Methimazole inhibits CXC chemokine ligand 10 secretion in human thyrocytes

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Abstract

CXC chemokine ligand 10 (CXCL10) plays a pivotal role in the self-perpetuation of the inflammatory processes in patients with autoimmune thyroid disease. Treatment with methimazole (MMI) reduces serum CXCL10 in patients with Graves' disease. In isolated human thyrocytes, tumor necrosis factor (TNF) α demonstrates a potent synergistic effect on interferon (IFN) γ -induced CXCL10 secretion. We investigated the mechanism underlying the synergism between IFN γ and TNF α and the effect of MMI on CXCL10 secretion in human thyrocytes. A peroxisome proliferator-activated receptor γ agonist, rosiglitazone (RGZ), a known inhibitor of T helper 1 (Th1)-mediated responses, was also studied for comparison. Experiments were carried out in human thyrocytes isolated from internodular parenchyma of thyroid tissues derived from patients who had undergone surgery for multinodular goiter. ELISA was used to measure CXCL10 levels in culture supernatant. Flow cytometry was

used to assess IFN γ membrane receptor expression. Specific mRNA analysis was performed by Taqman real-time PCR. Immunofluorescence was performed to detect nuclear translocation of nuclear factor- κ B (NF- κ B). In human thyrocytes, the synergistic effect of TNF α with IFN γ on CXCL10 secretion is due to the upregulation of IFN γ receptor expression. MMI decreased cytokine-induced CXCL10 secretion by reducing TNF α -induced upregulation of the IFN γ receptor. RGZ decreased the cytokine-induced CXCL10 secretion by impairing NF- κ B translocation, without affecting IFN γ receptor. MMI and RGZ targeted thyrocytes with the same pharmacological potency, likely acting throughout different mechanisms. Targeting T helper 1-mediated autoimmune thyroid disease with drugs that impair different intracellular pathways could be a novel pharmacological tool.

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Introduction

In the pathogenesis of human autoimmune thyroid disorders (AITDs), Hashimoto's thyroiditis and Graves' disease (GD), intrathyroidal lymphocytes seem to play a central role since thyroid antigen recognition is an essential step to T- and/or B-cell stimulation (Weetman & McGregor 1994). The T-cell pattern involved in GD might change throughout the disease course, with T helper 1 (Th1) as the predominant subtype of CD4⁺T cells in patients with recent disease onset and Th2 as the predominant subtype in patients with longer disease duration (Aniszewski *et al.* 2000).

In AITDs, a wide range of Th1-associated cytokines, such as interleukin (IL)-1, IL-2, IL-6, interferon (IFN) γ , and tumor necrosis factor (TNF) α , are produced. The cytokine production in AITDs has been ascribed to infiltrating T cells, macrophages (Weetman 2003), thyrocytes (Watson *et al.* 1995, Weetman 2004), and endothelial cells (Romagnani *et al.* 2002). The active phase of the disease is characterized by

the presence of proinflammatory and Th1-derived cytokines in the thyroid gland, whereas Th2-derived cytokines do not seem to be involved (Wakelkamp *et al.* 2003).

Chemokines, a group of low-molecular-weight peptides belonging to the cytokine family, are known to induce the chemotaxis of different leucocyte subtypes (Zlotnik & Yoshie 2000). The CXC chemokines inducible by IFN γ – CXCL9, CXCL10, and CXCL11 – are associated with Th1-mediated immune responses (Antonelli *et al.* 2006a). CXCL10, in particular, has been identified as a prototypic chemokine involved in the pathogenesis of glandular autoimmunity (Romagnani *et al.* 2002, Rotondi *et al.* 2003, Antonelli *et al.* 2004, 2005). Indeed, CXCL10 and its CXCR3 receptor play a pivotal role in the initial phases of AITDs (Romagnani *et al.* 2002, Kemp *et al.* 2003). In patients with GD, CXCL10 was detected in thyrocytes and endothelial and inflammatory cells, which also expressed peculiarly a large amount of CXCR3 (Garcia-Lopez *et al.* 2001, Romagnani *et al.* 2001, Aust *et al.* 2002, Kemp *et al.* 2003, Antonelli *et al.* 2004, 2005).

Furthermore, CXCL10 serum levels were significantly increased in GD patients with recent disease onset (Romagnani *et al.* 2002) and with active Graves' ophthalmopathy (Antonelli *et al.* 2006*d*). The CXCL10 secretion was synergistically induced by IFN γ and TNF α in isolated thyrocytes *in vitro* (Garcia-Lopez *et al.* 2001, Antonelli *et al.* 2006*d*).

Anti-thyroid drugs (ATDs) used in the treatment of hyperthyroidism due to GD, in addition to its effect on thyroperoxidase, inhibited cell-mediated and humoral immune reactions (Volpé 2001, Laurberg 2006). While some investigators supported an immunosuppressive effect on immune system cells (Mc Gregor *et al.* 1980), other authors favored a direct effect exerted primarily on the thyroid cells, with secondary effects on the immune system via reduced thyrocyte-immunocyte signaling (Volpé 2001, Laurberg 2006).

