Profound systemic alteration of the immune phenotype and an immunoglobulin switch in Erdheim-Chester disease in 78 patients from a single center

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SUPPLEMENTARY MATERIALS

Profound systemic alteration of the immune phenotype and an immunoglobulin switch in Erdheim–Chester disease in a single-center of 78 patients

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Running title: Alteration of the systemic immune cell phenotype in ECD.

Methods.

Analysis of blood immune cells by flow cytometry.

A 100- or 300-µl aliquot of fresh blood samples was used for immunostaining of monocytes, lymphocytes, or dendritic cells (DCs), respectively. Samples were blocked with 200 µl of 1/400 diluted Fc Blocking reagent (Miltenyi) and then incubated with corresponding antibodies for 30 min at 4°C, in the dark. If necessary, 50 µl of 1/200 diluted streptavidin PE Texas Red (BD Biosciences) was added and samples were incubated for a further 15 min at 4°C in the dark (final dilution 1/1400). Then, red blood cells were lysed and leukocytes were fixed with 700 µl (for lymphocytes and monocytes) or 1300 µl (for DCs) of Versafix solution (Beckman Coulter), according to the manufacturer's instructions. Distinctions among lymphocyte subsets were based on different expression patterns of surface markers, as previously described [11]: T helper cells ($CD45^+$, $CD3^+$, $CD4^+$, $CD8^-$, $CD25^-$, $CD127^+$), T regulatory cells (CD25+, $CD127^-$), cytotoxic lymphocytes ($CD45^+$, $CD3^+$, $CD4^-$, $CD8^+$), and B lymphocytes ($HLA\text{-}DR^+$, $CD19^+$). Monocyte subsets were distinguished as classical ($CD14^{++}/CD16^-$), intermediate ($CD14^{++}/CD16^+$), and nonclassical ($CD14^+/CD16^{++}$) monocytes. DC subsets were identified according to their plasmacytoid ($CD11c^-$, $CD123^+$, $BDCA2^+$(CD303)), myeloid 1 ($CD11c^+$, $BDCA1^+(CD1c^+)$, $BDCA3^-$ ($CD141^-$)), or myeloid 2 lineages ($CD11c^+$, $BDCA1^-(CD1c^-)$, $BDCA3^+(CD141^+)$). Samples were run on an LSR II FORTESSA SORP (BD Biosciences) and the results were analyzed using FACSDIVA software (BDBiosciences). Absolute quantification of leukocytes was assessed using the TRUCOUNT method (BDBiosciences).

Quantification of circulating chemokines and cytokines. Plasma was isolated from fresh blood samples collected in EDTA tubes, following centrifugation for 20 min at 3000 rpm at 4°C; the plasma samples were then immediately stored at -80°C. Circulating concentrations of cytokines and chemokines were quantified from 25-µl non-diluted aliquots of the plasma samples using a Milliplex 29-plex human cytokine/chemokine magnetic bead panel (Millipore) and a Luminex® analyzer (MAGPIX), according to the manufacturer's instructions.

Measurement of circulating immunoglobulins. Circulating concentrations of immunoglobulins were quantified from plasma samples (50-µl 1/16,000 diluted samples) using a Milliplex human immunoglobulin (IgA, IgM, IgG1, IgG2, IgG3, and IgG4) isotyping magnetic bead panel (Millipore) and a Luminex® analyzer (MAGPIX), according to the manufacturer's instructions. Plasma samples from healthy individuals were included as controls.

			n	Age (years)		n	Age (years)
Controls			17	53±25 (21-90)	Males Females	13 4	52±27 (21-90) 56±19 (28-70)
ECD		**All**	78	60±14 (18-84)	Males Females	60 18	61±13 (18-84) 58±17 (26-83)
	***BRAF*^{V600E} mutation**	**WT**	21	63±14 (30-79)	Males Females	15 3	65±12 (30-79) 51±18 (37-71)
		V600E	50	61±14 (18-84)	Males Females	37 13	60±14 (18-84) 62±15 (32-83)

Supplemental Table 1. Distribution of controls and ECD patients according to age and gender. Values are expressed as mean±S.D (range). WT and V600E, absence and presence of the *BRAF*^{V600E} mutation.

