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

4

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32 **Running Title**: CXCL10 and CCL2 in Graves' ocular myopathy

33

33 **Abstract**

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

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

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

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

55

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

57

57 **1. Introduction**

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

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

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

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

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

82 helper (Th)1-like cytokines predominate in EOM tissue in most patients, probably playing a
83 role on the development of eye muscle component of TAO in the acute stage [12].
84 Recent data have shown that C-X-C α-chemokines (Th1), in particular chemokine (C-X-C
85 motif) ligand (CXCL)9, CXCL10 and CXCL11, play an important role in the initial phases of
86 autoimmune disorders [13-15]. Serum CXCL10 levels are increased in GD, especially in
87 patients with active disease, and the CXCL10 decrease after thyroidectomy [16] or after
88 radioiodine [17] shows that it is more likely to have been produced inside the thyroid gland.
89 Furthermore, patients with newly diagnosed autoimmune thyroiditis show increased serum
90 CXCL10, in particular in the presence of a more aggressive thyroiditis and hypothyroidism
91 [13, 14, 18].
92 The secretion of CXCL10, CXCL9 and CXCL11 in primary cultures of TAO fibroblasts and
93 preadipocytes can be stimulated by IFNγ, and TNFα [19], suggesting that these cells
94 participate in the self-perpetuation of inflammation by releasing chemokines (under the
95 influence of cytokines) and inducing the recruitment of activated T cells in the thyroid. The
96 IFNγ-stimulated C-X-C chemokine secretion was significantly inhibited treating orbital cells
97 with peroxisome proliferator-activated receptor (PPAR)γ activators, at near-therapeutical
98 doses, strongly suggesting that PPARγ might be involved in the regulation of IFNγ-induced
99 chemokine expression in TAO [19].
100 Until now, no study has evaluated the chemokines expression in EOM in TAO. We aimed to:
101 1) compare serum CXCL10 and chemokine [C-C motif ligand (CCL)2] levels in patients with
102 active TAO (TAO-p) with prevalent EOM involvement (EOM-p) in comparison with those
103 with prevalent OF expansion (OF-p); 2) test the effect of IFNγ and/or TNFα stimulation on
104 the secretion of the prototype Th1 (CXCL10), and Th2 (CCL2) chemokines in primary
105 cultures of orbital EOM myoblasts; 3) assess the effect of PPARγ activation on CXCL10 and
106 CCL2 secretion in EOM myoblasts.

107

108 **2. Materials and Methods**

109 ***2.1 In vivo studies***

110 ***2.1.1 Patients***

111 We selected 26 consecutive Caucasian patients with GD and with active TAO and 26 age- and
112 sex-matched controls from our outpatient clinic (**Table 1**). The selection criteria included the
113 presence of exophthalmos, and: 1) expansion of OF, without evident EOM at orbital
114 computed tomography (CT); 2) EOM muscle enlargement without OF expansion at CT; 3) all
115 mixed forms (presence of both, OF expansion and EOM enlargement, were excluded). The
116 diagnosis of GD was established from the clinical presentation [20].
117 All TAO-p were clinically euthyroid on antithyroid drugs (16 patients), levo-thyroxine (6
118 patients) or spontaneously (4 patients), at the time of evaluation and eye disease activity was
119 assessed by the Clinical Activity Score [20]. A score of 5 (maximal score=10), including a
120 worsening over the previous 2 months, was considered indicative of active TAO. Inactive eye
121 disease was defined as no changes in eye status over the previous 6 months. Considering these
122 26 patients, 21 had never received immunosuppressive therapy, 3 had been previously treated
123 with corticosteroids, 1 with orbital irradiation, and 1 with both; a median of 11 months (range
124 6-42) had elapsed from the end of treatment. Total Eye Score was calculated as the sum of the
125 products of each NOSPECS class by its grade (to this purpose, we substituted 1, 2 and 3,
126 respectively, for grades a, b and c) [20]. We recorded the duration of both the eye and the
127 thyroid disease since their first signs and symptoms.