Methimazole (MMI), in particular, has been reported to interfere with immunological signals associated with GD hyperthyroidism (Mc Gregor *et al.* 1980, Laurberg 2006) and decrease CXCL10 serum levels (Antonelli *et al.* 2006*b,c*). The reduction in circulating CXCL10 levels induced by MMI might suggest that this drug could target thyrocytes as a source of CXCL10.

To our knowledge, the synergistic effect of TNF α on IFN γ -induced CXCL10 secretion in human thyrocytes has yet to be clarified and it has not been elucidated whether MMI is able to exert any direct effect on these cells. Since we hypothesized that MMI might hamper the cytokine-induced biological pathway(s) leading to CXCL10 secretion, we investigated the mechanisms underlying TNF α +IFN γ synergistic effect on CXCL10 secretion in cultured human thyrocytes, and the modulation, if any, by MMI on CXCL10 secretion in the cells stimulated with Th1-type cytokines. We utilized for comparison a peroxisome proliferator-activated receptor γ (PPAR γ) agonist, rosiglitazone (RGZ), a drug for type 2 diabetes treatment, since it has been recently reported to suppress IFN γ +TNF α -induced CXCL10 secretion in human thyroid follicular cells of GD patients (Antonelli *et al.* 2006*b,d*).

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (1:1) with and without phenol red, Ca²⁺/Mg²⁺-free PBS, BSA fraction V, glutamine, antibiotics, collagenase type IV, NaOH, Bradford reagent, and MMI were from Sigma-Aldrich Corp. The protein measurement kit was obtained from Bio-Rad Laboratories Inc. Fetal bovine serum was purchased from Unipath (Bedford, UK). IFN γ , TNF α , and ELISA kit for CXCL10 measurement were from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal anti-thyroglobulin antibody was from Cell Marque Corporation (Hot Spring, AR,

USA). Goat polyclonal anti-Pax8 antibody was from Abcam plc (Cambridge, UK). For flow cytometry analysis, PE-conjugated anti-CD119 (GIR-208, mouse IgG1) mAb was from BD Biosciences (Mountain View, CA, USA), the PE-conjugated anti-TNFR2 (22235.311, mouse IgG2a) mAb was purchased from R&D Systems, and conjugated isotype-matched control Abs were from Southern Biotechnology Associated Inc. (Birmingham, AL, USA; mouse IgG1:clone 15H6, mouse IgG2a:clone HOPC-1). β -Mercaptoethanol was purchased from Fluka Biochemika Ultra (Buchs, Switzerland). For RNA extraction, RNeasy Mini reagent kit was from Qiagen Italy. TaqMan Reverse Transcription Reagents kit, all primer/probe mixes (Taqman Gene Expression Assays), CXCL10 (ID number Hs00171042-m1), IFN γ R (ID number Hs00166223-m1), Pax8 (ID number Hs00247586-m1), and 1 $\times$ Universal Master Mix were from Applied Biosystems (Foster City, CA, USA). Quantitative PCR human reference total RNA was purchased from Stratagene (La Jolla, CA, USA). RGZ was from Glaxo (Welwyn, UK). For immunofluorescence, rabbit polyclonal antihuman primary antibody against nuclear factor- κ B (NF- κ B) p65 (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Alexa Fluor 488 goat anti-rabbit conjugate antibody was from Molecular Probes (Eugene, OR, USA). Plasticware for cell cultures and disposable filtration units for growth media preparation were purchased from Corning (Milan, Italy).

Cell cultures

Primary cultures of thyrocytes were obtained from inter-nodular parenchyma of thyroid tissues derived from 15 patients who underwent surgery for multinodular goiter (10 females: age range 37–83 years and 5 males: age range 32–81 years). Certificates of consent were obtained. Patients did not receive any specific treatment for thyroid disease; thyroid hormones and thyroid autoantibody measurements were in normal range. Thyrocytes were prepared as previously described (Garcia-Lopez *et al.* 2001) with some modifications. Briefly, tissues were minced to fragments as small as possible and treated with 2 mg/ml bacterial collagenase in PBS for 45 min at 37 °C. Digested tissues were mechanically dispersed until a homogeneous suspension was obtained. After washing with PBS, the cell suspension was cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 10% FBS, 2 mmol/l glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a fully humidified atmosphere of 95% air–5% CO₂ in plastic 100 mm dishes. Cells began to emerge within 48 h and were used within the fifth–sixth passage. Human thyrocytes expressed paired box (Pax)8, a thyroid-specific transcription factor (Kang *et al.* 2001), and were positively stained for thyroglobulin and Pax8 (data not shown).

