Extra-ocular muscle cells from patients with Graves' ophthalmopathy secrete α (CXCL10) and β (CCL2) chemokines under the influence of cytokines, that are modulated by PPARγ

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Running Title: CXCL10 and CCL2 in Graves' ocular myopathy

Abstract

To our knowledge, no study has evaluated the involvement of T helper (Th)1- and Th2-chemokines in extra-ocular muscle (EOM) myopathy in "patients with thyroid-associated ophthalmopathy" (TAO-p).

We tested the effects of interferon (IFN)γ and tumor necrosis factor (TNF)α stimulation, and of increasing concentrations of peroxisome proliferator-activated receptor (PPAR)γ agonists (pioglitazone or rosiglitazone; 0.1 μM-20 μM), on Th1-chemokine [C-X-C motif ligand (CXCL)10] and Th2-chemokine [C-C motif ligand (CCL)2] secretion in primary EOM cultures from TAO-p vs. control myoblasts. Moreover, we evaluated serum CXCL10 and CCL2 in active TAO-p with prevalent EOM involvement (EOM-p) vs. those with prevalent orbital fat expansion (OF-p).

Serum CXCL10 was higher in OF-p and EOM-p vs. controls, while serum CCL2 was not significantly different in controls, or in OF-p and EOM-p. We showed the expression of PPARγ in EOM cells. In primary EOM cultures from TAO-p: a) CXCL10 was undetectable in the supernatant, IFNγ dose-dependently induced it, whereas TNFα did not; b) EOM produced basally low amounts of CCL2, TNFα dose-dependently induced it, whereas IFNγ did not; c) the combination of TNFα and IFNγ had a significant synergistic effect on CXCL10 and CCL2 secretion; d) PPARγ agonists have an inhibitory role on the modulation of CXCL10, while stimulated CCL2 secretion.

EOM participate in the self-perpetuation of inflammation by releasing both Th1 (CXCL10) and Th2 (CCL2) chemokines under the influence of cytokines, in TAO. PPARγ agonists activation plays an inhibitory role on CXCL10, but stimulates the release of CCL2.

Keywords: CXCL10; CCL2; Graves' ophthalmopathy; extra-ocular muscles; chemokines

1. Introduction

During thyroid-associated ophthalmopathy (TAO) orbital tissues become inflamed and are remodeled. TAO occurs with a variable presentation: in some patients, extra ocular muscles (EOM) enlargement predominates, while in others, the connective/adipose tissue enlargement appears the most significant problem, or both EOM and the connective/adipose tissue are involved.

The frequency of EOM involvement and diplopia in patients with Graves' disease (GD) [1] ranges from 5–10% [2] to 49% of the patients [3]. There is also a minority of patients whose endocrine orbitopathy consists almost only of involvement of the EOM [4].

A complex interplay among orbital fibroblasts, myocytes, immune cells, cytokines, autoantibodies, genetics and environmental factors cause the dramatically enlarged EOM and increased orbital fat (OF) in TAO [5]. However, clear and indisputable identification of a target antigen has not been established. In this scenario, autoantibodies specific for fibroblast surface TSH-receptor (TSH-r) and IGF-1 receptor (IGF-1r) are proposed initiators of orbital inflammation [5]. Interestingly, TSH-r protein is expressed also in EOM [6]. However, increased TSH-r and IGF-1r expression occurs with adipogenesis, providing an alternative, non-causative explanation for their presence in TAO orbits [5, 7].

The nature and significance of antibodies targeting EOM and orbital connective tissue (OCT) antigens have also been studied by other studies, suggesting that autoimmunity against the EOM antigen calsequestrin and the OCT antigen collagen XIII has an important role in the pathogenesis of TAO [8, 9], or that eye-muscle stimulating antibodies were demonstrable in sera of patients with TAO [10].

EOM participate in the pathogenesis of inflammation producing cytokines, whatever the primary target antigen. In fact, interferon (IFN)γ, tumor necrosis factor (TNF)α, interleukin (IL)-1beta, and IL-6 mRNA were mainly detected in EOM tissue [11], suggesting that T

helper (Th)1-like cytokines predominate in EOM tissue in most patients, probably playing a role on the development of eye muscle component of TAO in the acute stage [12].

Recent data have shown that C-X-C α-chemokines (Th1), in particular chemokine (C-X-C motif) ligand (CXCL)9, CXCL10 and CXCL11, play an important role in the initial phases of autoimmune disorders [13-15]. Serum CXCL10 levels are increased in GD, especially in patients with active disease, and the CXCL10 decrease after thyroidectomy [16] or after radioiodine [17] shows that it is more likely to have been produced inside the thyroid gland. Furthermore, patients with newly diagnosed autoimmune thyroiditis show increased serum CXCL10, in particular in the presence of a more aggressive thyroiditis and hypothyroidism [13, 14, 18].

