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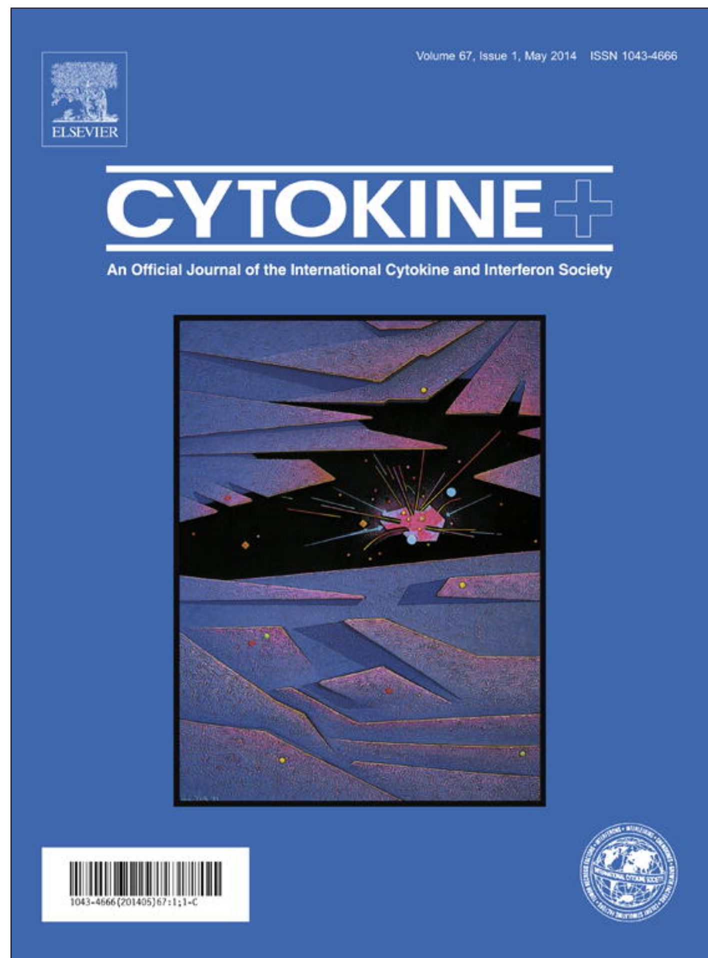

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Antimalarial drugs inhibit IFNα-enhanced TNFα and STAT4 expression in monocytes: Implication for systemic lupus erythematosus

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ABSTRACT

Objectives: To analyse the influence of IFNα on TNFα production by human peripheral blood mononuclear cells (PBMCs), as well as the possible interference of this cytokine on the effect of antimalarial drugs, TNFα regulators widely used in the treatment of systemic lupus erythematosus (SLE).

Methods: PBMCs, monocytes or T cells were treated with IFNα alone or simultaneously to cellular stimuli as well as in the presence or absence of chloroquine. Supernatants from such cultures were collected to quantify TNFα by ELISA. TNFα and STAT4 expression in cultured cells were analysed by intracellular flow cytometry. In addition, STAT4 gene expression and serum levels of TNFα and IFNα were quantified in 53 SLE patients and 45 controls.

Results: IFNα alone did not modify significantly TNFα production, but an increase was observed in stimulated PBMC. Further analyses showed that monocytes were the cellular population responsible for this effect. In addition, IFNα treatment increased STAT4 in stimulated monocytes, suggesting that TNFα upregulation could be mediated by STAT4. On the other hand, the analysis of the antimalarial effect showed that chloroquine was able to inhibit *in vitro* the expression of TNFα and STAT4 enhanced by IFNα. In antimalarial-treated SLE patients, however, only those with high IFNα serum levels presented lower expression of STAT4.

Conclusions: IFNα treatment enhances the induction of TNFα and STAT4 in stimulated monocytes, an effect inhibited *in vitro* by chloroquine treatment. However, the consequence of antimalarial treatment on SLE patients could be different depending on their IFNα serum levels.

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1. Introduction

Interferon alpha (IFNα) is a cytokine of the type I IFN family with a wide range of effects upon the immune system, including a potent host-protective antiviral response. In fact, IFNα can induce the activation, differentiation and maturation of monocytes into dendritic cells (DCs), increasing the expression of MHC and co-stimulatory molecules [1,2]. In addition, it can produce B cell activation, differentiation and antibody production [3]. These immune-stimulant functions place IFNα in a critical position bridging innate and adaptive immune responses, and suggest that IFNα could be important in the pathogenesis of autoimmunity. The pivotal role of IFNα in the pathogenesis of systemic lupus erythematosus (SLE) and other autoimmune diseases [4] is well established, since the majority of patients present increased production of type I IFN as well as IFNα-induced genes [5,6]. Moreover, Rönnblom and Alm demonstrated that immunocomplexes containing nucleic acids, characteristic of SLE serum, could trigger the production of high levels of IFNα [7] by plasmacytoid DCs through their interaction with the Toll-like receptor (TLR)-7 and TLR-9 [8,9].

