JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 33, Number 9, 2013

DOI: 10.1089/jir.2012.0080

Type I and Type II Interferons Inhibit Both Basal and Tumor Necrosis Factor-α-Induced CXCL8 Secretion in Primary Cultures of Human Thyrocytes

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Interferons (IFNs) and tumor necrosis factor-α (TNF-α) cooperate in activating several inflammation-related genes, which sustain chronic inflammation in autoimmune thyroid disease (AITD). Much is known about the positive signaling of IFNs to activate gene expression in AITD, while the mechanisms by which IFNs negatively regulate genes remain less studied. While IFNs inhibit CXCL8 secretion in several human cell types, their effects on thyroid cells were not evaluated. Our aim was to study the interplay between TNF-α and type I or type II IFNs on CXCL8 secretion by human thyroid cells. CXCL8 was measured in supernatants of primary cultures of thyroid cells basally and after a 24-h incubation with TNF-α. CXCL8 was detected in thyroid cell supernatants in basal conditions (96.2 ± 23.5pg/mL) being significantly increased (784.7 ± 217.3 pg/mL; $P < 0.0001$ vs. basal) by TNF-α. Twenty-four hour incubation with IFN-γ or IFN-β or IFN-α dose dependently and significantly inhibited both basal and TNF-α-induced CXCL8 secretion. The degree of the inhibitory effect was IFN-γ > IFN-β > IFN-α. This study demonstrates that type I and type II IFNs downregulate both basal and TNF-α-induced CXCL8 secretion by human thyrocytes, IFN-γ being the most powerful inhibitor. Future studies aimed at a better comprehension of the interplay between CXCL8 and thyroid diseases appear worthwhile.

Introduction

Interferons (IFNs) were first recognized for their antiviral properties (Isaacs and Lindenmann 1957), but they display a much broader spectrum of functions, including immunomodulating and cytostatic activities (Borden and others 1982; Biron 2001; Samuel 2001). IFN-γ is the cardinal Th1-oriented cytokine, playing a critical role in autoimmune thyroid disease (AITD) (Caturegli and others 2000; Lira and others 2005; Fang and others 2007). Tumor necrosis factor-α (TNF-α) is a critical proinflammatory mediator in both acute and chronic stages of systemic inflammatory disease states (Bradley 2008). TNF-α is a known activator of the nuclear factor kappa B (NF-κB) and the activator protein-1 (AP-1), 2 key modulators of the inflammatory response (Wajant and others 2003; Bradley 2008). Experimental evidence supports a synergic role of TNF-α and IFN-γ in sustaining chronic autoimmune inflammation. Indeed, the 2 proinflammatory cytokines largely cooperate in activating a great number of inflammation-related genes (Ohmori and others 1997). A good example of the synergism between IFN-γ and TNF-α is provided by the secretion of CXCR3-binding chemokines, which occurs in primary cultures of thyroid cells after stimulation with a combination of the 2 cytokines (García-López and others 2001; Rotondi and others 2005; Antonelli and others 2006c; Rotondi and others 2007; Lombardi and others 2008; Rotondi and Chiovato 2011). As shown by a recent study from our group, an exception to this rule is represented by the regulation of CXCL8 secretion (Rotondi and others 2013). CXCL8 is secreted by human thyroid cells in basal conditions (Weetman and others 1992) and its secretion is strongly enhanced by TNF-α (Roebuck 1999). Studies using rt-PCR demonstrated a significant expression of the mRNA for CXCL8 in cultured human thyroid cells. (Weetman and others 1992; Watson and others 1995; Roebuck 1999; García-López and others 2001). As demonstrated by *in vitro* experiments using several types of human cells, both type I and type II IFNs have an opposite effect, because they downregulate the secretion of CXCL8 (Oliveira and others 1992; Cassatella and others 1993; Nyhlén and others 2000; Nozell and others 2006). However, previous studies evaluated the inhibition of CXCL8 secretion produced by a single type of IFN; thus, the relative potency of different subtypes of IFNs was not investigated. IFNs are a class of

