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J Immunol 2003; 170:6320-6328; doi: 10.4049/jimmunol.170.12.6320
http://www.jimmunol.org/content/170/12/6320

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Chemokines Determine Local Lymphoneogenesis and a Reduction of Circulating CXCR4⁺ T and CCR7⁺ B and T Lymphocytes in Thyroid Autoimmune Diseases

Maria-Pilar Armengol,* Cristina B. Cardoso-Schmidt,* Marco Fernández,† Xavier Ferrer,* Ricardo Pujol-Borrell,‡* and Manel Juan*

Chemokines and their corresponding receptors are crucial for the recruitment of lymphocytes into the lymphoid organs and for its organization acting in a multistep process. Tissues affected by autoimmune disease often contain ectopic lymphoid follicles which, in the case of autoimmune thyroid disorders, are highly active and specific for thyroid Ags although its pathogenic role remains unclear. To understand the genesis of these lymphoid follicles, the expression of relevant cytokines and chemokines was assessed by real-time PCR, immunohistochemistry and by in vitro assays in autoimmune and nonautoimmune thyroid glands. Lymphotixin α, lymphotixin β, C-C chemokine ligand (CCL) 21, CXC chemokine ligand (CXCL) 12, CXCL13, and CCL22 were increased in thyroids from autoimmune patients, whereas CXCL12, CXCL13, and CCL22 levels were significantly higher in autoimmune glands with ectopic secondary lymphoid follicles than in those without follicles. Interestingly, thyroid epithelium produced CXCL12 in response to proinflammatory cytokines providing a possible clue for the understanding of how tissue stress may lead to ectopic follicle formation. The finding of a correlation between chemokines and thyroid autoantibodies further suggests that intrathyroidal germinal centers play a significant role in the autoimmune response. Unexpectedly, the percentage of circulating CXCR4⁺ T cells and CCR7⁺ B and T cells (but not of CXCR5) was significantly reduced in PBMCs of patients with autoimmune thyroid disease when they were compared with their intrathyroidal lymphocytes. This systemic effect of active intrathyroidal lymphoid tissue emerges as a possible new marker of thyroid autoimmune disease activity.


Antigens reach the lymph node transported by dendritic cells (DCs), which perform an initial selection of potentially dangerous Ags via pattern recognition receptors (1–3). In the secondary lymphoid organs, these Ags are presented to T cells with a repertoire that has already been purged from self-reacting cells before leaving the thymus. Naïve T cells recirculate only through the secondary lymphoid tissue, thus reducing the chances of these cells interacting with peripheral self-Ags (4). Lymph nodes are organized to favor the encounter between foreign Ags and lymphocytes bearing complementary receptors and also to facilitate crucial interactions between B and T cells on encounter with Ag. Within the lymph nodes, lymphoid follicles (LFs) are complex small structures in which many crucial processes take place such as somatic hypermutation, affinity maturation, isotype switch (5), and receptor revision. In the germinal centers (GCs), autoreactive B cells are presumably generated de novo, thus requiring specific mechanisms to maintain tolerance (6). LFs not only develop within the secondary lymphoid tissues, but also they sometimes form within the parenchyma of nonlymphoid organs when the immune response fails to eradicate an infectious agent (7), e.g., hepatitis C (8) or Helicobacter pylori gastritis (9), and in autoimmunity. Fully developed ectopic LFs are also found in several autoimmune diseases such as autoimmune thyroiditis (10), rheumatoid arthritis (11, 12), myasthenia gravis (13, 14), Sjögren’s disease (15), and cryptogenic fibrosing alveolitis (16). It has been suggested that ectopic lymphoid tissue constitutes a different compartment of the lymphoid system that should be called the tertiary lymphoid tissue (TLT) (17), but its specific functions have not been fully elucidated yet. Hashimoto’s disease (HT) and Graves-Based thyrotoxicosis (GD) are two clinical forms of autoimmune thyroid disease (AITD) (18) in which lymphoid infiltration commonly evolves into secondary LFs (19). Despite longtime suspicion (20), only recently it has been shown that these structures are fully functional, yet their role in pathogenesis is unclear (19). To sustain their activity, intrathyroidal LFs must be included in the migration circuits of B and T lymphocytes. However, migration by itself is not sufficient, because massive infiltration can occur in the thyroid without LF neogenesis. Different types of organizing factors must be present in the tissue. It is currently accepted that lymphotixin (LT) α, LTβ, C-C chemokine ligand (CCL) 21 (secondary lymphoid tissue chemokine), CXC chemokine ligand (CXCL) 12 (stromal cell-derived factor-1), CXCL13 (B cell-attracting chemokine-1), and

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²Abbreviations used in this paper: DC, dendritic cell; LF, lymphoid follicle; CCL, C-C chemokine ligand; CXCL, CXC chemokine ligand; LT, lymphotixin; GC, germinal center; HT, Hashimoto’s disease; GD, Graves-Based thyrotoxicosis; TLT, tertiary lymphoid tissue; AITD, autoimmune thyroid disease; MNG, multinodular goiter; TPO, thyroid peroxidase; PT, palatine tonsils; ITLs, intrathyroidal lymphocytes; rb, recombinant human.
CCL22 (macrophage-derived chemokine) are probably the most important mediators for GC formation (21). CCL21 selectively attracts naive lymphocytes and DCs via CCR7 (CD197) (22), and DCs in turn produce CCL22 that induces segregation of lymphocytes into T and B zones (23). CXCL12 has homeostatic chemotactic activity via CXCR4 (CD184) on most T and B cell subpopulations and contributes decisively to the development of GCs and maintenance of LFs (24). CXCL13 organizes the distribution of B and T cells within the GCs via CXCR5 (25).

