



Cytokines (IL-15, IL-21, and IFN- γ) in rheumatoid arthritis: association with positivity to autoantibodies (RF, anti-CCP, anti-MCV, and anti-PADI4) and clinical activity

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Received: 7 March 2019 / Revised: 23 May 2019 / Accepted: 5 July 2019 / Published online: 17 July 2019
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Abstract

Introduction Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial membrane damage and autoantibody production. RA is a heterogeneous disease, where cytokines such as IL-15, IL-21, and IFN- γ have been associated. However, their association with the autoantibodies has not been clearly described. The aim of this study was to evaluate the relationship between the cytokines IL-15, IL-21, and IFN- γ with the autoantibodies (RF, anti-CCP, anti-MCV, and anti-PADI4) in RA and disease activity.

Methodology This study included 153 RA patients and 80 control subjects (CS). The levels of IL-15, IL-21, IFN- γ , anti-CCP, anti-MCV, and anti-PADI4 were quantified by ELISA, whereas RF was quantified by turbidimetry. The disease activity was evaluated by the indices disease activity score 28-erythrocyte sedimentation rate (DAS28-ESR), clinical disease activity index (CDAI), and simple disease activity index (SDAI).

Results The serum levels of IL-15, IL-21, and IFN- γ , and autoantibodies were increased in RA patients, compared with CS ($p < 0.05$). A correlation was found between IL-21 and anti-CCP and anti-MCV ($p < 0.05$). According to RA evolution, RF, anti-CCP, and anti-MCV had higher levels in early RA. In addition, increased levels of IL-21 were observed in RA seropositive patients (RF/anti-CCP/anti-MCV). The higher levels of both cytokines and autoantibodies were observed in moderate activity, evaluated by the three indices.

Conclusions Our results suggest that the increased soluble levels of IL-15, IL-21, and IFN- γ are involved in the inflammatory network in RA. However, IL-21 serum levels are associated with higher titers of autoantibodies (RF, anti-CCP, and anti-MCV) and IL-15 with moderate activity.

Key Points

- IL-15, IL-21, and IFN- γ are associated with the immunopathology of RA, but not significantly with the evolution of the disease.
- RF, anti-CCP, and anti-MCV had higher levels in early than established RA.
- IL-21 has an association with RF, anti-CCP, and anti-MCV, for this reason, could be proposed as a disease biomarker.
- Patients with activity moderate of disease showed higher levels of RF, anti-CCP, anti-MCV, and IL-15.

Keywords Anti-CCP · Anti-MCV · Autoantibodies · Cytokines · Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is an autoimmune, inflammatory, chronic, and a systemic disease characterized by synovial membrane damage and autoantibody production. The interaction of genetic, environmental, and hormonal factors, as well as the exacerbated activation of the immune system, leads to the production of both humoral and cellular mediators that promote the disease progression [1].

The worldwide RA prevalence is approximately 1%, but a study conducted in Mexico reports a prevalence of 1.6% [2], while another described in Latin-American indigenous groups reports a prevalence of 1.3% [3]. The RA classification is based on the counting of damaged joints, acute-phase reactant values, evolution time, and the presence of autoantibodies [4].

Several cytokines have been associated with the development, establishment, and severity of RA. IL-15 is a cytokine that is expressed by myeloid cells and is responsible for the proliferation and survival of NK cells, memory CD8⁺ T cells, and invariant NKT cells [5]. In RA, IL-15 levels are elevated and have been associated with joint destruction and lymphocyte activation promoting a proinflammatory environment [6, 7]. Besides, there are cytokines such as IL-21 with a proinflammatory role, which is mainly produced by Th17 cells and Tfh cells. In RA, it has been involved in activation of Th17 cells, macrophages, B cells, and Tfh cells, as well as in stimulating them for the secretion of other cytokines by the activation of STAT-3, PI3K/Akt, and MAPK pathways. It has also been associated with inflammatory and osteoclastogenesis processes [8]. IL-21 intervenes in turn in B cell proliferation and autoantibody production [9, 10]. In addition, IFN- γ promotes antigenic presentation and participates in inflammatory and antiviral processes [11]. In RA, its central effect is to orchestrate the proinflammatory environment in acute stages. This involves the regulation of B cells and the production of antibodies. It also promotes the shift towards an IgG isotype [12].

With regard to the autoantibodies related to the pathogenesis of RA, the most studied are rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies [13, 14]. These autoantibodies have been used for the RA classification; moreover, particularly RF has allowed classifying these patients through an easy test; nevertheless, its specificity is not so high. On the other hand, anti-CCP has been associated with severity increase and has been proposed as a poor prognosis factor [15, 16]. Recently, anti-mutated citrullinated vimentin (anti-MCV) antibodies [17, 18] and antibodies against enzymes such as peptidyl arginine deaminase 4 (anti-PADI4) [19–21] have been evaluated in RA, and association with clinical and prognostic variables has been found. The

profile of these autoantibodies has already been described in our population, determining their high specificity and sensitivity for RA [22].

Based on this knowledge and to understand the landscape of the pathophysiology of RA and how these cytokines are involved in the production of autoantibodies and severity of diseases, we decided to investigate the relationship between the IL-15, IL-21, and IFN- γ cytokines with the autoantibodies (RF, anti-CCP, anti-MCV, and anti-PADI4) and disease activity in RA patients.

