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► To cite this version:

Fleur Cohen Aubart, Lucie Poupel, Flora Saint-Charles, Frederic Charlotte, Youssef Arsafi, et al.. Profound systemic alteration of the immune phenotype and an immunoglobulin switch in Erdheim–Chester disease in a single-center of 78 patients. *Haematologica*, 2022, 107 (6), pp.1347-1357. 10.3324/haematol.2021.279118 . hal-03388029v1

HAL Id: hal-03388029

<https://hal.sorbonne-universite.fr/hal-03388029v1>

Submitted on 20 Oct 2021 (v1), last revised 3 Feb 2023 (v2)

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Received: April 30, 2021.

Accepted: October 6, 2021.

Citation: Fleur Cohen Aubart, Lucie Poupel, Flora Saint-Charles, Frédéric Charlotte, Youssef Arsafi, Eric Frisdal, Damien Roos-Weil, Jean-François Emile, Zahir Amoura, Maryse Guerin, Philippe Lesnik, Julien Haroche, and Wilfried Le Goff. Profound systemic alteration of the immune phenotype and immunoglobulin switch in Erdheim-Chester disease in a single-center of 78 patients. Haematologica. 2021 Oct 14. doi: 10.3324/haematol.2021.279118. [Epub ahead of print]

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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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Financial support.

INSERM, Sorbonne Université, French National Agency (ANR-10-IAHU-05), and the Erdheim-Chester Disease Global Alliance.

Acknowledgments.

The authors are indebted to all the participants for their cooperation.

Author contributions.

FCA, LP, FS-C, YA, EF, and WLG performed the research. FCA, PL, JH, and WLG designed the research. FCA and JH recruited the patients. MG and WLG analyzed the data. JH and WLG funded the research. All authors gave critical comments on the manuscript. WLG supervised the study and wrote the manuscript.

Conflict-of-interest disclosure.

The authors declare no competing financial interests.

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

Abstract

Erdheim–Chester disease (ECD) is a rare, systemic, non-Langerhans cell histiocytosis neoplasm, which is characterized by the infiltration of CD63⁺ CD1a⁻ histiocytes in multiple tissues. The *BRAF*^{V600E} mutation is frequently present in individuals with ECD and has been detected in hematopoietic stem cells and immune cells from the myeloid and systemic compartments. Immune cells and pro-inflammatory cytokines are present in lesions, suggesting ECD involves immune cell recruitment. Although a systemic cytokine Th-1-oriented signature has been reported in ECD, the immune cell network orchestrating the immune response in ECD has yet to be described. To address this question, the phenotypes of circulating leukocytes were investigated in a large, single-center cohort of 78 patients with ECD and compared with a group of 21 control individuals. Major perturbations in the abundance of systemic immune cells were detected in patients with ECD, with a decrease in circulating plasmacytoid, myeloid 1, and myeloid 2 dendritic cells, mostly in *BRAF*^{V600E} carriers, in comparison with individuals in the control group. Similarly, a marked decrease in blood T-helper, cytotoxic, and B lymphocyte numbers was observed in patients with ECD, relative to the control group. Measurement of circulating immunoglobulin concentrations revealed an immunoglobulin G switch, from IgG1 to IgG4 subclasses, which are more frequently associated with the *BRAF* mutation. First-line therapies, including pegylated IFN α and vemurafenib, were able to correct most of these alterations. This study reports a profound disturbance in the systemic immune phenotype in patients with ECD, providing important new information and helping to understand the physiopathological mechanisms involved in this rare disease and in the therapeutic management of patients.

Introduction

Erdheim–Chester Disease (ECD) is a rare, systemic, non-Langerhans cell histiocytosis neoplasm, frequently caused by mutations in the MEK-extracellular signal-regulated kinase (ERK) signaling pathway; these are mostly *BRAF* mutations¹. ECD is characterized by the infiltration of tissues by foamy histiocytes expressing markers of the monocyte/macrophage lineage, including CD45, CD68, CD163, and CD14, whereas ECD histiocytes are negative for CD1a and CD207 dendritic cell markers. It is proposed that in ECD, histiocytes originate from myeloid CD34⁺ and CD14⁺ progenitor cells^{2,3}. The *BRAF*^{V600E} mutation has been detected in hematopoietic stem cells (HSCs), including common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs), in the bone marrow of patients with ECD², supporting a model in which *BRAF*-mutated myeloid cells disseminate from bone marrow to the periphery for tissue infiltration. Consistent with this model, the *BRAF*^{V600E} mutation was also found in circulating leucocytes, including classical (CD14⁺) and nonclassical (CD16⁺) monocytes and CD1c⁺ myeloid dendritic cells in individuals with ECD².

The accumulation of histiocytes within lesions in cases of ECD is accompanied by the expression of a chemokine and cytokine network favoring immune cell recruitment^{4,5}. Indeed, pro-inflammatory cytokines are highly expressed in ECD lesions, together with the infiltration of pro-inflammatory T-cell helper 1 (Th-1) lymphocytes. In addition, immunohistological examination of ECD biopsies revealed that infiltrated histiocytes express a large set of chemokines and chemokine receptors⁴. Consistent with these observations, patients with ECD exhibited a systemic immune Th1-oriented cytokine profile⁶, thereby providing important clues for the therapeutic management of these patients. However, the therapeutic management of patients with ECD remains difficult. First-line therapies are mostly determined by the severity of the disease. Thus, pegylated interferon- α (IFN α) is used to treat mild disease and nonrefractory ECD⁷, whereas drugs targeting the mutated *BRAF*, such as vemurafenib, are used in multisystemic and refractory ECD⁸.

The underlying mechanisms that orchestrate the immune response in ECD remain largely unknown, and a comprehensive characterization of systemic immune cells in ECD patients is lacking. Therefore, the goal of our study was to determine whether patients with ECD exhibit abnormalities in their systemic immune phenotype and whether this is affected by the presence of the *BRAF* mutation and therapeutic agents. We demonstrated that patients with ECD exhibited a profound alteration in their systemic immune cell phenotype, characterized by a low abundance of dendritic cell subsets and by specific lymphocyte populations, together with a switch in immunoglobulin (Ig) G subclasses, which may be partially corrected by first-line therapies.

Methods.

Patients. Fasting blood samples were obtained from 17 healthy individuals who formed the control group (13 male and 4 female; mean age, 53±25 years, range, 21–90 years) and 78 patients with ECD (60 male and 18 female; mean age, 60±14 years, range, 18–84 years) who were followed at the Pitié-Salpêtrière Hospital, Paris, France, between December 2012 and July 2015 (Supplemental Table 1). For all patients, ECD was diagnosed based on the consensus guidelines for the diagnosis and clinical management of ECD ⁹. The detection of the *BRAF*^{V600E} mutation was performed using multiplex picodroplet digital PCR (Raindance Technologies), as previously described ¹⁰. The prevalence of the *BRAF*^{V600E} mutation was 64% in the ECD group (50/71 patients, indeterminate for 7 patients). The absence of the *BRAF*^{V600E} mutation in ECD patients was referred to as the wild-type (WT) in this study. At the time of the blood sample collection, the patients were free of any treatment (n=42) or were receiving treatment, either pegylated IFN- α (n=31), vemurafenib (n=13), or others (n=17). It is noteworthy that we cannot determine which of the cases reported in the present study were previously reported. Blood samples were collected from 25 patients at several time points (free of any treatments and upon treatment). This study was approved by the ethics committee of Ile de France III (#2011-A00447-34) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients.

Analysis of blood immune cells by flow cytometry. Fresh blood samples were collected in EDTA tubes at the same time of the day for all patients and control individuals; the samples were used immediately for the flow cytometry analysis. Analysis of blood immune cells was carried out simultaneously in both patients and control individuals throughout the study (2013–2015). Similar blood immune cell counts were obtained when flow cytometry analysis was performed for the same control individual at different times of the study. Quantification of the immune cell subsets was described in details in *Online Supplementary Materials*.

Quantification of circulating chemokines, cytokines and immunoglobulins is described in *Online Supplementary Materials*.