The signal transducer and activator of transcription 4 (STAT4) is one of the gene products involved in the IFNα signaling, and it can be activated and phosphorylated upon ligation of the type I IFN receptor by IFNα in monocytes, macrophages, DCs, and T lymphocytes [10]. Following activation, human STAT4 binds to regulatory regions of several genes to induce proinflammatory cell-mediated immunity [11]. Interestingly, STAT4 variants are associated with susceptibility to SLE [12,13] and the STAT4 risk allele is associated with nephritis and antibodies to double-stranded DNA, two clinical features linked to the severity and activity of the disease [14].

Antimalarial drugs (hydroxychloroquine, chloroquine, and quinacrine) have been used for a long time as disease modifying antirheumatic agents in the treatment of SLE and other

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autoimmune diseases and they are still widely prescribed. It has been described that antimalarials prevent lupus flares and increase long-term survival of SLE patients [15,16], probably due to their ability to downregulate the production of TNFα and other proinflammatory cytokines, demonstrated *in vitro* by stimulated human monocytes [17–19], and in SLE patients [20,21]. Although the mechanisms for antimalarials are not completely known, it has been described they may block the TLR interaction with nucleic acid ligands by altering lysosomal acidification and disrupting endosomal maturation [22], constituting an interesting therapy for the IFNα blockade, which could prevent the activation of proinflammatory pathways. In fact, it has been previously observed a notable positive correlation between serum levels of IFNα and TNFα [23,24], both cytokines considered essential elements in the etiopathology of the SLE [5,25].

In view of all this evidence, in the present study we investigated the possible involvement of IFNα in the TNFα induction and the STAT4 upregulation, as well as the effect of the antimalarial treatment both *in vitro* and *in vivo*.

2. Material and methods

2.1. Cellular isolation

Human peripheral blood mononuclear cells (PBMCs) were obtained from standard buffy-coat preparations from routine healthy donors (Asturian Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll–Hypaque gradients (Lymphoprep, Nycomed, Norway). Monocytes and T lymphocytes were isolated by means of a Percoll gradient as previously described [2,26]. The monocyte-cell population obtained from the gradient was approximately 85% surface $CD14^+$, while T cells were more than 95% $CD3^+$, as determined by flow cytometry. Independent experiments were performed with cells from different individuals. All blood donors (the number is specified in each figure legend) were healthy adult volunteers between 18 and 60 years old without any pathology or treatment. Approval for this study was obtained from the Regional Ethics Committee for Clinical Investigation.

2.2. Culture conditions

PBMCs, monocytes and T lymphocytes were incubated at 2×10^6 cells in the presence of phytohemaglutinin (PHA, 2.5 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), chloroquine (30 μM), recombinant human IFNα2b (Intron-A, 1000 U/ml), or their combinations, in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Bio Whitaker, Belgium) supplemented with 10% heat-inactivated foetal calf serum and the antibiotics streptomycin and ampicillin (100 g/ml) in 24-well flat bottomed plates, at 37 °C in 5% CO_2.

IFNα was obtained from Schering-Plough (Canada) in vials containing 10^7 International Units (IU) that were dissolved in phosphate-buffered saline (PBS). LPS, PHA and chloroquine was provided by Sigma Chemical Co. (USA) and dissolved in culture medium.

2.3. Cytokine quantification

Culture supernatants and serum samples were collected and maintained at −80 °C until cytokine determination. TNFα levels were determined by an in-house ELISA as previously described [20,23]. IFNα serum levels were quantified by ELISA (PBL Biomedical, USA) following the manufacturer's instructions. This assay has been previously validated in 30 SLE patients and 8 healthy controls by the positive correlations observed between the amount of IFNα detected in the serum and the expression of IFNα-inducible genes in peripheral blood cells (Supplementary Table 1). The lower limit of detection was 7.5 pg/ml for TNFα and 3.12 pg/ml for IFNα.

2.4. Intracellular expression of TNFα and STAT4

Intracellular accumulation of TNFα and STAT4 was evaluated by flow cytometry in a FACSCanto II flow cytometer (Becton Dickinson, BD Biosciences, CA). PBMCs were cultured in the presence 2 μM of monensin (Sigma) for the last 8 h. Then, cells were washed and stained with the appropriate monoclonal antibody (MAb) or with the corresponding isotype-matched conjugated irrelevant MAb as a negative control. Extracellular staining of CD14 (Pacific Blue conjugated) was performed for 30 min at 4 °C. After that, cells were fixed/permeabilized (Cytofix/Cytoperm, BD Pharmingen) and intracellularly stained with anti-TNFα (peridinin chlorophyll protein [PerCP]-Cy5.5 conjugated) (eBiosciences Inc., CA) or anti-STAT4 (fluorescein isothiocyanate [FITC] conjugated) (BD Pharmingen) following the manufacturer's instructions. A minimum of 10,000 viable monocytes, gated according to the CD14 expression, were acquired and analyzed using the FACSDiva software 6.1.2 (BD Biosciences). Positive cells for each marker were determined using the corresponding isotype-matched conjugated irrelevant MAbs as negative control. The specific fluorescence intensity was quantified as the mean fluorescence intensity (MFI) calculated by subtracting the background of isotype matched control staining from the total fluorescence.

