

Supplemental Material; Chemokines IP-10/CXCL10 and IL-8/CXCL8 are novel biomarkers of warm autoimmune hemolytic anemia.

Supplementary Methods

Cytokine analysis

Thirty-eight cytokines were analyzed in thawed plasma samples using Luminex® technology with a MILLIPLEX MAP Human Cytokine Magnetic Bead Panel (EMD Millipore) using the HCYTMAG-60K-PX38 kit. Cytokines and chemokines examined included: IL-1RA, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFN α 2 (interferon- α 2) and IFN- γ , IP-10 (interferon- γ -inducible protein 10; CXCL10), TNF- α (tumour necrosis factor- α), TNF- β (lymphotoxin), TGF- α (transforming growth factor- α), EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), FGF (basic fibroblast growth factor), G-CSF (granulocyte-colony stimulating factor), GM-CSF (granulocyte-macrophage colony stimulating factor), MCP-1(monocyte chemo attractant protein;CCL2), MCP-3 (CCL7), MDC (CCL22), MIP-1 α (macrophage inflammatory protein;CCL3), MIP-1 β (CCL4), and Eotaxin (CCL11), Flt3L (fms-like tyrosine 3 kinase ligand), Fractalkine (CX3CL1), GRO (CXCL1), sDC40L (soluble CD40-ligand).

Sample handling

EDTA whole blood was stored by the American Red Cross (ARC) at 4°C and sample remaining after their confirmational testing was shipped on ice packs to Canadian Blood Services in Toronto within 48-72 hours of being received by ARC. Upon arrival in Toronto, samples were kept at 4°C until plasma was separated within 48 hours and stored at -80°C until tested. Total time that EDTA blood was stored at 4°C before plasma was separated and frozen was less than 6 days. For analysis, plasma samples were thawed on ice and centrifuged briefly to pellet any insoluble material.

Statistical analysis

Concentrations of cytokines in plasma samples were determined from standard curves generated for each analyte using five-parameter logistic (5-PL) regression. Five-fold dilutions of cytokine standards giving a range of 10,000–3.2 pg./mL were used to generate the curves. Values extrapolated from the logistic curves were used 'as-is'. Values that were flagged as 'low, out of range; <OOR) were assigned the lowest value detected for that cytokine. Overall detection was excellent: data was returned from over 80% of queries. Most out-of-range-low data arose from 3 cytokines; IL-4 (70% OOR), Flt-3L (63% OOR), IL-13 (53% OOR).

Unless otherwise noted, statistical analyses were done using SAS. All cytokines tested were found to be non-normally distributed using the Kolmogorov-Smirnov test, so were \log_{10} -transformed to reduce skew (Olivier, 2008). Where transformed cytokine distribution was log-normal, Student's t-test was used to compare cytokine concentrations between healthy controls and patients, otherwise the non-parametric Mann Whitney U test was used. Using these methods, we found 15 cytokines differed significantly ($p < 0.05$): elevation of IL-10, IL12p70, IL-1Ra, IL-6, TNF α , IL-8/CXCL8, IP-10/CXCL10, GRO/CXCL1, MCP-1/CCL2, and decrease in IFN α 2, IL-3, IL-13, TGF α , fractalkine/CX3CL1, MDC/CCL22. As discussed in the Letter, to focus on changes related to disease state and not sample storage, we reduced this number to 7 cytokines previously identified as stable in whole blood (Binnington, 2020). Figure 1 in the Letter was prepared using GraphPad Prism v9.3.1. Significance bars above the data show p-values for significant differences between HC and wAIHA, transfused-wAIHA, or non-transfused wAIHA.

For multivariable logistic regression model analyses, the \log_{10} -transformed data was used, while Spearman's rank correlation analysis was performed using z-score normalization of the \log_{10} -transformed data; $(x-\bar{x})/SD$. Normalization places the data from different cytokines on a common scale, to eliminate over-weighting cytokines with larger values for example. Spearman's rank correlation was used because all of the 7 cytokines were not log-normally distributed, and because Spearman's is more resistant to the effect of outliers, which are common in observed cytokine levels.