CXCL10 secretion assay

For CXCL10 secretion assays, 4000 cells were seeded onto 96-well plates in growth medium. After 24 h, the growth medium was removed and cells were washed in PBS and incubated in phenol red- and serum-free medium. After 24 h, different stimuli were added in phenol red- and serum-free medium with 0.1% BSA (200 µl/well). Cells in phenol red- and serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. For dose-response assays, cells were incubated for 24 h with TNFα at 0.1, 1, 10, 100, and 500 ng/ml or IFNγ at 10, 100, 1000, 5000, and 10 000 U/ml, alone or combined with 10 ng/ml of TNFα; or with a combination of IFNγ (1000 U/ml) + TNFα (10 ng/ml) in the presence or absence of MMI (1, 2.5, 5, 10, 25, 50, 100, 200, 300, 500, and 1000 ng/ml) or RGZ (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 30 µM). The supernatant was harvested and kept frozen at -20 °C until CXCL10 ELISA was performed. Experiments were performed in triplicate or quadruplicate with 4–7 different cell preparations.

CXCL10 ELISA

CXCL10 levels were measured in culture supernatants (diluted 1:20–1:30) using commercially available kits, according to the manufacturer's recommendations. The sensitivity ranged from 0.41 to 4.46 pg/ml; mean minimum detectable dose was 1.67 pg/ml. The intra- and inter-assay coefficients of variation were 3.1% and 6.7% respectively. Samples were assayed in triplicate or quadruplicate. Quality control pools of low, normal, or high concentrations for all parameters were included in each assay. The obtained results, expressed as pg/ml, were normalized by total cell protein amount. Protein extraction was performed on the cell layer in the 96-well plates using 1 M NaOH for 5 min, followed by Bradford reagent for 10–15 min. Protein concentration was measured by spectrophotometric analysis at 595 nm.

Flow cytometry analysis

For cytometry analysis, cells were seeded onto 100 mm dishes in growth medium. Since the cultures were near confluent (about 10⁶), the growth medium was removed and cells were washed in PBS and incubated in phenol red- and serum-free medium for 24 h. Thereafter, cells were stimulated for 24 h with IFNγ (1000 U/ml) or TNFα (10 ng/ml) in phenol red- and serum-free medium with 0.1% BSA. Cells in phenol red- and serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. Flow cytometry analysis on cell suspensions was performed as detailed elsewhere (Annunziato *et al.* 2002). Briefly, 10⁵ cells were incubated with the specific or the isotype

control mAb at +4 °C for 30 min; cells were then washed with PBS (pH 7.2) containing 0.5% BSA and analyzed on a BDLSRII cytofluorimeter using the Diva software (BD Biosciences). Ten thousand events for each sample were acquired. The area of positivity was determined using an isotype-matched mAb. Experiments were performed at least four times with 4–8 different cell preparations.

RNA extraction

For mRNA analysis, cells (500 000 in 60 mm dishes) were maintained in phenol red- and serum-free medium for 24 h. Then, cells were incubated in phenol red- and serum-free medium containing 0.1% BSA with different stimuli, according to the following protocol: IFNγ (1000 U/ml) or TNFα (10 ng/ml) alone or combined; TNFα (10 ng/ml) combined with MMI (300 ng/ml) or RGZ (5 µM). Cells in phenol red- and serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. Thereafter, cells were trypsinized, washed twice in PBS, and pelleted before lysis. Cell pellet (stored at -80 °C) was resuspended in 350 µl of RLT plus β-mercaptoethanol (10 µl β-mercaptoethanol per ml of RLT buffer). Total RNA from the cells was extracted with the RNeasy Mini reagent kit according to the manufacturer's recommendations. RNA concentration and quality were measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Experiments were performed three/four times with different cell preparations.

Real-time PCR

Total RNA (400 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents kit. Reverse transcription was performed in a final volume of 80 µl containing 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl (pH 8.3), 5.5 mM MgCl₂, 500 µM of each dNTP, 2.5 µM random examers, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe Reverse Transcriptase. The reverse transcription reaction was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 3 min. Measurement of gene expression was performed by quantitative real-time PCR (TaqMan). The amount of target, normalized to an endogenous reference (18s, pre-developed TaqMan Assay Reagents) and relative to a calibrator (Quantitative PCR human reference total RNA), was given by 2^{-ΔΔCt} calculation (Livak & Schmittgen 2001). The formula applied is $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{18s}}) - (C_{t_{\text{target gene calibrator}}} - C_{t_{18s \text{ calibrator}}})$. For each sample, 12.5 ng of cDNA were added to 10 µl of PCR mix containing each primer/probe mix and 1 × Universal Master Mix. The samples were then subjected to 40 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s in the ABI Prism 7700 Sequence Detector (Applied Biosystems).

