Decreased levels of CCR3 in CD4+ lymphocytes of rheumatoid arthritis patients

V. Aloush[1], J. George[2], O. Elkayam[1], I. Wigler[3], S. Oren[3], M. Entin-Meer[2], S. Maysel-Auslender[2], J.N. Ablin[1]

[1]Internal Medicine 6 and Institute of Rheumatology, Tel Aviv Sourasky Medical Centre, Israel; [2]Cardiology Institute, Tel Aviv Sourasky Medical Centre, Israel; [3]Institute of Rheumatology, Tel Aviv Medical Centre, Israel.

Abstract

Objective

To evaluate the expression of CCR3 receptors as well as CCR3 agonists, including eotaxin-2 and RANTES, among patients suffering from rheumatoid arthritis and healthy controls, as a possible pathogenetic mechanism in inflammatory joint disease.

Methods

Twenty-two patients and 13 healthy controls were recruited and clinically evaluated. CCR3 expression on CD4+ lymphocytes and mononuclear cells was evaluated by FACS analysis after staining with human CD4 APC (bioscience) and human CCR3 (CD193)PE. Levels of eotaxin-2 and RANTES were analysed by ELISA.

Results

A significant decrease was observed in the level of CD4+ cells expressing the CCR3 receptor in serum of RA patients (0.96±0.5) as compared with healthy controls (1.48±0.6) ($p<0.05$). A significant decrease in serum eotaxin-2 levels was evident among RA patients suffering from active disease, defined by a DAS-28 score above 5.5, compared with RA patients with lower activity scores (2.1±1.6 vs. 7.0±5.1; $p=0.01$). A significant decrease was evident in the number of CCR3 expressing Monocytes among RA patients treated with steroids and anti TNF-α medications as compared with RA patients not receiving such treatment.

Conclusions

CCR3 is differentially expressed on inflammatory cells in RA, while eotaxin-2, a potent CCR3 agonist, is differentially expressed in active disease. Anti-inflammatory medications may down-regulate CCR3 expression in RA. The CCR3-CCR3 agonist pathway may thus have a pathogenic role in RA and may be a future target for novel treatment modalities.

Key words

Rheumatoid arthritis, CCR3 receptors, eotaxin-2, RANTES

Valerie Aloush, MD
Jacob George, MD
Ori Elkayam, MD
Irena Wigler, MD
Shirly Oren, MD
Michal Entin-Meer, PhD
Sofia Maysel-Auslender, MSc
Jacob N. Ablin, MD

Please address correspondence and reprint requests to:
Jacob N. Ablin, MD,
Department of Rheumatology,
Tel-Aviv Sourasky Medical Centre,
Weitzman St. 6,
Tel-Aviv, Israel.
E-mail: ajacob@post.tau.ac.il

Received on November 7, 2008; accepted in revised form on January 19, 2010.

Competing interests: none declared.

Background

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterised by intense destructive infiltration of synovial tissue, mediated by a broad spectrum of inflammatory cells (1). Multiple cytokines and chemokines, derived from macrophages and fibroblasts partake in this destructive cascade (2). Chemokines, which are small chemoattractant cytokines, play a central role in the interaction between leukocytes and other inflammatory cells with both endothelial cells and adhesion molecules, during the highly regulated process of migration into inflammatory sites such as the rheumatoid joint (3). The accumulation of leukocytes in the joint space leads to secretion of tissue-degrading factors, including matrix degrading enzymes. Increased concentrations of several chemokines have been detected in the synovial tissue and synovial fluid of patients suffering from rheumatoid arthritis (4) and a number of chemokine receptors have been identified on the inflammatory cells infiltrating the synovium in RA (5). Differential expression of chemokine receptors has also been demonstrated in the peripheral blood of RA patients (6, 7). Eotaxin-1 was first discovered as a powerful eosinophil - chemoattractant in the broncho-alveolar lavage fluid of sensitised guinea-pigs exposed to aerosol allergen challenge (8). This 73 amino acid C-C chemokine actively stimulates eosinophils *in vitro* and *in vivo*. cDNA for this chemokine was subsequently cloned (9) and from this sequence eotaxin homologues have been identified in several species including mouse (10) and man (11).

