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Synovial-tissue resident macrophages play proinflammatory functions in the pathogenesis of RA while maintaining the phenotypes in the steady state

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ABSTRACT

Synovial tissue-resident macrophages (STRMs) maintain normal joint homeostasis in a steady state. However, it is unclear whether STRMs still play homeostatic roles or change the functions in the joint of rheumatoid arthritis (RA), where infiltrating peripheral blood monocyte-derived macrophages (PBMoMs) play proinflammatory roles. In the present study, we examined changes in the phenotypes and functions of STRMs in response to RA-related stimuli *in vitro*. STRMs were prepared from non-inflammatory osteoarthritis (OA) joint synovium, which is histologically indistinguishable from normal joint synovium. PBMoMs were prepared and used for comparison. After stimulation with plate-bound IgG, which mimics anti-citrullinated protein antibody immunocomplex formed in RA joints, or with combinations of RA-related inflammatory mediators, namely tumor necrosis factor-α (TNF-α) and prostaglandin E2 or interferon-γ, PBMoMs downregulated surface markers and genes associated with anti-inflammatory macrophages, and upregulated cytokine and marker genes of proinflammatory macrophages in RA. On the other hand, STRMs hardly changed the expression of surface molecules and marker genes but altered the pattern of cytokine gene expression after stimulation like PBMoMs. Furthermore, *in vitro* stimulated STRMs promote proinflammatory functions of cocultured synovial fibroblasts. Thus, STRMs might play proinflammatory roles in RA joints, while maintaining their phenotypes in the steady state.

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KEYWORDS
Rheumatoid arthritis; macrophage; anti-citrullinated peptide antibody; cytokine; synovium

Introduction

Tissue-resident macrophages (TRM) are generally involved in the maintenance of tissue homeostasis [1]. They often exert anti-inflammatory roles and, therefore, had once been categorized as the 'alternatively activated' M2, as opposed to the pro-inflammatory 'classically activated' M1, although M1/M2 polarization is an oversimplified concept that does not fit with *in vivo* situations. However, in some pathogenic conditions, TRMs are known to play proinflammatory roles [2]. Therefore, understanding the functions of TRMs in various conditions is important for the treatment of diseases with disturbed tissue homeostasis.

Synovial joints are one of the tissues abundant of TRMs. Synovial membrane in normal joints forms thin lining layers consisting of TRMs and fibroblasts. These synovial tissue-resident macrophages (STRM) are implicated in removing debris in the joint cavity [3]. Furthermore, a recent study demonstrated their barrier functions that maintains synovial tissue integration [4]. Hence disruption of the synovial lining leads to the development of arthritis in mice. Thus, STRMs are involved in maintaining homeostasis of normal joints.

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting multiple synovial joints. RA is an autoimmune disease characterized by the production of autoantibodies, such as anti-citrullinated protein antibody (ACPA), which precedes the disease onset and are implicated in the initiation of arthritis by triggering local inflammation. The histology of established RA synovium shows hyperplasia of the

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lining layer and massive infiltrates in the sublining. Macrophages are found in both layers, and those in the lining, if not all, are thought to originate from STRMs while those in the sublining are from peripheral blood monocytes (PBMo) that have infiltrated into the synovium and are the main producer of TNF-α [5,6]. The pathogenicity of PBMo-derived macrophages (PBMoM) was more clearly demonstrated in mouse models of arthritis [4,7,8]. Changes in the number of sublining, but not lining, macrophages, correlated with the reduction of disease activity after treatment of human RA, supporting the importance of PBMoM in the pathogenesis [9]. Rapid decrease of the synovial macrophages after initiation of anti-TNF-α therapy without evidence of increased apoptosis suggests an enhanced efflux of PBMoM [10–12]. On the contrary, the roles for synovial macrophages of STRM origin in the pathogenesis of RA are not well understood.