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cytokines with molecular weights of ~15,000–21,000 Da, which share a high degree of homology both in their amino acid sequence and 3-dimensional structure (Baron and others 1991). They are grouped into types I and II according to their immunogenicity and the specific plasma membrane-binding receptor (Pestka and others 2004). Type I IFNs include IFNα, IFN-β, and IFN-ω subtypes. All type I IFNs are coded by genes on chromosome 9 and bind to the same receptor, although with different affinities (Baron and others 1991; Pestka and others 2004). IFN-γ is the only type II IFN, it binds to a distinct receptor and it is coded by genes on chromosome 12 (Baron and others 1991).

Much is known about the positive signaling of IFNs to activate gene expression in AITD, while the mechanisms by which IFNs are able to negatively regulate genes remain by far less studied (Laver and others 2008). In a previous study, type I and type II IFNs were compared for their ability to promote CXCL10 secretion in human thyrocytes (Antonelli and others 2010), and IFN-γ was identified as the strongest stimulator. The present study was specifically designed to evaluate the interplay between TNF-α and IFN-α, β, or γ on the secretion of CXCL8 by human thyroid cells in primary culture. The results of these experiments could be helpful for understanding the regulation of basal and stimulated CXCL8 secretion by human thyroid cells.

Materials and Methods

Primary cultures of human thyroid cells

Normal human thyroid specimens were obtained from the controlateral disease-free lobe of 8 patients who underwent thyroidectomy for the differentiated thyroid cancer ($n=2$) solitary hyperfunctioning nodule ($n=3$) and the solitary nonfunctioning nodule ($n=3$). All patients were euthyroid at the moment of surgery; none of them had positive tests for either thyroglobulin antibodies (TgAb) and thyroperoxidase antibodies (TPOAb) and had a normo-echoic pattern of the thyroid parenchyma at a presurgery ultrasound scan. Signed informed consent was obtained from all patients. Surgical specimens were minced and then incubated with collagenase type II (Sigma, St. Louis, MO) 5 mg/mL, in 5 mL of the Coon's F12 medium, for 4 h at 37°C. Then, 20 mL of the Coon's F12 medium was added, following which, cells were filtered, spun at 1,000 *g* for 10 min, washed with the Coon's F12 medium, spun again, and finally resuspended in a complete medium [Coon's F12 containing 5% newborn calf serum and a mixture of 5 hormones plus bovine thyrotropin (TSH) 1 mU/mL].

Secretion of CXCL8 by cultured human thyroid cells in basal conditions and after incubation with type I and type II IFNs

For the CXCL8 secretion assays, 3,000 thyroid cells were seeded into 96-well plates in the complete medium. After adherence to the plastic surface, cells were incubated for 24 h in a serum-free medium either plain or with the addition of IFN-α (Roferon-A; Roche Diagnostic GmbH, Penzberg, Germany), IFN-β (Extavia; Novartis International AG, Basel Switzerland), or IFN-γ (Euroclone S.p.A, Milano, Italy). Dose–response experiments were performed with each IFN using the following concentrations: 1, 10, 100, and 1,000 U/mL. After 24 h, the growth medium was removed and used for the CXCL8 assay. Experiments were performed on 3 different cell preparations in triplicates.

Secretion of CXCL8 by cultured thyroid cells after stimulation with TNF-α alone or in combination with type I and type II IFNs

Using the experimental conditions described above, thyroid cells were incubated with TNF-α alone (10 ng/mL; R&D Systems, Minneapolis, MN) or with TNF-α + IFN-α, β, or γ. Dose–response experiments were performed with each IFN at the concentrations of 1, 10, 100, and 1,000 U/mL. A second set of experiments were performed by adding 1,000 U/mL of each IFN simultaneously with TNF-α or at 1, 2, 6 h, after TNF-α. After 24 h, supernatants were collected and used for the CXCL8 assay. In the attempt to partially evaluate whether the inhibitory effects of the type I and type II IFNs involve the same or different pathways, thyrocytes (3,000/well) were plated and incubated with either 1,000 U/mL of a given IFN or with mixtures of 2 IFNs (500 U/mL each). Cell supernatants were collected after 24 h for CXCL8 measurements.

