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Early chemokine expression induced by interferon-gamma in a murine model of Hashimoto's thyroiditis

Hiroaki Kimura, Miho Kimura, Noel R. Rose, Patrizio Caturegli*

Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD 21205, United States

Abstract

Chemokines represent a group of small, secreted proteins mainly involved in navigating leukocytes towards site of inflammation. Some chemokines have been implicated in the pathogenesis of autoimmune diseases, which are characterized by an ectopic retention of leukocytes within the target organ, ultimately leading to loss of function. To determine the chemokines profile expressed in the thyroid gland upon chronic exposure to interferon-gamma (IFNγ), we analyzed C57BL6 transgenic mice that aberrantly express IFNγ under control of the thyroglobulin promoter. We compared by reverse transcriptase PCR the thyroidal expression of 10 chemokines (CCL1 through 5 and CXCL9 through 13) in *thyr*-IFNγ transgenics and wild-type littermates. We found that transgenics exclusively expressed CCL4, CXCL9, and CXCL11, and showed increased expression of CCL5 and CXCL10. This chemokine profile was associated with moderate mononuclear cell infiltration of the thyroid stroma that, however, decreased significantly after 2 months of age and did not organize into lymphoid structures. Our findings indicate that the isolated expression of IFNγ is capable of recruiting mononuclear cells but they do not progress to full lymphoid transformation of the thyroid.
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Introduction

Chemokines (*chemo*attractant cyto*kines*) are a group (about 50) of small (8–12 kDa), secreted proteins, containing conserved cysteine motifs at the N-terminus (Rossi and Zlotnik, 2000; Rot and von Andrian, 2004). They are classified into two major structural subfamilies, based on the number and spacing of the first two N-terminal cysteines (Moser et al., 2004). The CC chemokines, consisting of CCL1 through CCL28 and encoded by a gene cluster on human chromosome 17, have the first two cysteines adjacent to each other. The CXC chemokines, from CXCL1 to CXCL16 and found on chromosome 4, have one amino acid between the two initial cysteines (Christopherson et al., 2004). The N-terminal region is essential for chemokine

function. It mediates docking to the glycosaminoglycans that decorate the luminal surface of endothelial cells and the extracellular matrix, and binding to specific plasma membrane receptors. Chemokines are released by numerous cell types, including leukocytes, fibroblasts, endothelial cells, and also epithelial cells, in response to a wide variety of stimuli, such as bacterial products, viruses, allergens, urate crystals, and silica.

Chemokine receptors are integral membrane proteins that contain seven membrane-spanning helices and are coupled to G proteins. These receptors are mainly found on leukocytes, although they have also been identified on endothelial cells and on some epithelial cells, especially those that have undergone malignant transformation (Muller et al., 2001). There are about 20 receptors that often bind to more than one chemokine (Balkwill, 2004). Chemokine binding modifies the extracellular portion of the receptor, so that the intracellular part can bind and activate the heterotrimeric G-protein, which dissociate into α- and βγ-subunits. The βγ-subunit mediates signaling by activating

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* Corresponding author. Johns Hopkins Medical Institutions, Department of Pathology, Ross Building, Room 656, 720 Rutland Avenue, Baltimore, MD 21205, United States. Fax: +1 410 614 3548.

E-mail address: pcat@jhmi.edu (P. Caturegli).

the phosphoinositide-3 kinaseγ, which is linked with small GTPases such as Rac and Cdc42 that eventually lead to unidirectional movement of the cell, the hallmark of chemotaxis.

Chemokines mediate several functions such as angiogenesis (Bernardini et al., 2003), leukocyte degranulation (Oliveira and Lukacs, 2003), and tumor metastasis (Balkwill, 2004), but their distinguishing feature is the induction of chemotaxis in leukocytes, during both innate and adaptive immune responses and during leukocyte development, differentiation, and anatomic distribution (Dong et al., 2003). For this reason, chemokines have been divided into two functional groups. Inflammatory chemokines control the recruitment of leukocytes to the site of infection, autoimmune response, or tumor spread. Homeostatic cytokines, on the contrary, guide leukocytes during their ontogeny in bone marrow and thymus and during migration through secondary and mucosa-associated lymphoid organs (Moser et al., 2004). Some chemokines highly selective for lymphocytes, such as CCL1, CCL22, CXCL9, and CXCL10, participate both in immune defense functions and in ontogeny and are therefore referred to as "dual-function" chemokines.

