The mRNA levels of TGF-β Type II receptor splice variants in monocytes are associated with disease activity in patients with rheumatoid arthritis

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Abstract

Objective

In rheumatoid arthritis (RA) patients, TGF-β exerts a singular effect on lymphocytes, macrophages, and polymorphonuclear leukocytes. Moreover, evidences indicate that TGF-β1 stimulation affects the expression levels of TGF-β receptors. Therefore, we analysed in different leukocyte subpopulations, whether the mRNA abundance of TGFBR2 splice variants might be related to RA.

Methods

We isolated different leukocyte subpopulations from peripheral blood from 9 healthy control volunteers and 9 RA patients, matched by gender and age (cohort 1), and 8 additional RA patients (cohort 2). Then we quantified, by RT-qPCR, the mRNA relative abundance of TGFBR2 splice variants (namely TGFBR2A and TGFBR2B) in PMNs, and PBMCs (monocytes and non-monocytes). We first checked whether the TGFBR2-splice variant mRNA profile could be associated with any particular blood cell type both, in healthy control volunteers and in RA patients. In addition, PBMC and PMN mRNA levels were correlated, using Spearman's rank-order correlation test, with clinical and biochemical determinations of RA patients.

Results

We have shown that TGFBR2 exhibits an alternative splicing pattern in different subpopulations of human leucocytes from healthy controls, and the lack of it in the same cell type from RA samples. Furthermore, our study yields initial evidence that TGFBR2 mRNA expression levels in monocytes might mirror RA disease activity.

Conclusion

mRNA abundance of TGFBR2 splice variants in monocytes shows changes linked to RA disease activity.

Key words

biomarkers, disease activity, gene expression, monocytes/macrophages, rheumatoid arthritis

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Competing interests: A. Carrea, J. Velasco Zamora and R.A. Dewey submitted a patent application related to this study with Priority Date November 13th 2013. Application no. 20130104170, pending in Argentina. All the authors submitted an additional patent application (no. 20160103481) related to this study with Priority date November 14th 2016, also pending in Argentina, and WIPO application PCT/ES2017/070752 (publication WO/2018/087416).

Introduction

Rheumatoid arthritis (RA) is a systemic, autoimmune disease characterised by chronic inflammation of peripheral joints resulting in their structural and functional damage. Although the pathogenesis of RA is still unclear, many factors such as pro-inflammatory cytokines, immune cells, and genetic factors were considered to be implicated in RA pathogenesis (1). The inflamed synovial membrane of the joints collects T and B-lymphocytes, macrophages, and plasma cells. This lining gradually thickens and forms the *pannus* that invades the joint cartilage and finally, cartilage and bone are destroyed (2). In a recent review regarding RA pathogenesis, the authors gathered relevant information indicating that monocytes have an important role during joint inflammation and bone erosion occurring in RA (3).

Transforming growth factor-β (TGF-β) is a multifunctional cytokine involved in critical processes, such as embryonic development, cell maturation and differentiation, wound healing, and immune regulation. It maintains immune homeostasis by acting as a potent immune suppressor through inhibition of proliferation, differentiation, and activation of immune cells. Paradoxically, and depending on the cell microenvironment, TGF-β may also display pro-inflammatory properties (4, 5). In mammals, three different TGF-β isoforms have been identified: TGF-β1, TGF-β2 and TGF-β3 (6).

It has been reported that TGF-β expression increases in various tissues with damage, especially when accompanied by inflammation (5). It was also demonstrated that TGF-β is a fibrotic agent that is involved in numerous fibrotic disorders such as diabetic nephropathy, Crohn's disease, RA, radiation-induced fibrosis, and myocarditis (7, 8). Zhu *et al*. have shown that TGF-β1 has an effect on RA-fibroblast-like synoviocytes and might lead to joint damage by inducing epithelial-mesenchymal transition (9). Therefore, TGF-β has become a promising target for the treatment of cancer, fibrosis, asthma, and autoimmune diseases (4, 5, 10).

There are three classes of cell surface TGF-β binding proteins known as TGF-β receptors (TGFBR): TGFBR1, TGFBR2 and TGFBR3. Unlike TGFBR1 and TGFBR2, which contains a kinase domain in order to transduce intracellular signals, TGFBR3 cannot transduce intrinsic signals (11).

Signalling through TGF-βs starts with TGF-β ligands binding to TGFBR2. TGFBR1 is then recruited into the complex and phosphorylated by TGFBR2. TGFBR1 associates temporarily with SMAD2/3 proteins, which are phosphorylated at their C-terminal ends and dissociate themselves from the receptor. These SMAD2/3 proteins combine with SMAD4 to form a complex that, finally, is translocated into the nucleus in order to regulate the transcription of a significant amount of relevant genes (12, and references therein).

The TGFBR2 gene encodes two characterised splice variants: TGFBR2 (hereafter TGFBR2A), and TGFBR2B. The latter involves an insertion of 75 bp coding for 25 amino acids at the extracellular domain of the receptor, with an isoleucine to valine exchange (13). Little is known about the function of this alternative receptor isoform, but previous studies showed that TGFBR2B signals through a slightly different mechanism than TGFBR2A (14, 15). So far, there is no information regarding expression patterns of TGFBR2 alternative splicing in human immune cells.

