Th1 chemokines in autoimmune endocrine disorders

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Short title: Th1 chemokines in endocrine autoimmunity

Keywords: CXCL10, CXCL9, CXCL11, autoimmune thyroiditis, Graves' disease, Type 1 diabetes

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Word Count: 5620

Disclosure: The Authors have nothing to disclose.

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Abstract

Context. The chemokine receptor (CXCR)3 and its chemokines CXCL10, CXCL9 and CXCL11 are implicated in the pathogenesis of autoimmune diseases. Here, we review these chemokines in "autoimmune thyroiditis" (AT), "Graves' disease" (GD), "thyroid eye disease (TED), "Type 1 diabetes" (T1D), "Addison's disease" (AAD).

Evidence Acquisition. A PubMed revision of the literature was conducted searching for the above mentioned chemokines in combitation with AT, GD, TED, T1D, AAD.

Evidence Synthesis. Thyroid follicular cells in AT and GD, retroorbital cells in TED (fibroblasts, preadipocytes, myoblasts), β cells and islets in T1D, and adrenal cells in AAD, respond to IFN-γ stimulation producing large amounts of these chemokines. Furthermore, lymphocytes and PBMC are in part responsible for the secreted Th1 chemokines. In AT, GD, TED, T1D, AAD circulating levels of these chemokines have been shown to be high. Furthermore these chemokines have been associated with the early phases of the autoimmune response in all the above mentioned disorders. High levels of these chemokines have been associated also with the "active phase" of the disease in GD, and also in TED. Other studies have shown an association with the severity of hypothyroidism in AD, of hyperthyroidism in GD, with severity of TED, or with fulminant T1D.

Conclusion. The above mentioned data have shown the importance of the Th1 immune response in different endocrine autoimmune diseases, and many studies have suggested that CXCR3, and its chemokines, might be considered potential targets of new drugs for the treatment of these disorders.

Precis

CXCR3 and its chemokines CXCL10, CXCL9, CXCL11 are implicated in the pathogenesis of AT, GD, TED, T1D, AAD. They can be considered potential targets of new drugs for the treatment of these disorders.

Introduction

Chemokines are small cytokines, called "chemotactic cytokines", able to induce chemotaxis in responsive cells. Some of them control cell migration during tissue development, or maintenance (homeostatic chemokines), whereas others are proinflammatory and are involved in the recruitment of cells of the immune system to sites of inflammation, or infection (1).

Chemokines can be classified into 4 subfamilies: CXC, XC, CX3C, or CC. After the interaction with chemokine receptors, that are G protein-linked transmembrane receptors, on the external membranes of the target cells, chemokines exert their biological effects (1).

The chemokine receptor (CXCR)3 is expressed by different cells (i.e., Natural Killer, some epithelial and endothelial cells, activated T lymphocytes, etc.), and in particular on Th1 cells. It has two isoforms, CXCR3-A or CXCR3-B. It binds the interferon (IFN)-γ inducible chemokines (or Th1-chemokines), that are: a) IFN-γ-inducible protein 10 (IP-10)/chemokine ligand 10 (CX-C motif) (CXCL)10; b) monokine induced by IFN-γ (MIG)/CXCL9; c) IFN-γ -inducible T-cell α chemoattractant (I-TAC)/CXCL11 (2).

CXCR3 and its ligands have a central role in inflammatory cells recruitment: Th1 lymphocytes are attracted by CXCL9, CXCL10, CXCL11, that are secreted by the damaged cells (3-5). The recruited Th1 lymphocytes enhance the IFN-γ and tumor necrosis factor (TNF)-α release in the inflamed tissues, that induces the production of Th1-chemokines by different cells, leading to an amplification feedback loop (6,7). Chemokines secreted into the inflamed tissues then enter the circulation. For this reason, the high level of Th1-chemokines in circulation is a marker of the host inflammatory response (5-7). In fact, increased levels of Th1-chemokines in tissues and serum are shown in different autoimmune diseases, as: organ specific autoimmune diseases [autoimmune

thyroiditis (AT) (8-14), Graves' disease (GD) (15), thyroid eye disease (TED) (16), type 1 diabetes (T1D) (17)]; or systemic rheumatological disorders [systemic sclerosis (18), systemic lupus erythematosus (19), rheumatoid arthritis (RA) (20), sarcoidosis (21), psoriatic arthritis (22), HCV-related cryoglobulinemia (11,23,24), other HCV immune mediated disorders (25,26)]; in other diseases, and also in cancers (27).

Here, we review Th1-chemokines in autoimmune endocrine disorders. A PubMed revision of the literature was conducted searching the terms: "Th1 chemokines", "CXCL10", "CXCL9", "CXCL11"; in combination with "autoimmune thyroiditis", "Graves' disease", "Graves' ophthalmopathy", "thyroid eye disease", "Type 1 diabetes", "Addison's disease", respectively.

Th1 chemokines in AT

AT [known also as Hashimoto's or chronic lymphocytic thyroiditis] derives from a dysregulation of the immune system that causes an immune attack to the thyroid. In AT the infiltrate of the thyroid is composed by CD4+ and CD8+ T cells, CD19+ B cells, macrophages and plasma cells. Infiltrating B cells are the source of antibodies against thyroid peroxidase (AbTPO), or thyroglobulin (AbTg), and they are also the source of proinflammatory cytokines/chemokines. B lymphocytes, by presenting thyroid autoantigens to autoreactive CD4+ T cells, may also act as antigen presenting cells (APCs) (8,28,29). Many studies have demonstrated the involvement of the Th1 immune response in the AT immunopathogenesis (6,8,30).

The selective migration of lymphocytes in autoimmune thyroid disorders (AITDs) was investigated analyzing thyroid samples obtained from AITD patients (31), showing an increased expression of CXCL10, by T lymphocytes. *In vivo*, thyroid follicular cells (TFCs) from AITD patients expressed

CXCL10 and CXCL9. The supernatants obtained from stimulated TFCs *in vitro* induced an increased migration of T lymphoblasts. This chemotactic response was blocked by specific antibodies against the chemokines, as well as against CXCR3 (31).

To define the profile of 10 chemokines (CCL1 through 5 and CXCL9 through 13) expression after a chronic exposure of thyroid to IFN-γ, a C57BL6 transgenic mice model (that under control of the thyroglobulin promoter presents an aberrant expression of IFN-γ) was used (32), in comparison with wild-type littermates. CCL4, CXCL9, and CXCL11 were exclusively expressed in the transgenic model, that showed also an over-expression of CXCL10 and CCL5, that induced a moderate mononuclear cell infiltration of the thyroid (32).