128 ***2.1.2 Controls***

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

132 A blood sample was collected in the morning after an overnight fasting, and serum was kept
133 frozen until the measurement of thyroid hormones, thyroid autoantibodies, CXCL10 and
134 CCL2, in both patients and controls. All study subjects gave their informed consent to
135 participate in the study, which was approved by the local Ethical Committee.
136
137 ***2.2 In vitro studies***
138 We investigated the effects of IFNγ, TNFα and PPARγ agonists on the release of CXCL10
139 and CCL2 in primary cultures of human myoblasts.
140 ***2.2.1 Human myoblasts cultures***
141 EOM samples were obtained from 5 patients operated on for EOM repair or decompression
142 (all previously treated with antithyroid medication and systemic corticosteroids, euthyroid at
143 the time of surgery; none treated with orbital radiotherapy). Control myoblasts were obtained
144 from M. rectus abdominis in 5 patients undergoing abdominal surgery. Human skeletal muscle
145 cells were prepared as previously reported [21].
146 Cells were isolated from EOM, or M. rectus abdominis, with trypsin followed by a
147 purification step with fibroblast-specific magnetic beads to prevent contamination with
148 fibroblasts. After two passages, the myoblasts were characterized by the manufacturer
149 (PromoCell, VWR International PBI S.r.l., Milan, Italy) using immunohistochemical detection
150 of sarcomeric myosin in differentiated cultures at 100% confluence (8 days). These cells were
151 grown to confluence in 25 cm^2 flasks, trypsinized, and subsequently $1x10^6$ cells were seeded
152 in 75 cm^2 flasks. After two passages, 5–7.5 x 10^7 cells were harvested and stored until further
153 use as frozen aliquots containing $2x10^6$ myoblasts. For each experiment, 10^5 cells per well
154 were seeded in six-well culture plates and cultured in α-modified Eagle's/Ham's F-12
155 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack (PromoCell) to
156 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***

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

189 ***2.2.5 Reverse transcription-polymerase chain reaction (RT-PCR) for PPARγ***

190 Total RNA from the cells was extracted with the RNeasy Mini reagent kit according to the
191 manufacturer's recommendations (QIAGEN S.r.l., Milan, Italy). TaqMan Reverse
192 Transcription Reagents kit and Universal PCR Master Mix were from Applied Biosystems -
193 Life Technologies (Grand Island, NY, USA). Quantitative PCR human reference total RNA
194 was purchased from Stratagene (La Jolla, CA, USA). Primers and probes for PPARγ were
195 from Applied Biosystems (TaqMan Gene Expression Assay; Hs00234592_m1). Total RNA
196 (400 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents kit as
197 reported previously [22]. The amount of target, normalized to the endogenous reference
198 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Pre-Developed TaqMan Assay
199 Reagents, Applied Biosystems) and relative to a calibrator (Quantitative PCR human
200 reference total RNA), was given by $2^{-\Delta\Delta Ct}$ calculation [22].

201

202 ***<u>2.3 Data analysis</u>***

203 Values are given as mean±standard deviation (SD) for normally distributed variables,
204 otherwise as median and [interquartile range]. Mean group values were compared by using
205 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

231 and IFNγ had a significant synergistic effect on CCL2 secretion (2760 ± 247 vs. 611 ± 53
232 pg/mL with TNFα alone, $P < 0.0001$) (**Fig. 4B**).
233 Treating EOM cells with RGZ (**Fig. 5A**), or pioglitazone (**Fig. 5B**), in combination with the
234 IFNγ+TNFα stimulation, dose-dependently stimulated CCL2 release.
235 Regarding the CCL2 secretion, the results obtained in muscle cells from M. rectus abdominis
236 tissue (data not shown) were not statistically different from those obtained in EOM cells.
237

238 **4. Discussion**

239 The increased levels of CXCL10 in active TAO agree with previous studies that showed a
240 predominant involvement of Th1 cytokines in GD and TAO [23, 24]. In fact, it has been
241 shown that the active phase in TAO is characterized by the presence of proinflammatory and
242 Th1-derived cytokines, while other cytokines, among them Th2-derived cytokines, do not
243 seem to be associated with a specific stage of TAO [24]. These results are in agreement with
244 those observed in a previous study showing that serum CXCL10 levels are increased in TAO-
245 p, especially in patients with active disease [19].
246 The increase in CXCL10 concentrations was unrelated to hyperthyroidism *per se,* as all our
247 patients were clinically euthyroid at the time of the study. CXCL10 levels were similar in OF-
248 p and EOM-p, both in the active phase of the disease, but higher than in normal controls,
249 suggesting that CXCL10 is involved in the active phase of TAO, during which the
250 inflammatory process is sustained by Th1-mediated immune responses, independently from
251 the prevalent involvement of OF or EOM.
252 A switch from a Th1 to Th2 phenotype appears to occur in TAO, in line with a previous report
253 showing that lymphocytes obtained from orbital tissue of TAO-p had a prevalent Th1 profile,
254 whereas patients with remote-onset hyperthyroidism had a large majority of Th2 lymphocytes
255 [23].