Eotaxin-1 is produced within the lung in a T-lymphocyte dependent process by various cell types (12). The gene encoding human eotaxin-1 is located on chromosome 17 at q21, contained within three exons (13). Tumour necrosis factor α (TNF-α) and IL-4 stimulate transcription of the eotaxin-1 gene in lung fibroblasts (14) and human airway epithelial cells (15). The promoter for the eotaxin-1 gene contains overlapping consensus binding sites for the transcription factors nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-6, which appear to mediate the responses to TNF-α and IL-4 (16), thus emphasising the intricate role of eotaxin in allergic and classic inflammatory pathways.

Two functional homologues of eotaxin-1, eotaxin-2 and eotaxin-3, have been identified (17-21). Despite bearing less than 40% identity to eotaxin-1 at the amino acid level, eotaxin-2 andeotaxin-3 possess similar eosinophil-selective properties to eotaxin-1 and also signal exclusively via CCR3. In humans, the genes encoding eotaxin-2 and eotaxin-3 localize within a 40 kilobases region on chromosome 7 at q11.2, distinct from the eotaxin-1 gene which is found on chromosome 17 at q21.1–21.2. In assays of eosinophil chemotaxis, eotaxin-1 and eotaxin-2 exhibit similar potencies, but eotaxin-3 appears to be approximately one order of magnitude less potent (22). Increased eotaxin-2 mRNA levels have been reported in bronchial biopsies taken from atopic and non-atopic asthmatics (23).

Like eotaxin-1, eotaxin-2 acts via highly specific activation of the CCR3 receptor, promoting chemotaxis and $Ca2^+$ mobilisation in human eosinophils (24). In classical animal models of allergy, eotaxin-1 mediates the rapid mobilisation of bone marrow eosinophils and trafficking into alveolar tissue (25). The chemotactic activity of eotaxin-1 is not however restricted to eosinophils; lymphocytes, monocytes, and basophils, as well as some stromal cells, such as endothelial and smooth muscle cells, are also recruited (26). In addition, eotaxin-1 has the capacity to induce diapedesis of inflammatory cells across vascular endothelium (27). CCR3, the eotaxin receptor, is expressed on a variety of inflammatory cells associated with allergic responses, including basophiles, mast cells, T-helper-2 lymphocytes, and resident tissue - cells such as airway epithelium (28). In the current study we have attempted to evaluate levels of eotaxin-2, RANTES and of the CCR3 receptor in RA patients and healthy controls.

Methods

Patients

Twenty-two patients, 4 male and 18 female, fulfilling the ACR criteria for RA

Table I. Basic demographic data of RA patients and controls.

Group	RA	Controls
n.	22	13
Age, mean (SD)	62.7 (12.4)	59.0 (13.7)
Male / Female	4/18	4/9

Table II. Clinical characteristics and use of medications among RA patients.

RF positive (%)	15 (68.2)
DAS-28 (mean; SD)	5.42 (1.44)
ESR (mean; SD)	43 (24.1)
Methotrexate, n. (%)	14 (63.6)
Hydroxychloroquine, n. (%)	3 (13.6)
Glucosteroids, n. (%)	7 (31.8)
Anti TNF-α, n. (%)	9 (40.9)
Aspirin, n. (%)	2 (9.1)

(29) were consecutively recruited from the rheumatology clinic of the Tel Aviv medical centre.

All patients gave written informed consent and the study was authorised by the institutional review board. Patients recruited included both active and inactive cases and received ongoing medical treatment at the discretion of the attending rheumatologist, including glucocorticosteroids, methotrexate and anti TNF-α medications. Three patients were receiving both glucocorticosteroids and methotrexate, while 4 were receiving both glucocorticosteroids and anti TNF-α treatment.

Patients underwent clinical evaluation on recruitment and disease activity was documented using the 28 joint disease activity score (DAS-28) (30). Patient's status regarding Rheumatoid factor and anti-CCP antibodies was documented from patient files as available and erythrocyte sedimentation rate (ESR) was measured on recruitment. Details regarding patient's co morbid conditions and medications were documented.

Thirteen healthy controls (4 male, 9 female) were recruited among the institute staff.

