Eotaxin/CCL11 in idiopathic retroperitoneal fibrosis

Domenica Mangieri¹, Domenico Corradi², Davide Martorana³, Giovanni Malerba⁴, Alessandra Palmisano¹, Irene Libri¹, Veronica Bartoli⁵, Maria L. Carnevali¹, Matteo Goldoni⁵, Paolo Govoni⁶, Rossella Alinovi⁵, Carlo Buzio¹ and Augusto Vaglio¹

¹Department of Clinical Medicine, Nephrology and Health Sciences, Section of Nephrology, University of Parma, Parma, Italy, ²Department of Pathology and Laboratory Medicine, Section of Pathology, University of Parma, Parma, Italy, ³Molecular Genetics Unit, University Hospital of Parma, Parma, Italy, ⁴Department of Life and Reproduction Sciences, Section of Biology and Genetics, University of Verona, Verona, Italy, ⁵Department of Clinical Medicine, Nephrology and Health Sciences, Section of Occupational Medicine, University of Parma, Parma, Italy and ⁶Department of Experimental Medicine, University of Parma, Parma, Italy

Correspondence and offprint requests to: Augusto Vaglio; E-mail: augustovaglio@virgilio.it

Abstract

Background. Idiopathic retroperitoneal fibrosis (IRF) is a rare fibro-inflammatory disorder characterized by a periaortic tissue which often encases the ureters causing acute renal failure. IRF histology shows fibrosis and a chronic inflammatory infiltrate with frequent tissue eosinophilia. We assessed a panel of molecules promoting eosinophilia and fibrosis in IRF patients and performed an immunogenetic study.

Methods. Serum levels of eotaxin/CCL11, regulated and normal T-cell expressed and secreted (RANTES), granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-5, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) were measured using a multiplex assay in 24 newly diagnosed, untreated IRF patients and 14 healthy controls. Retropertitoneal biopsies (available in 8/24 patients) were histologically evaluated to assess eosinophil infiltration, whereas mast cells (MCs) were identified by immunohistochemical analysis for human tryptase. Immunohistochemistry for eotaxin/CCL11 and its receptor CCR3 was also performed. Six single nucleotide polymorphisms (SNPs) within the CCL11 gene (rs6505403, rs1860184, rs4795896, rs17735961, rs16969415 and rs17809012) were investigated in 142 IRF patients and 214 healthy controls.

Results. Serum levels of eotaxin/CCL11 were higher in IRF patients than in controls (P = 0.009). Eotaxin/CCL11 drives tissue infiltration of eosinophils and MCs, which can promote fibrosis. Eosinophilic infiltration was prominent (>5 cells/hpf) in five (62.5%) cases, and abundant tryptase-positive MCs were found in all cases; notably, MCs were in a degranulating state. Immunohistochemistry showed that CCL11 was highly produced by infiltrating mononuclear cells and that its receptor CCR3 was expressed by infiltrating eosinophils, MCs, lymphocytes and fibroblasts. None of the tested CCL11 SNPs showed disease association, but the TTCCAT haplotype was significantly associated with IRF (P = 0.0005).

Conclusions. These findings suggest that the eotaxin/CCL11-CCR3 axis is active in IRF and may contribute to its pathogenesis; the TTCCAT haplotype within the CCL11 gene is significantly associated with IRF.

Keywords: chemokines; eosinophils; eotaxin/CCL11; haplotype analysis; idiopathic retroperitoneal fibrosis

Introduction

Idiopathic retroperitoneal fibrosis (IRF) is a rare disease, characterized by a fibro-inflammatory tissue that develops around the abdominal aorta and the iliac arteries, often encasing the ureters and may cause acute renal failure [1–3]. The pathogenesis of IRF is poorly understood, although immune-mediated mechanisms and genetic and environmental factors may play a role [1, 4–6]. IRF presents with abdominal or back pain and often with systemic symptoms such as fatigue and weight loss; laboratory tests usually show a high erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, and in some cases positive auto-antibodies [2, 3, 7, 8].

Histological examination of IRF biopsies show the coexistence of fibrous and inflammatory components. The fibrous fraction is rich in Type-I collagen, which is produced by fibroblasts and myofibroblasts [7]. The inflammatory infiltrate often aggregates around small vessels, but is also found in a diffuse pattern. The infiltrate consists mainly of CD4+ and CD8+ T cells, B cells and plasma cells, which are frequently IgG4+ [7, 9]. Tissue infiltration by eosinophils has been observed in many IRF studies [7–9], but has never been systematically characterized.