2.5. STAT4 gene expression

Sample mRNA (poli-A+) was isolated from healthy cultured monocytes or whole blood of SLE patients using the mRNA isolation kit for blood/bone marrow (Boehringer Mannheim, USA). Reverse transcription was carried out by standard procedures. Real time reverse transcriptase polymerase chain reaction (RT–PCR) (LightCycler, Roche Diagnostics) was used to quantify STAT4 mRNA by monitoring the fluorescence emitted by SYBR Green I dye, using an external standard (cDNA obtained from LPS stimulated peripheral blood mononuclear cells) to generate a calibration curve. β2-microglobulin was used as the housekeeping gene, enabling the determination of mRNA relative units. Primers employed were: CACCTGCCACAT TGAGTCAACTA and TAAGACCACCAACGTACGA for STAT4; and CCAGCAGAGAATGGAAAGTC and GATGCTGCTTA CATGTCTCG for β2-microglobulin.

2.6. SLE patients

All patients included in the study ($n = 53$) were on the Asturian SLE Register [27,28], were all Caucasian in origin, and fulfilled the American College of Rheumatology (ACR) criteria for SLE [29]. Information on clinical features during the disease course was obtained after a detailed review of clinical histories (Table 1). At the time of sampling any patient had flares of disease activity and they were asked precise questions regarding the treatment received over the previous 3 months. Sex and age-matched healthy controls ($n = 45$ (38 women and 7 men); mean age ± SD: 43.36 ± 15.74) were enlisted from the Asturian Blood Transfusion Center. Informed consent was obtained from all individuals prior to participation in the study, which was approved by the Regional Ethics Committee for Clinical Investigation.

2.7. Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data. Data from TNFα and STAT4 *in vitro* expression were represented by mean ± SD. Differences between culture

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Table 1
Demographic and clinical features of SLE patients.

Total SLE patients	53
Sex (female/male) (*n*)	50/3
Age at diagnosis, years (mean ± SD)	29.47 ± 11.60
Disease duration, years (mean ± SD)	15.19 ± 11.29
Clinical manifestations, n (%)	
Malar rash	28 (52.8)
Discoid lesions	13 (24.5)
Photosensitivity	30 (56.6)
Oral ulcers	23 (43.4)
Arthritis	43 (81.1)
Serositis	12 (26.6)
Renal disorder	14 (26.4)
Neurologicaldisorder	4 (7.5)
Haematologicaldisorder	31 (58.5)
Autoantibodies, n (%)	
ANAs	53 (100.0)
Anti-dsDNA/titer, U/ml (mean ± SD)	38 (71.7)/25.11 ± 42.09
Anti-SSa	22 (14.5)
Anti-SSb	12 (22.6)
Anti-Sm	4 (7.5)
Treatment, n (%)	
None or NSAIDs	10 (18.9)
Antimalarial drugs	29 (54.7)
Glucocorticoids/Dose, mg/day (mean ± SD)	28 (52.8)/8.14 ± 6.89
Immunosuppressive drugs[a]	6 (11.3)

dsDNA: double stranded DNA; NSAID: non-steroidal anti-inflammatory drug.
[a] Methotrexate, azathioprine, cyclophosphamide, cyclosporine A or mycophenolatemophetil.

conditions in the TNFα production in supernatants were assessed by the Wilcoxon test for paired data, and differences in the TNFα or STAT4 quantification by flow cytometry were tested by paired *T*-test. Non-parametric testing was used to determine differences between patient groups (Mann–Whitney *U*-test) and these data were represented by median (interquartil range). GraphPad Prism 5 software (GraphPad Software) and SPSS 18.0 software package (SPSS Inc.) were used for all determinations and a $p < 0.05$ was considered significant.

3. Theory/calculation

In spite of the proposed cross-regulation between type I IFNs and TNFα under homeostatic conditions, this equilibrium seems to be loss in SLE, since both cytokines are usually increased in these patients and probably involved in the etiopathology of the disease. It is known that antimalarial drugs may downregulate proinflammatory mediators, but only are able to control the disease in several patients. Thus, given the role of STAT4 in the induction of proinflammatory mediators and cell-mediated immunity through IFNα signaling in monocytes, analyzing the influence of IFNα levels and the *in vitro* and *in vivo* effect of antimalarials on STAT4 and TNFα expression should be of interest to detect a biomarker of response to treatment.

4. Results

4.1. IFNα enhances TNFα expression in LPS-stimulated monocytes

To analyse the possible influence of IFNα on the TNFα production, we firstly investigated the possible *in vitro* effect of IFNα on the expression of TNFα by human immune cells. To this end, freshly isolated PBMCs from healthy donors ($n = 24$) were incubated during 48 h with IFNα alone or in combination with LPS or PHA, and supernatants from such cultures were collected for the quantification of TNFα expression. Results suggest a faint effect on TNFα secretion of the treatment with IFNα alone compared with untreated cells (249.59 ± 425.15 *vs* 221.76 ± 254.15; n.s.). However, the presence of IFNα significantly increased TNFα secretion in PBMCs stimulated with PHA (3658.99 ± 3897.62*vs* 3276.32 ± 3364.36, $p = 0.03$) and with LPS (3247.07 ± 2844.25 *vs* 2931.73 ± 3183.25, $p = 0.01$). Then, we examined separately in monocytes and T cells the effect of IFNα at different times. As expected, IFNα as a single stimulant failed to promote significant production of TNFα in both cell types. However, the presence of IFNα during monocyte stimulation with LPS increased notably TNFα production, detecting a notable cytokine accumulation after 48 h of culture (Fig. 1a). Conversely, IFNα addition to PHA-stimulated T cells only showed a slight increase in TNFα expression (Fig. 1b).