Circulating CXCL10 was evaluated in 223 AT and 29 nontoxic multinodular goiter (MNG) patients, and 97 controls, showing higher levels in AT than in controls or MNG. Furthermore, in AT serum CXCL10 levels were significantly higher among those with hypothyroidism and a hypoechoic ultrasonographic pattern, suggesting that CXCL10 is a marker of a more aggressive thyroid inflammation leading to TFCs destruction (33).

Discordant results have been reported by another study that showed CXCL10 levels were similar in AT patients vs. MNG subjects (34). However, other studies confirmed the elevated levels of Th1-chemokines in AITD patients. Circulating CXCL9 and CXCL11 were measured in 141 AT and 35 MNG patients vs. 70 euthyroid controls (10). Serum CXCL9 and CXCL11 levels were significantly higher in AT than in controls, or MNG. Among AT, those with hypothyroidism, or with a hypoechoic ultrasonographic pattern had the highest levels of these chemokines. In a multiple linear regression model, only thyroid-stimulating hormone (TSH) was related to serum CXCL9, or CXCL11, levels. These results showed that also circulating CXCL9 and CXCL11 are increased in AT patients, overall in presence of hypothyroidism, such as CXCL10 (10). Other studies have subsequently confirmed these results (35).

These chemokines have been also evaluated also in 41 euthyroid AT non-pregnant women with a history of recurrent spontaneous abortions (RSA), and in 35 women with euthyroid AT vs. 29 controls (36). Serum CXCL9 and CXCL11 levels were higher in the antibody-positive women with RSA than in both controls. Furthermore, AT patients without RSA had higher CXCL9 levels than controls. In multiple linear regression analyses, abortions were significantly related to the chemokines levels in AT. These data suggested a more dominant Th1 cytokine profile in AT patients with RSA (36).

In order to define the association between the pathogenesis of AITD and polymorphisms in genes encoding certain chemokines [RANTES-403G/A, IL8-251T/A, -28C/G, IP-10-1596C/T, MIG rs2276886G/A, IL16-295T/C and Monocyte Chemoattractant Protein1 (MCP1)-2518G/A], polymorphisms were genotyped in GD patients [59 with intractable GD (euthyroid patients with GD who had been treated with methimazole (MMI) for at least five years and were still positive for anti-thyrotropin receptor antibody (TRAb); 53 GD patients in remission], AT patients (54 with severe; 46 with mild hypothyroidism), and 99 controls (37). MIG rs2276886 A and IL8-251TT genotypes were more frequent in AITD patients, while RANTES-403AA and -28GG genotypes were less frequent. In AT patients, the frequency of MCP1-2518GG genotype was higher, while in intractable GD the MIG rs2276886 AG genotype was less frequent (37).

The role of myeloid-related protein 14 (MRP14), an important factor in inflammatory reactions, and IL-1β-mediated regulation of MRP14 expression on the secretion of proinflammatory chemokine in TFCs, was evaluated in a further study (38). An elevated MRP14 expression was present in thyroid tissues and sera from AT patients. Besides, IL-1β remarkably increased the expression of MRP14 in TFCs, that induced the secretion of CXCL9, GRO-2, and CCL22, indicating a potential pathological mechanism of lymphocyte infiltration into the thyroid in AT (38).

Other studies have been conducted in AT patients with other autoimmune diseases, such as psoriatic arthritis, systemic sclerosis, and HCV-mixed cryoglobulinemia, confirming a strong association among high levels of circulating CXCL10 and AT, independently from the type of the associated autoimmune disease (20,22,39,40). These data suggest a common prevalent immunopathogenic Th1-chemokine pattern in patients with AT in presence of the above mentioned immune-mediated diseases (6,8) (**Table 1**).

Th1 chemokines in GD

GD is an autoimmune disorder caused by a complex interplay of genetic, environmental and endogenous factors (41). The key role of the autoimmune response and of thyroid stimulating autoantibodies (TSAb) has been shown in the pathogenesis of GD (42). Th1 and Th17/Treg lymphocytes infiltration producing Th1/Th2 cytokines and chemokines have been shown in the development of GD.

It has been shown that in the early phase of GD, the recruitment of CXCR3-expressing Th1 cells can be caused by the production of CXCL10 by TFCs (43). TFCs are not able to produce CXCL10 in basal conditions, but they are induced to secrete it under IFN-γ stimulation. The combination of IFN-γ and TNF-α induced a huge release of this chemokine (44).

Other papers investigated the role of CXCL9 and CXCL11 secretion in GD thyrocytes in primary cultures (45-47). The secretion of CXCL9 and CXCL11 was absent basally, IFN-γ stimulated their release dose-dependently, while TNF-α alone had no effect. The co-treatment with TNF-α+IFN-γ had a synergistic effect on their secretion. The PPAR-γ ligands, rosiglitazone or pioglitazone, in presence of TNF-α+IFN-γ, suppressed this effect dose-dependently. These data suggested that under the cytokines stimulation, thyrocytes from patients with GD are involved in the self-

protraction of inflammation secreting these Th1-chemokines, and that PPAR-γ are involved in the inhibition of this process. Among the 3 chemokines, GD TFCs secrete huge amounts of CXCL9, suggesting a leading role of this chemokine in the Th1 immune response (45).

The role of PPAR-α activation has been also studied in GD TFCs (47,48). The presence of PPAR-α in primary GD and control cells was shown. PPAR-α-agonists inhibited dose-dependently the cytokines-stimulated release of the three CXCR3 chemokines showing the strongest inhibitory activity on these chemokines in GD thyrocytes vs. PPAR-γ agonists (47,48).

The chemokines secreted by TFCs in GD enter the circulation increasing their serum levels. In fact, high levels of circulating CXCL10 are present in GD patients with active disease (44). In GD patients the chemokine level declined during anti-thyroid therapy with MMI reaching near normal levels when thyroid hormones normalized, or in GD patients in remission (49). When GD hyperthyroidism relapsed, serum CXCL10 increased again reaching levels comparable to recently diagnosed untreated hyperthyroid GD ones, suggesting that both in relapsing hyperthyroid patients, and in those with new diagnosis, the active phase of GD is associated with high circulating CXCL10 (49). Also other studies have demonstrated that circulating Th1-chemokines were associated with the active phase of GD and their decrease after treatment may be related to the immune-modulatory effects of MMI (50).

