INTRODUCTION

An increased prevalence of cardiovascular (CV) events, not fully explained by traditional risk factors, has been widely reported in rheumatoid arthritis (RA) patients. Therefore, other causes, such as disease activity, chronic inflammation, glucocorticoid treatment and genetic background, have been proposed as disease-related independent risk factors. Such factors could increase CV risk by promoting the development of early atherosclerotic lesions and impairing the endothelial repair mechanisms. Moreover, it has been reported that endothelial repair mechanisms were impaired in RA and other autoimmune diseases, partly due to the altered number or function of endothelial progenitor cells (EPC), a haematopoietic-derived population involved in vasculogenesis and vascular repair.

Although EPC levels have been considered as a surrogate marker of CV status in healthy subjects, different and even contradictory data about their role in RA patients have been reported. Recently, a novel T cell subset, the so-called angiogenic T cells (Tang), has been described that seems to cooperate with EPCs and enhance endothelial repair function, possibly through the secretion of proangiogenic cytokines. In fact, in vitro experiments showed that Tang depletion could abrogate EPC functionality. Animal models of ischaemia also highlighted the relevance of the Tang population in capillary formation. Tang cells are characterised by the coexpression of CD3, CD34 (platelet endothelial cell adhesion molecule) and CXCR4 (receptor for stromal-cells-derived factor-1) and may express CD4 or CD8. This subset is characterised by the coexpression of naive and memory markers, thus revealing its heterogeneous nature. A recent study in human patients revealed, for the first time, that lower Tang numbers are associated with vascular disease. Thus, Tang may be used as a novel putative biomarker for CV disease.

On the other hand, among other factors that could impair endothelial repair, type I interferons (IFNs) deserve to be noted. IFNα and related cytokines are a family of pleiotropic molecules with potent antiviral effects and an established relevance in systemic autoimmunity. However, increasing evidence points out their role in endothelial injury and repair failure. In fact, several mechanisms by which IFNα could damage the endothelium have been described. In addition, this cytokine has been associated with the occurrence of CV disease in systemic lupus erythematosus independent of traditional CV risk factors. Similar conclusions have been reached by our group when studying RA patients.

Given the very recent description of the Tang population, no such studies in RA patients have yet been reported. Thus, we hypothesised that the increased CV risk of RA patients could be related to altered numbers of Tang cells. Therefore, the main aims of the present study were: (i) to quantify Tang population in RA patients, (ii) to evaluate clinical parameters that could be associated with these cells and (iii) whether IFNα serum levels could influence Tang numbers in these patients.
Clinical Investigation, according to the Declaration of Helsinki the study was obtained from the Regional Ethics Committee for analysis were carried out for all the participants. Approval for T ejidos de Asturias. Automatised blood count and serum lipids were recruited from the Centro Comunitario de Sangre y

A CV event was considered if the patient suffered from heart failure, ischaemic heart disease or cerebrovascular accident since their RA diagnosis to the time of sampling. Simultaneously, matched healthy volunteers (n=18; 15 women; age range 23–63 years) without any pathology or treatment were recruited from the Centro Comunitario de Sangre y Tejidos de Asturias. Autamatised blood count and serum lipids analysis were carried out for all the participants. Approval for the study was obtained from the Regional Ethics Committee for Clinical Investigation, according to the Declaration of Helsinki and all the participants gave written informed consent.

Flow cytometry analyses
EPC and Tang frequencies were measured by flow cytometry. EPCs were quantified as previously described. Tang were stained with anti-CD3 PerCP-Cy5.5, anti-CD31 fluorescein isothiocyanate (FITC) and CXCR4 PE-Cy7, and those CD31/ CXCR4+ were considered Tang (figure 1A) (see online supplementary text).

Cytokine serum level quantification
Serum aliquots were stored at −80°C until cytokine measurements. IFNα and tumour necrosis factor (TNF)α serum levels were analysed by immunoassays (see online supplementary text).

In vitro cultures
Peripheral blood mononuclear cell (PBMC) cultures were carried out to investigate the effect of IFNα, TNFα and patients’ serum on Tang frequency in vitro (see online supplementary text).