256 This phenomenon has been reported in other long-standing autoimmune diseases. In multiple
257 sclerosis simultaneous measurements of CXCL10 in the serum and cerebrospinal fluid
258 showed elevated CXCL10 levels in acute phase, recent-onset disease or during exacerbations,
259 suggesting a pathogenetic role for the chemokine in mediating relapse [25]. The prognostic
260 value of increased, or rising, CXCL10 levels in patients with TAO remains to be established.
261 The difference between active and inactive TAO is the presence of a lymphocytic infiltrate
262 [26]; therefore the increased production of CXCL10 might be sustained by orbital
263 lymphocytes. However, our *in vitro* studies demonstrate that CXCL10 can be produced by
264 non-lymphoid cells in the orbit. In fact, we have previously shown that both fibroblasts and
265 preadipocytes from TAO-p secreted CXCL10 stimulated with increasing doses of IFNγ, and
266 that the combination of IFNγ and TNFα synergistically increased CXCL10 secretion [20].
267 In this study we first show that EOM cells secrete CXCL10 when stimulated with increasing
268 doses of IFNγ, and the combination of IFNγ and TNFα synergistically increases CXCL10
269 secretion. These results agree with previous studies showing that the idiopathic inflammatory
270 myopathies (dermatomyositis, polymyositis and sporadic inclusion body myositis) are
271 associated with CXCL10 upregulation [27]. A significant increase in CXCL10 and chemokine
272 (C-X-C motif) receptor (CXCR)3 mRNA levels in both thymus and muscle was observed also
273 in myasthenic patients [28]. Moreover, another study reported that IFNγ upregulated the
274 mRNA expression of CXCL9 and CXCL10 by human myotubes in a dose-dependent manner
275 [29]. It has been also recently shown that human fetal cardiomyocytes secreted CXCL10 in
276 response to IFNγ and TNFα, and that this effect was magnified by cytokine combination [30].
277 Different types of normal mammalian cells, as endothelial cells, thyrocytes [20], fibroblasts
278 [20], and others, can release IFNγ-inducible C-X-C chemokines. However, these cells do not
279 produce the C-X-C chemokines in basal condition, but only after the stimulation by cytokines,
280 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

330 phase of the disease, to a prevalent Th2 immunity, in the later phases. However, other studies
331 are needed to evaluate this point.
332 Recently, it has been shown that RGZ was associated with an increased risk of stroke, heart
333 failure, and all-cause mortality in elderly patients [41], and the European Medicines Agency
334 (EMA) recommended on September 2010 that RGZ be suspended from the European market.
335 More recently, EMA extended review of safety to pioglitazone [42]. Even if these arguments
336 cannot be automatically translated in TAO field, they do not advice PPARγ agonists for the
337 therapy of TAO.
338
339 In conclusion, CXCL10 serum levels were confirmed to be higher than in control subjects in
340 the active phase of TAO, without any significant difference between OF-p and EOM-p.
341 Moreover, the present study first shows that primary EOM cells from patients with TAO
342 produce both Th1 (CXCL10) and Th2 (CCL2) chemokines, under the influence of IFNγ
343 and/or TNFα, and may participate in the inflammatory process present in the orbit of patients
344 with TAO. PPARγ expression has been shown in EOM cells and PPARγ agonists have an
345 inhibitory role on the modulation of CXCL10, while stimulated CCL2 chemokine secretion,
346 suggesting a possible role in the switch from Th1 to Th2 immunity.
347
348 **Take-home messages:**
349
350 • We demonstrate elevated serum CXCL10 levels in the active phase of TAO
351 • Primary EOM cells, of TAO patients, treated with IFNγ and TNFα, release chemokines
352 • We have shown the PPARγ expression in EOM cells
353 • PPARγ agonists inhibit CXCL10, but stimulate CCL2, in EOM
354 • EOM cells are involved in the inflammatory process in the orbit of TAO patients
355

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487
488

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490 within three years of beginning the submitted work that could inappropriately influence, or be
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495

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496 Alessandro Antonelli, Silvia Martina Ferrari and Poupak Fallahi made substantial contribution
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498 Sellari Franceschini and Stefania Gelmini made substantial contribution in the acquisition and
499 analysis and interpretation of data. Ele Ferrannini revised the paper critically for important
500 intellectual content. All Authors gave the final approval of the version to be submitted.