In order to verify the original site of production of the Th1-chemokines, CXCL10 serum levels were also assessed in GD patients treated with total thyroidectomy or after radioactive iodine therapy. The reduction of CXCL10 levels after these treatments suggested that the site of production of this chemokine is into the thyroid gland itself (51-53). Moreover, in the paper by Leite et al. (53), serum CXCL10 levels were evaluated in 16 newly diagnosed GD patients who received MMI, 15 relapsed GD patients who were treated with radioactive iodine (RAI), with respect to 18 controls. In the MMI group, serum CXCL10 levels decreased following euthyroidism

196 at 6 and 12 months. In the RAI group, serum CXCL10 levels decreased after 3, 6, 9, and 12 months
197 of RAI administration. Elevated serum TRAb levels in the MMI group decreased at 6 months,
198 while in the RAI group they increased to a peak level at 6 months. After RAI therapy, the transient
199 increase in serum TRAb levels at 6 months was accompanied by the decrease in serum CXCL10
200 levels. These findings suggest that CXCL10, and thyroid autoantibodies, are associated with
201 different molecular pathways.
202 On the whole, the above mentioned data suggest that, even if GD was initially considered a Th2
203 immune predominant autoimmune disease, a prevalence of the Th1 immune response is present at
204 the beginning of the active phase of GD and during relapse (**Table 2**).
205
206 **Th1 chemokines in TED**
207 TED is of clinical relevance in about 50% of subjects with GD. The onset of ophthalmopathy and
208 GD are often concomitant. Actually TED primary prevention is not feasible. An early diagnosis,
209 and accurate control of thyroid function, the stop of smoking and the treatment of early mild eye
210 disease can avoid the progression of subclinical TED into overt and/or severe TED (54). The risk
211 of progression is higher in particular in patients who smoke, with a severe hyperthyroidism or
212 hypothyroidism, or with elevated levels of TSAb. An early immunosuppressive treatment
213 (corticosteroids, rituximab, and others), or orbital decompression, represent the tertiary prevention,
214 to avoid the deterioration and complications of TED (55,56).
215 The interactions between orbital fibroblasts and lymphocytes lead to the expansion and the
216 remodeling of the orbital tissues, and to the clinical manifestations of TED (57).
217 Serum CXCL10 levels were evaluated in patients both with active or inactive TED, investigating
218 also the effect of IFN-γ and TNF-α on the chemokine secretion in primary cell cultures of orbital
219 cells (fibroblasts and preadipocytes) (44). TED patients with an active disease had higher CXCL10

than those with an inactive one. Primary cell cultures of orbital fibroblasts or preadipocytes obtained from TED patients did not secrete CXCL10 in basal conditions, while the treatment with IFN-γ alone, or combined with TNF-α, stimulated its release. The above reported results indicated that TED retrobulbar cells participate in the perpetuation of the orbital inflammation by releasing Th1-chemokines under the influence of IFN-γ (44), as confirmed also by another study (58).

Similar data have been reported also for the chemokines CXCL9 and CXCL11 in TED. In fact, the production of these chemokines was absent basally, while it was dose-dependently stimulated by the treatment with IFN-γ, overall in presence of TNF-α. Such as in GD TFCs, the co-treatment of orbital cells with the PPAR-γ agonists showed an inhibitory role in this process (45).

PPAR-α, -δ and -γ are present in TED fibroblasts and preadipocytes, and in these cells PPAR-α activators showed the most potent inhibition of CXCL9 and CXCL11 secretion induced by IFN-γ+TNF-α, underlining the role of PPAR-α in the modulation of the immune response in TED (59).

A study evaluated the potential role of circulating chemokines CXCL9 and CXCL11 as markers of TED activity (60). Forty-two TED patients were studied: a. 10 euthyroid GD patients; b. 15 euthyroid TED patients treated with corticosteroids [teleradiotherapy (TR) and intravenous infusions of methylprednisolone (MP)]; c. 10 patients with GD hyperthyroidism; d. 7 controls. CXCL9 and CXCL10 significantly decreased after corticosteroids, and TR treatments. In corticosteroid-responders TED patients, CXCL9 was significantly reduced even after 24h from the first MP dose. These outcomes lead to hypothesize these chemokines may be used as a guideline in therapeutic decision-making in TED patients (60).

To evaluate if retrobulbar myoblasts might be involved in the immune-pathogenesis of the inflammation in TED, CXCL10 was investigated *in vivo* in active TED patients with prevalent extraocular muscle (EOM) involvement (EOM-p) vs. those with prevalent orbital fat expansion (OF-p). Serum CXCL10 was higher both in OF-p, or EOM-p, compared to controls. Furthermore,

the effects of IFN-γ and TNF-α stimulation, and of increasing concentrations of the PPAR-γ ligands, on CXCL10 secretion were investigated in primary EOM cell cultures from TED patients compared to control myoblasts (61). CXCL10 was undetectable in primary EOM cultures from TED patients, IFN-γ dose-dependently induced it, overall in presence of TNF-α. PPAR-γ was expressed in EOM cells, and PPAR-γ agonists showed an inhibitory action on the induction of CXCL10 by IFN-γ. These results showed that EOM are involved in the promotion and perpetuation of inflammation in TED by releasing CXCL10, and that PPAR-γ agonists play an inhibitory role in this process (61) (**Table 2**).

Th1 chemokines in T1D

The slow and continuous destruction of pancreatic β-cells in T1D is determined by autoimmune mechanisms, leading to a progressive shortage of β-cells and a final absence of endogenous insulin. Consequently, multiple hormonal abnormalities regarding insulin, glucagon and incretin occur, causing an extreme glycemic variability, and metabolic instability (62-64). T1D is caused both by autoantibodies and antigen-specific diabetogenic T cells against β-cells (65,66). Islet-specific CD8+ T cytotoxic cells (CTL) are responsible for destroying β-cells in T1D in a cytokine microenvironment provided by diabetogenic CD4+ Th1. An association between T1D and the predominance of the Th1 response has been observed, that leads to a derangement of the Th system, and to the development of autoimmune diseases (67).

CXCR3 chemokines have an important role in the immune-pathogenesis of T1D. High serum CXCL10 levels were observed by most of the studies in children and adults with T1D, in particular in children with newly onset of T1D (68-71). However, discordant results have been reported by other studies (72,73).

CXCL10 levels have been evaluated in children with T1D (74). The Th1-chemokine CXCL10 circulating levels were significantly higher in newly diagnosed T1D children than in controls, and declined during the follow-up. For comparison, the Th2-chemokine CCL2 levels were similar in patients, relatives and controls and did not change at follow-up. These data showed that in children with newly diagnosed T1D, a predominant Th1-driven autoimmune process is observed, which shifts toward a Th2 predominance in 1-2 years from diagnosis (74).