Statistical analyses. Values are given as medians and interquartile ranges (Q1–Q3). Comparisons of two groups were performed using the Mann–Whitney test. Comparisons of more than two groups were performed using the Kruskal–Wallis test followed by a Dunn’s comparison test. The impacts of the *BRAF*^{V600E} mutation and treatment with first-line therapies on blood leukocyte counts were tested using the Jonckheere-Terpstra trend test. Correlations were calculated using the Spearman rank-order test. A χ^2 test was performed to analyze the distribution of individuals with ECD around the median value of the indicated parameter, according to the *BRAF* status. For skewed variables, the raw data were logarithmically transformed prior to conducting the analyses. Statistical analyses were performed using R statistical software version 3.3.2 (R foundation for Statistical Computing) and Prism software from GraphPad (San Diego, CA USA). Principal component analysis was performed using the public MetaboAnalyst web server (<https://www.metaboanalyst.ca/>).

Results

Profound alteration of the systemic immune cell phenotype in patients with ECD. Flow cytometry analysis of blood immune cells in patients with ECD allowed the identification of the complete set of monocytes (classical, intermediate, and nonclassical), DCs (plasmacytoid (pDC), myeloid 1 (mDC1), and 2 (mDC2)), and lymphocytes (T helper (Th), cytotoxic (CT), T regulatory (Treg), natural killer (NK), and B) independently of the $BRAF^{V600E}$ mutation as observed in control individuals (**Supplementary Figure 1**). As shown in **Supplementary Figure 1**, no atypical population was detected in ECD patients irrespective of their $BRAF$ status in comparison with control individuals. Although the number of total blood monocytes was higher in patients with ECD who had the $BRAF$ -mutation in comparison with total blood monocytes in the controls (+58.9%, $P<0.05$), none of the monocyte subsets was found to be significantly increased in those individuals (**Table 1**). Instead, a trend for a decrease in nonclassical $CD14^+CD16^{++}$ monocytes was observed in ECD patients carrying the $BRAF^{V600E}$ mutation (-73.8%, $P<0.08$). More strikingly, a marked decrease in the absolute count of DCs, including pDCs (-63.6%, $P<0.0005$), mDC1s (-62.0%, $P<0.05$), and mDC2s (-72.6%, $P<0.005$), was observed in patients with ECD when compared with these values in healthy individuals; this effect mostly reflected the reduction in all DC subsets in patients with the $BRAF$ -mutation. Such effects were independent of a patient's sex (data not shown). Although the number of blood neutrophils, NK, NKT, and Treg cells was not altered in patients with ECD, we noticed a large decrease in CT (-80.8%, $P<0.0005$) and B (-66.5%, $P<0.005$) lymphocytes in ECD patients relative to the counts for these cells in controls. Finally, a substantial reduction in the absolute count of Th lymphocytes (-84.5%, $P<0.05$) was observed in patients with ECD who lacked the $BRAF$ mutation.

Principal component analysis of the blood immune cell populations of individuals in the control and ECD groups illustrated the peculiar systemic immune signature that characterized ECD (**Figure 1A**), as well as a potential effect of the $BRAF^{V600E}$ mutation, as was suggested by the analysis of individual cell populations (**Table 1**). Assessment of the impact of the $BRAF^{V600E}$ mutation on

populations of blood immune cells in ECD supports an enhancing effect of the mutation on the reduction in blood nonclassical CD14⁺CD16⁺⁺ monocyte ($P<0.03$) and DC (pDC, $P<0.0002$; mDC1, $P<0.05$, and mDC2, $P<0.0009$) numbers and on the increase in blood total monocytes ($P<0.04$) in ECD patients compared with control individuals (**Figure 1B-I**).

Analysis of the effect of first-line therapies on this disturbed systemic immune cell signature indicated that patients with ECD who also had the *BRAF* mutation and who were treated with first-line therapies, including pegylated IFN α (pegIFN α) and vemurafenib, did not exhibit such a massive alteration of the systemic immune cell phenotype when compared with control individuals (**Table 1**). As an illustration, the absolute counts of mDC1 and mDC2 populations in treated ECD patients carrying the *BRAF*^{V600E} mutation were not significantly different from those of control individuals. Assessment of the impact of first-line therapies in ECD patients with the *BRAF* mutation highlighted the capacity of treatments to partially correct or restore the circulating numbers of several altered leukocyte populations in ECD; this was observed for nonclassical CD14⁺CD16⁺⁺ monocyte ($P<0.03$), mDC1 ($P<0.03$), and mDC2 ($P<0.0006$) populations (**Figure 2**). However, treatments taken individually or as a whole were unable to restore the decrease in pDC populations in patients with ECD (**Table 1 and Figure 2**).

Taken together, these findings highlighted a major perturbation of the systemic immune cell phenotype in ECD cases, characterized by a deficit of DCs and lymphocytes, which could be partially restored by first-line treatments in patients with the *BRAF*-mutation.

Impact of first-line therapies on the systemic cytokine and chemokine network in patients with ECD.

To provide clues about the mechanism underlying the alteration of the systemic immune cell phenotype according to the *BRAF* status of patients with ECD, a comprehensive quantification of circulating chemokines and cytokines was performed on this single-center group of 78 patients with ECD (**Supplementary Table 2**). As previously reported ⁶, the levels of many circulating cytokines and

chemokines are highly heterogeneous among ECD patients (**Supplementary Table 2**). However, when we investigated the impact of the *BRAF*^{V600E} mutation on ECD patients' cytokine and chemokine profiles, we observed that the proportion of individuals with high levels of numerous circulating cytokines driving the Th1 response (IL-6, IL-8, IL-12p40, and TNF α) and chemokines (IP-10, CCL2, MIP-1 α , and CCL22) was higher in carriers of the *BRAF*^{V600E} mutation in comparison with noncarriers (**Figure 3**). It is noteworthy that patients with the *BRAF*-mutation also exhibited high levels of the anti-inflammatory cytokine, IL-10. In contrast, a higher proportion of patients with elevated circulating levels of eotaxin, EGF, and IL-15 was detected among patients lacking the *BRAF* mutation than in their counterparts with the *BRAF* mutation. Because of the specific mode of action of the treatments, i.e., vemurafenib and pegIFN α , no difference in the circulating concentrations of cytokines and chemokines was detected between ECD patients with the *BRAF* mutation who were treated or not treated with first-line therapies when taken as a whole (**Supplemental Table 2**). Conversely, compared with their nontreated counterparts, ECD patients with the *BRAF* mutation who were treated with pegIFN α exhibited higher circulating levels of cytokines that drove either a pro-inflammatory Th1 (IFN α , 5.6-fold, $P < 0.0001$; and IL-15, 1.8-fold, $P < 0.05$) or anti-inflammatory Th2 (IL-10, 1.9-fold, $P < 0.05$) response, as well as higher levels of chemokines (IP-10, 1.5-fold, $P < 0.05$; and CCL2, 1.3-fold, $P < 0.05$) and a cytokine involved in hematopoiesis (G-CSF; 2.2-fold, $P < 0.05$) (**Supplementary Table 2**). However, it is worth mentioning that there was a reduction in plasma CCL22 levels (-34%, $P < 0.05$) in ECD patients carrying the *BRAF*^{V600E} mutation, after they received pegIFN α treatment. A similar pegIFN α signature was observed when all patients with ECD were considered, irrespective of their *BRAF* status (data not shown). Finally, a significant reduction in plasma CCL2 levels was only observed in ECD patients upon vemurafenib therapy compared with untreated ECD patients carrying the *BRAF* mutation (-49.5%, $P < 0.05$) (**Supplementary Table 2**).

Taken together, our results show that patients with ECD and who carried the *BRAF*^{V600E} mutation exhibited an overall more pro-inflammatory cytokine and chemokine signature than ECD patients

who did not carry this mutation, and this signature appears to have been further exacerbated by pegIFN α treatment.

Interrelationship between blood immune cell phenotype and cytokine and chemokine network in patients with ECD.