In previous studies, CCL3 (macrophage-inhibitory protein-1a), CCL4 (macrophage-inhibitory protein-1b), CCL5 (26), CCL21, CXCL12, and CXCL13 (19) have been detected inAITD glands and elevated levels of macrophage chemotactrant protein-1 in the patient’s sera (27), whereas CXCR3 and CCR5 (28) were associated with GD. In contrast, in experimental autoimmune thyroiditis (29), several chemokines have been involved in the disease. There are no systematic studies on the expression of the chemokines responsible for the GC organization and their corresponding receptors inAITD.

We report here that autoimmune thyroid glands hyperexpress LTR, IFN-γ, CXCL12, CXCL13, and CCL22. Those containing secondary lymphoid follicles overexpress IFN-γ, CXCL12, CXCL13, and CCL22 and express LTB and CCL21, and the corresponding receptors CXCR4, CXCR5, LTBR, and CCR7. We found that thyroid epithelial cells produce CCL12 in vivo and in vitro; this may explain the relatively high frequency of LFs in thyroid glands. In patients with intrathyroidal LFs, which are rich in CXCR4+ T lymphocytes, we detected a concomitant reduction in circulating CXCR4+ T cells and also in CCR7+ T and B lymphocytes. This constitutes an unexpected effect on the periphery of intrathyroidal lymphoid neogenesis and suggests the existence of a general disturbance in lymphocyte recirculation circuit as part ofAITD.

Materials and Methods

Tissues and cell culture

Thyroid tissue was obtained from 34 patients (27 of them had been included in a previous study (19)): 4 HT, 12 GD with LFs, 11 GD without LFs, 1 multinodular goiter (MNG) with thyroiditis, 4 MNGs and 2 multiorgan donor glands. MNG and donor glands were used as nonautoimmune thyroid controls. Clinical diagnosis was made based on usual thyroid tests and confirmed by histopathology. Thyroid peroxidase (TPO) and thyroglobulin (Tg) Abs were measured by ELISA (Immunowell, San Diego, CA) and anti-thyroid-stimulating hormone receptor Abs were measured by RIA (Fundación Jiménez Díaz, Madrid, Spain). Platelets containing highly active lymphoid follicles were not in thyroid controls. Clinical diagnosis was made based on usual thyroid tests and confirmed by histopathology. Thyroid peroxidase (TPO) and thyroglobulin (Tg) Abs were measured by ELISA (Immunowell, San Diego, CA) and anti-thyroid-stimulating hormone receptor Abs were measured by RIA (Fundación Jiménez Díaz, Madrid, Spain). Platelets containing highly active lymphoid follicles were not in thyroid controls. Clinical diagnosis was made based on usual thyroid tests and confirmed by histopathology. Thyroid peroxidase (TPO) and thyroglobulin (Tg) Abs were measured by ELISA (Immunowell, San Diego, CA) and anti-thyroid-stimulating hormone receptor Abs were measured by RIA (Fundación Jiménez Díaz, Madrid, Spain).

PBMCs from 12 patients (obtained at the same time as the thyroid resection procedure) and 12 healthy donors were separated using a Ficoll (Sigma-Aldrich, St. Louis, MO) discontinuous density gradient.

Immunofluorescent histochemistry and FACS

Sources of Abs were: goat Abs to CXCL12, CCL21 and CXCL13, and murine mAbs to CXCR4 and CXCR5 from R&D Systems (Minneapolis, MN); mAb to β-catenin 8–18 from Novocastra Laboratories (Newcastle upon Tyne, U.K.); mAb to CD3 from T. F. Tedder (Duke University Medical Center, Durham, NC); mAbs to human CD19, CD20, CD3, and CD5 from R. Vilella (Hospital Clinic Barcelona, Barcelona, Spain), murine mAb to CD21L from P. Garrone (Laboratory for Immunological Research, Schering-Plough, Paris, France); mAb to CD4 and CD8 from M. Bo (Hospital Clinic Barcelona, Barcelona, Spain), murine mAb to cytokeratin 8 from Novocastra Laboratories (Newcastle upon Tyne, U.K.); mAb to CD83 from T. F. Tedder (Duke University Medical Center, Durham, NC); mAb to CD21L from P. Garrone (Laboratory for Immunological Research, Schering-Plough, Paris, France); mAb to CD4 and CD8 from M. Bo (Fundación IRSI-Caixa, London, U.K.); mAbs to CD3-PerCP, CCf, CD45RA-PE, and CD45RO-PE from BD PharMingen (San Diego, CA), and mAb to CD19-PECy5 from DAKO; and fluorescein-labeled secondary Abs from Southern Biotechnologie (Birmingham, AL). For thyroperoxidase detection, a high titer anti-TPO-positive human serum was used.

