Monokine Induced by Interferon γ (IFNγ) (CXCL9) and IFNγ Inducible T-Cell α-Chemoattractant (CXCL11) Involvement in Graves' Disease and Ophthalmopathy: Modulation by Peroxisome Proliferator-Activated Receptor-γ Agonists

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Context: CXC α-chemokine CXCL10/IP-10 plays an important role in the initial phases of Graves' ophthalmopathy (GO). Human thyrocytes, orbital fibroblasts, and preadipocytes are stimulated to produce CXCL10 when treated with interferon γ (IFNγ) and TNFα. Peroxisome proliferator-activated receptor-γ (PPARγ) activation plays an inhibitory role in this process.

Objective: Until now, no data are present in literature about the involvement of CXCL9 and CXCL11 in Graves' disease and GO, or of PPARγ activators' effect on these chemokines.

Methods: It has been studied how IFNγ and TNFα stimulation and PPARγ activation affect CXCL9 and CXCL11 secretion in primary cultures of thyrocytes, orbital fibroblasts, and preadipocytes.

Results: In primary cultures of thyrocytes, retrobulbar fibroblasts, and retrobulbar preadipocytes obtained from GO patients, CXCL9 and CXCL11 production was absent under basal conditions; CXCL9 and CXCL11 secretion was not induced by TNFα alone, whereas it was dose dependently stimulated treating cells with IFNγ. The treatment with TNFα plus IFNγ has a synergistic effect on CXCL9 and CXCL11 release. Treating all cell types with the PPARγ agonist, rosiglitazone, or pioglitazone, the IFNγ plus TNFα-induced CXCL9 and CXCL11 release was dose dependently (0.1–20 μM) suppressed.

Conclusions: We conclude that thyrocytes and retrobulbar cell types from patients with Graves' disease and ophthalmopathy participate in the self-perpetuation of inflammation, releasing CXCL9 and CXCL11 chemokines when stimulated with cytokines. PPARγ activation plays an inhibitory role in this process. The huge response of CXCL9 to the IFNγ plus TNFα-stimulation suggests its leading role among CXC chemokines. (***J Clin Endocrinol Metab* 94: 1803–1809, 2009**)

Chemokines constitute families of low-molecular weight proteins that induce chemotaxis of specific leukocyte subsets. They are classified into four or more groups; among them, the CXC chemokines have been widely investigated (1).

Although CXC chemokines with an N-terminal ELR (Glu-Leu-Arg) motif, such as IL-8, are specific chemoattractants for neutrophils, those lacking the N-terminal ELR motif, such as IP-10/CXCL10, monokine induced by interferon-γ (IFNγ) (Mig/CXCL9) and IFNγ-inducible T-cell α-chemoattractant (I-TAC/CXCL11), do not act as chemoattractants for neutrophils (2–4) but attract T helper (Th) 1 cells (2, 5).

A dominant Th1 profile has been shown by lymphocytes obtained from thyroid tissue of patients with Graves' disease (GD) (6), or orbital tissues (7) of patients affected by Graves' ophthal-

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Abbreviations: FBS, Fetal bovine serum; GD, Graves' disease; GO, Graves' ophthalmopathy; GO-p, Graves' ophthalmopathy patient; IFNγ, interferon γ; I-TAC, IFNγ inducible T-cell α-chemoattractant; Mig, monokine induced by IFNγ; NF-κB, nuclear factor-κB; PPARγ, peroxisome proliferator-activated receptor-γ; RGZ, rosiglitazone; Th, T helper; TZD, thiazolidinedione.

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mopathy (GO), whereas in patients with remote-onset GO, the vast majority of lymphocytes have a Th2 profile (8). In particular, the presence of proinflammatory and Th1-derived cytokines characterize the active phase in GO, whereas other cytokines, among them Th2-derived cytokines, do not seem to be associated with a specific stage of GO (9). Other studies confirm the importance of Th1 immunity in active GO (10, 11).

Furthermore, recent evidence has shown that CXC α-chemokines (Th1), especially CXCL10, play an important role in the initial phases of autoimmune thyroid disease (12–16). Serum CXCL10 levels are increased in GD, especially in patients with active disease (17), and the CXCL10 decrease after thyroidectomy (18) or after radioiodine (19) 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, overall in the presence of a more aggressive thyroiditis and hypothyroidism (13, 14, 17).

These clinical observations, along with the demonstration that human thyrocytes in primary culture produce large amounts of CXCL9 and CXCL10 when stimulated by IFNγ and TNF-α (15), suggest that thyroid follicular cells are able to modulate the autoimmune response through the production of CXCL9/Mig and CXCL10 (12–15). The migration of Th1 lymphocytes into the gland, where they secrete IFNγ, is stimulated by these chemokines. In turn, the autoimmune process is perpetuated by the IFNγ stimulation of follicular cells, which increase the production of chemokines (13–15).

This pathogenetic hypothesis may be applied to other autoimmune disorders. In particular, if orbital fibroblasts and orbital preadipocytes from GO patients (GO-ps) secreted CXCL10 under IFNγ/TNF-α stimulation, a similar mechanism may be involved in the pathogenesis of GO. In fact, recently we have shown (20) that CXCL10 serum levels were higher in patients with active GO, compared with those with inactive GO, and that the secretion of CXCL10 in primary cultures of thyrocytes, orbital fibroblasts, and preadipocytes can be stimulated by IFNγ and TNF-α, suggesting that GO thyrocytes and retrobulbar cell types participate in the self-perpetuation of inflammation by releasing chemokines under the influence of cytokines.

