Rivnak, A. J. *et al.* "*A Fully-automated Six-Plex Single Molecule Immunoassay for Measuring Cytokines in Blood*"

A
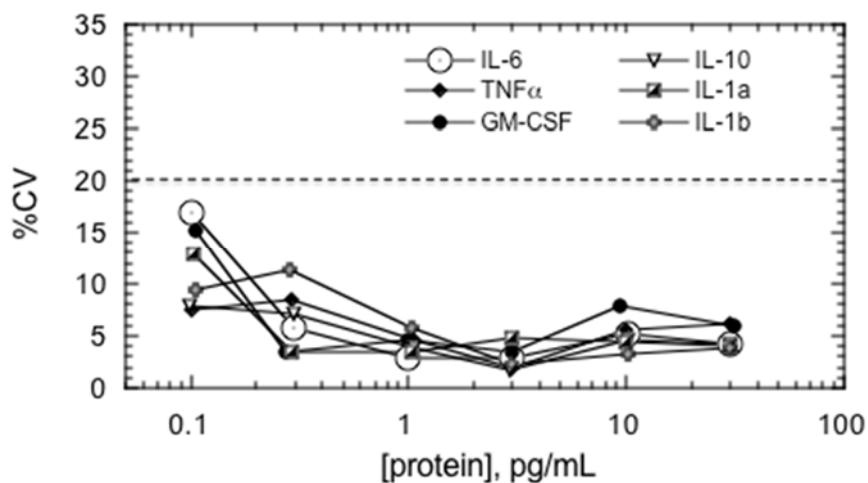
B
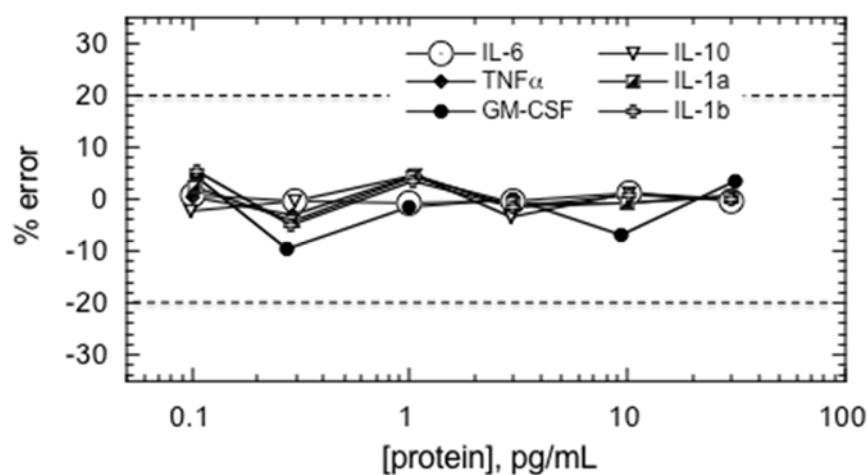
Figure S1. Plots of concentration of six cytokines spiked into bovine serum against: A) the imprecision (in %CV) in concentrations determined by reading back the calibrators off the calibration curve; and, B) the inaccuracy (in % error) in concentration value by reading back the calibrators off the calibration curve. These plots are based