Eosinophil recruitment is common in many fibro-inflammatory disorders, namely scleroderma, idiopathic pulmonary fibrosis and systemic vasculitides [10]. Additionally, eosinophil-rich disorders such as the idiopathic hypereosinophilic syndrome, the Churg–Strauss syndrome and chronic asthma often produce pronounced sclerotic lesions [11–13]. Eosinophils stimulate fibroblast
proliferation and differentiation into myofibroblasts, ultimately exaggerating the deposition of extracellular matrix (ECM) [14, 15]. Similarly, mast cells (MCs) have traditionally been recognized as key mediators of allergy but recent reports have shown that they can modulate other inflammatory processes [16, 17]; intriguingly, MCs also enhance fibroblast activation and collagen deposition through the release of their enzymes, particularly tryptase [18, 19]. Mobilization and recruitment of eosinophils and MCs into the affected tissues is governed by soluble factors such as the CC chemokines eotaxin/CCL11 and RANTES as well as interleukin (IL)-5 and granulocyte colony-stimulating factor (G-CSF) [20, 21].

The circulating levels of soluble mediators promoting fibrosis and tissue recruitment of eosinophils and MCs have never been investigated in IRF. In this study, we assessed the serum levels of eotaxin/CCL11, RANTES, IL-5 and G-CSF, and of the pro-fibrotic mediators platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) in newly diagnosed, untreated IRF patients, and analysed tissue infiltration by eosinophils and MCs in IRF biopsies. As eotaxin/CCL11 was found to be up-regulated in IRF, we investigated whether it was produced within the retroperitoneal lesions and also whether its receptor, CCR3, was expressed by infiltrating cells. Finally, we investigated the association with IRF of six single nucleotide polymorphisms (SNPs) tagging the most common haplotypes of the CCL11 gene.

Materials and methods

Study subjects

We enrolled 24 consecutive patients with newly diagnosed, untreated IRF, referred to or diagnosed at the Department of Clinical Medicine and Nephrology of Parma University Hospital between January 2007 and March 2010. IRF was diagnosed by means of computed tomography (CT) or magnetic resonance imaging (MRI) following commonly accepted criteria [22]. IRF thickness was measured on CT/MRI at the lower abdominal aorta and common iliac artery levels, and maximal IRF thickness at these two levels was included in the analysis. Retroperitoneal biopsy was performed in case of atypical mass localization, or when underlying infections or malignancies were suspected; biopsy was also performed in all patients undergoing surgical ureterolysis. At the time of diagnosis, medical and medication history was reviewed, and each patient underwent clinical examination and routine laboratory tests including ESR, CRP and a panel of auto-antibodies, as previously described [6]. The main characteristics of the 24 patients at the time of disease onset are summarized in Table 1. The control group consisted of 14 sex- and age-matched Caucasian healthy subjects. All patients received corticosteroids, mainly following the regimen published in a recent trial [23]; the percentage reduction in IRF thickness was assessed as previously described [23].

Another group consisting of 11 IRF patients (at the time of their disease onset and disease remission) seen at our department between April 2010 and January 2011, 12 anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) patients [seven with granulomatosis with polyangiitis (Wegener’s) and five with microscopic polyangiitis] and 8 healthy controls were included in a confirmatory ELISA assay. The characteristics of these 11 IRF patients were comparable to those of the 24 patients described above (data not shown). All of the AAV patients had an active disease.

Finally, we used DNA samples from 142 Caucasian IRF patients and 214 age- and sex-matched healthy Caucasian controls (all recruited at our centre) for the immunogenetic analysis.