The presence of peroxisome proliferator-activated receptor-γ (PPARγ) has been recently shown in thyroid tissue (20) and in orbital tissues from GO-ps (21). PPARγ has been demonstrated to be implicated in adipocyte differentiation, glucose homeostasis, and in the modulation of inflammatory responses. Treatment of endothelial cells with PPARγ activators inhibits: 1) IFNγ-induced mRNA and protein expression of CXCL10, CXCL9, and CXCL11/I-TAC (22); and 2) the release of chemotactic activity for CXCR3-transfected lymphocytes (22). For these reasons, PPARγ activity may be involved in the regulation of IFNγ-induced chemokine expression in human autoimmunity, and PPARγ activators might attenuate the recruitment of activated T cells at sites of Th1-mediated inflammation (22–25). Furthermore, we have shown that treatment of thyroid follicular cells, orbital fibroblasts, or preadipocytes, with a pure PPARγ activator, rosiglitazone (RGZ), at near-therapeutic doses, significantly inhibited IFNγ-stimulated CXCL10 secretion, strongly suggesting that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in human thyroid autoimmunity and GO (20).

Until now, to our knowledge, no data are present in the literature about the involvement of CXCL9 and CXCL11 in GO, or of PPARγ activators' effect on these chemokines.

To verify the hypothesis that CXCL9 and CXCL11 may participate in the induction and perpetuation of the inflammatory process at the orbital level, we have tested the effect of IFNγ stimulation on the secretion of the Th1 chemokines, CXCL9 and CXCL11, in primary cultures of cells obtained from the main tissues involved in the pathogenesis of GD and GO (thyrocytes, orbital fibroblasts, and preadipocytes). Furthermore, we have assessed a possible modulatory role of PPARγ activation on CXCL9 and CXCL11 secretion in these cell types.

Materials and Methods

It was investigated how IFNγ, TNF-α, and PPARγ agonists can affect the release of CXCL9 and CXCL11 in primary cultures of human thyroid follicular cells, fibroblasts, and preadipocytes (Fig. 1).

Thyroid follicular cells

Samples of thyroid tissue were obtained from five euthyroid GO-ps undergoing surgery. GO-ps were advised to have thyroidectomy (after a previous methimazole course in the presence of a large goiter and/or thyroid nodules), mainly due to a relapse of hyperthyroidism. In addition, control samples of normal thyroid tissue were collected from five patients (two undergoing parathyroidectomy and three laryngeal intervention). All study subjects gave their informed consent to the study, which was approved by the local ethical committee. Thyrocytes were prepared as reported previously (15, 20). The tissue samples were di-

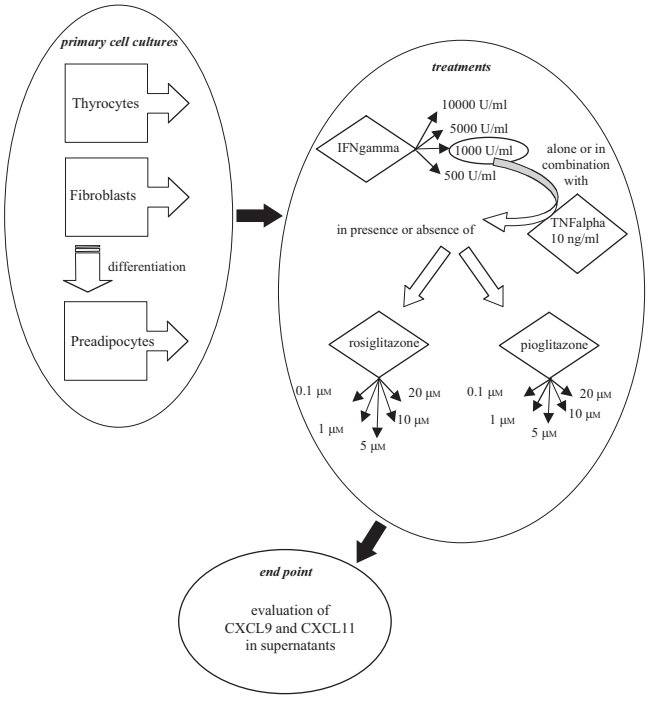

FIG. 1. Primary cultures of thyrocytes, orbital fibroblasts, or preadipocytes were treated with different concentrations of IFNγ alone, or in combination with TNF-α, to evaluate the secretion of CXCL9 and CXCL11 chemokines. The modulation exerted in this process by PPARγ agonists was evaluated adding increasing doses of RGZ or pioglitazone.

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gested by collagenase (1 mg/ml; Roche, Mannheim, Germany) in RPMI 1640 (Whittaker Bioproducts, Inc., Walkersville, MD) for 1 h at 37 C. Removed semi-digested follicles were sedimented for 2 min, washed, and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Seromed, Biochrom, Berlin, Germany), 2 mM glutamine, and 50 μg/ml penicillin/streptomycin at 37 C and 5% CO_2.

Fibroblast and preadipocyte cell cultures

Orbital adipose/connective tissue samples were obtained from five euthyroid patients (two on levo-thyroxine after thyroidectomy) undergoing orbital decompression for severe GO during the inactive phase of the disease (all previously treated with antithyroid medication and systemic corticosteroids; none treated with orbital radiotherapy). All subjects gave their informed consent to the study, which was approved by the local ethical committee. GO tissue explants were minced and placed directly in plastic culture dishes, to let preadipocyte fibroblasts to proliferate, as described previously (26, 27). Cells were propagated in medium 199 with 20% FBS (Life Technologies, Inc.- Invitrogen Ltd., Paisley, UK), penicillin (100 U/ml), and gentamycin (20 μg/ml) in a humidified 5% CO_2 incubator at 37 C, and maintained with medium 199 containing 10% FBS and antibiotics.

