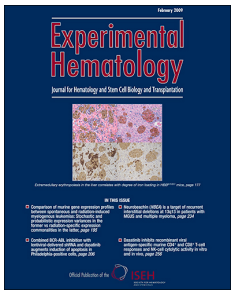
Accepted Manuscript



Title: Abnormal Regulation Of Soluble And Anchored Il-6 Receptor In Mononocytes From Patients With Essential Thrombocythemia

Authors: Nora P. Goette, Paola R. Lev, Paula G. Heller, Ana C. Glembotsky, Carlos D. Chazarreta, Juan P. Salim, Felisa C. Molinas, Rosana F. Marta

PII: S0301-472X(10)00242-0

DOI: 10.1016/j.exphem.2010.06.007

Reference: EXPHEM 2634

To appear in: *Experimental Hematology*

Received Date: 13 January 2010

Revised Date: 13 June 2010

Accepted Date: 15 June 2010

Please cite this article as: Goette NP, Lev PR, Heller PG, Glembotsky AC, Chazarreta CD, Salim JP, Molinas FC, Marta RF. Abnormal Regulation Of Soluble And Anchored Il-6 Receptor In Mononocytes From Patients With Essential Thrombocythemia, Experimental Hematology (2010), doi: 10.1016/j.exphem.2010.06.007

ABNORMAL REGULATION OF SOLUBLE AND ANCHORED IL-6 RECEPTOR IN MONONOCYTES FROM PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

Nora P. Goette, Paola R. Lev, Paula G. Heller, Ana C. Glembotsky, Carlos D. Chazarreta, Juan P. Salim, Felisa C. Molinas, Rosana F. Marta.

Sección Hematología Investigación, UE IDIM-CONICET, Instituto Alfredo Lanari, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

Corresponding author:

Nora Paula Goette, Ph.D.

Instituto de Investigaciones Médicas A. Lanari

Combatientes de Malvinas 3150

(1427) Ciudad autónoma de Buenos Aires, Argentina

Telephone/Fax: 54-11-45238947

E-mail: npgoette@gmail.com

Category: Cytokines

Paper word count: 4519

Keywords: Essential thrombocythemia , monocytes, sIL-6R, IL-6R

Abbreviations used in this paper: ET, essential thrombocythemia; DS-sIL-6R, soluble receptor for IL-6 originated from differential splicing; PC-sIL-6R, soluble receptor for IL-6 originated from proteolytic cleavage; ADAM, a disintegrin and metalloproteinase domain; FCS, fetal calf serum; TAPI, TNF-α protease inhibitor; PBMC, peripheral blood mononuclear cells.

ABSTRACT

OBJECTIVE. In a previous study, we found increased plasma sIL-6R levels in patients with essential thrombocythemia (ET) that could promote megakaryocytopoyesis through IL-6 binding and further interaction with the signal transducer gp130. Here we have searched for the cell source of sIL-6R within mononuclear cells in these patients and the underlying abnormalities involved in its overproduction.

PATIENTS AND METHODS. Thirty patients with the diagnosis of essential thrombocythemia were studied. sIL-6R levels were measured by ELISA technique in the supernatants of peripheral monocyte and lymphocyte cultures. Expression of membrane anchored IL-6 receptor was determined by flow cytometry. In order to study the mechanism of sIL-6R production, TNF-α protease inhibitor (TAPI) was added to specifically block IL-6R shedding. Gene expression of sIL-6R levels were evaluated by RT-PCR.

RESULTS. Monocytes were the main source of sIL-6R. Besides, in ET patients, monocyte sIL-6R release was higher than that of controls, $p = 0.0014$. Lymphocytes enhanced monocyte sIL-6R production by cell-mediated contact in normal controls, but this cooperation could not be seen in patients. Membrane expression of IL-6R was increased after monocyte adhesion in ET. sIL-6R synthesis was up-regulated in most patients while mRNA was normal.

CONCLUSION. Our results indicate that ET monocytes are responsible for sIL-6R overproduction within mononuclear cells through synthesis up-regulation. In addition, the lack of cooperation of lymphocytes in monocyte sIL-6R production in ET could be due to a monocyte abnormality. The agonistic effect of sIL-6R on IL-6 action could contribute to the exacerbated megakaryocytic growth in ET.

INTRODUCTION

IL-6 is a pleiotropic cytokine involved in a wide range of physiologic and pathologic states [1-4]. The ability of IL-6 to stimulate megakaryopoiesis has been largely described [5-7]. The receptor complex that mediates IL-6 intracellular signal transduction consists of two distinct membrane–bound glycoproteins, an 80 kDa specific receptor subunit (IL-6R, CD126) and a 130 kDa signal-transducing element (gp130, CD130). While gp130 is found in almost all cells, IL-6R expression is restricted to some cell types including hepatocytes and leukocyte subpopulations. In addition to the membrane-bound receptor, a soluble IL-6R form (sIL-6R) also possesses agonistic activity when bound to IL-6. The main feature of sIL-6R/IL-6 complex is its ability to interact with membrane-bound gp130 allowing the activation of cells that lack IL-6R in a process called trans-signaling [8-9].

sIL-6R is produced by two different mechanisms. On one hand, an alternative spliced mRNA gives rise to a 55 kDa protein (Differential Splicing-sIL-6R, DS-sIL-6R), lacking the transmembrane domain. On the other hand, a soluble IL-6R form can be generated by proteolytic cleavage (PC-sIL-6R) of the membrane bound receptor. The latter, also called shedding, has been proved to be inhibited by hydroxamic acid compounds [10-11]. Investigations concerning this issue showed that two members of the ADAM (a disintegrin and metalloproteinase domain) family of metalloproteases, ADAM10 and ADAM17, are involved in IL-6R shedding [12], although other proteases could have a minor role [13].

Recently published studies have shown *trans*-signaling through the IL-6/sIL-6R complex as a mechanism that maintains or even causes pathological states [14]. Essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by increased megakaryocyte proliferation and high platelet counts [15]. Although a

growing amount of data is available concerning its pathological features, the molecular abnormalities that cause this illness are not yet fully elucidated. The presence of either the activating somatic mutation of the regulatory domain of the Janus Kinase 2, JAK2V617F, or the gain-of-function *MPL* mutation, MPLW515L/K, would only account for the phenotypic profile in about 50% of ET patients [16, 17, 18, 19]. We have previously reported elevated sIL-6R plasma levels in this disorder [20]. Moreover, raised sIL-6R concentrations in conditioned media from ET mononuclear cell culture were found, indicating that these cells could be one of the sources of its increased plasma levels.

The aim of this study was to identify the cell type that gives rise to the increased sIL-6R levels among peripheral mononuclear cells in ET patients. In order to clarify the underlying abnormality responsible for its deregulation, we also studied the mechanism involved in sIL-6R production.

MATERIAL AND METHODS

Patients and controls. Thirty consecutive patients with ET were enrolled in the study. The diagnosis was based on clinical and laboratory features according to the Polycythemia Vera Study Group criteria [21]. Patients included in this study had no evidence of infectious or inflammatory diseases at the time of blood sample collection. The median age at the time of the study was 42 years old (range, 15-79), and twenty three were female. Only four patients were on treatment with anagrelide. Fifteen out of twenty-seven patients were positive for *JAK2V617F* mutation, which was analyzed using genomic DNA from total peripheral leukocytes by means of allele-specific PCR, modified from that described by Jones et al [22]. DNA from three patients was not available. Hematological parameters from ET patients were: leukocyte count, 8.8 10^9 /L

(5.4-15.8), granulocyte count, 5.95 10^9 /L (3.66-9.48), monocyte count, 0.41 10^9 /L (0.17-0.92), lymphocyte count, 2.25 10^9 /L (1.34-4.84), platelet count, 850 10^9/L (230-1365), hemoglobin levels, 13.3 g/L (10-15.8). Thirty-four healthy individuals with a similar age and gender were studied as controls. This investigation was approved by our institutional Ethics Committee (IDIM, A. Lanari) and informed consent according to the declaration of Helsinki was obtained from patients and healthy controls.