Recent progress in single-cell transcriptomics unveiled the presence of several macrophage subsets in RA synovium [13,14]: some resemble PBMoM, while others express the markers of STRMs, such as MerTK. Interestingly, a MerTK + macrophage subset with anti-inflammatory functions was identified in RA patients with sustained remission [15]. This suggests that STRM-derived macrophages exert anti-inflammatory functions in active RA as well. It is also possible that STRMs change their phenotypes and functions in the inflammatory milieu of RA, but these possibilities have not been addressed. On the other hand, there have been studies analyzing changes in the functions of human PBMoMs in response to RA-related stimuli. For instance, ACPA-immunocomplex induced tumor necrosis factor-α (TNF-α) production from PBMoMs *in vitro* [16], which might account for the amplification of synovial inflammation in established RA by ACPA. However, this does not explain the roles of ACPA in the initiation phase of arthritis, when only STRMs exist in the joints before the influx of PBMoMs. Stimulation of PBMoMs with TNF-α and synovial fibroblasts (SFs) induced genes upregulated in the HBGEF + inflammatory macrophage cluster in RA synovium, and prostaglandin E2 (PGE2) substituted the effect of SFs [13]. Another study comparing macrophage clusters in several inflammatory tissues, including RA synovium, discovered an expansion of a CXCL10 + CCL2+ population [17]. Stimulation of PBMoMs with interferon-γ (IFN-γ) and TNF-α induced differentiation of macrophages with similar phenotypes. Those experiments have contributed to understanding the development and functions of infiltrating PBMoMs in RA synovium. In the present study, we aim to verify whether STRMs also changes their functions in response to RA-related inflammatory stimuli *in vitro*.

Materials and methods

Patients

Twelve osteoarthritis (OA) (3 males and 9 females, mean age 77.1 ± 5.8 years) and 2 RA patients (both female, 70 and 76 years old, 3 and 4 years of disease duration) were included. Both of RA patients fulfilled 1987 criteria of the American College of Rheumatology and were seropositive and treated solely with methotrexate. This study complies with the Declaration of Helsinki, and the study protocol was approved by the Kyushu University School of Medicine Human Research Ethics Regional Committee (30-269). All subjects provided informed consent before participating in the study.

Isolation of macrophages from synovium

Synovial tissues were obtained at the time of joint replacement surgery for OA. The tissue was cut into 1–2 mm pieces and digested with 2 mg/ml collagenase (Wako, Osaka, Japan) and 10 μg/ml DNase (Roche, Basel, Switzerland) in culture medium (RPMI 1640 with 10% fetal bovine serum, 500 units/ml penicillin, 500 μg/ml streptomycin, 0.1 μM 2-mercaptoethanol) at 37 °C in a shaking incubator for 1.5 h and was filtered through a 70 μm cell strainer (Corning, NY, USA). To enrich for macrophages, cells were incubated for 1 h at 37 °C on Cepallet thermal detachment cell culture plates (DIC, Tokyo, Japan). After flushing with warm phosphate-buffered saline (PBS), the plates were placed on ice, and adherent cells were collected gently with a scraper. Macrophages were purified by using human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). We only used macrophages isolated from histologically confirmed non-inflammatory OA for the following experiments as STRMs (Supplementary Figure 1).

Differentiation of macrophages from PBMo

Peripheral blood mononuclear cells were collected by using Ficoll-Paque Plus separation media (GE Healthcare Bio-Science, Uppsala, Sweden), and PBMo were isolated by using human CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMo were differentiated into macrophages in the presence of 50 ng/ml of macrophage-colony stimulating factor (M-CSF) for 5 days and were further cultured with 50 ng/ml of

IFN-γ and 100 ng/ml of lipopolysaccharide or 20 ng/ml of IL-4 (PeproTech, Cranbury, New Jersey) to induce M1 or M2, respectively.