Pathway	Circulating biomolecules (pg/mL)	Untreated ECD			Treated ECD (V600E)		
			BRAF^{V600E} mutation				
		All (n=34)	WT (n=9)	V600E (n=21)	PegIFNα (n=16)	Vemurafenib (n=11)	All (n=27)
Th1	IL-1a	0.00 (0.00-30.04)	13.34 (0.00-30.34)	0.00 (0.00-35.88)	17.62 (1.34-53.51)	8.30 (0.00-53.53)	13.34 (0.00-53.53)
	IL-1β	1.38 (0.66-3.42)	0.97 (0.55-2.94)	1.38 (0.38-3.52)	1.37 (0.61-2.68)	1.58 (0.97-3.33)	1.58 (0.77-2.85)
	IL-6	0.00 (0.00-18.01)	0.00 (0.00-0.00)	0.00 (0.00-20.06)	0.00 (0.00-31.57)	8.71 (0.00-32.49)	8.27 (0.00-32.49)
	IL-7	12.15 (3.38-20.58)	5.91 (0.00-17.91)	7.95 (0.00-21.14)	10.57 (0.00-18.03)	0.00 (0.00-21.70)	9.75 (0.00-18.82)
	IL-12p40	2.28 (0.00-20.47)	0.00 (0.00-22.50)	10.00 (0.00-26.38)	9.98 (0.00-17.99)	13.66 (0.00-15.99)	11.24 (0.00-17.14)
	IL-12p70	4.48 (1.10-7.72)	4.48 (2.32-5.13)	4.48 (0.55-8.36)	4.15 (2.49-7.64)	7.08 (3.16-9.65)	6.43 (2.49-8.69)
	IL-15	5.07 (1.95-10.49)	6.41 (1.40-8.92)	4.88 (2.47-10.66)	**9.02 (5.25-12.93)**†	4.88 (0.00-13.38)	8.28 (3.70-13.38)
	IFNα2	53.67 (39.99-99.12)	57.63 (34.08-97.77)	53.67 (38.88-101.5)	**299.7 (122.6-466.8)**†††	49.61 (36.64-103.2)	**118.9 (53.67-330.7)**†
	IFNγ	6.11 (3.59-11.48)	6.40 (4.57-10.63)	5.83 (3.59-11.77)	6.11 (3.94-10.77)	9.78 (5.27-17.44)	6.68 (4.15-12.90)
	TNFα	36.27 (21.28-65.88)	21.28 (17.56-47.90)	44.33 (24.90-77.92)	53.89 (38.94-68.99)	38.73 (27.70-49.27)	49.27 (33.79-65.13)
Th2	IL-1ra	38.83 (21.54-74.85)	40.35 (10.97-71.35)	32.89 (20.83-48.37)	40.35 (27.03-88.54)	97.57 (24.33-157.2)	43.34 (26.36-121.4)
	IL-4	3.01 (0.00-17.07)	8.33 (0.00-20.96)	3.01 (0.00-24.09)	10.76 (3.70-21.15)	8.33 (0.00-26.59)	8.33 (0.00-22.26)
	IL-5	0.82 (0.04-1.75)	0.88 (0.15-1.88)	0.88 (0.09-1.62)	0.88 (0.34-1.60)	0.88 (0.06-1.83)	0.88 (0.31-1.73)
	IL-10	8.80 (4.67-18.63)	7.28 (3.91-16.12)	9.31 (5.08-18.24)	**18.07 (12.94-29.47)**††	7.28 (4.92-17.54)	16.68 (7.28-24.17)
Th17	IL-17a	2.94 (1.12-5.95)	3.27 (1.55-5.55)	2.94 (1.02-7.75)	2.25 (0.64-4.58)	3.93 (1.92-9.29)	2.94 (1.21-6.50)