Separation of mononuclear cells. Blood samples were drawn into sterile polypropylene tubes containing 342 mmol/L EDTA. Mononuclear cells were separated by Ficoll Hypaque gradient (density 1.077 g/cm^3) (SIGMA Laboratory, St. Louis, U.S.A.). Cells were washed once with PBS supplemented with 2 mmol/L EDTA and red blood cells were lysed with 0.113 mmol/L EDTA, 1 mmol/L $KHCO_3$, 42.2 mmol/L NH_4CL. Mononuclear cell subpopulations were statistically similar in patients and normal controls. In order to minimize platelet contamination, every washing step was performed at 200 g and the supernatants were discarded.

Purification of CD14 positive cells. Monocytes were isolated by positive selection from peripheral blood mononuclear cells (PBMC) using anti-CD14 antibody directly conjugated to magnetic beads (Miltenyi Biotec, Germany). CD14 is a molecule involved in LPS-mediated activation. To examine whether the positive selection or passage through the column promote monocyte activation, we tested two markers of monocyte activation, IL-1β release and IL-2Rα on the monocyte surface. We did not find any difference in IL-2Rα expression in purified monocytes compared to CD14-positive cells present in PBMC from the same individual (n = 4). Besides, we evaluated IL-1β release from monocytes after 20-hour culture purified either by negative or by

positive immunomagnetic selection and found identical results in both samples. Cell purity was usually more than 93% pure by FACS analysis after positive selection (Becton-Dickinson, San José, CA, USA). CD14-negative cells were taken as purified lymphocytes and residual contaminating monocytes in this fraction were 0.7% (range 0.6-3%).

Cell culture. $1x10^6$ cells were incubated in 1 ml of Iscove´s Modified Dulbecco´s Medium (IMDM), (Gibco BRL, Gaithersburg, MD, USA) containing 10 % heat inactivated fetal calf serum (FCS), 15 μg/ml penicillin and 15 μg/ml streptomycin (Gibco BRL) on polystyrene plaques (TPP, Trasadingen, Switzerland) at 37ºC in 5% CO2 atmosphere. Viability of cultured cells was over 92%. After 18 hours, the supernatants were collected, centrifuged and stored at –70ºC. Assays were performed in triplicate. For metalloprotease inhibition studies, TNF-α protease inhibitor (TAPI, Peptides International, USA), was added to culture media at a final concentration of 200 μmol/L, and samples were processed as previously described.

For studies of cell cooperation, purified fractions were incubated with 1% paraformaldehyde for 5 minutes at 4ºC. Albumin 1.5% was added to inactivate paraformaldehyde and after a washing step, cells were seeded as detailed in Table 1, and cultured as described above.

*sIL-6R measurement in cell culture supernatants. s*IL-6R levels in supernatants from PBMC and purified cells culture were assayed by ELISA technique (R&D Systems, Minneapolis, USA). The lower detection limit according to the manufacturers was 6.5 pg/ml. Intra-assay and inter-assay variations were lower than 5.2% and 9.2%, respectively.

Evaluation of IL-6R on cell membrane by flow cytometry. Monocytes and T-lymphocytes were individualized by the addition of specific monoclonal antibodies (mAbs) directed against CD45 labeled with PerCP and anti CD14 and CD3 labeled with FITC (Becton-Dickinson) respectively. IL-6R was identified by incubation with anti CD126-PE antibody (Immunotech, Coulter Co). Irrelevant mAbs of the same Ig subclass were used as negative controls. Samples were analyzed by a FACScan flow cytometer (Becton-Dickinson) and results we expressed as percentage of CD126-positive population in T-lymphocytes and monocytes compared to the corresponding isotype control.

Real Time RT-PCR for sIL-6R and IL-6R expression. mRNAs for *DS-sIL-6R* and *IL-6R* isoforms (*IL-6R+sIL-6R*) and for *DS-sIL-6R* alone were detected by real-time RT-PCR. Total monocyte RNA was isolated after culture using Trizol reagent (Gibco-BRL) from 1.2 x 10^6 cells according to Chomcynski and Sacchi [23]. cDNA was synthesized from 1μg RNA using the Super Script™ preamplification system (Gibco-BRL) according to the manufacturer's instructions. Real-time PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad, Life Science, Ca) in an iCycler iQ Real-Time PCR System (Bio-Rad). Quantification was carried out using *GAPDH* expression as an internal normalization control in both PCR studies. Annealing temperature was set at 64°C in all cases. Primer sequences for *sIL-6R* were: forward 5´-GCGACAAGCCTCCCAGGTTC-3´ (directed to the specific sequence generated by alternative splicing, spanning bases 1488 to 1503 followed by bases 1598 to 1601 in the membrane *IL-6R*, which corresponds to bases 1488 to 1506 in the *sIL-6R*) and reverse 5´-GTGCCACCCAGCCAGCTATC-3´ (bases 1746 to 1765), as previously described

[24]. To quantify *IL-6R+sIL-6R* mRNA, a fragment common to both, the anchored and soluble isoforms, was amplified using the following forward 5'-CCAGCATCACTGTGTCATCC-3' and reverse 5'-TCCTGGATTCTGTCCAAGGC-3 primers (bases 1256 to 1395). Primer sequences for *GAPDH* were: forward 5´-CGACCACTTTGTCAAGCTCA-3´ and reverse 5´-CCCTGTTGCTGTAGCCAAAT-3´. Quantitative normalization of cDNA in each sample was obtained by the Δ cycle threshold (ΔCt) method (*sIL-6R* Ct – *GAPDH* Ct) or [(*IL-6R+sIL-6R)* Ct – *GAPDH* Ct]. Melting curve analysis and electrophoresis in 2% agarose gel followed by ethidium bromide staining were performed to confirm the identity of PCR products. Each sample was assayed in triplicate, and a negative control was included in each assay.

Statistical analysis. All values were expressed as median and range. Correlations between two variables were analyzed using Spearman's rank correlation coefficient. Mann–Whitney *U*-test was used to compare variables between two groups (patients and normal controls). Wilcoxon matched-pairs signed-ranks test was performed to compare monocyte IL-6sR production from different samples of the same individual. $p < 0.05$ was considered statistically significant; all *p* values presented are two-sided.

RESULTS

ET monocytes are responsible for sIL-6R increase in mononuclear cell cultures

sIL-6R in the supernatant of mononuclear cell cultures

In accordance to our previous results [20], samples from ET patients (n = 20) displayed higher sIL-6R than those from normal controls (n = 18), 124.5 (74.0-357.1) pg/10^6 cells and 85.4 (35.7-161.0) pg/10^6 cells, respectively, $p = 0.0024$. Wells seeded with platelets in a concentration similar to that found contaminating mononuclear cells ($1x10^3$ to

$1x10^5$ platelets per well) yielded undetectable sIL-6R levels. There was no correlation between sIL-6R levels in conditioned media and monocyte (r = 0.24, *p* = 0.26) or lymphocyte (r = -0.16, *p* = 0.44) percentage in the culture fraction.

sIL-6R release by purified cells

With the aim of identifying the cellular source of sIL-6R among mononuclear cells in ET patients, we performed cell culture from purified monocytes and lymphocytes separately. sIL-6R released from monocytes was higher than that released from lymphocytes in both, patients (*p* = 0.0001) and controls (*p* = 0.0002), (Figure 1). Furthermore, sIL-6R production from purified monocytes was higher in all ET patients (*n* = 18) than controls (*n* = 13), 511.9 pg/ml (243.5-815.7) vs 212.7 pg/ml (130.0-499.7), *p* = 0.0014. Monocytes from patients under anagrelide treatment produced 465.5 pg/ml (296.0- 653.9), n = 4. Notably, the amount of sIL-6R released by purified lymphocytes was very low, in some cases below the detection limit of the ELISA, in patients 23.8 pg/ml, (6-56.6) as well as in controls, 22.2 pg/ml, (0-44.3), *p* = NS. This production could be attributed to residual monocyte contamination in purified lymphocytes. Taken together, these results indicate that monocytes are the main source of sIL-6R within mononuclear cells.