501

502

502 **Table 1. Characteristics of patients with active Graves' ophthalmopathy:** patients with
503 prevalent orbital fat expansion (OF), or with prevalent extra-ocular muscle (EOM)
504 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

505

505 **Figure Captions**

506 **Figure 1.** Serum CXCL10 and CCL2 levels in patients with active GO or controls. Serum
507 CXCL10 levels were higher in both patients with active GO with prevalent OF expansion (OF
508 patients) and with prevalent EOM involvement (EOM patients) than in controls ($P < 0.01$,
509 ANOVA, for both) (**A**), however no significant difference was observed between OF patients
510 and EOM patients. Serum CCL2 levels were not significantly different in controls, or in both
511 OF patients and EOM patients (**B**). The box indicates the lower and upper quartiles and the
512 central line is the median value; the horizontal lines at the end of the vertical lines are the
513 2.5% and 97.5% values. * = $P < 0.05$ or less vs. controls by Bonferroni-Dunn test.

514

515 **Figure 2.** Stimulation of CXCL10 release from EOM cells by IFNγ (1000 IU/mL) and TNFα
516 (10 ng/mL). CXCL10 release from EOM cells was absent under basal conditions (0) and was
517 significantly stimulated by increasing doses of IFNγ ($P < 0.0001$, by ANOVA) (**A**). Bars are
518 mean±SEM. * = $P < 0.05$ or less vs. 0 by Bonferroni-Dunn test. The combination of TNFα
519 and IFNγ had a significant synergistic effect on CXCL10 secretion (* = $P < 0.0001$, by
520 ANOVA) (**B**).

521

522 **Figure 3.** CXCL10 secretion from EOM cells treated with rosiglitazone or pioglitazone.
523 Treatment of EOM cells with rosiglitazone (**A**), or pioglitazone (**B**), added at the time of IFNγ
524 (1000 IU/mL) and TNFα (10 ng/mL) stimulation, dose-dependently inhibited CXCL10
525 release. Bars are mean±SEM. * = $P < 0.05$ or less vs. 0, and ° = not significantly different
526 from the preceding dose by Bonferroni-Dunn test.

527

528 **Figure 4.** Stimulation of CCL2 release from EOM cells by IFNγ (1000 IU/mL) and TNFα (10
529 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

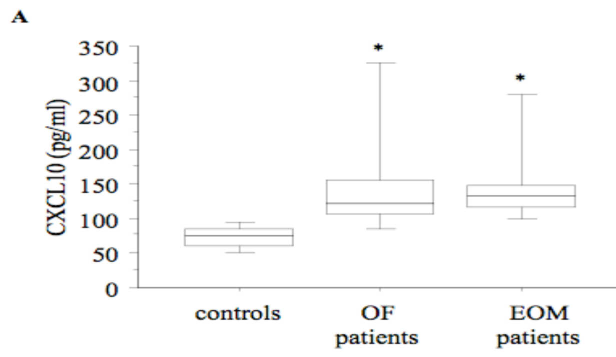
541 **Take-home messages:**

542

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

548

A



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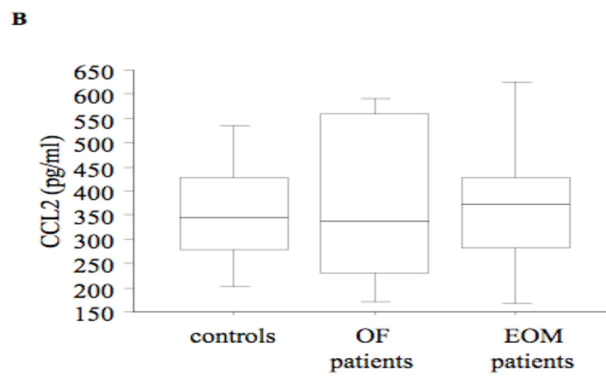

Figure 1

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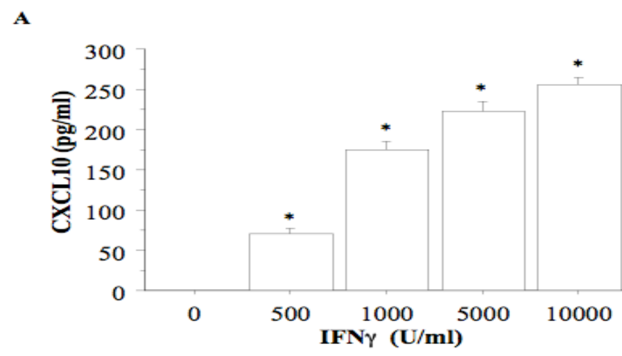

