CIRCULATING CXCL10 IS INCREASED IN NON-SEGMENTAL VITILIGO, IN PRESENCE OR ABSENCE OF AUTOIMMUNE THYROIDITIS.

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Abstract

Recently the importance of CXCL10 in the pathogenesis of non-segmental vitiligo (NSV) and autoimmune thyroid disorders (AITD) has been shown. No data are present about chemokines CXCL10 (Th1 prototype) and CCL2 (Th2 prototype) circulating levels in NSV patients with/without thyroiditis (AT).

Serum CXCL10 and CCL2 have been measured in 50 consecutive NSV patients, in 40 consecutive patients with NSV and AT (NSV+AT), in 50 sex- and age-matched controls without AT (control 1) and in 40 sex- and age-matched patients with AT without NSV (control 2).

Serum CXCL10 levels were significantly higher in control 2, than in control 1 (P = 0.001; ANOVA). NSV patients have serum CXCL10 levels significantly higher than control 1, or control 2 (P = 0.001). NSV+AT patients have serum CXCL10 levels higher than control 1, or 2 ($P < 0.001$), and than NSV ($P = 0.01$).

In conclusion, we first demonstrate high serum CXCL10 in NSV patients, overall in presence of AT and hypothyroidism, suggesting the importance of a common Th1 immune response in their immune-pathogenesis. To evaluate if serum CXCL10 might be used as a clinical marker of NSV and/or AT further studies are needed.

Keywords: Vitiligo, autoimmune thyroiditis, CXCL10, CCL2, chemokines.

Take-home messages:

- Vitiligo is a skin disease characterized by pale patchy areas of depigmentation on the face, wrists and hands, that are initially small, but often tend to grow and change in shape. Two type of vitiligo are known: 1- non-segmental vitiligo (NSV) that is usually symmetrical in the location of depigmentation; 2- segmental vitiligo (SV) that differs in appearance, and it is not associated with autoimmune diseases.
- NSV is an autoimmune disease arising from an autoimmune response against melanocytes in the skin, and it is often associated with other autoimmune disorders.
- The importance of the Th1 immune response in the development of vitiligo, and of (C-X-C Motif) Receptor 3 (CXCR3) receptor and its chemokine CXCL10, has been shown, suggesting these could be novel targets of future therapeutical approaches.
- Our study first demonstrates high serum levels of CXCL10 in patients with NSV and NSV+AT with respect to control 1 (without NSV or AT) and control 2 (without NSV) with thyroiditis. Serum CXCL10 levels of NSV+AT patients with thyroiditis were significantly higher than that of control 1, of NSV patients without thyroiditis or control 2 with thyroiditis; while no significant difference was observed about CCL2 in the four groups.
- This suggests a predominance of the Th1 immune response in these patients.
- Future studies in larger casistics will be needed to evaluate the potential usefullness of serum CXCL10 determination as prognostic marker of NSV or thyroiditis, and in the follow-up of NSV patients treated with different therapies.

1. Introduction

Vitiligo is a skin disease characterized by pale patchy areas of depigmentation on the face, wrists and hands, that are initially small, but often tend to grow and change in shape [1]. Two type of vitiligo are known: 1- non-segmental vitiligo (NSV) that is usually symmetrical in the location of depigmentation; 2- segmental vitiligo (SV) that differs in appearance, and it is not associated with autoimmune diseases [2]. NSV is an autoimmune disease arising from an autoimmune response against melanocytes in the skin, and it is often associated with other autoimmune disorders. NSV is a component of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Autoimmune polyendocrine syndrome type 1 - APS1) and Schmidt (APS2) multiple autoimmune syndromes, and it is associated with pernicious anemia and Addison's disease [3], too. Thyroid autoimmune disorders [4] or the presence of thyroid-specific autoantibodies have been shown in 15-25% of NSV patients [5-7].

Recently several studies have shown [8-10] the importance of the Th1 immune response in the development of vitiligo, and of (C-X-C Motif) Receptor 3 (CXCR3) receptor and its chemokine CXCL10, suggesting these could be novel targets of future therapeutical approaches.

Furthermore, recent reports have shown that the serum and/or the tissue expressions of CXCL10 are increased in organ specific autoimmune diseases, such as, type 1 diabetes (T1D), autoimmune thyroiditis, Graves' disease (GD), or Graves' ophthalmopathy (GO), or systemic rheumatological disorders, like rheumatoid arthritis, systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and HCV-related cryoglobulinemia [11].

Here we report our data about the prototype Th1 chemokine CXCL10 circulating levels in NSV patients with or without thyroiditis, in comparison with the prototype Th2 chemokine CCL2.

