Increase of Circulating CXCL9 and CXCL11 Associated with Euthyroid or Subclinically Hypothyroid Autoimmune Thyroiditis

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Context: Recently, CXCL9 and CXCL11 have been shown to be involved in autoimmune thyroid disorders; however, no data are present about CXCL9 and CXCL11 circulating levels in thyroid autoimmunity.

Objective: Our objective was to evaluate circulating CXCL9 and CXCL11 in autoimmune thyroiditis (AIT).

Design and Patients or Other Participants: Serum CXCL9 and CXCL11 have been measured in 141 consecutive patients with newly diagnosed AIT (AIT-p), 70 euthyroid controls, and 35 patients with nontoxic multinodular thyroid. The three groups were similar in gender distribution and age; among the AIT-p, 26% had subclinical hypothyroidism.

Results: Serum CXCL9 and CXCL11 levels were significantly ($P < 0.0001$ for both) higher in AIT-p (143 $\pm$ 164 and 121 $\pm$ 63 pg/ml, respectively) than in controls (68 $\pm$ 37 and 65 $\pm$ 19 pg/ml, respectively) or patients with multinodular thyroid (87 $\pm$ 43 and 71 $\pm$ 20 pg/ml, respectively). Among AIT-p, CXCL9 and CXCL11 levels were significantly higher in patients older than 50 yr or those with a hypoechoic ultrasonographic pattern or with hypothyroidism. In a multiple linear regression model including age, thyroid volume, hypoechogenicity, hypervascularity, TSH, anti-thyroglobulin, and anti-thyroid peroxidase, only age and TSH were significantly ($P < 0.05$) related to serum CXCL9 or CXCL11 levels. In a multiple linear regression model of CXCL9 *vs.* age, TSH, and CXCL11, TSH ($P = 0.032$) and CXCL11 ($P = 0.001$) were significantly and independently related to CXCL9.

Conclusions: We first show that circulating CXCL9 and CXCL11 are increased in patients with thyroiditis and hypothyroidism and are related to each other. These results underline the importance of a Th1 immune attack in the initiation of AIT. **(*J Clin Endocrinol Metab* 96: 1859–1863, 2011)**

Recent evidence has shown that CXC α-chemokine CXCL10 (Th1) plays an important role in the initial phases of autoimmune thyroid diseases (1–4).

Little is known about CXCL9 and CXCL11 in thyroid autoimmunity (5, 6). Transgenic mice that aberrantly express interferon (IFN)-γ overexpressed CXCL9 and CXCL11 (7). Furthermore, we have recently shown the secretion of CXCL9 and CXCL11 in primary cultures of human thyrocytes can be stimulated by IFN-γ and TNF-α (8, 9).

Only one study evaluated serum CXCL9 in 27 patients with autoimmune thyroiditis (AIT), showing no significant difference with respect to nontoxic nodular goiter (10).

No study evaluated CXCL11 together with CXCL9 circulating levels in AIT with respect to controls. The aim

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Abbreviations: AbTg, Anti-thyroglobulin; AbTPO, anti-thyroid peroxidase; AIT, autoimmune thyroiditis; FNA, fine-needle aspiration; FT_4, free T_4; IFN, interferon; MNT, multinodular thyroid; RC, regression coefficient.

of the present study therefore was to measure serum CXCL9 and CXCL11 levels in patients with AIT and to relate the findings to the clinical phenotype.

Patients and Methods

From the outpatient clinic, we selected 141 [114 females and 27 males (4.2:1); age, 47 ± 15 yr] consecutive Caucasian patients with newly diagnosed AIT. The diagnosis of AIT was established as previously reported (2).

TSH, free T_4 (FT_4), FT_3, anti-thyroglobulin (AbTg), and anti-thyroid peroxidase (AbTPO) were measured as previously described (2) (TSH = 3.2 ± 6.4 μU/ml; AbTPO = 374 ± 681 IU/ml; AbTg = 235 ± 349 IU/ml; AbTPO positivity = 81%; AbTg positivity = 74%; subclinical hypothyroidism = 26%).

Ultrasonography of the neck, thyroid blood flow by color-flow Doppler, and fine-needle aspiration (FNA) were performed as previously reported (2). The majority of these patients had a normal thyroid volume; some showed goiter (21%) or hypotrophic thyroiditis (14%). Hypoechogenicity was present in 82% of AIT and hypervascularization in 39%. Thyroid nodules were present in 15% of AIT patients. Nodules with a diameter larger than 10 mm (7% of patients) were submitted to FNA to exclude the presence of thyroid cancer or lymphoma (2); in these cases, cytology confirmed the presence of a lymphocytic infiltration.