Regulation of the TGF-β signalling pathway is important in determining cellular outcome, and the underlying mechanisms are complex. This pathway depends on several factors including the stoichiometric balance of TGF-β ligands and receptors expressed within the cell. There is some evidence that TGF-β and its receptors are potent regulators of the inflammatory process that occur in the synovial tissue in RA. Increased TGF-β1 levels have been found in synovial fluid as well as in cells derived from the joint structure of RA patients (16-19). In addition, augmented TGF-β1 serum levels have been correlated with larger joint damage in RA patients (20). Despite being contradictory, TGF-β can have anti-arthritic (21, 22), as well as pro-arthritic (23-25) activities. This could be explained considering TGF-β differential regulation of inflammation.

TGF-β ligand can induce the expression of its receptors, which is also predicted to enhance responsiveness (26). In addition, in mice lacking TGFBR2 signalling, macrophages polarisation towards an anti-inflammatory M2 phenotype is inhibited. This would explain macrophages defects to down regulate immune responses (27). Further studies on TGF-β1-null mutants or mice with bone marrow-specific deletion of TGFBR2, showed that TGF-β signalling is also critical for T-cell immunity (28-30).

Due to the above-mentioned reasons, we sought to analyse whether the mRNA levels of TGFBR2 splice variants produced by different immune cells might be affected in RA patients. Here, we showed TGFBR2 alternative splicing pattern in different white blood cell subpopulations from healthy donor controls, and the absence of that pattern in RA samples. Moreover, we give initial evidence that TGFBR2A and TGFBR2B mRNA levels in monocytes from patients show variations reflecting RA disease activity.

Table I. Volunteers' baseline characteristics.

Baseline characteristics	Cohort 1		Cohort 2
	HC (n=9)	RA (n=9)	RA (n=8)
Age (years old)	52.4 [22-75]	50.2 [23-78]	48.4 [30-64]
Gender (F/M)	7/2	7/2	8/0
Disease duration (years)	N/A	11.58 [0.25-21]	5.47 [0,5-15]
28TJC (0-28)	N/A	9.2 [0-20]	9.5 [0-24]
28SJC (0-28)	N/A	7.6 [0-19]	8.37 [0-19]
DAS28-ESR	N/A	4.77 [2.58-6.65]	5.3 [3.27-7.21]
HAQ	N/A	1.102 [0.125-2.8]	1.16 [0.25-2.8]
Pain VAS (mm)	N/A	43.6 [20-72]	42.6 [8-87]
Patients' global VAS (mm)	N/A	43.6 [29-78]	34 [4-83]
Physicians' global VAS (mm)	N/A	47 [5-80]	32.5 [2-83]
ESR (mm/hr)	17.3 [4-35]	36.3 [9-65]	42.6 [12-70]
Drug treatments			
NSAIDs	N/A	5/9	7/8
Methotrexate	N/A	6/9	5/8

Values are expressed as means and, in square brackets, minimum and maximum values.
HC: healthy controls. N/A: not applicable.

Materials and methods

Volunteers and samples

Peripheral blood was collected by venipuncture from 9 healthy control volunteers and 9 RA patients, matched by gender and age (cohort 1), and 8 additional RA patients (cohort 2) (Table I). Patients were diagnosed according to the ACR/EULAR 2010 criteria (31). All procedures were approved by CER Medical Institute Research Ethics Committee, and the *Comisión Conjunta de Investigación en Salud*, Department of Health, Buenos Aires Province, Argentina, registered under the number 2919/653/13. All procedures were performed after donor informed consent signature. Exclusion criteria included severe anaemia, autoimmune diseases different from RA, any other disease/condition able to increase ESR, treatment with biological drugs, treatment with disease-modifying anti-rheumatic drugs (DMARDs) except methotrexate, and with drugs with known effect on the TGF-β signalling cascade (losartan).

Clinical and laboratory data

Clinical determinations included swollen and tender joints, Health Assessment Questionnaire Disability Index (HAQ-DI), and visual analogue scale (VAS) scores, such as physician global VAS, patient global VAS, and pain VAS. ESR was determined by the Westergren Method (32). Disease activity of RA was determined by the 28 joint count disease activity score, including ESR values (DAS28-ESR with three variables) (33). Categories of disease activity based on the DAS28-ESR were defined as follows: remission = DAS28 ≤ 2.4, low = 2.4 <DAS28 ≤3.6, moderate = 3.6 <DAS28 ≤5.5, high = DAS28 >5.5 (34). The same rheumatologist assessed clinical determinations in all RA volunteers. Additional information regarding the volunteers is listed in Table I. The demographic and clinical characteristics of patients in cohort 2 were similar to those of patients in cohort 1 (Table I).

Purification of different subpopulations of leukocytes

Peripheral blood mononuclear cells (PBMCs) were initially separated from PMNs from both cohorts, by Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient centrifugation.

