Abstract

Objective: In Graves' disease (GD), stimulating anti-TSH receptor antibodies are responsible for hyperthyroidism. T-helper 2 (Th2) cells were expected to be involved in the underlying immune mechanism, although this is still controversial. The aim of this study was to examine the expression of CXCR6, a chemokine receptor that marks functionally specialized T-cells within the Th1 and T-cytotoxic 1 (Tc1) cell pool, to gain new insights into the running immune processes.

Methods: CXCR6 expression was examined on peripheral blood lymphocytes (PBLs) and thyroid-derived lymphocytes (TLs) of GD patients in flow cytometry. CXCR6 cDNA was quantified in thyroid tissues affected by GD (n = 16), Hashimoto's thyroiditis (HT; n = 2) and thyroid autonomy (TA; n = 11) using real-time reverse transcriptase PCR.

Results: The percentages of peripheral CXCR6⁺ PBLs did not differ between GD and normal subjects. CXCR6 was expressed by small subsets of circulating T-cells and natural killer (NK) cells. CXCR6⁺ cells were enriched in thyroid-derived T-cells compared with peripheral CD4⁺ and CD8⁺ T-cells in GD. The increase was evident within the Th1 (CD4⁺ interferon-γ⁺ (IFN-γ⁺)) and Tc1 (CD8⁺IFN-γ⁺) subpopulation and CD8⁺ granzyme A⁺ T-cells (cytotoxic effector type). Thyroid-derived fibroblasts and thyrocytes were CXCR6⁻. There was no significant difference between the CXCR6 mRNA levels in GD compared with HT and normal TA tissues. The lowest CXCR6 mRNA levels were obtained from thyroid nodules from TA patients and GD patients with low thyroid peroxidase autoantibody levels.

Conclusions: CXCR6 was overexpressed in Th1 and Tc1 TLs compared with PBLs in GD. CXCR6 could be a marker for lymphocytes that have migrated into the thyroid and assist in the thyroid, independently of the bias of the underlying disease.

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TSHR-adeno model, both IL-4 and IFN-γ are implicated in the development of hyperthyroidism (7).

Besides determining the ratio of type 1 and type 2 specific cytokine responses – in most cases IFN-γ/IL-4 – or the immunoglobulin subclasses of TSHR antibodies (IgG2a/IgG1), analysis of chemokine receptors and their ligands allows insights into the running autoimmune process (2, 3). Among the over 50 chemokines and 20 chemokine receptors, only a few are thought to mark functionally specialized T-cells within the total Th1 and Tc1 T-cell pool (8, 9).

CXCR6 (Bonzo, STRL33, TYMSTR) (10–12) and its ligand CXCL16 (SR-PSOX) belong to the few unique chemokine/receptor pairs that suggest special immune functions, whereas most other pairs show promiscuity and overlap in cell and tissue expression. CXCR6 seems to mark functionally specialized T-cells within the total Th1 and Tc1 cell pool (13). In contrast, the chemokine receptor is displayed by very few (1–2%) Th2 or Tc2 cells. CXCL16 (13–15) is structurally different from most other chemokines. It is a type I transmembrane protein with four distinct domains: one chemokine domain tethered to the cell surface via a mucin-like stack, which in turn is attached to transmembrane and cytoplasmic domains. CXCL16 acts as an adhesion molecule when expressed on the cell surface (16). Upon cleavage from the cell surface, the soluble chemokine shows chemotactic activity (17).

Recently published studies indicate a unique role for CXCR6 and its ligand in attracting activated lymphocyte subsets during inflammation, while also facilitating immune responses via cell contact (16, 18, 19).

There are no data on the distribution of CXCR6 in thyroid autoimmunity and few on autoimmune diseases at all (20). The aim of our study was to investigate CXCR6 and CXCL16 mRNA expression in thyroid tissues affected by GD compared with thyroid tissue from patients with HT and non-autoimmune thyroid autonomy (TA) to gain new insights into the running immune processes within the thyroid and to characterize the lymphocytic subpopulations involved. In GD, we isolated thyroid-derived lymphocytes (TLs) and compared the expression of CXCR6 with that of peripheral blood lymphocytes (PBLs) from the same patients. The potential thyroid-cell sources of the receptor and its ligand were defined.