Two control groups were used. The first control group [n = 70; 57 females and 13 males (4.4:1); age, 45 ± 16 yr] consisted of a random sample of the general population from the same geographic area in whom a complete thyroid work-up (physical examination, TSH, FT_3, FT_4, AbTg and AbTPO antibody measurements, and ultrasonography were normal) was available and excluded the presence of thyroid disorders. A second control group comprised 35 patients [26 females and nine males (2.9:1); age, 47 ± 14 yr] with nontoxic multinodular thyroid (MNT) (two or more nodules) extracted from the same random sample of the general population (TSH, FT_3, FT_4, AbTg, and AbTPO were in normal range). The majority of these patients had a normal thyroid volume; some showed goiter (37%). All these patients were submitted to FNA to exclude the presence of thyroid cancer; cytology confirmed the absence of a malignancy.

Exclusion criteria for patients and controls were 1) the presence of anti-TSH receptor antibodies; 2) clinical history of hyperthyroidism; 3) evidence of infectious diseases in the last 3 months; 4) treatment with drugs known to interfere with immune system, namely cytokines, IFN, corticosteroids, non-steroidal antiinflammatory drugs, amiodarone, and lithium; 5) pregnancy and lactation over the previous 6 months; 6) presence of acute or chronic systemic diseases or other autoimmune diseases.

All study subjects gave their informed consent to the study, which was approved by the local ethical committee. In all patients and controls, a blood sample was collected in the morning after overnight fasting, and serum was kept frozen until thyroid hormone, thyroid autoantibodies, CXCL11, and CXCL9 measurement.

Serum CXCL9 and CXCL11 levels were assayed by a quantitative sandwich immunoassay (R&D Systems, Minneapolis, MN). Sensitivity ranged from 1.2–11.5 pg/ml with a mean minimum detectable dose of 3.9 pg/ml for CXCL9; the intra- and interassay coefficients of variation were 4.3 and 5.9%. The CXCL11 sensitivity ranged from 0.5–3.5 pg/ml with a mean minimum detectable dose of 12.1 pg/ml; the intra- and interassay coefficients of variation were 4.0 and 7.1%.

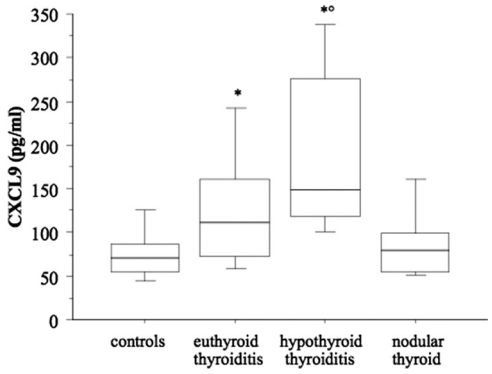

FIG. 1. The mean CXCL9 level was significantly (ANOVA, $P < 0.0001$) higher in patients with euthyroid thyroiditis or hypothyroid thyroiditis than in controls or MNT patients. The *box* indicates the lower and upper quartiles, and the *central line* is the median value; the *horizontal lines at the end of the vertical lines* are the 2.5 and 97.5 percentiles. *, $P < 0.05$ *vs.* controls or nodular thyroid; °, $P < 0.05$ *vs.* euthyroid thyroiditis, by Bonferroni-Dunn test.

Values are given as mean ± SD for normally distributed variables, otherwise as median and [interquartile range]. Statistical power (*ex post* analysis) was calculated.

Results

Gender and age distribution were similar in AIT, MNT, and controls. The mean CXCL9 level was significantly (ANOVA, $P < 0.0001$) (statistical power = 1) higher in patients with thyroiditis than in controls or MNT (143 ± 164, 68 ± 37, and 87 ± 43 pg/ml, respectively), although some overlap was evident in the low range of CXCL9 (Fig. 1).

In AIT, serum CXCL9 levels were significantly higher in patients older than 50 yr (117 ± 65 *vs.* 78 ± 43 pg/ml, $P = 0.001$), in patients with a hypoechoic pattern (141 ± 49 *vs.* 81 ± 32 pg/ml, $P = 0.008$), and in those with hypothyroidism (155 ± 73 *vs.* 101 ± 38 pg/ml, $P = 0.001$) (Fig. 1), whereas no significant difference was observed in relation to thyroid autoantibodies or other ultrasonographic parameters.