Another study evaluated sera cytokines [interleukin (IL)-12, IL-6, II-1β, TNF-α, IL-10] and chemokines (CXCL10, CXCL8, CXCL9, CCL2) levels in newly diagnosed T1D patients and healthy controls, reporting significantly higher levels of both cytokines in T1D (71).

Other studies are present in literature regarding the possible relationship between the secretion of CXCL10 and the stage of insulitis, i.e. the different time of appearance of specific autoantibodies. In a paper published in 2001 (68), serum CXCL10 levels were evaluated in diabetic patients positive for either one or both of the autoantibodies anti-glutamic acid decarboxylase (GAD) and insulinoma-associated protein-2 (IA-2) (Ab^+ type 1 group), and those negative for both (Ab^- type 1 group). There was no correlation between the autoantibody titer and CXCL10 levels. Moreover, GAD-reactive IFN-γ–producing $CD4^+$ cells were observed in all of the $CXCL10^+$ patients examined, but in only 21.4% of $CXCL10^-$ Ab^+ T1D patients examined ($P<0.002$). Another paper (69) reported elevated CXCL10 levels in both newly diagnosed T1D patients and in those with a high risk of disease [positive for the autoantibodies islet cell antibodies (ICA) and GAD], and the lack of correlation between the circulating concentrations of CXCL10 and those of ICA and GAD antibodies. The paper by Gabbay et al. (71) reported that GAD-65 autoantibodies were associated negatively with CXCL10 ($P=0.011$) and CCL2 ($P=0.000$), while IA-2A showed a negative correlation with IL-10 ($P=0.027$) in T1D patients. As cell-mediated immunity is primarily responsible for T1D development and because CXCL10 stimulates cell-mediated immunity to a

291 greater extent than humoral immunity, it is possible that CXCL10 has diabetogenic potential
292 without influencing antibody production from autoreactive B lymphocytes (69).
293 Increased CXCL9 and CXCL1 plasma levels were also reported in T1D Iranian patients in
294 comparison to controls, showing these chemokines were elevated in T1D patients suffering with
295 complications (75).
296 The main site of production of CXCL10 in T1D are peripheral blood monocytes and leukocytes
297 (76). However, CXCL10 is highly expressed in islet infiltrating lymphocytes. Moreover, β-cells
298 participate actively in the islet inflammation; in fact, when stimulated by cytokines (i.e., IFN-γ
299 and/or TNF-α), they secrete CXCL10, CXCL9 and CXCL11, that conduct to the invasion of Th1
300 lymphocytes into the islet themselves. The autoreactive cells secrete more cytokines, stimulating
301 the production of chemokines by the target cells, and reiterating the autoimmune cascade. Also
302 other studies have demonstrated that CXCL10 was the dominant chemokine expressed *in vivo* in
303 the islet environment of prediabetic animals and T1D patients (77-79).
304 In order to analyze the role between human leucocyte antigen (HLA) high-risk haplotypes
305 DQ2(DQA*0501-DQB*0201)-DQ8(DQA*0301-DQB*0302) and CXCL10 (rs8878, rs35795399
306 and rs3921) and CXCL9 (rs3733236, rs10336) polymorphisms in T1D, a combination of family
307 (221 patients) and case-control (447 cases and 300 controls) analysis was done (80). An
308 overtransmission of the alleles T and G of rs8878 and rs35795399 polymorphisms, in combination
309 with the HLA-high risk haplotypes in the family group was reported. The less transmitted
310 haplotype in affected offspring was rs8878A-rs35795399C and the most trasmitted was the
311 rs8878G-rs35795399T. The study did not find any association between T1D and CXCL9/CXCL10
312 polymorphisms in the German population, but they could not exclude it in other populations (80).
313 Another study evaluated the role of the natural vitamin D receptor (VDR) ligand, 1,25-
314 dihydroxyvitamin D(3) (1,25(OH)(2)D(3)) in interfering in T cell stimulatory capacity of

315 macrophages, in T1D (81). The 1,25(OH)(2)D(3) induces VDR and its downstream targets
316 expression, and macrophages constitutively expressed VDR. In control mice, macrophage
317 programming with 1,25(OH)(2)D(3) in part blocked the activation-stimulated expression of IL-
318 12p40, TNFα and iNOS and the effector T cell-recruiting chemokines, CXCL9, CXCL10 and
319 CXCL11 (81).
320 A paper assessed the protection exerted by Hypericum perforatum (St. John's wort, SJW) and its
321 component hyperforin (HPF) on isolated rat and human islets exposed to cytokines *in vitro* (83).
322 Both in rat and human islets, SJW and HPF down-regulated mRNA expression of iNOS, CXCL9,
323 CXCL10, COX2, reducing apoptosis and early β-cell damage (82).
324 A study profiled the transcriptome in human islets of Langerhans, basally or after the exposure to
325 pro-inflammatory cytokines, based on the RNA sequencing dataset downloaded from the NCBI
326 Short Read Archive (SRA) database (83). Sixty-three differentially expressed genes were reported,
327 of whom 60 upregulated and 3 downregulated. In the samples treated with cytokines, the two most
328 upregulated genes were CXCL9 and guanylate binding protein 5 (GBP5) (83).
329 In non-obese diabetic (NOD) mice, a decrease of CD4+ Foxp3+ regulatory T (Treg) cells, induced
330 by IL-2 deficiency in pancreas, is associated with Th1 autoimmunity and T1D development. Intra-
331 islet Treg cells expressed the ICOS costimulatory receptor and promoted self-tolerance delaying
332 the onset of T1D. During pre-diabetes in the NOD model the ICOS-dependent Treg cell homing to
333 the β-islets was associated with upregulation of CXCR3 (84). A CXCR3 expression occurred in the
334 islet-specific ICOS+ Treg cell subset in the pancreatic lymph nodes (pLN), that correlated with the
335 IFN-γ production by T effector (Teff) cells in pancreatic sites, which mediate the pancreatic
336 inflammation. *In vivo* studies reported a block of the Treg cell CXCR3 upregulation by
337 neutralizing IFN-γ (84).

The response of lymphocytes to cytokines can be modulated by fragments of glucagon-like peptide-1 (GLP1), GLP17-36 and GLP19-36 (85). In 34 patients with T1D and in 35 controls, hematologic parameters, the incretin axis and CXCR3 expression on Tregs were assessed. Flow cytometry revealed a higher CXCR3 expression on the CD25(-/)(low)Foxp3(+) with respect to CD25(+)Foxp3(+) Tregs independently from T1D, thus suggesting that CD25(-/)(low)Foxp3(+) Tregs are in a "standby" mode possibly waiting for orientational chemotactic stimuli (85).