Statistical analysis
All data are presented as median (IQR) unless otherwise stated. Mann–Whitney, Spearman’s ranks, analysis of the variance (ANOVA), χ² tests and multivariate regression analysis were used as appropriate. A p value<0.05 was considered statistically significant (see online supplementary text).

RESULTS
Angiogenic–T cells were reduced in RA patients
To evaluate Tang cells in RA patients in relation to EPC-mediated endothelial repair ability and traditional CV risk factors, blood samples from 103 RA patients and 18 healthy controls (HC) were analysed by flow cytometry, quantifying Tang population by means of their CD3, CD31 and CXCR4 expression (figure 1A), whereas EPC populations were determined according to their CD34, CD133 and VEGFR2 expression, as previously described. Demographic and clinical characteristics of patients were summarised in table 1. Results showed a strong decrease of Tang population in RA patients compared with HC, both in absolute numbers (figure 1B) and as a percentage of T cells (2.06 (1.89)% vs 5.52 (4.77)%, p=0.0002). Circulating EPCs, as previously reported, were also decreased in patients (figure 1C).

On the other hand, we observed interesting associations between Tang and CV risk factors in HC that were absent in RA patients (table 2). First, these cells exhibited a strong positive correlation with EPC levels. Of note, this association was found with CD34+CD133+VEGFR2+ cells (the so-called “true EPC”), but not with total CD34+ or CD34+CD133+ progenitor cells or with the CD34+VEGFR2+ population. In addition, Tang from HC were negatively associated with total cholesterol and low density lipoprotein (LDL)-cholesterol, but not with high density lipoprotein. Furthermore, EPC levels from HC showed similar correlations (total cholesterol: r=−0.573, p=0.013; LDL-cholesterol: r=−0.562, p=0.015), thus supporting the association of both Tang and EPC populations with CV risk factors. Nevertheless, these correlations were completely absent in RA patients. Moreover, male sex and the presence of diabetes, hypertension, dyslipidaemia or obesity did not significantly influence Tang in RA patients, although even lower levels were detected in smokers (p=0.037).

Finally, a stronger Tang–blood decrease was found in the subgroup of RA patients who had suffered a CV event since their RA diagnosis (n=19, time between RA diagnosis and CV event: 70.56 ±61.11 months) when compared with those without this complication (2.61 (1.88) vs 3.56 (3.75)·10³ cells/µL, p=0.014). Thus, we analysed the influence of traditional CV risk factors in RA patients with and without previous history of CV events using logistic regression modelling. We found that none of the variables included in the analysis were significantly associated with the occurrence of a CV event (age: p=0.704, male sex: p=0.074, obesity: p=0.958, hypertension: p=0.079, dyslipidaemia: p=0.569, diabetes: p=0.211 and smoking habit: p=0.840). Therefore, traditional CV risk factors did not appear to be the most relevant causes for Tang decrease in RA patients.

Disease activity and autoantibodies influenced Tang in RA patients
Therefore, we aimed to look for disease-specific features which may be involved in Tang reduction in peripheral blood. Among the analysed clinical parameters, the strongest association was detected with DAS28 score (figure 2A), indicating that disease activity plays an important role in Tang decrease. Moreover, Tang and EPC levels remained correlated, although at a lower degree than in HC, in patients with low disease activity (DAS28<2.6, n=27) (figure 2B). This association was completely lost in patients with active disease (DAS28≥2.6, n=76). Other clinical parameters such as tender joint counts (r=−0.260, p=0.009), erythrocyte sedimentation rate (r=−0.330, p=0.001) and age at diagnosis (r=−0.352, p<0.0001), but not disease duration (r=0.009, p=0.929), were negatively correlated with Tang population. In fact, the analysis of RA patients recruited at diagnosis and without treatment (n=7) indicated that Tang were strongly decreased even at early stage of the disease compared with HC (3.98 (4.13)·10³ vs 8.93 (6.63)·10³ cells/µL, p=0.006), showing similar levels as they established disease counterparts (3.30 (3.28)·10³ cells/µL).