Orbital cells were grown to confluence in six-well plates in medium 199 with 10% FBS, to initiate adipocyte differentiation, which was performed as reported previously (26). Cultures were changed to serum-free DMEM/Ham's-F-12 (1:1; Sigma-Aldrich Corp., St. Louis, MO) containing biotin (33 μM), pantothenic acid (17 μM), transferrin (10 μg/ml), T_3 (0.2 nM), insulin (1 μM), carbaprostacyclin (cPGI2; 0.2 μM; Calbiochem, La Jolla CA), and, for the first 4 d only, dexamethasone (1 μM) and isobutylmethylxanthine (IBMX) (0.1 mM). The differentiation protocol was continued for 10 d, and the medium was replaced every 3–4 d. Separately, fibroblasts obtained from the same patients' orbital tissues were maintained for the same period of time in medium lacking various components necessary for complete adipocyte differentiation (*i.e.* cPGI2, dexamethasone, and isobutylmethylxanthine).

Control fibroblasts and preadipocytes were obtained from unaffected dermal tissues of the same patients.

Orbital preadipocyte fibroblast cultures (26, 27) were plated in medium 199 with 10% FBS, grown to confluence, and subjected to either the differentiation protocol or nondifferentiation conditions. Cells were washed twice with 1× PBS, fixed in 10% formalin overnight at room temperature, and rinsed in 60% isopropanol before staining with filtered 0.21% Oil Red O in isopropanol/water for 1 h. Cells were then stained to Mayer's hematoxylin solution (MHS-32; Sigma-Aldrich) for 5 min, rinsed with tap water, visualized using an Olympus IX50 light microscope (Hamburg, Germany), and photographed at ×20 (20).

CXCL9 and CXCL11 secretion assays

For CXCL9 and CXCL11 secretion assays, cells were seeded in 96-well plates at a concentration of 30,000 cells/ml in a final volume of 100 μl per well, in growth medium, which was removed after 24 h. Cells were then washed in PBS, and incubated (24 h) in phenol red and serum-free medium with IFNγ (500, 1,000, 5,000, and 10,000 U/ml; R&D Systems, Inc., Minneapolis, MN) and 10 ng/ml TNF-α (R&D Systems), alone or in combination (22). The concentration of TNF-α was selected in preliminary experiments to obtain the highest responses. After 24 h the supernatant was collected and kept frozen at −20 C until CXCL10 assay.

To establish how the PPARγ activators affect the IFNγ-induced chemokine secretion, cells were treated (24 h) with IFNγ (1000 U/ml) and TNF-α (10 ng/ml) in the presence or absence of increasing concentrations (0, 0.1, 1, 5, 10, 20 μM) of the pure PPARγ agonist, RGZ (Glaxo, Welwyn, UK), or pioglitazone (Alexis Biochemicals, Lausen, Switzerland). Supernatants were assayed by ELISA for CXCL9 and CXCL11 concentrations. The experiments were repeated three times with the three different cell preparations.

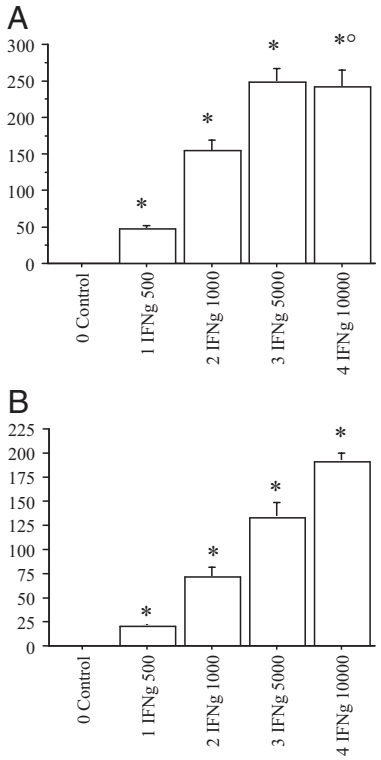

FIG. 2. CXCL9 (A) and CXCL11 (B) release from thyroid follicular cells was absent under basal conditions (0) and was significantly stimulated by increasing doses of IFNγ (IFNg) ($P < 0.0001$ by ANOVA). *Bars* are mean ± SEM. *, $P \leq 0.05$ *vs.* 0; °, not significantly different from the preceding dose by the Bonferroni-Dunn test.

Cell cultures and thiazolidinedione (TZD) treatment

Cultures of thyrocytes were treated (24 h) with 0.1, 1, 5, 10, or 20 μM RGZ or pioglitazone. Control cultures were grown (24 h) in the same medium containing vehicle (absolute ethanol, 0.47% vol/vol) without RGZ, or pioglitazone. Some cultures were examined by phase-contrast microscopy using an Olympus IX50.

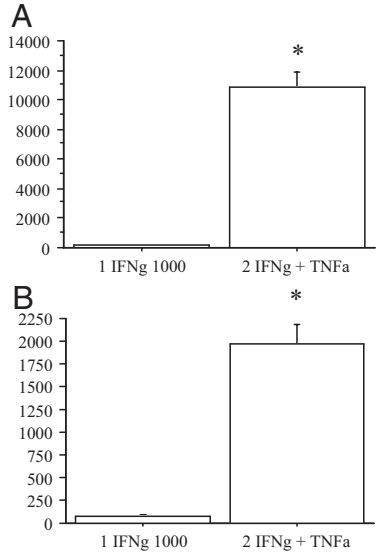

FIG. 3. The combination of TNF-α (TNFa) (10 ng/ml) and IFNγ (IFNg) (1000 U/ml) had a significant synergistic effect on CXCL9 (A) and CXCL11 (B) release from thyroid follicular cells ($P < 0.001$ by ANOVA), with respect to IFNγ alone. *Bars* are mean ± SEM. *, $P \leq 0.05$.