CHEMOKINES	CCL2	678.9 (444.7-864.1)	438.8 (362.9-765.5)	692.8 (482.2-876.0)	**924.3 (764.4-1189)**†	**467.5 (393.1-559.9)**†	760.7 (467.5-1026)
	CCL22	867.6 (603.7-1257)	683.7 (503.2-958.1)	975.3 (664.0-1397)	**644.6 (565.6-815.1)**†	1047 (694.3-1438)	748.3 (606.5-1047)
	Eotaxin	111.2 (87.06-156.3)	130.8 (102.5-185.8)	101.5 (78.48-135.2)	114.3 (95.34-156.5)	103.6 (82.87-177.1)	111.0 (88.70-158.9)
	Fractalkine	46.04 (15.85-62.62)	51.18 (20.71-71.14)	42.91 (15.85-59.54)	33.90 (26.67-50.68)	38.52 (17.96-45.73)	35.48 (24.68-48.49)
	GRO	1623 (879.5-1896)	1601 (448.0-1813)	1646 (1066-1934)	1474 (720.4-1610)	1712 (1226-2107)	1536 (787.9-1753)
	IL-8	7.88 (4.52-12.80)	6.40 (2.64-12.35)	8.67 (5.39-13.07)	10.73 (6.99-14.96)	10.72 (6.67-24.35)	10.72 (6.71-15.67)
	IP-10	877.0 (488.7-1244)	497.7 (449.1-721.7)	993.0 (522.9-1321)	**1529 (1083-1860)**†	1094 (689.6-1791))	1373 (787.5-1836)
	MCP-3	11.89 (0.00-53.18)	3.36 (0.00-56.24)	13.08 (2.30-53.38)	21.85 (7.80-84.57)	18.79 (0.00-70.61)	18.79 (5.73-70.79)
	MIP-1α	4.02 (0.01-5.73)	1.22 (0.00-4.75)	4.15 (0.75-6.06)	3.96 (2.48-5.34)	4.64 (2.36-7.23)	4.40 (2.36-5.47)
	MIP-1β	26.41 (19.08-31.87)	26.87 (16.06-28.10)	25.48 (19.07-32.62)	26.30 (21.64-31.36)	24.77 (22.04-32.03)	26.18 (22.04-31.83)
HEMATOPOIESIS	GCSF	31.31 (7.11-54.23)	31.31 (20.2-43.44)	31.31 (3.68-69.45)	**70.30 (57.12-85.04)**†	77.34 (55.08-136.6)	**77.34 (56.71-117.9)**†
	GM-CSF	8.79 (3.64-15.87)	6.70 (4.40-18.55)	9.37 (4.78-14.72)	9.75 (6.70-16.44)	11.28 (4.41-12.81)	9.75 (6.70-12.81)
	IL-2	0.81 (0.22-2.60)	0.81 (0.11-3.05)	0.81 (0.32-2.56)	1.00 (0.27-2.02)	1.19 -0.42-2.11)	1.19 (0.42-2.11)
	IL-3	2.07 (1.23-3.40)	1.90 (1.43-4.08)	2.11 (1.16-3.45)	2.45 (1.26-2.98)	1.90 (1.23-3.70)	2.04 (1.23-3.00)
GROWTH FACTORS	EGF	43.55 (15.66-67.88)	49.53 (18.04-87.72)	41.66 (15.02-54.28)	32.64 (19.74-72.13)	37.13 (28.86-55.61)	35.01 (23.57-55.61)
	VEGF	43.24 (12.33-98.03)	44.52 (9.33-105.0)	64.49 (12.33-95.68)	47.05 (28.92-73.01)	71.80 (28.92-98.03)	57.08 (28.92-83.83)

