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RAPID COMMUNICATION

Defective eosinophil chemotaxis to eotaxin in a patient with chronic lower baseline $CD4^+$ T-lymphocytes and elevated $CD8^+$ T cells

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Background: Idiopathic selective $CD4^+$ lower baseline cell count and an increase in $CD8^+$ cells is an unusual immune defect. Whether this is a true variant of idiopathic $CD4^+$ T lymphocytopenia (ICL) or a sequelae to recurrent infections is not clear.

Objectives: The primary objective of this study was to investigate the expression and function of the cc-chemokine receptor CCR3 in eosinophils from a female patient with this disorder. A secondary objective was to study the *in vitro* ability of different cytokines to modulate the reversed CD4:CD8 ratio in this syndrome.

Participants: A female patient suffering cellular immune defect with chronic lower baseline $CD4^+$ counts with persistent increase in $CD8^+$ cells, and positive skin test and radioallergosorbent test to pollens. Seven volunteers served as controls: five healthy subjects and two allergic volunteers (one asthmatic and one rhinitic).

Results: The patient's eosinophils had defective chemotaxis, shape changes, and F-actin reorganization against eotaxin, when compared to the controls. CCR3 surface and total expression was highest in allergic subjects and least in the patient with cellular immune defect. Culture of eosinophils with interleukin-7 (IL-7), but not IL-2 for 24 hours resulted in increased expression of CCR3 in both the patient and controls as evidenced by fluorescence-activated cell scanning (FACS) analysis. Confocal microscopy demonstrated cytoskeletal changes and F-actin reorganization of the patient's eosinophils only after treatment with IL-7. Culture of the patient's lymphocytes with IL-2, IL-7, IL-10, IL-17, IL-15, or phytohemagglutinin increased the number of the patient's $CD4^+$-expressing lymphocytes as measured by FACS analysis. The potency of correcting the CD4:CD8 ratio from a baseline of 0.5 was highest with IL-10, followed by IL-15, while the rest of the cytokines had a similar ratio, being 0.8, 0.7, and 0.6, respectively.

Conclusions: Taken collectively, our preliminary results may indicate a novel role for IL-7 in enhancing the CCR3 expression and function in patients of this syndrome and highlights the novel role of IL-10 in inducing a significant increase in $CD4^+$-expressing lymphocytes from the patient when compared to controls.

Keywords: eosinophil, defective chemotaxis, CCR3, eotaxin, $CD4^+$:$CD8^+$ Ratio, IL-7, IL-10

Introduction

Patients with idiopathic chronic selective lower baseline $CD4^+$ cell count with reversed $CD4^+$:$CD8^+$ ratio is underreported in the literature. Whether these patients represent a variant of idiopathic $CD4^+$ T lymphocytopenia (ICL)[1] is not clear.

ICL syndrome is characterized by selective $CD4^+$ cells depletion but without the presence of a recognizable cause of immunodeficiency. Negative tests for HIV1 and

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HIV2, $CD4^+$ cell count less than 300 mm^3 of blood, or a total $CD4^+$ cell number less than 20% of total lymphocytes count, on two different occasions with at least a six-week interval, are essential for ICL diagnosis. Nonetheless, the clinical spectrum of ICL may range from incidental asymptomatic laboratory findings to severe cellular immune responses and serious infections.[2–5]

In a recent cohort study[6] describing the clinical course of 39 patients with ICL with follow up for a median of 49.5 months, 32 patients remained with $CD4^+$ counts less than 300 mm^3 and the counts normalized in seven patients after an average of 31 months. In addition to the $CD4^+$ lymphocytopenia, several patients also have $CD8^+$ lymphocytopenia or an increased $CD8^+$ cell count while low B or NK cell counts have also been reported in other studies.[2,4,5,7]

Localized inflammation associated with the recruitment of lymphocytes and eosinophils play an important role in the host's cellular immune response to infections. The presence of these inflammatory cells participates in the resolution of various infections. Patients with acquired immunodeficiency syndrome (AIDS) may have defective lymphocyte chemotaxis[8] and an earlier report demonstrated a defective $p56^{Lck}$ tyrosine kinase activity in a patient with ICL with severe depletion of $CD4^+$ cells and increase in $CD8^+$ cells.[7] These studies also demonstrate the defective function of the remaining $CD4^+$ cells.