Next, to confirm this effect at the single cell level, we analyzed IFNα induced TNFα production in LPS-stimulated monocytes by intracellular flow cytometry, thus avoiding possible effects of IFNα on monocyte survival. To this end, PBMCs were incubated for 24 h with IFNα alone, or in combination with LPS, and then TNFα expression was analysed in $CD14^+$ gated cells (Fig. 1c). In agreement with the previous data, we observed an increase in the amount of TNFα-producer cells in the presence of LPS (untreated: 4.89 ± 3.34 *vs* LPS: 19.32 ± 9.90, $p = 0.01$), that was significantly enhanced in the presence of IFNα (29.78 ± 15.03, $p = 0.03$), an effect also observed analyzing the MFI (LPS: 117.60 ± 67.86 *vs* LPS + IFNα: 184.7 ± 99.72, $p = 0.04$).

4.2. Chloroquine inhibits IFNα-induced TNFα production and STAT4 expression

It is well known that antimalarial therapy inhibits the production of TNFα and other proinflammatory cytokines [20,21], thus we wanted to determine the influence of these drugs on the production of TNFα induced by IFNα. As is shown in Fig. 2a, chloroquine treatment was able to reduce the TNFα secretion by healthy cultured monocytes in all tested conditions. Also, the analysis of intracellular cytokine accumulation (Fig. 2b) showed that chloroquine inhibits TNFα production by with LPS stimulated monocytes in the presence of IFNα in a similar manner than in the absence of this cytokine (LPS: 117.60 ± 67.86 *vs* LPS + CQ: 26.40 ± 23.04, $p = 0.01$; LPS + IFNα:184.7 ± 99.72 *vs* LPS + IFNα + CQ: 46.05 ± 20.94, $p = 0.02$).

Next, to determine the possible involvement of STAT4 in the IFNα-induced TNFα production, we analysed STAT4 protein expression in LPS-stimulated monocytes. To this end, cells were cultured in the presence or absence of LPS, IFNα and chloroquine for 24 h and then STAT4 expression was analyzed by intracellular flow cytometry (Fig. 3). As expected, IFNα treatment induced an increase in the expression level of STAT4 by LPS-stimulated monocytes ($p = 0.03$), however, the addition of chloroquine was able to inhibit this IFNα-induced STAT4 expression (LPS: 189.10 ± 120.00 *vs* LPS + CQ: 78.88 ± 34.68, $p = 0.03$; LPS + IFNα: 262.81 ± 171.60 *vs* LPS + IFNα + CQ: 96.35 ± 89.65, $p = 0.03$). Furthermore, this inhibitory effect of chloroquine on the IFNα-induced STAT4 levels in LPS-stimulated monocytes was confirmed at transcriptional level quantifying STAT4 gene expression by real time RT–PCR (LPS + IFNα: 13.92 ± 5.65 *vs* LPS + IFNα + CQ: 7.53 ± 5.66, $p = 0.01$).

4.3. Circulating levels of IFNα affect the STAT4 expression in SLE patients treated with antimalarials

Given the chloroquine ability to inhibit *in vitro* the expression of STAT4 enhanced by IFNα, in an attempt to evaluate this effect *in vivo*, we studied STAT4 gene expression and TNFα serum levels in 53 SLE patients with ($n = 29$) and without ($n = 24$) antimalarial therapy and presenting different IFNα serum levels. As we have previously reported [23], serum levels of IFNα (18.11

