Clinicopathological Correlations of Podoplanin (gp38) Expression in Rheumatoid Synovium and Its Potential Contribution to Fibroblast Platelet Crosstalk

Manuel J. Del Rey, Regina Faré, Elena Izquierdo, Alicia Usategui, José L. Rodríguez-Fernández, Abel Suárez-Fueyo, Juan D. Cañete, José L. Pablos

1 Servicio de Reumatología, Instituto de Investigación Hospital 12 de Octubre, Madrid, Spain, 2 Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain, 3 Unidad d’Artritis, Servei de Reumatologia, Hospital Clínic de Barcelona and Institut d’Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain

Abstract

Introduction: Synovial fibroblasts (SF) undergo phenotypic changes in rheumatoid arthritis (RA) that contribute to inflammatory joint destruction. This study was undertaken to evaluate the clinical and functional significance of ectopic podoplanin (gp38) expression by RA SF.

Methods: Expression of gp38 and its CLEC2 receptor was analyzed by immunohistochemistry in synovial arthroscopic biopsies from RA patients and normal and osteoarthritic controls. Correlation between gp38 expression and RA clinicopathological variables was analyzed. In patients rebiopsied after anti-TNF-α therapy, changes in gp38 expression were determined. Platelet-SF coculture and gp38 silencing in SF were used to analyze the functional contribution of gp38 to SF migratory and invasive properties, and to SF platelet crosstalk.

Results: gp38 was abundantly but variably expressed in RA, and it was undetectable in normal synovial tissues. Among clinicopathological RA variables, significantly increased gp38 expression was only found in patients with lymphoid neogenesis (LN), and RF or ACPA autoantibodies. Cultured synovial but not dermal fibroblasts showed strong constitutive gp38 expression that was further induced by TNF-α. In RA patients, anti-TNF-α therapy significantly reduced synovial gp38 expression. In RA synovium, CLEC2 receptor expression was only observed in platelets. gp38 silencing in cultured SF did not modify their migratory and invasive properties but reduced the expression of IL-6 and IL-8 genes induced by SF-platelet interaction.

Conclusions: In RA, synovial expression of gp38 is strongly associated to LN and it is reduced after anti-TNF-α therapy. Interaction between gp38 and CLEC2 platelet receptor is feasible in RA synovium in vivo and can specifically contribute to gene expression by SF.

Introduction

Synovial fibroblasts (SF) are a heterogeneous cell population that represents the main resident cell component of synovial tissue. In rheumatoid arthritis (RA), SF expand and undergo phenotypic changes that contribute to the pathogenesis of chronic arthritis [1–3]. SF can respond to cytokines and, they maintain prolonged changes on the expression of genes involved in persistent inflammation and joint destruction in RA [4–6]. Crosstalk between SF and myeloid and lymphoid cell seems critical for persistent recruitment, survival and activation in chronic inflammation. These functions are associated to specific SF properties that resemble those of stromal cells in lymphoid tissues [7–10]. Lymphoid stromal cells play critical roles for the physiological trafficking and anatomico-functional compartmentalization of immune cells that supports normal immune responses [11,12].

Among the shared lymphoid and RA stromal features, the expression of the surface glycoprotein podoplanin or gp38 has been reported [12–14]. gp38 expression is normally restricted to lymphatic endothelium and in lymphoid organs, to stromal cells of the T-cell zone. Aberrant expression of gp38 in fibroblasts has also been observed in other pathological tissues where fibroblasts play diverse roles in cancer progression or fibrosis [12,15,16]. gp38(+) fibroblasts might emerge in inflammatory tissues due to either
specific cell proliferation of local gp38(+) progenitors or to induced expression in gp38(−) fibroblasts by inflammatory cytokines [14,16,17]. In a murine model of experimental autoimmune encephalomyelitis, a gp38 antagonist reduced inflammation-associated lymphoid neogenesis (LN) pointing to additional functions for gp38 in inflammation, although the precise mechanism remains unknown [18].

In cancer epithelial cells undergoing epithelial-mesenchymal transformation, gp38 expression confers enhanced cell migration and tumour invasiveness, consistently with the observation of gp38 up-regulation on the invasive front of tumors [19,20]. In cultured lymphatic endothelium gp38 knockdown has also shown to reduce cell migration by regulating the activities of RhoA and Cdc42 GTPases [21]. This effect has been studied ex vivo and it seems mediated by indirect mechanisms of intracellular interaction between gp38 intracellular domains and ERM proteins ezrin and moesin that result in modification of small GTPase activities involved in cancer cell motility. Whether gp38 can modify cell motility in stromal cells of lymphoid organs or in inflammatory fibroblasts is not known.