Material and methods

Patients

One hundred fifty-three RA patients were included and classified according to the American College of Rheumatology and the European League Against Rheumatism (ACR-EULAR) 2010 criteria. The RA patients were recruited in the period from September 2015 to June 2017, from the Rheumatology Department of the Hospital Civil de Guadalajara “Fray Antonio Alcalde,” and were stratified into two groups according to the disease evolution: early RA (< 1 year) and established RA (> 1 year). In parallel, we evaluated 80 control subjects (CS) without a family history of autoimmunity, who matched age and gender with RA patients. All individuals were from western Mexico, over 18 years of age; they have written an informed consent letter, in agreement with the Code of Ethics of the World Medical Association (Declaration of Helsinki, Brazil 2013). The ethics, research, and biosafety committees of Hospital Civil de Guadalajara “Fray Antonio Alcalde” (Reg. No. 037/16) approved this study.

Clinical assessment

Patients were interviewed and evaluated for the disease by a rheumatologist at the time of sampling. The clinical activity was evaluated by three indices: (a) disease activity score 28 (DAS28)-erythrocyte sedimentation rate (ESR) (DAS28-ESR), (b) clinical disease activity index (CDAI), and (c) simple disease activity index (SDAI) [23, 24].

Cytokine quantification

Blood and serum samples were obtained from individuals of both study groups. Serum cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BioLegend): IL-15 (detection limit 4 pg/mL), IL-21 (detection limit 16 pg/mL), and IFN- γ (detection limit 4 pg/mL),

according to the manufacturer's instructions. The analysis was carried out in a spectrophotometer (Synergy HT, Biotek).

ESR, CRP, and autoantibody quantification

Acute-phase reactants, ESR and CRP, and autoantibodies were measured in serum. ESR rate was performed by Wintrobe method (reported in mm/h), and CRP was assessed by turbidimetric assay (BioSystems) (reported in mg/L, detection limit 6 mg/L). Rheumatoid factor (RF; isotype IgM) detection was performed by turbidimetric assay (BioSystems) on automatic equipment; the cut-off value for positivity to RF was 30 IU/mL (detection limit 2 IU/mL). The anti-CCP antibodies (isotype IgG) were quantified by an ELISA kit (Axis-Shield Diagnostics/Euroimmune); the cut-off value for anti-CCP positivity was 5 U/mL (detection limit 1.04 U/mL). The anti-MCV antibodies (isotype IgG) were measured using an ELISA kit (Orgentec Diagnostika); the cut-off value for anti-MCV positivity was 20 U/mL (detection limit 1 U/mL). The anti-PADI4 antibodies were detected by an ELISA kit (MyBiosource); this assay does not have a cut-off value, so we use the 95th centile of the control group as a cut-off value (4.96 ng/mL) (detection limit 0.04 ng/mL). These protocols were performed according to the manufacturer's instructions.

Statistical analysis

The variables were analyzed according to their characteristics. Descriptive statistics were used for clinical data. The Mann-Whitney *U* test was used for comparison of non-parametric data, and chi-square test for comparison of frequencies. A *p* value < 0.05 was considered statistically significant. All the statistical analyses were performed using the GraphPad Prism 6 and SPSS 15.0 software.

Results

Demographic and clinical characteristics

In the RA group, the mean age was 47 (18–78) years old, 88% was female and 12% male; in the CS group, the mean age was 43 (18–64) years old; 81% was female and 19% male. The smokers were more frequent in the RA group than in the CS (27% vs 10%, *p* = 0.0004). The median value (p05–p95) of the inflammatory markers, ESR and CRP, was 31 (10–60) mm/h and 8.9 (1–62) U/mL, respectively, in RA vs 14.5 (6–35) mm/h and 2.8 (0.5–7) U/mL in CS (*p* < 0.0001). These data, as well as the time of disease evolution (years), clinical activity, and pharmacological treatment, are shown in Table 1.

Regarding the clinical assessment, the median for DAS28-ESR was 3.5, CDAI 8.0, and SDAI 9.5. Eighty-four percent of the patients were under treatment with disease-modifying anti-rheumatic drugs (DMARDs), 7% were under treatment with monotherapy, and 93% were under treatment with combination therapy. The most frequent treatment was methotrexate (MTX) + sulfasalazine (SSZ) + chloroquine/hydroxychloroquine (CQ/HQ). On the other hand, 28% of the subjects with RA reported a family history of autoimmunity.

In addition, RA patients were classified according to the years of evolution, as early RA (< 1 year) and established RA (> 1 year). No significant differences were observed in the comparison of these two subgroups in all these variables (Table 1).

Cytokine levels

The serum levels of IL-15, IL-21, and IFN- γ were evaluated in CS and RA patients. A significant increase in the levels of IL-15, IL-21, and IFN- γ was observed in RA patients compared with CS (mean 80.5 vs 31.5 pg/mL; 238.9 vs 21.7 pg/mL; 34.7 vs 12.2 pg/mL, respectively) (Fig. 1a–c). According to the evolution of the RA, no differences were found between early and established RA (data not shown). In addition, the frequencies of individuals were evaluated in whom the serum levels of cytokines were detected: in CS, 37 (46.2%), 14 (17.5%), and 78 (97.5%) vs 131 (85.6%), 106 (69.3%), and 148 (96.7%) in RA, for IL-15, IL-21, and IFN- γ , respectively. Significant differences only were observed in relation to IL-15 and IL-21 (*p* < 0.0001). This analysis was performed comparing early and established RA patients; no significant difference was observed.