Supplementary Results

Table S1: Clinical and laboratory characteristics of wAIHA patients

Patient ID	DAT		Hb (g/dL)	Transfusion history*	Diagnosis
	IgG	C3			
1	4+	4+	12.4	no	osteoarthritis
2	4+	micro	4.5	no	hemolytic anemia
3	3+	0	8.3	yes	anemia
4	3+	0	n/a	yes	n/a
5	4+	0	n/a	no	n/a
6	3+	0	7	yes	anemia, leukemia
7	3+	4+	11.7	no	intracranial hemorrhage
8	1+	0	8.4	yes	thrombocytopenia
9	2+	3+	n/a	yes	anemia, lymphoma
10	4+	1+	n/a	yes	anemia, chronic kidney disease
11	1+	1+	6	yes	anemia, MDS
12	4+	3+	7	no	vaginal bleeding
13	4+	0	6.6	no	anemia
14	3+	0	7.2	yes	anemia, liver failure
15	3+	3+	6	yes	hemolytic anemia
16	4+	4+	5.8	no	anemia
17	3+	3+	9.1	yes	CLL
18	3+	2+	12.1	no	pregnancy
19	4+	micro	n/a	no	CLL
20	4+	2+	10	yes	AIHA
21	3+	0	7.4	yes	anemia, prostate cancer
22	3+	4+	10.6	no	left side mass
23	3+	4+	n/a	yes	thalassemia
24	3+	4+	7.9	no	anemia
25	4+	3+	8.6	no	anemia, CLL
26	4+	0	8	yes	ALL
27	3+	0	15.1	yes	spinal injury
28	4+	0	9.1	no	n/a
29	2+	2+	8.9	n/a	n/a
30	4+	0	4.88	yes	anemia
31	3+	4+	3.7	n/a	anemia
32	4+	4+	7	no	anemia, chest pain, renal insufficiency
33	4+	0	8.3	no	AIHA
34	4+	0	5.2	n/a	pancytopenia
35	4+	2+	n/a	yes	n/a
36	2+	w+	11.2	no	anemia
37	4+	4+	6.1	no	n/a
38	4+	3+	8.7	yes	n/a
39	4+	2+	7.4	no	AIHA
40	3+	0	11.4	n/a	fall
41	4+	2+	7.9	yes	anemia
42	3+	micro	7.1	yes	n/a
43	4+	0	6.7	yes	anemia, elevated liver enzymes
44	4+	4+	5.5	no	anemia
45	2+	0	5.5	no	anemia and lower GI hemolysis
46	4+	4+	6.6	no	metastatic lung cancer
47	4+	1+	n/a	yes	n/a
48	4+	3+	10.5	n/a	brain neoplasm
49	4+	1+	5.8	n/a	anemia
50	4+	4+	7.6	no	acute leukemia
51	2+	1+	n/a	yes	n/a
52	4+	0	8.8	no	pulmonary nodular amyloidosis
53	4+	0	8.6	yes	anemia
54	4+	4+	7.5	no	hip fracture

DAT: direct antiglobulin test; CLL: chronic lymphocytic leukemia; ALL: acute lymphoma leukemia; AIHA: autoimmune hemolytic anemia.

*Transfusion history within last 3 months; n/a: data not made available

Table S2: Summary statistics of raw cytokine data

Cytokine	wAIHA (n=54)		HC (n=36)		P-value*
	Mean (SD)	Median	Mean (SD)	Median	
EGF	75.7 (153.0)	36.4	94.5 (88.9)	55.4	0.0868 ^a
Eotaxin	117.5 (69.2)	112.6	89.7 (37.7)	80.2	0.535 ^a
FGF_2	200.6 (497.2)	67.7	139.4 (119.5)	71.5	0.161 ^b
Flt_3L	48.0 (116.1)	1.9	12.3 (13.1)	3.1	0.604 ^b
Fractalkine	836.3 (5779.8)	29.6	66.7 (53.0)	42.3	0.010 ^b
G_CSF	189.6 (892.6)	52.9	60.2 (57.2)	36.6	0.341 ^a
GM_CSF	14.7 (20.3)	8.3	18.4 (18.8)	11.6	0.218 ^a
GRO	1869.8 (1566.0)	1067.1	1392.4 (1460.4)	690.3	0.040 ^b
IFN α 2	45.1 (40.5)	32.8	69.2 (59.7)	54.4	0.041 ^a
IFN γ	15.4 (23.6)	7.4	20.7 (25.0)	6.8	0.143 ^a
IL_10	214.4 (1035.9)	16.1	12.6 (21.6)	3.6	0.000 ^a
IL_12p40	17.6 (19.3)	13.2	23.2 (29.1)	10.8	0.888 ^b
IL_12p70	7.4 (12.3)	3.6	10.9 (11.2)	6	0.010 ^a
IL_13	20.5 (32.3)	1.3	53.4 (77.6)	3.6	0.014 ^b
IL_15	4.8 (3.8)	3.4	4.2 (4.0)	2.5	0.175 ^a
IL_17	4.7 (10.2)	1.8	5.0 (6.2)	2.5	0.132 ^a
IL_1a	50.5 (60.1)	27.6	109.1 (113.7)	65.9	0.055 ^b
IL_1b	4.9 (11.3)	1.4	3.3 (2.7)	2.4	0.203 ^a
IL_1RA	220.7 (448.3)	67.7	88.4 (110.4)	32	0.044 ^a
IL_2	2.7 (7.0)	0.6	1.9 (2.5)	0.9	0.195 ^b
IL_3	0.8 (0.8)	0.6	1.6 (1.3)	1.1	0.005 ^a
IL_4	5.5 (9.0)	0.9	9.0 (12.8)	1	0.903 ^b
IL_5	3.8 (5.2)	1.2	11.7 (21.9)	2.5	0.177 ^b
IL_6	15.1 (24.1)	5.5	5.2 (6.9)	1.5	0.008 ^b
IL_7	7.0 (7.6)	5	9.6 (7.4)	8.2	0.053 ^b
IL_8	22.7 (50.6)	9.9	9.7 (11.6)	3.3	0.000 ^a
IL_9	1.7 (2.1)	0.8	2.6 (2.7)	1.3	0.101 ^a
IP_10	1929.1 (4447.6)	745.7	295.4 (107.2)	265.8	0.000 ^b
MCP_1	830.4 (1863.6)	321.2	206.0 (92.0)	206.7	0.000 ^b
MCP_3	61.2 (80.0)	18.7	98.1 (121.8)	32.4	0.743 ^b
MDC	621.5 (1302.1)	321.5	532.3 (199.7)	524.8	0.002 ^b
MIP_1 α	8.5 (7.4)	6.4	9.0 (7.5)	6.2	0.881 ^a
MIP_1 β	47.3 (33.2)	42.1	33.9 (20.7)	33.5	0.060 ^b
sCD40L	1670.5 (2496.5)	589.1	1156.6 (1270.4)	704.3	0.824 ^b
TGF α	5.6 (7.0)	3.8	3.9 (5.1)	1.5	0.025 ^b
TNF α	32.6 (41.9)	17.1	7.7 (5.1)	5.7	0.000 ^a
TNF β	38.7 (64.3)	2.2	80.9 (110.5)	3.7	0.020 ^b
VEGF	310.6 (318.2)	205.1	332.8 (229.2)	279.5	0.199 ^a