Blood cell subpopulations from cohort 1 patients were further purified as follows. Erythrocyte-depleted PMNs were obtained by treatment with KCl 0.6 M to a purity of around 95%. Monocytes were separated from non-monocytes, according to their differential properties to adhere to plastic tissue culture plates. Briefly, PBMCs were cultured in RPMI 1640 (Thermo Fisher Scientific, Boston, MA.) supplemented with 10% heterologous human serum, and 1% penicillin/streptomycin for 2 hours. After that, supernatants containing mainly non-monocytes were collected, and the plastic adherent monocyte/macrophage fraction remained in culture for additional 16 hours. Cell purity was assessed by measuring and plotting Forward Scatter (FSC) versus Side Scatter (SSC) parameters in a FACS-Calibur platform (BD Biosciences, San Jose, CA) (Table II, and Supplementary Fig. S1A-B).

PBMCs from cohort 2 patients were labelled with magnetic conjugated anti- human CD14 mAb (IMag™) (BD Pharmigen, San Diego, CA.) following the indications of the manufacturer, and monocytes isolated by magnetic cell sorting using the BD IMagnet™ (BD Pharmigen, San Diego, CA.). Cell purity was assessed by plotting the percentage of APC conjugated Mouse Anti-Human CD14 (BD Pharmigen, San Diego, CA.) or PE conjugated Mouse Anti-Human CD3 (BD Pharmigen, San Diego, CA.) labelled cells *versus* Side Scatter (SSC) parameter, in a FACS-Calibur platform (BD Biosciences, San Diego, CA.) (Table II, and Suppl. Fig. S1A-B).

Table II. Purity of PBMC subpopulations.

Cohort 1						Cohort 2		
Healthy controls			RA			RA		
ID/gender	Purity (%)		ID/gender	Purity (%)		ID/gender	Purity (%)	
	Non-monocytes	Monocytes		Non-monocytes	Monocytes		$CD3^+$	$CD14^+$
04/F	63.70	52.52	**03/F**	51.77	58.63	**01/F**	97	92
07/M	92.24	66.83	**06/M**	78.96	43.31	**02/F**	60	89
10/M	79.92	84.29	**09/M**	76.34	90.16	**03/F**	65	96
11/F	81.67	83.15	**12/F**	74.37	67.48	**04/F**	90	87
13/F	84.16	71.88	**14/F**	83.80	89.35	**08/F**	93	87
16/F	89.27	77.30	**15/F**	82.02	77.89	**10/F**	80	70
18/F	90.03	78.48	**08/F**	73.59	35.84	**11/F**	91	74
19/F	90.86	67.73	**17/F**	86.88	81.77	**13/F**	70	62
20/F	69.89	79.92	**05/F**	71.49	69.15			

F: female; M: male. Age and gender matched volunteers belonging to cohort 1 are shown in the same row. No statistically significant differences were found between both cohorts, neither in non-monocytes ($CD3^+$) nor in monocytes ($CD14^+$) cell purity, analysed by Mann-Whitney U-test (*p*-values <0.05 were considered statistically significant).

RNA isolation and RT-qPCR

Total RNA from each blood cell population was obtained using the SV Total RNA Isolation System, and cDNA was generated using 1 μg RNA, M-MLV Reverse Transcriptase, and oligo $dT_{(15)}$ primers, according to the indications stated by the manufacturer (Promega Corporation, Madison, WI.). To measure the mRNA levels of TGF-β type II receptor splice variants (TGFBR2A and TGFBR2B), qPCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche Applied Science, Basel, Switzerland.), and the following primers: TGFBR2A forward (P1), 5´GCACGTTCAGAAGTCGGTTAA3´; TGFBR2B forward (P3), 5´CTGTAATAGGACTGCCCATC3´; and TGFBR2A and TGFBR2B reverse (P2), 5´TCTCTAGTGTTATGTTCTCGTC3´ (Fig. 1). Upon RT-qPCR, we obtained two cycle threshold (Ct) values: $Ct_{TGFBR2A}$ and $Ct_{TGFBR2B}$. The Ct value determined by RT-qPCR is the cycle number at which the fluorescence signal crossed the threshold line. These Ct values were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, giving rise to the following ΔCt values: ΔCt TGFBR2A ($Ct_{TGFBR2A} - Ct_{GAPDH}$) and ΔCt TGFBR2B ($Ct_{TGFBR2B} - Ct_{GAPDH}$). Relative mRNA abundances from triplicates, referred as $2^{(-\Delta\Delta CtTGFBR2A)}$ and $2^{(-\Delta\Delta CtTGFBR2B)}$ (35), were obtained by normalising ΔCt values between each patient, and the patient with the highest

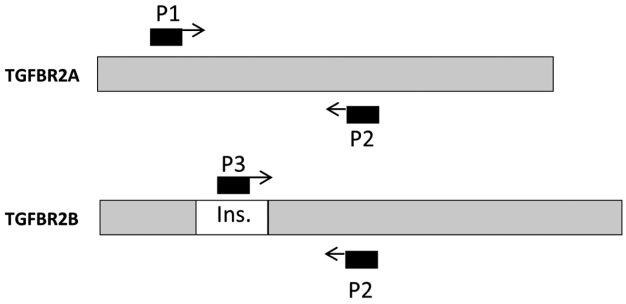

Fig. 1. Primer strategy employed to amplify TGFBR2A and B splice variants by RT-qPCR. P1: TGFBR2A forward primer; P3: TGFBR2B forward primer. P3: TGFBR2A and B reverse primer. Ins: 75 bp insertion in TGFBR2B mRNA.

mRNA levels of TGFBR2A and TGFBR2B, respectively, and log10 transformed.