We next investigated whether the modifications to the circulating cytokine and chemokine concentrations may translate to the major perturbation of the systemic immune cell phenotype, as well as its partial restoration following first-line therapy, in ECD patients carrying the *BRAF* mutation. For this purpose, correlations were explored between circulating immune cell numbers and the concentrations of cytokines and chemokines, in the entire ECD cohort. As shown in **Supplementary Table 3**, although none of these biomolecules were found to be correlated with blood B or Th lymphocytes levels, the results indicated that the absolute count of nonclassical monocytes (CD14⁺CD16⁺⁺) was positively correlated with the plasma concentrations of IFN α 2 ($r=0.31$, $P<0.005$), IL-6 ($r=0.30$, $P<0.05$), IL-8 ($r=0.23$, $P<0.05$), and IL-5 ($r=0.27$, $P<0.05$). Interestingly, plasma IP-10 levels were positively correlated with the abundance of both nonclassical monocytes ($r=0.31$, $P<0.05$) and mDC2 cells ($r=0.28$, $P<0.05$) in the blood, while a correlation was detected between TNF α and MIP-1 β levels with the number of mDC1 cells ($r=0.40$, $P<0.0005$) and CT lymphocytes ($r=-0.24$, $P<0.05$).

As a whole, these findings have led to the identification of a set of cytokines and chemokines that might account for the abundance of nonclassical monocytes and myeloid DCs following first-line therapies in the blood of ECD patients who carry the *BRAF* mutation.

Immunoglobulin switch toward immunoglobulin G4 in patients with ECD.

Finally, to determine if the disturbance of the systemic immune cell phenotype translates into a defect in immunoglobulin production, plasma concentrations of immunoglobulin isotypes (IgA, IgM, IgG1, IgG2, IgG3, and IgG4) were quantified in patients with untreated ECD. Although the quantities of IgA and IgM were within the reference ranges for adults ¹², those of IgG were more

elevated, which mostly reflected the high abundance of IgG4 and, to a lesser extent, high concentrations of IgG2¹³ (**Supplemental Table 4**). As a result, in patients with ECD, the proportion of IgG1 (IgG1/IgGs) was low, whereas that of IgG4 (IgG4/IgGs) was high; this effect appeared to be more pronounced in patients carrying the *BRAF* mutation. Analysis of the patient distribution according to their IgG4 levels (normal < 135 and high ≥ 135 mg/dL)¹⁴ indicated that whereas a roughly similar proportion of patients without the *BRAF* mutation displayed either normal or high levels of IgG4, the level of IgG4 in patients carrying the *BRAF*^{V600E} mutation was 1.7-fold higher than that of patients who lacked the mutation in the normal IgG4 group and up to 3-fold higher in the high IgG4 group (**Figure 4A**). On the whole, 64.7% of patients with ECD exhibited a high-IgG4 immune phenotype and this was predominantly among those patients who carried the *BRAF* mutation. Strikingly, first-line therapies corrected the IgG switch in this latter group, with a significant increase in IgG1 being observed upon pegIFN α therapy (untreated, 30.9 (25.3–43.8) versus pegIFN α , 50.3 (36.1–58.3), $P < 0.005$), while IgG4 returned to normal values following vemurafenib treatment (untreated, 16.5 (6.51–37.4) versus vemurafenib, 4.69 (2.02–7.94), $P < 0.05$) (**Figure 4**). A similar correction of the IgG profile was also observed for the entire ECD cohort treated with first-line therapies (**Supplemental Figure 2**). These findings revealed that patients with ECD exhibited an IgG switch, from IgG1 to IgG4, which was corrected by first-line therapies.

Discussion

The present study, involving a single-center series of 78 patients with ECD, revealed a profound perturbation of the blood immune phenotype in these patients, characterized by a decrease in the DC and lymphocyte populations and accompanied by a switch in IgG subclasses. This perturbation was exacerbated in patients carrying the *BRAF*^{V600E} mutation, who also exhibited a higher pro-inflammatory status than patients who lacked this mutation. First-line therapies were able to partially correct the altered immune cell phenotype and restore the IgG pattern.

This first comprehensive analysis of systemic immune cell populations in patients with ECD revealed a peculiar immune cell ECD signature, characterized by a very low abundance of DCs, including pDC, mDC1, and mDC2, in comparison with the abundance of these cells in matched control individuals. Although there is limited information available about the levels of immune cells in the blood of patients with histiocytosis, this observation contrasts with the increased quantity of DC precursors detected in the blood of patients with Langerhans cell histiocytosis (LCH), a histiocytic neoplasm that arises from the dendritic lineage¹⁵. Although a trend for such a decrease in DCs was observed in patients who lacked the *BRAF* mutation, a much stronger effect was detected in patients who did carry this mutation, suggesting that the activation of the ERK signaling pathway could underlie this phenotype. This perturbation in blood DC levels was unlikely to have resulted from the increased infiltration of these cells into tissues, as no CD123-positive cells (pDCs) have previously been detected in ECD lesions⁶. Rather, activation of the MEK/ERK signaling pathway was reported to inhibit the maturation of monocyte-derived DCs^{16,17}. More recently, Hogstad et al. elegantly demonstrated that the MAPK pathway, including the *BRAF*^{V600E} mutation, suppresses DC migration and traps DCs in LCH lesions¹⁸. *BRAF* mutations have been detected in myeloid progenitors in bone marrow from ECD patients²; therefore, our findings lead us to propose that the presence of the *BRAF*^{V600E} mutation in myeloid DC precursors might cause these cells to be retained in the bone marrow compartment and impede their migration to the blood circulation. This mechanism could

explain the paradoxical elevated systemic IFN α concentrations reported in patients with ECD ⁶, despite the low abundance of blood DCs presently described. Additional investigations are needed to determine if an increase in myeloid DC precursors can be detected in the bone marrow of patients with ECD.

Antigen-presenting cells such as DCs interact with lymphocytes and contribute to their proliferation and maturation and the establishment of an immune response. Together with the decrease in blood DCs, the systemic concentrations of helper, cytotoxic, and B lymphocytes were markedly reduced in patients with ECD in the present study. Moreover, decreased systemic levels of IL-7, a cytokine involved in B and T lymphocyte differentiation, have been reported in patients with ECD ⁶. The infiltration of Th1 cells into ECD lesions ⁴ could also contribute to the reduction in the abundance of circulating T lymphocytes. Indeed, CCL19/MIP-3 β , a chemo-attractant for B and T lymphocytes and DCs, was reported to be expressed in ECD lesions that were analyzed by immunohistochemistry ⁴. In contrast, the expansion of Treg lymphocytes alone in both the blood compartment and lesions has been reported in LCH, while monocyte and DC populations were not altered ¹⁹.

Despite the low abundance of circulating B cells, a recent study pointed out the high prevalence (42%) of autoimmunity in patients with ECD ²⁰. Here, we brought to light perturbations in the IgG profile characterized by high IgG4 levels and leading to an IgG1/IgG4 switch. A few case reports have documented high IgG4 levels in ECD patients, suggesting ECD mimics IgG4-related disease (IgG4-RD) ^{14,21}. In a review of a single-center cohort, Gianfreda et al. observed that high levels of IgG4 were present in 26.7% (4/15) of patients with ECD ¹⁴. In the present study, involving 78 patients, high levels of IgG4 (≥ 135 mg/dL) were observed more frequently, affecting 64.7% of patients. However, while ECD and IgG4-RD share some physiopathological characteristics, these diseases exhibit distinctive clinical features, suggesting they are distinct disorders. The increased production of IgG4 is frequently driven through a Th2 response to IL-4, IL-5, or IL-13 and by anti-

inflammatory IL-10 and TGF β cytokines²². Although ECD patients exhibit a Th1 immune response^{4,6}, the present study suggested that while patients who carry the *BRAF* mutation exhibit higher circulating IL-10 concentrations and are more likely to exhibit high IgG4 levels than patients who lack the mutation, suggesting that IL-10 might contribute to the IgG4 immune response in ECD. Moreover, IFN α , which is secreted by pDCs and initiates the Th1 response and whose systemic concentrations are elevated in ECD⁶, has been reported to increase IgG4 production by B lymphocytes²³. Thus, infiltration of pDCs in pancreatic lesions of patients with IgG4-related autoimmune pancreatitis has been proposed to induce IgG4 production by plasma cells via IFN α ²³. Similar to what is observed in cases of IgG4-RD, IgG4-positive plasma cell infiltrates were observed in ECD lesions at perirenal and subcutaneous sites¹⁴. To determine whether the reduction in circulating B cells in ECD detected in the present study reflects the infiltration of these cells into lesions or impaired B cell differentiation deserves further investigation.