Patients with Graves' disease

Serum samples were collected from 25 patients affected by Graves'disease (GD). GD had been diagnosed by measuring the serum concentrations of free thyroxine (FT4), free triiodothyronine (FT3), and TSH and by searching for anti-TgAb, anti-TPOAb, and anti-TSH receptor antibodies (TRAb). Twenty-five healthy volunteers from hospital staff and their relatives in whom a complete thyroid work-up (the history, physical examination, thyroid hormone, and antibody profile) ruled out the presence of thyroid disorders, served as controls. All GD patients were specifically selected for being euthyroid on methimazole because high levels of CXCL8 were previously reported in hyperthyroid patients with GD (Siddiqi and others 1999) being the consequence of hyperthyroidism *per se* rather than of the accompanying autoimmune inflammatory condition. None of them received corticosteroids. The serum levels of FT4, FT3, TSH, TgAb, TPOAb, TRAb, and CXCL8 were measured in all patients and controls. All subjects gave their informed consent to enter the study, which was approved by the local ethics committee.

ELISA for CXCL8

CXCL8 levels were measured in cell supernatants and serum using a commercially available kit (R&D Systems). The mean minimum detectable dose of CXCL8 was 3.5 pg/mL. The intra- and interassay coefficient of variation was 6.8%. Samples were assayed in duplicate.

Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS, Inc., Evanston, IL). Mean group values were compared by using one-way ANOVA for normally distributed variables. *Post hoc* analysis was performed applying the Bonferroni's correction. The Student's t test for paired data and the Wilcoxon test was used according to a normal and a nonparametric distribution of the analyzed variable. Correlation between 2 variables was ascertained by Pearson or Spearman's correlation tests, as appropriate. Values are

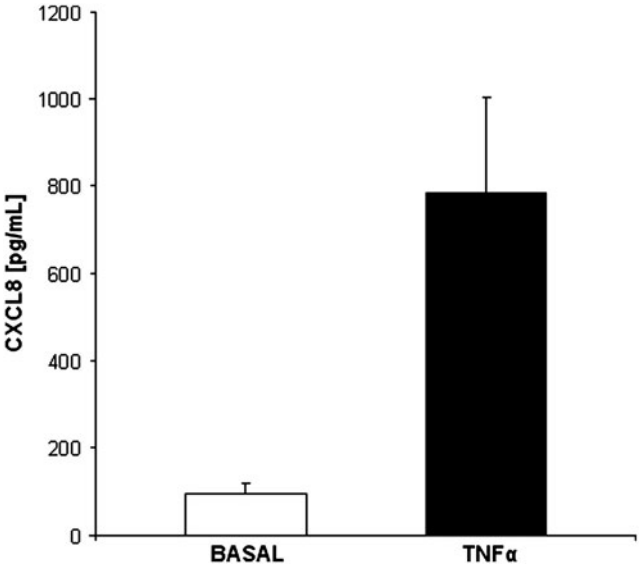

FIG. 1. Basal and tumor necrosis factor (TNF)-α-induced mean levels of CXCL8 in supernatants from primary cultures of human thyrocytes. Stimulation with TNF-α (10 ng/mL for 24 h) induces a significant increase in mean CXCL8 concentrations in the supernatants of primary cultures of human thyrocytes. Mean CXCL8 levels (basal 96.2 ± 23.5 pg/mL vs. TNF-α 784.7 ± 217.3 pg/mL; $P < 0.0001$).

given in the text as mean ± SD. A P value < 0.05 was considered statistically significant.