Flow cytometric analysis

Antibodies used for flow cytometric analysis were PE-conjugated anti-FOLR2 (94b) and anti-CD206 (15-2), APC-conjugated anti-CD163 (GHI/61), PE-Cy7-conjugated anti-MerTK (590H11G1E3,), BV421-conjugated anti-CD11b (M1/70), BV510-conjugated anti-HLA-DR (L243)(BioLegend San Diego, CA, USA), APC-Cy7-conjugated anti-CD45 (HI30; Tonbo Biosciences, San Diego, CA), unconjugated anti-TREM2 (237920; R&D Systems, Minneapolis, MN, USA), and Alexa Fluor 488-conjugated anti-mouse IgG (ThermoFisher Scientific, Waltham, MA, USA). Optimally diluted fluorescent antibodies were added to the single cell suspension in staining buffer (PBS containing 2% fetal bovine serum) for 20 min at 4 °C. After washing twice with the staining buffer, propidium iodide (2 μg/ml) was added to the cell suspension just before run on a FACSVerse flow cytometer (BD Biosciences, San Diego, CA, USA). The data were analyzed by using FlowLogic Software (Miltenyi Biotec). Analysis gate was set in the following manner. Doublets were excluded using forward light scatter (FSC) area and height, while dead cells were excluded based on Propidium Iodide staining. Myeloid cells were gated based on the side scatter (SSC) area and FSC area, and macrophages were defined as CD45+ CD11b+cell population.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol Reagent (ThermoFisher Scientific), and cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan). Quantitative real-time RT-PCR was performed using CFX Maestro (BIORAD, Hercules, CA, USA) and STBR Premix EX Taq II (Takara Bio). Data are presented as either 2-ΔCT, where ΔCT = CT for GAPDH minus CT for genes of interest, or fold-change, where ΔCT for the control = 1. The primers used for the experiments are shown in Supplementary Table 1.

In vitro stimulation of macrophages

To stimulate macrophages with plate-bound IgG, 24-well cell culture plates were precoated with purified human IgG (2 μg/ml) (Jackson ImmunoResearch, PA, USA). Macrophages were cultured in the plates at 50,000 cells/well for 24 h. To stimulate macrophages with cytokines, cells were cultured for 24 h with 20 ng/ml of TNF-α (Peprotech) and 1 μg/ml of PGE2 (Wako), or with 20 ng/ml of TNF-α (Peprotech) and 50 ng/ml of IFN-γ (Peprotech).

Co-culture of SFs with macrophages

Collagenase-dissociated synovial cells from OA or RA joints were cultured in the culture medium (RPMI 1640 with 10% fetal bovine serum, 500 units/ml penicillin, 500 μg/ml streptomycin, 0.1 μM 2-mercaptoethanol) in 10 cm plastic dishes (TPP, Trasadingen, Switzerland) for 2 days. After removing non-adherent cells by flushing with warm PBS, the remaining adherent cells were further cultured. Cells were harvested at passages 3–8 and were used as SFs for the coculture experiments, in which macrophages were incubated in 24-well cell culture plates, while the SFs were placed on cell culture inserts (pore size 0.45 μm) (Millicell HA, Merck Millpore, Darmstadt, Germany). Anti-TNF-α antibody, infliximab (10 μg/ml) (Mitsubishi Tanabe Pharma, Tokyo, Japan), was added to the culture in some experiments. After 48 h, RNA was extracted from the SFs to analyze gene expression.

Statistical analysis

Statistical significance was determined using paired *t*-test, Mann–Whitney test, or one-way ANOVA with Tukey's *post-hoc* analysis. All data analyses were performed using Prism version 5.0 software (GraphPad). *p*-Values less than 0.05 were considered significant.

Results

STRMs are distinct from M1 or M2 induced from PBMo

An obstacle in studying human STRMs is the ethical difficulty in obtaining synovium with sufficient number of cells from normal joints. In this regard, large pieces of synovium can be obtained at the time of joint replacement surgery for osteoarthritis (OA), which is widely accepted as a non-autoimmune, degenerative joint disorder and been used as the control for RA samples [4,8,14]. Nevertheless, since it was reported that a portion of OA patients show some features of inflammatory arthritis [18], we excluded samples showing cellular infiltrates from the analysis (Supplementary Figure 1). We confirmed that macrophages in the histologically non-inflammatory OA show phenotypes of STRMs in normal joint in that they express MerTK, CD206, FOLR2 and TREM2