Evaluation of CCR3-expressing inflammatory cells in the sera of RA patients

Ten ml of blood was loaded on ficoll gradient and centrifuged. The mononuclear layer was then removed, washed with PBS and stained with human CD4 APC (bioscience) and human CCR3 (CD193)PE for 30 min followed by FACS caliber analysis. Lymphocytes and monocytes were gated based on Forward & Side scatter and then analysed for CCR3 expression.

Detection of eotaxin-2 and RANTES levels in sera

Part of the lymphocytes recovered by ficoll gradient were lysed (50mM NaCl, 50mM TRIS ph7,5; 20 mM MgCl2;0,5% TritonX, and protease inhibitor cocktail) and loaded onto an ELISA-DuoSet Human-EOTAXIN-2 kit and onto ELISA Development kit RANTES, according to the manufacturer's instructions (R&D).

Due to non-normal distribution of the data, DAS-28 values and levels of eotaxin / RANTES were log-transformed for calculation of correlation by bivariate analysis.

Table III. Levels of CCR3 positive cells among RA patients, controls, patients treated with steroids and patients treated with anti TNF-α.

	Controls	Patients	*p*-value
CD4+/CCR3 from LYM	**1.5 (0.6)**	**1.0 (0.5)**	**0.020**
CD4-/CCR3 from LYM	**4.4 (3.4)**	**4.2 (3.6)**	**0.844**
MON/CCR3	5.2 (2.0)	5.6 (1.3)	0.445
Monocytes/CCR3	3.8 (1.6)	3.4 (1.5)	0.455
	Steroid treatment	No steroids	
CD4/CCR3 from LYM	**1.2 (0.5)**	**0.5 (0.2)**	**0.003**
CD4-/CCR3 from LYM	**5.0 (3.8)**	**1.7 (1.0)**	**0.007**
MON/CCR3	5.7 (1.2)	5.3 (1.4)	0.535
Monocytes/CCR3	**3.9 (1.1)**	**2.1 (1.0)**	**0.006**
	Anti TNF	No anti TNF	
CD4/CCR3 from LYM	**1.1 (0.5)**	**0.8 (0.6)**	**0.182**
CD4-/CCR3 from LYM	**3. (1.9)**	**4.6 (4.8)**	**0.968**
MON/CCR3	5.5 (1.3)	5.7 (1.2)	0.973
Monocytes/CCR3	**3.9 (1.4)**	**2.7 (1.1)**	**0.036**

Table IV. Levels of RANTES among RA patients, controls, patients treated with steroids and patients treated with anti TNF-α.

	Controls	Patients	*p*-value
RANTES pg in 1 μg	2.4 (1.9)	2.6 (3.0)	0.904
RANTES Serum	26.7 (9.0)	32.4 (12.4)	0.167
	Steroid treatment	No steroids	
RANTES pg in 1 μg	3.2 (3.5)	1.2 (0.4)	0.106
RANTES Serum	35.1 (12..8)	23.9 (8.5)	0.156
	Anti TNF	No anti TNF	
RANTES pg in 1 μg	3.7 (3.9)	1.2 (0.5)	0.028
RANTES Serum	33.0 (16.5)	31.2 (7.2)	0.780

Statistics

One-way ANOVA was used for comparing levels of serum and cell extract parameters (serum eotaxin-2, serum RANTES, pg eotaxin per 1ng, RANTES pg in 1ug of lysates, $CD4^+/CCR3^+$ lymphocytes, $CD4^-/CCR3$ lymphocytes, $CCR3^+$monocytes). Variables were log transformed to convert them into normal distribution. The level of significance was set at $p<0.05$. Spearman rank correlation coefficients were calculated to determine associations between log-transformed eotaxin/RANTES levels and DAS-28.

Results

Table I presents the demographic data of RA patients and healthy controls. As shown, patients and controls were similar regarding age although the proportion of females was higher among RA patients compared with controls.

Table II presents the clinical characteristics of the RA patients. As presented

in the table, RA patients were suffering from moderately active disease, as shown by the mean DAS-28 value (5.42) and the mean sedimentation rate (43). 63.6% of patients were on current methotrexate treatment, 31.8% were on steroid treatment and 40.9% were on current anti-TNF-α treatment.

Tables III, IV and V present the results of eotaxin-2, RANTES and CCR3 measurements obtained, demonstrating the effect of steroid and anti TNF-α on the expression of these markers.