### Materials and methods

#### Patients

Thyroid diseases were diagnosed on the basis of clinical, biochemical and immunological features as well as from scintiscans. Antibodies against the TSHR (TSH-binding-inhibiting immunoglobulin; TBI) and thyroid peroxidase (TPO) were measured in serum obtained up to 2 weeks before operation with commercial RIA kits (TRAKhuman DYNOTEST®, anti-TPO α DYNOSTEST®; Brahms Diagnostica GmbH, Berlin, Germany). The numbers of focal lymphocytic infiltrates and ectopic germinal centers were counted on hematoxilin/eosin-stained sections of thyroid samples within an area of 226 mm² as described in (21).

For mRNA quantitation, we used thyroid tissues from GD (n = 16, all females; mean age ± S.E.M. 35.6 ± 3.7 years), HT (n = 2, both females; 18 and 34 years) and TA (n = 11, five females; 55.7 ± 5.2 years) patients (same collective as described in detail in (22)). Four out of these 16 GD patients used for mRNA analysis were further included for flow-cytometric analysis (Table 1). All GD patients were treated with anti-thyroid drugs but not corticosteroids at the time of surgery. The duration of GD until enrolment was longer than 15 months in most cases. Peripheral blood samples from 10 normal adults (two male, eight female; mean age 33.7 ± 2.7 years) without any history of autoimmune disease were used as controls. The study was approved by the local Committee of Medical Ethics.

#### Isolation of cells and cell lines used in this study

Thyroid samples were obtained during operation. TLs were isolated after mechanical tissue disaggregation

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<th>Anti-thyroid drug treatment</th>
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and Ficoll density-gradient centrifugation. Thyrocytes were enriched after enzymatic digestion of the tissue as described in (23). Outgrowing fibroblasts were obtained by culturing small pieces of thyroid samples. PBLs were isolated by Ficoll density-gradient centrifugation. Anaplastic thyroid carcinoma cell lines SW-1736, Hth-74, C-634 (kindly provided by N.-E. Heldin, University of Uppsala, Uppsala, Sweden) and 8505 C, follicular lines FTC-133 and FTC-238 and papillary line B-CPAP (DSMZ, Braunschweig, Germany), as well as Chinese hamster ovary (CHO) cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium/10% fetal calf serum.

**Examination of the CXCR6 antibody**

Since we always measured a lower number of T-cells expressing CXCR6 than described in (10), the specificity of the monoclonal antibody (mAb) used for flow cytometry was examined. First, PBLs were cultured with IL-15 (Immunotools, Friesoythe, Germany) over 6 days, and the number of CXCR6+ cells was determined by flow cytometry. Secondly, the CXCR6-encoding cDNA was cloned in the vector pcDNA3.1 (Invitrogen GmbH, Karlsruhe, Germany) using the primer pair 5'-CCG GAA GCT TAC CAT GGC AGA GCA TGA TTA CC-3' (sense) and 5'-GGA ATT CGA ATG ACA TGC TGG TGT CCT CCA C-3' (antisense). CHO cells were transiently transfected with the construct using Lipofectamine (Invitrogen). mAb binding was measured after 48 h by flow cytometry.

**Analysis by flow cytometry**

Directly fluorochrome-labeled mAbs were supplied by R & D Systems GmbH (Wiesbaden, Germany; CXCR6-PE, IFN-γ-FITC), DakoCytomation Denmark A/S (Glostrup, Denmark; CD3-RPE-Cy5, CD8-RPE-Cy5, CD62L-FITC, CD4-FITC), Immunotech (Marseilles, France; CD45R0-FITC) or BD Biosciences Europe (Heidelberg, Germany; CD4-Cy5, CD19-PerCP, granzyme A-FITC, and CD62L-FITC, CD4-FITC), Immunotech (Marseilles, France; CD3-RPE-Cy5, CD8-RPE-Cy5, granzyme A-FITC). This antibody mix is suitable for flow cytometry. IL-15 increased the sensitivity of the monoclonal antibody (mAb) used for flow cytometry.