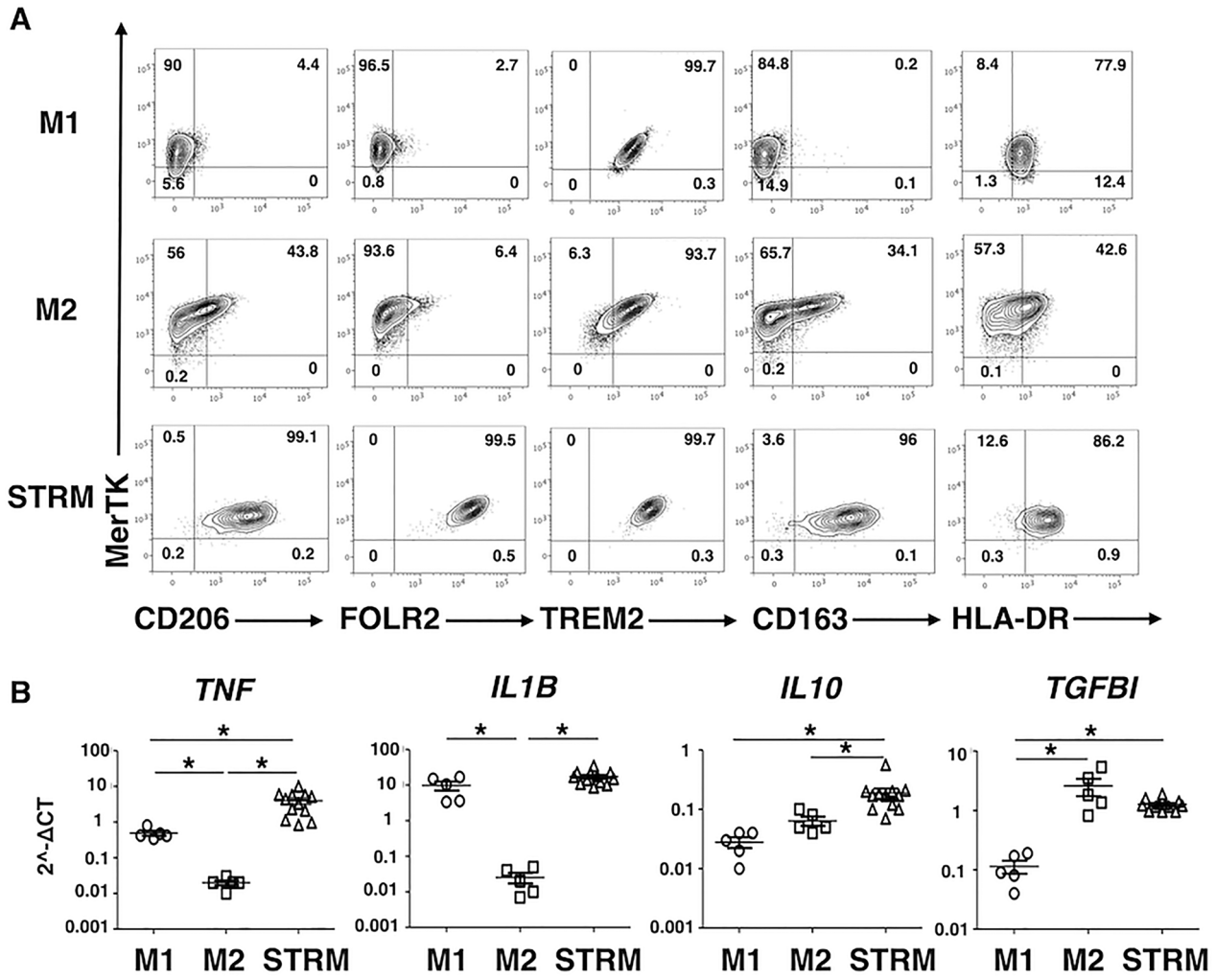

Figure 1. Phenotypes of STRMs in comparison to *in vitro* induced M1 and M2. (A) Representative dot plots of the expression of surface molecules on PBMo-derived M1 (upper panels), and M2 (Middle panels), and STRMs (lower panels) are shown after gating on CD45+ CD11b+cells. (B) Expression levels of mRNA for cytokines in M1, M2, and STRMs ($n=5$ for M1 and M2, $n=12$ for STRMs). *$p<0.05$.