Leukocytes migration out of the blood vessels is critical in the pathogenesis of autoimmune diseases. This relocation is in fact associated with tissue damage and subsequent loss of function of the target organ. Understanding, therefore, how leukocytes move under the direction of chemokines out of the vascular system, and are retained inside the target organ, has clear therapeutic implications for patients suffering from inflammatory (D'Ambrosio et al., 2003) and autoimmune (Santamaria, 2003) diseases.

Interferon gamma (IFNγ), the prototypic Th1 cytokine, induces the release of some chemokines, such as CXCL9, CXCL10, and CXCL11 (Rotondi et al., 2003), and has been involved in the pathogenesis of several autoimmune diseases (Barin et al., 2003; O'Shea et al., 2002). We have previously described transgenic mice that express IFNγ specifically in the thyroid gland and showed that they mimic some feature of the human Hashimoto's thyroiditis (Caturegli et al., 2000), one of the most prevalent autoimmune diseases in the United States (Jacobson et al., 1997). The goal of the present study was to characterize the chemokine profile expressed by thyroid glands chronically exposed to IFNγ.

Materials and methods

Mice

The *thyr*-IFN• transgenic mice were generated as previously described (Caturegli et al., 2000). They express the murine IFN• gene under control of the rat thyroglobulin promoter, to support transcription specifically in thyroid follicular cells. Transgenic mice were bred to normal

C57BL/6, and thus kept hemizygous, in specific pathogen-free facilities at Johns Hopkins University. Mice were maintained following protocols conformed to the Animal Care and Use Committee guidelines.

Thyroid histopathology

After euthanasia, tracheas with attached thyroids were removed and fixed for 48 h in the zinc-based Beckstead's solution. Specimens were then processed and embedded in paraffin. Six to eight nonsequential sections (5-μm thick) were cut from the tissue block and stained with hematoxylin and eosin (H&E). The same tissue block used for H&E histopathology was also used to cut sections for immunohistochemistry.

Thyroid immunohistochemistry

Sections were deparaffinated with xylene and rehydrated with decreasing concentrations of ethyl alcohol and finally distilled water. After blocking aspecific binding with 2% normal goat serum, sections were incubated with the following primary antibodies: rat anti-mouse CD45 (BD Pharmingen, San Diego, CA), rat anti-mouse CD4 (BD Pharmingen), rat anti-mouse CD8a (BD Pharmingen), and rat anti-mouse B220 (BD Pharmingen). Primary antibodies were diluted in phosphate-buffered saline (PBS), supplemented with 1% bovine serum albumin, at the following concentrations: 1:60 for CD45, 1:30 for CD4 and CD8, and 1: 2000 for B220, and incubated overnight at $4°C$ in a humid chamber.

After washing, the biotin-conjugated secondary antibody (affinity-purified goat anti-rat IgG, from Jackson ImmunoResearch, West Grove, PA, diluted 1:1000 in PBS–1% bovine serum albumin) was incubated for 1 h at room temperature. After additional washing, endogenous peroxidase was blocked by treating the sections with 3% H_2O_2 for 10 min at room temperature. After rinsing, peroxidase-conjugated streptavidin (Dako, Carpentaria, CA) was diluted 1:500 in PBS and incubated for 30 min at RT. Finally, sections were incubated for 10 min with DAB substrate (Sigma, St Louis, MO) for color development and then rinsed in distilled water. Sections were counter-stained with Meyer's modified hematoxylin (Polyscientific, Bay Shore, NY).

CD45 staining was assessed throughout the life of transgenics and controls, in particular on day 0, 8, 30, 60, 120, 240, and 480.

Thyroid chemokine expression assessed by semiquantitative reverse transcriptase PCR

Messenger RNA was extracted from homogenized thyroid lobes of 8-day-old mice, using oligo-(dT)$_{25}$ magnetic beads (Dynal, Lake Success, NY). After treatment with DNase I (Invitrogen), mRNA was reverse transcribed

into cDNA using Superscript II RNase H-Reverse Transcriptase (Invitrogen). PCR was performed with the forward primer 5′-GCATCTTGGGCTACACTGAG-3′ and the reverse primer 5′-TCTCTTGCTCAGTGTCCTTG-3′ for mouse G3PDH as a control. The amplified G3PDH of each sample (thyr-IFNγ transgenics thyroids and wild type littermates) were adjusted by using sequentially diluted templates to normalize the expression of chemokines to that of G3PDH. We analyzed 10 of the total 40 mouse chemokines using primers that yielded only one specific amplicon, as indicated in Table 1.