Supplemental Table 2. Circulating cytokines and chemokines concentrations according to the *BRAF*^{V600E} mutation. †p<0.05, ††p<0.005 and †††p<0.005 versus untreated BRAF mutated ECD patients. Expressed in median (Quartile 1- Quartile 3). Samples with nondetectable biomolecule concentrations were considered to be 0.0 pg/mL. WT and V600E, absence and presence of the *BRAF*^{V600E} mutation.

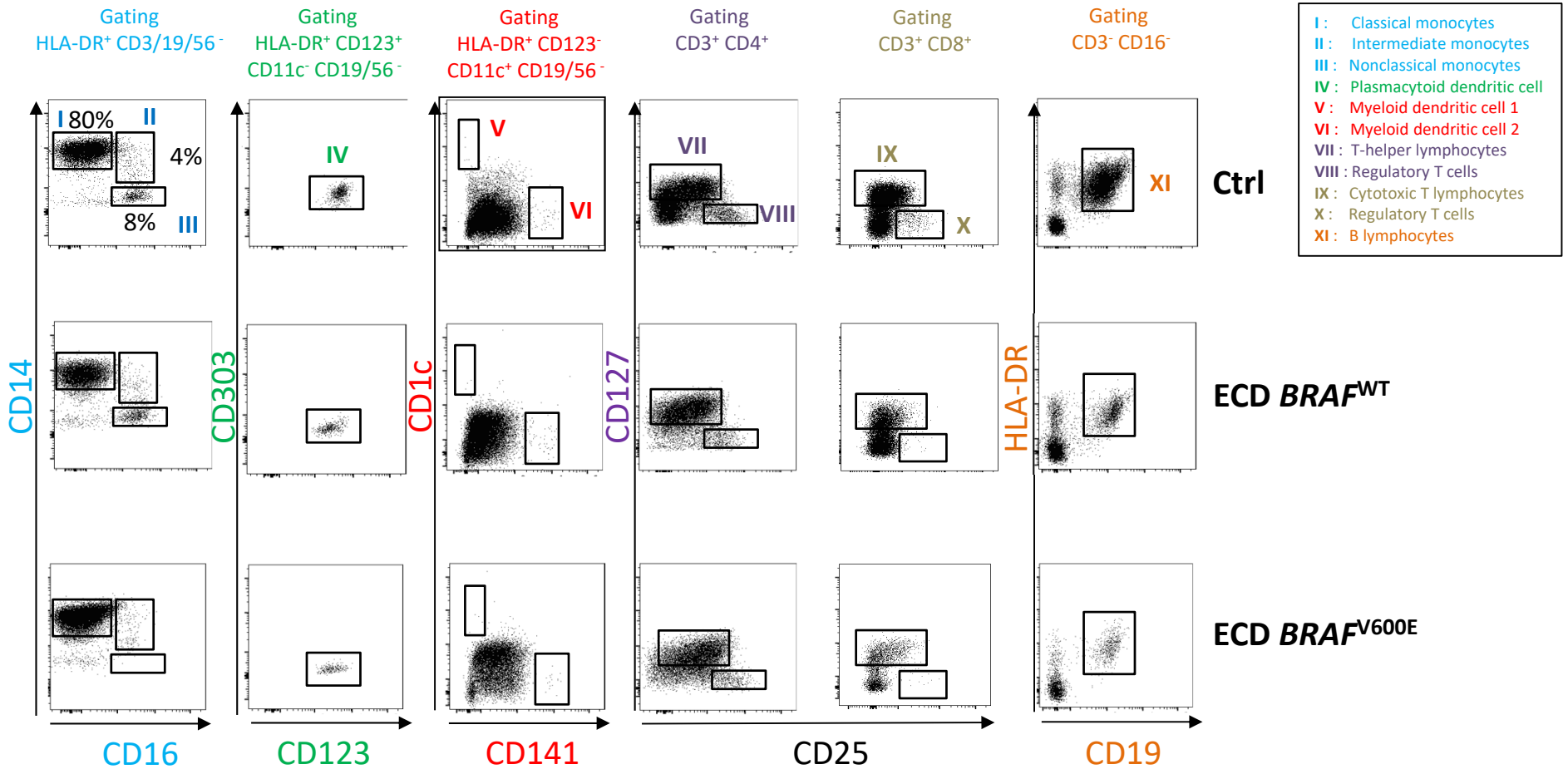
Pathway		Therapy	Monocytes				Dendritic cells			Neutrophils	Lymphocytes					
			Total	$CD14^{++}CD16^{-}$	$CD14^{++}CD16^{+}$	$CD14^{+}CD16^{++}$	pDC	mDC1	mDC2		NK	NKT	Treg	B	CT	Th
Th1	IL-1a		0.10	0.05	-0.10	-0.03	-0.06	-0.05	-0.02	-0.10	-0.13	-0.01	-0.06	-0.02	0.00	-0.05
	IL-1β		-0.01	-0.01	-0.14	-0.13	0.00	0.00	-0.06	-0.05	-0.11	0.04	-0.03	-0.04	-0.02	-0.09
	IL-6		0.02	0.00	0.09	**0.30***	-0.15	-0.07	0.08	-0.16	-0.18	-0.04	-0.01	-0.18	-0.07	-0.11
	IL-7		0.04	0.01	-0.03	-0.05	-0.15	-0.09	0.02	-0.09	-0.19	0.03	-0.12	0.01	-0.09	-0.15
	IL-12p40		0.14	0.12	-0.12	-0.05	0.13	0.06	-0.01	0.02	-0.04	0.03	0.08	0.05	0.13	0.02
	IL-12p70		-0.03	-0.06	0.05	-0.09	-0.12	-0.09	-0.07	-0.15	-0.20	0.03	-0.16	-0.02	-0.11	-0.14
	IL-15	P↑	-0.01	-0.02	-0.06	0.05	-0.07	-0.10	-0.06	-0.11	-0.21	-0.05	-0.04	0.00	-0.08	-0.11
	IFNα2	P↑	-0.03	-0.05	0.06	**0.31****	-0.13	-0.07	-0.06	-0.07	-0.07	-0.11	-0.06	0.03	-0.03	-0.14
	IFNγ		-0.03	-0.04	-0.01	0.10	-0.02	0.09	0.02	-0.08	-0.12	0.04	-0.06	0.00	-0.04	-0.05
	TNFα		-0.03	0.00	-0.06	0.02	-0.04	**0.3951*****	0.20	0.11	0.01	0.02	-0.02	-0.14	-0.03	-0.18
Th2	IL-1ra		0.06	0.11	-0.01	0.08	-0.06	-0.04	0.00	-0.09	-0.11	-0.07	-0.02	-0.09	-0.06	-0.09
	IL-4		0.06	0.03	0.07	-0.02	-0.10	-0.03	-0.03	-0.08	-0.03	0.04	0.04	0.15	-0.05	0.03