Immunofluorescence microscopy

Ten thousand cells were seeded onto glass coverslips in growth medium for 24 h and then incubated with serum-free medium overnight, before treatment with TNF α (10 ng/ml) alone or combined with IFN γ (1000 U/ml), in the presence or absence of MMI (300 ng/ml) or RGZ (5 μ M) for additional 24 h. Cells in phenol red- and serum-free medium containing 0.1% BSA and vehicle (absolute ethanol, 0.47%, vol/vol) were used as control. For method specificity, slides lacking the primary antibodies or stained with the corresponding nonimmune serum were processed. After washing twice with PBS, cells were fixed with 3.7% paraformaldehyde (pH 7.4) for 10 min and then permeabilized for 10 min with PBS with 0.1% Triton X-100. Immunostaining was performed as described previously (Vannelli *et al.* 1995) using primary antibody against NF- κ B p65 (1:100), followed by Alexa Fluor 488 conjugate secondary antibody (1:200). The slides were examined with a phase contrast microscope (Nikon Microphot-FX microscope, Nikon, Tokyo, Japan). Experiments were performed thrice with different cell preparations.

Statistical analysis

The statistical analysis was performed using SPSS 12.0 software package (SPSS for Windows 12.0; SPSS Inc. Chicago, IL, USA). The Kolmogorov–Smirnov test was used to test for normal distribution of the data. One-way ANOVA was applied. A *P* value <0.05 was considered significant and was corrected for comparisons using the Dunnett's *post hoc* test. Data are expressed as mean $\pm$ S.E.M. The computer program ALLFIT (NIH, Bethesda, MD, USA; De Lean *et al.* 1978) was used for analysis of sigmoid dose–response curves to obtain estimates of IC₅₀ of MMI and RGZ.

Results

In human thyrocytes, 24-h treatment with increasing concentration of IFN γ (10–10 000 U/ml) significantly increased CXCL10 protein secretion in the supernatant over undetectable basal levels in control cells (*P*<0.01 or *P*<0.05 versus control; Fig. 1A). This effect was dose dependent and did not achieve plateau (125.0 $\pm$ 16.96 pg/ μ g protein, mean value at highest dose). Treatment with increasing doses of TNF α (0.1–100 and 500 ng/ml) enhanced CXCL10 secretion over control levels as well (*P*<0.01 or *P*<0.05 starting from 10 ng/ml; Fig. 1B), although the secreted amount of chemokine was low (13.49 $\pm$ 4.49 pg/ μ g protein, mean value at plateau).

The simultaneous addition of a fixed dose of TNF α (10 ng/ml) to IFN γ (10–10 000 U/ml) magnified the IFN γ -induced CXCL10 secretion until the maximal cell response was evoked (2866.35 $\pm$ 229.43 pg/ μ g protein, mean value at plateau) at each tested dose (*P*<0.01 or *P*<0.05 versus

control and versus IFN γ alone corresponding dose), confirming the strong synergistic effect of the combined cytokines (Fig. 1C). Combination of TNF α (10 ng/ml) and IFN γ (1000 U/ml) yielded the highest response, very much in agreement with previous studies (Antonelli *et al.* 2006d) and, therefore, it was utilized for all subsequent experiments. Taqman real-time PCR analysis confirmed that either IFN γ (1000 U/ml) or TNF α (10 ng/ml) similarly upregulated CXCL10-specific mRNA, while cytokine combination resulted in about a 23-fold increase in CXCL10 mRNA, when compared with single cytokine-induced expression (*P*<0.01 or *P*<0.05 versus control, *P*<0.05 versus IFN γ -induced expression; Fig. 1D).

Cytokines are known to interact through the reciprocal modulation of their receptors. To verify whether the mechanism underlying TNF α and IFN γ synergism involved this mechanism in thyrocytes, we performed flow cytometry and mRNA analysis of IFN γ and TNF α receptors (IFN γ R and TNF α R, type I or II) in thyrocytes stimulated with TNF α (10 ng/ml) or IFN γ (1000 U/ml). As shown in Fig. 2A upper panel, IFN γ R protein membrane expression increased in thyrocytes treated with TNF α versus control cells (28.82 $\pm$ 3.11% vs 5.42 $\pm$ 0.63%, *P*<0.01), while IFN γ did not show significant effect. Figure 2A lower panel depicts a representative experiment out of eight. No significant effect on TNF α RI or II (data not shown) was observed. The mRNA analysis of IFN γ R also revealed a significant TNF α -induced increase in the IFN γ R gene over basal level (*P*<0.01 versus control) – although the increase in the protein was much higher – whereas IFN γ did not exert significant effect on the specific messenger, as shown in Fig. 2B.

To investigate the possible role of MMI in CXCL10 secretion induced by proinflammatory cytokines in human thyrocytes, cells were simultaneously incubated with a combination of IFN γ (1000 U/ml) and TNF α (10 ng/ml) and increasing concentrations of MMI (1, 2.5, 5, 10, 25, 50, 100, 200, 300, 500, and 1000 ng/ml) or RGZ (0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 30 μ M) used for comparison. The first two lowest doses of each molecule (1 and 2.5 ng/ml for MMI; 0.025 and 0.05 μ M for RGZ) were reported only in ALLFIT interpolation.

The drug concentrations were selected on the basis of their near therapy doses (300 ng/ml = 2.63 μ M for MMI and 5 μ M for RGZ respectively) according to their pharmacokinetics (*C*_{max} and area under the time–concentration curve, AUC).