The quantification of serum cytokines in a single-center series of 37 patients with ECD was previously reported; it included the identification of an ECD signature based on the concentrations of IFN α 2, IL-12, MCP-1, IL-4, and IL-7 that allowed ECD patients to be distinguished from control individuals⁶. The present study provides new information regarding the effect of the *BRAF*^{V600E} mutation on this ECD signature, as well as on the systemic immune Th1 phenotype that characterizes ECD. We found an exacerbated Th1-mediated systemic immune response in patients carrying the *BRAF* mutation, characterized by a higher proportion of participants carrying the *BRAF*^{V600E} mutation with elevated circulating concentrations of pro-inflammatory cytokines (IL-12p40, IL-6, IL-8, and TNF α) and chemokines (IP-10, CCL2, CCL22, and MIP-1 α) than participants who lacked this mutation. However, first-line therapies were unable to dampen this pro-inflammatory phenotype. Elevated levels of circulating chemokines in patients carrying the *BRAF* mutation are consistent with previous studies reporting that the presence of the *BRAF*^{V600E} mutation is a major determinant in histiocyte infiltration²⁴ and that vemurafenib shows a high efficacy in multisystemic and refractory ECD⁸. The decrease in CCL2 concentrations in ECD patients treated with vemurafenib in comparison with their

untreated counterparts carrying the *BRAF* mutation might account, at least in part, for the reduced infiltration upon receiving this therapy. An analysis of the systemic chemokine and cytokine network in 52 patients with LCH versus 34 control individuals revealed that patients carrying the *BRAF*^{V600E} mutation only showed elevated levels of MCP-3 in serum, with no other abnormalities detected ²⁵. Such an elevation of MCP-3 was not observed in patients carrying the *BRAF* mutation in the present study.

In the present study, we found that first-line therapies, although having a modest impact on the systemic chemokine and cytokine concentrations, were able to correct most of the alterations in blood immune cell counts, especially those of nonclassical CD14⁺CD16⁺⁺ monocytes, mDC1s, mDC2s, and B lymphocytes, whereas they failed to restore those of pDCs. While disturbances in the immune response were more frequent in ECD patients carrying the *BRAF* mutation, targeted therapy appeared to be less effective than pegIFN α to improve these patients. Nevertheless, vemurafenib, similarly to pegIFN α , corrected the IgG1/IgG4 switch. The major findings of the present study are summarized in **Figure 5**.

In conclusion, our study is the first report of the marked alteration of the systemic immune response in ECD and brings to light the involvement of DCs in this non-LCH neoplasm. This new information will help in our understanding of the mechanisms taking place in ECD pathophysiology and provides additional clues to the best approach to the therapeutic management of patients with ECD.

Limitations of the study. The limitations of this study include the relatively low number of patients with ECD on the different therapies (vemurafenib versus pegIFN α). Another limitation is the absence of data from ECD patients before and after treatment, which would be useful to investigate in more detail the impact of first-line therapies on the systemic disturbance of the immune cell phenotype and the IgG switch. Finally, the inclusion of a control group comprising patients with LCH would have helped as a comparison with the specific inflammatory patterns in ECD.

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Circulating Leucocytes (Absolute count/mL)	Control (n=17)	Untreated ECD			Treated ECD (V600E)		
		All (n=38)	<i>BRAF</i> ^{V600E} mutation		PegIFN α (n=17)	Vemurafenib (n=12)	All (n=29)
			WT (n=11)	V600E (n=23)			
Monocytes (x10³)							
Total	277.3 (131.9-441.7)	384.0 (181.6-921.9)	183.8 (106.7-965.5)	440.6 (248.5-984.9) *	416.3 (174.9-594.2)	399.2 (280.1-446.3)	413.3 (268.6-567.7)
CD14⁺⁺CD16⁻	196.8 (99.65-372.1)	281.3 (144.8-553.3)	203.2 (99.51-485.4)	288.2 (152.1-686.9)	241.2 (60.89-344.2)	260.6 (182.0-372.9)	241.2 (144.8-357.1)
CD14⁺⁺CD16⁺	11.50 (4.27-18.46)	11.62 (4.03-23.48)	13.22 (3.85-24.12)	13.22 (3.85-24.12)	18.41 (7.26-32.54)	10.19 (7.32-20.05)	13.75 (7.27-26.80)
CD14⁺CD16⁺⁺	18.57 (5.05-36.59)	6.72 (1.58-20.97)	11.10 (1.99-23.09)	4.86 (1.16-16.69)	15.33 (0.64-32.84)	9.15 (5.00-16.77)	13.82 (2.27-20.25)
Dendritic Cells							
pDC	4044 (2266-5531)	1472 (238.2-2479) ***	2092 (542.9-2710) **	1167 (186.1-2004) **	663.1 (180.5-2081) ***	1243 (590.0-1976) **	1030 (281.2-1997) ***
mDC1	303.4 (188.0-564.0)	115.2 (42.51-589.1) *	276.2 (42.51-890.0)	100.3 (41.13-595.5) *	186.3 (25.62-405.3)	250.5 (140.9-467.1)	229.7 (93.33-415.8)
mDC2	632.0 (377.5-2000)	172.9 (81.17-594.4) **	359.8 (91.27-872.2)	135.9 (63.60-399.9) **	233.0 (67.38-1114)	523.0 (285.3-863.2) †	327.1 (135.7-960.8)
Neutrophils (x10⁶)	11.93 (9.10-19.60)	20.12 (1.58-32.28)	17.53 (4.02-25.46)	20.12 (1.25-40.54)	1.04 (0.31-23.82)	11.23 (1.19-24.13)	3.55 (5.19-23.82)
Lymphocytes (x10³)							
NK	131.7 (57.52-204.2)	106.8 (25.67-228.4)	104.3 (22.05-214.3)	133.3 (39.61-267.8)	66.9 (27.72-187.3)	53.1 (4.90-101.7) *†	55.2 (27.09-162.5)
NKT	4.56 (2.07-11.13)	7.52 (3.41-14.77)	9.00 (4.23-15.01)	7.42 (2.83-13.85)	3.67 (1.44-5.80)	4.27 (1.67-8.52)	3.71 (1.49-8.31)
Treg	23.20 (12.75-35.01)	15.94 (7.48-30.94)	10.39 (5.45-27.19)	16.57 (7.65-43.81)	20.33 (17.51-37.90)	29.74 (4.82-41.13)	24.05 (11.39-40.64)
B	95.93 (52.14-174.30)	32.17 (14.98-84.32) **	31.73 (15.88-90.11) *	30.64 (13.30-84.51) *	67.34 (10.90-169.24)	9.87 (5.17-30.21) ***,†	21.65 (7.51-89.54) *
CT	200.9 (71.28-440.9)	38.63 (17.24-84.66) ***	29.13 (12.92-76.26) ***	45.69 (18.19-82.11) **	48.23 (13.47-114.9) **	46.36 (9.01-67.01) **	48.23 (10.36-75.44) ***
Th	235.8 (63.89-557.8)	88.77 (31.40-278.8)	36.44 (11.55-203.3) *	211.7 (51.47-506.0) †	174.5 (57.15-289.6)	95.12 (10.40-215.0) *	130.6 (33.74-256.6)

Table 1. Blood leucocytes phenotyping of Erdheim-Chester patients according to the *BRAF*^{V600E} mutation. pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; NK, Natural killer cells; NKT, Natural killer T cells; Treg, regulatory T cells; B, B lymphocytes; CTL, cytotoxic T lymphocytes; Th, T helper cells. * p<0.05, ** p<0.005 and *** p<0.005 versus the control individuals. † p<0.05 versus untreated ECD patients carrying the *BRAF*^{V600E} mutation. Expressed in median (Quartile 1-Quartile 3). WT and V600E, absence and presence of the *BRAF*^{V600E} mutation.