	IL-5		0.11	0.11	-0.06	**0.28***	-0.03	-0.07	-0.01	0.14	-0.07	-0.02	0.00	0.04	-0.04	-0.05
Th17	IL-10	P↑	0.05	0.10	0.04	0.06	-0.12	-0.05	0.04	-0.10	-0.14	-0.11	-0.06	-0.06	-0.10	-0.14
	IL-17a		0.05	0.03	0.14	-0.12	-0.08	-0.03	0.02	0.01	-0.12	0.10	-0.03	-0.05	-0.01	-0.12
CHEMOKINES	CCL2	P↑. V↓	0.01	0.04	0.03	0.15	-0.13	-0.13	-0.02	0.02	-0.04	-0.08	0.00	0.01	-0.13	-0.16
	CCL22	P↑	0.18	0.10	-0.11	-0.07	0.13	0.12	-0.07	0.06	-0.06	0.05	0.05	0.04	0.12	0.01
	Eotaxin		0.14	0.11	0.06	0.12	-0.04	-0.07	-0.11	-0.03	0.00	0.07	-0.09	-0.02	-0.08	0.00
	Fractalkine		0.14	0.21	0.25	0.16	0.19	0.04	-0.11	**0.30***	0.01	-0.13	0.07	-0.02	-0.09	0.11
	GRO		0.16	0.16	0.06	0.00	0.01	0.04	0.02	-0.13	0.03	0.02	0.00	0.16	0.04	0.10
	IL-8		0.08	0.08	0.14	**0.23***	-0.19	0.00	0.01	-0.11	-0.09	-0.11	-0.01	-0.13	-0.11	-0.12
	IP-10	P↑	-0.03	0.02	0.03	**0.31***	-0.14	-0.05	**0.28***	-0.06	-0.16	-0.15	0.00	-0.14	-0.17	-0.17
	MCP-3		0.00	0.00	-0.12	0.08	0.06	0.07	0.03	-0.06	**0.29***	-0.05	0.08	0.07	0.09	0.02
	MIP-1α		0.01	0.00	-0.10	-0.05	-0.10	0.00	-0.04	0.10	-0.18	-0.11	-0.14	-0.09	-0.10	-0.15
	MIP-1β		-0.06	-0.05	-0.09	0.06	-0.10	-0.06	0.00	-0.13	**-0.23***	-0.02	-0.17	-0.06	**-0.24***	-0.17
HEMATOPOIESIS	GCSF	P↑	0.04	-0.01	0.14	-0.03	-0.17	-0.05	0.11	-0.14	-0.17	-0.10	0.02	-0.13	-0.07	-0.15
	GM-CSF		-0.01	-0.02	-0.08	-0.07	-0.06	-0.04	-0.06	-0.09	-0.10	0.05	-0.06	0.01	-0.07	-0.09
	IL-2		0.00	-0.01	-0.10	-0.10	-0.02	-0.04	-0.06	-0.06	-0.13	0.03	-0.07	-0.03	-0.05	-0.11
	IL-3		0.02	0.01	-0.06	-0.05	-0.09	-0.07	-0.03	-0.10	-0.16	0.09	-0.07	-0.03	-0.07	-0.11
GROWTH FACTORS	EGF		0.05	-0.03	0.09	0.03	-0.05	0.00	0.02	-0.11	-0.09	0.05	-0.09	0.18	-0.07	0.05
	VEGF		0.03	0.02	-0.10	-0.12	-0.05	-0.01	0.03	-0.03	-0.11	0.04	-0.03	-0.05	0.02	-0.09