A female atopic patient presented to our clinic with recurrent otolaryngologic infections and a diagnosis of airway allergy to pollens confirmed by skin test and radioallergosorbent test (RAST). The patient had suffered several mixed infections in the past 10 years including severe viral, bacterial, and fungal microorganisms for which she received one antiviral protocol elsewhere (Cartolus, Lymphomyosot, taurine, vitamin C, iron tablets, Radinorm, and selenothionine) that increased her $CD4^+$ with improvement of the CD4:CD8 ratio to 1.3 temporarily, but her general condition worsened and the antiviral regimen had to be stopped. This may indicate that the problem is at the level of the function of the cells and not the low number only. Otherwise she was treated symptomatically with antibiotics and supportive measures when needed, and she never received specific immunotherapy. Her $CD4^+$ cells remained at 28%–30% of total lymphocytes with lower border $CD19^+$ B cells but no hypogammaglobulinemia. Her persistent elevation of $CD8^+$ cell count (55%–60%) resulted in chronic $CD4^+$:$CD8^+$ ratio fluctuating between 0.4–0.6 over several years. No other abnormalities of the subtypes of lymphocytes or obvious cause of immunodeficiency could be detected (Table 1). Clinical examination in several occasions showed rhinopharyngitis atypical of allergy with occasional oral candidiasis. Nasal biopsy showed nonspecific inflammation with absence of eosinophils and more CD8 than CD4 in the biopsy tissue, which is atypical of an allergy profile. It was our hypothesis that patients with idiopathic stable lower baseline CD4 cell count may be a variant of ICL and may also exhibit defective inflammatory cellular function. One important chemokine family involved in allergic and nonallergic inflammation is the cc-chemokine involved in calling both eosinophils and T_{H2} T-lymphocytes. Eotaxin is a classical cc-chemokine that signals through CCR3 receptor expressed largely by eosinophils and to a lesser extent in lymphocytes.[9,10] Recently Rzepecka and colleagues[11] reported that helminthic infection in a murine model of allergic asthma resulted in reduction in the total number and percentage of lung eosinophils that coincided with decreased levels of eotaxin in bronchoalveolar lavage fluid (BALF), lower expression of the CCR3 receptor on eosinophils, and impaired chemotaxis of these cells toward eotaxin. Accordingly and after obtaining the consent from all participants of the current study, we studied the chemotaxis of eosinophils from this patient and compared it to the normal immunocompetent volunteers. Several cytokines related to lymphocytes proliferation and activation was tested to see their ability to modify the reversed CD4:CD8 ratio.

Table 1 Phenotypic and functional studies of the patient's peripheral blood mononuclear cells

Phenotypes %	2003		2006	2008		2009
	Sep	Nov		April	July	
CD2					84	
T3			83	84 (1846 mm^3)	69	82
DR CD19+	4	5	5	4	4	4
T4 CD4+	28	28	29	31 (681 mm^3)	33	29
T8 CD8+	59	55	52	53 (1165 mm^3)	54	59
T4/T8 Ratio	0.47	0.51	0.56	0.58	0.61	0.49
CD4+/45RO+			9		17	18
T8 cytotoxic			36			30
T8 suppressor			19			30
T8c/T8s			1.89			1
CD3+DR+			18			1
CD8-CD57+			3		15	4
D3+CD16+/CD56+			7			7
D3-CD16+/CD56+			6		21	10

Materials and methods

Reagents

The following reagents were used: eotaxin (R&D Systems, Minneapolis, MN, USA), ammonium chloride solution (Acros Organics, Morris Plains, NJ, USA), and Percoll separating solution with a density of 1.124 g/ml (Biochrom AG, Berlin, Germany). CD16 and CD3 MicroBeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Isotypes CCR3 and CCR3-PE were purchased from R&D Systems. CD3-Pacific blue, CD4-FITC, and CD 8-PE were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Phosphate-buffered saline (PBS) and Hank's buffered salt solution (HBSS) with Ca^{2+} was purchased from Lonza (Verviers, Belgium). Interleukin-2 (IL-2), IL-10, IL-15, and IL-17 were purchased from R&D Systems. IL-7 was purchased from Peprotech EC, Ltd (London, UK). Diff-Quick was purchased from Baxter Scientific (Miami, FL, USA).

