS (Cary, NC).

Table S1. List of 56 proteins identified and quantified with ratio of cGVHD pool (labeled with TMT 127)/ no cGVHD pool (labeled with TMT 126) > 1.2 in at least one replicate.

Table S2. CCL15 according to cGVHD status and particular organ involvement in the diagnosis and prognosis cohort

Table S3. Organ involvement and NIH severity at cGVHD onset in the diagnostic cohort (n=114)

Supplementary Figures legends

Figure S1. CRK/CRKL-/- donor T cells result in improved survival and significantly higher body weight

Survival and weight were recorded after transplantation. CRK/CKRL$^{-/-}$ donor T recipients had 100% survival versus 75% in wt T recipients, which were not significantly different. At periodic time points, mean weights were significantly higher in CRK/CKRL$^{-/-}$ vs wt donor T recipients. Data shown are representative of 2 independent experiments with 5-8 mice per group. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

Figure S2: Neutralizing CXCL7 or CCL8 did not affect cGVHD

Transplanted mice were treated with anti CXCL7 (100mg or 200mg/dose) or CCL8 (100mg/dose) from 28-56 days post bone marrow transplant. Pulmonary function test suggest neither anti-CXCL7 nor anti-CCL8 significantly altered cGVHD lung disease. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

Figure S3: Histopathology scores from mice treated with the anti-CCL9 neutralizing antibody

Transplanted mice were treated with anti-CCL9 days 28-56 post bone marrow transplant. Six-micrometer cryosections taken d56 post-transplant from lung, liver and spleen were H&E stained to determine pathology. No significant difference with anti-CCL9 treatment for histopathology scores in any of the target organs. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

Figure S4: Anti-CCL9 treatment slightly improve survival and weight of cGVHD mice.

Survival and weight were recorded after transplantation. Anti-CCL9 treatment group had 100% survival versus cGVHD group 80%. No significant differences were seen in weight curves.

Figure S5: Neither CD45 hematopoietic cells or CD31 endothelial cells are the main producers of CCL9 in cGVHD spleen

(A). Staining of CD45 and CCL9 in spleen suggest hematopoietic cells were not the main producers of CCL9 during cGVHD. Images were taken at 200 x magnification. (B) CCL9 and CD31 staining did not co-localize suggesting CD31 endothelial cells were not the source of CCL9. Images were taken at 200 x magnification.

Figure S6: CCL9 did not involve in SRBC induced GC response

Naïve B6 mice were immunized by 100 microliter of 10% SRBC. CCL9 (100mg) was given on days -1, +1, and +3 of immunization. Mice were sacrificed on day 6. (A) Immunofluorescence staining of spleen sections suggests CCL9 was not increased in SRBC immunized mice. Images were taken at 200 x magnification (B) Flow cytometry analysis of GC reactions suggests anti-

CCL9 antibody had not effect on SRBC induced GC reactions. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

Figure S7: cGVHD mice have higher vascular SMCs volume that was not altered by anti-CCL9 antibody.

Immunofluorescence staining of αSMA in spleen at 100 x magnfication. cGVHD group had significantly higher aSMA positive areas than BM only group, which was not altered by anti-CCL9 treatment. Data shown are representative of 2 independent experiments with 5-8 mice per group. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

Figure S8: Spleen vascular smooth muscle cells and lung increase CCL9 expression early during the course of GVHD at day 28 post-HCT

(A). Costaining of αSMA with CCL9 in the spleen at day 28 post-HCT. Images were taken at 400x magnification. αSMA positive cells of cGVHD mice had increased CCL9 expression that was not altered by CCL9 blockade. Images were quantified by Fiji software "color segmentation" plugin. (B). CCL9 expression in lung. Quantification was done by Fiji software "measure" function. Data shown are representative of 2-3 independent experiments with 5-8 mice per group. Unpaired student T-test was used when comparing 2 groups. Significance: *P<0.05; **P<0.01; ***P< 0.001; ****P<0.0001.

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A. Diagnostic cohort: CCL15 according to cGVHD status and particular organ involvement

	Median (range)			P-value			
Organ	Control (no cGVHD)	cGVHD+		Kruskal-Wallis	cGVHD+		
		Organ -	Organ +		Organ + vs. Organ -	Organ – vs Control	Organ + vs Control
Skin	17.8 (10.9-43.8)	21.4 (9.6-53.8)	21.0 (9.6-63.9)	0.04	0.85	0.02	0.02
GI	17.8 (10.9-43.8)	20.1 (9.6-63.7)	24.6 (9.6-63.9)	0.005	0.03	0.05	0.003
Mouth	17.8 (10.9-43.8)	20.8 (9.6-63.8)	21.4 (10.7-63.9)	0.04	0.59	0.06	0.009
Eye	17.8 (10.9-43.8)	21.0 (9.6-63.9)	21.4 (9.6-55.3)	0.04	0.82	0.01	0.04
Lung	17.8 (10.9-43.8)	20.6 (9.6-63.7)	21.3 (10.1-63.9)	0.05	0.77	0.08	0.01
Liver	17.8 (10.9-43.8)	19.4 (9.6-63.7)	22.8 (10.1-63.9)	0.007	0.05	0.10	0.003

	N		
Organ	Control (No cGVHD)	cGVHD +	
		Organ -	Organ +
Skin	33	65	113
GI	33	121	57
Mouth	33	67	111
Eye	33	97	81
Lung	33	61	116
Liver	33	97	80

B. Day 100 prognostic cohort: CCL15 according to cGVHD status and particular organ involvement

	Median (range)			P-value			
Organ	Control (no cGVHD within 90 days)	cGVHD w/in 90 days		Kruskal-Wallis	cGVHD +		
		Organ -	Organ +		Organ + vs. Organ -	Organ – vs Control	Organ + vs Control
Skin	22.5 (1.0-78.5)	20.3 (7.8-47.4)	21.5 (5.7-57.3)	0.08	0.48	0.05	0.22
GI	22.5 (1.0-78.5)	20.6 (5.7-57.3)	23.6 (7.9-61.7)	0.14	0.42	0.05	0.59
Mouth	22.5 (1.0-78.5)	23.6 (7.4-47.4)	18.4 (5.7-57.3)	0.04	0.12	0.81	0.01
Eye	22.5 (1.0-78.5)	20.3 ((5.7-47.4)	21.0 (7.8-57.3)	0.10	0.65	0.04	0.42
Lung	22.5 (1.0-78.5)	22.6 (5.7-47.4)	20.7 (9.7-57.3)	0.15	0.82	0.08	0.33
Liver	22.5 (1.0-78.5)	20.2 (7.4-47.4)	22.6 (5.7-57.3)	0.11	0.55	0.06	0.29

	N		
Organ	Control	cGVHD+	
		Organ -	Organ +
Skin	677	45	64
GI	677	70	41
Mouth	677	50	61
Eye	677	77	33
Lung	677	76	33
Liver	677	52	56

Table S3. Organ involvement and NIH severity at cGVHD onset in the diagnostic cohort (n=114)

	0	1	2	3	Missing
Skin	45	28	22	14	5
Mouth	50	51	10	0	3
Eyes	77	27	5	1	4
GI	70	28	11	2	3
Liver	52	25	20	11	5
Lung	76	25	6	2	5
Joints	93	9	0	0	12
Genital	74	2	0	0	38