The physiological and developmental functions of gp38 have been dissected in knockout mice. gp38 lacks intracellular signalling domains and its function seems to depend on its monogamous signalling receptor CLEC2. gp38 and CLEC2 knockout mice display an identical phenotype characterized by an embryonal defect in blood-lymphatic vascular separation [22–24]. In mice, CLEC2 is only expressed by platelets and some myeloid cell types, notably dendritic cells (DC) [25]. gp38 triggering of CLEC2 receptor induces platelet activation through Syk and SLP-76 signaling and this pathway seems critical for blood-lymphatic vessel partitioning during development [26,27]. Crosstalk between lymphoid endothelial cells and platelets involves CLEC2 receptor triggering by gp38 and the release of specific platelet mediators that induce paracrine effects on endothelial cells [27].

To analyze the significance of increased gp38 expression in RA, we analyzed its correlation with clinical and pathological variables of the disease in a series of RA synovial tissues, including serial samples obtained before and after anti-TNF-α therapy. We also investigated the function of gp38 in SF by mean of RNA interference in different models of cell migration and platelet interaction relevant to RA pathogenesis.

Material and Methods

Patients and synovial biopsies

Synovial tissues were obtained from arthritic biopsies from the knee of patients who fulfilled the American Rheumatism Association revised criteria for RA (n = 38) [28]. All patients had active disease characterized by inflammation of at least one knee joint. Patients characteristics at biopsy were recorded and are shown in Table 1. Osteoarthritis (OA) synovial tissues were obtained by synovectomy at prostatic join replacement surgery (n = 15, age: 68±21, 60% female) and histologically normal synovial tissues were obtained from healthy individuals without joint disease at elective arthroscopy for minor traumatic lesions (n = 6, age: 59±19, 60% female). In a subgroup of patients (n = 16), a second biopsy was obtained after at least 6 months of anti-TNF-α therapy. The study was approved by ethics committees of Hospital Clinic, Barcelona, and Hospital 12 de Octubre, Madrid, and a written informed consent was obtained from all patients.

Immunolabeling of cells and synovial tissues

Immunohistochemical (IHC) staining was performed using a standard indirect avidin-biotin peroxidase method (ABC standard; Vector Laboratories, Burlingame, CA, USA) and developed by diaminobenzidine chromogen. The following antibodies and matched isotype controls were used: anti-gp38 mAb (D2/40 clone, Dako, Glostrup, Denmark) [29], anti-Cd61 mAb (SSZ1 IgG1clone, Immunotech, Marseille, France), and anti-CLEC2 polyclonal goat Ab (R&D Systems, Minneapolis, MN, USA). Pretreatments included 10 μg/ml proteinase K (Sigma-Aldrich Quimica SA, Madrid, Spain) for CD61 detection, and microwave heating in 1 mM EDTA pH 8 for gp38 and CLEC2 detection. Double CLEC2 and CD61 immunofluorescent labeling was performed using sequential incubation with anti-goat IgG Alexa-Fluor 488 (green) and anti-mouse IgG1 Alexa-Fluor 594 (red) secondary antibodies (Invitrogen Molecular Probes, Eugene, OR, USA).

Synovial tissue sections were photographed and digitalized using a Spot RT CCD camera and Spot 4.0.4 software (Diagnostic Instruments, Sterling Heights, Michigan) on a Zeiss Axioscop-2 fluorescence microscope (Zeiss, Jena, Germany). gp38 immunoperoxidase stained fractional area was quantified using ImageJ software [http://rsb.info.nih.gov/ij]. IHC analysis and quantification of CD3, CD20 CD68, hsp47, CD31 in RA synovial biopsies as well as characterization of lymphoid neogenesis was performed as previously described [30,31].

Fibroblasts grown on glass coverslips were immunolabeled with anti-gp38 mAb (D2/40 clone, Dako). Detection was performed with goat anti-mouse IgG Alexa-Fluor 594 labeled antibody and DAPI counterstaining. Photographs were obtained on a Zeiss LSM 510 META confocal microscopy (Zeiss).

Flow cytometric analysis of gp38 expression in cultured fibroblasts was performed with anti-gp38 mAb (clone 18H5, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) [32] and goat anti-mouse IgG Alexa-Fluor 647 (Invitrogen Molecular Probes). Cells were analyzed on a BD FACSCalibur instrument (Becton Dickinson, San José, CA, USA).

SF cultures and lentiviral siRNA transduction of fibroblasts

SF and normal dermal fibroblast (DF) cultures were established by explant growth of small biopsy fragments in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Lonza, Verviers, Belgium) and used after 3rd passage.