Autoantibody levels

As expected, the levels of all autoantibodies, RF, anti-CCP, anti-MCV, and anti-PADI4, were higher in the RA group than in the CS (*p* < 0.0001). In early RA, higher levels of RF (median of 90.2 vs 73.8 U/mL, *p* = 0.046), anti-CCP (median of 149.1 vs 80.1 U/mL, *p* = 0.030), and anti-MCV (median of 526.9 vs 178.5 U/mL, *p* = 0.011) were observed than in established RA. In contrast, the higher levels of anti-PADI4 were observed in established than in early RA (median of 3.4 vs 3.1 ng/mL, *p* = 0.15); however, it was not significant (Table 2).

In addition, we evaluated the frequency of seropositive individuals for each autoantibody in both study groups. In the RA group, the higher frequencies were anti-MCV (88%), anti-CCP (83%), and RF (77%), while anti-PADI4 was less frequent (17%). In CS, the frequencies were anti-CCP (13%), anti-MCV (4%), anti-PADI4 (3%), and RF (0%). In order to know the autoantibody profile in our RA population, we

Table 1 Demographic and clinical features in CS and RA patients

Variables	CS total <i>n</i> = 80	RA total <i>n</i> = 153	<i>p</i>	RA by years of disease evolution		<i>p</i>
				Early RA <i>n</i> = 20	Established RA <i>n</i> = 133	
Demographics						
Age mean (range)	43 (18–64)	47 (18–78)	NS	43 (26–57)	48 (18–78)	NS
F/M (%)	81/19	88/12	NS	95/5	82/18	NS
Ever smoking (%) (<i>n</i>)	10 (7)	27 (41)	0.0004	25 (5)	27 (36)	NS
Inflammation markers						
ESR (mm/h), median (p05-p95)	14.5 (6–35)	31 (10–60)	<0.0001	29 (10–63)	32 (8–64)	NS
CRP (U/mL), median (p05-p95)	2.8 (0.5–7)	8.9 (1–62)	<0.0001	7.3 (1.4–41.8)	9.5 (1–81.5)	NS
Clinical activity						
RA duration (years) mean (range)	–	6.8 (0.25–35)	NA	0.75 (0.5–1)	7 (1.1–35)	NA
DAS28 ESR median (p05-p95)	–	3.5 (1.7–6.5)	NA	3.8 (1.7–6.6)	3.4 (1.7–6.8)	NS
CDAI median (p05-p95)	–	8 (0–37.9)	NA	8.5 (0.2–40.3)	8 (0–38)	NS
SDAI median (p05-p95)	–	9.5 (0.9–41.0)	NA	12 (0.7–42.4)	9 (0.7–41.5)	NS
Pharmacological treatment						
Without treatment (%) (<i>n</i>)	–	16 (24)	NA	35 (7)	13 (17)	NA
With treatment (%) (<i>n</i>)	–	84 (129)	NA	65 (13)	87 (116)	NA
Monotherapy	–	7 (9)	NA	13 (2)	6 (7)	NA
Combined therapy	–	93 (120)	NA	87 (13)	94 (107)	NA

CS, control subjects; RA, rheumatoid arthritis patients; F, female; M, male; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, disease activity score 28; CDAI, clinical disease activity index; SDAI, simple disease activity index; NA, not apply; NS, not significant. Treatment: different combinations (methotrexate, sulfasalazine, chloroquine, prednisone). Statistical analysis was performed through the Mann-Whitney *U* test

formed groups according to the positivity/negativity to RF, anti-CCP, anti-MCV, and anti-PADI4 of each individual. The following groups were the most frequent and considered for analysis: (a) seronegative to the four autoantibodies (*n* = 7, 5%); (b) seropositive to RF and anti-MCV (*n* = 6, 4%); (c) seropositivity to anti-CCP and anti-MCV (*n* = 14, 9%); (d) seropositive to RF, anti-CCP, and anti-MCV (*n* = 85, 55%); and (e) individuals seropositive to the four autoantibodies (*n* = 19, 12%). The rest of the RA patients (15%) were seropositive to different combinations of autoantibodies in a range of 0.6 to 2%. In CS, we did not find any combination of positivity for these autoantibodies. According to evolution, the percentages of seropositivity were similar between early and established RA patients.

Cytokines and autoantibodies

Cytokine levels were analyzed in subgroups of seropositive and seronegative for each autoantibody: RF, anti-CCP, anti-MCV, and anti-PADI4. The IL-21 levels were higher in anti-CCP seropositive RA patients as well as in anti-MCV seropositive patients (*p* = 0.003 and *p* = 0.047, respectively), meanwhile, the higher levels of IFN- γ were observed in anti-PADI4 seronegative RA patients (*p* = 0.025) (Fig. 2). IL-15 did not show differences with any autoantibody (data not shown). Moreover, we evaluated the levels of cytokines

according to most frequent groups of the autoantibody combinations described in the previous section. The levels of IL-21 were significantly higher in the RF/anti-CCP/anti-MCV seropositive RA patients than in the other groups. The levels of IL-15 and IFN- γ were also higher in this group compared with the other groups, but it was not statistically significant (Fig. 3).