Likewise, the analysis of immunological features showed that presence of autoantibodies was also related with Tang–blood population, since patients presenting rheumatoid factor (RF), anticyclic citrullinated peptide antibody (anti-CCP) or antinuclear antibody (ANA) exhibited lower Tang levels than their negative counterparts (figure 2C). In fact, Tang were negatively

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risk factors, are implicated in Tang numbers in RA patients. Smoking, disease-specificities of IFN-\(\alpha\) (95% CI) are associated with endothelial damage. Results showed that serum levels of IFN-\(\alpha\) were significantly increased in patients \((p=0.004)\) (see online supplementary table S1) and correlated inversely with Tang (figure 3A). Although IFN-\(\alpha\) was undetectable in the 49.3% of patients, this association remained significant in the IFN-\(\alpha\)-detectable subgroup \((r=-0.233, p=0.048)\), which displayed lower Tang levels \((2.64 \times 10^3 \text{cells/\mu L}, p=0.004)\). This relationship was completely absent in HC \((r=-0.252, p=0.011)\), respectively. Additionally, patients with previous CV events presented higher frequency of anti-CCP (88.9% vs 62.5%, \(p=0.031\)) and a trend for RF positivity (83.3% vs 62.5%, \(p=0.091\)).

Therefore, in order to evaluate the relevance of clinical and immunological features in determining Tang frequencies, a multivariate linear regression analysis were performed. After adjusting for traditional CV risk factors (age, sex, dyslipidaemia, diabetes, hypertension, obesity and smoking), only disease activity (B (95% CI) −0.546 (−1.008 to −0.339), \(p=0.0001\)), age at diagnosis (−0.041 (−0.088 to −0.018), \(p=0.003\)), ANA positivity (−1.046 (−1.959 to −0.184), \(p=0.019\)) and smoking (−0.732 (−2.189 to −0.374), \(p=0.006\)) showed a significant effect in predicting Tang levels, thus supporting that, except for smoking, disease-specific parameters rather than traditional CV risk factors, are implicated in Tang numbers in RA patients.

IFN-\(\alpha\) levels were associated with Tang decrease in peripheral blood

In addition to clinical parameters, other factors involved in RA pathogenesis and inflammation burden could have a role in Tang frequency reduction. Thus, to evaluate possible serum markers associated with endothelial damage, we quantified circulating IFN-\(\alpha\) and TNF-\(\alpha\), two cytokines involved in the pathogenesis of several autoimmune diseases. Results showed that serum levels of IFN-\(\alpha\) were significantly increased in patients \((p=0.004)\) (see online supplementary table S1) and correlated inversely with Tang (figure 3A). Although IFN-\(\alpha\) was undetectable in the 49.3% of patients, this association remained significant in the IFN-\(\alpha\)-detectable subgroup \((r=-0.233, p=0.048)\), which displayed lower Tang levels \((2.64 \times 10^3 \text{cells/\mu L}, p=0.004)\). This relationship was completely absent in HC \((r=-0.252, p=0.011)\), probably because IFN-\(\alpha\) was undetectable in most of them (88%). No associations with total CD3 or CD31 cells were found, suggesting that detrimental effects were specific to Tang population rather than a generalised effect on T cells. Moreover, RA patients with previous CV events exhibited higher IFN-\(\alpha\) serum levels compared with those without them \((p=0.019, 78\% \text{ of IFN-\(\alpha\) positive})\). On the other hand, TNF-\(\alpha\) levels, also increased in patients \((p=0.014)\), failed to exhibit a significant association with Tang numbers (see online supplementary figure S1A), although patients with the highest levels (>80th percentile, 39.49 pg/mL) showed a trend to lower Tang counts \((2.49 \times 10^3 \text{vs 3.92 \times 10^3 cells/\mu L, p=0.081})\). No significant differences were detected in patients with previous CV events \((p=0.573)\).