In order to compare sIL-6R production rate in monocytes from mononuclear cell culture and the purified fraction, the amount of sIL-6R produced by 10^4 monocytes was calculated in both samples (mononuclear cells and purified monocytes) taking into account the percentage of CD14+ cells and the total number of seeded cells. Considering that CD14 negative cell culture produced less than 10% of that of monocytes, sIL-6R levels in wells with mononuclear cells was exclusively attributed to monocytes. sIL-6R production rate from purified monocytes was lower than that

obtained from monocytes cultured with autologous lymphocytes 4.30 (2.0-9.1) pg/10^4 cells vs 9.8 (6.2-76.1) pg/10^4 cells, $p = 0.0004$, (eight patients and six normal controls were analysed) suggesting that interaction between both cell types promotes sIL-6R release.

Cooperation between monocytes and lymphocytes in sIL-6R production

To further investigate if interaction between monocytes and lymphocytes could promote sIL-6R release we designed a cell culture protocol where monocytes were co-cultured in the presence of lymphocytes, either viable or formolized. Wells with living or formolized lymphocytes were also seeded. Four normal controls and five patients were studied (Table 1). These results clearly confirm that monocytes are the main cell source of sIL-6R. In normal samples, the sum of sIL-6R produced by isolated monocytes and isolated lymphocytes cultured separately (wells 1+4, Table 1) was lower than that obtained when both cell types were co-cultured (well 2), $p = 0.005$, demonstrating a cooperation between monocytes and lymphocytes in sIL-6R production. Moreover, the presence of either living or formolized lymphocytes in monocyte culture induced an increase in sIL-6R to a similar extent, suggesting that direct cell contact instead of soluble mediators release, is the mechanism involved in monocyte-lymphocyte interaction. Surprisingly, cooperation between monocytes and lymphocytes was not evident in patients' samples revealing an abnormal sIL-6R regulation in ET. In order to find out whether this abnormality was due to a low lymphocyte stimulation or a decrease of monocyte response to lymphocyte stimulation, ET monocytes were co-cultured in the presence of formolized autologous lymphocytes or formalized heterologous normal lymphocytes and *vice versa*. Samples from 3 patients and 3 normal controls were tested. As shown in Table 2, sIL-6R levels produced by ET monocytes in the presence of both, autologous or normal lymphocytes were similar. The same results

were found when normal monocytes were evaluated. These data strongly suggest that the lack of cooperation between monocytes and lymphocytes in ET is due to a monocyte abnormality. With the aim of testing whether soluble mediators produced by monocytes could induce lymphocyte sIL-6R production, viable lymphocytes were cultured in the presence of monocytes' conditioned media. sIL-6R levels measured in the supernatant of cultured lymphocytes in these conditions were similar to those found in monocytes' conditioned media (two samples were tested, one from a normal control and one from a patient) indicating that lymphocytes are not stimulated by monocytes' soluble mediators in our culture conditions. Finally, we analyzed whether monocytes could induce lymphocyte sIL-6R production through direct cell contact. For this purpose we cultured living lymphocytes with autologous formolized monocytes from three normal individuals. sIL-6R levels in these wells, 33 pg/ml, 20-45, were similar to those found in wells containing isolated lymphocytes (Table 1, well 4), disregarding this possibility.

Evaluation of the mechanism of sIL-6R production

IL-6R on the monocyte membrane

IL-6R expression on cell surface reflects the balance between synthesis and proteolytic cleavage. When monocytes are stimulated by shedding inducers, membrane expression of IL-6R decreases [25]. In an attempt to evaluate whether IL-6R cleavage was increased in ET patients, we studied its expression on monocyte surface by flow cytometry using anti-CD126-PE. In basal conditions, IL-6R, expressed as percentage of CD126-positive monocytes in ET patients, was similar to that found in normal counterparts, 4.7 % (1.8-32.5) (n = 11) and 6.4% (0.9-33.2) (n = 12), respectively, p = 0.22. When evaluated after mononuclear cell culture, ET monocytes had higher IL-6R

membrane expression than normal controls, 9.5% (1.5-27.0%) and 4.1 % (0.10-16.0) respectively, p = 0.03. We then calculated the difference between IL-6R membrane expression after culture (T2) and in basal conditions (T1): Δ CD126 (T2 – T1) in both, patients and normal controls. ET patients showed a positive Δ CD126, 6.5% [(-9.6)-(13.5)], compared to a negative Δ CD126 in normal controls, -4.5 [(-17.3)-(10.7)], p = 0.0364 (Figure 2). These results demonstrate IL-6R up-regulation in ET monocytes and IL-6R down-regulation on normal monocytes during culture, disregarding shedding as a mechanism underlying the increase in sIL-6R in ET. On the contrary, T-lymphocytes from both ET patients (n = 8) and normal controls (n = 7) displayed similar IL-6R membrane levels in basal conditions, 30.1 (12.5-47.5) % vs 29.5 (4.9-68.1) % respectively, p=NS, as well as after PBMC culture, 13.4 (2.1-41.7) % and 19.2 (10.3-35.9) %, respectively, p = NS. These results suggest that the IL-6R abnormalities are restricted to the monocyte lineage and that other immune cells are unaffected.

sIL-6R production after inhibition of shedding by TAPI

To confirm that shedding is not responsible for the higher production of sIL-6R found in ET, we tested the effect of direct inhibition of metalloproteases involved in IL-6R cleavage on monocyte sIL-6R release. For that purpose, we performed monocyte cultures with the addition of the metalloprotease inhibitor TAPI [11]. In these conditions, sIL-6R production would be mainly due to *de novo* synthesis although minor contribution of other proteases not inhibited by TAPI cannot be excluded [13]. When TAPI was added, conditioned media from ET monocytes (n = 11) had higher sIL-6R concentrations than those obtained from normal monocytes (n = 10), 340.7 (121.0-731.5) pg/ml vs 193.9 (82.5-335.0) pg/ml, respectively, p = 0.011 (figure 3), indicating that *de novo* synthesis of sIL-6R is increased. We then calculated the difference between

sIL-6R produced when monocytes were cultured alone (total sIL-6R) and with the addition of the metalloprotease inhibitor (TAPI-inhibited sIL-6R production), which mainly represents sIL-6R produced by shedding due to ADAM 10 and ADAM17 activity [12]. Results obtained from ET patients were similar to those of normal controls, 134 pg/ml (35.5-494.0) and 92.5 pg/ml (21.0-215.0) respectively, p=0.35, supporting the fact that IL-6R shedding is not increased in ET monocytes. Figure 4 illustrates the contribution of each mechanism to sIL-6R production in every sample (patients, left; controls, right). As shown, nine out of eleven patients had higher sIL-6R levels in the presence of TAPI than the normal median value. However, contribution of both mechanisms to sIL-6R increase was observed in patient 5.