As shown in Fig. 3A, 24-h cell incubation with MMI decreased, in a dose-dependent manner, cytokine-induced CXCL10 secretion, significantly from 10 ng/ml (*P*<0.01 or *P*<0.05 versus IFN γ + TNF α -treated cells).

Similarly, RGZ dose-dependently inhibited CXCL10 secretion significantly (Fig. 3B) from 1 μ M (*P*<0.01 versus IFN γ + TNF α -treated cells).

MMI and RGZ exerted a comparable inhibitory effect on CXCL10 secretion, as confirmed by the simultaneous fitting using ALLFIT program (De Lean *et al.* 1978) of the inhibitory

curves, showing no statistically significant difference in 50% effective concentration ($IC_{50} = 0.29 \pm 0.05 \mu M$, $P = 0.21$), as shown in Fig. 3C. To verify whether the inhibitory effect of both drugs somehow affected the mechanism of synergy between the two cytokines, we analyzed IFN γ R expression induced by TNF α (10 ng/ml) in the presence of MMI (300 ng/ml) and RGZ (5 μM) respectively.

Flow cytometry demonstrated that the treatment of thyrocytes with MMI for 24 h significantly reduced IFN γ R membrane protein expression induced by TNF α ($17.4 \pm 3.8\%$ vs $30 \pm 1.5\%$ respectively, $P < 0.05$), while RGZ did not exert any significant effect (Fig. 4A). Real-time PCR analysis of specific mRNA confirmed that MMI significantly reduced IFN γ R gene expression induced by TNF α ($P < 0.01$ versus TNF α -induced expression), although to a lower extent with respect to protein, while RGZ showed no effect (Fig. 4B).

In order to assess whether MMI or RGZ impaired the pathway of NF- κB , a pleiotropic transcriptional factor tightly associated with several cellular biological functions (Gilmore 2006) and specifically activated by TNF α , we set up a nuclear translocation assay.

Stimulation with TNF α alone (10 ng/ml; Fig. 5B) or combined with IFN γ (1000 U/ml; Fig. 5C) resulted in the same compact increase (nuclear staining with TNF α : $91.42 \pm 2.13\%$; nuclear staining with TNF α + IFN γ : $89.82 \pm 2.48\%$) of NF- κB translocation from the cytoplasmic to the nuclear compartment. In Fig. 5A, control cells are depicted, showing $1.14 \pm 0.64\%$ nuclear staining for NF- κB . IFN γ alone did not exert any effect on NF- κB (data not shown). Since the effect of each drug with TNF α alone or

Figure 1 CXCL10 secretion is induced by IFN γ and TNF α in human thyrocytes and potentiated by synergism between cytokines. (A) Increasing concentrations of IFN γ (10–10 000 U/ml) significantly enhanced dose-dependently CXCL10 secretion versus almost undetectable levels in control cells ($*P < 0.05$, $**P < 0.01$) and did not reach a plateau. Amount of CXCL10 secretion ranged from 16.94 ± 1.01 to 125.0 ± 16.96 pg/ μ g protein, lowest to highest dose. (B) Treatment with increasing concentration of TNF α (0.1–100 and 500 ng/ml) significantly increased CXCL10 secretion ($*P < 0.05$, $**P < 0.01$ versus control), although to a low extent (3.49 ± 0.98 – 13.49 ± 4.49 pg/ μ g protein, lowest to highest dose). (C) A fixed dose of TNF α (10 ng/ml) enhanced CXCL10 secretion induced by IFN γ ($*P < 0.05$, $**P < 0.01$ versus control cells, or versus IFN γ alone corresponding dose, $^oP < 0.05$ versus preceding dose). CXCL10 levels ranged from 1225.50 ± 41.09 to 2866.35 ± 229.43 pg/ μ g protein, lowest to highest dose. Synergism between the two cytokines effect, initially dose dependent, reached a plateau at the combination of IFN γ (1000 U/ml) and TNF α (10 ng/ml). (D) mRNA expression of CXCL10 in thyrocytes treated with IFN γ (1000 U/ml) and TNF α (10 ng/ml) alone or combined. CXCL10 mRNA was upregulated by the two single cytokines and it was potentiated by their combination ($*P < 0.05$, $**P < 0.01$ versus control cells, $^oP < 0.05$ versus IFN γ -induced expression). (A–C: ordinate) CXCL10 secretion expressed as pg/ μ g protein; columns are mean $\pm$ S.E.M. Data are derived from 4–7 separate experiments using distinct cell preparations ($n = 4$ and 7). (D: ordinate) CXCL10 mRNA fold increase versus expression in control cells, taken as 1. Data are derived from three separate experiments using distinct cell preparations ($n = 3$).

Figure 2 IFN γ receptor (IFN γ R) protein and gene expression are upregulated by TNF α in human thyrocytes.