**Real-time reverse transcriptase (RT)-PCR**

Total cellular RNA was isolated with the Qiagen total RNA isolation kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized from 1 μg RNA in a 20 μl standard reaction mixture containing 200 U Superscript II RNaseH reverse transcriptase (Invitrogen). Quantitative PCR was performed on a Rotorgene (Corbett Research, Mortlake, Australia) real-time machine by using SYBR® Green I as a double-strand DNA-specific binding dye and continuous fluorescence monitoring. Amplification was carried out in a total volume of 20 μl containing 0.5 μM of each primer, 1–3 mM MgCl2, 1 U Taq polymerase (Roche), 10 × Taq buffer (500 mM KCl/100 mM Tris/HCl, pH 8.3; Roche), 2 mM of each dNTP and 1 μl SYBR® Green (1:2000; Molecular Probes Europe, Leiden, The Netherlands) dissolved in DMSO and 1 μl cDNA diluted 1:5.

The PCR primers were CXCR6 sense (5'-CCT GCC TTA ACC CTG TGC TCT AT-3') and antisense (5'-ATG TCA TCC CCC TTG GTT TCA-3'), CXCL16 sense (5'-GGG GGC AGT CAC CGC AGT CCT-3') and antisense (5'-ATT AGC CGG GTG TGG TGG TGA GCA-3') or CD18 sense (5'-GGC GCA CAA GCT GGC TGA AAA CAA-3') and antisense (5'-AGC GCC CGG ATG ATC AAC GAC TG-3'), respectively. CD18 mRNA was measured in order to quantify leukocytic infiltration (25). Sizes of PCR products were: CXCR6 459 bp; CXCL16 518 bp, and CD18 372 bp. Glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) transcripts were measured with a ready-to-use quantitative PCR test kit (Roboscreen, Leipzig, Germany) (26). External cDNA standards were constructed by blunt-end cloning of PCR fragments into pGEM-T vector (Promega, Mannheim, Germany). Plasmid DNA was prepared by free access to plasmid DNA standards.

**Results**

**Flow cytometry**

We first examined whether the available CXCR6 mAb was suitable for flow cytometry. IL-15 increased the number of CXCR6+ PBLs by about 5-fold as shown in a typical case in Fig. 1A. Moreover, transiently transfected CHO cells expressing human CXCR6 were

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CXCR6 expression of TLs and PBLs from patients with GD and normal controls (n = 10) analyzed by flow cytometry using multicolor analysis and lymphocyte gating.

Table 2

<table>
<thead>
<tr>
<th>Marker expression</th>
<th>Relative number of cells expressing the marker (%) in each lymphocyte subpopulation</th>
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<tr>
<td></td>
<td>TLs (GD)</td>
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<tr>
<td>Characterization of lymphocytes</td>
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<tr>
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<tr>
<td></td>
<td>CD4⁺</td>
</tr>
<tr>
<td></td>
<td>CD8⁺</td>
</tr>
<tr>
<td>100% CD8⁺</td>
<td>Granzyme A⁺</td>
</tr>
<tr>
<td></td>
<td>IFN-γ⁺</td>
</tr>
<tr>
<td>100% CD4⁺</td>
<td>IFN-γ⁺</td>
</tr>
<tr>
<td>Characterization of CXCR6⁺ lymphocytes</td>
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<td>100% CD4⁺ IFN-γ⁺</td>
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*Significant differences between TLs and PBLs in GD patients; **significant differences between PBLs from GD patients and controls; percentage of labeled cells (means ± S.E.M.).
Granzymes are important effector molecules for cytotoxic lymphocytes and induce granule-mediated apoptosis in target cells. Some 40–50% of CD8\(^+\) cells expressed intracellular granzyme A in both PBLs and TLs from GD patients. Within these peripheral CD8\(^+\) granzyme A\(^+\) cells, only a few (<4%) expressed CXCR6, whereas nearly 20% carried the receptor in the thyroid (Fig. 2C). The majority of CD8\(^+\)CXCR6\(^+\) peripheral and thyroid-derived cells were positive for granzyme A.