The Ethics Committee of Parma University Hospital approved the study, and a written informed consent was obtained from all the study subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at disease onset, median (IQ range), years</td>
<td>54 (50–60)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>13 (54)</td>
</tr>
<tr>
<td>IRF localization, n (%)</td>
<td>22 (92)</td>
</tr>
<tr>
<td>Periaortoiliac/pericaval</td>
<td></td>
</tr>
<tr>
<td>Other localization</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Systemic symptoms*, n (%)</td>
<td>16 (67)</td>
</tr>
<tr>
<td>Abdominal or lumbar pain, n (%)</td>
<td>20 (83)</td>
</tr>
<tr>
<td>Ureteral obstruction, n (%)</td>
<td>1 (63)</td>
</tr>
<tr>
<td>Acute renal failure, n (%)</td>
<td>8 (33)</td>
</tr>
<tr>
<td>Associated immune-mediated diseases, n (%)</td>
<td>10 (42)</td>
</tr>
<tr>
<td>Serum creatinine, median (IQ range), mg/dl</td>
<td>0.95 (0.9–2.1)</td>
</tr>
<tr>
<td>WBC, median (IQ range), cells/mm³</td>
<td>7010 (6135–8352)</td>
</tr>
<tr>
<td>ESR, median (IQ range), mm/h</td>
<td>64 (51–92)</td>
</tr>
<tr>
<td>CRP, median (IQ range), mg/L</td>
<td>27 (16–47)</td>
</tr>
<tr>
<td>ANA positivity, n (%)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>Other auto-antibody positivity, n (%)</td>
<td>14 (58)</td>
</tr>
<tr>
<td>Serum IgG4 level², median (IQ range)- mg/dL</td>
<td>33 (19–55)</td>
</tr>
<tr>
<td>Maximal periaortic IRF thickness, median (range), mm</td>
<td>21 (15–26)</td>
</tr>
<tr>
<td>Maximal peri-iliac IRF thickness, median (range), mm</td>
<td>15 (10–21)</td>
</tr>
</tbody>
</table>

*Systemic symptoms include fatigue, anorexia, weight loss, diffuse myalgias and arthralgias.
²IgG4 levels were available in 15 patients.

Chemokine and growth factor measurement

Sera were obtained at the time of diagnosis and stored at −80°C until use. Serum levels of chemokines, cytokines and growth factors were measured using the Bio-Rad Luminex assay technologies (Bio-Rad Laboratories, Life Science Group, Hercules, CA). This assay was developed to measure simultaneously eotaxin/CCL11, RANTES, IL-5, G-CSF, bFGF and PDGF-BB. Standard curves for each cytokine were generated using the reference cytokine concentrations supplied with the kit. Each sample was diluted 1:2 with sample diluent, according to the manufacturer’s instructions, and incubated for 2 h with fluorescent beads coated with the primary antibodies directed against the above cytokines and growth factors. All samples and standards were performed in duplicate. The wells were then washed using a vacuum manifold and a biotinylated detector antibody was subsequently added. After 1 h, the beads were washed again and then incubated for 30 min with streptavidin conjugated to the fluorescent protein R-phycocerythrin (Streptavidin-RPE). After washing to remove the unbound Streptavidin-RPE, the beads were analysed using a Luminex 100 instrument and the results examined using the Bio-Rad Manager Software version 4.0.

To confirm the increased eotaxin/CCL11 production in IRF patients, and to assess its specificity and levels during disease remission, we performed an eotaxin/CCL11 ELISA (R&D Systems, Minneapolis, MN), using the sera obtained from an additional 11 active IRF patients, 10 of which in remission, 8 healthy controls and 12 AAV patients. All samples and standards were performed in duplicate.

Histology and immunohistochemistry

Eight of the 24 patients included in the multiplex analysis had undergone retroperitoneal biopsy; biopsy specimens were available for this study in all of these eight cases. The specimens were fixed in a 10% buffered formalin solution, paraffin-embedded and sectioned at 5 μm. For histological analyses and eosinophil counts, the sections were stained with haematoxylin and eosin, and observed through a light microscope (Olympus BX 51; Olympus, Tokyo, Japan). The number of infiltrating eosinophils was measured by counting the average number of eosinophils per microscopic field after evaluating 30 histological fields (total square area examined 1.2 mm²) per case.
Tryptase-positive infiltrating MCs and eotaxin/CCL11 expression were evaluated by immunohistochemistry, using the following mouse monoclonal antibodies: anti-human tryptase, dilution 1:150 (Abcam, Cambridge, UK) and anti-human eotaxin/CCL11, dilution 1:100. Briefly, the sections were deparaffinized in xylene, rehydrated in a graded alcohol scale and stained with the avidin–biotin–immunoperoxidase technique. Heat-induced antigen retrieval was performed in citrate buffer and then the sections were incubated for 1 h at room temperature with the primary antibodies. After washing with PBS, they were treated for 30 min at room temperature with a biotinylated horse anti-mouse

**Fig. 1.** Serum profile of eotaxin/CCL11, RANTES, IL-5, G-CSF, PDGF-BB and bFGF obtained using a multiplex assay. The central horizontal line and the whiskers indicate the median and the IQ ranges. P values were calculated using the Mann-Whitney U test. In the X axis, IRF denotes idiopathic retroperitoneal fibrosis.