Supplemental Table 3. Correlation of circulating cytokines and chemokines concentrations with blood leucocytes. Pearson correlation (r) was calculated in the whole ECD cohort (n=75). P. pegylated IFNα. V. vemurafenib. Arrow indicated the effect of the therapy on circulating concentrations of the corresponding biomolecule in *BRAF*-mutated ECD patients; ↑. increase; ↓. decrease. *p<0.05. **p<0.005 and ***p<0.005.

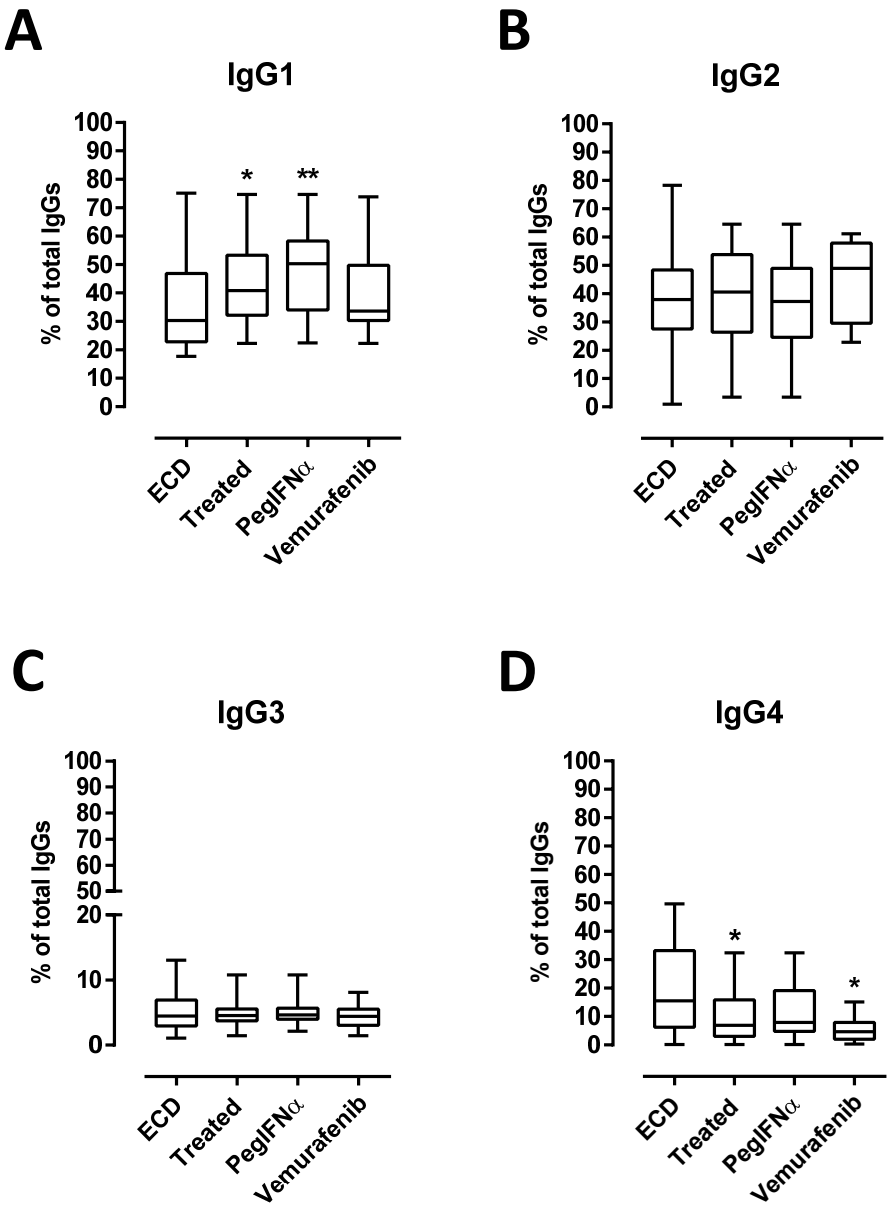
Circulating immunoglobulins (mg/dL)	Reference Values[12-13]	Untreated ECD		
			BRAF^{V600E} mutation	
		All (n=34)	WT (n=9)	V600E (n=22)
IgG1	500.0 (280.0-800.0)	589.4 (410.2-755.9)	529.1 (351.5-744.3)	620.9 (471.1-942.7)
IgG2	300.0 (115.0-570.0)	642.6 (437.9-1063.0)	660.2 (433.3-100.2)	695.4 (491.5-1068.0)
IgG3	64.0 (24.0-125.0)	53.0 (44.1-77.4)	46.6 (41.0-71.9)	56.3 (35.5-78.1)
IgG4	34.9 (52.0-125.0)	266.5 (60.7-747.4)	278.8 (21.1-727.0)	334.0 (71.6-907.7)
IgGs	898.9 (471.0-1620.0)	1880.0 (1126.0-2365.0)	1622.0 (1044.0-2425.0)	1905.0 (1440.0-2987.0)
IgA	239 (87-576)	444.3 (286.3-595.1)	318.0 (272.9-635.7)	481.6 (294.4-595.1)
IgM	134 (46-386)	152.2 (68.6-219.9)	121.3 (52.9-283.1)	158.5 (70.1-230.3)
IgG1/IgG (%)	55.6 (31.1-89.0)	30.3 (22.8-46.9)	23.3 (18.9-49.5)	30.9 (25.3-43.8)
IgG2/IgG (%)	33.4 (12.8-63.4)	37.9 (27.5-48.4)	45.4 (29.8-54.5)	37.0 (27.5-44.9)