CD3\(^+\)CD56\(^-\) T-cells are effector cells with strong cytotoxic activity against target cells. In this regard, CXCR6 was expressed on higher percentages of CD3\(^+\)CD56\(^-\) than on less cytotoxic CD3\(^-\)CD56\(^+\) cells.

Most peripheral CD3\(^+\)CXCR6\(^+\) cells have extralymphoid tissue-homing potential since 60% failed to express CD62L. Approximately 90% of thyroid-derived CXCR6\(^+\)CD3\(^+\) T-cells lack CD62L (Table 2, Fig. 2D). Peripheral and thyroid-derived B-cells did not express CXCR6 (not shown).

**CXCR6 and CXCL16 mRNA in thyroid tissues and thyroid-derived cells**

Real-time PCR revealed that thyroid tissues from all GD patients with high TPO levels and both HT patients...
were positive for CXCR6 mRNA, whereas no signal was present within some tissues from GD patients with low or no TPO levels and TA tissues (Fig. 3A). We found a slight correlation between CXCR6 and (1) CD18 mRNA levels ($r = 0.51; P = 0.044$) and (2) TPO antibody levels ($r = 0.66; P = 0.05$) in GD patients. There was no correlation between CXCR6 mRNA levels and TBII ($P = 0.60$) or the number of lymphocytic infiltrates ($P = 0.60$) in these patients. We found no difference in CXCR6 mRNA levels in GD compared with normal TA tissue. Lowest levels were present in TA nodules. Here, three out of nine samples were negative for CXCR6 mRNA.

Using simple RT-PCR, PBLs and TLs were CXCR6 mRNA-positive, whereas no signal was obtained from thyroid-derived fibroblasts (Fig. 3C). Thyrocytes showed only a very faint signal. Thyroid carcinoma cell lines were positive for CXCR6 mRNA. The expression level varied considerably.

Quantifying CXCL16 cDNA in thyroid tissues (Fig. 3B), the levels were somewhat high compared with other chemokines quantified (21, 25, 28). Surprisingly, CXCL16 cDNA levels within the two HT tissues were within the same range as those of many GD and normal TA tissues. This suggests that cells other than the known CXCL16 producers such as dendritic cells and macrophages may express CXCL16 mRNA. Indeed, besides TL, thyrocytes were strongly positive for this chemokine mRNA (Fig. 3C). Thus, the CXCL16 mRNA levels tend to represent the presence of this chemokine within several cell types of the thyroid than a specific expression by immunocompetent cells.

**Discussion**

We have demonstrated here that CXCR6, a chemokine receptor present on a small subset of specialized circulating Th1 and Tc1 cells, is expressed on a higher percentage of TLs than PBLs from GD patients. The data raise the question of the CXCR6’s function in the thyroid. The result agrees with our own previous study on CXCR3 and CCR5 (29), chemokine receptors that are thought to be predominantly, but not exclusively, found on Th1 cells. Both receptors were expressed on a higher percentage of TLs compared with PBLs, and may be involved in the recruitment of lymphocytes to the thyroid in GD (30, 31). We suggest that, in addition to CXCR3 and CCR5, CXCR6 may be a marker for cells that have migrated into thyroid tissue and assist there, independently of the bias of the underlying disease. This is confirmed by the fact that most of the CXCR6$^+$ T-cells in the thyroid lost their lymph-node-homing capabilities since they were CD62L$^-$. The antigen is downregulated in response to migration and activation by proteolytically cleaving from the cell surface (32). Moreover, our assumption is supported by recent studies demonstrating that CXCR6$^+$ T-cells were enriched in tissue sites of inflammation, such as rheumatoid joints and inflamed livers (13, 18).

Although a higher percentage of TLs compared with PBLs were CXCR6$^+$ in GD, we did not find a difference between CXCR6 mRNA levels in the thyroid tissues from different patients groups. Mistakes in CXCR6 mRNA determination were unlikely since quantitation of the chemokine receptor/ligand pair CXCR5/CXCL13 in the same cDNAs samples resulted in significantly higher levels in GD compared with TA samples and the highest levels in HT tissues (21). Our results suggest that the number of CXCR6$^+$ and thus Th1 and Tc1 cells in HT may not be higher than in other thyroids, including non-autoimmune TA. There are several possible explanations for this result. As in GD tissues, the chronic lymphocytic infiltrate does not reflect the initiation, but rather the maintenance or perturbation, of the immune process in HT tissues.