Cell preparation

Eosinophils and lymphocytes were purified by Percoll solution separation from the patient with cellular immune defects and chronic lower baseline counts of $CD4^+$ cells. Seven volunteers served as controls. Two of them were allergic. One was mild asthmatic not taking inhaled corticoids and the other was rhinitic not on allergy medication. Briefly, 60 ml of heparin-anticoagulated peripheral blood were obtained by venopuncture. The blood was diluted with PBS containing 2% fetal calf serum (FCS) in the ratio of 1:1. The Percoll solution at concentration of 60% was then placed carefully in the bottom of the tube by a pipette. After centrifugation for 30 minutes at 20 °C and 800 g, a band and a pellet were obtained. The band above the Percoll layer is composed of peripheral blood mononuclear cells (PBMCs) while the pellet is a mixture of eosinophils and neutrophils. Sedemented red blood cells were removed by hypodense lysis (NH_4CL, 155 mM; $KHCO_3$, 10 mM; and EDTA, 0.1 mM). CD16 is expressed on neutrophils, but not on normal resting eosinophils. Eosinophils were further purified by negative selection immunomagnetic cell separation (MACS; Miltenyi Biotec), using anti-CD16 as described previously.[12] Eosinophils purity consistently exceeded 98%.

Chemotaxis assay

Chemotaxis assays were performed in triplicate (and in six wells from the patient) in a 48-well microchemotaxis Boyden chamber incubated in 5% CO_2 at 37 °C for 90 min. Aliquots of 29 µl of the chemotactic agent eotaxin were placed in the lower wells and 50 µl of eosinophil suspension (10^6 cells/ml) were placed in the upper wells. The two chambers were separated by a polycarbonate membranes with pore size 5.0 µm polyvinyl pyrrolidone free filter (Nuclepore, Whatman, Middlesex, UK). The controls consisted of a solution of HBSS with Ca^{2+} (pH 7.4). The filters were fixed in methanol and stained with Diff-Quick. Migrated cells adherent to the lower surface were counted in five high-power fields per well under a light microscope (5 hpf; × 400).

Actin reorganization assessment with phalloidin-FITC and cytoskeletal changes

Thirty microliters of cell suspensions at a concentration of 10^6 cells/ml were placed in 1 µ-Slide VI coated (collagen IV) cell microscopy chamber (ibidi Integrated BioDiagnostics, Munich, Germany) and left to adhere for 30 minutes. Stimulation with either buffer only or eotaxin 100 ng/ml were then performed for 15 minutes. After two washes with PBS, the cells were fixed in 4% formaldehyde for 20 minutes and permeabilized with 0.1% Triton X-100, for another 30 minutes. The cells were then stained with Alexa Fluor 488®–Phalloidin diluted 40× (Invitrogen Molecular probes; Eugene, OR, USA) for 30 minutes in the dark, washed twice with PBS and conserved in Prolong® Gold Antifade with DAPI (Invitrogen Molecular Probes) and analyzed by confocal microscopy (TCS SP2; Leica, Solms, Germany).

Cell culture

Eosinophils or PBMC (1×10^6 cells) were incubated at 37 °C–5% CO_2 with 1 ml RPMI-1640 supplemented with 100 U penicillin/ml and 100 µg streptomycin/ml (Lonza, Verviers, Belgium), 10% of inactivated fetal calf serum (Lonza) in 24-well plate (BD biosciences). Cells were stimulated with 10 µg/ml phytohemagglutinin (PHA; Biochrom AG, Berlin, Germany) or with IL-2 (1 ng/ml), or Il-7, IL-10, IL-15, IL-17 (15 ng/ml). After 24 hours of eosinophils culture or 120 hours of PBMC culture, eosinophils and nonadherent cells of PBMC culture (>90% lymphocytes) were washed twice with PBS containing 0.2% bovine serum albumin (Sigma Aldrich, St Louis, MO, USA) and analyzed for cytometry.