(A: upper panel) Flow cytometry revealed that TNF α (10 ng/ml) upregulated IFN γ R membrane expression expression versus control cells ($28.82 \pm 3.11\%$ vs $3.42 \pm 0.63\%$, $**P < 0.01$ versus basal expression in control cells); the treatment with its cognate ligand (1000 U/ml) did not show significant effect. (A: lower panel) IFN γ R (CD119) membrane expression in thyrocytes after 24-h culture in the indicated conditions is depicted (one representative experiment out of eight). Dotted histograms represent the staining obtained with control isotype-matched mAbs. In each, the percentage of positive cells is indicated. (B) Quantitative analysis using real-time RT-PCR of IFN γ R gene expression showed that TNF α (10 ng/ml) significantly increased IFN γ R gene expression ($**P < 0.01$ versus control cells), while IFN γ (1000 U/ml) did not exert any significant effect. (A: upper panel ordinate) IFN γ R (CD119) membrane protein expression (percentage of positive cells over total), columns are mean $\pm$ S.E.M. Results are derived from eight separate experiments using distinct cell preparations ($n=8$). (B: ordinate) IFN γ R mRNA expression (fold increase over expression in control cells, taken as 1); columns are mean $\pm$ S.E.M. Results are derived from four separate experiments using distinct cell preparations ($n=4$).

combined with IFN γ was the same, only the condition with cytokine combination has been reported.

Co-incubation with MMI (300 ng/ml) did not influence nuclear translocation as demonstrated by $83.23 \pm 3.21\%$ of nuclear stain cell positivity (not significant versus cytokine-treated cells; Fig. 5D). Conversely, simultaneous incubation with RGZ (5 μ M) significantly reduced cytokine-stimulated NF- κ B translocation, although more than 40% of cell nuclei still showed positivity (nuclear staining $42.13 \pm 6.85\%$, $P < 0.01$ versus cytokine-treated cells; Fig. 5E). Columns in Fig. 5F summarize the results obtained with all treatments.

Discussion

This study shows that in human thyrocytes the synergistic effect of TNF α with IFN γ on CXCL10 secretion is associated with a significant upregulation of IFN γ R driven by TNF α , both at protein and gene levels. We, for the first time, provide evidence in thyrocytes that MMI decreases cytokine-induced CXCL10 protein secretion by reducing TNF α -induced upregulation of the IFN γ membrane receptor. RGZ similarly decreases cytokine-induced CXCL10 secretion without affecting IFN γ R expression.

Interestingly, RGZ blocked TNF α -mediated nuclear translocation of NF- κ B, pointing out that both drugs abrogated CXCL10 secretion likely by different pathways.

CXCL10 secretion potentiates the Th1 immunity-promoting inflammatory loop, driven by the recruited CD4⁺T lymphocytes at inflammation sites, supporting T-cell proliferation and IFN γ secretion (Campbell *et al.* 2004, Romagnani *et al.* 2004). It is well known that TNF α synergizes with IFN γ , modulating a number of different biological effects (Krakauer & Oppenheim 1993). In particular, the synergy between the two cytokines is essential in potentiating inflammatory and Th1 immune responses promoted by IFN γ . To date, previous studies reported that CXCL10 secretion is triggered only by IFN γ in different resident cell types (Rotondi *et al.* 2005, Antonelli *et al.* 2006d), while in microvascular endothelium TNF α has been reported to upregulate cytokines other than CXCL10 (Sana *et al.* 2005).

In our study, TNF α alone elicited a dose-dependent CXCL10 protein secretion in thyrocytes, although to a low extent. Accordingly, TNF α alone was able to upregulate CXCL10 mRNA similarly to IFN γ , suggesting that TNF α , in addition to its synergistic effect with IFN γ , could contribute to initiating the Th1-mediated response.

Furthermore, the synergic action between the two cytokines in thyrocytes seems to be exerted mainly at a receptor level. A number of cytokines are known to amplify their biological responses both *in vivo* and *in vitro* via regulation of the expression of other cytokine receptors (Krakauer & Oppenheim 1993).

We clearly demonstrated that TNF α upregulated IFN γ R at both protein and gene levels, while no significant effect was observed on TNFR type I or II expression (data not shown). Since dose-response curves with IFN γ did not reach a