A small-interfering RNA (siRNA) targeting human gp38 and a scrambled non-silencing construction in pGIPz vector lentiviral were purchased from Open Biosystems (Thermo Fisher Scientific, Waltham, MA, USA). Lentiviral particles were obtained by co-transfection of 293T cells with pGIPz vector lentiviral, pPAX2 packaging vector, and pMD2-VSVg envelope vector as previously described [33]. Supernatants were harvested 48 hours after transfection, filtered through a 0.45 μm filter and diluted 1/2 in DMEM medium for fibroblast transduction. Silencing efficiency was monitored by analyzing gp38 mRNA and protein expression by qRT-PCR and flow cytometry.

SF migration and invasiveness studies

SF migration was analyzed in wound-healing assays. Cells were grown to confluence on culture chambered coverslides (Lab-Tek II 4, NUNC, Rochester, NY, USA) and a wound was made by a single scrape using a sterile pipette tip. The wound area was sequentially photographed at 0, 18 and 24 h. The area covered by
cells migrating into the wound area was determined using ImageJ software as the average closed area of the wound at 18 and 24 h after scraping.

The invasive capability of RA SF was analyzed using a matrigel coated transwell system (Becton Dickinson). SF were resuspended in DMEM 0.1% BSA, plated on the upper chamber of the transwell insert (8 µm pores) and allowed to migrate through the matrigel membrane for 4 days. DMEM with 10% FBS and 10% human serum was added to the bottom well and used as a chemoattractant. The membrane was fixed with 4% paraformaldehyde and stained with DAPI for direct fluorescent microscopy. Ten random fields per membrane were photographed and digitalized and the number of cells per area was counted using ImageJ software.

The capacity of SF to invade cartilage was indirectly determined as the release of glycosaminoglycan (GAG) degradation products from cartilage SF cocultures. Fresh frozen human cartilage slices of fixed diameter and height (6 x 2 mm) were collected from cartilage obtained during joint replacement surgery for hip fracture and attached to 24-well plates. SF were dropwise added to the slices. After incubation for 3 h at 37°C and 5% CO₂, wells were filled with 1 ml DMEM 10% FBS. Supernatants were removed after 4 and 7 days of culture and GAG determined using Blyscan assay (Biocolor Ltd, Belfast, N. Ireland) according to the manufacturer’s protocols.

SF platelet co-cultures
Platelets were isolated from peripheral whole blood in EDTA from healthy donors. Platelet-rich plasma (PRP) was obtained by centrifugation at 150 g at room temperature for 10 minutes. Platelets were pelleted, resuspended on DMEM and added to SF cultures at 1:1000 ratio (SF:Platelets). SF were seeded into 6-well plates in 1% FBS DMEM one day before platelet co-culture. Cocultures were maintained at 37°C for 5 h. Cells and supernatants were collected for RNA and protein quantification. Parallel SF cultures without platelets were established and used as controls.

Quantitative Real Time-PCR (qRT-PCR) of RNA extracts from SF was carried out on Applied Biosystems 7500 Fast Real-Time PCR System using either Power Sybr Green PCR Master Mix or TaqMan Gene Expression assays (Applied Biosystems). The sequences of PCR primers used are listed in Table S1. For relative quantification, we calculated the amount of target gene normalized to the endogenous reference gene (β-actin) using 2^{-ΔΔCt} formula, where Ct is the mean of threshold cycle at which the amplification of the PCR product is first detected. We performed a previous validation experiment comparing the standard curve of the reference and the target to demonstrate that the primers efficiencies were approximately equal.

IL-6 concentration in supernatants from SF platelets co-cultures was determined by ELISA (Biolegend Inc, San Diego, CA, USA).

Statistical Analysis
Data were analyzed using Prism software v5.0 (GraphPad Software, San Diego, CA, USA). Results are expressed as mean±SD and means were compared by Student’s t-test, Mann-Whitney U-test, and Wilcoxon tests as appropriate. Correlation between different numerical variables was analyzed by Spearman’s or Pearson’s tests as appropriate. Categorical variables were analyzed by Chi-square test. In all experiments, p value <0.05 was considered statistically significant.

Results
Clinicopathological correlates of gp38 expression in RA synovial tissues
To extend previous observations by Ekwall et al. in advanced RA disease undergoing joint replacement [14], to RA patients biopsied at earlier stages, we performed quantitative IHC analyses in arthroscopic biopsies from a series of RA patients with active arthritis of the knee, heterogeneous regarding other characteristics of the disease (Table 1). Abundant gp38 expression was observed in synovial lining and sublining fibroblasts in RA biopsies (n = 38), whereas minimal expression was observed in OA (n = 15), and it was undetectable in normal synovial tissues (n = 6). In all RA