As noted, a significant decrease was observed in the level of $CD4^+$ cells expressing the CCR3 receptor in serum of RA patients (1.5±0.6) as compared with healthy controls (1.0±0.5)($p<0.05$) (Fig. 1).

No significant difference was found in the number of CCR3 expressing cells among $CD4^-$ lymphocytes. (This population of $CD4^-$ cells includes both CD8 cells as well as B cells). Similarly, no significant difference in the number of CCR3 expressors was observed among monocytes. No significant difference was observed in serum levels of eotaxin-2 between healthy controls and RA patients (Table I). No difference was observed in serum levels of RANTES between RA patients and healthy controls.

A significant decrease in serum eotaxin-2 levels was evident among RA patients suffering from active disease, defined by a DAS-28 score above 5.5, compared with RA patients with lower activity scores (2.1±1.6 *vs*. 7.0±5.1; $p=0.01$).

Correlation between concentration of eotaxin-2, RANTES and DAS-28

No significant correlation was found between concentration of eotaxin-2, RANTES and DAS-28 levels.

Effect of medications on CCR3 expression on inflammatory cells

As shown in Table IV, a significant decrease was evident in the number of CCR3 expressing monocytes among RA patients treated with steroids (3.9±1.1) as compared with RA patients not receiving such treatment (2.1±1.0)($p<0.01$).

Similar results were obtained for $CD4^+$/CD4- lymphocytes.

Table V. Levels of eotaxin-2 among RA patients, controls, patients treated with steroids and patients treated with anti TNF-α.

	Controls	Patients	*p*-value
Eotaxin per 1ng*	2.1	0.5	0.058
Eotaxin Serum*	2.6	2.5	0.991
	Steroid treatment	No steroids	
Eotaxin per 1ng*	0.4	0.9	0.291
Eotaxin Serum*	2.8	2.4	0.823
	Anti TNF	No anti TNF	
Eotaxin per 1ng*	0.6	0.5	0.633
Eotaxin Serum*	3.4	2.1	0.604

*variables were log transformed to convert them into normal distribution; Geometrical mean is displayed.

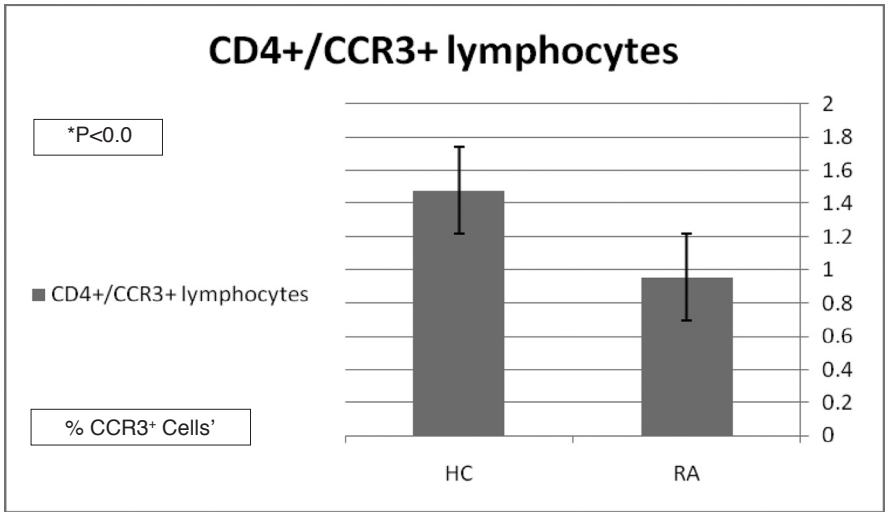

Fig. 1. Expression of CCR3 on CD4+ T cells in RA patients and healthy controls. RA: rheumatoid arthritis; HC: healthy controls; Mean ± SE.

A significant decrease in the number of CCR3 expressing monocytes was also evident in RA patients treated with anti TNF-α medications (2.7±1.1), as compared to RA patients not receiving such treatment (3.9±1.4; $p<0.05$) (Table V).