Legends to Figures

Figure 1. Patients with ECD are characterized by a peculiar systemic immune cell signature. A. Principal component analysis and blood counts of total (B) and nonclassical (C) monocytes, T helper (D), cytotoxic (E) and B (F) lymphocytes, and plasmacytoid (G), and myeloid 1 (H) and 2 (I) dendritic cells in untreated ECD patients according to their *BRAF* status in comparison with individuals in the control group. Controls, n=17; ECD patients without the *BRAF* mutation (WT), n=11; and ECD patients carrying the *BRAF*^{V600E} mutation (V600E), n=23. *P*-value for the trend was assessed using the Jonckheere-Terpstra trend test.

Figure 2. Impact of first-line therapies on the systemic immune cell signature in ECD patients carrying the *BRAF* mutation. A. Principal component analysis and blood counts of total (B) and nonclassical (C) monocytes, T helper (D), cytotoxic (E) and B (F) lymphocytes, and plasmacytoid (G), and myeloid 1 (H) and 2 (I) dendritic cells in untreated or treated ECD patients carrying the *BRAF*^{V600E} mutation in comparison with individuals in the control group. Controls (n=17), untreated (n=23), and treated (n=29) ECD patients carrying the *BRAF*^{V600E} mutation (V600E). Treatments included pegylated interferon α and vemurafenib. *P*-value for the trend was assessed using the Jonckheere-Terpstra trend test.

Figure 3. Impact of the *BRAF*^{V600E} mutation on the systemic chemokine and cytokine network in patients with ECD. Analysis of the repartition of untreated ECD patients according to their *BRAF* status around the median values of systemic concentrations of IL-6 (A), IL-12p40 (B), IL-15 (C), TNF α (D), IL-10 (E), CCL2 (F), CCL22 (G), eotaxin (H), IL-8 (I), IP-10 (J), MIP-1 α (K), and EGF (L). ECD patients without the *BRAF* mutation (WT), n=9 and ECD patients carrying the *BRAF*^{V600E} mutation (V600E), n=21. Statistical significance was tested using a χ^2 test.

Figure 4. Correction of the IgG1/IgG4 switch by first-line therapies in patients carrying the *BRAF* mutation. Prevalence of the high-level of IgG4 phenotype in untreated ECD patients according to the

presence of the *BRAF*^{V600E} mutation. Normal IgG4 < 135 mg/dL, high IgG4 ≥ 135 mg/dL. ECD patients without the *BRAF* mutation (WT), n=9 and ECD patients carrying the *BRAF*^{V600E} mutation (V600E), n=22. Impact of first-line therapies on the percentage of IgG1 (B), IgG2 (C), IgG3 (D), and IgG4 (E). Untreated (n=22) and treated (n=27; pegIFNα=16 and vemurafenib=11) ECD patients carrying the *BRAF*^{V600E} mutation (V600E). Differences between groups were tested using the Kruskal–Wallis test. * $P < 0.05$ and ** $P < 0.005$ versus untreated ECD patients carrying the *BRAF*^{V600E} mutation.

Figure 5. Major alterations of the systemic immune cell phenotype in patients with ECD. Flow cytometry analysis of blood leukocytes in patients with ECD revealed a marked decrease in dendritic cells (pDC, mDC1, and mDC2) and lymphocytes (CTL and BL), as well as a reduction in NC monocytes in comparison with levels of these cells in individuals in the control group. Such a reduction in antigen-presenting cells might impair the activation of CTLs and BLs and the production of IgG, leading to an IgG switch toward IgG4. These alterations were mostly observed in ECD patients carrying the *BRAF*^{V600E} mutation (in red), who exhibited a more pronounced systemic inflammation. First-line therapies partially corrected the systemic immune cell phenotype and normalized blood IgG concentrations. BL: B lymphocytes; CTL: cytotoxic T lymphocytes; IgG: immunoglobulin G; NC: nonclassical; mDC: myeloid dendritic cells; pDC: plasmacytoid dendritic cells.