Statistical analyses

Correlation results were achieved using the Spearman's rank-order correlation test, and medians were compared using the non-parametric Mann-Whitney U-test. Both tests were performed using the software OriginPro 8.5.1 (Origin Lab Corporation, Northampton, MA). *p*-values <0.05 were considered statistically significant.

Results

We first aimed to check whether the TGFBR2-splice variant mRNA profile could be associated with any particular blood cell type in healthy control volunteers. To this end, we analysed by RT-qPCR mRNA purified from PMNs, PBMC non-monocytes and PBMC monocytes from peripheral blood of 9 healthy control volunteers (Fig. 2A, C, and E). In PMNs we found that TGFBR2B was the most abundant splice variant in all donor samples (Fig. 2A). Conversely, in PBMC non-monocytes, TGFBR2A represented the most abundant splice variant (Fig. 2C). Therefore, TGFBR2-splice variant profile in PBMC non-monocytes and PMNs seem to display cell subpopulation specificity. Contrarily, in monocytes (Fig. 2E), we observed that there was no predominance of either splice variant, being TGFBR2A or TGFBR2B mRNA levels more abundant in some samples, and equally abundant in others.

We next investigated whether the TGFBR2-splice variant mRNA pattern observed in different leucocyte subpopulations from healthy control donors is affected in RA donors, matched by age and gender (Fig. 2B, D and F). Here we observed no predominance of any TGFBR2 mRNA splice variant not only in monocyte from different patients (Fig. 2F), but also in PMNs (Fig. 2B) and PBMC non-monocytes (Fig. 2D). These results show that the splice variant profile specificity found in PMNs and PBMC non-monocytes