Discussion

In the current study we have demonstrated differential expression of the CCR3 receptor in CD4 lymphocytes, in RA patients compared with healthy controls. In addition, we have demonstrated a significantly reduced CCR3 expression on CD4 lymphocytes and monocytes from RA patients treated with corticosteroids or anti TNFα medications. In interpreting these results, we must consider the potential role ascribed to the eotaxin / CCR3 system in the pathogenesis of RA. Activation of the CCR3 receptor, either by eotaxin-1, eotaxin-2, RANTES or other ligands, can lead to changes in the adhesive capacity of inflammatory cells. Thus, through down-regulation of VCAM-1, eotaxin-2 stimulates eosinophils to detach from endothelial cells and migrate into tissue (31). Acting through Mitogen-Activated Protein-kinases (MAP-kinases), eotaxin-2 has also been shown to alter eosinophil adhesion patterns, shifting from a predominantly VCAM-1 to an ICAM-1 dominated pathway (32). Inhibition of the CCR3 receptor has been shown to inhibit eosinophil chemotaxis and thus is a potential therapeutic target in allergic conditions (33). It appears that this system is responsible for early changes in adhesion and migration of inflammatory cells, a conclusion which

is also supported by animal models of inflammatory arthritis.

In the Adjuvant Induced Arthritis (AIA) model, inhibition of RANTES, a CCR3 agonist, reduced joint inflammation, bone destruction, and cell recruitment (34). In this model, which is a commonly used animal model of RA, CCR3 and CCR2 have been shown to play a role in early leucocytes recruitment to synovial tissue (35).

Recently, attention has been drawn to the role played by CCR3 receptor agonists, in the pathogenesis of inflammatory joint disease including RA. RANTES (CCR5) has long been identified as differentially expressed and possibly pathogenic in RA (36, 37). MCP4/CCL13, a ligand for CCR2 and CCR3 is highly expressed in the cartilage of RA patients (38). The CC chemokine eotaxin-2/CCL11 binds to CCR3, acting as a strong chemoattractant for eosinophils (39), basophils (40) and Th2-type lymphocytes (41). Levels of eotaxin have been shown to be increased in serum of patients with early RA (42) as well as in plasma of patients with juvenile idiopathic arthritis (JIA) (43).

In view of this background it is possible that decreased CCR3 expression by CD4 cells observed in this study is a manifestation of down-regulation cause by increased activity of CCR3 agonists. Our results, demonstrating differential expression of CCR3 on inflammatory cells in RA patients, further validate the concept that activation of the CCR3/CCR3 agonist matrix is at work in RA; furthermore the observed effect of anti-inflammatory medications on CCR3 expression may be involved in the therapeutic mechanism of these medications. While we were not able in the current study to demonstrate differential expression of eotaxin-2 in RA patients compared with controls, possibly due to a relatively small sample of patients, the possibility remains that this chemokine plays a regulating role in cell trafficking in inflammatory joint disorders.

The finding of decreased expression of CCR3 on inflammatory cells among RA patients treated with corticosteroids is in line with recent evidence which indicates that both anti-CCR3 and dexamethasone are capable of down regulating CCR3 expression, while mitigating eotaxin-induced migration and differentiation (44). On the other hand, the number of CD4 T cells and CD14 monocytes expressing CCR3 has previously been shown to increase during Infliximab treatment in RA patients (45). Since only 40.1% of our patients were on anti TNF-α treatment, it is difficult to directly compare this finding with ours.

Similarly, TNF-α leads to expression of the CCR3 receptor (46) and thus neutralisation of TNF-α may be expected to decrease CCR3 expression. Anti-TNF-α treatment has the potential of inducing *in vitro* lymphocyte apoptosis in a subset of RA patients (47) and may be accompanied by down-regulation of the activating Fc-gamma receptor I on monocytes (48). It remains to be studied to what extent the manipulation of chemokine expression by corticosteroids and anti-TNF-α treatment may be significant in the anti inflammatory activity of these classes of medications in RA.

In conclusion, in the current study we have demonstrated differential expression of the CCR3 receptor on inflammatory cells in RA and the reaction of this receptor to therapeutic manipulation in this patient population. We have also demonstrated a differential expression of eotaxin-2, a potent CCR3 agonist in active versus less active RA. CCR3 and its ligands appear to have a pathogenetic role in RA and may serve in the future as therapeutic targets.

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