2. Methods

2.1. Patients

2.1.1. NSV patients

We studied 50 NSV patients (**Table 1**), not previously treated with immunosuppressive treatments, such as corticosteroids, calcineurin inhibitors and phototherapy, in whom a thyroid screening [history, physical examination, thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4), anti-thyroglobulin (AbTg) and anti-thyroid peroxidase (AbTPO) antibodies measurements, and ultrasonography] excluded the presence of associated thyroid autoimmune disorders (a well known cause of high serum CXCL10), and without other well-known systemic disorders, such as immuno-rheumatic, neoplastic, and infectious diseases. Patients were diagnosed with NSV with body surface area (BSA) involvement of $> 5\%$ [12].

2.1.2. NSV patients with autoimmune thyroiditis (NSV+AT)

Patients (n=40) with NSV at first presentation, consecutively referred to our Department were recruited into this group. Only NSV patients who were not previously treated with immunosuppressive treatments, such as corticosteroids, calcineurin inhibitors and phototherapy, and in whom a thyroid screening (history, physical examination, TSH, FT3, FT4, AbTg and AbTPO antibodies measurements, and ultrasonography) demonstrated the presence of associated thyroid autoimmune disorders were included in this group (**Table 1**).

2.2. Controls

Two control groups were included.

2.2.1. Control 1

The first control group (control 1) consisted of 50 subjects (**Table 1**), extracted from a random sample of the general population from the same geographic area, coupled by sex and age (that is a well known counfounding factor [13]) with NSV patients, in whom a complete thyroid work-up (history, physical examination, TSH, FT3, FT4, AbTg, AbTPO, and ultrasonography) was available, and excluded the presence of thyroid or autoimmune disorders, or any kind of immunomodulant therapy.

2.2.2. Control 2

The second control group (control 2) consisted of 40 subjects (**Table 1**), extracted from a random sample of the general population from the same geographic area [14], coupled by sex and age (that is a well known counfounding factor [13]) with NSV+AT patients, in whom a complete thyroid work-up (history, physical examination, TSH, FT3, FT4, AbTg, AbTPO, and ultrasonography) was available, and demonstrated the presence of thyroid autoimmune disorders, but excluded the presence of other autoimmune disorders and any kind of immunomodulant therapy.

In all patients and controls, a blood sample was collected in the morning, after overnight fasting, and serum was kept frozen until CXCL10 and CCL2 measurement.

All study subjects gave their informed consent to the study, which was approved by the local Ethical Committee.

Thyroid ultrasonography [15] was performed both in patients as in controls as previously reported, and thyroid blood flow (TBF) by color-flow doppler (CFD) was studied in all patients [16].

2.3. Laboratory evaluation

Laboratory evaluation included measurement of serum levels of TSH (reference range 0.3-3.6 μIU/mL), FT3, FT4, AbTg and AbTPO titers. Circulating FT3 and FT4 were measured by commercial RIA kits (AMERLEX-MAB FT3/FT4 Kit; Amersham, UK). Serum TSH (DiaSorin, USA), AbTPO and AbTg (ICN Pharmaceuticals, USA) were evaluated by IRMA methods. For AbTg, AbTPO, positivity was set at >100 UI/ml and >100 IU/mL, respectively. Values are given as mean ± SD for normally distributed variables.

2.4. Chemokines Assay

Serum CXCL10 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Minneapolis, MN), with a sensitivity ranging from 0.40-4.45 pg/mL. The intra- and inter-assay coefficients of variation were 3.1% and 6.7%. Serum CCL2 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Minneapolis, MN), with a sensitivity of less than 5.0 pg/mL. The intra- and inter-assay coefficients of variation were 4.5% and 5.6%.

2.5. *Data analysis*

Values are given as mean ± SD for normally distributed variables. Mean group values were compared by using one-way analysis of variance (ANOVA) for normally distributed variables, otherwise by the Mann-Whitney *U* test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test. Univariate analysis was performed by simple regression.

3. Results

NSV, NSV+AT and controls 1 and 2 were not significantly different (due to the matching procedure) in relation to the demographic characteristics (**Table 1**).

As expected, NSV+AT patients, and controls with AT (control 2) showed significantly higher thyroid autoantibodies levels, as well as hypoechogenicity and hypervascularity of the thyroid gland, and subclinical hypothyroidism in comparison to control 1, and NSV patients without AT.