### Table 1. Clinicopathological data of RA patients at biopsy.*

| RA characteristics | Value |
|--------------------|-------|
| Age, years         | 60.6±12.5 [40–89] |
| Female             | 61.8% |
| RA duration, months| 103.2±111.4 [4–441] |
| CD3+T cells/mm²    | 736±557 [9–2374 | 2374 range] |
| CD20+B cells/mm²   | 282±265 [2–1040] |
| CD68+cells/mm²     | 2267±1416 [45–1016] |
| DAS28               | 5.2±1.5 [2.34–8.35] |
| Lymphoid neogenesis positive (%) | 63.9% |
| CRP (mg/dl)        | 3.5±2.8 [0.1–10.1] |
| Erosive disease    | 79.4% |
| RF positive a      | 61.2% |
| ACPA positive b    | 80.6% |
| Previous DMARD     | 100% |
| Previous anti-TNF-α| 48.6% |

*Quantitative data are expressed as mean±SD and [range]. CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; DMARD, disease-modifying antirheumatic drug; aRF>30 IU/ml; anti-citrullinated protein antibodies ACPA>50 IU/mL.

doi:10.1371/journal.pone.0099607.t001

---

**GP38 Expression and Functions in Synovial Fibroblasts**

---

---
tissues, gp38 was observed in lining cells, whereas in 54% of the cases it extended to sublining fibroblasts, and stromal cells within LN structures (Figure 1A). Lymphatic gp38(+) vessels were extremely rare in the superficial lining and sublining areas used for quantitative evaluation, but were readily observed in deeper tissues.

The level of gp38 expression in RA, expressed as the fractional labeled area, did not correlate with age, disease duration, activity evaluated as DAS28, C-reactive protein, macrophagic, T-cell or B-cell infiltration, and fibroblast or vascular density (data not shown). gp38 expression was found significantly increased in the group of patients (64%) with ectopic LN (Table 2). We also found significantly increased gp38 expression in the groups of patients with rheumatoid factor or anti-citrullinated protein (ACPA) autoantibodies compared to seronegative patients (Table 2). No correlation between LN and the presence of autoantibodies was found.

In a subgroup of 16 patients that initiated therapy with a TNF-α antagonist after biopsy, we analyzed the changes in gp38 expression after therapy. In synovial tissues obtained after 12.3±6.1 months of anti-TNF-α therapy, a significant reduction in gp38 expression compared to pretreatment tissues was observed (20.2±9.0% to 6.9±6.0%, p = 0.0001) (Figure 1B). EULAR good or moderate responses [34] had been achieved in 12 of 16 patients at second biopsy. A lower decrease on gp38 expression in the second biopsy was observed in non-responders but the difference was not statistically significant.

gp38 expression and function in cultured fibroblasts

Cultured SF displayed abundant gp38 expression irrespective of their source (RA, OA or healthy synovium) but this was not a general feature of cultured human fibroblasts since DF did not show gp38 expression (Figure 2). Membrane expression of gp38 was also confirmed by labeling of unpermeabilized SF by flow cytometry and confocal microscopy (Figure 2). Treatment of cultured SF with TNF-α induced a significant up-regulation of gp38 mRNA and protein expression, as determined by qRT-PCR (data not shown) and flow cytometry (Figure 2). In cultured DF, TNF-α also induced gp38 mRNA and protein expression but to a lower extent compared to SF.

To analyze the potential role of gp38 on the cell behaviour of SF, we performed RNA interference experiments in cultured RA fibroblasts. In SF transduced with a lentiviral gp38-specific siRNA construct, a profound downregulation of gp38 mRNA and
membrane protein was observed compared to control siRNA transduced SF (Figure S1A). Cell viability was similar in gp38 (82.51 ± 15.29%) and control (84.09 ± 11.88%) transduced cells.

Since the best described function of gp38 is a gain of function in cell migratory and invasive properties of epithelial cancer cells [19,20], we compared cell migration and invasive capacities of SF transduced with gp38 or control siRNA lentivirus. Cell migration through matrigel coated transwells was similar in gp38 silenced and control SF (Figure S1B). Invasion of wound area on plastic monolayer was also similar in gp38 silenced or control SF (Figure S1C), therefore excluding a relevant function for gp38 in cell adhesion and motility on matrigel or plastic surfaces in SF.

Previous studies have shown that RA SF attach and invade the cartilage causing degradation of the extracellular matrix. Therefore, to analyze the invasive capacity of SF in a model relevant to RA, SF were seed onto human cartilage slices [35,36]. Transduced RA SF expressing GFP and siRNA efficiently attached and invaded cartilage ex vivo, as observed by direct fluorescent microscopy, and induced an increase on GAG release to the media. GAG release at 4 and 7 days after SF seeding was similar in gp38 silenced compared to control RA SF, excluding an important role for gp38 membrane expression in this process (Figure S1D).