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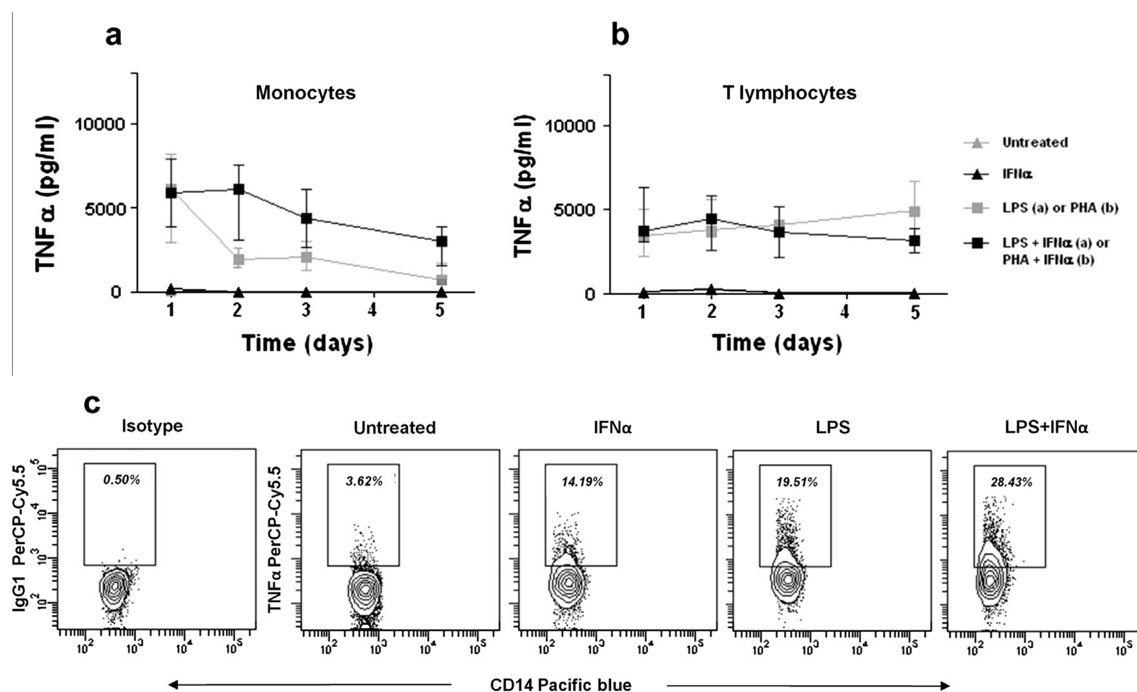

Fig. 1. Induction of TNFα secretion by IFNα in monocytes and T cells. Isolated monocytes (a) and T cells (b) were incubated at different times with IFNα alone or at the same time of LPS or PHA stimulation and supernatants from such cultures were collected for the quantification of TNFα production by ELISA. Points represent the median and interquartile range of TNFα (pg/ml) obtained in five independent experiments performed with different blood donors. Intracellular TNFα expression in IFNα- treated monocytes (c). Dot-plots represent the percentage of TNFα-producer $CD14^+$ cells after incubation for 24 h with IFNα alone or in combination with LPS. Analysis of the intracellular TNFα accumulation was performed by flow cytometry. A representative experiment was shown.

(45.46) pg/ml) and TNFα (39.24 (116.03) pg/ml) in SLE patients were elevated with respect to controls (IFNα: 5.54 (2.80); TNFα: 19.84 (71.91); pg/ml) and positively correlated in SLE patients ($r = 0.291$, $p = 0.04$, Spearman's rank correlation test). Thus, patients were classified in IFNα-low and IFNα-high using the percentile 80 obtained in healthy controls ($n = 45$) as cut off (P80 = 29.25 pg/ml). In agreement with previous results [20,21], antimalarial treated SLE patients showed strikingly lower levels of TNFα (81.14%) compared to those without such treatment (86.88 (107.03) *vs* 16.39 (60.94) pg/ml)). STAT4 gene expression tends to decrease in patients under antimalarial treatment (64.90 (52.46) *vs* 57.24 (69.49), n.s.), but, interestingly, the lowest levels, compared to the rest of patients, were observed in IFNα-high antimalarial-treated patients (68.74 (56.05) *vs* 27.72 (65.95), $p = 0.02$), whereas this treatment seems to be ineffective in reducing STAT4 expression in patients with low IFNα serum levels (Fig. 4). This reduction of STAT4 levels was not observed in patients under glucocorticoid or immunosuppressive treatments. Moreover, presence of these drugs in combination with antimalarial therapy did not influence STAT4 levels (Supplementary Table 2).

5. Discussion

During the last few years, the existence of a cross-regulation between type I IFNs and TNFα has been proposed, so that these cytokines can be regarded as opposite vectors in the immune response, remaining balanced under homeostatic conditions but losing equilibrium in specific pathological states, such as autoimmunity. In fact, several *in vitro* and *in vivo* studies showed an inhibitory effect of TNFα on type I IFNs, whereas a reciprocal suppressive effect of IFNα/β on TNFα production has also been suggested. Nevertheless, this counterbalance is controversial in several autoinflammatory diseases, in which both cytokines may exert a pathological effect.

In this work we observed that IFNα treatment increases TNFα secretion and the number of TNFα-producer cells after *in vitro* monocyte stimulation with LPS. Although several works reported a suppressive effect of type I IFNs in the TNFα production [30–34], other cellular studies suggested that this proposed inhibitory effect may be dependent on both cell type and inflammatory conditions. In this sense, it has been observed that IFNβ diminishes *in vitro* TNFα production in T-cell contact-activated monocytes, while the same cytokine enhances TNFα production in LPS-activated monocytes [35]. Moreover, previous exposure to low doses of type I IFNs enhances subsequent response to proinflammatory cytokines such as TNFα by several immune cells [36].

These data are in line with the elevated and directly correlated serum levels of TNFα and IFNα detected in SLE patients that were previously described in a large group [23,24]. Other authors observed that the -308ATNFα allele, associated with an increased production of this cytokine, was positively correlated with higher IFNα serum levels in untreated patients of dermatomyositis at disease onset, suggesting that the relationship between both cytokines could be influenced by the treatment or the disease progression [37]. It has also been described that anti-IFNα monoclonal antibody treatment down-modulated TNFα mRNA expression in peripheral blood and skin lesions in SLE patients [38].