Finally, culture assays were performed in order to evaluate the effects of these cytokines on Tang population. Thus, PBMCs were cultured for 4 days in medium alone or in the presence of IFN-\(\alpha\) (1000 U/mL). Tang frequency was significantly reduced (up to a 26.6%) in IFN-\(\alpha\)-treated cells (figure 3B), although the total amount of viable T lymphocytes was similar in both cultures \((p=0.516)\). Therefore, to determine the possible effect of the IFN-\(\alpha\) present in RA serum, PBMCs were cultured in medium supplemented with 10% of pooled sera from either HC or RA patients and with increasing concentrations of anti-IFN-\(\alpha\) or control rabbit IgG antibodies added to RA serum. At day 4, RA serum-treated cells displayed a strong Tang decrease compared with those HC-treated that was partially restored, dose-dependently, by anti-IFN-\(\alpha\) blockade. No differences in total CD3 counts were observed after IFN-\(\alpha\) or RA serum treatment. Moreover, the Annexin V/7-Actinomycin D (AAD) staining showed that both early and late Tang apoptosis was not different

Figure 1  Tang and endothelial progenitor cell (EPC) are decreased in peripheral blood of rheumatoid arthritis (RA) patients. (A) Representative CD31 versus CXCR4 dot-plots of a healthy controls (HC) and a RA patient. Gated CD3 lymphocytes were analysed for CD31 and CXCR4 expression by flow cytometry. Tang population was identified as the triple-positive CD3/CD31/CXCR4 cells in the lymphocyte gate. Quadrants were set according to the fluorescence signal provided by the isotype controls. Box plots represent Tang (B) and EPC (C) peripheral blood reduction in RA patients compared with HC. Differences were evaluated by Mann–Whitney U test.

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in IFN-treated cultures than in the negative control (early: 0.53 ±0.21 vs 0.82±0.28, p=0.114; late: 0.16±0.20 vs 0.08±0.07, p=0.771). However, IFNα seemed to be able to downregulate CXCR4 expression (mean fluorescence intensity (MFI) in Tang: 7630.50±1575.8 vs 5318.50±3253.80; in CD3 cells: 4359 ±799.23 vs 3606.25±454.77), thus being one potential mechanism to explain lower T ang numbers.

These results point out the detrimental role of IFNα on Tang subset and the involvement of other serum factors in this effect. In fact, TNFα was also able to slightly decrease Tang frequencies (7.4%) in vitro (see online supplementary figure S2B), although no associations were observed in patients.

**DISCUSSION**

In recent years, several studies have been performed to explore the mechanisms underlying the increased CV risk and endothelial damage observed in RA patients. The results presented herein are the first reporting a new factor that seems to be implicated in this condition: the recently described subpopulation of immune cells, the so-called Tang.

It has been suggested that Tang cells may be used as a biomarker for CV risk and endothelial function. Accordingly, the evaluation of Tang cells in healthy individuals performed in this work showed that this population was negatively correlated with total and LDL-cholesterol levels. In addition, Tang were positively associated with CD34+VEGFR2+CD133+ cells, the true EPC population, but not with other phenotypes (CD34+VEGFR2+ or CD34+CD133+). This result highlights the special relevance of CD133 labelling for EPC measurements by flow cytometry. Although a positive correlation between Tang and EPC colonies in vitro has been previously reported, this is the first study showing a correlation between EPC and Tang in human peripheral blood. These findings suggest a connection between decreased Tang numbers and increased CV risk.

The most important finding of our work, however, was the striking circulating Tang decrease detected in RA patients, even at diagnosis, which, additionally, was unrelated to EPC levels and traditional CV risk factors, except for smoking. Instead, disease activity and presence of autoantibodies seemed to have detrimental effects on the Tang population. These cells were decreased in a disease activity-dependent manner in RA patients, thus suggesting that specific disease features were implicated in Tang decrease. In fact, the association between EPC and Tang was partially recovered in patients with low disease activity. These findings are in line with the idea that an accurate control of the disease will have a positive impact on CV risk management. However, our data could support an alternative role of Tang in chronic