Results

CXCL8 secretion by human thyroid cells

In basal conditions, significant amounts of CXCL8 were found in all supernatants of cultured thyroid cells. The addition of TNF-α led to a significant increase in mean CXCL8 concentrations, nearly an 8-fold increase over basal conditions (96.2 ± 23.5 pg/mL vs. 784.7 ± 217.3 pg/mL; $P < 0.0001$) (Fig. 1).

Modulation of both basal and TNF-α-induced CXCL8 secretion by type I and type II IFNs

All IFNs decreased in a statistically significant and dose-dependent manner the secretion of CXCL8, as measured in the cell supernatants (Fig. 2). However, the degree of such inhibition differed among IFNs, as shown by a considerable difference in the significance level by ANOVA: IFN-α: $F = 4.207$; $P < 0.01$; IFN-β: $F = 11.193$; $P < 0.0001$; IFN-γ: $F = 18.115$; $P < 0.00001$).

Similar results were observed in dose–response experiments performed by coincubating thyroid cells with TNF-α (10 pg/mL) and each of the IFNs at increasing concentrations (1, 10, 100, and 1,000 U/mL) (Fig. 3). Also, in these experiments, the significance of inhibition differed among IFNs as assessed by separate ANOVA analysis: IFN-α: $F = 4.854$; $P < 0.01$; IFN-β: $F = 11.201$; $P < 0.001$; IFN-γ: $F = 18.958$; $P < 0.0001$). It is interesting to note that the inhibitory effect of each IFN was similar on both basal and TNF-α-induced secretion of CXCL8. From a quantitative point of view, ANOVA analysis showed that IFN-γ was the most potent

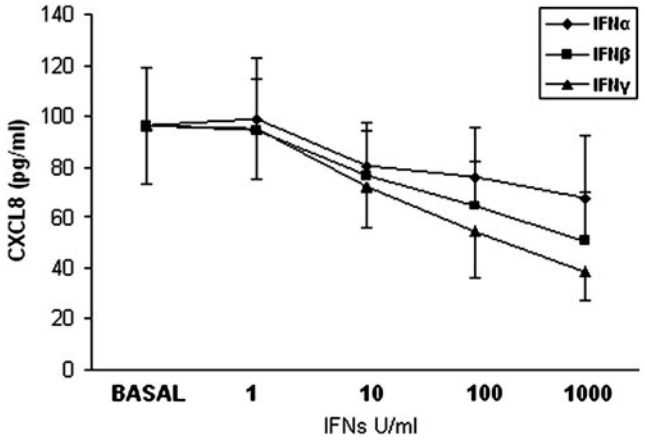

FIG. 2. Type I and type II interferons (IFNs) dose dependently inhibit the basal secretion of CXCL8 in primary cultures of human thyroid cells. Inhibition of CXCL8 secretion in thyroid cells by increasing doses (1, 10, 100, and 1,000 U/mL) of IFN-α (◆), IFN-β (■), and IFN-γ (▲).The mean (bars are the ± SD) CXCL8 concentrations in the supernatants of primary cultures of thyrocytes decreased significantly after incubation with increasing doses of IFN (IFN-α: $F = 4.207$; $P < 0.01$; IFN-β: $F = 11.193$; $P < 0.0001$; IFN-γ: $F = 18.115$; $P < 0.00001$). *Post hoc* comparisons by Bonferroni showed: (IFN-α $P < 0.05$ for basal vs. 1,000 U/mL); (IFN-β $P < 0.01$ for basal vs. 100 U/mL and $P < 0.001$ for basal vs. 1,000 U/mL); (IFN-γ $P < 0.05$ for basal vs. 10 U/mL and $P < 0.0001$ for basal vs. 100 U/mL and $P < 0.0001$ for basal vs. 1,000 U/mL).