Quantification of mRNA isoforms generated from the IL-6R gene

In order to evaluate monocyte sIL-6R production by differential splicing, *sIL-6R* RNA levels were measured in purified monocytes from 7 ET patients and 7 normal controls by real time RT-PCR. The median ΔCt value for ET monocytes was similar to that of controls, 7.54 (5.24-9.86) and 6.75 (5.97-8.98), respectively, $p = 0.8$ (Figure 4). We also investigated the expression of the total mRNA isoforms arising from *IL-6R* gene, corresponding to the soluble and anchored isoforms, by using primers that amplify a sequence common to both. The median ΔCt were 3.82 (-1.85-5.70) and 4.82 (1.5-6.3) for patients (n = 8) and normal controls (n = 8) respectively, $p = 0.28$. These results demonstrate that sIL-6R and IL-6R mRNA levels were not affected in ET patients, thus raising the possibility of a post-transcriptional abnormality.

Correlation between clinical and experimental parameters

No correlation was found between either haemoglobin levels, platelet, leukocyte, granulocyte, monocyte and lymphocyte counts and any of the experimental parameters measured in this cohort, including sIL-6R levels in mononuclear and monocyte culture supernatants, IL-6R in monocyte membrane or IL-6sR production in the presence of TAPI. sIL-6R levels in mononuclear cell culture in *JAK2V617F*-positive and *JAK2V617F*–negative patients were 139.5 pg/ml (88.0-357.1) and 123.0 pg/ml (74.7-197.3), respectively, while sIL-6R levels in monocyte conditioned media were 641.9 pg/ml (296.0-815.7) and 417.9 pg/ml (243.5-772.0), respectively. Individual sIL-6R levels and clinical data are listed in Table 3 as Supplemental Material. Monocyte sIL-6R production in the presence of TAPI was 346.7 pg/ml (212.5-731.5) and 307.7 pg/ml (121.0-524.0) in patients with or without the mutation, respectively, while IL-6R on the monocyte surface was 12.2 % (5.7-27.0) and 8.1% (1.5-22.9), respectively. Although none of these experimental parameters were significantly different in both groups of patients, it is remarkable that minimum, maximum and median values were always higher in the group of patients with the mutation (Figure 5).

DISCUSSION

Trans-signaling through sIL-6R is a key contributing pathologic mechanism in immune disorders, such as rheumatoid arthritis and inflammatory bowel disease, [1, 2, 3) as well as in non-immune entities like cancer or hypertension [26, 27]. In the present study, we have shown that monocytes from patients with ET are at least one of the sources of the raised plasma sIL-6R we have previously described in this myeloproliferative neoplasm. The elevated sIL-6R levels could contribute to the high platelet production by increasing megakaryocyte response to IL-6 [28, 29]. It is interesting to note that

thrombocytosis, the hallmark of ET, is a common feature in inflammatory and neoplastic entities, and high sIL-6R levels are also present in some of these disorders [30].

A number of reports have described sIL-6R production from normal monocytes or monocytic cell lines [12, 31]. Indeed, in normal controls, monocytes are the main source of sIL-6R in mononuclear cell culture, in the absence of stimulus other than FCS. However, lymphocytes are able to release sIL-6R under specific conditions such as TCR activation [32] and HTLV-transformation [33]. Therefore, the possible contribution of ET lymphocytes to sIL-6R increase was also considered. According to our results, sIL-6R production by non-stimulated lymphocytes was low, and contact with monocyte soluble or membrane factors was not efficient to trigger sIL-6R release by these cells. On the contrary, monocytes accounted for the sIL-6R release and reproduced the abnormal behavior seen in ET mononuclear cells in culture.

Noteworthy, sIL-6R released by normal isolated monocytes was lower than that found when monocytes were co-cultured with autologous lymphocytes, suggesting that these cells could potentiate sIL-6R release. Therefore, cooperation between both cell types was considered. We clearly demonstrate here for the first time that contact-mediated monocyte stimulation by lymphocytes increases monocyte sIL-6R release. Our results are in agreement with previously published data indicating that, contact-mediated signaling of monocytes by stimulated T cells is a potent mechanism that induces up-regulation of pro-inflammatory cytokines. However, it is important to remark that, in our study, lymphocytes were not treated with any activating agent. Interestingly, cooperation of lymphocytes in monocyte sIL-6R release was more evident in normal controls than in ET patients. When normal or ET fixed lymphocytes were tested to show their capacity to induce monocyte stimulation, we found the same response to both

stimuli, either in ET or normal monocytes, indicating a normal behavior of ET lymphocytes, and pointing to monocytes as responsible for the abnormal interaction.

In order to clarify the mechanism responsible for sIL-6R production, and taking into account that the anchored receptor could be the source of its soluble form by proteolytic cleavage, we also assessed IL-6R membrane expression in basal conditions and after cell culture. We found abnormal changes on monocyte IL-6R expression in ET patients; while the anchored receptor tended to decrease during culture in normal monocytes, as already described [34], IL-6R expression increased after 18 hours of cell culture in samples from ET patients, suggesting synthesis up-regulation. In addition, we performed monocyte culture in the presence of TAPI, a well-known inhibitor of ADAM10 and ADAM17. These metalloproteases are the main enzymes involved in IL-6R shedding. In ET patients, sIL-6R released by ADAM activity was found normal. However, a minor contribution to sIL-6R production by proteolytic cleavage due to proteases that are not inhibited by TAPI must also be considered [13]. This possibility seems unlikely as, if the latter mechanism took place in ET monocytes, a decrease in IL-6R should be noted on the cell membrane, in contrast to our findings. Overall, the absence of surface IL-6R down-regulation in ET monocytes during adhesion, together with the persistence of high sIL-6R in the culture supernatant after metalloprotease inhibition by TAPI, suggest that proteolytic cleavage is not responsible for the raised sIL-6R levels in conditioned medium, and point to synthesis up-regulation as the possible mechanism involved in the increased sIL-6R production. Considering that the elevated sIL-6R protein could be the consequence of an enhanced transcription rate, we also assessed the expression of DS-sIL-6R at the RNA level. Surprisingly, real time PCR analysis indicated that monocyte *sIL-6R* RNA was not increased, ruling out this hypothesis. Similarly, anchored *IL-6R* expression was also normal in cDNA from

adherent ET monocytes when semi-quantitative PCR analysis was carried out (data not shown) and mRNA corresponding to both *IL-6R* isoforms (*IL-6R+sIL-6R*) was not increased by Real Time RT-PCR. Of note, both proteins, sIL-6R and IL-6R, arising from the *IL-6R* gene, were found up-regulated during culture in monocytes from ET patients, while increased transcription rates were disregarded. It could be hypothesized then that IL-6R and sIL-6R protein levels are enhanced due to post-transcriptional regulation. Concerning this issue, IL-6R and/or sIL-6R protein modulation at this level has, to our knowledge, not been reported. However, post transcriptional regulation was described for other receptors, such as CXCR-4 [35]. Another possibility is that post-translational changes in both proteins lead to decreased turnover. Four N-glycosylation sites have been described for membrane IL-6R [36] but their role in protein stability has not been studied yet. Interestingly, N-linked glycosylation has been proved to be essential for the stability in gp130 [37]. Studies on IL-6R and sIL-6R stability might shed light in this matter.

During their life-time, circulating monocytes are recruited to tissues, where they adhere to matrix proteins triggering their transformation into macrophages. Cellular adhesion in culture mimics these events and macrophages can be obtained after 10 days of culture. Our *in vitro* observations of overexpression of the membrane-bound IL-6 receptor and up-regulated soluble form release could take place *in vivo* in tissue macrophages, enhancing their response to IL-6 and increasing the sensitivity of surrounding cells to this cytokine by the trans-signaling mechanism.