(Figure 1(A)) [15]. Thereafter, these were used as STRMs.

To characterize STRMs more in detail, we compared them with M1 or M2 induced *in vitro* from PBMo. We found that STRMs have an intermediate phenotype: STRMs express MerTK and CD163 like M2, but also express HLA-DR like M1. Gene-expression analysis showed the STRMs expressed proinflammatory *TNF* and *IL1B* like M1, but also expressed anti-inflammatory *IL10* and *TGFBI* like M2 (Figure 1(B)). Thus, it was revealed that STRMs in non-inflammatory joints are phenotypically and functionally different from M1 or M2 induced *in vitro* from PBMo.

Phenotypical and functional changes of STRMs in response to plate-bound IgG mimicking ACPA immune-complex

In order to test the hypothesis that ACPA activates STRMs that triggers joint inflammation at the initiation phase of RA, we examined *in vitro* responses of the STRMs in non-inflammatory joints to stimuli mimicking ACPA immune-complex, namely plate-bound IgG [19]. We also examined PBMoMs as the control for positive response. While PBMoMs clearly downregulated the expression of MerTK, CD163, FOLR2, and TREM2 after stimulation, STRMs did not show changes in the expression of surface molecules except for downregulation of TREM2 (Figure 2(A)). We next examined the expression of cytokine and marker genes for four synovial macrophage clusters identified by single-cell transcriptome analysis in previous studies [13,15]. PBMoMs upregulated *TNF* and *IL1B*, but downregulated *IL10* and *TGFBI* in response to plate-bound IgG (Figure 2(B)). STRMs also upregulated *TNF* and *IL1B*, though to a smaller extent than the PBMoMs. Expression of *IL10* was downregulated in PBMoMs but upregulated in STRMs by IgG stimulation. For the expression of cluster markers,