**Table 1** Demographic and clinical parameters of RA patients

| **RA patients (n=103)** |
|-------------------------|
| **Gender (female: male)** | 83:20 |
| **Age at sampling, years (mean±SD)** | 54.81±14.37 |
| **Disease features** |
| Disease duration, years (mean range) | 5.58 (0–30.00) |
| Age at diagnosis, years | 47.83 (13.92) |
| Disease activity (DAS28) | 3.49 (1.90) |
| Tender joint count | 2.00 (4.00) |
| Swollen joint count | 1.00 (3.00) |
| Patient global assessment (0–100) | 37 (36.50) |
| ESR, mm/h | 13.00 (22.50) |
| CRP, mg/L | 2.00 (3.90) |
| HAQ (0–3) | 0.87 (1.21) |
| RF (+), n (%) | 65 (63.1) |
| αCCP (+), n (%) | 66 (64.0) |
| ANA (+), n (%) | 51 (49.1) |
| Shared epitope, n (%) | 41 (39.8) |
| Erosive disease, n (%) | 48 (46.6) |
| **Traditional CV risk factors, n (%)** |
| Dyslipidaemia | 36 (34.9) |
| Hypertension | 35 (33.9) |
| Diabetes | 9 (8.7) |
| Obesity (BMI >30) | 20 (19.4) |
| Smoking habit | 34 (33.0) |
| CV events, n (%) |
| Previous CV events | 18 (17.4) |
| Ischaemic heart disease | 8 (7.7) |
| Heart failure | 8 (7.7) |
| Cerebrovascular accidents | 2 (1.9) |
| Treatments, n (%) |
| None or NSAIDs | 7 (6.7) |
| Glucocorticoids | 56 (54.3) |
| Methotrexate | 77 (74.7) |
| TNFα blockers | 44 (42.7) |
| Tocilizumab | 12 (11.6) |
| Statins | 20 (19.4) |

**Table 2** Associations of angiogenic T cells with EPC populations and CV risk factors

| **RA** | **HC** |
|---|---|
| **Traditional CV risk factors** |
| Total cholesterol (mg/dL) | r=−0.688 | p=0.040 |
| HDL-cholesterol (mg/dL) | r=−0.099 | p=0.112 |
| LDL-cholesterol (mg/mL) | r=−0.670 | p=0.325 |
| Male sex | p=0.360 | p=0.282 |
| Diabetes | p=0.712 |
| Hypertension | p=0.570 |
| Obesity (BMI >30) | p=0.119 |
| Smoking habit | p=0.037 |

**Categorical variables are summarised as n (%), and continuous one as median (IQR).**
inflammation, since Tang behaviour under this situation is yet unknown. In fact, it might be expected that Tang–blood cells would migrate to the inflamed tissues, thus explaining the low circulating counts and the inverse relationship with inflammatory markers. Therefore, we cannot exclude that Tang cells in patients with an active inflammatory disease could be involved in the chronic inflammation rather than in angiogenic repair. On the other hand, Tang were also associated with late age at diagnosis and the presence of ANA, RF and anti-CCP antibodies, all of them previously associated with poor prognosis and CV risk in RA.27 28 Moreover, autoantibody positivity has been previously related to CV events in RA29 and other clinical conditions.30 31 Therefore, our data suggest that the negative effect of autoantibodies on Tang could be one of the underlying mechanisms by which they influenced CV risk, at least in RA.

Figure 2  Disease activity and autoantibody positivity were associated with Tang decrease. (A) Tang cells were decreased in rheumatoid arthritis (RA) patients in a disease activity-dependent manner and (B) were positively correlated with endothelial progenitor cells (EPC) populations in inactive patients (Disease Activity Score (DAS)<2.6, n=27) but not in active ones (DAS≥2.6, n=66). (C) Autoantibodies positivity (rheumatoid factor (RF); n=65, α cyclic citrullinated peptide antibody (CCP); n=66 and antinuclear antibody (ANA); n=51) was associated with Tang reduction. Correlations were assessed by Spearman ranks test and differences were evaluated by Mann–Whitney U test.