**Fig. 2.** Correlations between eotaxin/CCL11 serum levels and the maximal thickness of idiopathic retroperitoneal fibrosis taken at the levels of the lower abdominal aorta (left panel) and common iliac arteries (right panel). These measures were obtained at the time of disease onset by means of CT or MRI scans and were available in 21 of the 24 patients studied. The r and P values were calculated using Spearman’s correlation. In the y-axis, IRF denotes idiopathic retroperitoneal fibrosis.
secondary antibody (Dako, Hamburg, Germany) diluted 1:150 in PBS, followed by Diaminobenzidine staining for eotaxin/CCL11 and by 3-amino-9-ethylcarbazole for trypstatase. Afterwards, the sections were counterstained with Mayer’s haematoyxlin (Bio-Optic, Milan, Italy) and mounted in buffered glycerine. A pre-immune mouse serum replacing the primary antibodies served as negative control. The sections were examined with a Leica photomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland).

To detect the eotaxin receptor CCR3 in the retroperitoneal biopsies and to evaluate which cell types express it, we used an anti-CCR3 primary antibody; each CCR3-treated histological section was then incubated with the following monoclonal primary antibodies: anti-myeloperoxidase (Ventana, dilution 1:400) to detect granulocytes (essentially eosinophils, because basophils and neutrophils are usually absent in IRF lesions), anti-human tryptase (Abcam, dilution 1:150) to detect MCs, eosinophils, because basophils and neutrophils are usually absent in IRF bated with the following monoclonal primary antibodies: anti-myeloperoxidase (Ventana, dilution 1:400) to detect granulocytes (essentially eosinophils, because basophils and neutrophils are usually absent in IRF lesions), anti-human tryptase (Abcam, dilution 1:150) to detect MCs, eosinophils, because basophils and neutrophils are usually absent in IRF.

Immunogenetic analysis

As eotaxin/CCL11 proved to be up-regulated in IRF, we tested the association between some CCL11 gene polymorphisms and IRF. Six SNPs mapping in the region of the CCL11 gene and able to tag the most common haplotypes of the HapMap CEU population sample (Utah residents with ancestry from northern and western Europe) were selected (http://www.hapmap.org [24]) and genotyped.

The six SNPs (rs6505403, rs1860184, rs4795896, rs17735961, rs16969415 and rs17809012) were amplified from the genomic DNA of 142 IRF patients and 214 healthy controls. Two SNPs (rs6505403 and rs1860184) were genotyped using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with a TaqMan 5’-allele discrimination method. The remaining four SNPs (rs4795896, rs17735961, rs16969415 and rs17809012) were analysed on an automated sequencer (CEQ 2000XL DNA Analysis System, Beckman Coulter, Fullerton, CA). Sequence data were aligned using SeqMan II (DNAStar, Madison, WI).

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). Data were presented as median and interquartile (IQ) ranges due to significant deviations from normality (Kolmogorov–Smirnov test). Comparisons between groups were performed using the Mann–Whitney or Wilcoxon test, as appropriate. Correlations between serological and clinical parameters were assessed by Spearman’s test or Pearson’s test, depending on the normality of residues.

In the immunogenetic analysis, all SNPs were tested for the Hardy–Weinberg equilibrium. Differences in allele frequencies between IRF patients and controls were tested by chi-square test. Two-sided P values <0.05 were considered significant.

The haplotype structure of the CCL11 gene region in the CEU population was investigated using the information provided in the Hapmap database (version #27). A small number of SNPs able to tag the most common haplotypes (frequency > 5%) each of the region in the CEU population was then selected. Association analysis between haplotypes and IRF was conducted using PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) [25]. An association was considered statistically significant when the global test (omnibus test) showed P < 0.05. In this case the association of each of the estimated haplotypes was tested and the association was considered statistically significant when showing a P < 0.05.