Thyroid CXCL10 expression assessed by Northern blot

To validate the results obtained from reverse transcriptase PCR, we selected CXCL10, one of the classical IFNγ-inducible chemokines, and assessed its expression in 8-day-old and 1-year-old transgenics and in wild-type controls. Total RNA was extracted by Trizol (Invitrogen), following the manufacturer's recommendations. Ten micrograms RNA were then separated on 1% denaturing agarose gel, containing 2 M formaldehyde, and transferred to Zeta Probe GT membrane (Biorad, Hercules, CA). The membrane was hybridized overnight with a RNA ^{32}P-UTP probe prepared by in vitro transcription (Strip-EZ system from Ambion, Austin, TX) using the entire 296-bp coding sequence of the murine CXCL10. After proper washing, the membrane was wrapped in plastic and exposed overnight at −70°C to BioMax MS autoradiographic film (Eastman Kodak, Rochester, NY).

Statistical analysis

Thyroid histology and immunohistochemistry were evaluated qualitatively. Chemokine RNA levels were first assessed by imaging the bands obtained from PCR and Northern blot experiments with the free software Image J (http://rsb.info.nih.gov/ij). Then, after adjusting each band for the G3PDH band obtained from the corresponding genotype (wild type or transgenic), the expression ratios of transgenics over wild type were calculated.

Results

Mononuclear cells infiltration in thyroid of young thyr-IFNγ transgenic mouse

Thyr-IFNγ transgenic mice developed, as previously reported, a striking disruption of the thyroid architecture (Caturegli et al., 2000). They also showed a moderate inflammatory infiltrate of mononuclear cells throughout the thyroid interstitium (Fig. 1B), not present in wild-type littermates (Fig. 1A). The mononuclear infiltrate consisted of hematopoietic cells, as demonstrated by the expression of CD45 (Fig. 2), a transmembrane tyrosine phosphatase expressed on all nucleated cells of the hematopoietic lineage (Sasaki et al., 2001). The infiltrate never organized into secondary follicles, with their characteristic central B cell-rich area (the germinal center and the mantle zone) and outer T cell-rich paracortex. The infiltrate was clearly evident in younger mice, up to 2 months of age, and decreased after puberty (Fig. 2).

Identification of infiltrating mononuclear cells

Subsequent staining with markers for the major cell population showed that, in addition to macrophages, there were a few CD4+ T lymphocytes (Fig. 3B), and B220+ B lymphocytes (Fig. 3D). CD8+ lymphocytes were absent (data not shown).

Chemokine expression in thyr-IFNγ transgenic mouse thyroids

To characterize the chemokines produced by thyrocytes upon exposure to IFNγ and responsible for recruiting the hematopoietic cells described above, we extracted mRNA from young mouse thyroids (young *thyr*-IFNγ transgenics and wild-type littermates) and performed semiquantitative PCR. CCL4, CXCL9, and CXCL11 were detected only in *thyr*-IFNγ transgenic mice but were absent in age-matched controls (Fig. 4). CXCL10 and CCL5 were detected in both genotypes, but were significantly up-regulated in transgenic thyroids. CCL1, CCL2, CCL3, CXCL12, and CXCL13