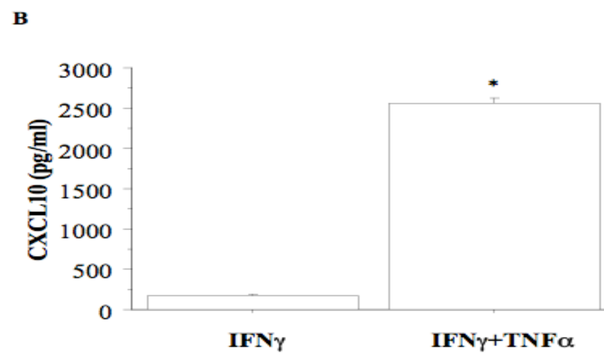

Figure 2

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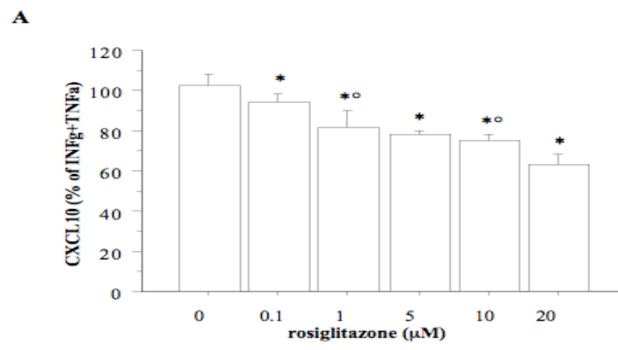

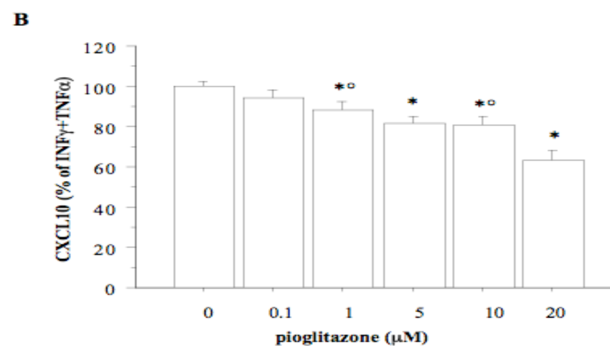

Figure 3

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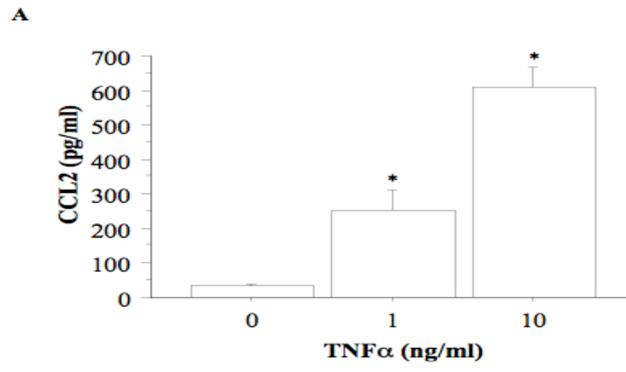

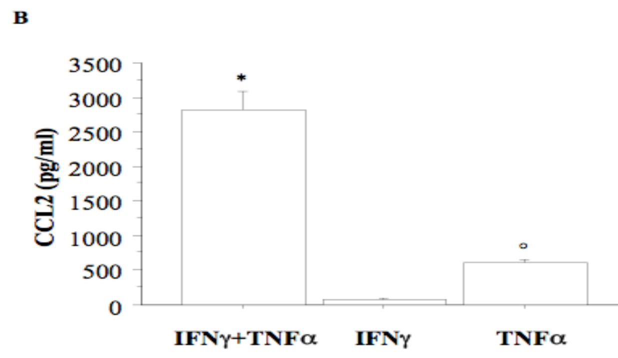

Figure 4

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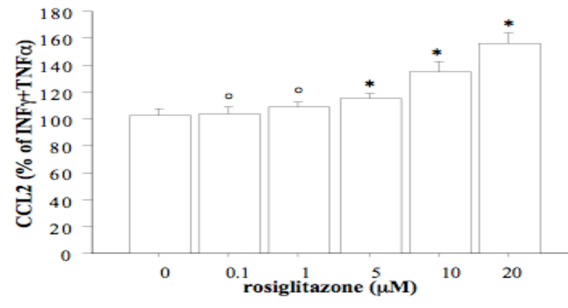

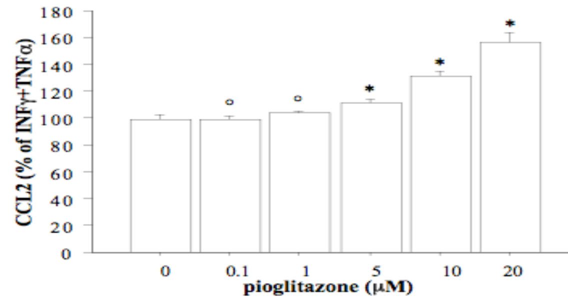

Figure 5