In a simple regression analysis, CXCL9 levels were significantly related to TSH values in AIT patients (r = 0.561; $P = 0.001$). In a multiple linear regression model including age, thyroid volume, TSH, AbTg, AbTPO, hypoechoic pattern, and the presence of hypervascularity, only age [β-coefficient = 0.272; regression coefficient (RC) = 0.09; 95% lower = 0.001; 95% upper = 0.022; $P = 0.007$] and TSH (β-coefficient = 0.311; RC = 0.035; 95% lower =

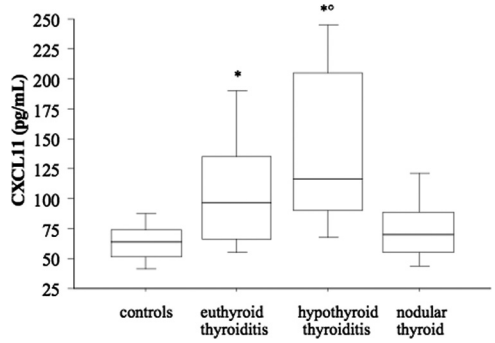

FIG. 2. The mean CXCL11 level was significantly (ANOVA, $P < 0.0001$) higher in patients with euthyroid thyroiditis or hypothyroid thyroiditis than in controls or MNT patients. The *box* indicates the lower and upper quartiles, and the *central line* is the median value; the *horizontal lines at the end of the vertical lines* are the 2.5 and 97.5%. *, $P < 0.05$ *vs.* controls or nodular thyroid; °, $P < 0.05$ *vs.* euthyroid thyroiditis, by Bonferroni-Dunn test.

0.010; 95% upper = 0.071; $P = 0.006$) were significantly related to serum CXCL9 levels.

CXCL9 values higher than 142 pg/ml (mean + 2 SD of CXCL9 levels in controls) were found in 39 of 102, in two of 68, and in two of 33, respectively, AIT, control, and MNT subjects ($\chi^2 = 23.8$; $P < 0.0001$).

The mean CXCL11 level was significantly ($P < 0.0001$) (statistical power = 1) higher in patients with thyroiditis, than in controls or MNT patients (121 ± 63, 65 ± 19, and 71 ± 20 pg/ml, respectively), although some overlap was evident in the low range of CXCL11 values (Fig. 2). In AIT patients, serum CXCL11 levels were significantly higher in patients older than 50 yr (116 ± 65 *vs.* 76 ± 35 pg/ml, $P = 0.001$), in patients with a hypoechoic pattern (142 ± 56 *vs.* 92 ± 31 pg/ml, $P = 0.008$), and in those with hypothyroidism (151 ± 79 *vs.* 106 ± 53 pg/ml, $P = 0.001$), whereas no significant difference was observed in relation to the other parameters.

In a simple regression analysis, CXCL11 levels were significantly related to TSH values in AIT patients (r = 0.463; $P < 0.0001$). In a multiple linear regression model (see above), only age (β-coefficient = 0.209; RC = 0.009; 95% lower = 0.001; 95% upper = 0.015; $P < 0.001$) and TSH (β-coefficient = 0.218; RC = 0.016; 95% lower = 0.005; 95% upper = 0.042; $P < 0.001$) were significantly related to serum CXCL11 levels.

CXCL11 values higher than 103 pg/ml (mean + 2 SD of CXCL11 levels in controls) were found in 33 of 108, one of 69, and in two of 33, respectively, AIT, control, and MNT subjects ($\chi^2 = 20.6$; $P < 0.0001$).

Higher CXCL9 or CXCL11 levels were observed 1) in AIT with goiter and nodules *vs.* MNT with goiter (CXCL9 was 134 ± 161 and 91 ± 46 pg/ml, respectively, $P < 0.01$; CXCL11 was 125 ± 67 and 75 ± 23 pg/ml, respectively; $P < 0.01$) and 2) in AIT having nodules in a normally sized thyroid *vs.* MNT with a normally sized thyroid (CXCL9 was 126 ± 158 and 82 ± 41 pg/ml, respectively, $P < 0.01$; CXCL11 was 117 ± 62 and 67 ± 19 pg/ml, respectively, $P < 0.01$).

After grouping controls and MNT, simple regression analysis showed that CXCL9 (r = 0.384; $P = 0.02$) or CXCL11 (r = 0.352; $P = 0.04$) serum levels were significantly related to age.