Cytoskeletons were fixed in 4% parafomaldehyde (CXCL12 and CXCL13), or cold acetone (CCL21). Independent observers blindly examined slides. Images were acquired with a high resolution video camera and analyzed using Openlab software (Improvison, Coventry, U.K.).

Three-color flow cytometry was performed using a FACScan (BD Biosciences, San Jose, CA) to assess chemokine receptors expression in ITLs and PBMCs. Data were acquired and analyzed with the CellQuest software (BD Biosciences). Intrathyroidal T and B cells were sorted in a FACS-Vantage cell sorter (BD Biosciences); their purity ranged from 94 to 99%.

CXCL12 ELISA

CXCL12 levels were measured in supernatants from HT93 cells and from thyroid cultures by an in-house sandwich ELISA (T. Gallart, Hospital Clinic Barcelona, Barcelona, Spain). Plates containing 96 wells were coated with Abs to human CXCL12a (R&D Systems). Soluble CXCL12a was detected by a biotinylated Ab to human CXCL12a, followed by streptavidin-peroxidase incubation. The ELISA detection range was 0.39–200 ng/ml (human CXCL12a; Peprotech, Rocky Hill, NJ).

Table 1. Thyroid samples used

<table>
<thead>
<tr>
<th>Sample</th>
<th>Anti-Tg (IU/ml)</th>
<th>Anti-TPO (IU/ml)</th>
<th>Anti-TSHR (IU/L)</th>
<th>% LI</th>
<th>ILFs</th>
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<tr>
<td>GD1</td>
<td>774.0</td>
<td>105.00</td>
<td>96.6</td>
<td>76</td>
<td>6</td>
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<tr>
<td>HT2</td>
<td>ND</td>
<td>ND</td>
<td>60.2</td>
<td>62</td>
<td>0</td>
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<td>ND</td>
<td>80.2</td>
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<td>0</td>
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<td>HT4</td>
<td>85.1</td>
<td>ND</td>
<td>61.6</td>
<td>2.0</td>
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<tr>
<td>MNG1</td>
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<td>1.2</td>
<td>3.5</td>
<td>5.2</td>
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<tr>
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<td>ND</td>
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<tr>
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<td>ND</td>
<td>3.5</td>
<td>5.2</td>
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</tr>
<tr>
<td>HD2</td>
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<td>1.4</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Tg, Thyroglobulin; TSHR, thyroid-stimulating hormone receptor; LI, lymphocytic infiltration; ILFs, intrathyroidal lymphoid follicles; −, negative.
### Table II. Primers, probes, and conditions used to amplify/hybridize cytokines/chemokines using semiquantitative and real time PCR

<table>
<thead>
<tr>
<th>Primers and Oligoprobes</th>
<th>Semiquantitative PCR</th>
<th>Real Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>Sense 5′-TCTTCTTTTGGTGTCGCCAG-3′</td>
<td>95°C 7 min + 28 × (95°C 20 s/68°C 15 s/72°C 15 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-AGCCCCAGCTTCTCTCA-3′</td>
<td></td>
</tr>
<tr>
<td><strong>LTα</strong></td>
<td>Sense 5′-ACGGCGATGCGGCTGATG-3′</td>
<td>95°C 7 min + 30 × (94°C 30 s/70°C 15 s/72°C 40 s) + 72°C 7 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-ACTGACCCACGCTCCAC-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-GGCGATGGCGCTGATGT-3′</td>
<td></td>
</tr>
<tr>
<td><strong>LTβ</strong></td>
<td>Sense 5′-ATTACCCACCTCTACCTCTCCCT-3′</td>
<td>95°C 7 min + 30 × (94°C 30 s/68°C 15 s/72°C 25 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-GCTGTTCTATCCCCTCCATTTCTCT-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-CCACCCAGCCCCCA-3′</td>
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<tr>
<td><strong>LTβR</strong></td>
<td>Sense 5′-GGTGCTCTCGTGCGTC-3′</td>
<td>95°C 7 min + 28 × (95°C 20 s/68°C 15 s/72°C 15 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-GGGGACGACGAGTGGTGT-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-TTTTTCCTGGTGCTCGCA-3′</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Sense 5′-GTITTTGGTTCTTCTTTGG-3′</td>
<td>95°C 7 min + 30 × (94°C 30 s/60°C 15 s/72°C 20 s) + 72°C 7 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-ATTACTGGGAGCTCTCTT-3′</td>
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</tr>
<tr>
<td></td>
<td>Probe 5′-GGGAGGAAAAAGGAGTCA-3′</td>
<td></td>
</tr>
<tr>
<td><strong>CXCL12</strong></td>
<td>Sense 5′-GTGGTGTTGGTCTCTGGTC-3′</td>
<td>95°C 7 min + 30 × (95°C 20 s/68°C 15 s/72°C 15 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-CGGGCTCACATCTCGAGG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-GCCTTATAAGGCCGG-3′</td>
<td></td>
</tr>
<tr>
<td><strong>CCL21</strong></td>
<td>Sense 5′-AAGGACGTAGTGGAGGGG-3′</td>
<td>95°C 7 min + 35 × (94°C 15 s/63°C 10 s/72°C 10 s) + 68–95°C</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-CTGGCGTGTGTTCTGGG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-CTGTGGTTCTGCTTCGG-3′</td>
<td></td>
</tr>
<tr>
<td><strong>CXCL13</strong></td>
<td>Sense 5′-CGACATCTGCTCTCTCCCT-3′</td>
<td>95°C 7 min + 35 × (94°C 20 s/68°C 15 s/72°C 15 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-ACCCATACATCTCTGAGG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe 5′-ACGACATTCTCCAGCGG-3′</td>
<td></td>
</tr>
<tr>
<td><strong>CCL22</strong></td>
<td>Sense 5′-GAGACATAAGGACAGACATGGAT-3′</td>
<td>95°C 7 min + 30 × (95°C 20 s/68°C 15 s/72°C 15 s) + 72°C 10 min</td>
</tr>
<tr>
<td></td>
<td>Antisense 5′-ATGGAGATCACCCCATGGAACGAG-3′</td>
<td></td>
</tr>
</tbody>
</table>
RT-PCR and quantitative real time PCR