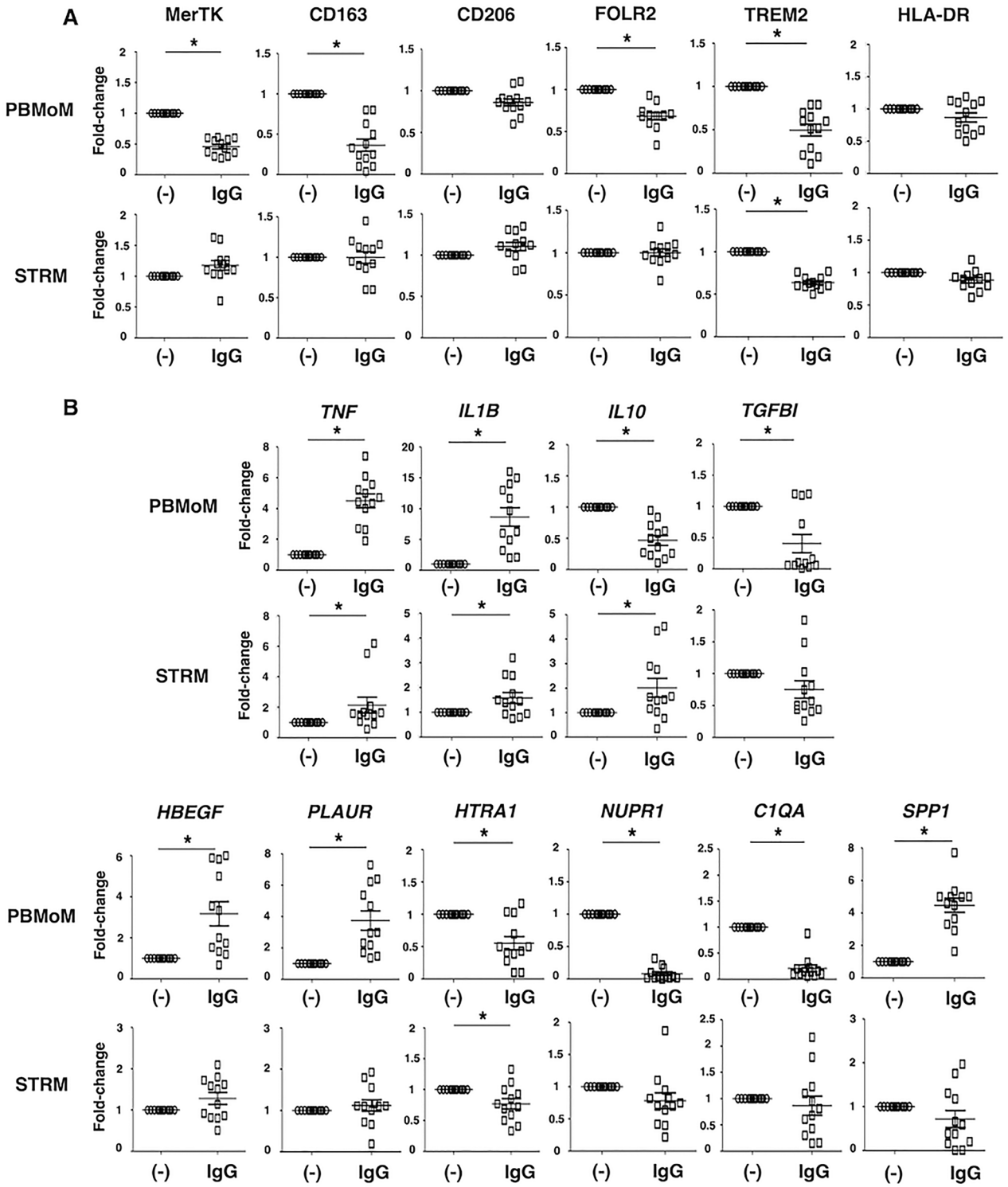

Figure 2. Changes in the phenotypes and functions of STRMs in response to plate-bound IgG stimulation. (A) Changes in the expression of surface molecules on PBMoM (upper panels) and STRMs (lower panels) after stimulation with plate-bound IgG (IgG) or without stimulation (–) ($n = 12$ per group). (B) Changes in the expression levels of mRNA for cytokines or cluster markers in PBMoMs (upper panels) and STRMs (lower panels) after IgG-stimulation (IgG) or unstimulated control (–) ($n = 12$ per group). *$p < 0.05$.

PBMoMs upregulated *HBGEF*, *PLAUR* and *SPP1*, which are associated with the proinflammatory macrophage clusters [13,17], while downregulating non-inflammatory cluster-associated *HTRA1* and *NUPR1*. STRMs showed little changes in the expression of the marker genes except for the downregulation of *HTRA1*.

To test whether the IgG-stimulated STRMs, although they showed relatively small changes in gene expression, affect the functions of SFs, we cocultured SFs from the non-inflammatory OA patients with STRMs (Figure 3(A)). Upregulation of the expression of proinflammatory genes, such as *IL6* and *ICAM1*, with downregulation of *COL1A1*,