Figure 3 MMI and RGZ dose-dependently inhibited CXCL10 secretion in human thyrocytes, with a similar potency. (A) Increasing doses of MMI (5, 10, 25, 50, 100, 200, 300, 500, and 1000 ng/ml) decreased CXCL10 secretion induced by IFN γ (1000 U/ml) and TNF α (10 ng/ml; * P <0.05, ** P <0.01 versus IFN γ + TNF α -treated cells). The maximum inhibitory concentration was 300 ng/ml corresponding to 2.63 μ M (max inhibition 46.28 $\pm$ 3.04%). (B) Incubation of thyrocytes with different doses of RGZ (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 30 μ M) similarly inhibited cytokine-induced CXCL10 secretion (** P <0.01 versus IFN γ + TNF α -treated cells). The maximum inhibitory concentration was 5 μ M (max inhibition 54.44 $\pm$ 5.34%). (C) After ALLFIT interpolation, MMI (open circles) and RGZ (closed circles) exerted a similar inhibitory effect in thyrocytes, fitting the same curve (IC₅₀ = 0.29 $\pm$ 0.05 μ M, P = 0.21). The two lowest extra doses reported in ALLFIT interpolation corresponded to 1 and 2.5 ng/ml for MMI and to 0.025 and 0.05 μ M for RGZ respectively. Drug concentrations were selected on the basis of their near therapy doses according to their pharmacokinetics ($C_{\max}$ and area under the time–concentration curve, AUC). (A and B: ordinate) CXCL10 secretion expressed as pg/ μ g protein; columns are mean $\pm$ S.E.M. Data derived from seven separate experiments using distinct cell preparations (n = 7). (C: ordinate) CXCL10 secretion expressed as percent variation (mean $\pm$ S.E.M.) over the maximal IFN γ + TNF α -induced stimulation.

Figure 4 Modulation of IFN γ R protein and gene expression by MMI and RGZ in human thyrocytes. (A) Twenty-four-hour treatment with MMI (300 ng/ml) significantly reduced IFN γ R membrane protein expression induced by TNF α (10 ng/ml; 17.4 $\pm$ 3.8% vs 30 $\pm$ 1.5%, ° P <0.05 versus TNF α -induced expression, ** P <0.01 versus control). Conversely, RGZ (5 μ M) did not exert any significant effect in TNF α -treated cells. (B) MMI (300 ng/ml) inhibited TNF α -induced IFN γ R mRNA upregulation (° P <0.01 versus TNF α -induced expression, * P <0.05, ** P <0.01 versus control), while RGZ (5 μ M) showed no effect. (A: ordinate) IFN γ R (CD119) membrane protein expression (percentage of positive cells over total). Results are derived from four separate experiments using distinct cell preparations (n = 4). (B: ordinate) IFN γ R mRNA expression (fold increase versus expression in control cells, taken as 1); columns are mean $\pm$ S.E.M. Results are derived from three separate experiments using distinct cell preparations (n = 3).

plateau, the increase, induced by TNF α , of IFN γ R expression may account for the raised magnitude of the response to IFN γ , in terms of CXCL10 protein secretion. Accordingly, TNF α was able to potentiate the amount of CXCL10 protein secretion (ng/ml versus pg/ml) dose-dependently induced by IFN γ , until the maximal cell response was evoked. CXCL10 mRNA specific amount resulted significantly increased in the presence of both cytokines. Since cells with higher number of specific receptors are able to respond to lower concentrations of IFN γ , TNF α could be critical in the pathogenesis of AITDs, in line with previous work highlighting the relevance of the TNF α pathway in GD (Diez *et al.* 2002).

Our data support the thyroid itself as the main site of CXCL10 secretion perpetuating the Th1-mediated autoimmune cascade in AITDs (Garcia-Lopez *et al.* 2001, Romagnani *et al.* 2002, Kemp *et al.* 2003, Antonelli *et al.* 2004). This is very much in agreement with recently published studies in patients showing the reduction of high CXCL10 serum levels in patients with GD after

Figure 5 Microscopy assessment of NF- κ B activation in human thyrocytes. The nuclear accumulation of NF- κ B p65 subunit in the presence or absence of $\text{TNF}\alpha$ (10 ng/ml) alone or with $\text{IFN}\gamma$ (1000 U/ml) and with MMI (300 ng/ml) or RGZ (5 μ M) was assessed by microscopy of thyrocytes stained with antihuman antibody against p65. (A) In control cells, p65 was distributed throughout the cytosol. (B and C) After stimulation with $\text{TNF}\alpha$ alone (B) or combined with $\text{IFN}\gamma$ (C), p65 consistently disappeared from the cytosol and accumulated in the nucleus, in most of the cells. (D) In cells stimulated by both cytokines and MMI, p65 subcellular distribution pattern was similar to that in cells stimulated with cytokines. (E) The simultaneous presence of RGZ in cells treated with cytokines reduced more than 50% p65 nuclear translocation induced by $\text{TNF}\alpha + \text{IFN}\gamma$. Since the effect of each drug with $\text{TNF}\alpha$ alone or combined with $\text{IFN}\gamma$ was the same, only the condition with cytokine combination is reported. Bar: 100 μ m. (F) Cells were scored as either positive or negative for nuclear p65 and results were expressed as the percentage of positive cells $\pm$ S.E.M. (calculated by counting the number of stained cells over total in 18 separate fields in each stained slide). Positive cells for nuclear p65 were virtually absent in control cells ($1.14 \pm 0.64\%$). The percentage of positive cells after stimulation was $91.42 \pm 2.13\%$ with $\text{TNF}\alpha$, $89.82 \pm 2.48\%$ with $\text{TNF}\alpha + \text{IFN}\gamma$, $85.12 \pm 2.58\%$ with $\text{TNF}\alpha$ and MMI, $83.23 \pm 3.21\%$ with $\text{TNF}\alpha + \text{IFN}\gamma$ and MMI, $51.42 \pm 6\%$ with $\text{TNF}\alpha$ and RGZ, and $42.13 \pm 6.85\%$ with $\text{TNF}\alpha + \text{IFN}\gamma$ and RGZ. ** $P < 0.01$ versus control; ° $P < 0.01$ versus $\text{TNF}\alpha$; # $P < 0.01$ versus $\text{TNF}\alpha + \text{IFN}\gamma$. (F: ordinate) p65 nuclear positive cells (percentage over total cells); columns are mean $\pm$ S.E.M. Results are derived from three separate experiments using distinct cell preparations ($n=3$).