RNA was obtained using a modified Chomczynski method (32); genomic DNA was removed and then was retrotranscribed with oligo(dT) 15 (Pharmacia Biotech, Peapack, NJ) and SuperScript-II (Pharmacia Biotech). cDNA duplicates were amplified by semiquantitative PCR using Dynazyme II (Finnzymes OY, Helsinki, Finland) and specific primers for LTA, LTβR, and IFN-γ (conditions listed in Table II). Amplimers were hybridized with [γ-32P]ATP oligoprobes and quantified in a Bio-Rad Molecular Imager-FX (Bio-Rad, Hercules, CA) by using Quantity-One. Results were normalized by GAPDH expression.

For real time PCR, standards and controls for CCL21, CXCL12, CXCL13, and CCL22 were obtained by PCR from thymus, PT, and PHA-stimulated PBMC cDNAs. Amplification products from conventional PCR were quantified in serial dilutions from 10^1 to 10^7 molecules. Real time PCR were performed in a LightCycler (Roche, Mannheim, Germany) using the master mix containing 4 mM MgCl₂, 0.5–0.3 μM primers, and 1 μl LightCycler Fast Start DNA Master SYBR Green I (Roche) (the experimental run protocols are described in Table II). The amount of cDNA was calculated by the second derivative method after confirming the specificity of the amplification with the melting curve profiles. The samples and standards were run four times for chemokines/cytokines and six times for GAPDH in nondependent runs. The relative abundance of each chemokine was calculated by normalizing the mean of mRNA sample copies (mean Chem-Sample) with the corresponding GAPDH one (mean GAPDH-Sample), referred to average levels of PTs, and expressed in arbitrary units (Fig. 1) by the formula

\[
\text{Index}_{\text{Sample}} = \left( \frac{\text{mean}_{\text{CHEM-Sample}}}{\text{mean}_{\text{GAPDH-PTs}}} \right) \left( \frac{\text{mean}_{\text{GAPDH-Sample}}}{\text{mean}_{\text{CHEM-PTs}}} \right).
\]

Chemotaxis

The transmigration assay was conducted on Transwell plates (5-μm pore size; Corning, Corning, NY), by triplicate or duplicate, depending on cell availability. 3 × 10⁶–10⁸ sorted CD3⁺ and CD19⁺ cells were plated in the upper chamber; and the lower chamber was filled with medium containing 10–1000 ng/ml human CXCL12 (R&D Systems). Supernatants from thyrocytes cultured with IL-1β, IFN-γ, or TNF-α (at 200, 500, and 500 IU/ml, respectively) taken at peak CXCL12 protein (quantified by the previous ELISA) or control medium were also used to measure spontaneous migration. In some experiments, cells were preincubated with blocking anti-CXCR4 (12G5, 20 μg/ml). Migrated cells were counted using Flow-Count Fluospheres (Coulter, Miami, FL).

Statistical analysis

Pearson’s correlation coefficient, together with parametric (Student t test) and nonparametric (Mann-Whitney) tests, were applied to normal and non-normal distributed data, respectively, using the SPSS software package (Microsoft, Seattle, WA). A level of significance of 5% was used in all the statistical evaluations.