Monocytes have been shown to play a role in thrombosis in ET [38] as well as in other myeloproliferative neoplasms, such as primary myelofibrosis [39]. Indeed, evidence of monocyte activation, such as increased levels of membrane tissue factor [38] and elevated levels of IL-2Rα on the cell surface [40] were found in these entities. This

activated status could be the consequence of the clonal origin of ET monocytes or be triggered by monocyte interaction with activated platelets. The increased monocyte sIL-6R production we describe here is another evidence of the abnormal behavior of these cells in this disease.

In summary, the present study unveils monocytes as one of the sources of sIL-6R increase in ET patients. Abnormal regulation of the IL-6 receptor is demonstrated by up-regulation of anchored IL-6R on cultured monocytes, increased sIL-6R synthesis and abnormal monocyte response to cooperation with lymphocytes on sIL-6R production. These findings highlight the pathogenic role of monocytes in myeloproliferative neoplasms. The agonistic nature of sIL-6R on IL-6, a well-known megakaryopoietic inducer, could contribute to the exacerbated megakaryocytic growth in essential thrombocythemia.

ACKNOWLEDGEMENTS

This work was supported by grants from CONICET (PIP 5711), Agencia Nacional de Promoción Científica y Tecnológica (05-PICT 11229) and Fundación Pedro Mosoteguy.

CONFLICT OF INTEREST DISCLOSURE

The authors state that they have no conflict of interest.

REFERENCES

1- Park JY, Pillinger MH. Interleukin-6 in the pathogenesis of rheumatoid arthritis. Bull NYU Hosp Jt Dis 2007; 65: S4-10.

2- Mudter J, Neurath MF. IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm Bowel Dis 2007; 13: 1016-1023.

3- Barillé S, Bataille R, Amiot M. The role of interleukin-6 and interleukin-6/interleukin-6 receptor-a complex in the pathogenesis of multiple myeloma. Eur Cytokine Netw 2000; 11: 546-551.

4- Trikha M, Corringham R, Klein B, Rossi JF. Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. Clin Cancer Res 2003; 9: 4653-4665.

5- Nakashima K, Taga T. gp130 and the IL-6 family of cytokines: signaling mechanisms and thrombopoietic activities. Semin Hematol 1998; 35: 210-221.

6- Pang L, Weiss MJ, Poncz M. Megakaryocyte biology and related disorders. J Clin Invest 2005; 115: 3332-3338.

7- Zheng C, Yang R, Han Z, Zhou B, Liang L, Lu M. TPO-independent megakaryocytopoiesis. Crit Rev Oncol Hematol 2008; 65: 212-222.

8- Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol 2006; 80: 227-236.

9- Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling: the in vivo consequences. J Interferon Cytokine Res 2005; 25: 241-253.

10- Müllberg J, Schooltink H, Stoyan T, et al. The soluble interleukin-6 receptor is generated by shedding. Eur J Immunol 1993; 23: 473-480.

11- Mullberg J, Durie FH, Otten-Evans C, et al. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. J Immunol 1995; 155: 5198-5205.

12- Matthews V, Schuster B, Schütze S, et al. Cellular cholesterol depletion triggers shedding of the human interleukin-6 receptor by ADAM10 and ADAM17 (TACE). J Biol Chem 2003; 278: 38829-38839.

13- Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM. C-reactive protein: a physiological activator of interleukin 6 receptor shedding. J Exp Med 1999; 189: 599-604

14- Mitsuyama K, Sata M, Rose-John S. Interleukin-6 trans-signaling in inflammatory bowel disease. Cytokine Growth Factor Rev 2006; 17: 451-461.

15- Levine RL, Gilliland DG. Myeloproliferative disorders. Blood 2008; 112: 2190-2198.

16- Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005; 365: 1054-1061.

17- Kralovics R, Passamonti F, Buser et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005; 352: 1779-1790.

18- Levine RL, Wadleugh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer cell 2005; 7: 387-397.

19- Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 2006; 108: 3472-3476.

20- Marta R, Goette N, Lev P, et al. Increased levels of plasma interleukin-6 soluble receptor in patients with essential thrombocythemia. Haematologica 2004; 89: 657-663.

21- Murphy S, Peterson P, Iland H, Laszlo J. Experience of Polycythemia Vera Study Group with essential thrombocythemia: a final report on diagnostic criteria, survival, and leukemic transition by treatment. Semin Hematol 1997; 34: 29-39.

22- Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, et al. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 2005; 106: 2162-8.

23- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159.

24- Nakanishi H, Yoshioka K, Joyama S, et al. Interleukin-6/soluble interleukin-6 receptor signaling attenuates proliferation and invasion, and induces morphological changes of a newly established pleomorphic malignant fibrous histiocytoma cell line. Am J Pathol 2004; 165: 471-480.

25- Jones SA, Horiuchi S, Novick D, Yamamoto N, Fuller GM. Shedding of the soluble IL-6 receptor is triggered by Ca2+ mobilization, while basal release is predominantly the product of differential mRNA splicing in THP-1 cells. Eur J Immunol 1998; 28: 3514-3522.

26- Murakami-Mori K, Taga T, Kishimoto T, Nakamura S. The soluble form of the IL-6 receptor (sIL-6R alpha) is a potent growth factor for AIDS-associated Kaposi's sarcoma (KS) cells; the soluble form of gp130 is antagonistic for sIL-6R alpha-induced AIDS-KS cell growth. Int Immunol 1996; 8: 595-602.

27- Coles B, Fielding CA, Rose-John S, Scheller J, Jones SA, O'Donnell VB. Classic interleukin-6 receptor signaling and interleukin-6 trans-signaling differentially control angiotensin II-dependent hypertension, cardiac signal transducer and activator of

transcription-3 activation, and vascular hypertrophy in vivo. Am J Pathol 2007; 171: 315-325.

28- Sui X, Tsuji K, Ebihara Y, et al. Soluble interleukin-6 (IL-6) receptor with IL-6 stimulates megakaryopoiesis from human CD34(+) cells through glycoprotein (gp)130 signaling. Blood 1999; 93: 2525-2532.

29- Marta RF, Goette NP, Molinas FC. Megakaryopoietic cytokine levels in patients with essential thrombocythemia and their relationship with clinical and biochemical features. Medicina 2006; 66: 540-546.

30- Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. The FASEB J 2001; 15: 43-58.

31- Gallea-Robache S, Morand V, Millet S, et al. A metalloproteinase inhibitor blocks the shedding of soluble cytokine receptors and processing of transmembrane cytokine precursors in human monocytic cells. Cytokine 1997; 9: 340-346.

32- Briso EM, Dienz O, Rincon M. Soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. J Immunol 2008;180: 7102-7106.

33- Horiuchi S, Yamamoto N, Dewan MZ, et al. Human T-cell leukemia virus type-I Tax induces expression of interleukin-6 receptor (IL-6R): shedding of soluble IL-6R and activation of STAT3 signaling. Int J Cancer 2006; 119: 823-830.

34- Bauer J, Bauer TM, Kalb T, et al. Regulation of interleukin 6 receptor expression in human monocytes and monocyte-derived macrophages. Comparison with the expression in human hepatocytes. J Exp Med 1989; 170:1537-1549.

35- Labbaye C, Spinello I, Quaranta MT, et al. A three-step pathway comprising PLZF/miR-146a/ CXCR4 controls megakaryopoiesis. Nat Cell Biol 2008; 10: 788-801.