Taking into consideration our results and the published cellular, experimental, and human data, it seems clear that IFNα and TNFα have pleiotropic effects that depend on the time, dosage and cell type, so cross-regulation between both cytokines may occur in physiological conditions, where every cytokine controls another, but is certainly not a universal principle in pathological conditions, such as autoimmunity or inflammation [39]. In fact, if cross-regulation was effective, type I IFN response program might

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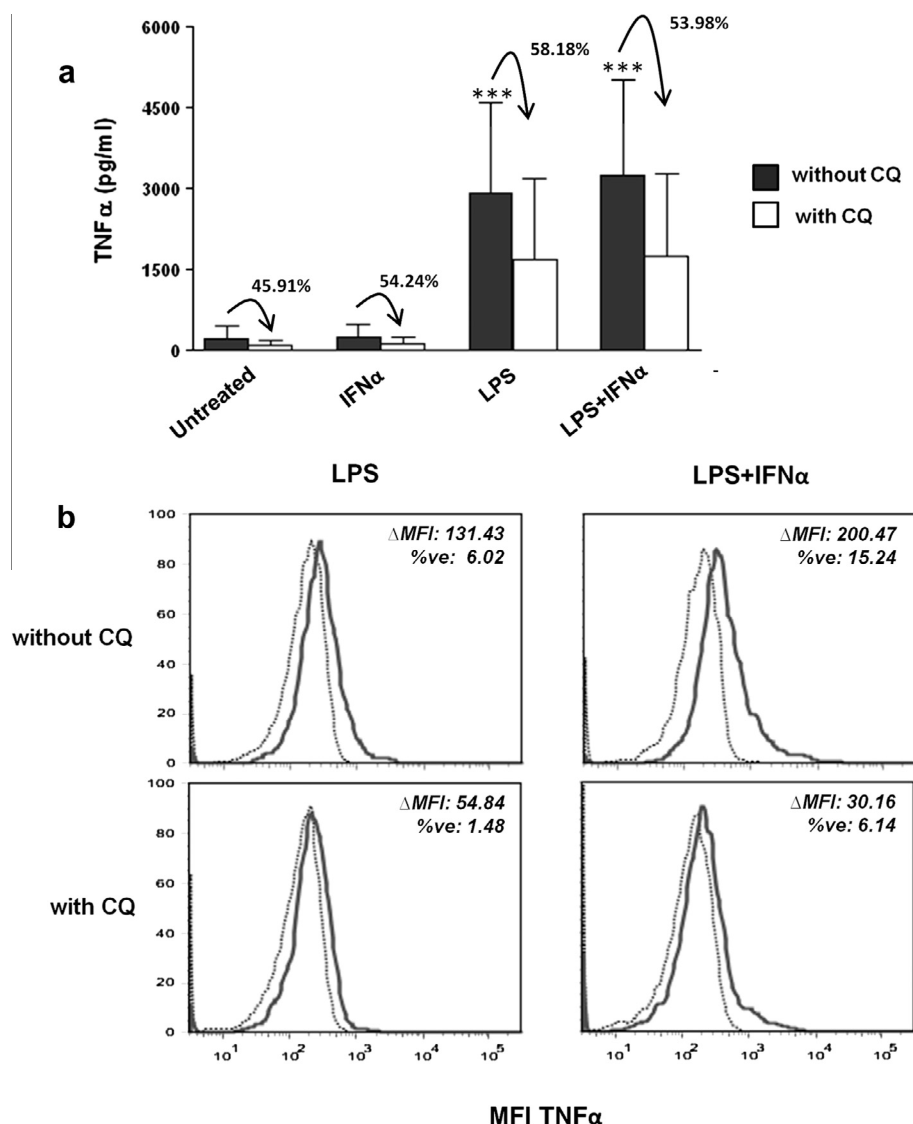

Fig. 2. Effect of the chloroquine treatment on the IFNα-induced TNFα production. Monocytes cultured for 24 h with IFNα, alone or in combination with LPS, were collected for flow cytometric quantification of intracellular TNFα while supernatants from such cultures were used for TNFα quantification by ELISA. Bars represent mean and SD of TNFα levels in the supernatants of 6 healthy blood donors. Statistical significance between presence/absence of chloroquine in each culture was assessed by Wilcoxon test for paired data ($^{***}p < 0.0001$) (a). Histograms represent the intracellular TNFα expression in a representative donor (shaded) with the respective isotype matched control antibody (black dotted line). Numbers indicate the median fluorescence intensity of TNFα staining with the matched irrelevant control value subtracted (ΔMFI) and percentage of positive cells (% ve) (b).

hamper the elevated TNFα production. However, it is well known that both IFNα and TNFα are over-expressed in many patients with SLE and other autoimmune diseases, sometimes associated with disease activity [40,41]. In such patients, the presence of nucleic acid-containing immune-complexes induces plasmacytoid DCs, and probably other cell types, to produce IFNα [42] after stimulation across TLR-7/9 [9,43]. Similarly, immune-complexes can induce TNFα production [44]. In addition, TNFα may directly stimulate the interferon pathway by inducing IFNβ [45].