Fluorescence-activated cell scanning analysis

CCR3 expression on eosinophils and CD4 and CD8 expression on lymphocytes ($CD3^+$ cells) were analyzed by FACS (FACS CANTO II; BD Systems) as reported previously.[13] Briefly, stimulated or unstimulated cells were fixed with 4% paraformaldehyde in the presence (total expression)

or the absence (surface expression) of 0.1% saponin (Sigma Aldrich) for 15 minutes. The cells were then washed and incubated with the corresponding antibody for 60 minutes in ice. After two additional washes, cells were conserved in paraformaldehyde 1% and then analyzed for their fluoresence intensity.

Results and discussion

Eosinophils chemotaxis against eotaxin

Eotaxin chemoattracts eosinophil in Boyden chambers at concentrations ranging from 50 ng/ml to 500 ng/ml with optimal activity at 100 ng/ml.[14,15] Figure 1A, shows that eosinophils from the patient with idiopathic low baseline $CD4^+$ lymphocytopenia did not show chemotactic function to the optimal dose of eotaxin, while cells from five control healthy subjects demonstrated a significant chemotactic effect. The patient's eosinophils showed similar chemotactic response to eotaxin concentrations at 50 ng/ml and 500 ng/ml (data not shown). Cell migration following exposure to chemoattractants is preceded by many processes, including cytoskeletal reorganisation and cell shape changes. Rapid and reversible polymerisation of globular monomeric actin into filamentous polymeric actin (F-actin) initiates shape changes. Therefore we