CLEC2 expression in RA synovial tissues

To identify expression of CLEC2 receptor in cells potentially interacting with gp38+SF, we performed IHC studies in RA synovial tissues. CLEC2 expression was only detected in small particles or aggregates lacking nuclei located in intra- and perivascular areas of the sublining area. In some cases, CLEC2 positive aggregates were associated to amorphous fibrin deposits attached to the lining surface. In these locations we demonstrated the presence of platelets by specific CD61 IHC (Figure 3). Double immunofluorescent labeling confirmed colocalization of CLEC2 and CD61+ platelets (Figure S2).

Although IHC observations did not support CLEC2 expression by myeloid cells, to exclude lower levels of gp38 signalling receptor CLEC2 expression in human myeloid DC as recently described in murine DC [21], we performed western blot analysis of platelet and human DC under different maturation and activation status. Our data confirmed CLEC2 expression in platelet extracts but failed to detect CLEC2 in human DC (Figure S3). By flow

---

**Table 2. Clinicopathological correlations of gp38 expression* in RA synovial tissue.**

| RA variables          | gp38 expression* | p value |
|-----------------------|------------------|---------|
| RF(+/−)               | 20.1 ± 10.6/13.0 ± 7.9 | 0.0078  |
| ACPA(+/−)             | 20.4 ± 10.2/12.3 ± 7.9 | 0.0450  |
| LN(+/−)               | 21.9 ± 10.4/10.6 ± 5.6 | 0.0004  |
| Erosive disease(+/−)  | 18.9 ± 10.6/15.0 ± 8.0 | 0.22    |
| Earlier disease(+/−)  | 19.5 ± 7.4/18.2 ± 10.8 | 0.55    |

*Fractional immunolabeled gp38 area (%), mean ± SD. RF, rheumatoid factor; ACPA, Anti-citrullinated protein antibodies; LN, lymphoid neogenesis. †Patients with RA duration less than 1 year. Mean ± SD.

doi:10.1371/journal.pone.0099607.t002

---

**Figure 2. gp38 expression of cultured synovial fibroblasts.** SF from RA, OA and normal synovial tissues, and normal skin dermal fibroblasts (DF) were cultured on glass coverslips and immunolabeled (red) for gp38 expression. TNF-α treated DF cultures are also shown (DAPI nuclear counterstaining). Flow cytometric detection of surface gp38 by in RA SF and DF untreated (basal) or treated with TNF-α for 24h. Data are representative of 6 SF and 3 DF lines (*p = 0.03 basal vs TNF-α treated). MFI: Mean fluorescence intensity.
doi:10.1371/journal.pone.0099607.g002
cytometry, we also failed to detect binding of gp38-Fc protein to mature or immature DC (data not shown).

**gp38 function in fibroblast CLEC2 platelet interaction**

Since CLEC2 was not detected in any other cellular element of RA synovium, we focused on platelet SF functional interactions to analyze the potential function of gp38 in this system. Gene expression of cytokines, chemokines and metalloproteinases factors previously associated to proinflammatory or tissue destructive functions of RA SF was analyzed in SF platelet co-cultures compared to parallel SF cultures in the absence of platelets. Platelet co-culture induced a strong increase in the expression of IL-6 and the chemokines IL-8, CXCL2 and CXCL3 mRNA (Figure 4A), whereas MMP (MMP-1, MMP-3 and MMP-9 mRNA) and LN stroma related factors were non-significantly increased (IL-7, CXCL13 mRNA) or decreased (CCL21 mRNA) (data not shown).

In platelet cocultured SF, gp38 silencing significantly reduced IL-6 and IL-8 mRNA expression, whereas CXCL2 and CXCL3 mRNA expression was not significantly modified (Figure 4A). Protein levels of IL-6 in the supernatant of SF platelet co-cultures were also significantly reduced in gp38 silenced SF compared to non-silenced controls (Figure 4B).

**Discussion**

Our data confirm that fibroblast gp38 expression is characteristic of RA inflammatory tissue, in contrast to normal synovial tissue, where it is undetectable. Interestingly, regardless in vivo expression, both normal and arthritic SF acquire similar membrane gp38 expression in culture. Therefore, exposure to growth factors and tissue culture conditions seem to induce gp38 in normal SF that do not express the protein in vivo but not in normal DF pointing to different tissue-specific fibroblast pheno-
types. TNF-α and inflammatory cytokines may also induce gp38 expression in SF as previously reported [14] but again at much higher levels in SF compared to DF. Therefore, the higher capacity of SF to express gp38 represents a tissue specific property potentially relevant to their participation in joint specific inflammatory diseases. Recent studies have identified the proliferation of specific perivascular stromal precursors characterized by constitutive expression of gp38 and ADAM12 as the source of gp38 fibroblasts in different inflammatory mouse models [16,17]. We failed to detect perivascular gp38(+) cells in human healthy skin (data not shown) and normal synovium. This observation, together with the uniform acquisition of gp38 expression by cultured SF and its induction by TNF-α, suggest that growth factor or cytokine induced expression may underlie the observed gp38 stromal expansion in RA synovium. In addition, gp38 was significantly downregulated in patients treated with TNF-α antagonists further supporting this mechanism.