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The effect of RGZ or pioglitazone was examined both in differentiated cells (after 10 d) and in cells grown for the same period in medium lacking various components necessary for complete adipocyte differentiation (nondifferentiated cultures) (26). Cultures of fibroblasts and preadipocytes were treated (24 h) with 0.1, 1, 5, 10, or 20 μM RGZ, or pioglitazone after the initial 10-d period. Control cultures were grown (24 h) in the same medium containing vehicle (absolute ethanol, 0.47% vol/vol) without RGZ or pioglitazone. Some cultures were examined by phase-contrast microscopy using an Olympus IX50. Parallel cultures were stained with Oil Red O, and examined under light microscopy.

Lysis and homogenization of cell preparations were performed, and the sample was immediately assayed for its protein concentration by conventional methods (20).

ELISA for CXCL9, CXCL11

CXCL9 and CXCL11 levels were measured in culture supernatants by commercially available kits (R&D Systems). The mean minimum detectable dose was 1.37 pg/ml for CXCL9 and 3.4 pg/ml for CXCL11; the intraassay and interassay coefficients of variation were 3.3 and 6.2% for CXCL9, and 4.7 and 7.6%, respectively, for CXCL11. Quality control pools of low, normal, or high concentration for all parameters were included in each assay.

Data analysis

Values are given as mean ± SD for normally distributed variables, otherwise as median and (interquartile range). Mean group values were compared using one-way ANOVA for normally distributed variables, otherwise by the Mann-Whitney *U* or Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post hoc* comparisons on normally distributed variables were performed using the Bonferroni-Dunn test.

Results

CXCL9 and CXCL11 were undetectable in the supernatants collected from primary thyrocyte cultures. IFNγ dose dependently induced the CXCL9 and CXCL11 release (Fig. 2), TNF-α alone had no effect (chemokines remaining undetectable), whereas the combination of IFNγ and TNF-α had a significant synergistic effect on the CXCL9 and CXCL11 secretion (Fig. 3). Treating thyrocytes with RGZ (Fig. 4), or pioglitazone (Fig. 5), together with IFNγ and TNF-α stimulation, CXCL9 and CXCL11 release was dose dependently inhibited, whereas RGZ or pioglitazone alone had no effect and did not affect cell viability or total protein content (data not shown). The data obtained with thyrocytes from normal thyroid tissue were not statistically different from those obtained from GO-ps (data not shown).

The results obtained in cultured thyrocytes were similar to those seen in retrobulbar fibroblasts and preadipocytes. In fibroblasts (CXCL9: 0, 32 ± 8, 78 ± 17, 163 ± 23, and 172 ± 34, pg/ml, respectively, with IFNγ 0, 500, 1,000, 5,000, and 10,000 IU/ml; ANOVA, $P < 0.001$) (CXCL11: 0, 27 ± 11, 91 ± 13, 184 ± 27, and 291 ± 43, pg/ml, respectively, with IFNγ 0, 500, 1,000, 5,000, and 10,000 IU/ml; ANOVA, $P < 0.001$) and preadipocytes (CXCL9: 0, 51 ± 17, 178 ± 25, 305 ± 45, and 327 ± 56, pg/ml, respectively, with IFNγ 0, 500, 1,000, 5,000, and 10,000 IU/ml; ANOVA, $P < 0.001$) (CXCL11: 0, 43 ± 16, 88 ± 15, 179 ± 28, and 281 ± 49, pg/ml, respectively, with IFNγ 0, 500, 1,000, 5,000, and 10,000 IU/ml; ANOVA, $P < 0.001$), IFNγ dose dependently induced CXCL9 or CXCL11 release.

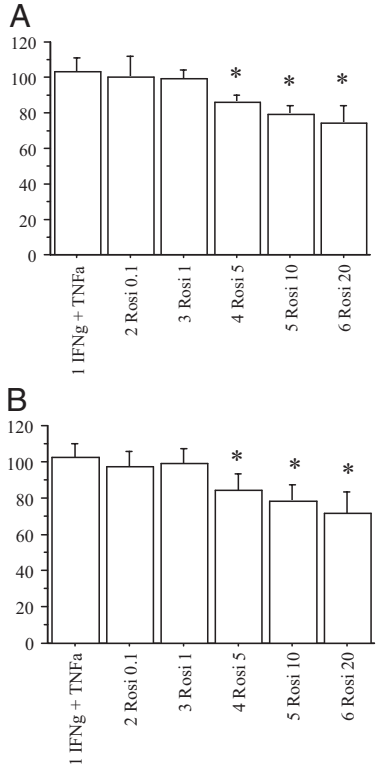

FIG. 4. Increasing doses of RGZ (Rosi) (0.1, 1, 5, 10, and 20 μM) inhibit CXCL9 (A) and CXCL11 (B) release from thyroid follicular cells stimulated with IFNγ (IFNg) (1000 U/ml) and TNF-α (TNFa) (10 ng/ml) (IFNγ plus TNF-α) ($P < 0.0001$ by ANOVA). *Bars* are mean ± SEM. *, $P \leq 0.05$ *vs.* IFNγ plus TNF-α by the Bonferroni-Dunn test.