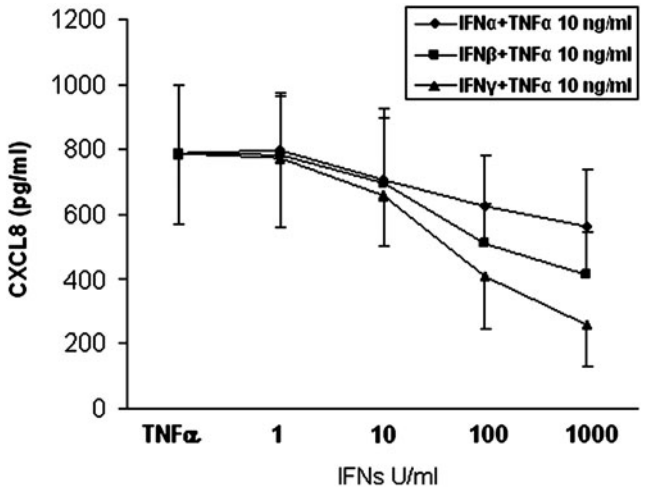

FIG. 3. Type I and type II IFNs dose dependently inhibit the TNF-α-induced secretion of CXCL8 in primary cultures of human thyroid cells. Modulation of TNF-α induced (10 ng/mL for 24 h) CXCL8 secretion in thyroid cells by increasing doses (1, 10, 100, 1,000 U/mL) of IFN-α (◆), IFN-β (■), and IFN-γ (▲). The mean (bars are the ± SD) CXCL8 concentrations in the supernatants of primary cultures of thyrocytes decreased significantly with increasing doses of IFN (IFN-α: $F = 4.854$; $P < 0.01$; IFN-β: $F = 11.201$; $P < 0.001$; IFN-γ: $F = 18.958$; $P < 0.0001$). *Post hoc* comparisons by Bonferroni showed (IFN-α $P < 0.05$ for basal vs. 1,000 U/mL); (IFN-β $P < 0.01$ for basal vs. 100 U/mL and $P < 0.001$ for basal vs. 1,000 U/mL); (IFN-γ $P < 0.05$ for basal vs. 10 U/mL and $P < 0.0001$ for basal vs. 100 U/mL and $P < 0.0001$ for basal vs. 1,000 U/mL).

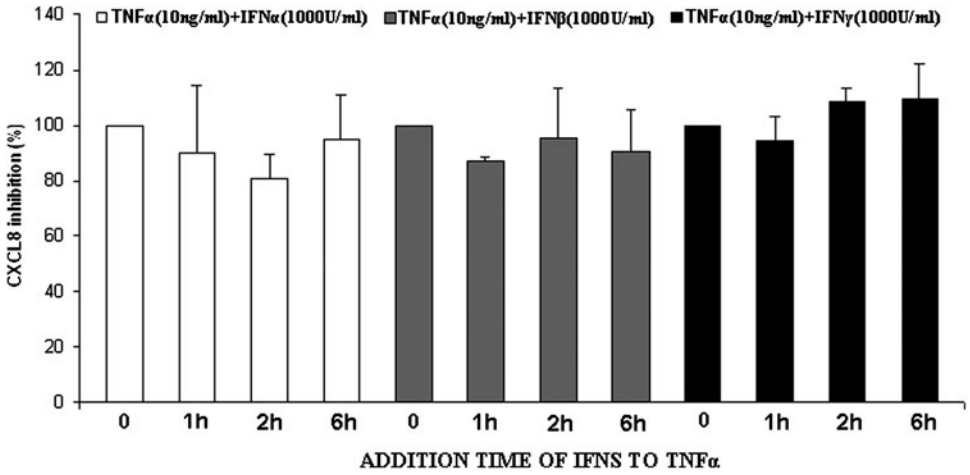

FIG. 4. Effect of type I and type II IFNs on TNF-α-induced CXCL8 secretion in supernatants of primary cultures of human thyroid cells in relation to the timing of IFN addition. IFN-α, IFN-β, and IFN-γ (1,000 U/mL) were added simultaneously (0) and at 1, 2, and 6 h after the stimulation with TNF-α. No significant differences in the degree of CXCL8 inhibition (expressed as a percentage of the inhibition obtained when IFNs and TNF-α were added simultaneously) were found in relation to the timing of IFN addition.

inhibitor of CXCL8 secretion, whereas IFN-α had the lowest inhibitory effect (IFN-γ > IFN-β > IFN-α).