36- Varghese JN, Moritz RL, Lou MZ, et al. Structure of the extracellular domains of the human interleukin-6 receptor alpha -chain. Proc Natl Acad Sci U S A. 2002 ; 99:15959-64

37- Waetzig GH, Chalaris A, Rosenstiel P, et al. N-linked glycosylation is essential for the stability but not the signaling function of the interleukin-6 signal transducer glycoprotein 130. J Biol Chem. 2010; 285: 1781-9.

38- Arellano-Rodrigo E, Alvarez-Larrán A, Reverter JC, Villamor N, Colomer D, Cervantes F. Increased platelet and leukocyte activation as contributing mechanisms for thrombosis in essential thrombocythemia and correlation with the JAK2 mutational status. Haematologica 2006; 91: 169-175.

39- Alvarez-Larrán A, Arellano-Rodrigo E, Reverter JC, et al. Increased platelet, leukocyte, and coagulation activation in primary myelofibrosis. Ann Hematol 2008; 87:269-276.

40- Rameshwar P, Chang VT, Gascón P. Implication of CD44 in adhesion-mediated overproduction of TGF-beta and IL-1 in monocytes from patients with bone marrow fibrosis. Br J Haematol 1996; 93:22-29.

FIGURE LEGENDS

Figure 1. sIL-6R levels in the supernatant of 18-hr cell cultures. sIL-6R released from purified monocytes and lymphocytes. CD14 positive cells from 18 ET patients and 13 normal controls (C) were obtained by immunomagnetic selection and cultured for 18 hs. Negative fractions containing total lymphocytes were cultured separately in the same conditions. M and L indicates sIL-6R levels in conditioned media from monocytes and lymphocytes respectively. The median values are shown as horizontal lines in the box. sIL-6R production from ET monocytes was significantly higher than that of their normal counterparts, $p = 0.0014$.

Figure 2. Variations in IL-6R monocyte expression before and after culture. IL-6R on monocyte membrane was evaluated by flow cytometry using a specific antibody (CD126-PE). The difference between IL-6R membrane expression after culture (T2) and in basal conditions (T1), ΔCD126 (T2-T1), in ET patients (n=11) and controls (n=12) are shown, $p = 0.0364$.

Figure 3. Monocyte sIL-6R production in the presence of TAPI. a) Monocytes from 11 ET patients (ET) and 10 normal controls (C) were cultured in the presence of TAPI. sIL-6R was measured by ELISA. Y axis indicates sIL-6R levels during metalloprotease inhibition. Mann–Whitney U-test show a significant difference between groups ($p = 0.011$). b) Contributing mechanisms to sIL-6R production in each patient or c) normal control. Dark bars refer to sIL-6R released during metalloprotease inhibition, mainly produced by *de novo* synthesis. Light bars show sIL-6R produced by shedding, calculated as the difference between total and TAPI-inhibited sIL-6R release. In the

presence of TAPI, median values of released sIL-6R were 340.7 pg/ml and 193.9 pg/ml, for patients and controls, respectively.

Figure 4. Expression levels of *sIL-6R* and *IL-6R* mRNA in monocytes. Purified monocyte samples were tested for *sIL-6R* mRNA and for both isoforms (*IL-6R+sIL-6R*) by real time RT-PCR using *GAPDH* as an internal control. Results are expressed as ΔCt. a) *sIL-6R* mRNA levels in 7 ET patients and 7 normal controls, p=0.8. b) *IL-6R+sIL-6R* mRNA levels in 8 ET patients and 8 normal controls, p = 0.2.

Figure 5. Experimental data according to *JAKV617F* status. sIL-6R production by a) mononuclear cells, b) purified monocytes and c) purified monocytes in the presence of TAPI from patients with wild type *JAK2* (WTJAK2) and from patients with the J*AK2V617F* mutation. d) CD126 expression on monocyte membrane after culture in patients with *JAK2* mutation and patients without the mutation. The number of patients included in each group is shown above the box.