The initial triggers in the autoimmune process in T1D are unknown, but a leukocytic infiltration, that precedes islet β-cell death and dysfunction is present. A study (86) demonstrated that the genes encoding the Th1-chemokines were primary response genes in pancreatic β-cells and raised in rat, mouse, and human islets, as part of the inflammatory response. STAT-1 was involved in the transcription control of the genes of these chemokines in response to the Th1 cytokines IL-1β and IFN-γ. Moreover, serum levels of chemokines activating CXCR3 were elevated in NOD mice. Mice harbouring a genetic deletion of CXCR3 reported a delay in diabetes development after the injection of multiple low doses of streptozotocin. The above reported data indicate that chemokines, released by β-cells, can control leukocytes migration and activity into pancreatic tissues, that influenced the mass and function of β-cells (86).

It has been recently reported that influenza A virus might be involved in the etiology of diabetes (87). *In vitro* and *in vivo* studies were carried out to test the damage induced by H1N1 virus in human β-cells, or pancreatic islets and on glucose metabolism. The Human H1N1 A/California/2009-derived viruses could infect human islets, with an increase of the release of CXCL9 and CXCL10. In the infected mice, the virus was observed, and the virus was also found in extrapulmonary organs, such as pancreas (87).

A recently published paper investigated the involvement of miRNAs in the early T1D development in NOD mice (88). Islet insulitis was associated with an exosome-mediated transfer of a specific

group of miRNAs (miR-142-3p, miR-142-5p, miR-155), from lymphocytes to β-cells, inducing the selective insulin secreting cells death. The *in vitro* inactivation of these miRNAs inside the β-cells protected them against T lymphocyte exosomes-induced apoptosis and prevented development of T1D in NOD mice *in vivo*. In β-cells, T lymphocyte exosomes induced the expression of genes involved in chemokine signaling (i.e., CCL2, CCL7, and CXCL10), β-cell apoptosis, and promoted the recruitment of immune cells during the autoimmune attack (88).

Recently, in order to better define the pathological changes in the endocrine and exocrine pancreas after onset of diabetes in fulminant type 1 diabetes (FT1DM), the pancreas of 3 FT1DM and 17 controls were assessed for infiltrating mononuclear cell (MNC) CD subtype, enterovirus capsid protein 1 (VP1) localization, CXCL10, or CXCR3 expressions (90). In islet cells, pancreatic acinar or exocrine ductal cells of FT1DM patients, a strict association was observed between VP1 and CXCL10 expression. CXCL10+ exocrine cells were surrounded by CXCR3+ T cells. The Authors concluded that suppressing the activated CXCL10-CXCR3 axis in exocrine and endocrine pancreas could represent a novel therapeutic target in FT1DM, and perhaps also in enterovirus-associated acute-onset T1D (89) (**Table 3**).

Th1 chemokines in Addison's disease (AAD)

AAD is a disease mainly caused by the autoimmune destruction of the adrenal cells (90,91). AAD is often concomitant with other autoimmune diseases, such as T1D, vitiligo, hypothyroidism, celiac disease, and pernicious anaemia in polyglandular autoimmune syndrome (92).

The adrenalitis in AAD is considered, at least in part, having a cell mediated, and thus Th1-driven, pathogenesis (93), however other studies showed the involvement of both Th1 and Th2 cytokines/chemokines in AAD (94). Plasmatic levels of Th1-chemokines [CXCL10, macrophage inflammatory proteins 1α (CCL3/MIP-1α)], and Th2-chemokines [macrophage inflammatory

proteins 1 β (CCL4/MIP-1β)], and adrenocortical antibodies, have been evaluated in AAD patients (94). Serum CXCL10, MIP-1α, and MIP-1β were significantly increased vs. controls (94).

A further study evaluated the circulating Th1 and Th2 chemokine in 15 AAD patients compared to 7 controls (90). The Th1-chemokines, CXCL10 and CXCL11 were higher in AAD patients, than in controls, and partially correlated to a reduced quality of life (90).

In vitro, CXCL10 can also be induced in adrenal cells stimulated with IFNs, other cytokines, or microbial components (95). Serum CXCL10 and CXCL9 levels were significantly higher in AAD patients vs. controls, but IFN-stimulated patients' peripheral blood mononuclear cells (PBMC) produced significantly less CXCL10, or CXCL9, vs. control PBMC. The Authors concluded that AAD PBMC have a deficient response to IFNs, and that the adrenal cortex itself is responsible for the increased circulating levels of CXCL10 (95).

Attempts to target CXCR3, or CXCL10, in autoimmune diseases

Different autoimmune disorders could benefit from agents blocking CXCR3 or its ligands.

A first study showed that neutralization of CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis (96).

Other studies have shown the importance of CXCL10 in the pathogenesis of ulcerative colitis (UC), in animal models, and in humans (97,98). A phase II study assessed the efficacy and safety of BMS-936557, a fully human, anti-CXCL10 monoclonal antibody, in the treatment of moderately-to-severely active UC, showing that BMS-936557 is a potentially effective therapy for UC, and that higher drug exposure correlated with increasing clinical response and histological improvement. As side effect, infections occurred in 12.7% BMS-936557-treated vs. 5.8% placebo-treated patients (99).

CXCL10 is involved in the recruitment of inflammatory T cells into the liver in patients with primary biliary cholangitis. The fully human anti-CXCL10 monoclonal antibody NI-0801 has been evaluated in these patients. Despite clear pharmacologic responses in the blood, no therapeutic benefit of multiple administrations of NI-0801 could be demonstrated. The Authors suggested that the high production rate of CXCL10 makes it difficult to achieve drug levels that lead to sustained neutralization of the chemokine, thus limiting its targetability (100).

Many studies have shown that CXCL10 and its receptor CXCR3 regulate synovial fibroblast invasion in RA, and that blocking CXCR3 ameliorates arthritis progression in animal models (101-104). A phase II study evaluated the efficacy and safety of MDX-1100, a fully human, anti-CXCL10 monoclonal antibody, in RA patients whose disease responded inadequately to methotrexate. The American College of Rheumatology 20% improvement criteria (ACR20) response rate was significantly higher among MDX-1100-treated patients than among placebo-treated patients (54% vs. 17%). No study drug-related serious adverse events were reported (105).

In T1D, it has been shown in NOD mice that CXCL10 DNA vaccination prevents spontaneous diabetes through enhanced β-cell proliferation, reverses hyperglycemia, and that anti-CD3/anti-CXCL10 antibody combination induces persistent remission of T1D in mouse models. However clinical trials are needed (106-109).