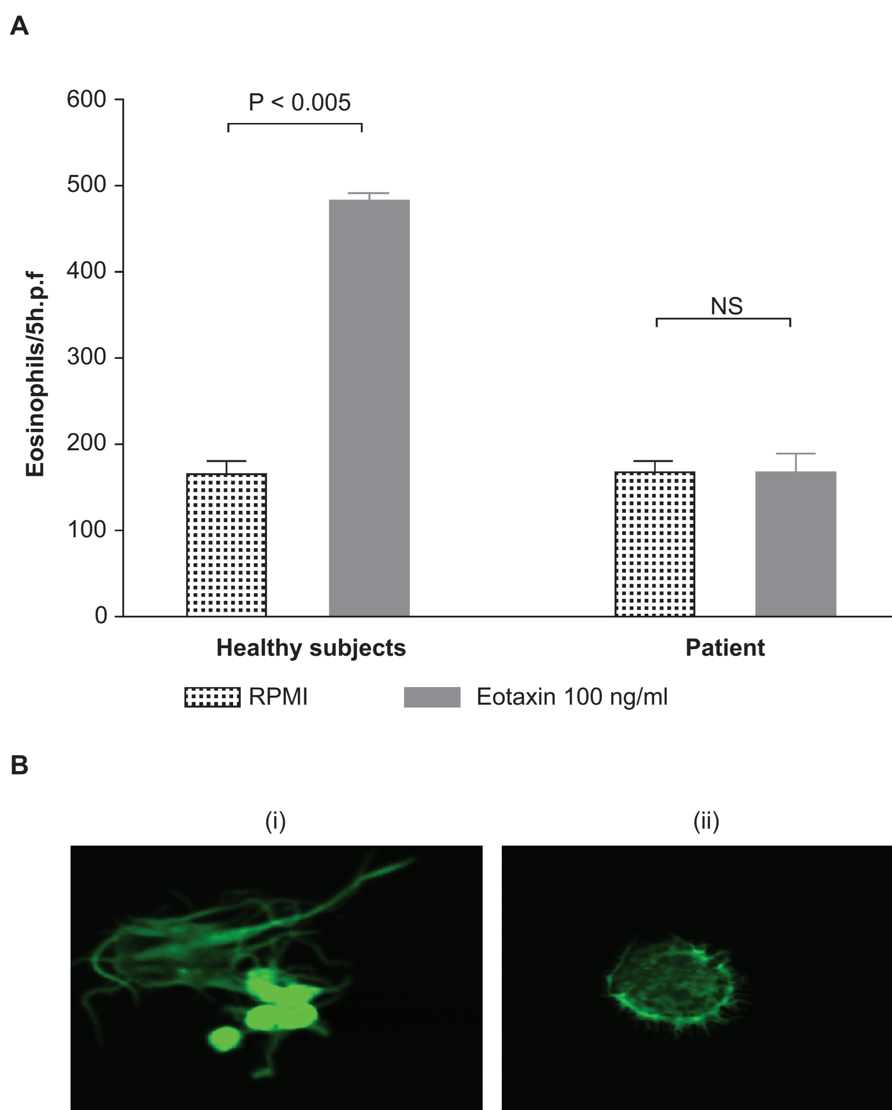

Figure 1 A) Eosinophilotactic activity of eotaxin. Results are number of eosinophils per five selected high-power fields (5 hpf; × 400) ± SEM of five independent experiments performed in triplicate from five healthy subjects. Patient results are the number of eosinophils per 5 hpf (×400) ± SEM of three independent experiments performed in six wells from the same patient (n = 1). Statistical analysis was performed by paired *t*-test. **B**) Confocal microscopy images of eosinophil cytoskeletal changes and F-actin (green color) reorganization from (i) healthy subject representative of the five healthy subjects and (ii) the single patient. Stimulation is with eotaxin 100 ng/ml. Images are from one representative of 3–6 independent experiments which all show similar images.

performed further experiments to check these cellular events. Interestingly, as demonstrated in Figure 1B, cells adherent to the collagen type 4-coated slides from control healthy subjects responded quickly to eotaxin stimulation by shape changes and F-actin reorganization while the patient's cells did not respond neither by changing their shape nor reorganizing the intracellular F-actin.

CCR3, the eotaxin receptor, is the major chemokine receptor expressed on eosinophils but also a subpopulation of Th2 lymphocytes.[16–19] CCR3 has been shown to be upregulated on monocytes and monocytoid U937 cells by interferons *in vitro*.[20] The only cells that consistently accumulate following eotaxin administration *in vivo* are myeloid cells.[21] The lack of the patient's eosinophils to respond to eotaxin may indicate that the patient's cells either express suboptimal CCR3 receptor or the receptor does not function by transmitting the signal transduction. We next explored this possibility in the following FACS analysis.

Surface and total CCR3 expression on eosinophils

As shown in table two all studied subjects including the patient expressed the CCR3 surface receptor. However, the total expression was much higher in the control subjects than the patient and highest in the allergic individuals. After 24 hours culture in buffer medium only, 2.3% of the patient's eosinophils expressed surface CCR3 and 74.7% had total expression. However, in the control subject the surface expression was 5.1% and the total was 93.3% while in the asthmatic subject the CCR3 surface expression was 11.8% and total expression was 94.7%. These results indicate that our studied patient actually had suboptimal expression of total CCR3. In the site of allergic inflammation there is a pool of cytokines that prime and activate eosinophils. This may explain the increased expression of the surface receptor in allergic individuals.

Patients with CD4 depletion due to ICL have been shown to respond well to IL-2 therapy.[22–25] Few articles also demonstrated a positive response of lymphocytes to IL-7 therapy.[26,27] Next we tested the ability of both IL-2 and IL-7 to induce the expression and/or function of CCR3 receptor on patient's eosinophils. Culture of the patient's eosinophils for 24 hours with IL-7 did modify both the total and surface expression while IL-2 increased only the total expression (Table 2). Coculture of both IL-2 and IL-7 increased the total CCR3 expression (Table 2). Only IL-7 enhanced the function of CCR3 as judged by confocal images for

Table 2 CCR3 surface and total mean fluoresence from eosinophils. Results are of three subjects representative of seven subjects. Results from the patient represent the mean values of two independent experiments. Similar results to the healthy subjects were obtained from another four controls. Similar results to the asthmatic subject were obtained from the allergic rhinitis patient

	Patient		Healthy subject		Asthmatic subject	
CCR3	**Surface**	**Total**	**Surface**	**Total**	**Surface**	**Total**
Buffer	530.5	966.5	388	1559	471	1834
IL-2 1 ng/ml	556.5	1226	465	1416	461	1414
IL-7 15 ng/ml	696.5	1495	814	906	588	2197
IL-2 + IL-7	505	1207	431	1558	499	1129

cytoskeletal changes and F-actin reorganization (Figure 2). Intriguingly, IL-7 also enhanced the surface expression of CCR3 in control subjects and demonstrated a potent effect in upregulating both the surface and the total expression of CCR3 in eosinophils from asthmatic and allergic rhinitis subjects (Table 2). Neither IL-2 alone nor IL-2 and IL-7 together modifed the CCR3 surface or total expression in controls (Table 2). Taken collectively, this may indicate a defect of the cytokine regulating the expression of total CCR3 receptor in this patient's cells or a bone marrow derived defect.[28] Further, the Dynamics of CCR3 internalization following eotaxin stimulation on human eosinophils highlights the importance of the intracellular CCR3 receptors expression.[29]