Figure 1

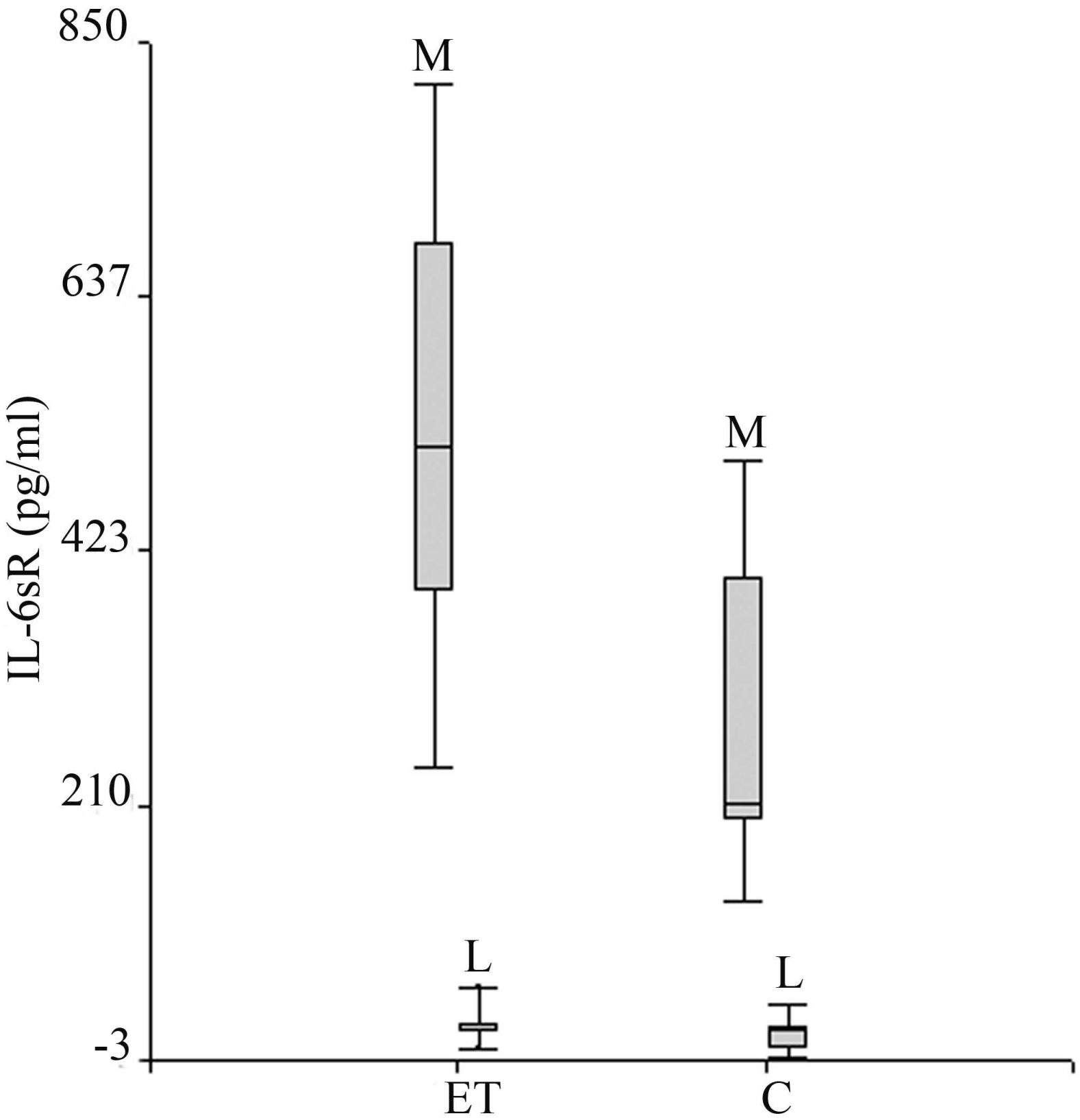
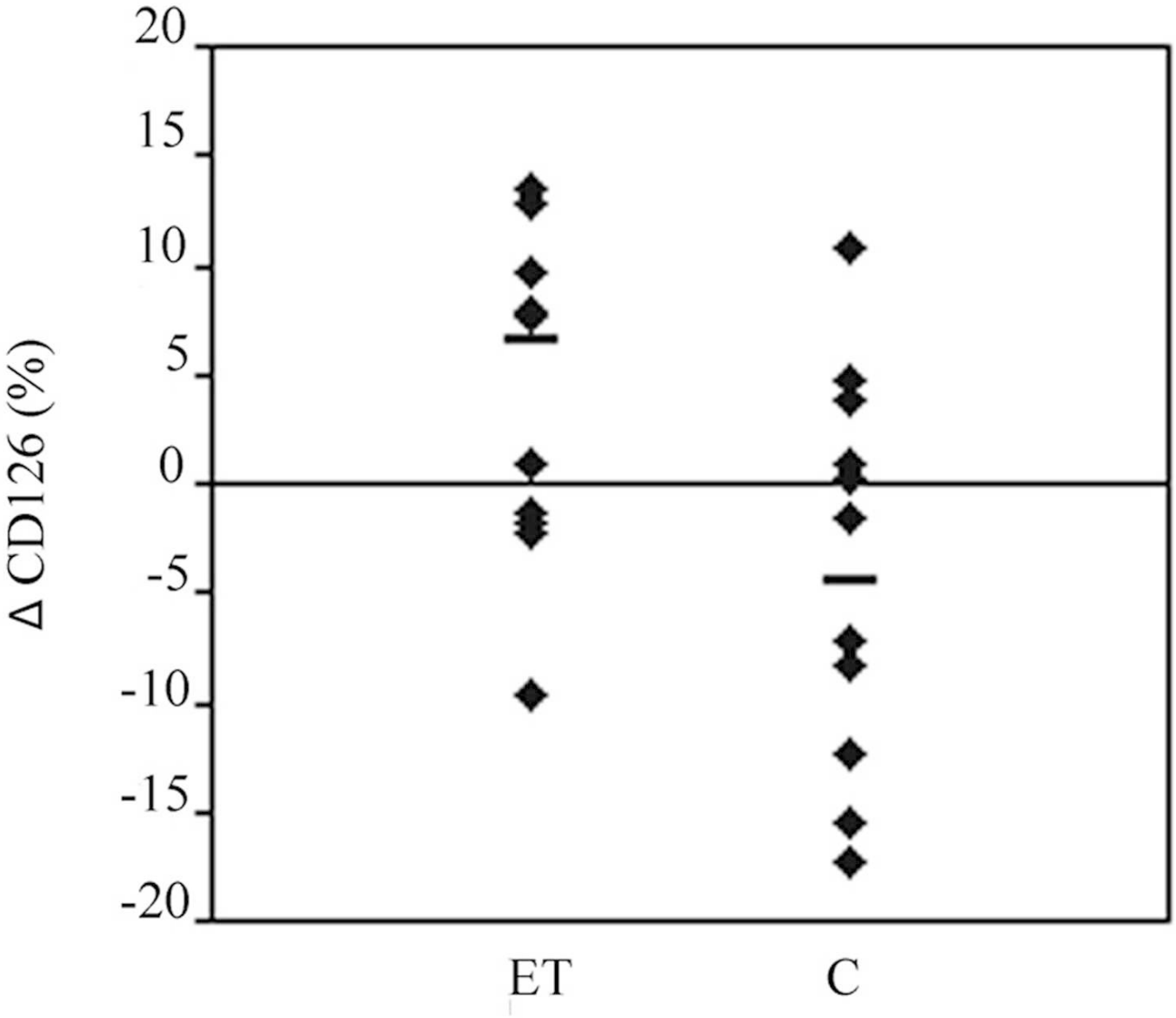