Results

Analysis of serum chemokines and growth factors by multiplex assay

We found significantly higher levels of eotaxin/CCL11 [median (IQ range), 193.1 pg/mL (145.6–266.9) versus 117.2 pg/mL (107.8–125.2); P = 0.009] and, to a lesser extent, RANTES [12 004.1 pg/mL (6889.2–24 843.7) versus 8355.9 pg/mL (7891.9–8875.4); P = 0.034] in IRF patients than in controls (Figure 1). We also found that eotaxin/CCL11 significantly correlated with the fibrogenic markers PDGF-BB (r = 0.50, P = 0.03) and bFGF (r = 0.44, P = 0.03), but not with the remaining molecules (data not shown). There was no association or correlation between eotaxin/CCL11 and the following parameters: age at disease onset, gender, localization of IRF (periaortoiliac versus other localization), presence of systemic symptoms or pain, ureteral involvement, acute renal failure or presence of an associated autoimmune disease (data not shown). Moreover, no correlation was found between eotaxin/CCL11 and ESR, CRP, white blood cell count and auto-antibody positivity (data not shown). In contrast, we observed a significant inverse correlation between eotaxin/CCL11 and IRF thickness, measured at the level of the lower abdominal aorta (r = −0.51, P = 0.02) and common iliac
arteries \( r = -0.68, \ P = 0.001 \) (Figure 2). No correlations were found between the other five tested biomarkers and IRF thickness (data not shown).

Twenty-one of the 24 patients had follow-up CT or MRI, which allowed us to calculate the percentage reduction in thickness of IRF after treatment; the median reduction was 50% (range 0–90%). Eotaxin/CCL11 levels inversely correlated with the percentage reduction in IRF thickness \( r = -0.46, \ P = 0.035 \).

IgG4 levels were available in only 15 cases, and were within the normal range (<135 mg/dL) in all cases (Table 1). There was an almost significant inverse correlation between eotaxin/CCL11 and IgG4 levels \( r = -0.51, \ P = 0.062 \).

**Eotaxin/CCL11 ELISA**

Eotaxin/CCL11 ELISA was performed using sera from 11 active IRF patients, 10 of them at the time of remission, 8 healthy controls and 12 AAV patients. This test confirmed the significant increase in serum eotaxin/CCL11 in active IRF patients when compared with healthy controls.
cytoplasmic positivity for eotaxin/CCL11 (original magnification ×10 in A and ×40 in B and C).

Tissue infiltration by eosinophils and MCs

Eotaxin/CCL11 drives eosinophil chemotaxis and recruitment into the inflamed tissues [26], a process to which RANTES may also contribute [27]. Therefore, we reviewed the available IRF biopsies in order to quantify and characterize tissue eosinophilic infiltration.

Histological examination of the eight retroperitoneal biopsies showed in all cases an admixture of fibrous and inflammatory components. Inflammatory infiltration was graded as mild, moderate or severe, as previously described [7]. One of the eight cases (12.5%) had mild, 2/8 (25%) moderate and 5/8 (62.5%) severe inflammatory infiltration. The infiltrate consisted of lymphocytes, plasma cells, macrophages and eosinophils, and was diffuse or organized in pseudo-nodular aggregates resembling lymphoid follicles with germinal centres (Figure 4A). A measurable eosinophilic infiltration was found in all cases; the median number of eosinophils per high-power field on haematoxylin and eosin-stained slides was 5.1 (range 3–13). Eosinophilic infiltration was considered prominent when the average number was higher than 5, as reported in other histopathologic studies on IRF [9, 28]. Five (62.5%) of our cases showed a prominent eosinophilic infiltration (Figure 4B).

Eosinophils were generally more abundant in the more inflammatory cases and predominated in the areas with diffuse inflammation; in most cases they were found at the periphery of the lymphoid follicle-like aggregates (Figure 4C). The cases with a more pronounced sclerotic component showed only rare infiltrating eosinophils (Figure 4D).

Eotaxin/CCL11 also regulates MC chemotaxis and infiltration into target tissues [29, 30]. Thus, we also evaluated whether MCs participate in the retroperitoneal inflammatory response associated with IRF; MCs were detected by immunohistochemistry using an anti-human tryptase monoclonal antibody. Intriguingly, in all of the available biopsies, we found numerous tryptase-positive MCs, whose amount and pattern of tissue distribution strongly resembled that of eosinophils. In fact, MCs were more abundant in the more inflammatory cases, and particularly within the areas of diffuse inflammation (Figure 4E); in some cases, they accumulated at the periphery of the lymphoid-like inflammatory aggregates. Noteworthy, most MCs were in a degranulating state (Figure 4F).