The secretion of CXCL10, CXCL9 and CXCL11 in primary cultures of TAO fibroblasts and preadipocytes can be stimulated by IFNγ, and TNFα [19], suggesting that these cells participate in the self-perpetuation of inflammation by releasing chemokines (under the influence of cytokines) and inducing the recruitment of activated T cells in the thyroid. The IFNγ-stimulated C-X-C chemokine secretion was significantly inhibited treating orbital cells with peroxisome proliferator-activated receptor (PPAR)γ activators, at near-therapeutical doses, strongly suggesting that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in TAO [19].

Until now, no study has evaluated the chemokines expression in EOM in TAO. We aimed to: 1) compare serum CXCL10 and chemokine [C-C motif ligand (CCL)2] levels in patients with active TAO (TAO-p) with prevalent EOM involvement (EOM-p) in comparison with those with prevalent OF expansion (OF-p); 2) test the effect of IFNγ and/or TNFα stimulation on the secretion of the prototype Th1 (CXCL10), and Th2 (CCL2) chemokines in primary cultures of orbital EOM myoblasts; 3) assess the effect of PPARγ activation on CXCL10 and CCL2 secretion in EOM myoblasts.

2. Materials and Methods

2.1 In vivo studies

2.1.1 Patients

We selected 26 consecutive Caucasian patients with GD and with active TAO and 26 age- and sex-matched controls from our outpatient clinic (**Table 1**). The selection criteria included the presence of exophthalmos, and: 1) expansion of OF, without evident EOM at orbital computed tomography (CT); 2) EOM muscle enlargement without OF expansion at CT; 3) all mixed forms (presence of both, OF expansion and EOM enlargement, were excluded). The diagnosis of GD was established from the clinical presentation [20].

All TAO-p were clinically euthyroid on antithyroid drugs (16 patients), levo-thyroxine (6 patients) or spontaneously (4 patients), at the time of evaluation and eye disease activity was assessed by the Clinical Activity Score [20]. A score of 5 (maximal score=10), including a worsening over the previous 2 months, was considered indicative of active TAO. Inactive eye disease was defined as no changes in eye status over the previous 6 months. Considering these 26 patients, 21 had never received immunosuppressive therapy, 3 had been previously treated with corticosteroids, 1 with orbital irradiation, and 1 with both; a median of 11 months (range 6-42) had elapsed from the end of treatment. Total Eye Score was calculated as the sum of the products of each NOSPECS class by its grade (to this purpose, we substituted 1, 2 and 3, respectively, for grades a, b and c) [20]. We recorded the duration of both the eye and the thyroid disease since their first signs and symptoms.

2.1.2 Controls

We enrolled a control group of 26 sex- and age (± 5 years)-matched subjects extracted from a random sample of the general population from the same geographic area of the patients, in whom the presence of thyroid disorders was excluded by a complete thyroid work-up [18].

A blood sample was collected in the morning after an overnight fasting, and serum was kept frozen until the measurement of thyroid hormones, thyroid autoantibodies, CXCL10 and CCL2, in both patients and controls. All study subjects gave their informed consent to participate in the study, which was approved by the local Ethical Committee.

2.2 In vitro studies

We investigated the effects of IFNγ, TNFα and PPARγ agonists on the release of CXCL10 and CCL2 in primary cultures of human myoblasts.

2.2.1 Human myoblasts cultures

EOM samples were obtained from 5 patients operated on for EOM repair or decompression (all previously treated with antithyroid medication and systemic corticosteroids, euthyroid at the time of surgery; none treated with orbital radiotherapy). Control myoblasts were obtained from M. rectus abdominis in 5 patients undergoing abdominal surgery. Human skeletal muscle cells were prepared as previously reported [21].

Cells were isolated from EOM, or M. rectus abdominis, with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination with fibroblasts. After two passages, the myoblasts were characterized by the manufacturer (PromoCell, VWR International PBI S.r.l., Milan, Italy) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100% confluence (8 days). These cells were grown to confluence in 25 cm^2 flasks, trypsinized, and subsequently $1x10^6$ cells were seeded in 75 cm^2 flasks. After two passages, 5–7.5 x 10^7 cells were harvested and stored until further use as frozen aliquots containing $2x10^6$ myoblasts. For each experiment, 10^5 cells per well were seeded in six-well culture plates and cultured in α-modified Eagle's/Ham's F-12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack (PromoCell) to near confluence. The cells were then differentiated and fused by culture in modified Eagle's

157 medium supplemented with 2% fetal calf serum (FCS) for 7 days. The myocytes were
158 cultured in differentiation medium without FCS for 24 h before being used for any experiment
159 [21].