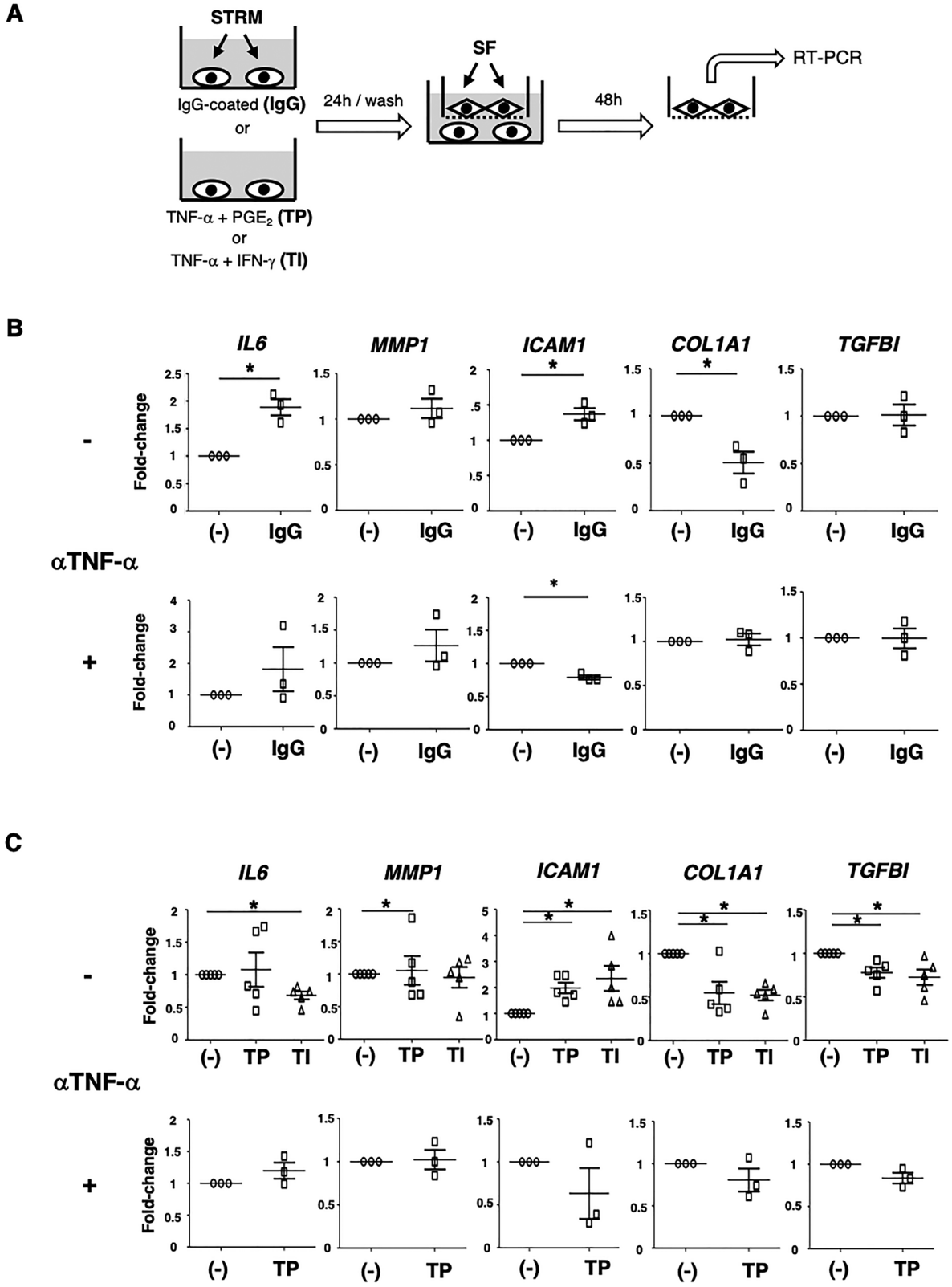

Figure 3. Changes in the functions of FLs cocultured with the *in vitro* stimulated STRMs. (A) Overview of the experimental design. (B) Changes in the expression of mRNA levels in SFs from non-inflammatory OA joints cocultured with STRMs with (IgG) or without IgG (–) ($n = 3$ per group). Lower and upper panels show data of cultures with or without anti-TNF-α antibody. (C) Changes in the expression levels of mRNA in SFs from active RA joints cocultured with STRMs stimulated with TNF-α + PGE2 (T + P), TNF-α + IFN-γ (T + I) or without stimulation (–) ($n = 5$ per group). Lower and upper panels show data of cultures with or without anti-TNF-α antibody (T + P not included). *$p < 0.05$.

which is involved in tissue repair, was detected in the SFs cocultured with STRMs stimulated with plate-bound IgG (Figure 3(B)). As TNF-α was upregulated the stimulated STRMs, we added anti-TNF-α antibody to the coculture. The proinflammatory effect of STRMs was reduced by

blocking TNF-α, indicating that the effect was largely dependent on TNF-α. Thus, STRMs might be involved in the onset of RA by responding to ACPA to promote inflammatory functions of SFs.