Disease activity: cytokines and autoantibodies

Once the levels of both cytokines and autoantibodies were determined, we proceeded to estimate if there was any association between these levels and the disease activity evaluated by DAS28-ESR, CDAI, and SDAI. First, the frequencies of patients with detectable levels of cytokines were evaluated, and patients with low activity were more frequent than remission, moderate, and high activity by SDAI (*p* = 0.038). In another index, it did not show differences in relation to detectable cytokine levels. IL-15 and IFN- γ did show association with the activity of the disease; however, IL-21 showed no relationship with the activity of the disease in any index.

In disease activity evaluated by DAS28-ESR, IL-15 was found in higher levels in patients with moderate activity than in those with low and high activity (median 65.6 pg/mL vs 31.5 pg/mL (*p* = 0.025) and vs 35.1 ng/mL (*p* = 0.02), respectively). IFN- γ showed higher levels in patients with low

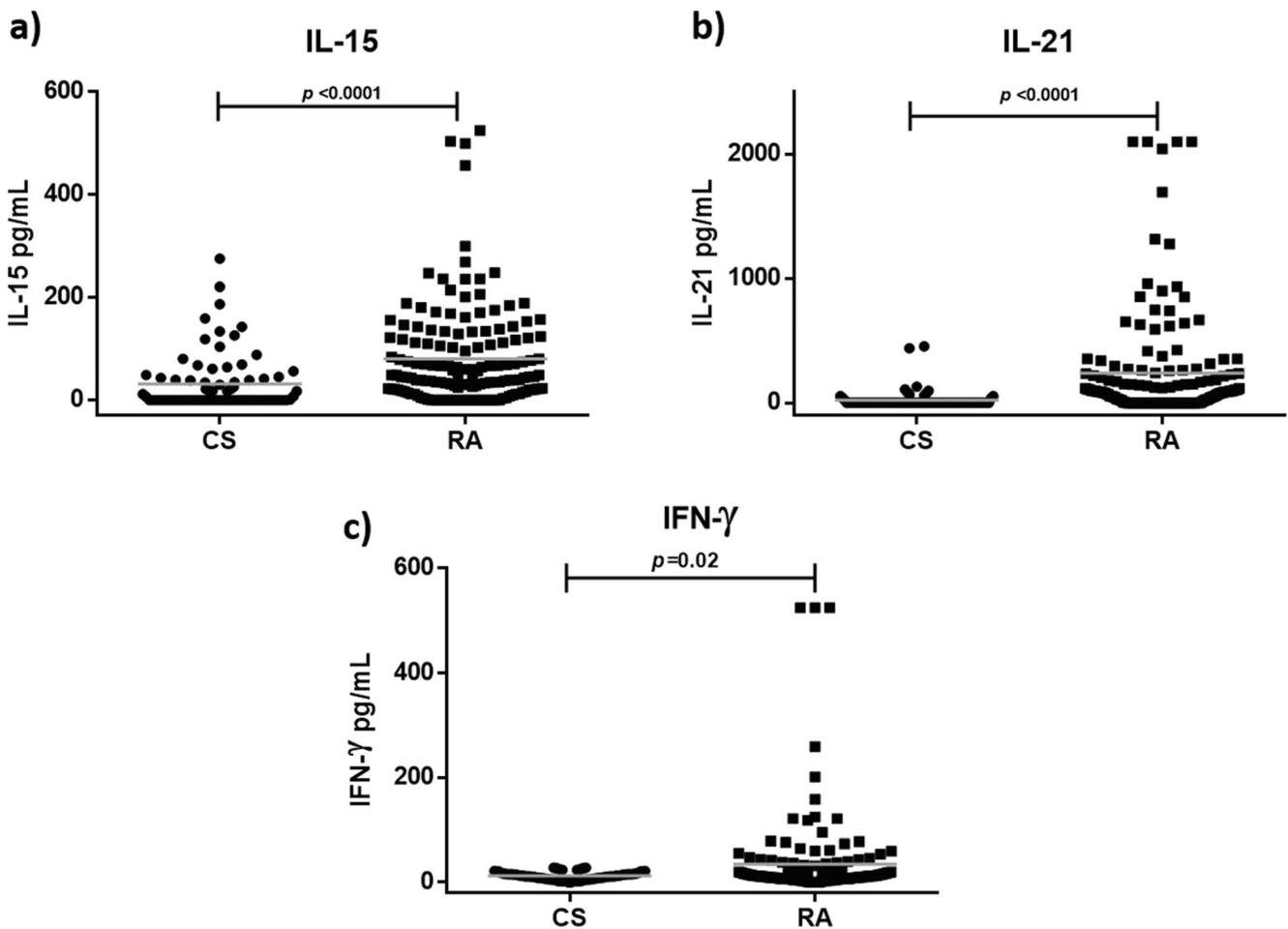


Fig. 1 Cytokine levels in CS and RA patients. **a** IL-15. **b** IL-21. **c** IFN- γ . The data are shown in mean (gray); the statistical analysis was performed through the Mann-Whitney *U* test

activity than in those with high activity (18.4 pg/mL vs 11.1 pg/mL, $p = 0.022$).