160 ***2.2.2 CXCL10 and CCL2 secretion assay***

161 We seeded 3000 cells onto 96-well plates in growth medium and after 24 h the growth
162 medium was removed and cells were accurately washed in phosphate-buffered saline, and
163 incubated in phenol red and serum-free medium. Cells were treated with IFNγ (R&D Systems,
164 Minneapolis, MN, USA; 0, 500, 1000, 5000, 10000 IU/mL) and 10 ng/mL TNFα (R&D
165 Systems), alone or in combination [19], for 24 h. The concentration of TNFα was selected in
166 preliminary experiments to yield the highest responses. Then, the supernatant was removed
167 and frozen at –20°C until assays.
168 Moreover, cells were stimulated with IFNγ (1000 IU/mL) and TNFα (10 ng/mL) for 24 h in
169 the absence or presence of increasing concentrations (0, 0.1, 1, 5, 10, 20 μM) of the pure
170 PPARγ agonists, rosiglitazone (RGZ, GlaxoSmithKline, Brentford, UK), or pioglitazone
171 (Alexis Biochemicals, Lausen, Switzerland). Conditioned medium was assayed by enzyme-
172 linked immunosorbent assay (ELISA) for CXCL10 and CCL2 concentrations. All
173 experiments were repeated 3 times with the 10 different cell preparations.

174 ***2.2.3 Cell cultures and PPARγ agonists treatment***

175 Myoblasts were treated with 0.1, 1, 5, 10, or 20 μM RGZ or pioglitazone for 24 h, while
176 control cultures were grown in the same medium containing vehicle (absolute ethanol, 0.47%
177 v/v) without RGZ or pioglitazone for 24 h. Some cultures were examined by phase contrast
178 microscopy by an Olympus IX50 light microscope (New Hyde Park, NY).
179 For quantitation of total protein in cell preparations, lysis and homogenization were performed
180 and the sample was assayed for its protein concentration by conventional methods [19].

181 ***2.2.4 ELISA for CXCL10 and CCL2***

CXCL10 and CCL2 levels were measured in serum and culture supernatants, by a quantitative sandwich immunoassay with a commercially available kit (R&D Systems). The mean minimum detectable dose for CXCL10 was 1.35 pg/mL; the intra- and inter-assay coefficients of variation were 3.1% and 6.8%. The mean minimum detectable dose for CCL2 was 4.6 pg/mL; the intra- and inter-assay coefficients of variation were 4.6% and 5.7%. Quality control pools of low, normal, or high concentration for all parameters were included in each assay.

2.2.5 Reverse transcription-polymerase chain reaction (RT-PCR) for PPARγ

Total RNA from the cells was extracted with the RNeasy Mini reagent kit according to the manufacturer's recommendations (QIAGEN S.r.l., Milan, Italy). TaqMan Reverse Transcription Reagents kit and Universal PCR Master Mix were from Applied Biosystems - Life Technologies (Grand Island, NY, USA). Quantitative PCR human reference total RNA was purchased from Stratagene (La Jolla, CA, USA). Primers and probes for PPARγ were from Applied Biosystems (TaqMan Gene Expression Assay; Hs00234592_m1). Total RNA (400 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents kit as reported previously [22]. The amount of target, normalized to the endogenous reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Pre-Developed TaqMan Assay Reagents, Applied Biosystems) and relative to a calibrator (Quantitative PCR human reference total RNA), was given by $2^{-\Delta\Delta Ct}$ calculation [22].

2.3 Data analysis

Values are given as mean±standard deviation (SD) for normally distributed variables, otherwise as median and [interquartile range]. Mean group values were compared by using analysis of variance (ANOVA) for normally distributed variables, otherwise by the Mann-

206 Whitney *U* or Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post-hoc*
207 comparisons of normally distributed variables were performed with the Bonferroni-Dunn test.
208
209 **3. Results**
210 ***3.1 In vivo studies***
211 Serum CXCL10 levels were higher in both OF-p and EOM-p, than in controls (**Fig. 1A**),
212 however no significant difference was observed between OF-p and EOM-p. Serum CCL2
213 levels were not significantly different in controls, or in both OF-p and EOM-p (**Fig. 1B**).
214
215 ***3.2 In vitro studies***
216 In primary EOM cell cultures, CXCL10 was undetectable in the supernatant, IFNγ dose-
217 dependently induced its release (**Fig. 2A**), while TNFα alone had no effect. The combination
218 of IFNγ (1000 IU/mL) and TNFα (10 ng/mL) had a significant synergistic effect on CXCL10
219 secretion (2644 ± 114 vs. 205 ± 43 pg/mL with IFNγ alone, $P < 0.0001$) (**Fig. 2B**).
220 PPARγ mRNAs were detectable in all primary EOM cells. PPARγ expression vs. the
221 reference gene (GAPDH) ranges from 0.39 to 1.11 in EOM cells and from 0.21 to 2.03 in
222 control muscle cells.
223 Treating EOM cells with RGZ (**Fig. 3A**), or pioglitazone (**Fig. 3B**), in combination with the
224 IFNγ+TNFα stimulation, dose-dependently inhibited CXCL10 release. RGZ or pioglitazone
225 alone had no effect and did not affect cell viability or total protein content (data not shown).
226 Regarding the CXCL10 secretion, the results obtained in muscle cells from M. rectus
227 abdominis tissue (data not shown) were not statistically different from those obtained in EOM
228 cells.
229 In primary EOM cells, CCL2 was detectable in the supernatant, TNFα dose-dependently
230 induced CCL2 release (**Fig. 4A**), while IFNγ alone had no effect. The combination of TNFα

and IFNγ had a significant synergistic effect on CCL2 secretion (2760 ± 247 vs. 611 ± 53 pg/mL with TNFα alone, $P < 0.0001$) (**Fig. 4B**).