on the data from the three calibration runs presented in Figure 2 and Tables S1-S3.
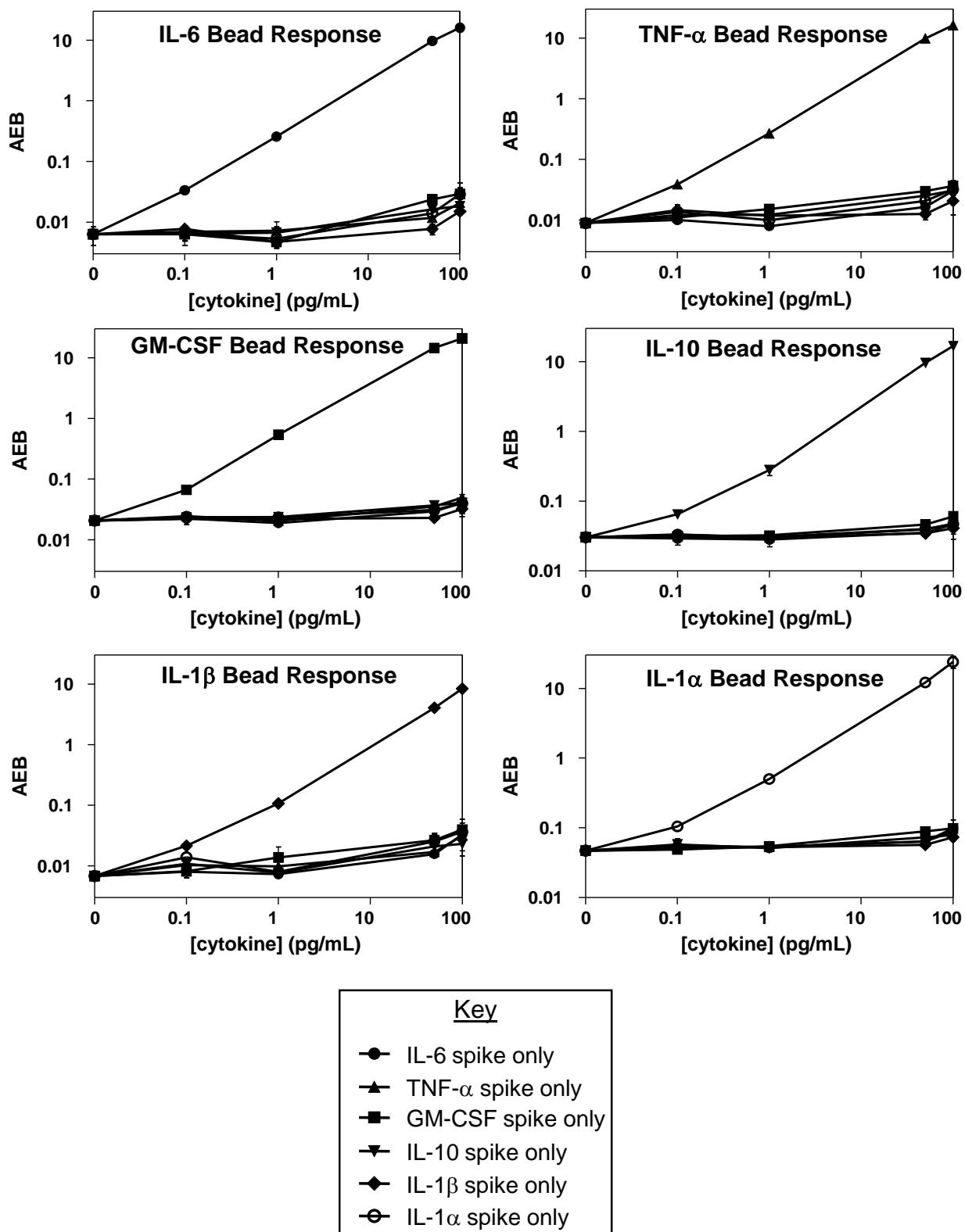
Figure S2. Plots of AEB against concentration of cytokine for six different specific bead types in a 6-plex Simoa assay. In these experiments, individual cytokines were separately spiked into bovine serum from 0 to 100 pg/mL to assess cross-reactivity.
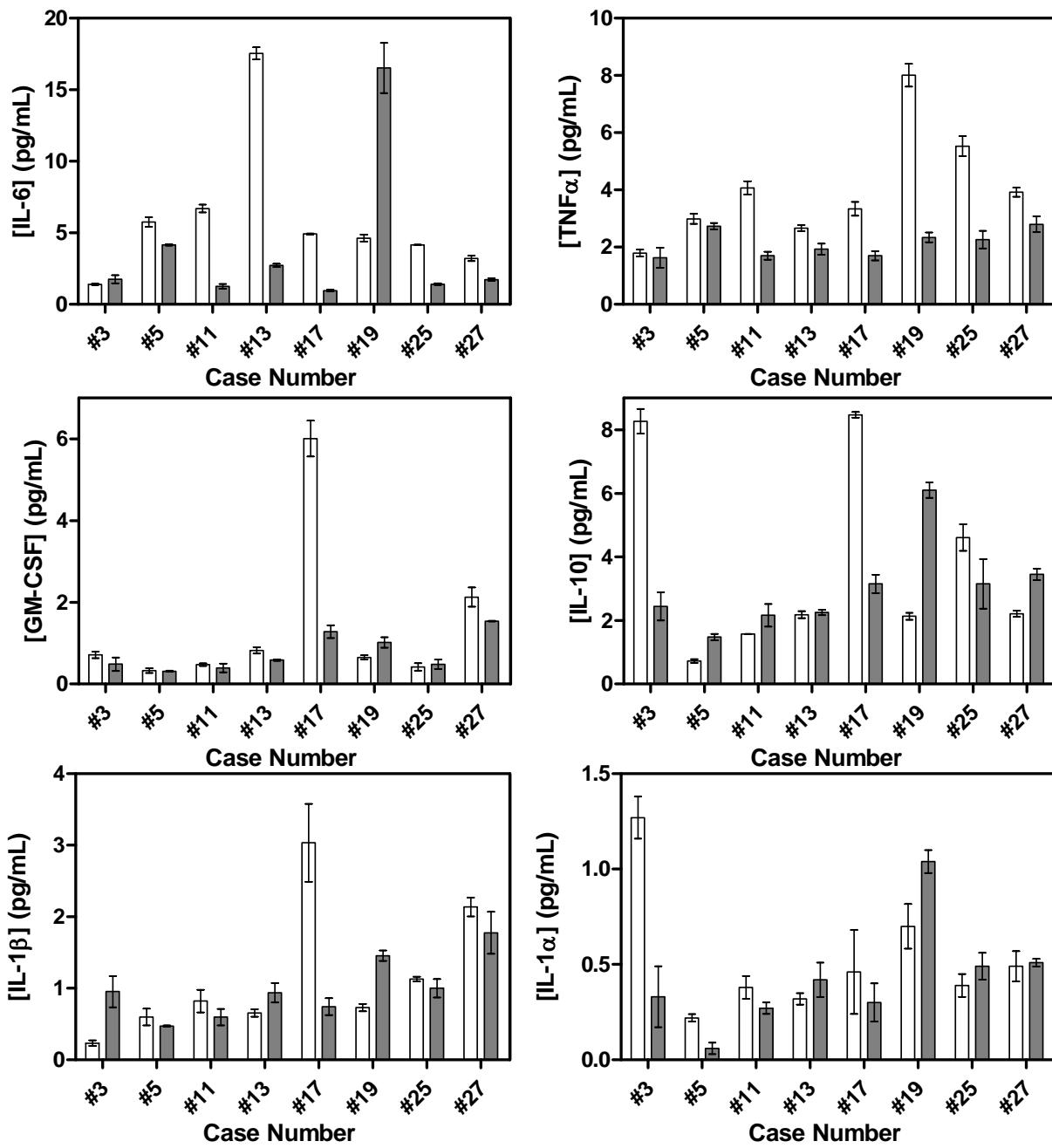
Figure S3. Plots of the concentrations of 6 cytokines in the plasma of eight CD patients before treatment with anti-TNF-α