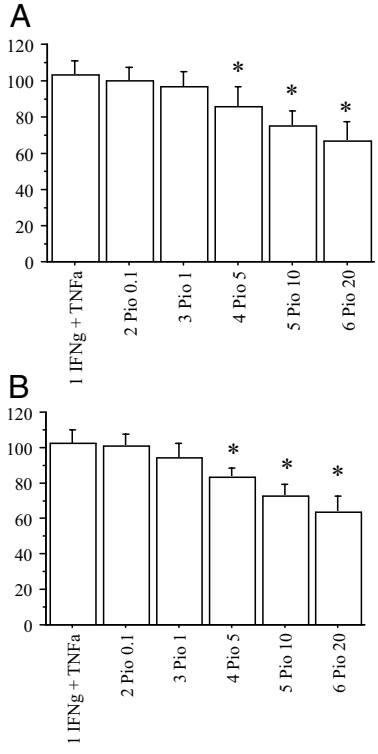

FIG. 5. Increasing doses of pioglitazone (Pio) (0.1, 1, 5, and 10, 20 μM) inhibit CXCL9 (A) and CXCL11 (B) release from thyroid follicular cells stimulated with IFNγ (IFNg) (1000 U/ml) and TNF-α (TNFa) (10 ng/ml) (IFNγ plus TNF-α) ($P <$ 0.0001 by ANOVA). *Bars* are mean ± SEM. *, $P \leq 0.05$ *vs.* IFNγ plus TNF-α by the Bonferroni-Dunn test.

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The combination of TNF-α and IFNγ had a significant synergistic effect on the chemokines' secretion (in fibroblasts: CXCL9, 13,124 ± 1,642 *vs.* 70 ± 19 pg/ml with IFNγ alone, ANOVA, $P < 0.0001$; CXCL11, 2,357 ± 271 *vs.* 98 ± 16 pg/ml with IFNγ alone, ANOVA, $P < 0.0001$) (in preadipocytes: CXCL9, 15,897 ± 2,283 *vs.* 61 ± 12 pg/ml with IFNγ alone, ANOVA, $P < 0.0001$; CXCL11, 2,781 ± 245 *vs.* 80 ± 13 pg/ml with IFNγ alone, ANOVA, $P < 0.0001$).

Treating retrobulbar fibroblasts with RGZ, together with IFNγ and TNF-α stimulation, CXCL9 and CXCL11 release was dose dependently inhibited (Table 1), such as treating them with pioglitazone (data not shown; $P < 0.01$, at concentrations of 10 and 20 μM pioglitazone, by ANOVA).

Treating retrobulbar preadipocytes with pioglitazone, combined with IFNγ and TNF-α stimulation, CXCL9 and CXCL11 release was dose dependently inhibited (Table 1), such as treating them with RGZ (data not shown; $P < 0.01$, at concentrations of 5, 10, and 20 μM RGZ, by ANOVA).

RGZ or pioglitazone alone had no effect, did not affect cell viability or total protein content, and did not cause significant adipogenic changes (as judged by Oil Red O staining) in retrobulbar fibroblasts and preadipocytes after a 24-h period of incubation (data not shown).

The data obtained with fibroblasts or preadipocytes from unaffected dermal tissues of the same patients were not statistically different from those obtained with their retrobulbar fibroblasts and preadipocytes (data not shown).

Discussion

To our knowledge, this is the first demonstration of CXCL9 production by IFNγ and TNF-α in orbital fibroblasts and preadipocytes, and of CXCL11 in thyrocytes, orbital fibroblasts, and preadipocytes of GO-ps. Furthermore, a dose-dependent inhibition has been shown by the PPARγ agonists, RGZ and pioglitazone, in this process.

IFNγ alone dose dependently induced CXCL9 and CXCL11 release in thyrocytes, fibroblasts, and preadipocytes. Moreover, the combination of IFNγ and TNF-α resulted in a huge response of CXCL9, in all the three cell types, which is definitively higher with respect to that obtained in similar conditions and in the same cells for CXCL11 and CXCL10 (20), suggesting that CXCL9 may play a leading role among CXC chemokines.

The finding of increased levels of CXCL9 or CXCL11 in GO is in agreement with previous studies that showed an involvement of Th1 cytokines in GD and GO (6–8, 28, 29), with the recent finding that (20) CXCL10 serum levels were higher in patients with active GO (compared with those with inactive GO), and that the secretion of CXCL10 in primary cultures of thyrocytes, orbital fibroblasts, and preadipocytes can be stimulated by IFNγ and TNF-α (20). These results suggest that, in GO, thyrocytes and retrobulbar cell types participate in the self-perpetuation of inflammation by releasing CXC chemokines under the influence of Th1 cytokines.

IFNγ-inducible CXC chemokines can be produced by several types of normal mammalian cells, such as endothelial cells (22), thyrocytes (15), fibroblasts (20), colon epithelial cells (24), islet cells (30), dendritic cells (23), and others. However, these cells are not able to produce the CXC chemokines in the basal condition, but only after the stimulation by cytokines, such as IFNγ and TNF-α, which are secreted in a Th1 type inflammatory site, such as the orbit at the beginning of GO, by T-helper 1 activated lymphocytes. This process has been suggested to be involved in the initiation and perpetuation of the inflammation in several autoimmune diseases (15, 20, 22–24, 30), and on the basis of our results, can be applied to the orbit too in GO.