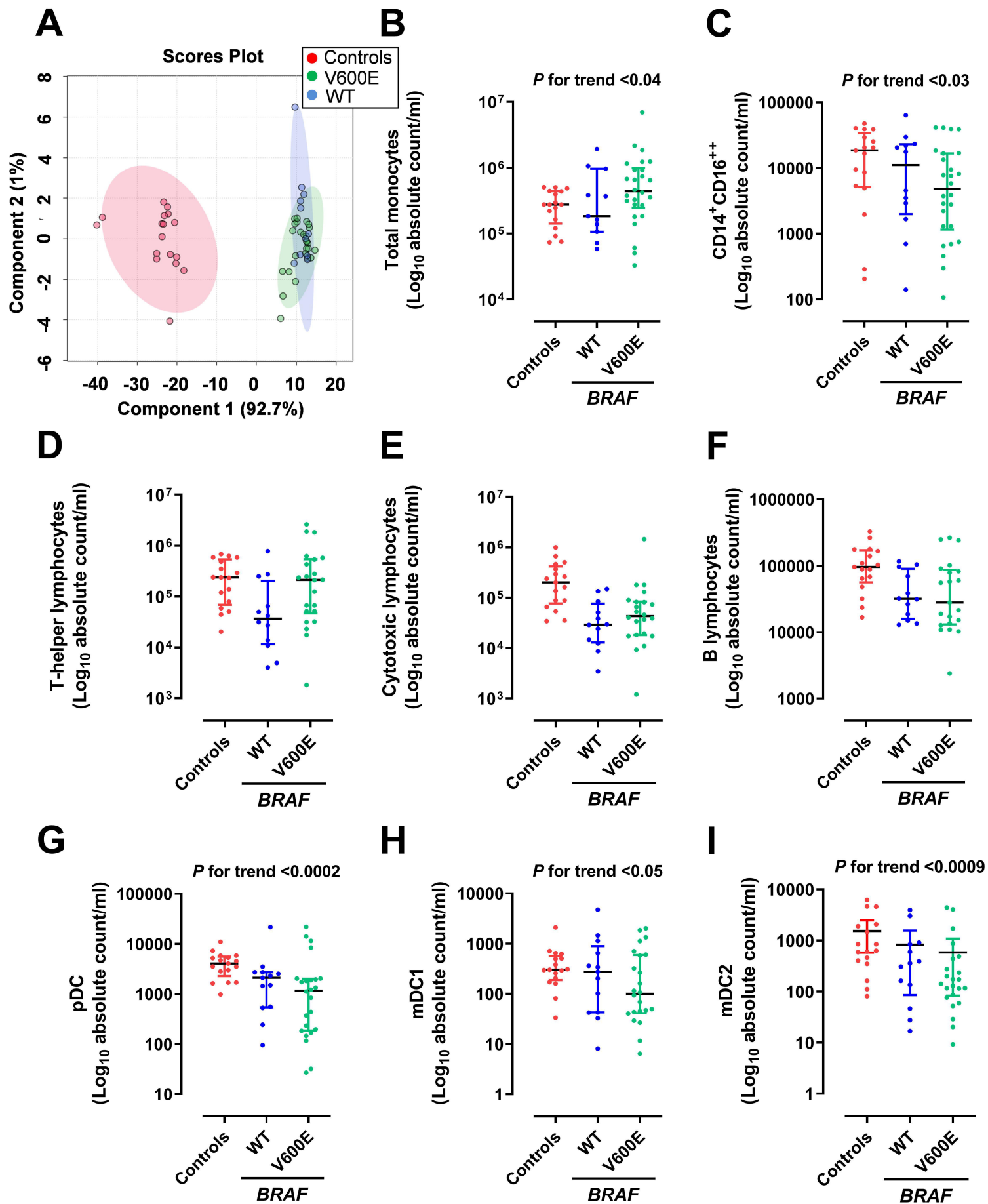


Figure 1

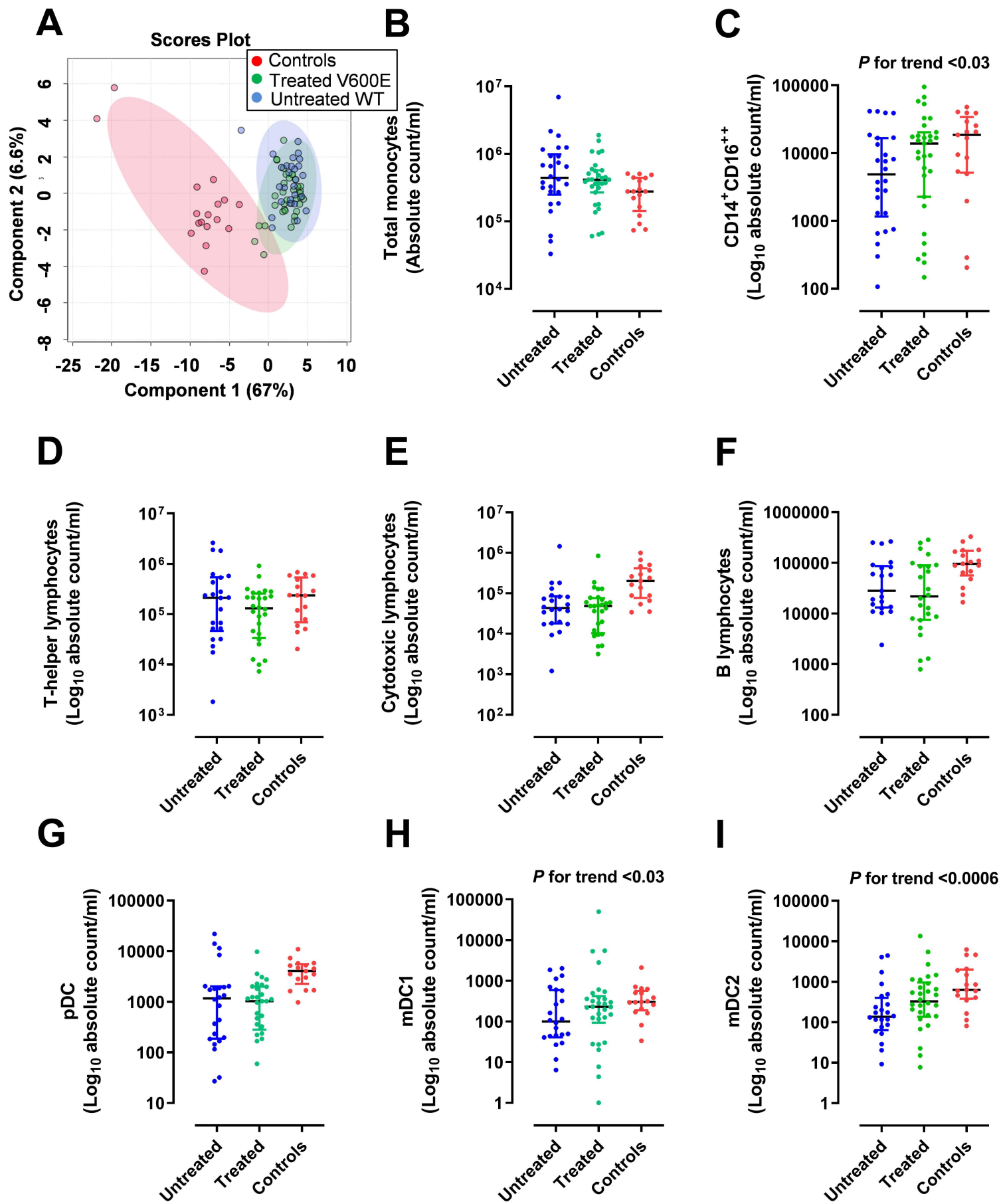


Figure 2

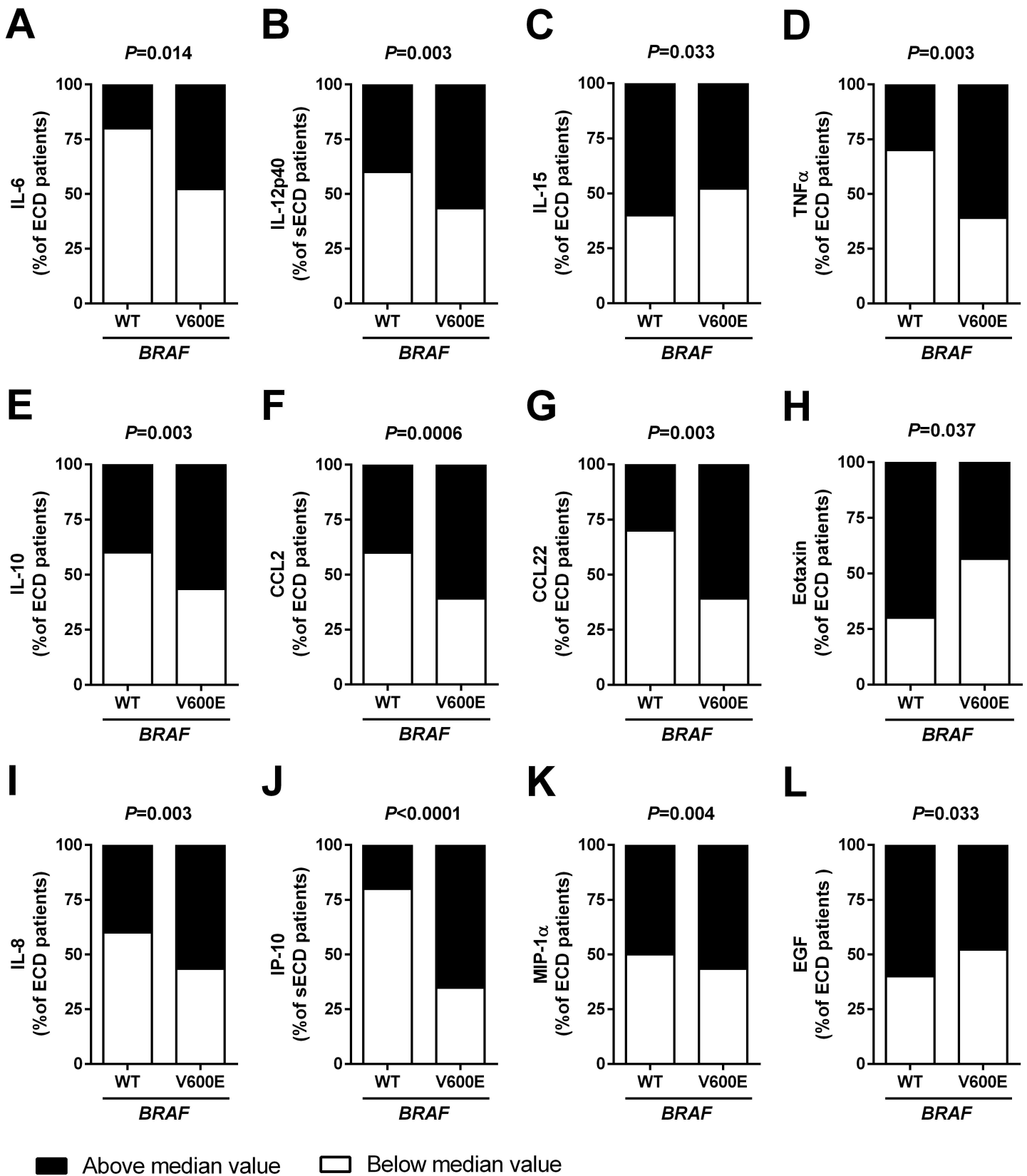
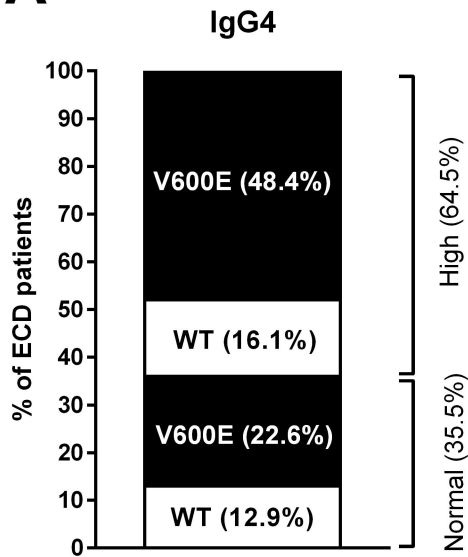
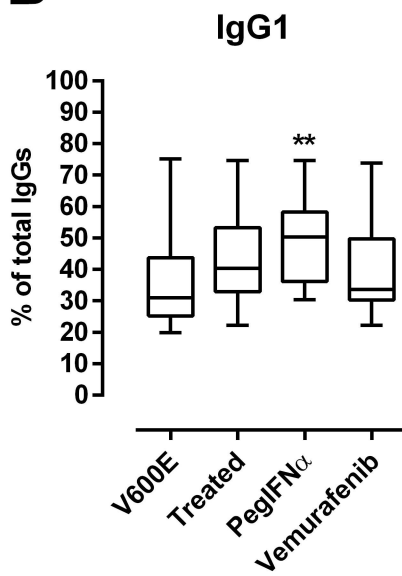
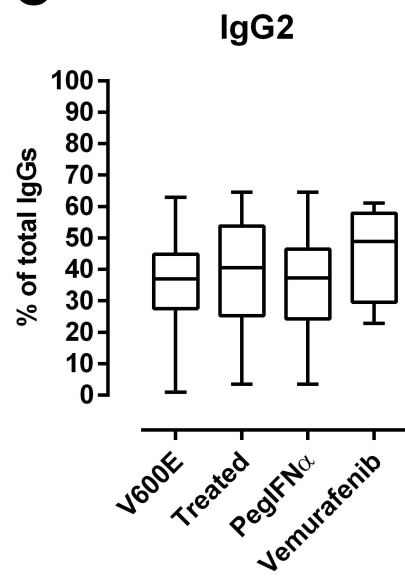
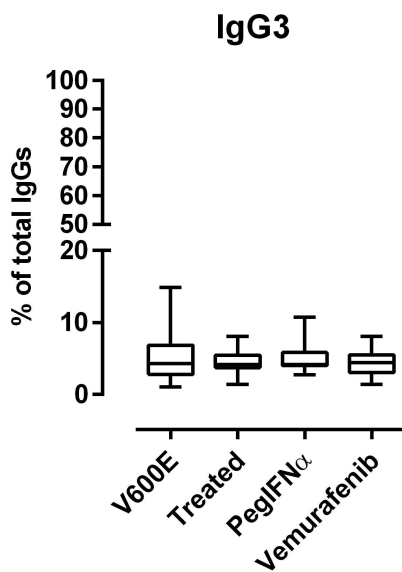
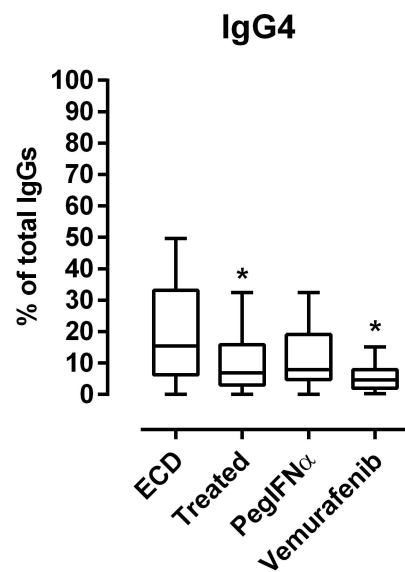