Eotaxin/CCL11 and CCR3 expression in IRF biopsies

In order to investigate whether the high serum levels of eotaxin/CCL11 could also be accounted for by an increased synthesis within the retroperitoneal tissue, we analysed eotaxin/CCL11 expression in retroperitoneal biopsies. We observed a remarkable expression of eotaxin/CCL11, especially in the mononuclear cells aggregated in inflammatory lymphoid-like follicles (Figure 5A); the strong peripheral cytoplasmic staining observed within such cells indicated true, specific staining (Figure 5B and C). Some mononuclear inflammatory cells found in the areas with diffuse inflammation also showed mild eotaxin/CCL11 staining (data not shown); mild staining was also observed in scattered spindle-shaped cells (likely fibroblasts) as well as in the walls of small retroperitoneal vessels (data not shown).

CCR3 was also found to be abundantly expressed within the retroperitoneal lesions. Myeloperoxidase-positive granulocytes (essentially eosinophils, given the absence of neutrophils and basophils in the lesions) and tryptase-positive MCs consistently showed CCR3 positivity (Figure 6A and B); however, other cell types also expressed CCR3, namely fibroblasts (Figure 6C), T-cell subsets and B cells (Figure 6D through E) and rare macrophages (Figure 6F).
Immunogenetic analysis

Genotypes of both IRF patients and controls were in Hardy–Weinberg equilibrium for each of the SNPs investigated. None of the individual CCL11 SNPs was significantly associated with IRF in our cohort (Table 2). The association analysis between cases and control individuals for the nine most common estimated haplotypes showed a significant result (global test: \( \chi^2 = 27.8 \), degree of freedom = 8; \( P = 0.0005796 \)); in particular, the TTCCAT haplotype was significantly associated with IRF, its frequency being 22.92% in IRF patients and 12.65% in controls.

Fig. 6. CCR3 expression in retroperitoneal biopsies. CCR3 is stained red, while the proteins used to detect the different cell types are green. Yellow staining is due to green and red signal superimposition. (A) An eosinophilic granulocyte with a bilobated nucleus and markedly granular cytoplasm containing myeloperoxidase. (B) A degranulating mast cell (whose cell edges are not well defined) intensely positive for tryptase. (C) A highly cellular area showing several vimentin-positive fibroblasts/myofibroblasts. CD4+ (D), CD8+ (E) and CD20-positive (F) lymphocytes in a perivascular inflammatory infiltrate. (G) CD68-KP1-positive macrophages in a fibro-adipose tissue area. Original magnification (A–G): ×63. Bar scale, A and B: 10 μm, C–G: 50 μm.
enhance eosinophil chemotaxis and eosinophils themselves may contribute to the development of tissue eosinophilia [7]. Recent studies have demonstrated a causal link between tissue eosinophil infiltration and the development of fibrosis, given that chemokines regulating eosinophil chemotaxis and eosinophils themselves may enhance fibrogenic responses [20, 21, 31]. In the present study, we have shown that the serum levels of eotaxin/CCL11 and, to a lesser extent RANTES, are increased in IRF patients; both chemokines are potent chemoattractants for eosinophils. The increase in serum eotaxin/CCL11 was not specific for IRF, as patients with active AAV also had high levels; however, the raised eotaxin/CCL11 levels in IRF were confirmed in an additional ELISA. We have also confirmed that tissue eosinophilia is common in IRF and described, for the first time, the presence of numerous infiltrating MCs, a cell type traditionally involved in allergy and capable of promoting fibrosis. Interestingly, we observed that the lymphocytes forming the retroperitoneal lymphoid-like aggregates are a source of eotaxin/CCL11, and that the eotaxin receptor CCR3 is also abundantly expressed within the retroperitoneal lesions. Taken together, these findings suggest that the eotaxin/CCL11–CCR3 axis is active in IRF.