Regarding CDAI, IL-15 showed higher levels in patients with moderate activity than in those with low and high activity (median 77.6 pg/mL vs 37.5 pg/mL ($p = 0.026$) and vs 33.5 ng/mL ($p = 0.014$), respectively). IFN- γ showed lower levels in patients with high activity than in those with low and moderate activity (10.3 pg/mL vs 15.6 pg/mL ($p = 0.016$) and 13.9 pg/mL ($p = 0.035$)).

In SDAI evaluation, IL-15 showed higher levels in patients with moderate activity than in those with low and high activity (median 80.2 pg/mL vs 37.5 pg/mL ($p = 0.022$) and vs 31.1 ng/mL ($p = 0.007$), respectively). IFN- γ showed lower levels in patients with high activity than in those with low and moderate activity (9.4 pg/mL vs 17.4 pg/mL ($p = 0.003$) and 14.3 pg/mL ($p = 0.008$)).

With respect to autoantibody levels, RF was found to have significantly increased levels in patients with moderate

Table 2 Autoantibodies in CS and RA patients

	CS	RA	<i>p</i>	Early RA	Established RA	<i>p</i>
Autoantibodies						
RF (U/mL)	5.7 (4.2–15.5)	77.5 (8.5–98.7)	< 0.0001	90.2 (6.3–99.0)	73.8 (9.0–98.6)	0.046
Anti-CCP (U/mL)	0.4 (0–16.5)	83.9 (1.2–296.6)	< 0.0001	149.1 (0.7–654.7)	80.1 (1.5–210.0)	0.030
Anti-MCV (U/mL)	4.9 (0.6–19.35)	202.4 (13.9–1538)	< 0.0001	526.9 (13.9–5264)	178.5 (11.3–1191)	0.011
Anti-PADI4 (ng/mL)	2.6 (0.01–5.4)	3.4 (1.3–7.1)	< 0.0001	3.1 (1.2–6.2)	3.4 (1.3–7.6)	NS

The data are shown in the median and p05-p95. CS, control subjects; RA rheumatoid arthritis patients; RF, rheumatoid factor; anti-CCP, antibodies against cyclic citrullinated peptides; anti-MCV, antibodies against mutated citrullinated vimentin; anti-PADI4, antibodies against peptidyl arginine deiminase 4. NS, not significant. Mann-Whitney *U* test