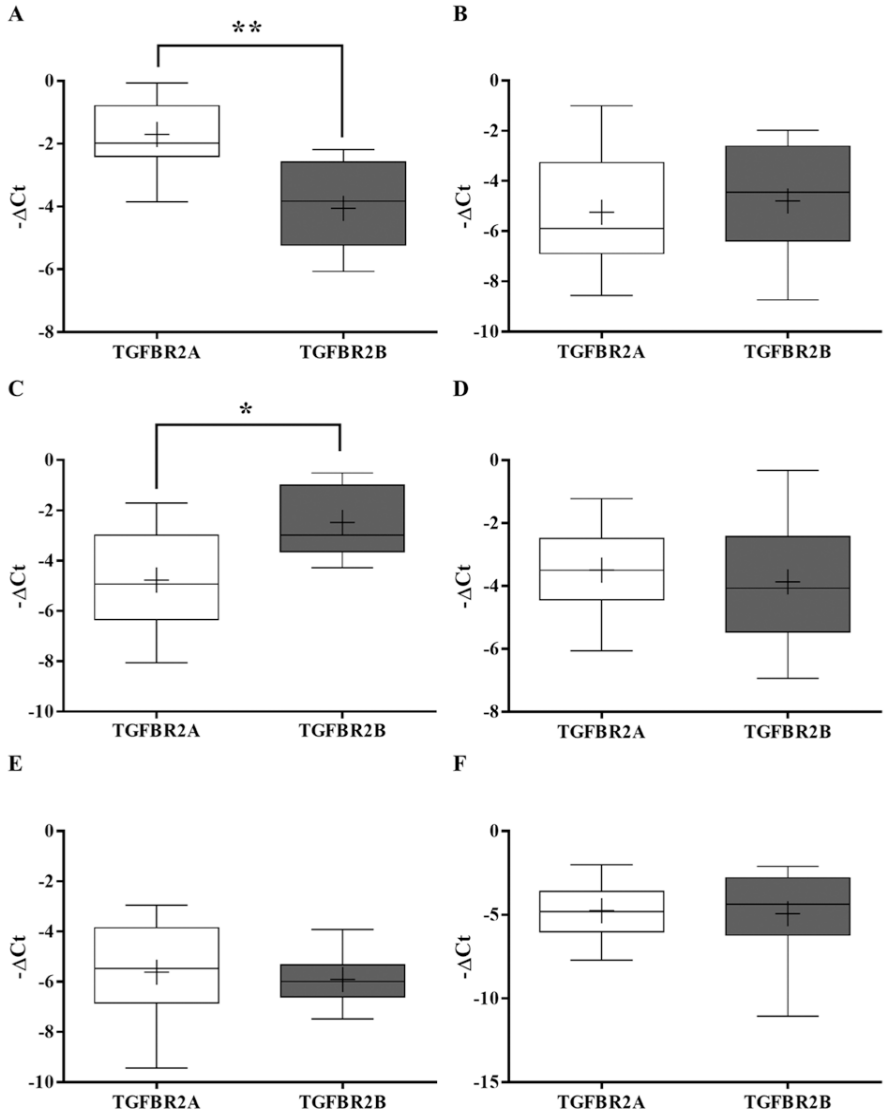

Fig. 2. Box and whisker plots of relative TGFBR2A and TGFBR2B mRNA expression levels (expressed as -ΔCT) in different leukocyte populations from peripheral blood.
Healthy control volunteers (**A**, **C** and **E**). RA patients (**B**, **D** and **F**). PMNs (**A** and **B**), PBMC non-monocyte (**C** and **D**); PBMC monocytes (**E** and **F**). The horizontal lines depict median values, boxes show 5th and 95th percentile (interquartile) ranges. Whiskers show the range, and the plus symbol denotes the mean. Mann-Whitney U-test results are denoted in the plot with horizontal bars and asterisks (*p<0.05, **p<0.01).

from healthy control volunteers is no longer preserved in the same leucocyte subpopulation of RA donors. This observation suggested a deregulation of the TGFBR2 splice variant mRNA expression in peripheral blood subsets derived from RA patient.

To check whether TGFBR2 splice variant mRNA levels in leucocytes of RA patients would be link to clinical/biochemical parameters, we correlated the relative mRNA abundance of both, TGFBR2A and TGFBR2B either in monocytes, PBMC non-monocytes, or PMNs, and the clinical and biochemical parameters determined in patients with RA from cohort 1 (Table III).

In monocytes from RA patients (Fig. 3), we found statistically significant positive correlations between TGFBR2A and TGFBR2B mRNA levels and both, 28 swollen joint count (28SJC) (Fig. 3A-B) and 28 tender joint count (28TJC) (Fig. 3C-D). In addition, we also detected statistically significant positive correlations between DAS28-ESR (which includes in its calculation 28TJC and 28SJC) and the mRNA levels of both splice variants separately (Fig. 3E-F). Furthermore, we found no correlation between the monocyte purity and the mRNA abundance of both splice variants (see Suppl. Fig. S2), helping us to discard a major influence of cell concentration on our results.

In PBMC derived non-monocytes, no statistically significant correlations were found between the mRNA abundance of both splice variants and clinical/biochemical parameters (Table III). On the other hand, in PMNs we only found statistically significant negative correlations between TGFBR2B mRNA levels and both, HAQ-DI and physician global VAS (Table III, and Suppl. Fig. S3). However, these two scores are measures of the overall patient well-being that reflect RA disease activity, together with the influence of comorbid illness and fixed damage. Therefore, no further analysis was made on PMNs, and we focused only on the correlations found in monocytes.

To check whether monocyte purification by magnetic bead cell separation would represent an option to plastic adherence, we analysed an additional panel of 8 RA patients (cohort 2). The demographic and clinical characteristics of patients in cohort 2 were similar to those of patients in cohort 1 (Table I). Furthermore, no statistically significant differences were found between cohort 1 and cohort 2, regarding monocytes ($CD14^+$) purity (Table II, and Suppl. Fig. S1B). In these patients, monocytes were purified by positive selection using anti CD14 mAb conjugated with magnetic beads (Fig. 4). Just like in cohort 1 monocytes, we observed statistically significant positive correlation between DAS28-ESR and the mRNA level of TGFBR2A (Fig. 4A). However, in this new analysis, the correlation between DAS28-ESR and the mRNA abundance of TGFBR2B was statistically non-significant (Figure 4B). This observation indicates a lack of major influence of the monocyte purification method, and confirms the link between TGFBR2 splice variant mRNA abundance and RA disease activity.

Discussion

In this paper we firstly documented the presence of both TGFBR2A and B splice variants in all the analysed leu-

Table III. Summary of correlation analyses between mRNA abundance of TGFBR2A and TGFBR2B in peripheral blood cell subpopulations from AR patients (cohort 1), and clinical or biochemical determinations.

	Spearman's rank correlation coefficient (rs)					
	Monocytes		Non-monocytes		PMNs	
	TGFBR2A	TGFBR2B	TGFBR2A	TGFBR2B	TGFBR2A	TGFBR2B
Disease duration	-0.37657	-0.47699	-0.10879	-0.02941	0.14469	0.27615
28TJC	0.77825*	0.71967*	0.42678	0.16807	0.1532	-0.32636
28SJC	0.8452*	0.77825*	0.31799	0.06722	0.13618	-0.38494
ESR	0.13446	0.05042	-0.10925	-0.10549	-0.40172	-0.15967
DAS28-ESR	0.7*	0.73333*	0.48333	0.07531	-0.01695	-0.21667
HAQ-DI	0.38494	0.26778	-0.23431	-0.04202	0.01702	-0.8703*
Patients' global VAS	0.16952	0.45769	0.64416	0.05106	0.03448	-0.0847
Pain VAS	0.39522	0.52696	0.43115	0.1747	0	-0.29941
Physicians' global VAS	0.41841	0.54394	0.15063	-0.15966	-0.03404	-0.7113*