Treating EOM cells with RGZ (**Fig. 5A**), or pioglitazone (**Fig. 5B**), in combination with the IFNγ+TNFα stimulation, dose-dependently stimulated CCL2 release.

Regarding the CCL2 secretion, the results obtained in muscle cells from M. rectus abdominis tissue (data not shown) were not statistically different from those obtained in EOM cells.

4. Discussion

The increased levels of CXCL10 in active TAO agree with previous studies that showed a predominant involvement of Th1 cytokines in GD and TAO [23, 24]. In fact, it has been shown that the active phase in TAO is characterized by the presence of proinflammatory and Th1-derived cytokines, while other cytokines, among them Th2-derived cytokines, do not seem to be associated with a specific stage of TAO [24]. These results are in agreement with those observed in a previous study showing that serum CXCL10 levels are increased in TAO-p, especially in patients with active disease [19].

The increase in CXCL10 concentrations was unrelated to hyperthyroidism *per se,* as all our patients were clinically euthyroid at the time of the study. CXCL10 levels were similar in OF-p and EOM-p, both in the active phase of the disease, but higher than in normal controls, suggesting that CXCL10 is involved in the active phase of TAO, during which the inflammatory process is sustained by Th1-mediated immune responses, independently from the prevalent involvement of OF or EOM.

A switch from a Th1 to Th2 phenotype appears to occur in TAO, in line with a previous report showing that lymphocytes obtained from orbital tissue of TAO-p had a prevalent Th1 profile, whereas patients with remote-onset hyperthyroidism had a large majority of Th2 lymphocytes [23].

This phenomenon has been reported in other long-standing autoimmune diseases. In multiple sclerosis simultaneous measurements of CXCL10 in the serum and cerebrospinal fluid showed elevated CXCL10 levels in acute phase, recent-onset disease or during exacerbations, suggesting a pathogenetic role for the chemokine in mediating relapse [25]. The prognostic value of increased, or rising, CXCL10 levels in patients with TAO remains to be established. The difference between active and inactive TAO is the presence of a lymphocytic infiltrate [26]; therefore the increased production of CXCL10 might be sustained by orbital lymphocytes. However, our *in vitro* studies demonstrate that CXCL10 can be produced by non-lymphoid cells in the orbit. In fact, we have previously shown that both fibroblasts and preadipocytes from TAO-p secreted CXCL10 stimulated with increasing doses of IFNγ, and that the combination of IFNγ and TNFα synergistically increased CXCL10 secretion [20].

In this study we first show that EOM cells secrete CXCL10 when stimulated with increasing doses of IFNγ, and the combination of IFNγ and TNFα synergistically increases CXCL10 secretion. These results agree with previous studies showing that the idiopathic inflammatory myopathies (dermatomyositis, polymyositis and sporadic inclusion body myositis) are associated with CXCL10 upregulation [27]. A significant increase in CXCL10 and chemokine (C-X-C motif) receptor (CXCR)3 mRNA levels in both thymus and muscle was observed also in myasthenic patients [28]. Moreover, another study reported that IFNγ upregulated the mRNA expression of CXCL9 and CXCL10 by human myotubes in a dose-dependent manner [29]. It has been also recently shown that human fetal cardiomyocytes secreted CXCL10 in response to IFNγ and TNFα, and that this effect was magnified by cytokine combination [30]. Different types of normal mammalian cells, as endothelial cells, thyrocytes [20], fibroblasts [20], and others, can release IFNγ-inducible C-X-C chemokines. However, these cells do not produce the C-X-C chemokines in basal condition, but only after the stimulation by cytokines, such as IFNγ and TNFα, that are secreted in a Th1 type inflammatory site, such as the orbit at