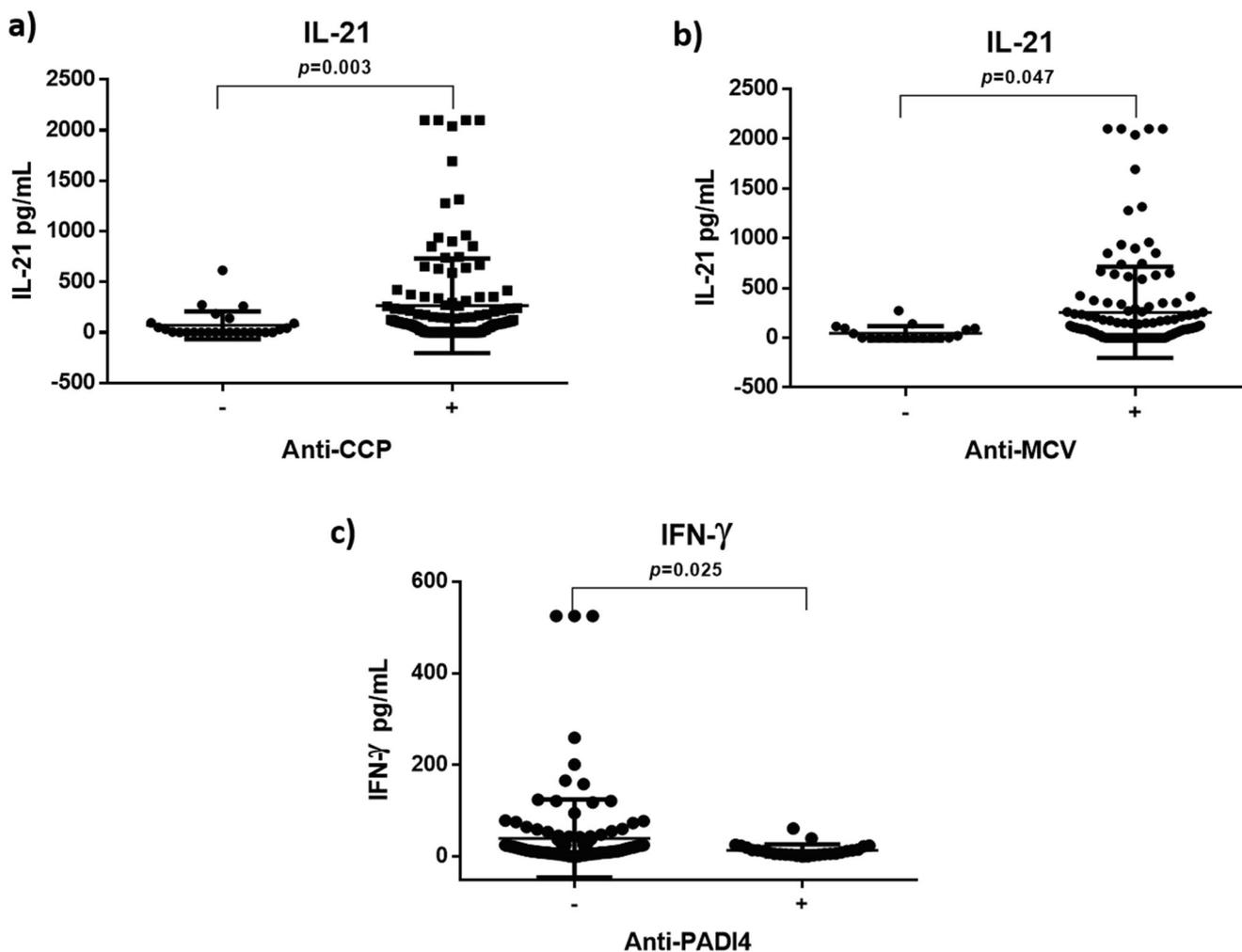


Fig. 2 IL-21 and IFN- γ serum levels in seronegative and seropositive RA patients. **a** Levels of IL-21 in anti-CCP seronegative and seropositive patients. **b** Levels of IL-21 in anti-MCV seronegative and seropositive

patients. **c** Levels of IFN- γ in anti-PADI4 seronegative and seropositive patients. The data are shown in mean and standard deviation. The statistical analysis was performed through the Mann-Whitney U test

activity than in those with low activity evaluated by DAS28-ESR and by SDAI ($p=0.02$) and also in moderate activity compared with low and high activity evaluated by CDAI ($p<0.05$). Anti-CCP only showed significantly higher levels in moderate activity evaluated by CDAI ($p<0.01$). Anti-MCV showed higher levels in moderate activity compared with remission evaluated by DAS28-ESR ($p=0.02$) and in moderate activity compared with low and high activity evaluated by CDAI ($p<0.05$). Anti-PADI4 showed higher levels in patients with moderate activity than with low and high activity evaluated by CDAI ($p<0.05$). Anti-CCP, anti-MCV, and anti-PADI4 levels did not show significant differences in evaluation by DAS28-ESR and SDAI.

Discussion

Rheumatoid arthritis is a multifactorial disease based on clinical, radiological, and functional parameters for its

classification; moreover, several serological factors have been described in the pathogenesis of RA. The study of the dichotomy that exists between the cellular and humoral immune responses in RA has allowed identifying some molecules (such as cytokines and autoantibodies) that are associated with the progression of the disease, and some of them have culminated until their application as therapeutic targets, and others are used as biomarkers [1]. The secretion of cytokines is a factor that can intervene in the production of autoantibodies, so it is necessary to define which could be involved in this process. For this reason, we decided to evaluate IL-15, IL-21, and IFN- γ , relevant cytokines in RA, and their relationship with the autoantibody production.

In this study, the levels of IL-15, IL-21, and IFN- γ were higher in individuals with RA compared with CS, which demonstrates their participation in the pathogenesis of RA and is consistent with previous reports. In relation to this, IL-15 is secreted in proinflammatory environments and helps mediate the function of T cells and NK cells, which are involved in the