*Correlations with p-values <0.05.

cocyte subtypes. Moreover, we found a splice variant pattern in healthy donor samples with more representation of TGFBR2A transcript over TGFBR2B in PMNs, and the opposite, in a broad white blood cell subpopulation named PBMC non-monocytes. Interestingly, the alternative splicing pattern found in those two leukocyte subtypes was not evident in the same populations from RA patients, suggesting a deregulation of the TGFBR2 splice variant expression. In monocytes, we found a totally different scenario, there was no splice variant predominance, neither in healthy control donors nor in RA patients.

So far, it is difficult to speculate how these two TGFBR2 splice variants contribute to cell physiology. One reason is that, although human TGFBR2B was described in 1996 (13), there is scant information in the literature regarding its expression pattern and/or physiological activity. Konrad *et al*. found that the expression of TGFBR2B was found in all prostate cell lines studied with a preferential localisation in epithelial cells in some human prostatic glands (36). Parker *et al*. documented that TGFBR2B is expressed in normal and OA human chondrocytes. Besides, they found that the regulation of TGF-β signalling by TGFBR2B in chondrocytes reveal that transient overexpression of TGFBR2B results in enhanced TGF-β-induced responses (37). More recently, Bee and coworkers, working with dermal fibroblast from both, healthy controls and thoracic aortic aneurysm patients with a mutation in TGFBR2B, found that TGF-β2 signalling is decreased in cells expressing TGFBR2B, and mutations in this receptor result in increased TGF-β2 signalling (38). Another reason is that, in the literature, most RT-PCR studies evaluating human TGFBR2 receptor transcripts mainly used primers directed to amplify the region encoding the intracellular domain of the receptors. Therefore, they do not discriminate splice variant contributing to the entire receptor transcript quantity.

In this work, we were able to individualise either TGFBR2A or TGFBR2B, by using primers targeting the mRNA region encoding the extracellular domain of the receptor. To distinguish them, we took advantage of the 75 bp insertion present in TGFBR2B but not in TGFBR2A (Fig. 1). To our knowledge, our study is the first to show initial evidence regarding TGFBR2 splice variant pattern in human leukocytes from healthy controls and the lack of it, in RA patients. However, the functional significance of TGFBR2B expression and its potential regulation of TGF-β signalling in white blood cells need to be addressed in further studies.

In order to get representative result, in this study, we recruited RA patients from low to high disease activity based on DAS28-ESR, and with short to long disease duration (m≠ean 11.58 years and 5.47, for cohort 1 and 2, respectively). In addition, here we included both, patients with and without NSAIDs treatment, and patients with and without methotrexate (DMARD). To get treatment-naïve patients with established RA is rather difficult in the clinical practice, owing to NSAIDs and/or low-dose methotrexate (MTX) constitute the standard first-line therapeutic strategy for RA (39). In this preliminary evaluation, we were not able to detect any bias in the results due to both, NSAIDs and MTX treatment. Moreover, it is known that MTX therapy of patients with RA is accompanied by a variety of changes in serum cytokine expression, which in turn correlate strongly with clinical disease activity and MTX pharmacokinetics (40). Furthermore, in this study, we also documented that TGFBR2 splice variant mRNA levels in peripheral blood monocytes showed changes associated with RA disease activity. Peripheral blood monocytes are the crucial innate effectors in RA pathogenesis (41). Reports describing changes in the properties of both PBMCs (42-44) and purified monocytes (45), suggested some kind of activation in RA. Whether the circulating cells are inherently different due to prior exposure to another stimulus (microorganism or an endogenous pathogen-associated molecular pattern), or whether these changes are dependent upon signals emanating from arthritic lesions (for instance, cytokines), is unknown.

Based on CD marker CD14 and CD16 expression, monocytes are classified into three types of human subsets: classical monocyte ($CD14^{++}CD16^{-}$), intermediate monocyte ($CD14^{++}CD16^{+}$), and non-classical monocyte ($CD14^{+}CD16^{++}$) (46). Monocytes show upregulated expressions of CD14 and CD16 expressions on their cell surface in RA patients. In peripheral blood and synovia of RA patients, intermediate monocyte ($CD14^{++}CD16^{+}$) highly upregulates in number and quantity. However, the reports on the composition of classical and non-classical monocytes in RA have been variable. In addition, migration and infiltration of circulating monocytes into RA synovium are vital in the propagation of synovial inflammation. The increased level of interme-