therapy (white bar) and 12 weeks after therapy was started (grey bar). Patients were anti-TNF-α naïve, except Case #19 who had previously been treated with infliximab. Error bars are based on one standard deviation of triplicate measurements.
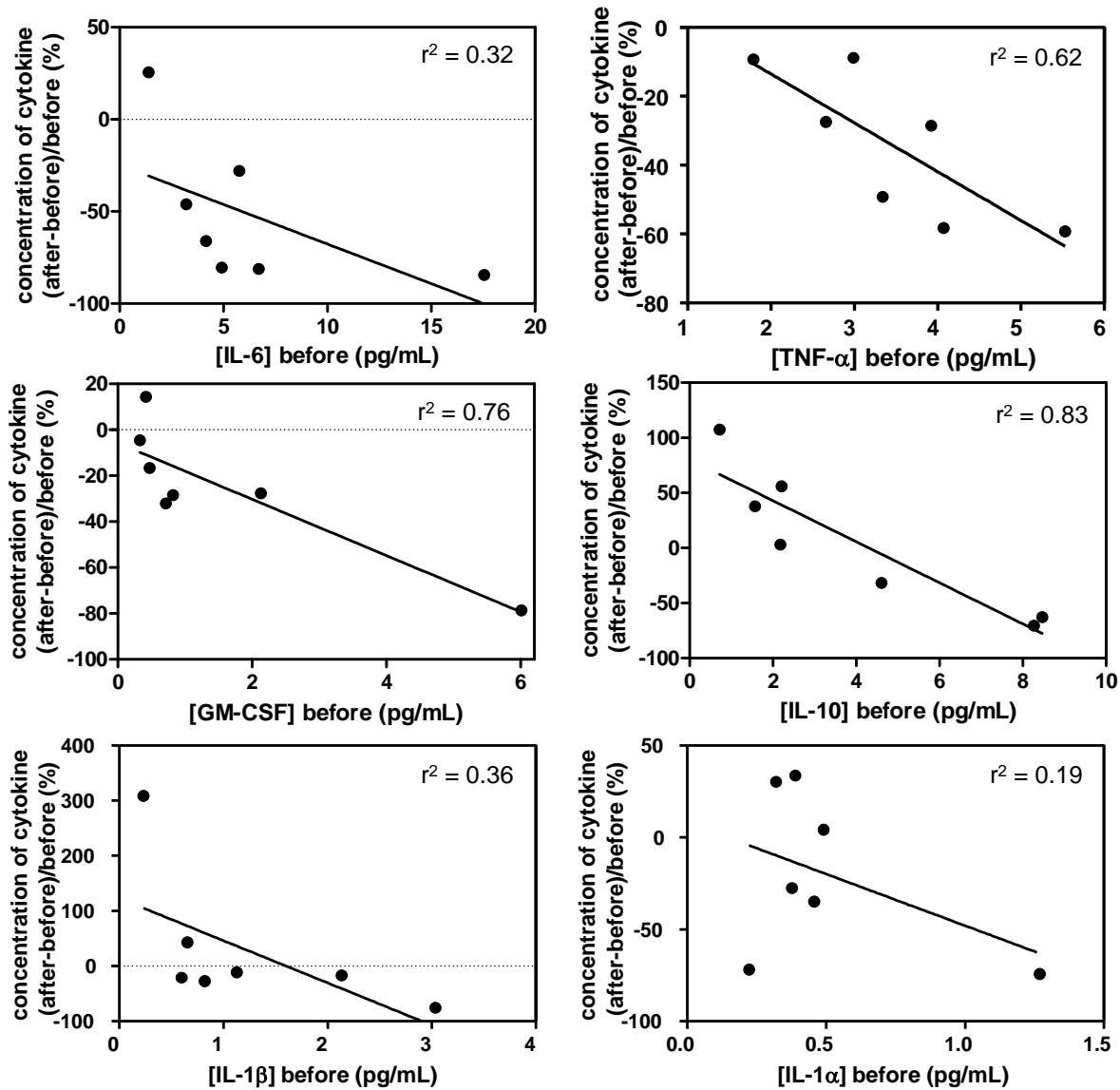
Figure S4. Plots determined using multiplex Simoa of the percentage change in concentration of 6 cytokines in the plasma of CD patients against the concentration of the cytokine before treatment with anti-TNF-α drug. The correlation of each data set is provided.