An up-regulation of eotaxin/CCL11 is typical of many Th2-dominant disorders [31]; interestingly, a Th2-skewed response has also been postulated in IRF [32], and increased tissue levels of Th2-cytokines such as IL-4 and IL-10 were detected in sclerosing pancreato-cholangitis, a condition that shares with IRF key histopathological features and the presence of IgG4+ plasma cells [33]. Eotaxin/CCL11 was originally described as the main chemotactic and activating factor for eosinophils; through the interaction with CCR3, it leads to differentiation of hematopoietic CD34+ progenitor cells into eosinophils and promotes eosinophil migration, activation and degranulation [26, 34, 35]. It also drives the recruitment and activation of other inflammatory cells such as T lymphocytes, macrophages and MCs [29, 36, 37]. Eotaxin/CCL11 immunoreactivity has been detected in different cell types including eosinophils themselves, lymphocytes, fibroblasts, smooth muscle cells, epithelial and endothelial cells [38, 39]. A number of disorders, including atherosclerosis [40], chronic asthma [41] and chronic liver disease [42], as well as rat models of heart transplant rejection [43] are characterized by an up-regulation of eotaxin/CCL11. In our study, we found that eotaxin/CCL11 was locally produced by the inflammatory cells forming the retroperitoneal aggregates, and that its receptor CCR3 was expressed by a variety of infiltrating cells, including not only eosinophils and MCs, but also lymphocytes and fibroblasts; this suggests that eotaxin/CCL11 may have pleiotropic effects and that it may also act directly on fibrous tissue-producing cells.

Different mechanisms may explain how eotaxin/CCL11 contributes to the pathogenesis of fibro-inflammatory diseases such as IRF. Eotaxin/CCL11 directly promotes the proliferation and migration of fibroblasts, their collagen synthesis and matrix metalloproteinase (MMP)-2 activity

### Table 2. Association study of six CCL11 single nucleotide polymorphisms (SNPs) with idiopathic retroperitoneal fibrosis (IRF) patients and healthy controls

<table>
<thead>
<tr>
<th>CCL11 SNP</th>
<th>Location (kB)</th>
<th>Alleles (m/M)a</th>
<th>IRF patients (n = 142) m/(m + M), %</th>
<th>Healthy controls (n = 214) m/(m + M), %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6505403</td>
<td>29627078</td>
<td>C/T</td>
<td>59/284 (20.8)</td>
<td>88/428 (20.6)</td>
<td>0.945</td>
</tr>
<tr>
<td>rs4795986</td>
<td>2963635</td>
<td>C/T</td>
<td>38/284 (13.4)</td>
<td>54/428 (12.6)</td>
<td>0.766</td>
</tr>
<tr>
<td>rs17735961</td>
<td>2963653</td>
<td>A/C</td>
<td>57/284 (20.1)</td>
<td>93/428 (21.7)</td>
<td>0.595</td>
</tr>
<tr>
<td>rs16969415</td>
<td>29636515</td>
<td>T/C</td>
<td>9/284 (3.2)</td>
<td>18/428 (4.2)</td>
<td>0.478</td>
</tr>
<tr>
<td>rs17809012</td>
<td>29636557</td>
<td>G/A</td>
<td>116/284 (40.8)</td>
<td>196/428 (45.8)</td>
<td>0.174</td>
</tr>
<tr>
<td>rs1860184</td>
<td>29637245</td>
<td>A/T</td>
<td>75/284 (26.4)</td>
<td>99/428 (23.1)</td>
<td>0.319</td>
</tr>
</tbody>
</table>

*Alleles: m and M correspond to the minor and major alleles, respectively.

### Table 3. CCL11 haplotype association analysis in idiopathic retroperitoneal fibrosis (IRF) patients and healthy controls

<table>
<thead>
<tr>
<th>CCL11 Haplotypea</th>
<th>Estimated haplotype frequency IRF patients (n = 142)</th>
<th>Estimated haplotype frequency healthy controls (n = 214)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTCCAA</td>
<td>0.1009</td>
<td>0.0924</td>
<td>0.7142</td>
</tr>
<tr>
<td>TTCCAA</td>
<td>0.1424</td>
<td>0.1316</td>
<td>0.6872</td>
</tr>
<tr>
<td>TCTCGT</td>
<td>0.0189</td>
<td>0.0377</td>
<td>0.1642</td>
</tr>
<tr>
<td>CTACGT</td>
<td>0.0479</td>
<td>0.0114</td>
<td>0.003531</td>
</tr>
<tr>
<td>TTACGT</td>
<td>0.1462</td>
<td>0.1911</td>
<td>0.1328</td>
</tr>
<tr>
<td>TCCGCT</td>
<td>0.1731</td>
<td>0.2074</td>
<td>0.2712</td>
</tr>
<tr>
<td>CTCACAT</td>
<td>0.0198</td>
<td>0.0173</td>
<td>0.8164</td>
</tr>
<tr>
<td>CTTCAT</td>
<td>0.1215</td>
<td>0.1845</td>
<td>0.0291</td>
</tr>
<tr>
<td>TCATTTCC</td>
<td>0.2392</td>
<td>0.1265</td>
<td>0.0004752</td>
</tr>
</tbody>
</table>