281 the beginning of TAO, by Th1 activated lymphocytes. This process has been suggested to be
282 involved in the initiation and the perpetuation of the inflammation in several autoimmune
283 diseases, and on the basis of our results can be applied to the orbit in TAO, too.
284 IFNγ stimulated EOM to express human leukocyte antigen (HLA)-DR. EOM cells treated
285 with IFNγ were more susceptible to lysis in antibody dependent cell-mediated cytotoxicity
286 assays than untreated targets [31]. It could be hypothesized that chemokines might be
287 important in the above mentioned immune process.
288 PPARγ modulate inflammatory responses in many kinds of cells: endothelial cells, thyrocytes,
289 fibroblasts, preadipocytes [17, 19, 32], and in others. Furthermore, the role of PPARγ has been
290 shown to be of importance in TAO; in fact, the IFNγ-stimulated CXCL9, CXCL10 and
291 CXCL11 [19, 20, 22] secretion was significantly inhibited treating thyroid follicular cells,
292 orbital fibroblasts or preadipocytes with a pure PPARγ activator, RGZ, strongly suggesting
293 that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in
294 human thyroid autoimmunity and TAO.
295 In this study we have shown the expression of PPARγ in EOM cells. Furthermore, the results
296 of our study are the first to demonstrate that the IFNγ-stimulated CXCL10 secretion was
297 significantly inhibited by the treatment of EOM with two pure PPARγ activators, RGZ and
298 pioglitazone. The drug concentrations were selected on the basis of their near therapy doses (5
299 μM for RGZ and pioglitazone) according to their pharmacokinetics (Cmax and area under the
300 time-concentration curve, AUC) [19]. These results strongly reinforce the hypothesis that
301 PPARγ might be involved in the regulation of the IFNγ-induced chemokine expression in
302 human thyroid autoimmunity and TAO.
303 Regarding the mechanism of these actions, PPARγ activators may act in different way. For
304 example, by decreasing CXCL10 promoter activity and inhibiting protein binding to the two
305 nuclear factor-kB (NF-kB) sites [17, 19], or reducing CXCL10 protein levels in a dose-

306 dependent manner at concentrations (nanomolar) that did not affect mRNA levels or NF-kB
307 activation. This effect is not only mediated by activating the NF-kB and Stat1 classic
308 pathways, but also involves a rapid increase in phosphorylation and activation of ERK1/2
309 [33].
310 The role of CCL2 in TAO is not yet completely clear. A first study showed that the expression
311 of CCL2 was higher in orbital fat of TAO patients than in controls [34]. The expression of
312 CCL2 in TAO fibroblasts was upregulated treating cells with CD154, the ligand for CD40,
313 which failed to do so in control cultures [35]. Moreover, CCL2 production by orbital
314 fibroblasts was increased by platelet-derived growth factor-BB stimulation [36]. To the best of
315 our knowledge, this study first shows that IFNγ and TNFα induce CCL2 secretion in EOM
316 cells. These results comply with the ones of previous studies in skeletal muscle cells, that
317 showed that IFNγ and TNFα were able to induce CCL2 secretion [37], which was involved in
318 the immune response in idiopathic inflammatory myopathies [27].
319 PPARγ activators have been shown to be able to suppress CCL2 expression in various cell
320 types, such as astrocytes and monocytes, via different pathways (mitogen-activated protein
321 kinase phosphatase-1, Toll-like receptor) [38, 39]. However, until now, no study has evaluated
322 the effect of PPARγ agonists on CCL2 secretion in skeletal and EOM muscles. Moreover, we
323 have recently shown that PPARγ agonists may have different effects in normal thyroid cells
324 (inhibiting CXCL10 secretion), or in papillary thyroid cancer cells (stimulating CXCL10),
325 suggesting that other pathways could be implicated in the PPARγ regulation of chemokine
326 secretion, that remain to be investigated [40]. According to our data, it could be hypothesized
327 that PPARγ agonists (that have an inhibitory role on the secretion of the Th1 CXCL10
328 chemokine, while stimulated the Th2 CCL2 chemokine) may be involved during the
329 progression of the disease in the switch from a prevalent Th1 immune response, in the first

phase of the disease, to a prevalent Th2 immunity, in the later phases. However, other studies are needed to evaluate this point.

Recently, it has been shown that RGZ was associated with an increased risk of stroke, heart failure, and all-cause mortality in elderly patients [41], and the European Medicines Agency (EMA) recommended on September 2010 that RGZ be suspended from the European market. More recently, EMA extended review of safety to pioglitazone [42]. Even if these arguments cannot be automatically translated in TAO field, they do not advice PPARγ agonists for the therapy of TAO.

In conclusion, CXCL10 serum levels were confirmed to be higher than in control subjects in the active phase of TAO, without any significant difference between OF-p and EOM-p. Moreover, the present study first shows that primary EOM cells from patients with TAO produce both Th1 (CXCL10) and Th2 (CCL2) chemokines, under the influence of IFNγ and/or TNFα, and may participate in the inflammatory process present in the orbit of patients with TAO. PPARγ expression has been shown in EOM cells and PPARγ agonists have an inhibitory role on the modulation of CXCL10, while stimulated CCL2 chemokine secretion, suggesting a possible role in the switch from Th1 to Th2 immunity.