**Table S1.** AEB, standard deviations and CVs for 6 cytokines spiked into calibrator samples generated during the first of three runs using a 6-plex Simoa assay on the fully-automated system.

[cytokine] (pg/mL)	IL-6			TNF-α			GM-CSF		
	AEB	SD	CV	AEB	SD	CV	AEB	SD	CV
30	10.596	0.340	3.2%	4.493	0.024	5.4%	6.212	0.257	4.1%
10	4.294	0.200	4.7%	1.622	0.087	5.3%	2.478	0.164	6.6%
3	1.397	0.038	2.7%	0.513	0.010	1.9%	0.930	0.028	3.1%
1	0.491	0.014	2.8%	0.192	0.008	4.3%	0.345	0.014	4.0%
0.3	0.160	0.008	5.2%	0.0622	0.0043	6.9%	0.118	0.003	2.7%
0.1	0.0618	0.0086	13.9%	0.0287	0.0013	4.7%	0.061	0.006	9.3%
0	0.0099	0.0015	15.2%	0.0106	0.0019	17.9%	0.021	0.002	9.7%

[cytokine] (pg/mL)	IL-10			IL-1β			IL-1α		
	AEB	SD	CV	AEB	SD	CV	AEB	SD	CV
30	6.668	0.240	3.6%	7.483	0.263	3.5%	9.178	0.296	3.2%
10	2.537	0.109	4.3%	2.742	0.114	4.2%	3.516	0.104	3.0%
3	0.785	0.014	1.7%	0.862	0.040	4.6%	1.124	0.024	2.2%
1	0.305	0.011	3.6%	0.311	0.011	3.4%	0.430	0.022	5.1%
0.3	0.104	0.006	5.6%	0.0942	0.003	3.1%	0.149	0.012	8.3%
0.1	0.0489	0.0022	4.4%	0.0412	0.0037	9.1%	0.0804	0.0039	4.8%
0	0.0213	0.0027	12.8%	0.0122	0.0016	13.3%	0.0387	0.0035	9.0%

Table S2. AEB, standard deviations and CVs for 6 cytokines spiked into calibrator samples generated during the second of three runs using a 6-plex Simoa assay on the fully-automated system.