Table 1
Sequence of primers used to amplify 10 murine chemokines

| Chemokine	Forward primer	Reverse primer	Amplicon size (bp)
CCL1	5′-CCGTGTGGATACAGGATGTTG-3′	5′-TCAGGACAGGAGGAGCCC-3′	133
CCL2	5′-TTTTGTCACCAAGCTCAAGAGAG-3′	5′-TCACTGTCACACTGGTCACTCC-3′	252
CCL3	5′-ACCACTGCCCTTGCTGTT-3′	5′-TCTGCCGGTTTCTCTTAGTCAG-3′	292
CCL4	5′-CTCTCCTCTTGCTCGTGGC-3′	5′-GTACTCAGTGACCCAGGGCTC-3′	230
CCL5	5′-GCTGCCCTCACCATCATCC-3′	5′-GTATTCTTGAACCCACTTCTTCTCTG-3′	237
CXCL9	5′-CTTCCTGGAGCAGTGTGGAG-3′	5′-CGACTTTGGGGGTGTTTTGG-3′	321
CXCL10	5′-CTTGAAATCATCCCTGCGAGC-3′	5′-TAGGACTAGCCATCCACTGGG-3′	254
CXCL11	5′-GGTCACAGCCATAGCCCTG-3′	5′-AGCCTTCATAGTAACAATCACTTCAAC-3′	193
CXCL12	5′-CACATCGCCAGAGCCAAC-3′	5′-CTGTTGTGCTTACTTGTTTAAAGC-3′	168
CXCL13	5′-AGGCTCAGCACAGCAACG-3′	5′-TCTTTGTAACCATTTGGCACG-3′	252
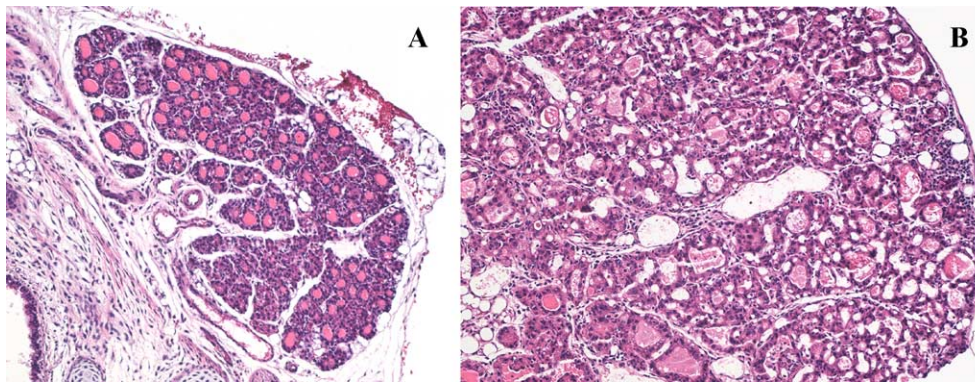
Fig. 1. Histopathology and mononuclear infiltration in the thyroids of young *thyr*-IFNγ transgenic mice. Note the disruption of the thyroid architecture and the moderate, diffuse infiltration of mononuclear cells in the thyroid of transgenic (B), not present in control littermates (A).

were not expressed in transgenics or controls (data not shown).

Northern blot analysis for CXCL10 confirmed the results of the more sensitive reverse transcriptase PCR, by revealing up-regulated levels in transgenic thyroids (Fig. 5).

Discussion

Hashimoto's thyroiditis is a chronic inflammatory disease of the thyroid gland characterized by local infiltration of mononuclear cells, with subsequent disruption of thyroid follicles and hypothyroidism (Pearce et al., 2003). In his original description, Hashimoto described four grossly enlarged thyroids that were heavily infiltrated by lymphocytes, organizing in some areas in true lymphoid follicles with germinal centers (Hashimoto, 1912). Having the appearance of a lymph node, he named this lymphoid transformation of the thyroid "lymphadenomatous goiter". Some features of Hashimoto's thyroiditis can be reproduced by immunization of experimental animals with thyroglobulin (Rose and Witebsky, 1956), and have greatly contribute to our understanding of its pathogenicity. Development of

Hashimoto's thyroiditis is now thought to occur through activation of thyroid-reactive T cells, up-regulation of adhesion molecules and chemokines, migration of leukocytes from the circulation into the thyroid stroma, retention of leukocytes within the thyroid followed by de novo formation of lymphoid tissue, destruction of the thyrocytes, and loss of thyroidal function. The influx of leukocytes into the thyroid is therefore a crucial early step in the pathway leading to clinical disease. For this reason, chemokines and their receptors are receiving a great attention in autoimmunity as possible novel diagnostic, prognostic, and therapeutic tools (Christopherson et al., 2004).