In TED fibroblasts and preadipocytes, PPAR-α and PPAR-γ are overexpressed and have a key role in inhibiting the production of CXCR3 chemokines by target cells (58,110).

The involvement of EOM in the promotion and perpetuation of inflammation in TED has been shown, and PPAR-γ agonists had an inhibitory role in the process (61). Actually there is disagreement on the role of PPAR-γ in the evolution of TED (111-113). However PPAR-α needs to be evaluated in TED patients.

In a prospective, randomized trial to compare the efficacy and safety of two protocols of iv 4.5 g methylprednisolone in a total of 80 TED patients (methylprednisolone weekly, or daily) TED-associated serum cytokines were measured. Among 10 different cytokines/chemokines evaluated it was observed sustained decreased levels only of serum CXCL10 in the 12th week compared to the baseline level in the patients on the weekly protocol, suggesting that CXCL10 might be used as a biomarker of the effect of the therapy in TED patients (114).

Anti CXCR3, or anti-CXCL10 agents need to be studied also *in vitro*, and *in vivo* in animal models, of AT, GD, and TED, with the view to a future evaluation in patients with different endocrine autoimmune diseases (115).

The studies above reported in humans show mostly an efficacy of drugs directed against CXCL10 or CXCR3. However the effect is limited (for example in UC), or not significant (for example in primary biliary cholangitis), and for this reason other more potent, and more safe, molecules need to be evaluated, or discovered (116,117).

Furthermore, most of the above reported drugs evaluated in human autoimmune diseases block CXCR3, or CXCL10, irrespective if they are produced by circulating or infiltrating lymphocytes, or by the cells target of the autoimmune process. Other molecules able to block selectively these chemokines, on lymphocytes, in circulation, or their production by target cells, need to be discovered and researched.

Targeting IFN-γ dependent chemokines, or their CXCR3 receptor, might be important overall in the initial/active phase of the diseases (for example in GD at the first appearance of hyperthyroidism, or in GO in the initial active inflammatory phase, or in T1D at the initial presentation of hyperglycemia), or at moment of the recurrence of the diseases (for example in GD, when a relapse of hyperthyroidism is present), since a prevalence of the Th1 immune-response has been shown during these phases. The Th2 immune-response might be instead targeted in the later

phases of the disorders [for example in T1D after more than 2 years from the appearance of hyperglycemia (74)], or in the inactive phases (for example in GD during the euthyroid phase, or in GO in advanced inactive disease). If targeting IFN-γ dependent chemokines might prevent the appearance of endocrine autoimmune disorders should be also evaluated in experimental studies, and in specific subgroups of patients [for example in GD patients with subclinical hyperthyroidism and positive TRAb at the initial presentation, or in subjects at high risk of T1D (positive for the autoantibodies ICA, and/or GAD), and initial dysglycemia].

Conclusion

Chemokines are small cytokines, able to induce directed chemotaxis in nearby responsive cells. Some of them (called proinflammatory chemokines) are enhanced during an immune response to recruit immune system cells to a site of inflammation, or infection (1).

The chemokine receptor (CXCR)3 is expressed by various cells (i.e., activated T lymphocytes, Natural Killer cells, macropahges, endothelial and some types of epithelial cells, etc.), and it binds the IFN-γ-dependent chemokines, CXCL10, CXCL9 and CXCL11 (2).

At the initial phases of an inflammatory process the Th1 lymphocytes produce IFN-γ and TNF-α cytokines that lead to the secretion of Th1-chemokines in inflammatory cells, such as in the target cells of epithelial or mesenchimal origins. The target cells produce themselves (under the pressure of IFN-γ stimulation) large amounts of these Th1-chemokines that recruit other effector lymphocytes and immune-cells into the site of inflammation, creating an amplification feedback loop (6,7) that induces and perpetuates the inflammatory process. The Th1-chemokines secreted into the tissues enter than into the circulation. For this reason, circulating chemokines are high in

478 many different autoimmune or inflammatory diseases, and the elevated level of these chemokines
479 in peripheral fluids can be considered a marker for the host immune response (5-7).

480 Many studies have shown that also TFC in AT and GD, retroorbital cells in TED (fibroblasts,
481 preadipocytes, myoblasts), β-cells and islets in T1D, and adrenal cells in AAD, respond to IFN-γ
482 stimulation producing large amounts of these chemokines (**Figure 1**). Furthermore, lymphocytes
483 and PBMC are in part responsible for the secreted Th1-chemokines.

484 Furthermore, Th1-chemokines have been associated with the early phases of the autoimmune
485 response. High levels of these chemokines have been associated also with the "active phase" of the
486 disease in GD, and also in TED. Other studies have shown an association with the severity of
487 hypothyroidism in AD, of hyperthyroidism in GD, with severity of TED, or with FT1DM.

488 In most of the above mentioned disorders, a Th1 immune predominance has been shown in the
489 early phases, with a switch to a Th2 immune response in the late, not active, phases of the
490 disorders.

491 The above mentioned data have shown the importance of the Th1 immune response in different
492 endocrine autoimmune diseases, and many studies have suggested that CXCR3, and its
493 chemokines, might be considered potential targets of new drugs for the treatment of these
494 disorders. Targeting IFN-γ dependent chemokines, or their CXCR3 receptor, might be important
495 overall in the initial/active phase of the diseases (in the hyperthyroid phase of GD, or in active GO,
496 or at the appearance of T1D), or at moment of the recurrence of the diseases (for example in
497 relapsing GD), when a prevalence of the Th1 immune-response has been shown. If targeting IFN-
498 γ dependent chemokines might prevent the appearance of endocrine autoimmune disorders should
499 be also evaluated (for example in subjects at high risk of T1D).

However, additional studies are necessary to better evaluate the role of Th1-chemokines, and their potential therapeutic implications, in endocrine autoimmunity.