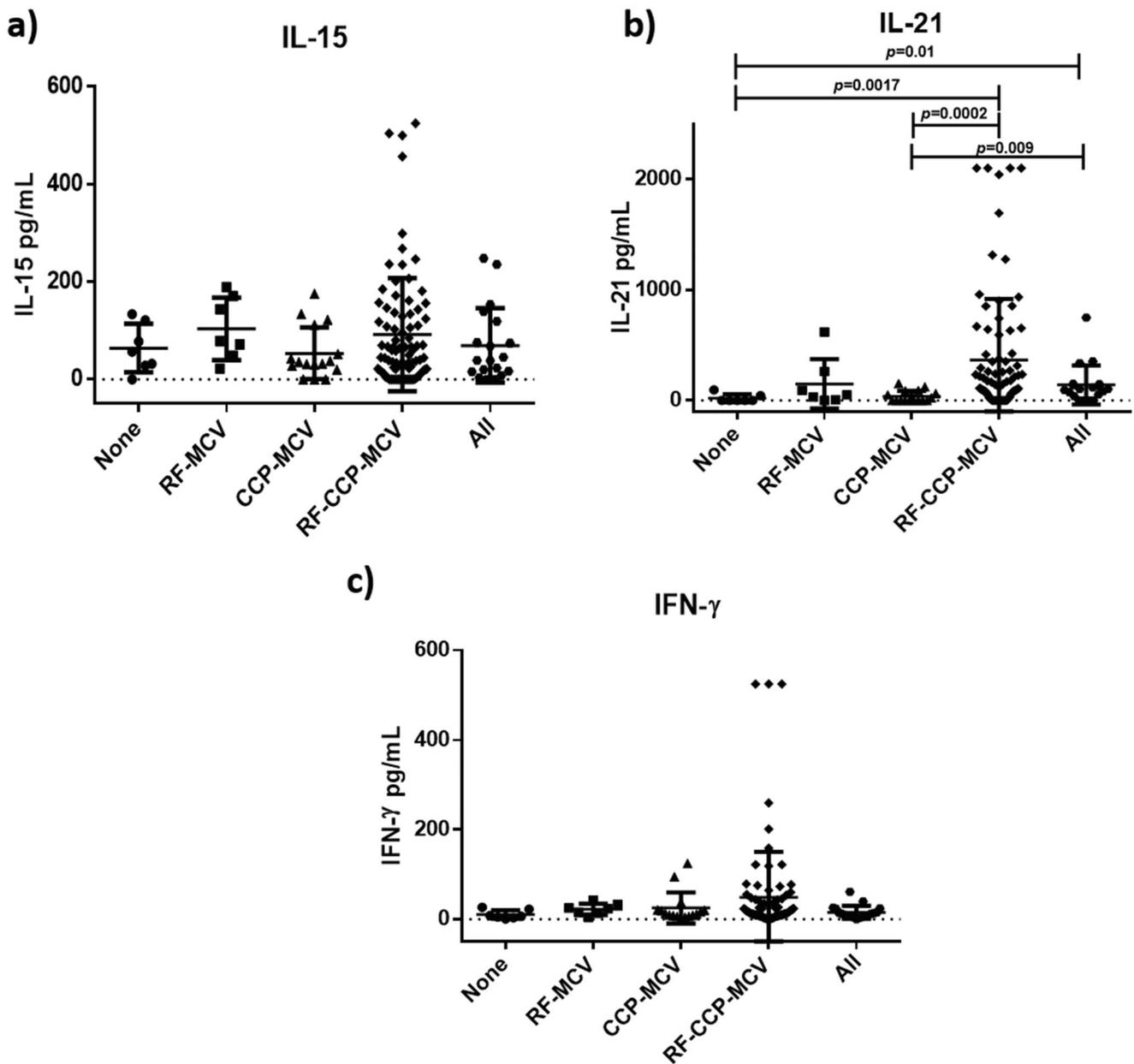


Fig. 3 Cytokine levels of RA patients in groups of autoantibody combinations. **a** IL-15. **b** IL-21. **c** IFN- γ . None, seronegative to all autoantibodies; RF/MCV, RF and anti-MCV seropositive; CCP/MCV, anti-CCP and anti-MCV seropositive; RF/CCP/MCV, RF, anti-CCP,

and anti-MCV seropositive; All, seropositive to all autoantibodies. The data are shown in mean. The statistical analysis was performed through the Mann-Whitney *U* test

immunopathology [6, 7]. Also, osteoclastogenesis and bone loss that occur in RA are some of the processes that are related to the high production of IL-15 [25]. IL-21 has characterized it as an important molecule in the development and progression of RA, because it not only favors the proliferation and activation of immune cells and fibroblast-like synoviocytes but also promotes the humoral response [8, 26, 27]. Other autoimmune diseases, such as systemic lupus erythematosus and Sjögren’s syndrome, and inflammatory pathologies have been associated with high levels of IL-21 [28–30]. Only one study indicates that the serum levels of this cytokine were lower in RA

patients compared with CS related to an increase in the population of regulatory B cells [31]. The elevated levels of IFN- γ suggest that the proinflammatory profile is prevalent even at a systemic level. The T cells and NK cells mediate the secretion of this proinflammatory cytokine mainly. These subpopulations are actively involved in the RA immunopathology [32, 33]. Consistent with our results, Pavlovic et al. reported higher levels of this cytokine in RA than in CS ($p < 0.001$) [34].

On the other hand, the cellular response mediated by the secretion of cytokines can lead to regulation of humoral response mechanisms [33, 35, 36]. Thus, we analyze whether

the increased levels of IL-15, IL-21, and IFN- γ could be related to the production of autoantibodies. In our results, the levels of RF, anti-CCP, anti-MCV, and anti-PADI4 were higher in RA patients than in CS. In addition, RF, anti-CCP, and anti-MCV levels were higher in early RA. Some studies have suggested the role of anti-CCP as a biomarker in early RA [37, 38], while other studies emphasize that there is no association of these autoantibodies with established RA severity [39]. Our results regarding RF and anti-CCP seropositivity frequencies are higher than those previously reported by Reyes-Castillo et al. [40]. It has been shown that these autoantibodies can act as predictors of poor prognostic factors for RA, such as erosions, even with levels that develop in early stages [41]. Unfortunately, we could not associate these variables with progression or severity, because other data of poor prognosis like erosions were not obtained.

The anti-MCV autoantibodies have been strongly associated with specificity in RA, as well as their role in early RA has gradually become known as a severity and progression biomarker [42]. In our study, patients with RA showed high levels of anti-MCV, even more than reported in other studies [43, 44]. Likewise, the seropositivity frequencies in our study were higher. These data agree with the percentages of seropositivity in other countries where it ranges from 42 to 96% [45]. Which suggests that in our population could be considered as a RA biomarker.