Serum CXCL10 levels were significantly (**Table 1; Fig. 1**) higher in control 2, than in control 1 (P = 0.001; ANOVA). NSV patients have serum CXCL10 levels significantly higher than control 1, or control 2 (P = 0.001). NSV+AT patients have serum CXCL10 levels higher than control 1, or 2 (P < 0.001), and than NSV (P = 0.01).

In order to better define the role of increased serum chemokines in AT, CXCL10 was studied in relation to clinical features of AT (age; gender; thyroid volume < 6 mL; thyroid hypoechoic pattern, or hypervascularity; AbTg or AbTPO positivity; subclinical hypothyroidism) in NSV+AT patients and control 2. Serum CXCL10 levels were significantly increased in patients NSV+AT with a thyroid hypoechoic pattern with respect to those without a hypoechoic pattern (483 ± 165 vs. 364 ± 111; P = 0.01; ANOVA) and hypothyroidism (445 ± 137 vs. 351 ± 103; P = 0.04; ANOVA), and in control 2 with a thyroid hypoechoic pattern with respect to those without a hypoechoic pattern (178 ± 145 vs. 109 ± 121; P = 0.02; ANOVA), and hypothyroidism (184 ± 129 vs. 112 ± 119; P = 0.03; ANOVA).

By defining a high CXCL10 level as a value at least 2 SD above the mean value of the control group (> 196 pg/mL), 4% of control 1, 32% of control 2, 82% of NSV and 93% of NSV+AT, had high CXCL10 (P = 0.001; $\chi 2$).

Serum CCL2 levels were not significantly different (**Table 1; Fig. 2**) in control 1 and control 2, NSV patients with or without thyroiditis. In order to better define the role of serum chemokine CCL2, it was studied in relation to clinical features of AT (age; gender; thyroid volume < 6 mL; thyroid hypoechoic pattern, or hypervascularity; AbTg or AbTPO; subclinical hypothyroidism) in NSV+AT patients and control 2, but no significant association was found (data not shown).

No association was found between serum CXCL10 or serum CCL2 levels by simple regression.

4. Discussion

Our study first demonstrates high serum levels of CXCL10 in patients with NSV with respect to control 1 and control 2 with thyroiditis. Serum CXCL10 levels of NSV+AT patients with thyroiditis were significantly higher than control 1, or NSV patients without thyroiditis or control 2 with thyroiditis. No significant difference was observed about CCL2 in the four groups.

The most important cytokine associated with the Th1 immune response, interferon (IFN)-γ [17], acts through chemokines, that are small glycoproteins active for different cell types. CXCL10 (also called IP-10) is a chemokine, induced by IFN-γ in various cell types, as neutrophils, lymphocytes, endothelial cells, thyrocytes and other cells [18,19]. CXCL10 binds to its specific receptor CXCR3 (expressed by immune cells and also endothelial cells, mesangial cells, thyrocytes and other epithelial cells), regulating immune responses by the recruitment and activation of T cells, monocytes, and NK cells. Tissue CXCR3 and CXCL10 expressions are increased in different autoimmune diseases, and are determinant in leukocyte homing into the inflamed tissues, being involved in tissue damage [19]. Elevated CXCL10 levels in peripheral

liquids are a marker of host immune response, particularly of a T helper (Th)1 orientated immune response. In inflamed tissues, Th1 lymphocytes are recruited enhancing IFN-γ and tumor necrosis factor (TNF)-α production, that in turn stimulate CXCL10 secretion from the above reported cells, in this way creating an amplification feedback loop [11].

It has been recently shown that both human vitiligo and a mouse model of vitiligo reflect a IFN-γ-specific Th1 immune response in the skin, involving the IFN-γ-dependent chemokines (CXCL9, 10, and 11) [8]. A gene expression profile was conducted to evaluate the key cytokine pathways involved in vitiligo. Such gene expression profile in lesional skin patches from vitiligo patients, that contained a T cell infiltrate, demonstrated a significant loss of melanocyte-specific transcripts in lesional skin with respect to healthy controls, while chemokine expression showed a predominantly Th1-mediated signature (*IFNG*-specific). Th1 chemokines (CXCL9, CXCL10, CXCL11, and CCL5) were strongly induced in vitiligo, with respect to adhesion molecules (as ICAM1 and VCAM1). CXCL10 was high in serum from patients with vitiligo, while CXCL9 and CXCL11 were not significantly different compared to healthy controls. Melanocyte-specific $CD8^+$ T cells expressed CXCR3, differently from healthy controls [8].