Antimalarial agents, which have long been used in the treatment of rheumatic diseases, are currently prescribed to SLE patients and they have been associated with a flare reduction and severe disease exacerbations [16,46]. Despite some uncertainty regarding the exact mechanisms underlying their antiinflammatory effects, *in vitro* studies showed that chloroquine interferes with the expression of TNFα and other proinflammatory cytokines [17,19], probably by affecting the cellular lysosomal acidification [18]. Also, studies in SLE patients showed that antimalarial treatment reduces serum levels of TNFα [20,21], thus suggesting that cytokine inhibition may be involved in a beneficial outcome. Accordingly, our results confirmed that chloroquine treatment inhibits the *in vitro* production of TNFα by healthy LPS-stimulated monocytes, but, in addition, we demonstrated this inhibitory effect even when TNFα induction is enhanced by IFNα, an expected situation in most SLE patients.

Interestingly, lysosomal acidification, an effect of antimalarial agents, is necessary for the TLR activation, so these drugs have been described as antagonists of the TLR7/9 stimulation [47]. Therefore, hydroxychloroquine blocks TLR-mediated activation of pDC *in vitro*, as shown by inhibition of IFNα synthesis, either after induction by immune-complexes [9] or upon stimulation with TLR9 agonists [48,49]. Recently, it has been observed that the significant clinical improvement in SLE patients after hydroxychloroquine therapy correlated with reductions in IFNα levels [50].

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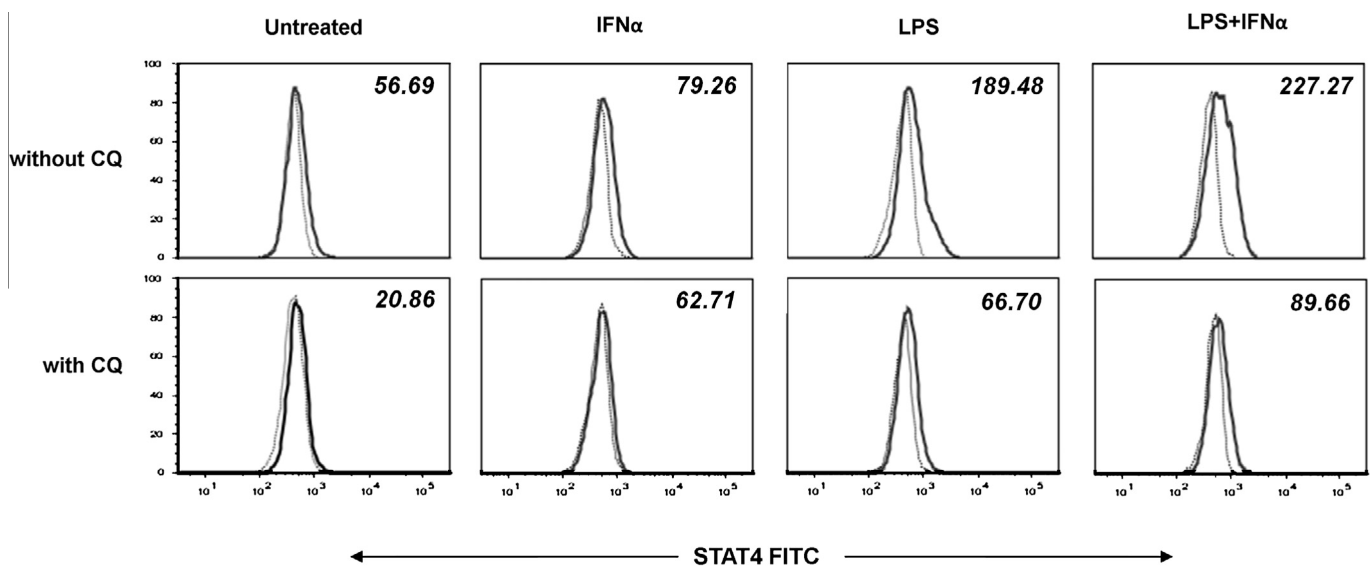

Fig. 3. Effect of IFNα and chloroquine on the STAT4 expression in stimulated monocytes. Cells from healthy donors cultured for 24 h in the presence or absence of LPS, IFNα and chloroquine were collected and intracellularly stained for STAT4-FITC expression by flow cytometry. Histograms represent the intracellular STAT4 expression in a donor as example (shaded) with the respective isotype matched control antibody (black dotted line). Numbers indicate the median fluorescence intensity of STAT4 staining with the matched irrelevant control value subtracted (ΔMFI) from one representative experiment ($n = 6$).