Take-home messages:

- We demonstrate elevated serum CXCL10 levels in the active phase of TAO
- Primary EOM cells, of TAO patients, treated with IFNγ and TNFα, release chemokines
- We have shown the PPARγ expression in EOM cells
- PPARγ agonists inhibit CXCL10, but stimulate CCL2, in EOM
- EOM cells are involved in the inflammatory process in the orbit of TAO patients

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Alessandro Antonelli, Silvia Martina Ferrari and Poupak Fallahi made substantial contribution in the conception and design of the study and in drafting the article. Alda Corrado, Stefano Sellari Franceschini and Stefania Gelmini made substantial contribution in the acquisition and analysis and interpretation of data. Ele Ferrannini revised the paper critically for important intellectual content. All Authors gave the final approval of the version to be submitted.

Table 1. Characteristics of patients with active Graves' ophthalmopathy: patients with prevalent orbital fat expansion (OF), or with prevalent extra-ocular muscle (EOM) involvement.

	OF	EOM	*P*
n	16	10	
Sex (M/F)	4/12	3/7	ns
Age (years)	40 ± 10	36 ± 12	ns
Smoking (no/yes)	7/9	5/5	ns
Duration GO (months)	7 (1-32)	5 (1-29)	0.001
Duration thyroid disease (months)	8 (2-39)	8 (1-44)	0.001
TSH (mIU/L)	1.1 ± 2.4	1.4 ± 1.3	ns
Free T_3 (FT_3) pg/mL (pmol/L)	3.7 ± 2.3 (5.7 ± 3.5)	3.9 ± 2.2 (6 ± 3.4)	ns
Free T_4 (FT_4) ng/dL (pmol/L)	1.4 ± 0.9 (18 ± 11.6)	1.2 ± 1.5 (15.4 ± 19.3)	ns
Anti-thyroid peroxidase antibodies (AbTPO) (kIU/L)	342 ± 276	297 ± 314	ns
Anti-thyroglobulin antibodies (AbTg) (kIU/L)	325 ± 529	187 ± 372	ns
Anti-thyrotropin receptor autoantibodies (TRAb) (kIU/L)	21 ± 42	32 ± 39	ns
Past immunosuppression (no/yes)	13/3	9/1	ns
Clinical Activity Score	5.3 ± 1.9	6.7 ± 1.6	ns
Total Eye Score	23.0 ± 7.2	25.4 ± 8.7	ns

Figure Captions

Figure 1. Serum CXCL10 and CCL2 levels in patients with active GO or controls. Serum CXCL10 levels were higher in both patients with active GO with prevalent OF expansion (OF patients) and with prevalent EOM involvement (EOM patients) than in controls ($P < 0.01$, ANOVA, for both) (**A**), however no significant difference was observed between OF patients and EOM patients. Serum CCL2 levels were not significantly different in controls, or in both OF patients and EOM patients (**B**). The box indicates the lower and upper quartiles and the central line is the median value; the horizontal lines at the end of the vertical lines are the 2.5% and 97.5% values. * = $P < 0.05$ or less vs. controls by Bonferroni-Dunn test.

Figure 2. Stimulation of CXCL10 release from EOM cells by IFNγ (1000 IU/mL) and TNFα (10 ng/mL). CXCL10 release from EOM cells was absent under basal conditions (0) and was significantly stimulated by increasing doses of IFNγ ($P < 0.0001$, by ANOVA) (**A**). Bars are mean±SEM. * = $P < 0.05$ or less vs. 0 by Bonferroni-Dunn test. The combination of TNFα and IFNγ had a significant synergistic effect on CXCL10 secretion (* = $P < 0.0001$, by ANOVA) (**B**).

Figure 3. CXCL10 secretion from EOM cells treated with rosiglitazone or pioglitazone. Treatment of EOM cells with rosiglitazone (**A**), or pioglitazone (**B**), added at the time of IFNγ (1000 IU/mL) and TNFα (10 ng/mL) stimulation, dose-dependently inhibited CXCL10 release. Bars are mean±SEM. * = $P < 0.05$ or less vs. 0, and ° = not significantly different from the preceding dose by Bonferroni-Dunn test.

Figure 4. Stimulation of CCL2 release from EOM cells by IFNγ (1000 IU/mL) and TNFα (10 ng/mL). CCL2 release from EOM cells was present under basal conditions (0) and was

530 significantly stimulated by increasing doses of TNFα ($P < 0.0001$, by ANOVA) (**A**). Bars are
531 mean±SEM. * = $P < 0.05$ or less vs. 0 by Bonferroni-Dunn test. The combination of TNFα
532 and IFNγ had a significant synergistic effect on CCL2 secretion (**B**). * = $P < 0.05$ or less vs.
533 IFNγ or TNFα by Bonferroni-Dunn test; ° = $P < 0.05$ or less vs. IFNγ by Bonferroni-Dunn
534 test.
535
536 **Figure 5.** CCL2 secretion from EOM cells treated with rosiglitazone or pioglitazone.
537 Treatment of EOM cells with rosiglitazone (**A**), or pioglitazone (**B**), added at the time of IFNγ
538 (1000 IU/mL) and TNFα (10 ng/mL) stimulation, dose-dependently stimulated CCL2 release.
539 Bars are mean±SEM. * = $P < 0.05$ or less vs. 0, and ° = not significantly different from the
540 preceding dose by Bonferroni-Dunn test.
541