In a simple regression analysis, CXCL9 and CXCL11 serum levels were significantly related to each other (r = 0.525; $P < 0.001$) in AIT patients.

In a multiple linear regression model of CXCL9 [ln-(picograms per milliliter)] *vs.* age, TSH, and CXCL11 [ln-(picograms per milliliter)], only TSH (β-coefficient = 0.134; RC = 0.011; 95% lower = 0.001; 95% upper = 0.032; $P = 0.031$) and CXCL11 (β-coefficient = 0.262; RC = 0.141; 95% lower = 0.029; 95% upper = 0.267; $P = 0.001$) were significantly (r = 0.428; $P < 0.001$) and independently related to CXCL9.

Discussion

This study first shows circulating CXCL9 and CXCL11 levels were clearly elevated in AIT patients as compared with normal controls or patients with MNT; within the AIT group, higher CXCL9 and CXCL11 levels were associated with older age, hypothyroidism, and a hypoechoic gland.

Moreover, to the best of our knowledge, this is the first study reporting a correlation between CXCL9 and CXCL11 levels in an immune-related disorder, such as AIT.

These results are in agreement with recent evidences that have shown that CXCL10 (Th1) plays an important role in the initial phases of autoimmune thyroid disease, especially in the presence of hypothyroidism (1–3).

However, our results are in disagreement with those of another study that evaluated serum CXCL9 in 27 patients with AIT showing no significant difference with respect to nontoxic nodular goiter (10). The discordance between the two studies may be due to different factors: 1) a larger number of AIT patients included in our study (141 *vs.* 27), 2) the presence of a control group of normal subjects that was not present in the other study, and 3) more importantly, the different methods of evaluation of serum CXCL9 levels. In fact, in that study (10), the chemokines were assayed by Fluorokine MAP Multiplex Assays, and the values of CXCL9 were above 1000 pg/ml in AIT and nodular goiter. These values are exceptionally high in relation to the values found in normal controls that range from 40–125 pg/ml (11), suggesting a bias in the method of CXCL9 determination (10). Interestingly, the mean value of circulating CXCL9 in our normal control is 68

pg/ml and in nodular goiter patients is 87 pg/ml, fully in the midrange of the CXCL9 values found in the study by Shurin *et al.* (11) (that first showed a positive relation between CXCL9 and age, in agreement with the results of our study).

T lymphocytes play a central role in the induction of the AIT response. Recent observations have indicated that specific combinations of different inflammatory cytokines (IFN-γ and/or TNF-α) transform nondestructive into destructive thyroiditis in murine experimental autoimmune thyroiditis (12, 13). Expression of CXCL9 and CXCL11 is induced by the prototypical Th1 cytokine, IFN-γ, and is dramatically enhanced by addition of TNF-α in a wide range of cells and tissues (14, 15) and in primary thyroid cells (8, 9). Moreover, IFN-α and IFN-β were able to stimulate CXCL9 and CXCL10 secretion in normal thyrocytes (16).

The elevated levels of circulating CXCL9 or CXCL11 in patients with AIT may result from secretion by both lymphocytes and thyroid follicular cells modulated through IFN-γ. However, serum CXCL10 levels are increased in patients with Graves' disease, especially in those with active disease, and the CXCL10 decrease after thyroidectomy or after radioiodine shows that it is more likely to have been produced inside the thyroid gland (17–19). These data suggest that thyrocytes may be an important source of CXCL9 or CXCL11 (18, 19).

CXCL9 and CXCL11 recruit activated Th1 lymphocytes to sites of inflammation (20). It can be speculated that CXCL9 and CXCL11 induced recruitment of Th1 lymphocytes, which secrete IFN-γ, which in turn stimulates chemokine production by follicular cells, thus maintaining the autoimmune process, reinforcing the effect of CXCL10.

These hypotheses are in agreement with the results reported by Kimura *et al.* (7). They found that transgenic mice that aberrantly express IFN-γ exclusively expressed CCL4, CXCL9, and CXCL11 and showed increased expression of CCL5 and CXCL10.

In conclusion, high circulating levels of CXCL9 and CXCL11 have been shown in AIT patients, especially in the presence of hypothyroidism. Longitudinal prospective observations in large patient cohorts will be needed to evaluate whether circulating CXCL9 and CXCL11 levels may serve as a clinical prognostic marker in AIT.

Acknowledgments

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