It has recently been shown that PPARγ modulates inflammatory responses in many kinds of models: endothelial cells (22–24); murine models of colitis (24, 31); dendritic cells (23); and in other kinds of cells (32). Furthermore, the role of PPARγ has been of great importance in GO (25). In fact, we have recently shown (20) that the IFNγ-stimulated CXCL10 secretion was significantly inhibited while treating thyroid follicular cells, or-

TABLE 1. Increasing doses of RGZ (0.1, 1, 5, 10, and 20 μM) or pioglitazone (Pio) (0.1, 1, 5, 10, and 20 μM) significantly (ANOVA; $P < 0.01$) inhibit CXCL9 and CXCL11 release from retroorbital fibroblasts, or preadipocytes (respectively), stimulated with IFNγ (1000 U/ml) and TNF-α (10 ng/ml)

	Fibroblasts				
	RGZ 0.1	**RGZ 1**	**RGZ 5**	**RGZ 10**	**RGZ 20**
CXCL9 (% of basal IFNγ + TNF-α stimulation)	99 ± 12	98 ± 5	86 ± 4	79 ± 4.5[a]	74 ± 11[a]
CXCL11 (% of basal IFNγ + TNF-α stimulation)	99 ± 6	97 ± 5	81 ± 9[a]	70 ± 4[a]	68 ± 12[a]
	Preadipocytes				
	Pio 0.1	**Pio 1**	**Pio 5**	**Pio 10**	**Pio 20**
CXCL9 (% of basal IFNγ + TNF-α stimulation)	99 ± 12	95 ± 6	96 ± 9	77 ± 6[a]	60 ± 6[a]
CXCL11 (% of basal IFNγ + TNF-α stimulation)	99 ± 7	97 ± 8	81 ± 4[a]	74 ± 7[a]	68 ± 5.5[a]

The results are expressed as percentage (%) (mean ± SEM) with respect to the chemokine secretion obtained with treatment with IFNγ and TNF-α in the absence of PPARγ agonists.

[a] $P \leq 0.05$ *vs.* IFNγ plus TNF-α by the Bonferroni-Dunn test.

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bital fibroblasts, or preadipocytes with a pure PPARγ activator, RGZ, strongly suggesting that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in human thyroid autoimmunity and GO. The results of our study are the first to demonstrate that the IFNγ-stimulated CXCL9 and CXCL11 secretion was significantly inhibited by the treatment of thyroid follicular cells, orbital fibroblasts, or preadipocytes with two pure PPARγ activators, RGZ and pioglitazone. The drug concentrations were selected on the basis of their near-therapy doses (5 μM for RGZ and pioglitazone) according to their pharmacokinetics (maximum concentration and area under the time concentration curve) (20, 33). These results strongly reinforce the hypothesis that PPARγ might be involved in the regulation of IFNγ-induced chemokine expression in human thyroid autoimmunity and GO.

Regarding the mechanism of these actions, PPARγ activators may act differently: first, by decreasing CXCL10 promoter activity and inhibiting protein binding to the two nuclear factor-κB (NF-κB) sites (22); and second, TZDs reduce CXCL10 protein levels in a dose-dependent manner at concentrations (nanomolar) that did not affect mRNA levels or NF-κB activation (24). It has been recently shown that the TZD effect is not only mediated by activating the NF-κB and signal transducer and activator of transcription 1 classical pathways but also involves a rapid increase in phosphorylation and activation of ERKs 1 and 2 (34).

GO is an autoimmune condition in which orbital tissue remodeling is caused by intense inflammation; it also involves the accumulation of extracellular macromolecules and fat. Disease progression is dependent on interactions between lymphocytes and orbital fibroblasts. The tissue characteristics of GO are produced by these cells engaged in a cycle of reciprocal activation. PPARγ may play divergent roles in this process, both attenuating and promoting disease progression. PPARγ has antiinflammatory activity, suggesting that it could interrupt intercellular communication. However, PPARγ activation is also critical to adipogenesis, making it a potential culprit in the pathological fat accumulation associated with GO (35). The finding that the expression of the PPARγ gene is higher in orbital adipose/connective tissue from patients in the active stage of GO compared with that obtained in tissues from controls or inactive GO-ps supports this hypothesis (21). Thus, the overexpression of PPARγ caused by the inflammatory process could lead to the increased orbital fat tissue observed in GO. About this, a type 2 diabetic patient who experienced exacerbation of GO and expansion of the orbital fat during treatment with the PPARγ agonists was described in two recent case reports (36, 37). In cultured retrobulbar preadipocytes, PPARγ agonists caused a 2- to 13-fold increase, and a PPARγ antagonist a 2- to 7-fold reduction, in adipogenesis (36). The adipogenic potential of PPARγ agonists on orbital preadipocytes has been confirmed by other studies (26, 38), suggesting that PPARγ antagonists could provide a novel therapy for GO-ps in the active stage of the disease. In the current studies, any significant adipogenic effect of RGZ or pioglitazone in fibroblasts or preadipocytes could be found, mainly because of the short incubation period (24 h) because TZDs need 10 d or more to exert their adipogenic effects (26). However, these results do not provide sufficient evidence that PPARγ agonists may be useful in the treatment of GO. It has been suggested that adipogenesis is a mechanism for stanching chronic inflammation (39), but whether it is helpful to abate or amplify inflammation, the associated increase in orbital tissue mass is undesirable. Despite antiinflammatory actions of PPARγ, its proadipogenic functions in the orbit might worsen the disease, contraindicating the use of these agents in GO (36). Thus, further studies are necessary to establish whether in active GO the antiinflammatory effects of PPARγ activation, mediated by RGZ or pioglitazone, can be exploited without the risk of expanding retrobulbar fat mass, *e.g.* combining PPARγ agonists and nonsteroidal antiinflammatory drugs or glucocorticoids, which may synergize to exert their antiinflammatory action.