Figure 3

A**B****C****D****E****Figure 4**

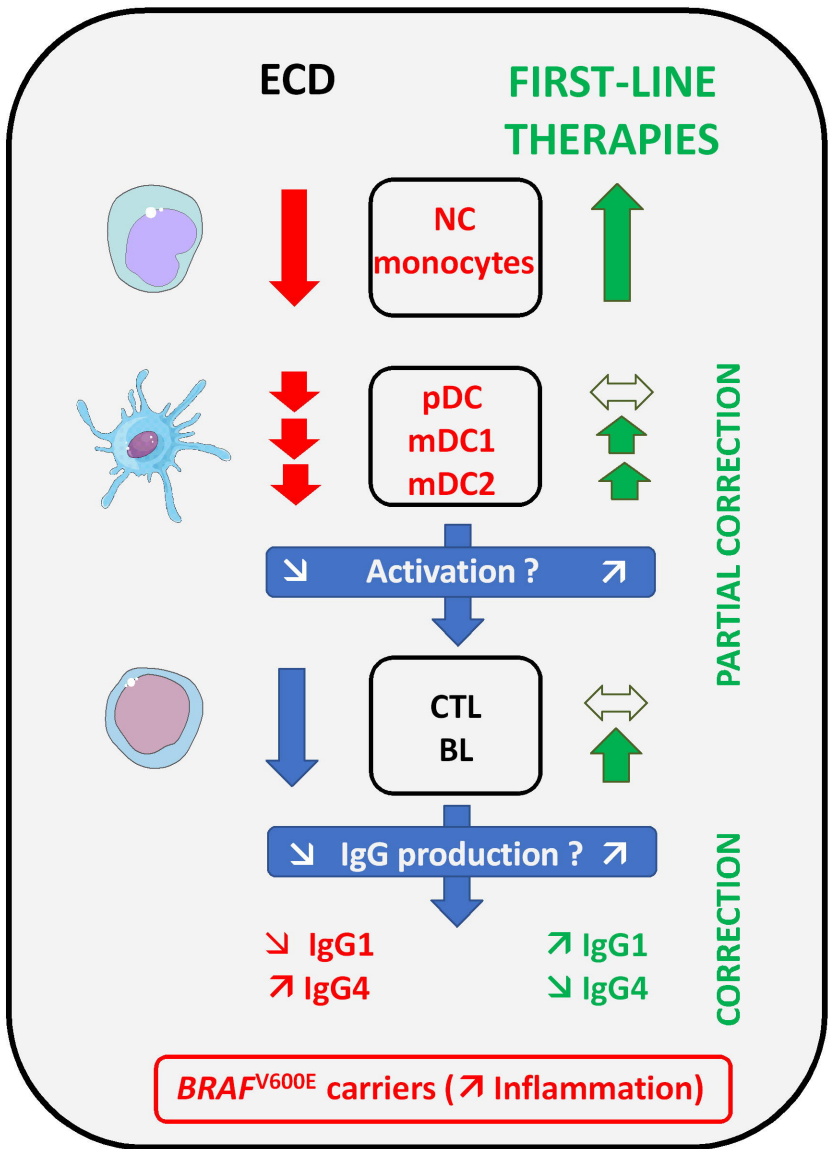


Figure 5

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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Financial support: INSERM, Sorbonne Université, French National Agency (ANR-10-IAHU-05), and the Erdheim-Chester Disease Global Alliance.

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

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

Methods.

Analysis of blood immune cells by flow cytometry.

A 100- or 300- μ l aliquot of fresh blood samples was used for immunostaining of monocytes, lymphocytes, or dendritic cells (DCs), respectively. Samples were blocked with 200 μ l 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 μ l 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 μ l (for lymphocytes and monocytes) or 1300 μ l (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-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 FORTRESSA 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- μ l 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- μ l 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	13	52±27 (21-90)		
				Females			4	56±19 (28-70)
ECD	<i>BRAF</i>^{V600E} mutation	All	78	60±14 (18-84)	Males	60	61±13 (18-84)	
					Females			18
		WT	21	63±14 (30-79)	Males	15	65±12 (30-79)	
					Females			3
		V600E	50	61±14 (18-84)	Males	37	60±14 (18-84)	
Females	13				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)		
		All (n=34)	<i>BRAF</i> ^{V600E} mutation		PegIFN α (n=16)	Vemurafenib (n=11)	All (n=27)
			WT (n=9)	V600E (n=21)			
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-1 β	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)
	IFN α 2	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)[†]
	IFN γ	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)
	TNF α	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-1 α	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-1 β	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.