Figure 3  Interferon (IFN)α exhibited negative effects on Tang population. (A) IFNα serum levels were negatively correlated with Tang frequency in rheumatoid arthritis (RA) patients. (B) Peripheral blood mononuclear cell (PBMC) cultured in the presence or absence (medium) of 1000 U/mL of recombinant human IFNα prompted an in vitro Tang reduction, which was totally abrogated by IFNα blockade. A similar decrease was observed when culturing in the presence of RA pool serum (IFNα: 159.65 pg/mL; TNFα: 88.63 pg/mL) compared with those healthy controls sera-treated (IFNα: undetectable; TNFα: 5.09 pg/mL). Serum RA-treated reduction was partially recovered by IFNα blockade in a dose-dependent fashion: 1 μg/mL (a) and 10 μg/mL (w). Independent cultures were performed with freshly isolated PBMC from different blood donors (n=7). Correlations were analysed by Spearman ranks test and differences between among treatments were evaluated by a repeated measures analysis of the variance (ANOVA) and Bonferroni post hoc test. Horizontal bars represent the mean value ***p<0.001, **p<0.01.
According to our results, disease-specific features rather than traditional CV risk factors, apart from smoking, appear to be associated with Tang reduction in RA, thus supporting the idea that new factors should be taken into account in CV risk assessment in RA. In line with this, an interesting result of this work was the suggested harmful role played by IFNα on Tang cells. Type I IFN signature has been widely associated with the pathogenesis of autoimmunity, first in systemic lupus erythematosus (SLE) 32 but currently also in a subset of RA and other disorders. 15–18 Moreover, type I IFNs have been associated with disease activity 35 and clinical features 28–29 as well as with atherosclerosis markers 39 and CV disease. 25 In fact, different ways by which IFNα could damage the endothelium have been described. 36 Additionally, IFNα treatment has been associated with increased CV events in non-RA subjects. 41–43 Thus, the role of IFNα in Tang decrease reported here may suggest a new way by which this cytokine could have a negative impact on endothelial repair and CV risk, in the subgroup of RA patients with the IFNα ‘signature’. 15–18 Recently, systemic disease has been associated with the occurrence of CV events in RA patients. 44 Our results are in line with all these findings, since patients with higher IFNα levels are characterised by a higher rate of CV events and lower Tang frequencies. Thus, in addition to inflammatory and disease-specific markers, high IFNα levels might be helpful in the identification of RA patients with high CV risk.

Finally, the analysis of patients with a history of CV events may support the use of Tang as a putative marker of endothelial damage and CV disease in RA, as was suggested in other pathologies. 34 These patients exhibited lower Tang counts than those CV-free, highlighting the role of Tang cells in vascular repair. These patients also displayed increased levels of IFNα, previously associated with the development of premature atherosclerosis 30–31 and CV disease 19 in lupus. Accordingly, type I IFN signature has been found to be upregulated even several years after CV event occurrence. 17 Therefore, Tang cells could be an interesting target in RA and CV disease.

In conclusion, our data indicate that peripheral Tang decrease, in addition to an altered EPC function, is associated with the increased CV risk in RA patients, probably by impairing endothelial repair. These low Tang levels are closely related to disease-specific parameters. Specifically, high disease activity and autoantibody positivity are strong indicators of Tang reduction, whereas presence of high IFNα levels could be considered an additional factor in a subgroup of patients. We cannot exclude, however, that severe disease, chronic inflammation and IFNα can directly promote endothelial dysfunction, thus increasing CV risk independently of Tang population. In any case, these disease features could be interesting tools to account for CV risk in RA patients. Although further studies are needed to investigate the functionality of these cells in inflammatory conditions, increasing Tang number and/or function might be a promising intervention in RA patients, mainly in those with high risk or history of CV disease. In this sense, IFNα blockade 35 could be a valuable therapy for patients with high levels of this cytokine.

Contributors JR-C performed most of the flow cytometry analyses and data collection as well as wrote the manuscript. PL participated in immunoassays measurements and experimental procedures. MA-L, SA-C and JB-G were in charge of patients’ recruitment and clinical data collection. AS conceived and coordinated the study, collected the data, performed the statistical analyses and corrected the manuscript. All the authors read and approved the final version of the manuscript.

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