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889 **Legend to Figure**
890

891 **Figure 1.** Th1 lymphocytes produce IFN-γ and TNF-α cytokines that lead to the secretion of Th1-
892 chemokines in thyroid follicular cells (TFC) in autoimmune thyroiditis (AT) and Graves' disease
893 (GD), retroorbital cells in thyroid eye disease (TED) (fibroblasts, preadipocytes, myoblasts), β-
894 cells and islets in type 1 diabetes (T1D), and adrenal cells in Addison's disease (AAD). The target
895 cells produce large amounts of these Th1-chemokines that recruit other effector lymphocytes and
896 immune-cells into the site of inflammation, creating an amplification feedback loop that induces
897 and perpetuates the inflammatory process. *In vitro*, or *in vivo*, studies have evaluated the activity of
898 different drugs, that block CXCL10 (BMS-936557, MDX-1100, NI-0801), or the CXCR3 receptor,
899 or decrease the stimulatory effect of IFN-γ in combination with TNF-α on the Th1 chemokines
900 secretion by target cells [PPAR-γ, or PPAR-α (in thyrocytes, or retro-orbital cells), methimazole
901 (MMI) (in thyrocytes), methylprednisolone (in orbital cells), hypericum perforatum and CXCL10
902 vaccination (in β-cells)], in different autoimmune disorders.
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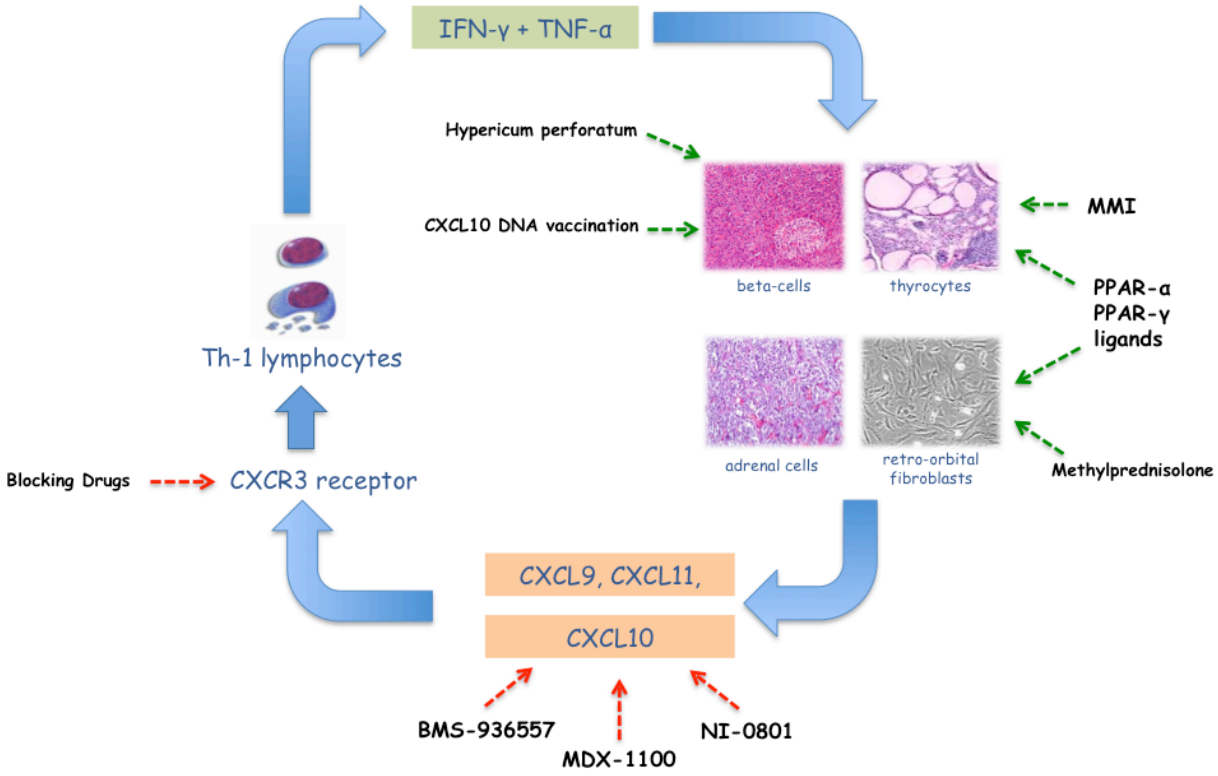

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Table 1. Th1 chemokines in autoimmune thyroiditis (AT).

CXCL10	*CXCL9*	*CXCL11*
• *In vivo*, TFCs from AITD patients express CXCL10 (31) • A C57BL6 transgenic mice model (with an aberrant expression of IFN-γ under control of the thyroglobulin promoter) over-expressed CXCL10 (32) • Circulating CXCL10 was significantly higher in AT patients than in controls or MNG, overall in the presence of hypothyroidism and a hypoechoic ultrasonographic pattern (33) • CXCL10 was higher in MC+AT patients than in patients with AT without MC or patients with MC without AT (35) • Circulating CXCL10 was higher in: HCV+AT patients than in HCV patients without AT, AT patients or healthy controls without AT; in AT patients than in healthy controls (39) • Serum CXCL10 levels were higher in patients with MC+HCV compared to healthy controls, overall in the presence of AT (40)	• Serum CXCL9 levels were significantly higher in AT patients than in controls, or MNG subjects, overall in the presence of hypothyroidism, or a hypoechoic ultrasonographic pattern (10) • *In vivo*, TFCs from AITD patients express CXCL9 (31) • A C57BL6 transgenic mice model (with an aberrant expression of IFN-γ under control of the thyroglobulin promoter) expressed CXCL9 (32) • CXCL9 was higher in MC+AT patients than in patients with AT without MC or patients with MC without AT (35) • Serum CXCL9 levels were higher in the antibody-positive women with RSA than in AT patients without RSA or healthy controls. Furthermore, AT patients without RSA had higher CXCL9 levels than controls (36) • An elevated MRP14 expression was present in thyroid tissues and sera from AT patients. IL-1β remarkably increased the expression of MRP14 in TFCs, that induced the secretion of the chemokine CXCL9 (38)	• Serum CXCL11 levels were significantly higher in AT patients than in controls, or MNG subjects, overall in the presence of hypothyroidism, or a hypoechoic ultrasonographic pattern (10) • A C57BL6 transgenic mice model (with an aberrant expression of IFN-γ under control of the thyroglobulin promoter) expressed CXCL11 (32) • Serum CXCL11 levels were higher in the antibody-positive women with RSA than in AT patients without RSA or healthy controls (36)

autoimmune thyroiditis (AT); autoimmune thyroid disorders (AITD); hepatitis C virus (HCV); mixed cryoglobulinemia (MC); multinodular goiter (MNG); myeloid-related protein 14 (MRP14); recurrent spontaneous abortions (RSA); thyroid follicular cells (TFCs)

927 **Table 2. Th1 chemokines in Graves' Disease (GD) and Thyroid Eye Disease (TED)**