Lymphocytes modification of CD4:CD8 ratio *in vitro* by different cytokines

Finally, we tested the ability of hemopoietic cytokines to modify the reversed CD4:CD8 ratio *in vitro*. Studied cytokines included IL-10, IL-15, IL-17 in addition to IL-2 and IL-7. We also included PHA, a plant derivative that is known for its strong *in vitro* mitogenic effect on lymphocytes. Results summarized in Table 3 indicate that all tested cytokines and HPA did actually increase the percentage of the patient's cells expressing CD4, however, IL-10 and IL-15 were the most effective in correcting the CD4:CD8 ratio to 0.8 and 0.7, respectively. Nonetheless, IL-10 and IL-15 also enhanced significantly lymphocytes co-expressing both receptors ($CD4^+CD8^+$). Interestingly, lymphocytes from the control and allergic patients did not show similar kinetics of lymphocytes to IL-10, however IL-15 also increased the CD4 and the co-expression of $CD4^+CD8^+$ in the control subject much higher than

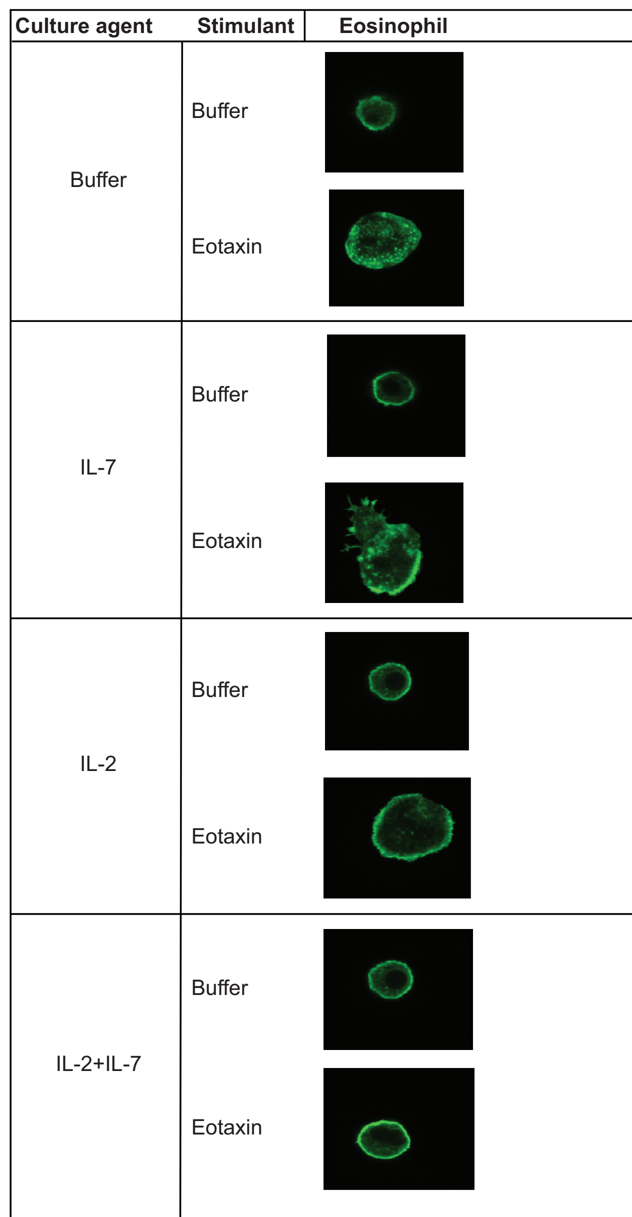

Figure 2 F-actin dynamics and cell shape changes induced by different stimulants. The time of stimulation is 15 minutes. Doses of IL-2 = 1 ng/ml, IL-7 = 15 ng/ml, or buffer (RPMI). Images are of two independent experiments from the same patient which show similar results.