Results

LTA, IFN-γ, CXCL12, CXCL13, and CCL22, but not LTβ or LTβR, are overexpressed in autoimmune thyroid glands

The levels of chemokine transcription were analyzed in relation to the diagnosis and presence of intrathyroidal GCs. LTA, IFN-γ,
CXCL12, and CCL22 mRNA levels were higher in AITD glands than in nonautoimmune glands \((p = 0.022, p = 0.024, p = 0.030, p = 0.010, \text{respectively})\). Elevated CXCL13 was a constant and exclusive feature of AITD glands \((p < 0.01)\) whereas CCL21 showed a nonstatistically significant association with disease \((p = 0.09)\). Presence of GC was associated to higher levels of CXCL12, CXCL13, CCL22, and IFN-\(\gamma\) \((n = 20; p = 0.022, p < 0.001, p = 0.007, \text{and} p = 0.024, \text{respectively})\). Lymphocytic infiltration was also associated with CXCL13 and CCL22 levels \((CXCL13, r = 0.972, p < 0.0009; \text{CCL22,} \ r = 0.983, p < 0.0009)\). Finally, expression of LT\(\beta\) and/or of LT\(\beta\)R did not bear a clear relation to diagnosis \((p = 0.6 \text{and} p = 0.54)\) (Fig. 1).

The immunohistochemistry tests showed CCL21\(^{+}\) cells around the GCs in up to 30% of capillaries with features of high endothelial venules \((\text{CLA}^{+}; \text{Fig. 2})\). CXCL12 was found in thyroid epithelium \((\text{TPO}^{+} \text{and cytokeratin}^{+}; \text{Fig. 2})\) and also in clusters of nonidentified cells amid the lymphocytic infiltration. By contrast, CXCL13\(^{+}\) cells were distributed in the mantle zone of LFs. Although we were unable to clearly identify CD83\(^{+}\) mature DCs or follicular DCs as CXCL13\(^{+}\) producers \((\text{due to nonappropriate reagents})\), the pattern of staining points to the former as the CXCL13 source (Fig. 2).

**Thyrocytes are the source of CXCL12**

Because production of CXCL12 by thyrocytes has pathogenic implications, we tested the effects of stimuli \((\text{IL1}\beta, \text{IFN}-\gamma, \text{or TNF}-\alpha)\) presumably acting in vivo on primary thyroid cell cultures. These cytokines clearly induced a raise of CXCL12\(\alpha\) secretion \((\text{peak at 6 h})\) over baseline levels more marked for IL-1\(\beta\), which produced a 2-fold increase: \(7.56 \pm 0.75 \text{vs} 3.72 \pm 1.4 \text{ng/ml (Fig. 3A)}\). To rule out the possibility that CXCL12\(\alpha\) was derived from contaminating macrophages or fibroblasts, it was also measured in the supernatants of HT93 cells, and similar results were obtained (Fig. 3A). Semiquantitative RT-PCR and hybridization detected CXCL12 mRNA in both tissues, confirming ELISA results (Fig. 3A). Interestingly, these supernatants from cytokine-stimulated thyrocytes showed a chemotactic activity that was proportional to CXCL12 levels, and chemotaxis was blocked with anti-CXCR4 Abs (Fig. 3B).

**FIGURE 2.** Chemokine expression in autoimmune thyroids. Upper left panel, Double immunofluorescent staining of endothelial cells with anti-CCL21 (green) and anti-CLA-1 (red) around the lymphoid follicle (dashed yellow line). Upper right panel, Amplified insets of the previous panel; middle left panel, results of a double exposure for CXCL12 (green) in formalin-fixed sections from GD patients colocalizing with epithelial cells, detected as TPO\(^{+}\) (red); middle right panel, superimposed images of follicular DCs stained using anti-CD21L (red) and with anti-CXCL13 (green); lower left and right panels, nonconsecutive sections stained with anti-CXCL13 (green) and anti-CD83\(^{+}\) (red) show a similar reticular staining pattern and distribution. Image sizes, referred to 100 \(\mu\)m, are represented as yellow lines inside the panels.

**FIGURE 3.** Regulation of CXCL12 expression in thyrocytes and its chemotactic activity. A, CXCL12 secretion and mRNA expression in thyroid follicular cells and in the HT93 thyroid-derived cell line in baseline conditions \((B)\) and after cytokine stimulation. Results are expressed as an index referred to the baseline conditions \((\text{index} = 1)\) from four different thyroid glands. B, Chemotaxis of PBMCs from healthy donors using supernatants from the aforementioned stimulated thyrocytes \((\text{x-axis stimuli: IL1}\beta + \text{aCXCR4}, \text{supernatant from thyrocytes stimulated with IL1}\beta\) and blocked with anti-CXCR4 Ab; IFN-\(\gamma\), supernatant from thyrocytes stimulated with IFN-\(\gamma\); TNF-\(\alpha\), supernatant from thyrocytes stimulated with TNF-\(\alpha\)). The migration results of a representative experiment are expressed as an index \((\text{x-axis})\) and calculated as the ratio between the numbers of migrating cells in the sample and the spontaneous migration.
Because CCL21 and CXCL13 were not detected by PCR in thyrocytes (either in baseline conditions or after stimulation), no further secretion studies were undertaken.