[cytokine] (pg/mL)	IL-6			TNF-α			GM-CSF		
	AEB	SD	CV	AEB	SD	CV	AEB	SD	CV
30	13.265	0.099	0.7%	4.913	0.040	0.8%	8.043	0.083	1.0%
10	5.260	0.142	2.7%	1.174	0.067	3.9%	3.174	0.052	1.6%
3	1.665	0.159	9.6%	0.562	0.013	2.3%	1.125	0.039	3.4%
1	0.597	0.019	3.1%	0.205	0.009	4.4%	0.404	0.023	5.7%
0.3	0.209	0.014	6.8%	0.072	0.005	7.2%	0.153	0.011	7.0%
0.1	0.0735	0.0075	10.2%	0.0312	0.0010	3.2%	0.0625	0.0022	3.5%
0	0.0127	0.0018	14.1%	0.011	0.0018	16.3%	0.0212	0.0026	12.4%

[cytokine] (pg/mL)	IL-10			IL-1β			IL-1α		
	AEB	SD	CV	AEB	SD	CV	AEB	SD	CV
30	5.727	0.104	1.8%	7.810	0.069	0.9%	8.777	0.180	2.0%
10	2.080	0.065	3.1%	2.785	0.089	3.2%	3.219	0.098	3.0%
3	0.679	0.027	4.0%	0.943	0.024	2.5%	1.108	0.023	2.1%
1	0.251	0.008	3.3%	0.320	0.012	3.7%	0.403	0.012	3.1%
0.3	0.090	0.002	2.3%	0.111	0.004	4.0%	0.156	0.018	11.8%
0.1	0.0434	0.0032	7.3%	0.0425	0.0049	11.6%	0.0750	0.0017	2.3%
0	0.0220	0.0026	11.6%	0.0117	0.0014	12.0%	0.0362	0.0039	10.9%

Table S3. AEB, standard deviations and CVs for 6 cytokines spiked into calibrator samples generated during the third of three runs using a 6-plex Simoa assay on the fully-automated system.

[cytokine] (pg/mL)	IL-6 AEB	IL-6 SD	IL-6 CV	TNF-α AEB	TNF-α SD	TNF-α CV	GM-CSF AEB	GM-CSF SD	GM-CSF CV
30	10.975	0.308	2.8%	4.584	0.058	1.3%	6.796	0.258	3.8%
10	4.132	0.078	1.9%	1.514	0.070	4.6%	2.496	0.147	5.9%
3	1.357	0.085	6.3%	0.552	0.019	3.3%	0.996	0.058	5.8%
1	0.511	0.011	2.2%	0.198	0.012	5.9%	0.384	0.006	1.5%
0.3	0.172	0.008	4.7%	0.070	0.005	6.9%	0.129	0.004	3.4%
0.1	0.0661	0.0047	7.1%	0.0328	0.0004	1.4%	0.0578	0.0019	3.3%
0	0.0131	0.0033	25.3%	0.0140	0.0018	12.9%	0.0227	0.0026	11.5%

[cytokine] (pg/mL)	IL-10 AEB	IL-10 SD	IL-10 CV	IL-1β AEB	IL-1β SD	IL-1β CV	IL-1α AEB	IL-1α SD	IL-1α CV
30	6.217	0.213	3.4%	7.079	0.161	2.3%	7.354	0.149	2.0%
10	2.173	0.083	3.8%	2.388	0.093	3.9%	2.645	0.079	3.0%
3	0.748	0.025	3.3%	0.878	0.031	3.5%	0.961	0.025	2.6%
1	0.266	0.003	1.0%	0.317	0.015	4.8%	0.361	0.005	1.5%
0.3	0.103	0.005	4.6%	0.105	0.006	5.3%	0.145	0.009	6.4%
0.1	0.0499	0.0024	4.8%	0.0435	0.0002	0.4%	0.0816	0.0026	3.2%
0	0.0247	0.0023	9.4%	0.0137	0.0017	12.5%	0.0512	0.0039	7.6%

Table S4. Limit of detection (LOD) values of cytokines in the automated 6-plex Simoa assay.