* P-value shown if for comparison of log-transformed data for HC vs wAIHA

^a cytokine is log-normal/P-value from t-test

^b cytokine is not log-normal; P-value from Wilcoxon rank-sum test

Parameter	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
IL-10	-0.1700	0.9009	0.0356	0.85
IL-6	0.00680	0.5649	0.0001	0.99

Parameter	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
IL-8/CXCL8	-2.0497	0.9754	4.4161	0.036
IP-10/CXCL10	-3.6554	2.0183	3.2802	0.070
MDC/CCL22	6.9310	2.3850	8.4455	0.0037
MCP-1/CCL2	4.0698	1.6008	6.4639	0.011
TNF α	-11.0948	3.4797	10.1663	0.0014

Figure S1 Logistic Regression Model Analysis

We used multivariable logistic regression analysis to identify which of the 7 significant cytokines best contributed to a biomarker model distinguishing wAIHA patients from healthy controls. TNF α , MCP-1/CCL2, MDC/CCL22 and IL-8/CXCL8 contributed to the model. IP-10/CXCL10 did not achieve significance ($p=0.07$) which we interpret as due to the strong correlation to TNF α (addition of IP-10 does not add significantly to the logistic regression model).

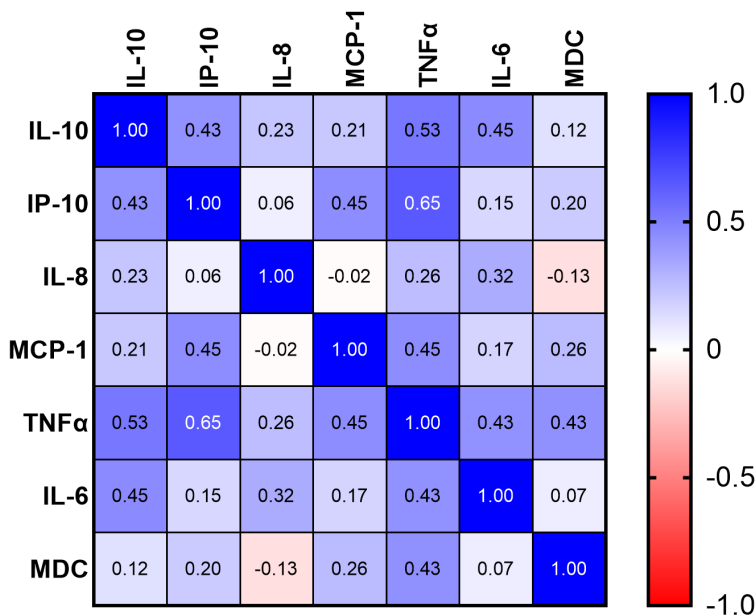


Figure S2 Correlation analysis of significant cytokines

Spearman's correlation coefficients (r_s) shown in the above heat-map were calculated from Z-score normalized data using GraphPad Prism v9.3.1. Strong positive correlation was found between IP-10/CXCL10 and TNF α ($r_s = 0.65$, confidence interval 0.45-0.78).

Supplemental References

Olivier, J., Johnson W.D., Marshall, G.D. 2008. The logarithmic transformation and the geometric mean in reporting experimental IgE results: what are they and when and why to use them? *Ann Allergy Asthma Immunol.* 2008; 100(4);333-7

Binnington B, Sakac D, Yi Q, et al. Stability of 40 cytokines/chemokines in chronically ill patients under different storage conditions. *Cytokine.* 2020 Mar 14;130:155057.