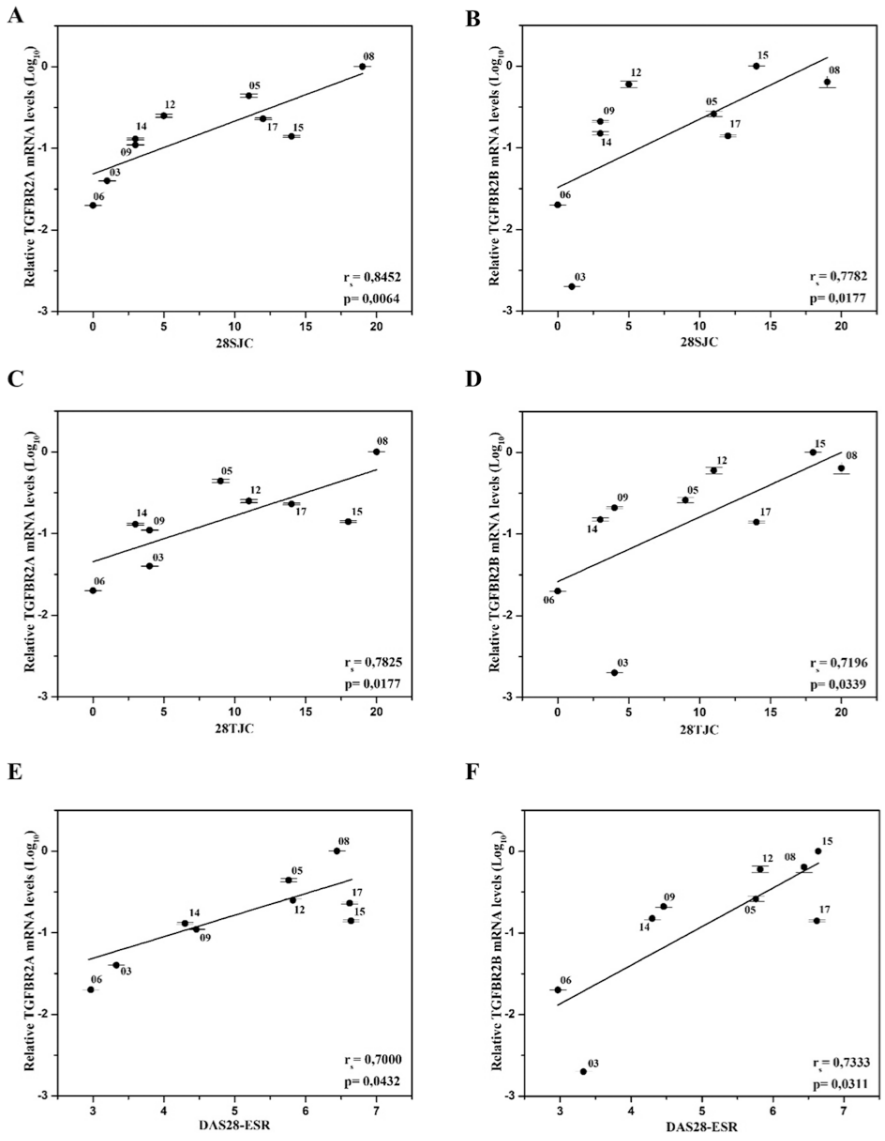

Fig. 3. Statistically significant correlations detected in monocytes derived from cohort 1 RA patients. (**A**-**F**), the mRNA abundance of both, TGFBR2A (**A**, **C**, **E**) and TGFBR2B (**B**, **D**, **F**), were correlated with the number of tender (28SJC) (**A** and **B**), and swollen joints (28TJC) (**C** and **D**), and disease activity score (DAS28-ESR) (**E** and **F**). Data are displayed as the mean ± SEM of triplicates. Numbers beside each data point correspond to the number assigned to volunteers in this study. rs: Spearman's rank correlation coefficient.

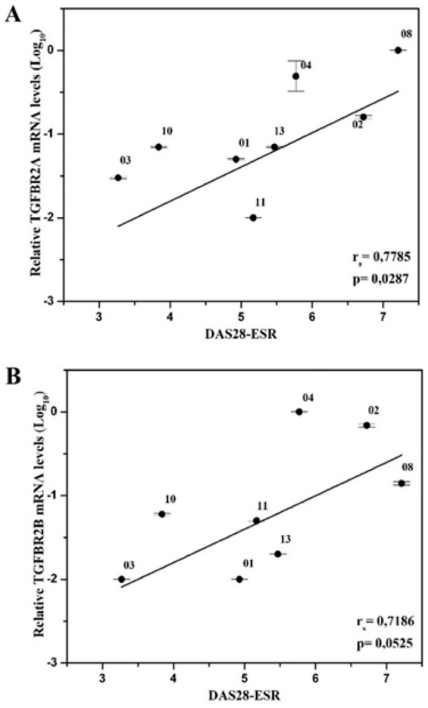

Fig. 4. Correlation analyses between disease activity scores and TGFBR2 splice variants mRNA abundance in monocytes derived from cohort 2 RA patients. (**A**-**B**), the mRNA levels of TGFBR2A (**A**), and TGFBR2B (**B**), were correlated with DAS28-ESR.
Data are displayed as the mean ± SEM of triplicates. Numbers beside each data point correspond to the number assigned to volunteers in this study. rs: Spearman's rank correlation coefficient.

diate monocytes and decreased level of classical monocytes in peripheral blood of RA patients reflect the constant recruitment of classical monocytes into the local inflamed synovium in order to promote inflammatory process. Additionally, the increased level of intermediate and classical monocytes in RA synovium suggests their active involvement in the local inflammation (39 and references therein).