Cytokine	Day 1 LOD (fg/mL)	Day 2 LOD (fg/mL)	Day 3 LOD (fg/mL)	Average LOD (fg/mL)
IL-6	7.2	7.4	15.7	10.1
TNF-α	26.3	22.3	24.0	24.2
GM-CSF	13.3	15.9	18.6	15.9
IL-10	24.7	29.9	23.2	25.9
IL-1β	14.0	11.3	14.3	13.2
IL-1α	20.9	25.3	31.8	26.0

Table S5. Inter-run precision of AEB values for 6 cytokines over the three runs presented

[cytokine] (pg/mL)	IL-6 CV	TNF-α CV	GM-CSF CV	IL-10 CV	IL-1β CV	IL-1α CV
30	12%	5%	13%	8%	5%	11%
10	13%	6%	15%	11%	8%	14%
3	11%	5%	10%	7%	5%	8%
1	10%	3%	8%	10%	1%	9%
0.3	14%	8%	13%	8%	8%	4%
0.1	9%	7%	4%	7%	3%	4%
0	15%	15%	4%	8%	8%	19%

Table S6. Concentrations (in pg/mL) of plasma samples from patients with Crohn's disease and control healthy subjects. Key: CD before = Crohn's Disease patient before treatment; CD after = Crohn's Disease patient after treatment.