**Thyroid autoantibody levels correlate with CXCL13 and CCL22**

In a previous study, we reported the association between thyroid autoantibody titers and the presence of intrathyroidal GCs. Here, we describe a correlation between thyroid autoantibody titers and chemokine levels: anti-thyroglobulin Abs correlated with CXCL13 (r = 0.951, p < 0.0001) and CCL22 (r = 0.906, p < 0.0001) levels; anti-TPO Abs correlated with CXCL13 (r = 0.940, p < 0.0001) and CCL22 (r = 0.910, p < 0.0001) levels. When GD were analyzed vs nonautoimmune glands, anti-thyroid-stimulating hormone receptor Abs correlated with IFN-γ (n = 14, r = 0.726, p = 0.025) and CCL21, CXCL13 and CCL22 levels (n = 18; r = 0.829, p < 0.0001; r = 0.831, p < 0.0001; and r = 0.877, p < 0.0001, respectively).

**Infiltrating leukocytes express the corresponding chemokine receptors**

In ITLs, chemokine receptors were expressed in a higher proportion of B cells than T cells following the hierarchy CXCR4—CXCR5—CCR7 (Table III and Fig. 4A); a higher density was reached in B cells than in T cells (MFI of CXCR4 109.9 vs 38.2; CXCR5 140 vs 49.7; and CCR7 27.8 vs 20.7). Preliminary experiments excluded a significant effect of the trypsin-collagenase solution on their membrane expression on ITLs but confirmed an overall 30% reduction in CD45 expression; therefore, the relative (but not absolute) distribution of chemokine receptor among the CD45RA and CD45RO subsets could be analyzed. Taking the predominance of CD45RO over CD45RA cells into account (ratio, 4:3), the distribution of CXCR4 (ratio, 3:1) indicates its preferential expression in the CD45RO+ subset. The same applies to CXCR5 and CCR7 (Table III). The immunohistochemistry studies confirmed the flow cytometry results: CXCR4+CD3+ or CXCR4+CD19+ cells were mainly located outside the LFs (Fig. 4A). CXCR5+CD3+ or CXCR5+CD19+ lymphocytes were mainly distributed in the mantle zone and within the GC, whereas only some CD4+ T cells were occasionally CXCR5+ in areas of diffuse infiltration (Fig. 4A). In contrast, CCR7 was little expressed by few T cells and B cells, possibly because those cells lose their receptor on arrival to the GC. In addition, mature DCs (CD83+) outside the lymphoid follicles were brightly stained (Fig. 4A).

**Table III. Chemokine receptor expression in T cell subsets from ITL, PBMCs from patients (AITDs) and from HD defined by RO and RA expression**

<table>
<thead>
<tr>
<th>Gate CD3+</th>
<th>ITLs</th>
<th>AITDs, PBMCs</th>
<th>HDs, PBMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4+</td>
<td>59.6 ± 15.0</td>
<td>21.9 ± 15.1</td>
<td>58.3 ± 9.6</td>
</tr>
<tr>
<td>CXCR4+ RO+</td>
<td>32.6 ± 10.7</td>
<td>5.1 ± 8.6</td>
<td>28.4 ± 10.6</td>
</tr>
<tr>
<td>CXCR4+ RA+</td>
<td>10.4 ± 9.1</td>
<td>19.0 ± 18.4</td>
<td>30.6 ± 9.5</td>
</tr>
<tr>
<td>CXCR5+</td>
<td>10.5 ± 8.7</td>
<td>10.2 ± 3.4</td>
<td>8.5 ± 4.2</td>
</tr>
<tr>
<td>CXCR5+ RO+</td>
<td>7.1 ± 3.4</td>
<td>6.2 ± 4.1</td>
<td>6.1 ± 3.3</td>
</tr>
<tr>
<td>CXCR5+ RA+</td>
<td>2.1 ± 1.9</td>
<td>6.9 ± 3.5</td>
<td>3.6 ± 1.8</td>
</tr>
<tr>
<td>CCR7+</td>
<td>2.5 ± 1.4</td>
<td>39.3 ± 13.6</td>
<td>67.3 ± 7.9</td>
</tr>
<tr>
<td>CCR7+ RO+</td>
<td>2.3 ± 1.6</td>
<td>8.9 ± 5.0</td>
<td>31.7 ± 13.0</td>
</tr>
<tr>
<td>CCR7+ RA+</td>
<td>0.8 ± 0.7</td>
<td>32.6 ± 14.5</td>
<td>41.2 ± 12.6</td>
</tr>
</tbody>
</table>

*Values are given as mean ± SD of eight patients. Due to the effect of trypsin, which strips out a proportion of CD45RO and CD45RA molecules (see Materials and Methods), the percentage of CXCR4+ in ITLs is higher than the sum of the RO and RA subsets. HDs, healthy donors; RO, CD45RO; RA, CD45RA.*