A few studies have analyzed the thyroidal expression of chemokines in patients with autoimmune thyroid diseases, which are represented by Graves' disease and Hashimoto's thyroiditis. Pujol–Borrell's laboratory reported overexpression of CCL3 and CCL4 in thyroid from patients with Graves' disease (Ashhab et al., 1999), later expanded to CCL21 and CXCL13 in Graves' and Hashimoto's thyroiditis (Armengol et al., 2001), and recently to CCL22 and CXCL12 (Armengol et al., 2003). Interestingly, the authors showed that in patients with autoimmune thyroid diseases, CXCL12, CXCL13, and CCL22 levels were significantly
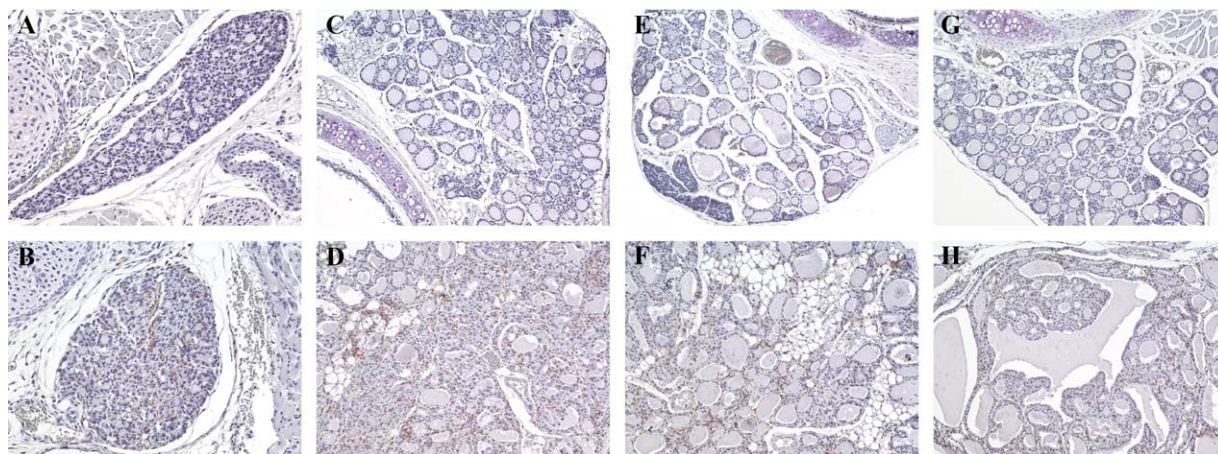

Fig. 2. Kinetics of CD45^{+} cells staining. The infiltrating cells in the thyroids of *thyr*-IFNγ transgenic mice (lower panels) are of the hematopoietic lineage because they express CD45. Only few infiltrating cells can be seen in wild-type thyroids (upper panels). Infiltrating cells in transgenics slightly decreased in number over time.
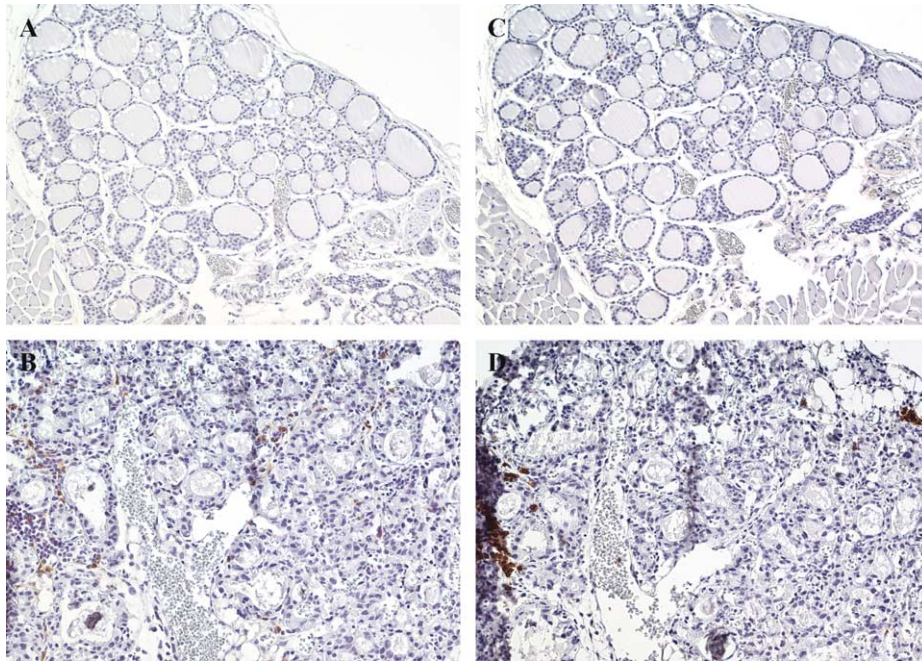
Fig. 3. Identification of CD45$^+$ cells. Transgenic thyroids (lower panels) included some CD4+ T lymphocytes and some B220+ B lymphocytes.

higher in thyroids with newly formed lymphoid follicles than in those without (Armengol et al., 2003). Aust et al. (2004) reported first increased CCL5 in Graves' thyroids (Simchen 2000) and then increased CXCL13 in Graves' and especially Hashimoto's thyroid glands. Garcia-Lopez et al. (2001) analyzed 8 patients with Hashimoto's thyroiditis and 16 with Graves' disease and found increased thyroidal expression of CXCL9, CXCL10, and CCL5. Their findings were confirmed by Romagnani et al. (2002), and by Kemp et al. (2003), who also found increased CCL2, CCL3, and CCL4.