Case	Type	IL-6			TNF-α			GM-CSF			IL-10			IL-1β			IL-1 α		
		Conc	SD	CV	Conc	SD	CV	Conc	SD	CV	Conc	SD	CV	Conc	SD	CV	Conc	SD	CV
#3	CD before	1.40	0.04	2.7%	1.79	0.12	6.8%	0.71	0.08	11.3%	8.27	0.39	4.7%	0.23	0.04	16.8%	1.27	0.11	8.5%
#3	CD after	1.76	0.29	16.3%	1.63	0.35	21.4%	0.48	0.17	34.7%	2.44	0.44	18.0%	0.95	0.22	22.9%	0.33	0.16	48%
#5	CD before	5.76	0.33	5.7%	2.99	0.18	5.9%	0.33	0.06	18.0%	0.72	0.06	8.6%	0.60	0.12	19.4%	0.22	0.02	9.7%
#5	CD after	4.15	0.04	1.0%	2.73	0.12	4.5%	0.31	0.01	3.9%	1.48	0.10	6.8%	0.47	0.01	1.7%	0.06	0.03	51%
#7	CD before	9.72	0.56	5.8%	4.03	0.02	0.5%	0.33	0.09	26.0%	2.16	0.07	3.3%	0.63	0.09	14.2%	0.36	0.06	17%
#8	CD before	12.65	0.42	3.3%	22.28	1.50	6.7%	19.0	0.53	2.8%	67.3	2.4	3.5%	47.2	1.69	3.6%	14.7	0.47	3.2%
#11	CD before	6.70	0.28	4.2%	4.07	0.23	5.8%	0.47	0.03	6.5%	1.57	0.01	0.4%	0.82	0.16	19.4%	0.38	0.06	17%
#11	CD after	1.26	0.17	13.4%	1.70	0.14	8.2%	0.39	0.11	26.7%	2.16	0.36	16.6%	0.60	0.11	19.0%	0.27	0.03	10%
#13	CD before	17.55	0.42	2.4%	2.66	0.11	4.0%	0.82	0.08	9.8%	2.18	0.11	5.2%	0.66	0.05	8.3%	0.32	0.03	9.0%
#13	CD after	2.73	0.12	4.5%	1.93	0.19	9.7%	0.59	0.02	3.0%	2.25	0.09	4.1%	0.94	0.14	14.6%	0.42	0.09	23%
#15	CD before	8.43	0.29	3.4%	5.10	0.31	6.1%	0.94	0.05	5.1%	1.91	0.11	5.6%	0.87	0.04	4.6%	0.49	0.16	34%
#17	CD before	4.92	0.04	0.8%	3.34	0.24	7.3%	6.01	0.44	7.4%	8.47	0.09	1.0%	3.03	0.54	17.9%	0.46	0.22	48%
#17	CD after	0.96	0.07	7.1%	1.70	0.16	9.4%	1.28	0.16	12.2%	3.15	0.29	9.3%	0.74	0.12	15.9%	0.30	0.10	35%
#19	CD before	4.63	0.24	5.1%	8.01	0.40	5.0%	0.65	0.05	7.6%	2.13	0.11	5.2%	0.73	0.05	7.2%	0.70	0.12	17%
#19	CD after	16.53	1.75	10.6%	2.34	0.17	7.4%	1.02	0.13	12.7%	6.10	0.24	3.9%	1.45	0.07	5.1%	1.04	0.06	5.4%
#21	CD before	46.80	1.80	3.8%	5.21	0.13	2.4%	0.42	0.09	22.6%	6.04	0.29	4.7%	0.77	0.00	0.2%	0.22	0.05	23%
#23	CD before	9.94	0.35	3.5%	6.98	0.11	1.6%	1.85	0.15	8.2%	3.45	0.18	5.2%	0.60	0.06	10.3%	0.25	0.08	32%
#25	CD before	4.16	0.01	0.3%	5.53	0.35	6.3%	0.42	0.09	21.8%	4.61	0.42	9.0%	1.13	0.03	3.0%	0.39	0.06	16%
#25	CD after	1.41	0.06	3.9%	2.26	0.31	13.9%	0.48	0.12	24.3%	3.15	0.78	24.7%	1.00	0.13	12.9%	0.52	0.08	16%
#27	CD before	3.21	0.19	5.9%	3.92	0.16	4.1%	2.13	0.23	11.0%	2.21	0.10	4.6%	2.14	0.13	6.2%	0.49	0.08	17%
#27	CD after	1.73	0.09	5.2%	2.80	0.28	10.2%	1.54	0.01	0.5%	3.45	0.18	5.1%	1.77	0.29	16.5%	0.51	0.02	4.2%
#29	CD before	3.45	0.09	2.5%	4.15	0.17	4.0%	0.38	0.03	7.6%	1.53	0.16	10.2%	0.78	0.08	10.8%	0.49	0.07	14%
#31	CD before	5.49	0.10	1.7%	2.00	0.06	3.1%	0.39	0.00	0.2%	0.99	0.12	11.8%	0.77	0.06	7.3%	0.22	0.05	25%
#35	CD before	7.82	0.43	5.5%	2.77	0.04	1.4%	0.58	0.04	6.2%	2.61	0.13	4.9%	0.86	0.06	6.8%	1.38	0.21	16%
#4	Control	1.01	0.08	7.7%	3.64	0.22	6.1%	0.29	0.04	13.2%	1.57	0.08	5.3%	1.14	0.09	7.5%	0.33	0.02	5.1%
#6	Control	2.53	0.14	5.7%	5.76	0.06	1.0%	2.23	0.03	1.1%	7.11	0.16	2.2%	2.51	0.14	5.4%	3.45	0.20	5.7%
#9	Control	5.33	0.21	3.9%	3.81	0.14	3.7%	0.43	0.05	12.2%	3.46	0.13	3.6%	0.61	0.04	6.5%	0.29	0.01	2.8%
#10	Control	1.45	0.06	3.9%	3.47	0.32	9.2%	0.26	0.01	3.9%	1.93	0.20	10.2%	0.79	0.04	4.4%	0.27	0.08	28.6%
#12	Control	5.53	0.13	2.4%	5.53	0.11	2.0%	0.43	0.07	16.6%	5.45	0.24	4.4%	1.37	0.02	1.2%	1.77	0.02	0.9%
#14	Control	1.64	0.10	6.1%	3.80	0.04	1.0%	0.47	0.05	10.5%	2.90	0.08	2.6%	0.61	0.05	8.7%	0.46	0.24	53%
#16	Control	1.96	0.13	6.5%	2.94	0.08	2.7%	0.81	0.04	4.6%	2.72	0.08	2.9%	1.94	0.06	2.9%	2.06	0.58	28%
#18	Control	6.52	0.29	4.5%	8.34	0.40	4.8%	3.75	0.18	4.9%	9.46	0.10	1.0%	4.74	0.35	7.3%	5.21	0.41	7.9%
#20	Control	1.33	0.02	1.5%	3.09	0.01	0.5%	0.19	0.12	60.7%	5.59	0.15	2.7%	0.59	0.22	36%	0.40	0.00	0.7%
#22	Control	0.81	0.26	31.8%	2.40	0.55	22.8%	0.39	0.10	24.6%	3.33	0.18	5.3%	0.32	0.10	33%	0.17	0.07	41%
#24	Control	3.15	0.04	1.1%	6.95	0.57	8.3%	2.58	0.04	1.6%	14.4	1.7	12.1%	4.96	0.28	5.7%	3.89	0.82	21%
#26	Control	2.94	0.19	6.5%	9.45	0.59	6.3%	7.31	0.71	9.7%	7.46	0.52	7.0%	9.38	0.77	8.2%	7.09	0.37	5.2%
#28	Control	3.64	0.13	3.5%	5.59	1.77	31.6%	2.62	1.01	38.4%	14.5	2.7	18.7%	2.06	0.86	42%	0.27	0.06	24%
#30	Control	2.17	0.10	4.8%	2.97	0.28	9.3%	0.33	0.05	16.6%	3.08	1.24	40.4%	0.38	0.00	1.0%	0.32	0.17	53%
#32	Control	5.35	0.36	6.7%	1.86	0.25	13.7%	0.36	0.04	9.7%	2.80	0.38	13.7%	0.56	0.14	25%	0.57	0.14	25%
#36	Control	2.72	0.05	1.7%	3.72	0.28	7.6%	1.36	0.09	6.9%	8.90	1.36	15.3%	2.21	0.23	10.5%	2.07	0.31	15%