To evaluate whether the effect of IFNs was produced by inhibiting the generation of a signal required for CXCL8 stimulation by TNF-α, IFNs were added to thyroid cell cultures at various time intervals after the addition of TNF-α. As shown in Fig. 4, the inhibitory effect exerted by 1,000 U/mL of a given IFN occurred at a similar degree at any time of IFN addition (simultaneously or at 1, 2, 6 h after TNF-α). Mixtures of type I and type II IFNs were used to evaluate the possibility of synergism between IFNs, which would suggest that IFNs act through different inhibitory pathways. The comparisons of the percentages of CXCL8 inhibition obtained with a single versus a mixture of 2 IFNs failed to demonstrate a synergic effect (data not shown), thus indirectly supporting that IFNs act through similar pathways.

Serum levels of CXCL8 in patients with GD and healthy controls

No significant difference in the mean serum levels of CXCL8 was observed in euthyroid patients with GD compared with healthy controls (17.4 ± 27.6 pg/mL vs. 26.2 ± 28.0 pg/mL, respectively; NS). The circulating levels of CXCL8 in GD patients largely overlapped those found in healthy controls and high SD were found. No significant correlation was found between the serum levels of CXCL8 and the concentrations of circulating FT4, FT3, TSH, TgAb, TPOAb, and TR Ab. Similarly, no relationship between CXCL8 levels and age was found (Antonelli and others 2005).

Discussion

The results of this study confirm that human thyrocytes in primary cultures secrete CXCL8 and that TNF-α is a strong inducer of CXCL8 secretion. Our results provide the first evidence that both type I and type II IFNs produce a dose-dependent inhibitory effect on basal and TNF-α-induced secretion of CXCL8 by human thyroid cells. In a previous study, type I and type II IFNs were compared for their ability to promote CXCL10 secretion in human thyrocytes (Antonelli and others 2010). The present study, being specifically designed to allow such a comparison for CXCL8 secretion, led to the following results: (1) type I and type II IFNs downregulate in a dose–response manner the secretion of CXCL8, although with a different power; (2) both basal and TNF-α-induced secretion of CXCL8 are inhibited by type I and type II IFNs; (3) IFNs do not completely revert neither the basal nor the TNF-α-induced secretion of CXCL8. Previous experimental evidence suggested a role for TNF-α in AITD because it strongly synergizes IFNs in promoting the secretion of CXCR3-binding chemokines (Antonelli and others 2006c, Rotondi and others 2007). Thus, the described antagonism between these proinflammatory cytokines could be regarded as unexpected. However, previous data obtained in human umbilical vein endothelial cells (HUVEC) (Nyhlén and others 2000), fibroblasts (Oliveira and others 1992), leucocytes (Cassatella and others 1993), and astroglioma cells (Nozell and others 2006) consistently reported a significant inhibitory effect exerted by type I and type II IFNs on both basal and cytokine-induced CXCL8 secretion.

The observation that IFNs inhibit the basal and the TNF-α-induced secretion of CXCL8 by human thyroid cells is cumbersome to interpret because little is known on the role of CXCL8 in AITD. However, some considerations can be done.