the allergic patients. Taken collectively, our results indicate that IL-15 is the only tested cytokine that increased the CD4:CD8 ratio in both the patient and the control, while IL-10 showed a novel and specific improvement in the CD4:CD8 ratio in the patient only. Of note PHA increased significantly both CD4 and CD8 cells as well as the cells expressing both receptors ($CD4^+CD8^+$), therefore it did not correct the ratio significantly. PHA also increased the CD8 cells in controls and this was much higher in asthmatic than the control. This resulted actually in reduction of the CD4:CD8 ratio in controls.

The most important infective cause of $CD4^+$ lymphocytopenia nowadays is human immunodeficiency virus (HIV) infection. However, other pathogenic viral, bacterial and fungal diseases may also depress CD4 cell counts but without reversed $CD4^+$:$CD8^+$ ratio.[30] Acute cytomegalovirus (CMV) infection can cause similar reversed CD4:CD8 ratio as in our studied patient. Although one of the severe infections this patient had was CMV seven years prior, usually CMV infection induced deranged lymphocytes counts normalizes after resolution of the infection with no differences in the counts between CMV seropositive and CMV seronegative persons.[31] It is possible that this patient actually fulfilled the criterion of ICL prior to the first time the T-lymphocytes subsets were checked, and that her counts improved but never normalized. The possibility that this could be a distinct variant of ICL syndrome that present from the start with lower baseline CD4 counts and increased CD8 counts resulting in chronic reversed CD4:CD8 ratio should not be excluded. Another possibility of this chronic reversed ratio and the defective of eosinophil migration to eotaxin is the recurrent mixed infections. Infection down-regulated the immune response as production of Th1 (IFN-γ)-, Th2 (IL-4, IL-5)-, and Treg (IL-10)-related cytokines as well as IL-6 and tumor necrosis-α was diminished upon nematode infection and resulted in defective eosinophil chemotaxis in a murine model.[11]

In conclusion, we provide the first evidence of defective eosinophil chemotaxis to eotaxin in a patient with what looks like a variant of ICL. Eosinophils treatment with IL-7 increased eotaxin receptor and eosinophil cellular response to eotaxin. To our best knowledge this study provides the first evidence that show *in vitro* activity of IL-10 in correcting the reversed CD4:CD8 ratio in patients with ICL similar syndromes. Nonetheless, it is difficult to perform a statistical analysis of our preliminary but significant findings due to the low number of studied subjects. Further studies are required with a larger number of patients to obtain a reliable statistical analysis and to further characterize the immunoregulatory role of these cytokines in this cellular immune defective syndrome.

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Table 3 Effect of different cytokines on lymphocytes subtypes proliferation. Results are of one representative of two independent experiments showing similar results. The allergic rhinitis patient showed similar results to the asthmatic. The stimulants were added to the culture medium for 120 hours

Stimulant	CD4%			CD8%			CD4:CD8			CD4 + CD8 co-expression		
	A	B	C	A	B	C	A	B	C	A	B	C
RPMI	28	57	55	62	32	32	0.5	1.8	1.7	3.1	11.2	4.9
IL-2	35	58	48	58	32	34	0.6	1.8	1.4	15	12.6	5.9
IL-7	37	58	48	58	32	32	0.6	1.8	1.5	13.2	8.8	5.2
IL-10	50	57	45	60	33	36	0.8	1.7	1.3	41	11.6	8.2
IL-15	45	79	58	65	35	34	0.7	2.3	1.7	31	27.9	15.9
IL-17	35	57	48	59	31	34	0.6	1.8	1.4	12.5	8.3	5.2
PHA	42	45	52	76	39	70	0.6	1.2	0.7	32	20.7	44.2
IL-2 + IL-7	33	62	45	58	28	34	0.6	2.2	1.3	14.6	9.6	7.4

Notes: Buffer (RPMI); IL-2, 1 ng/ml; IL-7, 15 ng/ml; IL-10, 15 ng/ml; IL-15, 15 ng/ml; IL-17, 15 ng/ml; PHA, 10 μg/ml.
Abbreviations: A, the patient; B, healthy subject; C, asthmatic (N = 3).

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