The expression of gp38 in RA tissues from patients with advanced destructive disease had suggested its potential role in structural damage [14]. We have also identified high gp38 expression in patients at earlier phases of the disease, excluding a correlation between disease progression and the level of gp38 expression and indicating that gp38 expression may be an early feature in RA. No significant correlations between the level of gp38 expression and most clinical or pathological RA features were found, with the striking exceptions of LN and RA autoantibodies positive groups. The finding of increased gp38 expression in patients with synovial LN, together with the previous observation of defective lymphoid development in gp38 deficient mice, point to a mechanistic link between both processes [18]. In several mouse models of inflammation also characterized by LN, the expansion of gp38(+) fibroblasts has been demonstrated [17]. The dependence of inflammatory LN on gp38 has been demonstrated in the mouse model of autoimmune encephalomyelitis, where anti-gp38 antibodies abrogated LN development [18]. However, the role of gp38(+) stromal cells is uncertain in that study, since gp38 expression and function was attributed to Th17 cells.

Whether ontogenic or pathological LN is dependent on functional gp38/CLEC2 receptor interaction is unknown. Such interaction is feasible in RA, where we observed both gp38(+) SF and CLEC2(+) platelets in the synovial tissue. Previous studies had also demonstrated the presence of abundant platelet microparticles in the synovial fluid of patients with RA and their capacity to induce potent paracrine proinflammatory effects on SF [37,38]. Platelets respond to CLEC2 triggering by phosphorylating its cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) which, in turn, recruits and activates tyrosin kinases Syk/Btk resulting in platelet activation. This process leads to the generation of platelet microparticles and the release of mediators stored in platelet granules such as IL-1 with potent paracrine effects on SF [27,38]. ITAM-dependent platelet activation may also be induced by GPVI receptor and therefore, both pathways might participate in platelet-stromal inflammatory crosstalk. CLEC2 specific effects, not triggered by GPVI receptor activation, have been proposed using CLEC2 deficient platelets but are difficult to dissect out in inflammatory models where platelet activation is required to maintain vascular integrity [27,39].

Our data confirm that platelet-SF co-culture induces strong changes in the expression of multiple genes by SF, similar to those induced by platelet microparticles [37,38]. Among these genes, IL-6 and IL-8 were specifically reduced by gp38 silencing in SF in contrast with other platelet-induced genes, providing evidence of a contribution of CLEC2 to proinflammatory platelet-SF crosstalk. IL-6 plays a pivotal role in RA pathogenesis and its therapeutic targeting is highly effective in modifying the course of the disease [40]. Although macrophagic cytokines IL-1β and TNF-α are also potent stimuli for IL-6 expression in RA SF, our and previous data suggest that platelets may also play an important role [37]. However, the link between this observation and the potential role of gp38 expression in rheumatoid LN is unclear. IL-6 has pleiotropic functions on lymphoid cells including the development of Th17 cells as well as T follicular helper and B cells involved in the development of orthotopic germinal centres in autoimmune arthritis but its influence on ectopic LN development is unknown [41-43]. We did not detect gp38 dependent changes on the expression of stromal factors associated to ontogenic or inflammatory LN such as IL-7 or lymphoid homing chemokines CXCL13 or CCL21 [10,44].

![Figure 4. Expression of cytokines and chemokines in gp38 silenced RA SF platelet co-cultures. (A) Magnitude of the up-regulation on IL-6, IL-8, CXCL2 and CXCL3 mRNA expression induced by platelets and expressed as the ratio (FC) between SF and platelet co-cultures compared to parallel SF only cultures in gp38 siRNA silenced SF and non-silenced controls (si-CTRL) as measured by qRT-PCR. (B) IL-6 protein levels in supernatants of platelet cocultured gp38 siRNA silenced SF compared to non-silenced controls as measured by ELISA. Mean±SD data are representative of 3 independent experiments (*p=0.0002, **p=0.01, ***p<0.0001).](image_url)
We failed to confirm an intrinsic role for gp38 in an ex vivo model of SF invasion and cartilage degradation, in contrast to that observed in cancer cells [19,20]. In cancer epithelial cells, gp38 is associated to EMT, a process that provides mesenchymal cell migratory properties to epithelial cells otherwise lacking motile or invasive capacity. It is therefore possible that gp38 does not provide these functionalities to fibroblasts which are highly mobile cells. An alternative explanation is that siRNA silencing of gp38 expression is not complete and remaining gp38 expression might be sufficient to maintain its function.