In conclusion, the results of the present study, in GO, demonstrate that: 1) IFNγ in combination with TNF-α induces the release of CXCL9 and CXCL11, not only by primary thyrocytes, but also from retrobulbar fibroblasts and preadipocytes obtained from GO-ps; and 2) the PPARγ agonists, RGZ and pioglitazone, exert a dose-dependent inhibition on IFNγ- and TNF-α-induced CXCL9 or CXCL11 secretion in these cells. The huge response of CXCL9 to the IFNγ plus TNF-α-stimulation suggests its leading role among CXC chemokines.

Acknowledgments

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References

1. **Luster AD** 1998 Chemokines—chemotactic cytokines that mediate inflammation. N Engl J Med 338:436–445
2. **Mackay CR** 1996 Chemokine receptors and T cell chemotaxis. J Exp Med 184:799–802
3. **Dewald B, Moser B, Barella L, Schumacher C, Baggiolini M, Clark-Lewis I** 1992 IP-10, a γ-interferon-inducible protein related to interleukin-8, lacks neutrophil activating properties. Immunol Lett 32:81–84
4. **Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B** 1996 Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med 184:963–969
5. **Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, Matsushima K, Kelvin DJ, Oppenheim JJ** 1993 Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J Exp Med 177:1809–1814
6. **Mariotti S, Del Prete GF, Mastromauro C, De Carli M, Romagnani S, Ricci M, Pinchera A** 1991 The autoimmune infiltrate of Basedow's disease: analysis of clonal level and comparison with Hashimoto's thyroiditis. Exp Clin Endocrinol 97:139–146
7. **De Carli M, D'Elios MM, Mariotti S, Marcocci C, Pinchera A, Ricci M, Romagnani S, Del Prete G** 1993 Cytolytic T cells with Th1-like cytokine profile predominate in retroorbital lymphocytic infiltrates of Graves' ophthalmopathy. J Clin Endocrinol Metab 77:1120–1124
8. **Aniszewski JP, Valyasevi RW, Bahn RS** 2000 Relationship between disease duration and predominant orbital T cell subset in Graves' ophthalmopathy. J Clin Endocrinol Metab 85:776–780
9. **Wakelkamp IM, Bakker O, Baldeschi L, Wiersinga WM, Prummel MF** 2003 TSH-R expression and cytokine profile in orbital tissue of active vs. inactive Graves' ophthalmopathy patients. Clin Endocrinol (Oxf) 58:280–287
10. **Heufelder AE, Smith TJ, Gorman CA, Bahn RS** 1991 Increased induction of HLA-DR by interferon-γ in cultured fibroblasts derived from patients with