Take-home messages:

- We demonstrate elevated serum CXCL10 levels in the active phase of TAO
- Primary EOM cells, of TAO patients, treated with IFNγ and TNFα, release chemokines
- We have shown the PPARγ expression in EOM cells
- PPARγ agonists inhibit CXCL10, but stimulate CCL2, in EOM
- EOM cells are involved in the inflammatory process in the orbit of TAO patients

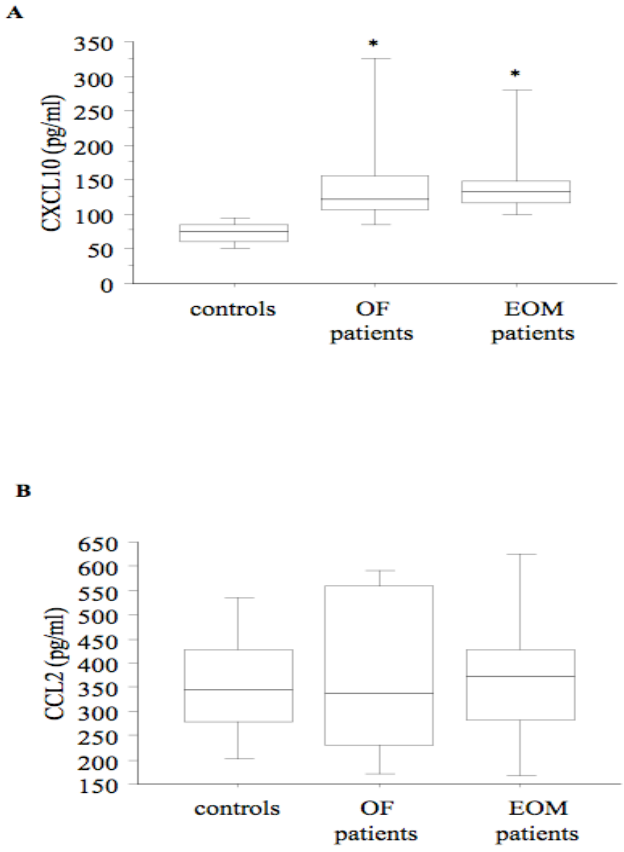

Figure 1

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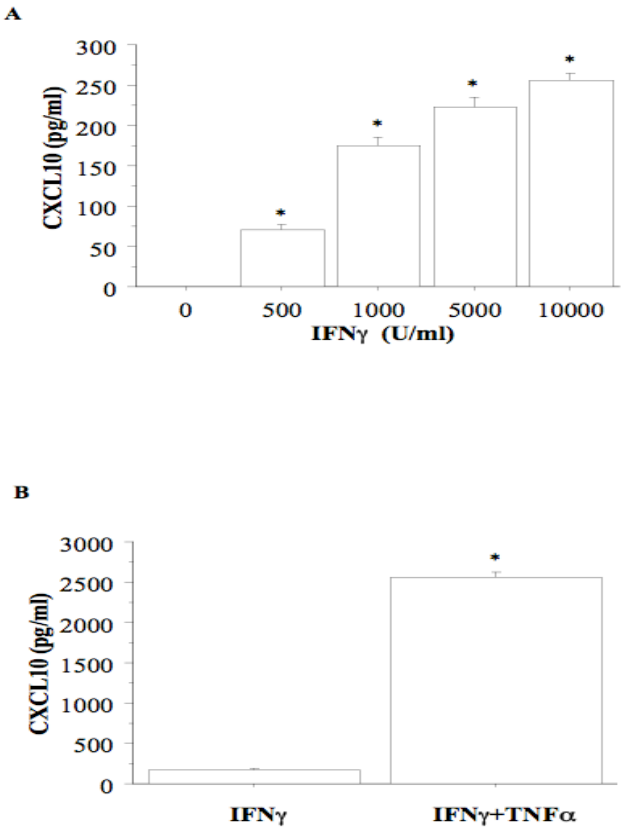