Table S7. Mean concentration (± SEM) of 6 cytokines in plasma of CD patients and matched healthy controls. Key: s = means are significantly different; ns = means are not significantly different

Cytokine	CD Patients (pg/mL)	Healthy Controls (pg/mL)	P value of t-test
IL-6	9.5±2.7	3.0±0.4	0.022; s
TNF-α	5.3±1.2	4.6±0.5	0.59; ns
GM-CSF	2.2±1.2	1.5±0.5	0.57; ns
IL-10	7.3±4.1	5.9±1.0	0.75; ns
IL-1β	3.9±2.9	2.1±0.6	0.56; ns
IL-1α	1.4±0.9	1.8±0.5	0.71; ns

Table S8. Summary of percentage changes in cytokine concentrations in the plasma of CD patients before and after treatment with anti-TNF-α drugs, and mean concentrations before and after treatment.

Case	IL-6		TNF-α		GM-CSF	IL-10	IL-1β	IL-1α
	Multiplex	Singleplex[b]	Multiplex	Singleplex[b]	Multiplex	Multiplex	Multiplex	Multiplex
#3	+26%	+20%	−9.3%	−32%	−32%	−71%	+309%	−74%
#5	−28%	−32%	−8.8%	−31%	−4.6%	+107%	−21%	−72%
#11	−81%	−81%	−58%	−43%	−17%	+38%	−27%	−28%
#13	−84%	−89%	−27%	−47%	−28%	+3%	+43%	+30%
#17	−81%	−81%	−49%	−73%	−79%	−63%	−76%	−35%
#19[a]	*+257%*	*+200%*	*−71%*	*−58%*	*+56%*	*+187%*	*+99%*	*+49%*
#25	−66%	−71%	−59%	−36%	+14%	−32%	−11%	+34%
#27	−46%	−48%	−29%	−43%	−28%	+56%	−17%	+4.2%
Mean % change	−52%	−55%	−34%	−43%	−25%	+6%	+29%	−20%
Mean conc. before (pg/mL)	6.2	7.3	3.5	3.2	1.6	4.0	1.2	0.50
Mean conc. after (pg/mL)	2.0	2.1	2.1	1.8	0.73	2.6	0.93	0.34

[a] Case #19 was excluded from mean calculations for all cytokines as this patient had previously been treated with anti-TNF-α, contrary to the original study design. This patient did not respond to either drug, as described in ref. 7.
[b] Data taken from ref.7. Case #1 was not retested in this study as sufficient volume of plasma was not available.

Table S9. Mean concentration (± SEM) of 6 cytokines in the serum of patients with Type 1 diabetes and healthy controls. Key: s = means are significantly different; ns = means are not significantly different

Cytokine	Type 1 diabetics (pg/mL)	Healthy Controls (pg/mL)	P value of t-test
IL-6	4.2±0.8	3.3±0.6	0.29; ns
TNF-α	5.9±1.4	2.0±0.1	0.001; s
GM-CSF	1.8±0.7	0.34±0.0.05	0.009; s
IL-10	12.6±2.4	4.8±1.4	0.007; s
IL-1β	2.4±1.2	0.20±0.05	0.020; s
IL-1α	3.0±1.6	0.72±0.30	0.075; ns