A mouse model of vitiligo was also used [9]. Krt14-Kitl* mice have several epidermal melanocytes, as human skin. Krt14-Kitl* mice develop epidermal depigmentation on their foot pads, ears, and tails, upon transfer of premelanosome protein-specific $CD8^+$ T cells (PMELs) and in vivo activation recombinant vaccinia virus expressing their cognate antigen (PMEL). Gene expression profile of lesional skin from this mice with vitiligo showed a gene chemokines exspression similar to human vitiligo, with a Th1-specific response in the skin, and expression of the IFN-γ-dependent chemokines

(CXCL9, CXCL10, and CCL5). CXCR3 is expressed by autoreactive T cells, as in humans with this disease [8]. In order to evaluate whether CXCR3 is required for the development of depigmentation, either wild-type (WT) or *Cxcr3−/−* PMEL T cells were transferred to induce vitiligo, and it was observed that *Cxcr3−/−* T cells were unable to induce depigmentation [8]. In this mouse model of vitiligo, *Cxcl10−/−* hosts developed minimal depigmentation, leading to hypothesize that vitiligo depends on this chemokine. CXCL10 is determinant in directing migration of T cells within the skin, and it was required for T cell function more than simple recruitment [8]. Upon treatment of mice with either CXCL9 or CXCL10 neutralizing antibodies, neutralization of CXCL10 significantly reduced depigmentation in this model, while neutralization of CXCL9 did not. Interestingly, CXCL10 neutralization in mice with established, widespread depigmentation reverses the disease, as shown by repigmentation. These results evidence a critical role for CXCL10 in both the progression and maintenance of vitiligo, supporting inhibiting CXCL10 as a targeted treatment strategy [8].

On the base of the results of the above mentioned study [8] it was evaluated the effect of inhibition of IFN-γ in vitiligo. STAT1 activation is required for IFN-γ signaling and recent studies revealed that simvastatin, an FDA-approved cholesterol-lowering medication, inhibited STAT1 activation in vitro. Therefore, it was hypothesized that simvastatin may be an effective treatment for vitiligo. In fact, simvastatin both prevented and reversed depigmentation in the mouse model of vitiligo, and reduced the number of infiltrating autoreactive CD8+ T cells in the skin. Treatment of melanocyte-specific, CD8+ T cells in vitro decreased proliferation and IFN-γ production, suggesting additional effects of simvastatin directly on T cells. Based on

these data, it has been suggested that simvastatin may be a safe, targeted treatment option for patients with vitiligo [10].

Interestingly, our data are in agreement with the results of another recent study reporting that serum CXCL9 and CXCL10 were significantly elevated in vitiligo patients and were higher in patients in progressive stages than those in stable stages, while CCL2 levels were not significantly different from controls [20].

NSV+AT patients have significantly increased serum CXCL10 than NSV patients. These data suggest that in presence of thyroiditis the further increase of serum CXCL10 levels may be due to the thyroiditis itself, and suggest a predominance of the Th1 immune response in presence of thyroiditis in NSV patients. In GD it has been shown that removal of the thyroid itself by surgery [21] or by radiodione [22] reduces the CXCL10 serum levels suggesting that the intrathyroidal lymphocytes and/or thyrocytes [22] may be the source of CXCL10 [23].

So it could be speculated that a superimposed Th1 response is active in the thyroid of patients with NSV+AT, explaining the higher levels of CXCL10 in NSV+AT with respect to NSV. These data agree to what found in autoimmune thyroid disorders (AITD) of patients with hepatitis C virus (HCV) infection or mixed cryoglobulinemia [24, 25].

Our data are also in agreement with our results in patients with AT, confirming higher levels of CXCL10 in presence of hypothyroidism and thyroid hypoechogenicity, thus suggesting that also in these patients CXCL10 may be regarded as a marker of a more aggressive thyroiditis [4, 26].

Longitudinal studies evaluating serum CXCL10 in large casistics of NSV will be necessary to evaluate if serum CXCL10 measurement could represent an easily assayable marker for clinical management of these patients.

The association of autoimmune disorders is known [27], though its pathogenetic base is still under investigation [28, 29]. A prevalent Th1 immune pattern is present in target organs of patients with chronic AT, or GO, or type 1 diabetes, at the beginning of these diseases, as shown also by animal models and by data available in humans [23,30,31]. For example, also in the initial and active phase of mixed cryoglobulinemia, a prevalent Th1 immune profile is shown, that switches to Th2 in the inactive phase [25,32]. This Th1 immune reactivity at the onset of mixed cryoglobulinemia and AT, under the influence of genetic and environmental conditions, might lead to the appearance of autoimmune phenomena in different organs in the same subject [4].