**Figure 3** Quantitation of (A) CXCR6 and (B) CXCL16 mRNA in thyroid tissues of GD patients with high (>1000 U/ml; TPOhigh; $n = 8$) and low or no antibodies against TPO (TPOlow; $n = 8$), HT patients (HT; $n = 2$) and in TA patients (normal and nodular tissue; $n = 11$). $*P < 0.005$. (C) Simple RT-PCR for CXCR6 and CXCL16 mRNA in thyroid-derived cell populations (thyrocytes, fibroblasts, lymphocytes = TLs), thyroid carcinoma cell lines and PBLs.
since a substantial number of thyrocytes are always destroyed at the time of surgery (33, 34). The time of action of specialized Th1 and Tc1 cells may be over during HT. This assumption is confirmed by reports showing a significant increase in IFN-γ-inducible chemokines CXCL10 (IP-10) and CXCL9 (Mig) strongly associated with Th1-mediated immune responses in thyroid tissue specimens obtained from subjects affected by recent-onset GD (2, 35, 36). An increase in serum CXCL10 levels has only been found in newly diagnosed GD patients, compared with healthy subjects and patients with long-standing disease. An inverse correlation between circulating CXCL10 and TPO antibodies. TPO antibodies better reflect the immune response in long-standing disease compared with TBII (38). Additionally, thyroid autoantibodies might not be produced exclusively by the thyroid itself (39–41).

The correlation between CXCR6 mRNA and CD18 mRNA is weak. CD18 mRNA also includes the mRNA of those lymphocytes that were not located within infiltrates or germinal centers, but scattered throughout the tissue and, additionally, the mRNA of non-lymphoid leukocytes, the macrophages. We do not know where CXCR6+ lymphocytes are located within Graves’ thyroids or whether or not they accumulate on certain structures. This may explain why we missed a correlation between CXCR6 and defined lymphocytic infiltrates and germinal centers.

Does the ligand of CXCR6, CXCL16, yield any additional information on the function of this chemokine/receptor pair in the thyroid? CXCL16 not only attracts CXCR6 expressing T-cells, natural killer (NK) cells and NK/T-cells to the site of inflammation and injury, but also mediates firm adhesion of CXCR6-expressing cells in its transmembrane form (16, 19). The chemokine is induced by pro-inflammatory stimuli such as tumour necrosis factor-α and IFN-γ (17). Surprisingly, we did not find any differences between CXCL16 mRNA levels in the thyroid tissues analyzed. Normally, CXCL16 is only expressed by macrophages, dendritic cells and CD19+ B-cells (14, 42). In our study, a strong CXCL16 mRNA signal was obtained from thyrocytes and thyroid carcinoma cell lines. Thus, CXCL16 also seems to be produced by epithelial cells, which confirms older studies showing that thyrocytes were potential cytokine producers (43). Untreated thyrocytes secrete CXCL8 (IL-8) and the other chemokine that binds to the CXCR2, CXCL1 (GRO-α) (28). CCL2 (monocyte chemoattractant protein-1; MCP-1) is a chemokine that attracts T-lymphocytes as well as monocytes, and can be also produced by thyrocytes (44). Thyrocytes from autoimmune thyroid disorders produce the chemokines CXCL9 and CXCL10 after stimulation (36). All these studies suggest a possible role for thyrocytes on accumulation of leukocytes in the tissue from the blood. Unfortunately, useful CXCL16 antibodies for immunohistology or establishment of protein screening assays were not available.

In summary, our study demonstrates that the percentages of Th1 (CD8+IFN-γ+), and Th1 (CD4+IFN-γ+) cells as well as cytotoxic effector cells (CD8+ granzymeA+) expressing CXCR6 were increased in TLI compared with PBLs in GD patients. However, we did not find any difference between CXCR6 mRNA levels in thyroid tissues from different patient groups. CXCR6 could be a marker for cells that have migrated into the thyroid and assist there, independently of the bias of the underlying disease.

Acknowledgements

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