Figure 2

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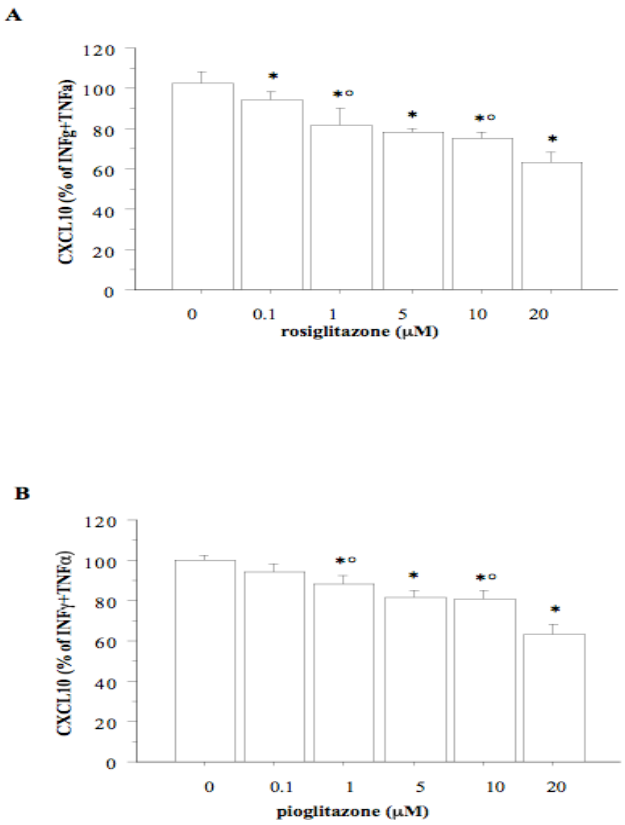

Figure 3

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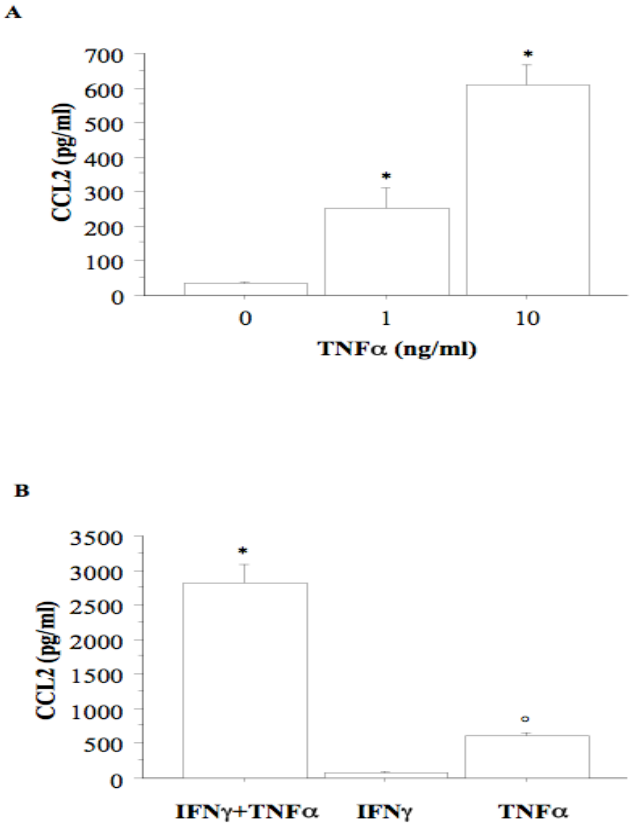

Figure 4

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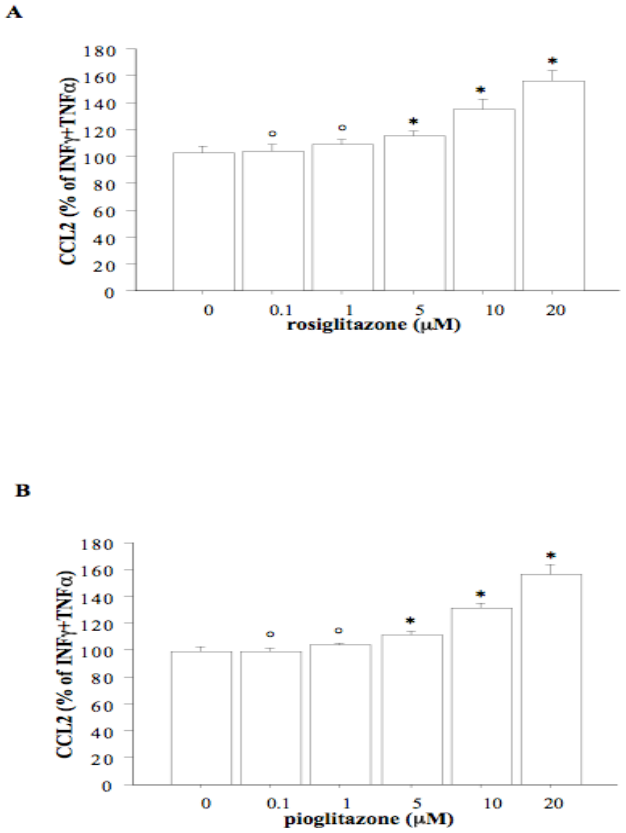
A
CCL2 (% of INFγ+TNFα)
180
160
140
120
100
80
60
40
20
0
0
0.1
1
5
10
20
rosiglitazone (μM)
B
CCL2 (% of INFγ+TNFα)
180
160
140
120
100
80
60
40
20
0
0
0.1
1
5
10
20
pioglitazone (μM)

Figure 5

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