**FIGURE 4.** Expression of chemokine receptors in CD3+, CD19+, and CD83+ thyroid-infiltrating cells. A. Dot plots of CXCR4, CXCR5, and CCR7 chemokine receptors and representative sections of their corresponding distribution by immunofluorescence in the intrathyroidal lymphoid follicles. Chemokine receptors are shown in red and CD markers are shown in green. A higher magnification is given in the inset of each panel; arrows indicate double-positive cells. B. CXCR4-mediated chemotaxis of sorted intrathyroidal T and B cells in a dose-dependent response to the CXCL12 chemokine (1–1000 ng/ml) and anti-CXCR4 blocking (100 ng/ml). The migration results are expressed as an index and are calculated as the ratio between the numbers of migrating cells in the sample referred to the spontaneous migration (mean ± SD from four independent experiments).
The CXCL12 receptor on ITLs is functional

Given that several factors (extracellular matrix, cell activation, soluble cytokines, and others) can determine molecular functions, we assessed the functionality of CXCR4 molecules on the surface of intrathyroidal B and T lymphocytes by chemotaxis assays with sorted CD3+ and CD19+ ITLs. Both sorted intrathyroidal T and B lymphocytes dose-dependently migrated in a CXCL12 gradient, thereby confirming the functionality of CXCR4; maximal migration index was obtained at 100 ng/ml (33) for T cells and at 1000 ng/ml for B cells (Fig. 4B).

Marked peripheral reduction in CCR7+ and CD3+ CXCR4+ lymphocytes ofAITD-GC+ patients

The comparison between ITLs and PBMCs showed that CXCR4+ lymphocytes were more abundant in the former (63.6 ± 12.9% vs 31.6 ± 12.9%; Fig. 5). This was due to their low proportion among circulating T cells (59.6 ± 15.0% vs 21.9 ± 15.1%; p < 0.0001); no such differences were found for B cells. This reduction of circulating CXCR4+ lymphocytes dose dependently migrated in a CXCL12 gradient, thereby confirming the functionality of CXCR4; maximal migration index was obtained at 100 ng/ml (33) for T cells and at 1000 ng/ml for B cells (Fig. 4B).

FIGURE 5. Chemokine receptor expression in ITLs and PBMCs from AITD patients and healthy donors (CTR). Data indicate the percentage of positive chemokine receptor expression. ITLs and PBMCs from the same AITD patient (□) are linked by a line; △, PBMCs from healthy donors; horizontal bars, mean of 8–10 independent experiments; *, p < 0.05; **, p < 0.01; ns, nonsignificant. As an additional reference, the mean ± SD of CXCR4-positive cells in lymph nodes from healthy donors is given with the symbol I.

FIGURE 6. Relative representation of the distribution of CD45RO+ and CD45RA+ CD3+ cells in relation to CXCR4 and CCR7 expression in ITLs and PBMCs from autoimmune thyroid disease patients and from healthy donors. The small pie charts (left) show the relative proportion of chemokine receptor-positive (gray) and -negative (white) T cells. The large pie charts (right) show the percentage of CD45RO+ and CD45RA+ cells. Percentages are reported to the total number of CD3+ cells.

Discussion

In rheumatoid arthritis, lymphoid neogenesis has been under intense scrutiny for clues that might explain the perpetuation of these diseases (11). We became interested in intrathyroidal lymphoid neogenesis as a part of TLT development, because it may help to explain how AITDs arise and are maintained despite a T cell repertoire tolerant to thyroid Ags (34). In a previous publication, we provided evidence suggesting that intrathyroidal LFs were functional and highly activated, and also that their B cells were specific for thyroid autoantigens (19). Our results clearly demonstrate that the main chemokines/cytokines involved in the generation of LFs are present at normal or above normal levels in intrathyroidal LFs, thus confirming the similitude to canonical LFs. Working with human tissues imposes several limitations, but we have tried to overcome them by studying a large number of samples from a
broad selection of patients and by testing in vitro the inferences derived from the observation of the pathological samples. Overall, our results suggest that the development of ectopic lymphoid follicles in AITDs does not depend on a sole molecule but on the interplay of multiple factors. Despite the parallelism between canonical and intrathyroidal LFs, three differences merit discussion.

1. The low number of CCR7⁺-infiltrating lymphocytes which cannot be explained by the lack of high endothelial venules expressing CCL21, the initial event in LFs formation and prime chemotactic of naive T cells (35), because we detected CCL21 in intrathyroidal high endothelial venules to an extent that is similar to those in other autoimmune (36, 37) or infectious diseases (38). The most plausible explanation is that once in the tissue, CCR7 is down-regulated; this would indicate indirectly that the lymphocyte turnover in intrathyroidal LFs is relatively low at this stage. By contrast, CXCR4 and CXCR5 receptors remain expressed by both T and B cells, probably because of their role in maintaining the distribution of the different cell types in distinct areas of the intrathyroidal LFs.

2. The lack of correlation of LTβ and LTβR expression, factors known to play a fundamental role in the initiation of LFs, with the presence of ectopic LFs. This difference, as the former, may be due to the late stage of maturation of the intrathyroidal LFs in our material. In fact, by the time patients undergo surgery, the disease has been active for years, and therefore early events are not necessarily present.