Figure 2



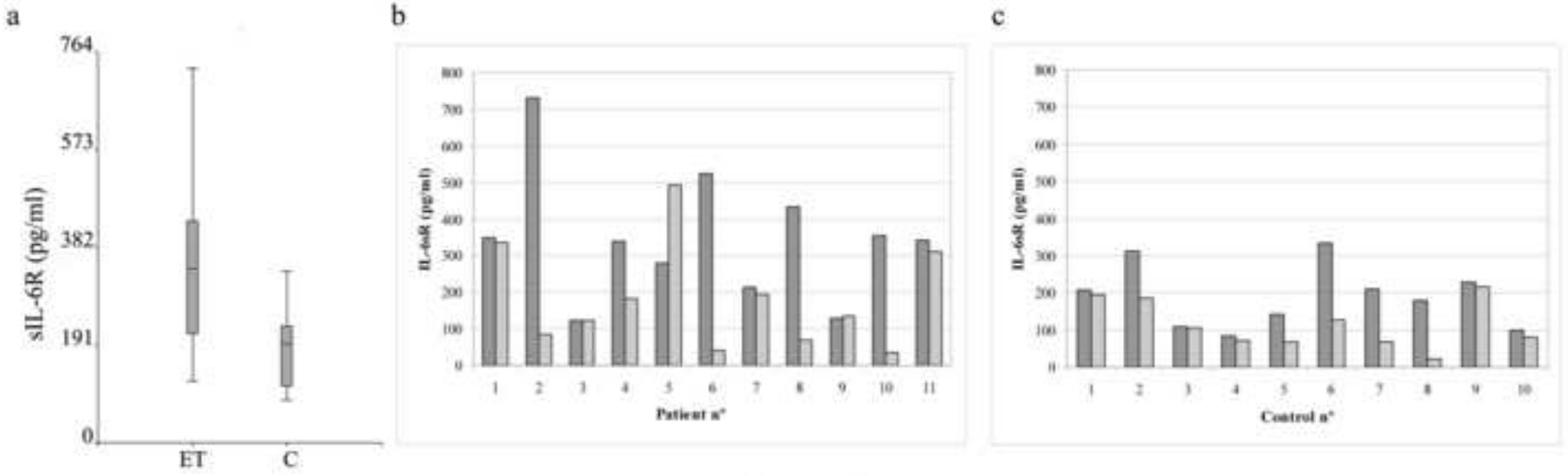


Figure 4

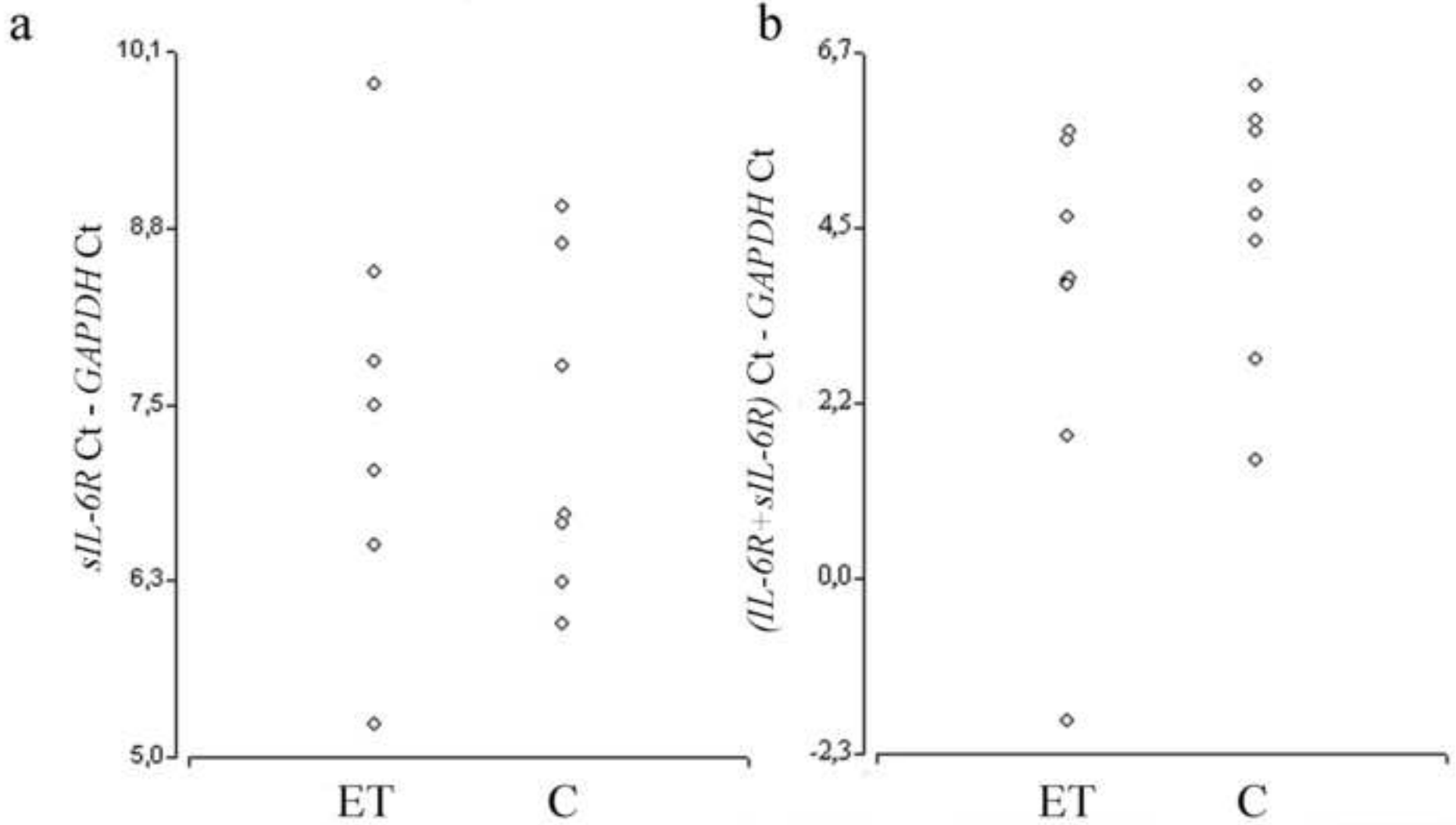


Figure 5

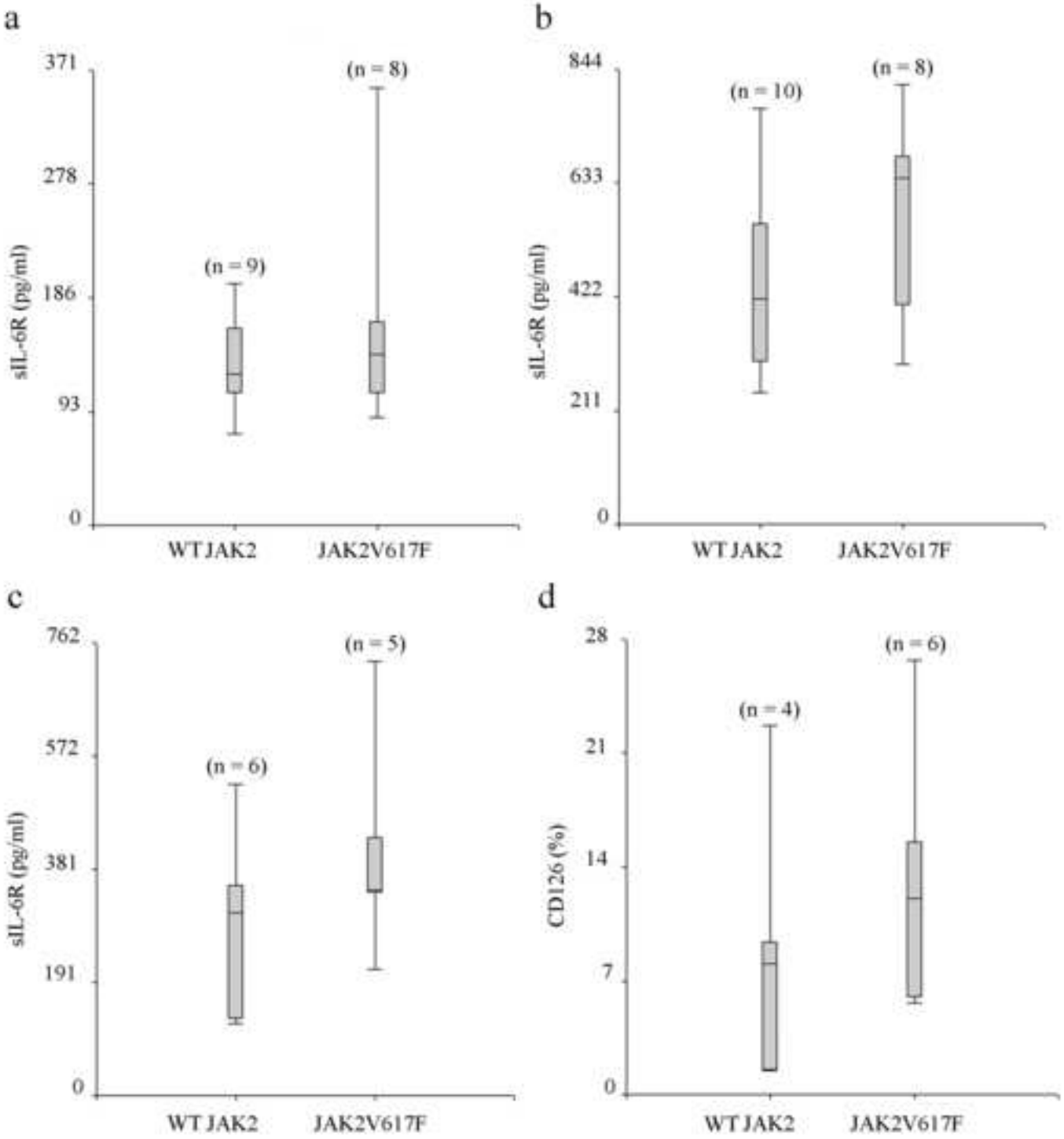


Table 1: Study of cellular interaction in sIL-6R production

Well	Cell type seeded	Patients sIL-6R (pg/ml)	Controls sIL-6R (pg/ml)
1	Monocytes	389 (296-654)	163 (108-306)
2	Monocytes + lymphocytes	414 (366-673)	276 (185-370)
3	Monocytes + formolized lymphocytes	430 (343-665)	273 (181-360)
4	Lymphocytes	42 (26-44)	10 (2-27)
5	Formolized lymphocytes	11 (2-22)	3 (0-10)

Table 2: Monocyte stimulation by autologous and heterologous lymphocytes

Patient	ET Mo+fixed autologous Lym (sIL-6R pg/ml)	ET Mo+fixed normal Lym (sIL-6R pg/ml)
P1	466.7	480.2
P2	656.8	686.3
P3	854.0	811.0
Normal control	**Normal Mo+fixed autologous Lym (sIL-6R pg/ml)**	**Normal Mo+ET Lym (sIL-6R pg/ml)**
N1	272.2	328.0
N2	441.4	431.9
N3	541.0	529.1

Abreviations: Mo, monocytes; Lym, lymphocytes; P, patient; N, normal control.

***Statement of Authorship**

This piece of the submission is being sent via mail.