	GD	*TED*
CXCL10	• In the early phase of GD, the recruitment of CXCR3-expressing Th1 cells can be caused by the production of CXCL10 by TFCs (43) • TFCs are not able to produce CXCL10 in basal conditions, but they secrete it under IFN-γ stimulation. The combination of IFN-γ and TNF-α induced a huge release of this chemokine (44) • Higher serum CXCL10 has been reported in GD patients compared to sex- and age-matched controls (44) • Circulating Th1-chemokines were associated with the active phase of GD (newly diagnosed or relapsing), they decreased after treatment in relation to the immune-modulatory effects of MMI (49,50) • The reduction of CXCL10 levels, after total thyroidectomy or radioactive iodine therapy, suggests that the site of production of this chemokine is the thyroid gland (51-53)	• Retrobulbar cell types participate in the self-perpetuation of inflammation by releasing CXCL10 under the influence of cytokines (44) • A relationship between the increased concentrations of CXCL10 and the activity of orbital inflammation was shown, leading to hypothesize these chemokines may be used as a guideline in therapeutic decision-making in TED patients (60) • Serum CXCL10 was higher in OF-p and EOM-p compared to controls (61); EOM participates in the self-perpetuation of inflammation by releasing CXCL10, and PPARγ agonist activation plays an inhibitory role on it (61)
CXCL9	• Under the cytokines stimulation, thyrocytes from patients with GD participate to the self-protraction of inflammation by releasing CXCL9, and PPAR-γ inhibited this effect (45) • PPAR-α-agonists inhibited dose-dependently the cytokines-stimulated release of CXCL9, showing the strongest inhibitory activity on these chemokines in GD thyrocytes vs. PPAR-γ agonists (47,48)	• Retrobulbar cell types from patients with TED participate in the self-perpetuation of inflammation, releasing CXCL9 and CXCL11 chemokines when stimulated with cytokines (45) • PPAR-α, -δ and -γ are present in TED fibroblasts and preadipocytes, and in these cells PPAR-α activators showed the most potent inhibition of CXCL9 secretion induced by IFN-γ+TNF-α, underlining the role of PPAR-α in the modulation of the immune response in TED (59) • A relationship between the increased concentrations of CXCL9 and the activity of orbital inflammation was shown, leading to hypothesize these chemokines may be used as a guideline in therapeutic decision-making in TED patients (60)
CXCL11	• Under the cytokines stimulation, thyrocytes from patients with GD participate to the self-protraction of inflammation by releasing CXCL11, and PPAR-γ inhibited this effect (45) • PPAR-α-agonists inhibited dose-dependently the cytokines-stimulated release of CXCL11 showing the strongest inhibitory activity on these chemokines in GD thyrocytes vs. PPAR-γ agonists (47,48)	• Retrobulbar cell types from patients with TED participate in the self-perpetuation of inflammation, releasing CXCL11 when stimulated with cytokines (45) • PPAR-α activators showed the most potent inhibition of CXCL11 secretion by IFN-γ+TNF-α in retrobulbar cells, underlining the role of PPAR-α in the modulation of the immune response in TED (59)

928 patients with extraocular muscle (EOM) involvement (EOM-p); methimazole (MMI); patients with orbital fat
929 expansion (OF-p)

930
931 **Table 3. Th1 chemokines in Type 1 Diabetes (T1D)**
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	Ref
T1D is caused both by autoantibodies and antigen-specific diabetogenic T cells against β-cells	65,66
High serum CXCL10 levels were observed in children and adults with T1D, in particular in children with newly diagnosed T1D	68-71
Significantly higher levels of serum cytokines (IL-12, IL-6, II-1β, TNF-α, IL-10) and chemokines (CXCL10, CXCL8, CXCL9, CCL2) were shown in newly diagnosed T1D patients compared to controls	71
Circulating CXCL10 levels were significantly higher in newly diagnosed T1D children than in controls, and declined during the follow-up, while CCL2 levels were similar in patients, relatives and controls and did not change at follow-up, suggesting a predominant Th1-driven autoimmune process, which shifts toward a Th2 predominance over the first 1-2 years from diagnosis	74
An Iranian study demonstrated a strictly association between chemokines and T1D complications	75
The main site of production of CXCL10 in T1D are peripheral blood monocytes and leukocytes	76
CXCL10 is highly expressed in islet infiltrating lymphocytes. When stimulated by cytokines, β-cells secrete Th1-chemokines, that induce the invasion of Th1 lymphocytes into the islet themselves. The autoreactive cells secrete more cytokines, stimulating more chemokines production by the target cells, and reiterating the autoimmune cascade	77-79
In the islet environment of T1D patients and prediabetic animals, CXCL9 and RANTES were present at lower levels, while CXCL10 was the dominant expressed chemokine	79
The study did not find any association between T1D and CXCL9/CXCL10 polymorphisms in the German population	80
1,25(OH)(2)D(3) induces VDR and its downstream targets expression, and macrophages constitutively expressed VDR. In control mice, macrophage programming with 1,25(OH)(2)D(3) in part blocked the activation-stimulated expression of IL-12p40, TNFα and iNOS and the effector T cell-recruiting chemokines, CXCL9, CXCL10 and CXCL11	81
In rat and human islets, the extract of Hypericum perforatum (SJW) and its component HPF downregulated the mRNA expression of pro-inflammatory genes (i.e., iNOS, CXCL9, CXCL10 and COX2)	82
GBP5 and CXCL9 were the two most upregulated genes in human islets of Langerhans treated with cytokines	83
A study suggested an ICOS-dependent mechanism of Treg cell homing to the β-islets during pre-diabetes in the NOD model through the upregulation of the CXCR3 receptor	84
In T1D patients vs. controls, flow cytometry revealed a higher CXCR3 expression on the CD25(-/)(low)Foxp3(+) compared to CD25(+)Foxp3(+) Tregs independently from T1D, suggesting that CD25(-/)(low)Foxp3(+) Tregs are in a "standby" mode possibly waiting for orientational chemotactic stimuli	85
The genes encoding CXCL9, CXCL10 and CXCL11 chemokines were primary response genes in pancreatic β-cells and raised in rat, mouse, and human islets, as part of the inflammatory response	86
The Human H1N1 A/California/2009-derived viruses could infect human pancreatic islets, increasing CXCL9 and CXCL10 release	87
In β cells, T lymphocyte exosomes induced the expression of genes involved in chemokine signaling (i.e., CCL2, CCL7, and CXCL10), β cell apoptosis, and promoted the recruitment of immune cells during the autoimmune attack	88
In islet cells, pancreatic acinar or exocrine ductal cells of FT1DM patients, a strict association was observed between VP1 and CXCL10 expression	89

933 enterovirus capsid protein 1 (VP1); hyperforin (HPF); St. John's wort (SJW); Type 1 diabetes mellitus (T1D); vitamin
934 D receptor (VDR)
935