3. Thyrocytes are the main source of CXCL12 in AITD. This is important but not totally unexpected because CXCL12 is an ancestral chemokine produced by a variety of cell types (24). Thyrocyte production of biologically active CXCL12 and infiltrating lymphocyte responsiveness to it are conclusively demonstrated by a combination of RT-PCR, immunofluorescent histochemistry, and chemotaxis techniques. Thyrocytes can also produce CCL2 (macrophage chemotactic protein-1) (39), CCL19 (monokine induced by IFN-γ), and CCL10 (IFN-γ-inducible protein-10) (40, 41), and this may explain why LFs arise so often in the thyroid gland even in MNG, a clinical entity of uncertain etiology. It is conceivable that the initial event leading to the formation of intrathyroidal LFs is local nonspecific stress, e.g., iodine overload as in nonobese diabetic mouse thyroiditis (42). Iodine may induce thyrocyte necrosis, which would stimulate resident macrophages to produce IL-1 and TNF, which may in turn induce CXCL12 synthesis by adjacent thyrocytes.

The finding of a correlation between the levels of CCL21, CCL22, and CXCL13 and thyroid autoantibodies is well in agreement with our previous report of a correlation between the presence of intrathyroidal LFs and thyroid Ab titers (19). The capability of the thyroid tissue to express such a diversity of chemokines may contribute decisively to the local differentiation of autoreactive B cells and to the pathogenesis of AITDs. This concept is also in line with the geographical view of immune response proposed by Zinkernagel et al. (4); i.e., the normal regional recirculation and distribution of lymphocytes would be crucial to prevent autoimmunity, and therefore its disruption, as here reported, may be a decisive event in the development of autoimmunity.

The question of the formation of intrathyroidal LFs is part of a more general question of the reasons for the development of TLTs in many conditions. Secondary lymphoid organs are designed to maximize the chances of a scarce Ag meeting a rare lymphocyte bearing the appropriate receptor. When the immune system fails to create an efficient immune response against a highly localized Ag (e.g., a microorganism), it may be advantageous to move a critical anatomical element of the immune response, the lymphoid follicle, to the site of the infection. The stimulus that triggers lymphoid neogenesis may be the continuous production of cytokines and other inflammatory mediators by the stromal cells (43). TLTs lack the intricate circulation channels that distribute scarce incoming Ag through the lymph node. They are probably not needed in TLT because their LFs are responding to local Ags that are both abundant and readily accessible (and this may be the main difference between TLTs and secondary lymphoid tissue). Lymphoid neogenesis in autoimmunity may be an undesirable consequence of this mechanism and may arise as results of chronic local stress (either mechanical stress in the joints or chemical stress in the thyroid). The formation of extranodal LFs does not inevitably lead to autoimmunity, because there is tolerance to local self-Ags, but breaking tolerance may be easier within these extranodal LFs because of the huge excess of Ag and the abundance of costimulatory signals (such as intrathyroidal chemokines) that may activate ever present low affinity T cells and B cells. Unrestrained receptor revision occurring within these LFs may eventually lead to the formation of high affinity autoreactive B cells. Intrathyroidal LFs are highly activated (19), and this is not surprising because there is plenty of Ag and inflammatory mediators to keep the reaction going.

Finally, one of the most striking results from this work is the marked decrease in the proportion of circulating CXCR4⁺ T cells and CCR7⁺ lymphocytes, and the less marked but still significant reduction in CXCR5⁺ B cells in AITD patients with ectopic LF. These phenotypic changes may constitute good markers of the activity of the cellular immune response to the thyroid Ags and may be useful tests for the clinical assessment of the patients. Ongoing work at our laboratory will determine whether this pattern of chemokine receptor expression in PBLs of AITD patients is a feature of the group whose glands contain LFs and also whether its is present in patients suffering other autoimmune diseases characterized by the formation of TLT.

Autoimmune diseases are the end result of a multistep process in which genetic and environmental factors interact for a long time. In humans, it is very difficult to define the main checkpoints, but organization of TLT may be an important step in the consolidation of an autoimmune response. The data presented in this article define a broad and complex network of chemotactic molecules determining the migration of lymphocytes to the thyroid and the formation of intrathyroidal lymphoid tissue, a process that, given its correlation with thyroid autoantibody levels and its repercussion in the phenotype of circulating lymphocytes, we believe constitutes a decisive checkpoint in the development of AITD.

Acknowledgments

We thank the colleagues cited in the text for their generous donations of Abs and other reagents. We thank Dr. T. Gallart for invaluable comments and conceptual discussion in several moments of our work, and also Dr. C. Martinez for the generous gift of reagents and protocol to perform CXCL12 ELISA. We acknowledge the help of Dr. A. Lucas, Dr. A. Alasrud, and Dr. J. Escalante and their teams in collecting materials; Dr. L. Alcalde, Dr. M. Sospedra, Dr. F. Vargas, and L. Muixí in tissue processing; and A. Esteve for helping with statistics.

References