With regard to anti-PADI4, the analyses showed increased levels in patients with RA, which agrees with a study conducted in the same population; nevertheless, our seropositivity percentages are lower [40], which could be explained by the difference of cut points and ELISA kits used for their quantification. However, the specificity (95%) and sensitivity (17%) agree with those reported in other studies [40, 46, 47].

The frequency of seropositivity to the different combinations of autoantibodies were analyzed, and triple seropositive (RF/anti-CCP/anti-MCV) was the most frequent with 55%. This is similar to that reported by Gonzalez-Lopez et al. with 53.5% [44] and higher than reported by Reyes-Castillo et al. with 38% [40], both studies conducted in the same population. The frequency of seronegative patients was only 5%. In a study that only included anti-CCP and anti-MCV, they report a 34% seronegativity in Europeans [48] and in western Mexicans, 12% seronegativity to RF, anti-CCP, anti-MCV, and anti-PADI4 [40].

In the analysis of cytokine levels and their relationship with autoantibodies, higher levels of IL-15 only in triple seropositive patients (RF/anti-CCP/anti-MCV) were observed; however, it was not statistically significant. This could be explained by mechanisms that have been described in the development of memory B cells in vitro [49].

Exclusively in the case of seropositivity to anti-CCP and anti-MCV, higher levels of IL-21 were found in RA patients compared with CS, as well as a positive correlation with anti-

CCP but not with RF [26]. Contrary to expectations, IL-21 did not obtain its highest levels in those patients seropositive to all autoantibodies; these were in the triple seropositivity RF/anti-CCP/anti-MCV, so this cytokine is probably more involved in the production of these autoantibodies.

A study concluded that IL-21 promotes the differentiation of plasmablasts and with it an increase in IgG and IgM levels [26]. Therefore, this would be promoting the increase in the production not only of anti-CCP but also of other autoantibodies such as anti-MCV. This suggests their participation in the loss of tolerance and the formation of antibodies against their own proteins, B cell activation, and radiographic progression in RA [10, 50, 51]. IFN- γ , in addition to being considered as a cytokine with the immunoregulatory role, has studied the mechanisms by which when produced by B lymphocytes, could promote the development of arthritis in murine models and therefore the activation of T cells and an increase of autoantibodies [52]. Also, in humans, it can intervene in the change of isotype to IgG in general. However, increased levels of IFN- γ were found in seronegative anti-PADI4 patients, and no difference was found with any other autoantibody. So, its role in this autoantibody production is not clear. On the other hand, a study suggests that this cytokine has no association with anti-PADI4 [40].

In seropositive patients to RF, anti-CCP, and anti-MCV, we found higher levels of IL-15, compared with seronegative patients, however, without significant differences. In contrast, the serum levels of the three cytokines were lower in those individuals seronegative to RF, anti-CCP, anti-MCV, and anti-PADI4; however, only IL-21 obtained significant differences. To date, the phenotypes of seropositive and seronegative RA have not been established molecularly, so that cytokines such as IL-21 could give an overview of the humoral behavior in the pathology and its association with the production of autoantibodies, as suggested in other diseases [53–55].

According to cytokine levels and disease activity, increased levels of IL-15 and IFN- γ were found in moderate activity, evaluated by the three indices. IL-15 has already been related to high disease activity [6]. In contrast, some authors have reported that there is a negative correlation of this cytokine with the DAS28 [34]. With respect to IL-21, we did not find any association with the activity of the disease; however, some studies have reported that high levels of IL-21, as well as high titers of autoantibodies against IL-21, correlate with DAS28 [56, 57]. This could be because IL-21 remains in constant production for the maintenance of the humoral response in our patients. It is important to highlight that the seronegative group did not show differences between treatment and cytokine levels, nor in the activity of the disease, so it will be important to deepen our understanding in its pathophysiology.

These findings will allow us to evaluate more accurately the RA population, which will allow an adequate diagnosis and improve the patient's approach. It is still necessary to

define more specific criteria in our population, as well as the proper mechanisms for the transfer of this information to the clinic. So, we can suggest that IL-15, IL-21, and IFN- γ have an important role in the RA immunopathology. On the other hand, the increase of IL-21 promotes the production of autoantibodies; this is reflected in RF/anti-CCP/anti-MCV seropositive individuals; for this reason, the quantification of IL-21 and the co-detection of RF, anti-CCP, and anti-MCV could be a better complement for RA diagnosis. Likewise, these three autoantibodies are the same as those found in early stages, suggesting that IL-21 could be a marker of humoral progression in RA. Moreover, further studies are required to understand the possible involvement of IL-21 on memory B cell compartments (IgG, IgM, and IgA) and plasmablast cells (IgG, IgA and, IgM) in the context of RA immunopathology and production of autoantibodies.

Acknowledgments RPIV was a Ph.D. CONACYT fellow (Reg. 575070).

Funding This research was performed with the financial support of the PRO-SNI 2016–2017 program from Universidad de Guadalajara granted to SHPE and RDMG.

Compliance with ethical standards

All individuals have written an informed consent letter, in agreement with the Code of Ethics of the World Medical Association (Declaration of Helsinki, Brazil 2013). The ethics, research, and biosafety committees of Hospital Civil de Guadalajara “Fray Antonio Alcalde” (Reg. No. 037/16) approved this study.

Disclosures None.

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