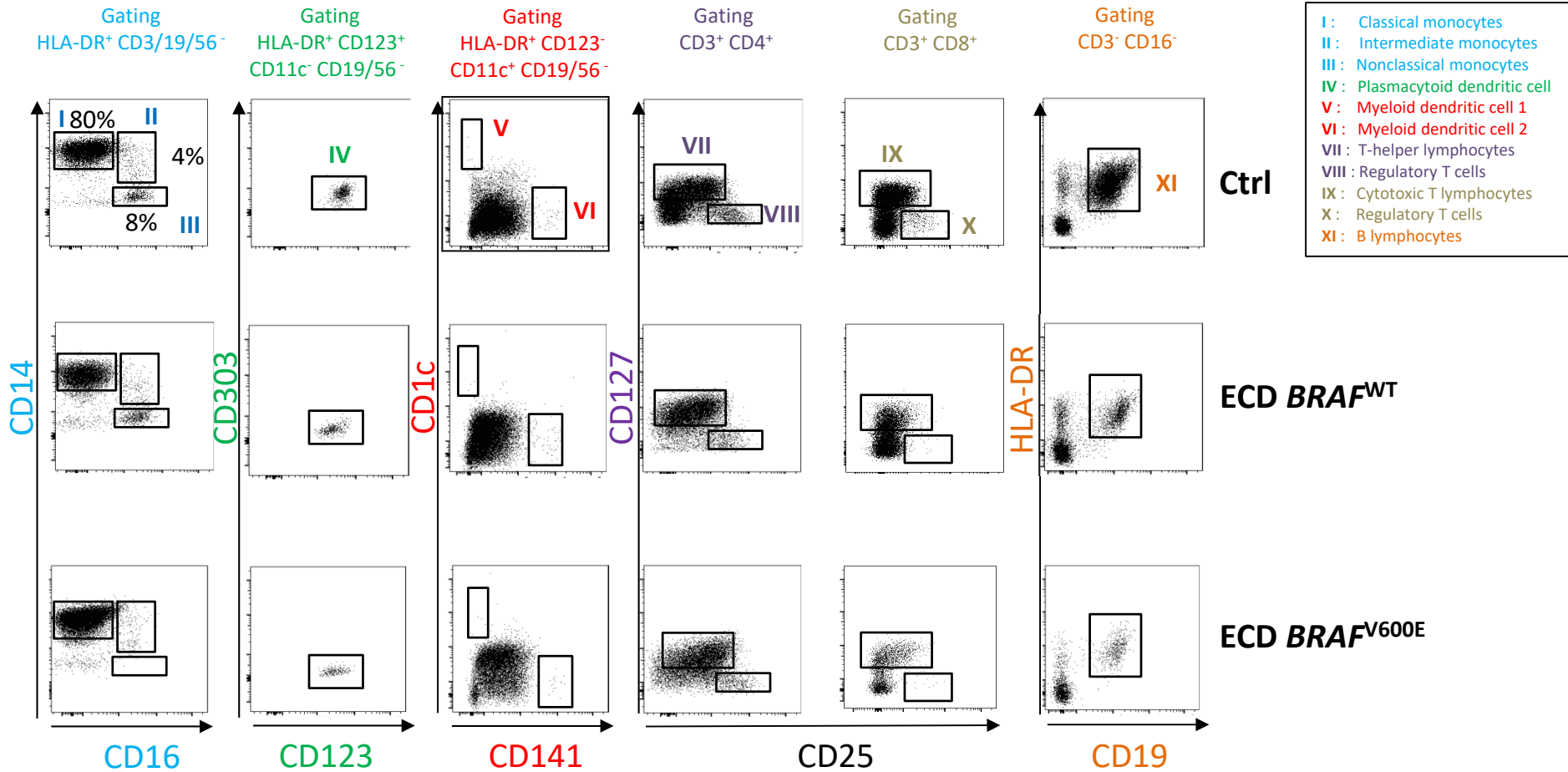
Pathway	Therapy	Monocytes				Dendritic cells			Neutrophils			Lymphocytes				
		Total	CD14 ⁺⁺ CD16 ⁻	CD14 ⁺⁺ CD16 ⁺	CD14 ⁺ CD16 ⁺⁺	pDC	mDC1	mDC2	NK	NKT	Treg	B	CT	Th		
Th1	IL-1a	0.10	0.05	-0.10	-0.03	-0.06	-0.05	-0.02	-0.10	-0.13	-0.01	-0.06	-0.02	0.00	-0.05	
	IL-1β	-0.01	-0.01	-0.14	-0.13	0.00	0.00	-0.06	-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	0.02	-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	-0.11	-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
	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
CHEMOKINES	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
	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
HEMATOPOIESIS	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.

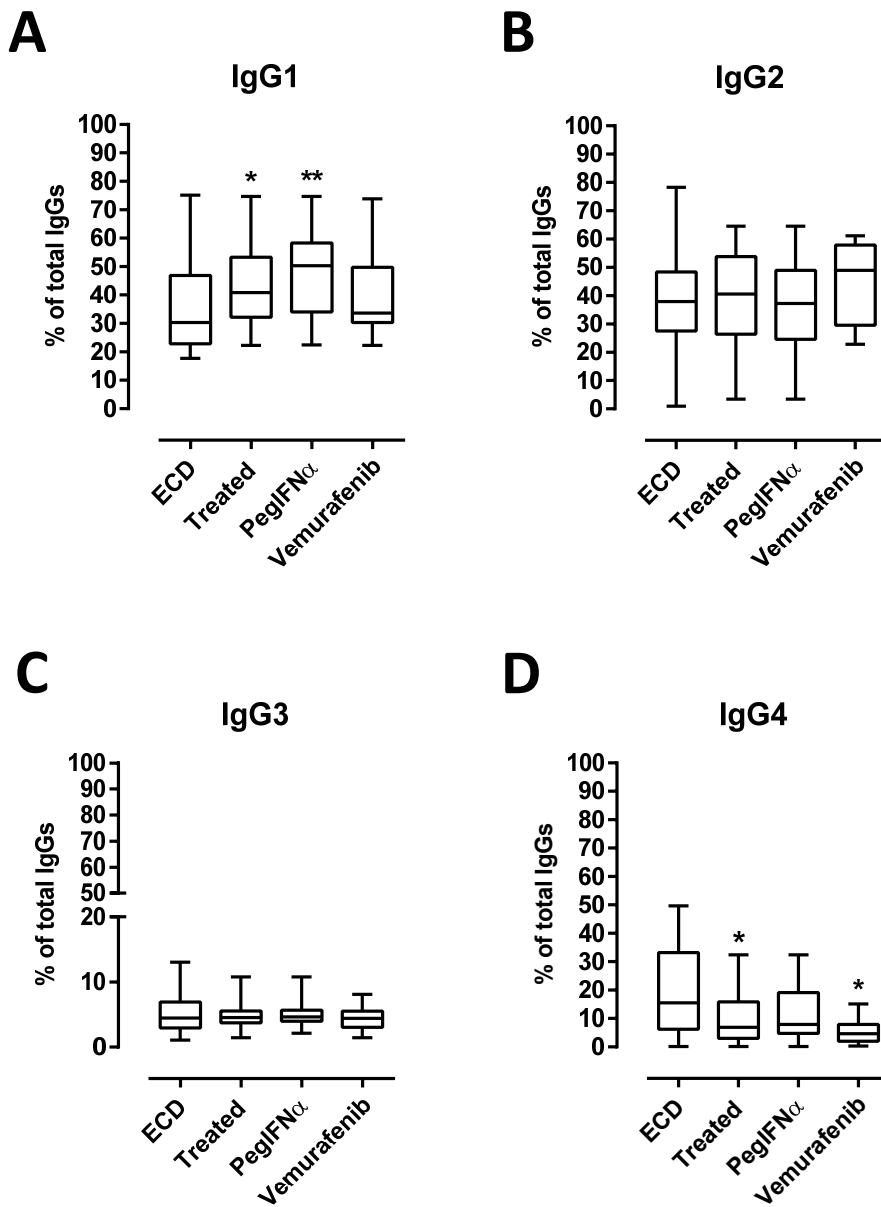
Circulating immunoglobulins (mg/dL)	Reference Values ¹²⁻¹³	Untreated ECD		
		All (n=34)	<i>BRAF</i> ^{V600E} mutation	
			WT (n=9)	V600E (n=22)
IgG1	500.0 (280.0-800.0)	589.4 (410.2-755.9)	529.1 (351.5-744.3)	620.9 (471.1-942.7)
IgG2	300.0 (115.0-570.0)	642.6 (437.9-1063.0)	660.2 (433.3-100.2)	695.4 (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 IgGs¹³, IgA¹², and IgM¹² are expressed in median (2.5-97.5 Percentiles). WT and V600E, absence and presence of the *BRAF*^{V600E} mutation.

Supplemental Figure 1



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; pegIFN α =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.