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Graves' ophthalmopathy and pretibial dermopathy. J Clin Endocrinol Metab 73:307–313
11. **Bartalena L, Pinchera A, Marcocci C** 2000 Management of Graves' ophthalmopathy: reality and perspectives. Endocr Rev 21:168–199
12. **Romagnani P, Rotondi M, Lazzeri E, Lasagni L, Francalanci M, Buonamano A, Milani S, Vitti P, Chiovato L, Tonacchera M, Bellastella A, Serio M** 2002 Expression of IP-10/CXCL10 and MIG/CXCL9 in the thyroid and increased levels of IP-10/CXCL10 in the serum of patients with recent-onset Graves' disease. Am J Pathol 161:195–206
13. **Antonelli A, Rotondi M, Fallahi P, Romagnani P, Ferrari SM, Buonamano A, Ferrannini E, Serio M** 2004 High levels of circulating CXCL10 are associated with chronic autoimmune thyroiditis and hypothyroidism. J Clin Endocrinol Metab 89:5496–5499
14. **Antonelli A, Rotondi M, Fallahi P, Romagnani P, Ferrari SM, Paolicchi A, Ferrannini E, Serio M** 2005 Increase of interferon-γ inducible α chemokine CXCL10 but not β chemokine CCL2 serum levels in chronic autoimmune thyroiditis. Eur J Endocrinol 152:171–177
15. **Garcià-Lòpez MA, Sancho D, Sànchez-Madrid F, Marazuela M** 2001 Thyrocytes from autoimmune thyroid disorders produce the chemokines IP-10 and Mig and attract CXCR3+ lymphocytes. J Clin Endocrinol Metab 86: 5008–5016
16. **Kemp EH, Metcalfe RA, Smith KA, Woodroofe MN, Watson PF, Weetman AP** 2003 Detection and localization of chemokine gene expression in autoimmune thyroid disease. Clin Endocrinol (Oxf) 59:207–213
17. **Antonelli A, Fallahi P, Rotondi M, Ferrari SM, Romagnani P, Grosso M, Ferrannini E, Serio M** 2006 Increased serum CXCL10 in Graves' disease or autoimmune thyroiditis is not associated with hyper- or hypothyroidism per se, but is specifically sustained by the autoimmune, inflammatory process. Eur J Endocrinol 154:651–658
18. **Antonelli A, Fallahi P, Rotondi M, Ferrari SM, Serio M, Miccoli P** 2006 Serum levels of the interferon-γ-inducible α chemokine CXCL10 in patients with active Graves' disease, and modulation by methimazole therapy and thyroidectomy. Br J Surg 93:1226–1231
19. **Antonelli A, Rotondi M, Fallahi P, Grosso M, Boni G, Ferrari SM, Romagnani P, Serio M, Mariani G, Ferrannini E** 2007 Iodine-131 given for therapeutic purposes modulates differently interferon-γ-inducible α-chemokine CXCL10 serum levels in patients with active Graves' disease or toxic nodular goiter. J Clin Endocrinol Metab 92:1485–1490
20. **Antonelli A, Rotondi M, Ferrari SM, Fallahi P, Romagnani P, Franceschini SS, Serio M, Ferrannini E** 2006 Interferon-γ-inducible α-chemokine CXCL10 involvement in Graves' ophthalmopathy: modulation by peroxisome proliferator-activated receptor-γ agonists. J Clin Endocrinol Metab 91:614–620
21. **Mimura LY, Villares SM, Monteiro ML, Guazzelli IC, Bloise W** 2003 Peroxisome proliferator-activated receptor-γ gene expression in orbital adipose/connective tissues is increased during the active stage of Graves' ophthalmopathy. Thyroid 13:845–850
22. **Marx N, Mach F, Sauty A, Leung JH, Sarafi MN, Ransohoff RM, Libby P, Plutzky J, Luster AD** 2000 Peroxisome proliferator-activated receptor-γ activators inhibit IFN-γ-induced expression of the T cell-active CXC chemokines IP-10, Mig, and I-TAC in human endothelial cells. J Immunol 164:6503–6508
23. **Gosset P, Charbonnier AS, Delerive P, Fontaine J, Staels B, Pestel J, Tonnel AB, Trottein F** 2001 Peroxisome proliferator-activated receptor γ activators affect the maturation of human monocyte-derived dendritic cells. Eur J Immunol 31:2857–2865
24. **Schaefer KL, Denevich S, Ma C, Cooley SR, Nakajima A, Wada K, Schlezinger J, Sherr D, Saubermann LJ** 2005 Intestinal antiinflammatory effects of thiazolidenedione peroxisome proliferator-activated receptor-γ ligands on T helper type 1 chemokine regulation include nontranscriptional control mechanisms. Inflamm Bowel Dis 11:244–252
25. **Lehmann GM, Garcia-Bates TM, Smith TJ, Feldon SE, Phipps RP** 2008 Regulation of lymphocyte function by PPARγ: relevance to thyroid eye disease-related inflammation. PPAR Res 2008:895901
26. **Valyasevi RW, Harteneck DA, Dutton CM, Bahn RS** 2002 Stimulation of adipogenesis, peroxisome proliferator-activated receptor-γ (PPARγ), and thyrotropin receptor by PPARγ agonist in human orbital preadipocyte fibroblasts. J Clin Endocrinol Metab 87:2352–2358
27. **Bahn RS, Gorman CA, Johnson CM, Smith TJ** 1989 Presence of antibodies in the sera of patients with Graves' disease recognizing a 23 kilodalton fibroblast protein. J Clin Endocrinol Metab 69:622–662
28. **Watson PF, Pickerill AP, Davies R, Weetman AP** 1994 Analysis of cytokine gene expression in Graves' disease and multinodular goiter. J Clin Endocrinol Metab 79:355–360
29. **Hiromatsu Y, Yang D, Bednarczuk T, Miyake I, Nonaka K, Inoue Y** 2000 Cytokine profiles in eye muscle tissue and orbital fat tissue from patients with thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 85:1194–1199
30. **Schulthess FT, Paroni F, Sauter NS, Shu L, Ribaux P, Haataja L, Strieter RM, Oberholzer J, King CC, Maedler K** 2009 CXCL10 impairs β cell function and viability in diabetes through TLR4 signaling. Cell Metab 9:125–139
31. **Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD** 1999 A novel therapy for colitis utilizing PPAR-γ ligands to inhibit the epithelial inflammatory response. J Clin Invest 104:383–389
32. **Yang XY, Wang LH, Farrar WL** 2008 A role for PPARγ in the regulation of cytokines in immune cells and cancer. PPAR Res 2008:961753
33. **Crescioli C, Cosmi L, Borgogni E, Santarlasci V, Gelmini S, Sottili M, Sarchielli E, Mazzinghi B, Francalanci M, Pezzatini A, Perigli G, Vannelli GB, Annunziato F, Serio M** 2007 Methimazole inhibits CXC chemokine ligand 10 secretion in human thyrocytes. J Endocrinol 195:145–155
34. **Lombardi A, Cantini G, Piscitelli E, Gelmini S, Francalanci M, Mello T, Ceni E, Varano G, Forti G, Rotondi M, Galli A, Serio M, Luconi M** 2008 A new mechanism involving ERK contributes to rosiglitazone inhibition of tumor necrosis factor-α and interferon-γ inflammatory effects in human endothelial cells. Arterioscler Thromb Vasc Biol 28:718–724
35. **Smith TJ, Koumas L, Gagnon A, Bell A, Sempowski GD, Phipps RP, Sorisky A** 2002 Orbital fibroblast heterogeneity may determine the clinical presentation of thyroid-associated ophthalmopathy. J Clin Endocrinol Metab 87:385–392
36. **Starkey K, Heufelder A, Bakerm G, Joba W, Evans M, Davies S, Ludgate M** 2003 Peroxisome proliferator-activated receptor-γ in thyroid eye disease: contraindication for thiazolidinedione use? J Clin Endocrinol Metab 88:55–59
37. **Lee S, Tsirbas A, Goldberg RA, McCann JD** 2007 Thiazolidinedione induced thyroid associated orbitopathy. BMC Ophthalmol 7:8
38. **Kumar S, Coenen MJ, Scherer PE, Bahn RS** 2004 Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmopathy. J Clin Endocrinol Metab 89:930–935
39. **Feldon SE, O'loughlin CW, Ray DM, Landskroner-Eiger S, Seweryniak KE, Phipps RP** 2006 Activated human T lymphocytes express cyclooxygenase-2 and produce proadipogenic prostaglandins that drive human orbital fibroblast differentiation to adipocytes. Am J Pathol 169:1183–1193

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