In conclusion, our study shows a new mechanism involved in platelet-SF proinflammatory interactions in RA synovium, supporting an important role for the acquisition of gp38 expression in SF. We also identify a strong association between gp38 expression and synovial LN, consistent with its reported role in developmental and experimental LN, but the mechanistic basis of this association requires further studies.

Supporting Information

Figure S1 Migratory and invasive capabilities of gp38 silenced RA SF. (A) Silencing efficiency of siRNA lentiviral transduction of RA SF as analyzed by qRT-PCR (si-CTRL mRNA gp38/β-actin ratio set to 100%) and flow cytometry (percentage of gp38+ RA SF is indicated, *p = 0.03). MFI: Mean fluorescence intensity. (B) Invasive capability of RA SF on matrigel coated transwells expressed as the number of cells per field that migrated through the matrigel at 4 days. (C) SF migration in wound assays expressed as the percentual closure of the wound area 18 h and 24 h after scraping. A representative image is shown (100x). (D) GAG release into the supernatant at 4 and 7 days of RA SF cartilage co-culture. Basal: GAG release by cartilage in the absence of SF. A representative image of siRNA GFP transduced SF attached to cartilage is shown (200x). Results are representative of three independent experiments (NS: not significant). (TIF)

Table S1 Primer sequences used for quantitative real-time PCR analysis.

| Primer Sequences | Function |
|------------------|----------|
|                 |          |

Acknowledgments

We are grateful to the Servicio de Traumatología y Cirugía Ortopédica (Hospital 12 de Octubre) for providing normal and osteoarthritic synovial tissues. We also acknowledge Jordi Monfort (IMIM, Barcelona) for providing healthy cartilage slices, and Vanesa Miranda and Juan Carlos Armas (Hospital 12 de Octubre) for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: MJRD JLP. Performed the experiments: MJRD RF JLP. Analyzed the data: MJRD JDC JLP. Wrote the paper: MJRD JLP.

References

1. Bartok B, Firestein GS (2010) Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 233: 233–255.
2. Naylor AJ, File A, Buckley CD (2013) The role of stromal cells in the persistence of chronic inflammation. Clin Exp Immunol 171: 30–55.
3. Neumann E, Leffèvre S, Zimmermann B, Gay S, Müller-Ladner U (2010) Rheumatoid arthritis progression mediated by activated synovial fibroblasts. Trends Mol Med 16: 456–468.
4. Zhang Q, Gao Y, Grigoriev G, Chen J, Park-Min KH, et al. (2013) Tumor necrosis factor α induces sustained signaling and a prolonged and unremitting inflammatory response in rheumatoid arthritis synovial fibroblasts. Arthritis Rheum 65: 928–938.
5. Muller-Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, et al. (1996) Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. Am J Pathol 149: 1607–1615.
6. del Rey MJ, Izquierdo E, Caja S, Usetape S, Santamaria M, et al. (2009) Human inflammatory synovial fibroblasts induce enhanced myeloid cell recruitment and angiogenesis through a hypertrophic-inducible transcription factor 1alpha/vascular endothelial growth factor-mediated pathway in immunodeficient mice. Arthritis Rheum 60: 2926–2934.
7. Bradfield PF, Amid N, Vernon-Wilson E, Edley AE, Parsonage G, et al. (2003) Rheumatoid fibroblast-like synoviocytes overexpress the chemokine stromal cell-derived factor 1 (CXCL12), which supports distinct patterns and rates of CD4+ and CD8+ T cell migration within synovial tissue. Arthritis Rheum 48: 2472–2482.
8. Benito-Miguel M, García-Carmona Y, Balsa A, Baustista-Caro MB, Arroyo-Villa I, et al. (2012) IL-15 expression on RA synovial fibroblasts promotes T cell survival. PLoS One 2012; 7: e60620.
9. Lindhout E, van Eijk M, van Pel M, Lindenau J, Dinant HJ, et al. (1999) Fibroblast-like synoviocytes from rheumatoid arthritis patients have intrinsic properties of follicular dendritic cells. J Immunol 162: 5949–5956.
10. Manzo A, Paolotti S, Cardulli M, Blades MG, Barone F, et al. (2003) Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. Eur J Immunol 35: 1347–1359.
11. Rajewsky M, Egen GI, Koo LY, Laugier JP, Beauf B, et al. (2006) Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity 25: 989–1001.
12. Link A, Hardie DL, Favre S, Brinchgi MR, Adams DH, et al. (2011) Association of T-zone reticular vessels and conduits with ectopic lymphoid tissues in mice and humans. J Pathol 178: 1662–1675.
13. Farr AG, Berry ML, Kim A, Nelson AJ, Welch MP, et al. (1992) Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. J Exp Med 176: 1477–1482.
14. Ekwall AK, Eider T, Anderberg C, Jin C, Karlsson N, et al. (2011) The tumour-associated glycoprotein podoplanin is expressed in fibroblast-like synoviocytes of the hyperplastic synovial lining layer in rheumatoid arthritis. Arthritis Res Ther 13: R40.
15. Kawase A, Ishii G, Nagai K, Ito T, Nagano T, et al. (2008) Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. Int J Cancer 123: 1053–1059.
16. Dulauroy S, Di Carlo SE, Langa F, Eberl G, Peduto L, et al. (2009) Lineage tracing and genetic ablation of ADAM12(+)+ perivascular cells identify a major source of profibrotic cells during acute tissue injury. Nat Med 15: 1267–1270.
17. Peduto L, Dulauroy S, Lochner M, Spath GF, Morales MA, et al. (2009) Inflammation recapitulates the ontology of lymphoid stromal cells. J Immunol 182: 5798–5799.
18. Peters A, Patchett LA, Sullivan JM, Misdorffler M, Acton SE, et al. (2011) Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity 35: 996–996.
19. Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, et al. (2006) Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. Cancer Cell 9: 261–272.
20. Martin-Villar E, Megías D, Castel S, Vuittia MM, Vilaprò S, et al. (2006) Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. J Cell Sci 119: 4541–4553.
21. Navarro A, Perez RE, Rezaiekhah MH, Mahry SM, Elekezea II (2011) Polarized migration of lymphatic endothelial cells is critically dependent on podoplanin regulation of Cdc42. Am J Physiol Lung Cell Mol Physiol 300: L32–42.

22. Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, et al. (2003) T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J 22: 3546–3556.

23. Uhrin P, Zaujec J, Bressa JM, Olczady D, Chrenek P, et al. (2010) Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. Blood 115: 3997–4005.

24. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, et al. (2012) Platelet activation preferentially expressed in vascular endothelium. Biochem J 341 (Pt 2): 277–284.

25. Acton SE, Astarita JL, Malhotra D, Lukacs-Kornek V, Franz B, et al. (2012) Podoplanin-rich stromal networks induce dendritic cell motility via activation of the C-type lectin receptor CLEC-2. J Biol Chem 287: 24494–24507.

26. Bertozzi GC, Schmaier AA, Mericko P, Hess PR, Zou Z, et al. (2010) Platelets regulate lymphatic vascular development through CLEC-2/SLP-76 signaling. Blood 116: 661–670.

27. Osada M, Inoue O, Ding G, Shirai T, Ichise H, et al. (2011) Synovial fibroblast hyperplasia in rheumatoid arthritis critically depends on follicular dendritic cells. Immunity 30: 130–142.

28. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 231–245.

29. Zimmer G, Oeffner F, Von Messling V, Tschernig T, Groeness HJ, et al. (1999) Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. J Biol Chem 285: 24494–24507.

30. Genovese MC, Rubbert-Roth A, Smolen JS, Kremer J, Khraishi M, et al. (2013) Bruton’s Tyrosine Kinase mediates platelet receptor-induced generation of microparticles: a potential mechanism for amplification of inflammatory responses in rheumatoid arthritis synovial joints. Immunol Lett 150: 97–104.

31. Kopf M, Herren S, Wiles MV, Pepsys MB, Kosco-Vilbois MH (1998) Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. J Exp Med 188: 1893–1906.

32. Zufferey R, Doll T, Mandel RJ, Bukovsky A, Quiroz D, et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72: 9873–9880.

33. Pretzel D, Pohlers D, Weinert S, Kinne RW (2009) In vitro model for the analysis of synovial fibroblast-mediated degradation of intact cartilage. Arthritis Res Ther 11: R25.

34. Scacht V, Ramírez MI, Hong YK, Indukawa S, Feng D, et al. (2003) T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. EMBO J 22: 3546–3556.

35. Neidhart M, Seemayer CA, Hummel KM, Michel BA, Gay RE, et al. (2003) Functional characterization of adherent synovial fluid cells in rheumatoid arthritis: destructive potential in vitro and in vivo. Arthritis Rheum 46: 1873–1890.

36. Pretzel D, Pohlers D, Weinert S, Kinne RW (2009) In vitro model for the analysis of synovial fibroblast-mediated degradation of intact cartilage. Arthritis Res Ther 11: R25.

37. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327: 580–583.

38. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327: 580–583.

39. Zufferey R, Doll T, Mandel RJ, Bukovsky A, Quiroz D, et al. (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 72: 9873–9880.