IgG3/IgG (%)	7.1 (2.67-13.9)	4.45 (2.93-6.92)	4.81 (3.15-7.43)	4.29 (2.81-6.86)
IgG4/IgG (%)	3.88 (0.58-13.9)	15.5 (6.22-33.2)	10.9 (2.41-35.1)	16.5 (6.51-37.4)

Supplemental Table 4. Circulating immunoglobulin concentrations in untreated ECD patients according to the *BRAF*^{V600E} mutation. Expressed in median (Quartile 1- Quartile 3) Reference values for IgGs[13], IgA[12], and IgM[12] are expressed in median (2.5-97.5 Percentiles). WT and V600E, absence and presence of the *BRAF*^{V600E} mutation.

Supplemental Figure1



Supplemental Figure 1. **Blood leucocyte cell profiling.** Analysis of blood leucocyte populations from control individuals (Ctrl) and Erdheim-Chester (ECD) patients carrying or not the *BRAF*^{V600E} mutation. A representative panel was shown for each leucocyte subset in Ctrl and ECD.



Supplemental Figure 2. Correction of the IgG1/IgG4 switch by first-line therapies in patients with ECD. Impact of first-line therapies on the percentage of IgG1 (B), IgG2 (C), IgG3 (D) and IgG4 (E). Untreated (n=34) and treated (n=35; pegIFNα=24 and vemurafenib=11) patients with ECD. Difference between groups was tested using a Kruskal-Wallis test. **P*<0.05 and ***P*<0.005 versus untreated patients with ECD.

Supplemental Figure 2