thyroidectomy (Antonelli *et al.* 2006b) or after treatment with ^{131}I (Antonelli *et al.* 2007).

Treatment with thionamide in AITDs, in addition to its effect on hormone synthesis, is accompanied by a gradual remission of the autoimmune aberration in the majority of patients (Pinchera *et al.* 1969, Mc Gregor *et al.* 1980, Laurberg 2006). A recent report describes a reduction in CXCL10 circulating levels in hyperthyroid patients with GD under MMI therapy (Antonelli *et al.* 2006c).

We showed that MMI decreased proinflammatory cytokine-induced CXCL10 secretion in thyrocytes *in vitro*, throughout a strong reduction in TNF α -induced IFN γ R membrane protein expression. This effect, matching a reduction in specific amount of IFN γ R mRNA, seems to be particularly relevant because it counteracts the magnitude of the CXCL10 secretion in response to IFN γ , as previously emphasized. Thus far, it turned out that MMI exerts its immunomodulatory effect on thyrocytes and this should not be surprising since it is well known that it concentrates in follicular cells and not in the surrounding interstitial tissue, where the infiltrating immunocompetent cells are present (Marchant *et al.* 1972).

Hyperthyroidism *per se* plays a minimal role in determining CXCL10 levels that, indeed, are strongly associated with the autoimmune process of the hyperthyroid phase in GD (Romagnani *et al.* 2002, Antonelli *et al.* 2006a), but not with toxic nodular goiter (Antonelli *et al.* 2007). In this light, MMI emerged as a particularly relevant therapy, since it targets the thyroid by dual action, both restoring euthyroidism and decreasing CXCL10 release by thyrocytes.

Concerning drug doses, we want to highlight that the concentrations used in our experiments were selected according to drug plasma or intraglandular concentration range following therapeutic doses, differently from previous *in vitro* studies that have used far higher drug concentrations than the concentration reached in the thyroid tissue *in vivo* (Mc Gregor *et al.* 1980).

Our studies on MMI were performed in comparison to RGZ, a pure PPAR γ agonist, because it has been recently showed to reduce CXCL10 levels in thyroid follicular cells in GD patients (Antonelli *et al.* 2006d). PPAR γ agonists are known to play an inhibitory role in the self-perpetuation of chemokine-mediated inflammatory processes in several human cell types (Su *et al.* 1999, Marx *et al.* 2000, Gosset *et al.* 2001, Antonelli *et al.* 2006d).

RGZ exerted its inhibitory effect on Th1-polarized response in thyrocytes without affecting IFN γ R expression. In human thyrocytes, RGZ significantly inhibited CXCL10 secretion at micromolar concentrations by inhibiting NF- κ B activation, as demonstrated by the decrease of TNF α -induced NF- κ B translocation in the cell nucleus. On the contrary, in other cellular models (colon cells transfected with an NF- κ B-luciferase reporter element), the mechanism of CXCL10 decrease induced by PPAR γ agonists seems to be NF- κ B independent (Schaefer *et al.* 2005). Further studies are necessary to provide details on the RGZ

mechanism of action, intended to elucidate the potential effect on Graves' ophthalmopathy. Indeed, while some authors have suggested (Antonelli *et al.* 2006d) its beneficial effect, there is experimental and clinical evidence that PPAR γ agonists may impair the clinical course of Graves' ophthalmopathy by adipogenesis stimulation (Valyasevi *et al.* 2002, Starkey *et al.* 2003).

In summary, we have demonstrated a novel anti-inflammatory effect of MMI that is able to revert Th1 cytokine-mediated CXCL10 secretion directly in thyroid cells by damping the mechanism underlying cytokine synergy. Although further investigations are needed to understand its signaling pathway, MMI targeted thyrocytes with the same pharmacological potency as RGZ, a regulator of the inflammatory response (Campbell 2005), likely acting through different mechanisms.

Given the pivotal role of IFN γ -induced chemokines in Th1-mediated autoimmune thyroid diseases (Rotondi *et al.* 2007), a combination of different drugs with the same inhibitory effect targeting different intracellular pathways could be a novel pharmacological tool, intended to decrease therapeutic doses and thus minimizing side effects, similar to immunosuppressive protocols for the treatment of other autoimmune diseases or allograft rejection.

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