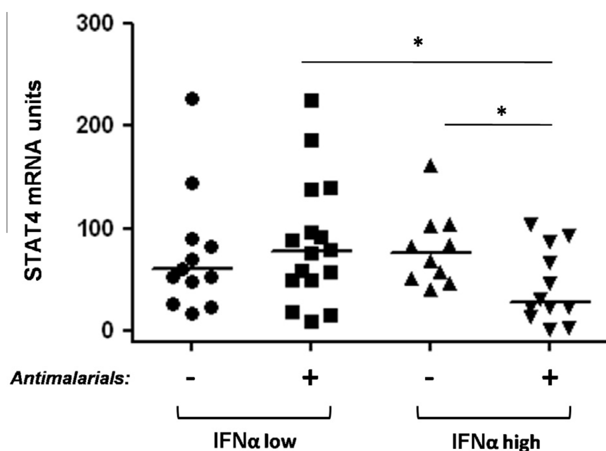

Fig. 4. STAT4 gene expression in SLE patients with and without antimalarial treatment and classified by their IFNα serum levels. SLE patients were classified as IFNα-low or IFNα-high using the percentile 80 in healthy controls as cut off. Scatter plots represent STAT4 mRNA relative units from peripheral blood cells of SLE patients with or without antimalarials. Horizontal bars show the median. Statistical significance was assessed by Mann–Whitney *U* test ($^*p < 0.05$).

Furthermore, treatment with hydroxychloroquine was associated with an impaired ability of pDCs from subjects with SLE to produce IFNα and TNFα upon stimulation with TLR7/9 agonists [51].

On the other hand, in agreement with the functional relationship between STAT4 and type I IFN receptor signaling, our results showed an upregulation of STAT4 protein expression after IFNα treatment in LPS-stimulated monocytes, in parallel to the increase in the production of TNFα. Since the binding of IFNα to its receptor leads to STAT4 phosphorylation and the subsequent transcription of several IFNα-response proinflammatory molecules in humans [10,11], our results might suggest that the IFNα-induced TNFα secretion by monocytes could be mediated by STAT4. In accordance with this hypothesis, our *in vitro* results revealed, for the first time, that chloroquine treatment was able to reduce STAT4 levels in IFNα and LPS-stimulated monocytes. Actually, IFNα is an inductor of the differentiation and maturation of monocytes into DCs, and it has been suggested that STAT4 expression could reflect a signal transduction pathway in response to IFNα in this monocyte cellular progression [52].

Finally, to confirm our *in vitro* results, we analyzed the effect of the antimalarial therapy on STAT4 gene expression and TNFα serum levels in SLE patients. As expected, antimalarial treated SLE patients showed strikingly lower levels of TNFα than those without such treatment, but, surprisingly, no significant differences were observed in STAT4 expression. We found, however, that patients under antimalarial treatment and high IFNα levels, showed diminished STAT4 expression, whereas this therapy seemed to be ineffective in reducing STAT4 in patients with low IFNα levels. Thus, STAT4 expression in patients treated with antimalarials depends on the circulating amounts of IFNα, those patients with high levels of IFNα presenting the lowest expression. A possible explanation for the reduced antimalarial-mediated down regulation of STAT4 in the IFNα low group of patients should be related to the presence of certain allelic variants of STAT4 known to constitute a risk factor for SLE and that has been associated with low IFNα serum activity but greater sensitivity to type I IFN signaling in SLE patients [53]. On the other hand, SLE patients with the STAT4 non-risk genotype displayed the commonly observed high IFNα production, which seems to be required for STAT4 downregulation after antimalarial treatment. Therefore, this therapy might be more effective in patients carriers of the wild STAT4 genotype presenting high IFNα levels.

Moreover, gene polymorphisms at the interferon regulatory factor 5 (IRF5), a major mediator of TLR-triggered expression of type I IFN [53] and other proinflammatory cytokines, including TNFα [54], act additively with STAT4 genotype to increase the risk for SLE [13,55]. This fact could be explained by the functional link between both molecules, since the risk allele of STAT4 determines a higher frequency of anti-dsDNA antibodies in SLE patients, allowing a continued type I IFN stimulation of the STAT4-dependent autoimmune response [13,53].

All these data suggest that antimalarial treatment could be able to reduce STAT4 expression by blocking the interaction between TLR and immune-complexes, thus avoiding the IRF5 action and interfering with IFNα induction. However, in SLE patients with low IFNα production, a feature associated with a low presence of autoantibodies [24] and TNFα [23,56], antimalarial therapy could

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be ineffective in reducing STAT4 expression, thus suggesting the use of IFNα as a possible biomarker of the clinical response to antimalarials.

6. Conclusions

The present study showed an enhanced *in vitro* effect of IFNα on the TNFα induction in LPS-activated monocytes from healthy individuals, which could explain the positive correlation observed in SLE patients between both cytokines. This IFNα upregulatory effect was also observed in the levels of STAT4, being both TNFα and STAT4 inhibited when chloroquine was added during stimulation of healthy monocytes. However, results obtained in SLE patients suggest that STAT4 expression is not always decreased during antimalarial treatment, but depends on the circulating levels of IFNα, which could contribute to an immune activation situation resulting in STAT4 and TNFα induction. Therefore, antimalarial therapy may be especially beneficial to improve SLE pathogenesis in patients with an overactive IFNα pathway, which thus can be blocked.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2014.02.002.

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