In conclusion, our study first demonstrates higher serum levels of CXCL10 in patients with NSV and NSV+AT than in controls. Serum CXCL10 levels in NSV+AT patients with thyroiditis are significantly higher than those of NSV patients without thyroiditis, suggesting a predominance of the Th1 immune response in these patients.

Future studies in larger casistics will be needed to evaluate the potential usefullness of serum CXCL10 determination as prognostic marker of NSV or thyroiditis, and in the follow-up of NSV patients treated with different therapies.

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Figure Captions

Fig. 1 Serum CXCL10 levels were significantly higher in control 2 thyroiditis (AT), than in control 1 (Control; $P < 0.01$; ANOVA). NSV without thyroiditis have serum CXCL10 levels significantly higher than control 1, or control 2 ($P < 0.001$). NSV+AT patients with thyroiditis have serum CXCL10 levels higher than, control 1, control 2 with thyroiditis ($P < 0.001$), and than NSV ($P < 0.05$).

Fig. 2 Serum CCL2 levels were similar in control 1 (control) and control 2 (AT), NSV, and NSV+AT patients ($P > 0.05$).

Table 1. Thyroid status of control subjects (control 1), control with autoimmune thyroiditis (control 2), non-segmental vitiligo patients without (NSV) or with autoimmune thyroiditis (NSV+AT).

	control 1	*control 2 thyroiditis*	*NSV without thyroiditis*	*NSV+AT with thyroiditis*	*p*
n	50	40	50	40	
Age (years)	52 ± 11	57 ± 12	54 ± 12	57 ± 13	ns
Gender (M/F)	18/32	13/27	18/32	13/27	ns
Thyroid volume (mL)	11 ± 10	12 ± 11	13± 11	11± 10	ns
Hypoechoic (%)	0	76	0	69	0.0001
Hypervascular (%)	0	36	0	41	0.0001
Serum TSH (μIU/mL)	1.1 ± 0.8	1.7 ± 1.7	1.2 ± 0.9	3.3 ± 3.1*	0.01
AbTPO (IU/mL)	15 ± 12	231 ± 373°	15 ± 14	189 ± 432°	0.0001
AbTg (IU/mL)	9 ± 12	145 ± 314°	13 ± 9	314 ± 423°	0.0001
Subclin. hypothyroidim (%)	0	11	0	14	0.01
CXCL10 (pg/mL)	90 ± 53	152 ± 128§	221 ± 137§^	329 ± 147§*	0.0001
CCL2 (pg/mL)	386 ± 179	378 ± 152	360 ± 161	432 ± 360	ns

Antithyroperoxidase antibody = AbTPO

Antithyroglobulin antibody = AbTg

Thyroid-stimulating hormone = TSH

Antithyrotropin-receptor antibody = TRAb

* $P < 0.05$ or less vs. control 1 or vs. autoimmune thyroiditis control 2, or vs. NSV.

§ $P < 0.05$ or less vs. control 1

^ $P < 0.05$ or less vs. control 1 or vs. autoimmune thyroiditis control 2.

° $P < 0.05$ or less vs. control 1 and vs. NSV

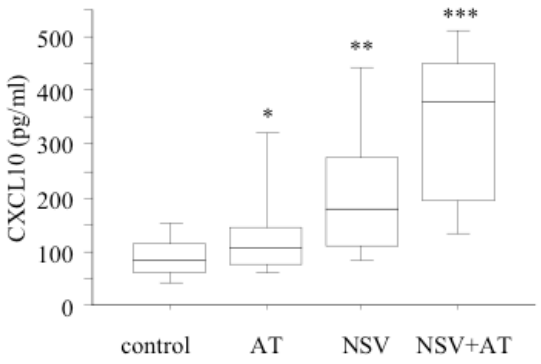

Figure 1

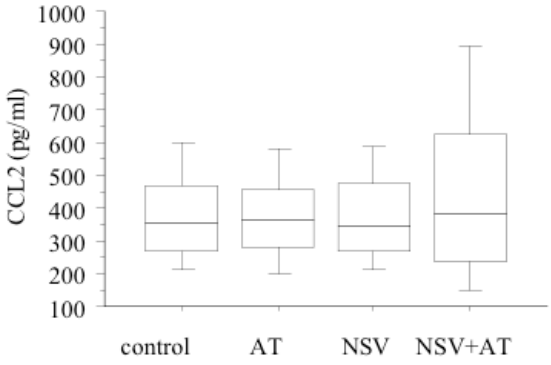
1000
900
800
700
600
500
400
300
200
100
CCL2 (pg/ml)
control
AT
NSV
NSV+AT

Figure 2