IFN is known to play a crucial role in AITD due to its capacity to activate the expression of several proinflammatory genes. The results of the present study suggest a double effect of IFNs. On one hand, IFNs stimulate the secretion of CXCL10 (which plays a role in the pathogenesis of AITD, but it is not constitutively present), on the other hand, it inhibits the secretion of CXCL8 (which is constitutively secreted). The fact that cultured thyroid cells secrete CXCL8 in basal conditions together with the recent demonstration that iodide significantly increases the expression levels of mRNA for CXCL8 in a time- and concentration-dependent manner suggests that CXCL8 might have a role in influencing the clinical course of AITD (Yamazaki and other 2010). The following scenario can be envisaged: In the early stages of AITD, when high levels of IFN are present, chemokines like CXCL10 would predominate (Antonelli and others 2006b; Antonelli and others 2007; Rotondi and others 2013), while CXCL8 expression would be downregulated.

As AITD progress, TNF-α levels increase with subsequent stimulation of CXCL8 secretion. Indeed, clinical studies indicate that an increased production of TNF-α, a major stimulator

of CXCL8 secretion, is required to initiate or maintain the remission of GD (Yamamoto and others 2012). This observation would indirectly support our hypothesis. In the present study, the circulating levels of CXCL8 were similar in euthyroid patients with GD and controls. This finding is in agreement with a previous report (Krassas and others 2000). The reason for investigating only euthyroid patients with GD stems from the observation of Siddiqi (Siddiqi and others 1999). These authors found that hyperthyroidism *per se*, rather than the accompanying autoimmune inflammatory condition, is responsible for elevated serum levels of CXCL8 at a difference with what was observed for CXCL10 (Antonelli and others 2006a). On the other hand, the demonstration that a significantly higher expression of the gene encoding CXCL8 was observed in thyroid cancer specimens compared with both normal thyroid and thyroiditis samples would suggest the involvement of CXCL8 in thyroid cancer (Muzza and others 2010).

The design of the present study does not allow drawing conclusions as to the mechanism by which IFNs negatively regulate CXCL8 secretion, but some speculations can be done. The inhibitory action of IFNs was observed even when these cytokines were added after TNF-α, similarly to what was reported by Oliveira (Oliveira and others 1992). This observation indicates that IFNs do not exert their inhibitory effect at an early stage of the stimulation pathway of CXCL8 gene expression. Moreover, the fact that the inhibitory effect of IFN-β decreased with the length of preincubation, being virtually absent when the IFN was added more than 4 h before TNF-α, would rule out the possibility that the inhibitory effect is mediated by IFN-induced proteins (Oliveira and others 1992). Previous data also showed that IFNs specifically inhibit the expression of CXCL8, while activating other NF-kB-regulated genes, as assessed by increased secretion of other chemokines such as CXCL10 (Nozell and others 2006). This would indicate that IFNs do not merely disturb NF-κB activation, but may more likely alter specific transcription regulatory elements at the CXCL8 promoter level. Other possibly involved mechanisms include (1) actions on post-translational modifications of NF-κB p65, which in turn alters the ability of NF-κB p65 to associate with proteins at certain promoters (Nozell and others 2006); (2) suppression of steady-state mRNA stability and accumulation (Nozell and others 2006). More recently, it was demonstrated that IFN-β inhibits CXCL8 through the ISGF3 complex as demonstrated using cell lines deficient for ISGF3 components (Laver and others 2008). Taken together, the available data support the concept that several mechanisms may be involved in IFN-mediated inhibition of CXCL8. This concept is in line with the frequent observation that a single agent may inhibit the synthesis of a protein at more than one level (Beutler and others 1986).

In conclusion, the results of this study confirm that human thyrocytes in primary cultures secrete CXCL8 both basally and after stimulation with TNF-α and demonstrate that type I and type II IFNs are able to downregulate in a dose-dependent manner CXCL8 secretion by thyrocytes, IFN-γ being the most powerful inhibitor. Future basic and clinical studies aimed at a better comprehension of the interplay between CXCL8 and thyroid diseases appear worthwhile.

Author Disclosure Statement

The authors have nothing to disclose.

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Received 28 July 2012/Accepted 22 February 2013