Studies in humans, however, suffer from the limitation that thyroid chemokines are typically analyzed at the time of surgery, when the disease is already in a somewhat advanced stage and has already been manipulated with some forms of treatment. Animal models can therefore be useful in deciphering the complex message of the many cytokines and chemokines generated during an autoimmune response, allowing us, for example, to isolate the contribution of individual molecules and to perform longitudinal analyses. Numerous recent studies have reported an association between chemokine expression and experimentally induced models of autoimmunity. In experimental autoimmune encephalomyelitis, CCL2, CXCL10, and CCL1 have been observed before symptoms appearance, and their level was shown to remain elevated throughout the course of the disease (Dogan and Karpus, 2004; Glabinski et al., 2003). In experimental autoimmune myocarditis, Fuse et al. (2001) have shown increased cardiac and serum levels of CCL2, and
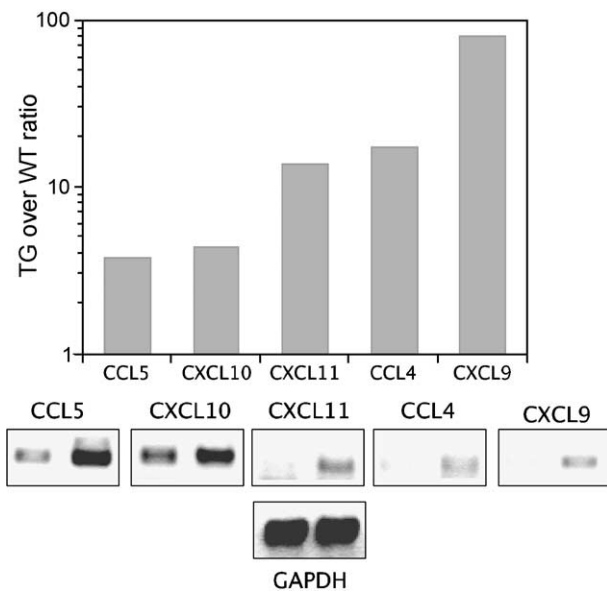

Fig. 4. Chemokine expression in thyroids of young *thyr*-IFNg transgenic mice, as assessed by reverse transcriptase PCR. Note the increased levels of CCL5 and CXCL10 and the appearance of CXCL11, CCL4, and CXCL9.
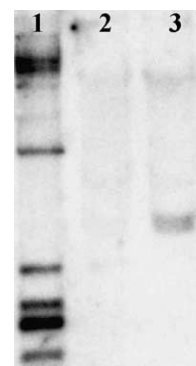

Fig. 5. Northern blot for CXCL10, to confirm the reverse-transcriptase PCR results. Note the increased expression of CXCL10 in *thyr*-IFNγ transgenic thyroids (lane 3), as compared to controls (lane 2).

Toyozaki et al. (2001) increased CCL3. In experimental autoimmune uveoretinitis, CCL3 (Crane et al., 2003) as well as CCL2, CLL5, and CXCL10 (Keino et al., 2003) have been described. Finally, in experimental autoimmune thyroiditis, Goulvestre et al. (2002) described an early increase of CCL2 and CCL5 that waned 1 month after the initial immunization.

We found in this study that the isolated expression of IFNγ within the thyroid gland induces the thyrocytes to synthesize CCL4, CXCL9, and CXCL11, and to increase their production of CCL5 and CXCL10. This set of chemokines recruits lymphocytes to the thyroid but is apparently insufficient to alter their behavior and allow their long-term retention, proliferation, and association into secondary lymphoid structures within the target organ.

Our results show that lymphoid tissue development during autoimmune diseases requires the coordinated action of several cytokines and chemokines in order to mediate recruitment and clustering of the cells involved in lymphoid neogenesis and germinal center formation. The ability to create lymphoid structures within nonlymphoid organs is a crucial in transforming a benign autoimmune response into a pathogenic autoimmune disease. IFNγ alone induces a non-organized mononuclear infiltrate that does not progress to full lymphoid transformation of the thyroid.

It will take some time before the molecular interactions responsible for matching the about 40 chemokines and their 20 receptors will become clear, because these interactions are likely to vary for each receptor and ligand. Delineating the events that lead to the retention of immune cells within the target organ, and their evolution into sophisticated anatomical structures, is similar to learning the music of an entire orchestra: the process is greatly facilitated by learning the part of each individual instrument.

Acknowledgment

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