*Haplotype: string of characters indicating the allele at marker rs6505403, rs4795986, rs17735961, rs16969415, rs17809012 and rs1860184, respectively.
Moreover, it acts synergistically with RANTES—whose levels were also increased in IRF—in orchestrating tissue trafficking of inflammatory cells such as eosinophils and MCs. Eosinophils can affect fibroblast functions through the release of their basic granule proteins and of an array of mediators including PDGF, FGF, transforming growth factor-β (TGF-β) and nerve growth factor [10, 14]; in addition, they also contain pre-formed MMPs (e.g. MMP-9) and their inhibitors TIMP-1 and TIMP-2, which suggests that they can modulate the ECM turnover [10]. Likewise, MCs may also release different molecules (e.g. TNF-α, TGF-β, FGF) capable of regulating ECM turnover and fibroblast function [18, 44]. MC-derived tryptase is a mitogen for lung and dermal fibroblasts [45] and stimulates their synthesis of function Type I collagen [19]. Of note, the tryptase-positive MCs we observed in IRF biopsies were degranulating, which indicates that they actively contribute to this fibro-inflammatory process.

The observed up-regulation of eotaxin/CCL11 in IRF prompted us to investigate whether this was genetically determined. Thus, we studied the potential association of IRF with six SNPs of the CCL11 gene; these SNPs were selected from the HapMap database and allowed us to infer nine different haplotypes of the CCL11 gene. None of the tested SNPs proved to be independently associated with IRF; however, haplotype analysis showed a strongly significant association with the TTCCAT haplotype. Further studies are needed to explore whether this haplotype ultimately leads to an increased synthesis of eotaxin/CCL11. Nevertheless, the protein up-regulation found at serological and tissue levels together with the strong association of IRF with the aforementioned CCL11 haplotype lend support to the hypothesis that the CCL11-related pathway is pathogenetically relevant in IRF.

IRF is thought to arise as a primary inflammatory aortic/periaortic reaction which subsequently elicits an exaggerated fibrous response [1, 46]. We found an inverse correlation between eotaxin/CCL11 levels and IRF thickness, suggesting that this chemokine is overproduced particularly in patients who present early during the course of the disease. More importantly, we found that eotaxin/CCL11 levels inversely correlated with response to steroid therapy in IRF; a similar inverse correlation has been demonstrated in patients with early rheumatoid arthritis, as those with high eotaxin/CCL11 levels had less radiographic progression [47]. When we examined eotaxin/CCL11 levels before and after steroid therapy, we found that they tended to increase. This has also been observed in asthmatic patients’ exhaled breath condensate, where eotaxin/CCL11 levels slightly increased after inhalation of corticosteroid during their treatment [48]. These data are difficult to interpret, but it is plausible that steroid therapy does not effectively suppress the pathogenetic mechanisms of IRF; this is also supported by the observation that IRF frequently relapses after steroid withdrawal, as it occurred in the majority of the patients enrolled in this study (data not shown).

In conclusion, the eotaxin/CCL11-CCR3 axis may play a pathogenetic role in IRF, and likely contributes to tissue recruitment of different cell types, including eosinophils and MCs. In addition, the TTCCAT haplotype within the CCL11 gene is significantly associated with IRF.

Acknowledgements. The authors thank Maria Nicastro and Michele Reina for their assistance with the ELISA test, Dr Francesco Bonatti for contributing to the immunogenetic analysis, Gabriella Becchi for her help in the immunohistochemical studies, and Prof. Rita Gatti for confocal microscopy analysis.

The authors are grateful to Prof. Domenico Ribatti (Department of Human Anatomy and Histology, University of Bari) for his critical revision of the manuscript. The study was supported by a MIUR (Ministero dell’Università e della Ricerca) grant (PRIN project no. 2007YA9BXE).

Conflict of interest statement. The authors declare that they have no conflicts of interest with the publication of this paper.

References


Received for publication: 16.4.2012; Accepted in revised form: 7.6.2012