Monocytes can be isolated either by plastic adherence or by positive selection of $CD14^+$ cells using magnetic bead cell separation. The latter method is rapid and produces high yield of pure cells, however, it is known that different isolation methods affect cytokine production in monocyte-derived dendritic cells (47). Therefore, despite we initially used plastic adherence isolation of monocytes (cohort 1), we then analysed whether magnetic immune-separation (cohort 2) might have an impact on RT-qPCR determinations. Here, we report that the correlation between the mRNA of TGFBR2 splice variants and DAS28-ESR remained similar, regardless the method used to isolate monocytes.

In RA, TGF-β1 is upregulated in most cellular components of the synovium, including interstitial macrophages, endothelial cells, and lining cells. Besides, increased TGF-β1 serum levels in RA are correlated with joint damage and may reflect the development of the autoimmune inflammation (20). TGF-β is a potent chemotactic factor for leucocytes, promoting monocyte adhesion and, possibly, resulting in excessive inflammatory infiltration in chronic inflammatory sites, with the potential for leukocyte-mediated tissue damage (25). Information linking increased TGF-β type II receptor levels and RA is scarce in the literature. Few studies have shown a relationship between RA and TGFBR2 mRNA abundance. In one study, the TGFBR2 mRNA levels were found to be upregulated in RA synovial fibroblasts (48). Similarly, studies of the synovial biopsy tissues of RA patients, by *in situ* hybridisation, have revealed markedly increased signals for TGF-

BR2 mRNA in tissues with an active inflammatory process, when compared with tissues with less active inflammation (49). Although, RA is mainly manifested in the joints, there are indications that disease-specific transcriptional imprints are already detectable at the level of peripheral monocytes (50, 51). In line with this, Maas and coworkers identified, by gene expression profiling by cDNA microarray technology, that TGFBR2 is overexpressed in RA patients PBMCs (N=20) compared to control individuals (52). This observation, together with our data, give clues that, in PBMC/monocytes from RA patients, TGFBR2 transcript levels might be reflecting some kind of involvement in inflammation.

Two main human phenotypes of macrophages differentiated from monocytes are; classically activated M1 (pro-inflammatory phenotype) producing high levels of TNF-α, IL-1, IL-6, IL-12, IL-23, and reactive oxygen species, and, alternatively activated M2 Mϕ (anti-inflammatory phenotype) producing high levels of IL-10, IL-1Ra, decoy IL-1RII, TGF-β, and low levels of IL-12. Both types are necessary for correct resolution of inflammation (Reviewed in 40). In this respect, Amoruso and coworkers suggested that intermediate monocyte subsets ($CD14^{++}$ $CD16^{+}$) are the predominant monocytes in RA synovium and they are the major subsets to undergo differentiation into inflammatory macrophages (M1) (53). Interestingly, transgenic mice that lack TGFBR2 signalling in macrophages, showed inhibition of the polarisation of macrophages to an anti-inflammatory M2 state. This result suggested that modulation of the TGF-β signalling pathway might be a method to medically regulate macrophage polarisation (27).

Current RA disease activity indices are commonly a combination of laboratory measurements, physician assessment of symptoms, and patient reported measures. Both, physician and patient reports are critical components of patient assessment and management, but they can be confounded by comorbid illness and fixed joint damage, resulting from long-standing disease, in addition to intra- and inter-observer variability (54-56). Therefore, several laboratories worldwide are making efforts to find objective inflammation biomarkers to be used to assess RA disease activity. In this context, monocyte subsets can act as a biomarker of the RA disease activity and may play a role in the evaluation of the efficacy of therapies. According to that, Tsukamoto and coworkers show that the proportion of circulating intermediate monocytes positively correlates with RA disease activity and their level in peripheral blood elevates during increased disease activity of RA (57). Similarly, our approach of TGFBR2 transcript quantification in monocytes might also represent a disease activity biomarker for RA, but this possibility should be thoroughly analysed in larger studies with RA patient derived samples. Besides, with the information available to date, we are not entitled to assert whether intermediate monocyte quantities and TGFBR2 transcript levels might be related in peripheral blood of RA patients. Therefore, further studies are warranted to assess the relationship between mRNA abundance of TGFBR2 splice variants and intermediate monocytes, including its link with monocyte migration, and M1/M2 polarisation in the synovial tissue of RA patients.

In sum, here we show that TGFBR2 exhibits an alternative splicing pattern in different subpopulations of human leucocytes from healthy controls, and the lack of it in the same cell type from RA samples. Furthermore, we give initial evidence that TGFBR2 mRNA expression levels in monocytes might mirror RA disease activity.

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