Phenotypical and functional changes of STRMs in response to inflammatory mediators associated with the pathogenesis of active RA

We next examined the changes of STRMs in response to inflammatory milieu, in order to verify their phenotypes and functions in the established phase of RA. We selected two culture conditions, namely TNF-α plus PGE2 and TNF-α plus IFN-γ, which were shown to induce inflammatory macrophages resembling those in RA joints from PBMo [13,17]. In fact, both combination of inflammatory mediators downregulated the expression of MerTK, CD163, and CD206 on PBMoMs (Figure 4(A)). Expression of TREM2 and HLA-DR was upregulated upon stimulation with TNF-α plus IFN-γ. On the contrary, STRMs did not show any changes in the expression of surface molecules in both culture conditions, similar to the case of IgG stimulation. The two combinations of inflammatory mediators affect differently the gene expression of PBMoMs: TNF-α plus PGE2 upregulates the expression of *IL1B*, *HBGEF* and *PLAUR* while TNF-α plus IFN-γ upregulated *TNF*, *PLAUR* and *SPP1* but downregulated *IL10*, *TGFBI* and *HTRA1* (Figure 4(B)). Stimulation with the combinations of cytokines induced similar in pattern but small changes of cytokine gene expression in STRMs, but the expression of the macrophage cluster marker genes were hardly changed.

We lastly tested whether STRMs are anti- or pro-inflammatory in the pathogenesis of established RA by coculturing STRMs with SFs isolated from the joint of RA, but not OA, patients. SFs upregulated *ICAM1* but downregulated *COL1A1* and *TGFBI* expression when cocultured with STRMs stimulated with the inflammatory mediators (Figure 3(C)). Blocking TNF-α, which was upregulated in STRMs stimulated with TNF-α plus IFN-γ, reduced the effects, similar to the case of IgG-stimulated STRMs. Taken together these data suggest that STRMs play proinflammatory roles in established RA while maintaining their phenotype in the steady state.

Discussion

STRMs are implicated in maintaining tissue homeostasis in normal joints [4,7], but their functions in active RA have not been well understood. We here demonstrated that human STRMs hardly changed the phenotypes but upregulated proinflammatory functions in response to inflammatory stimuli relevant to the pathogenesis of RA.

Although STRMs are implicated in tissue homeostasis in normal conditions, their phenotypes and functions in pathogenic conditions have not been explored until recently when Alivernini et al. reported distinct pattern of surface molecule expression on synovial macrophages between healthy subjects and RA [15]. They firstly showed by flow cytometric analysis that STRMs in healthy joint express MerTK, CD206 and CD163. We confirmed the macrophages in non-inflammatory OA joint showed similar phenotypes and used them as STRM afterwards. We also examined the expression of surface molecules and cytokines by comparing with M1 and M2 induced from PBMoMs and found that STRMs are different from either M1 or M2. Thus, we confirmed that the M1/M2 polarization is an oversimplified concept and is not applicable for *in vivo* differentiated macrophages.

ACPA has been implicated in the initiation of arthritis at the preclinical stage of RA. In animal studies, STRMs are activated by immunocomplex, resulting in disruption of barrier functions and the development of arthritis [4]. TNF-α production of human PBMoMs in response to ACPA immunocomplex has also been shown [16]. However, this only accounts for the amplification of synovial inflammation by ACPA, because PBMoMs infiltrate into the joint synovium after RA onset. Our study, for the first time, demonstrated that human STRMs activate SFs from non-inflamed joints in response to stimuli mimicking ACPA-immunocomplex [19]. This might occur in the joints of ACPA + individuals before the onset of RA. We failed to induce macrophage activation with ACPA purified from the serum of RA patients and citrullinated proteins. However, it is not surprising if ACPA does not induce strong inflammation, because it usually takes years from the detection of serum ACPA until RA onset.

PBMoMs infiltrating into synovial sublining are thought to play critical roles in the pathology of established RA. However, hyperplasia of lining layer, which likely includes STRMs, is also a feature of RA synovium. It is possible that these are homeostatic macrophages counteracting to inflammation or converted to inflammatory type macrophages adapting to the milieu. We found in this study that STRM upregulated TNF-α or IL-1β in response to RA-related inflammatory stimuli. Importantly, the stimulated STRMs promoted proinflammatory functions and downregulated anti-inflammatory functions of SF from RA patients, suggesting the inflammatory milieu in the joint of established RA

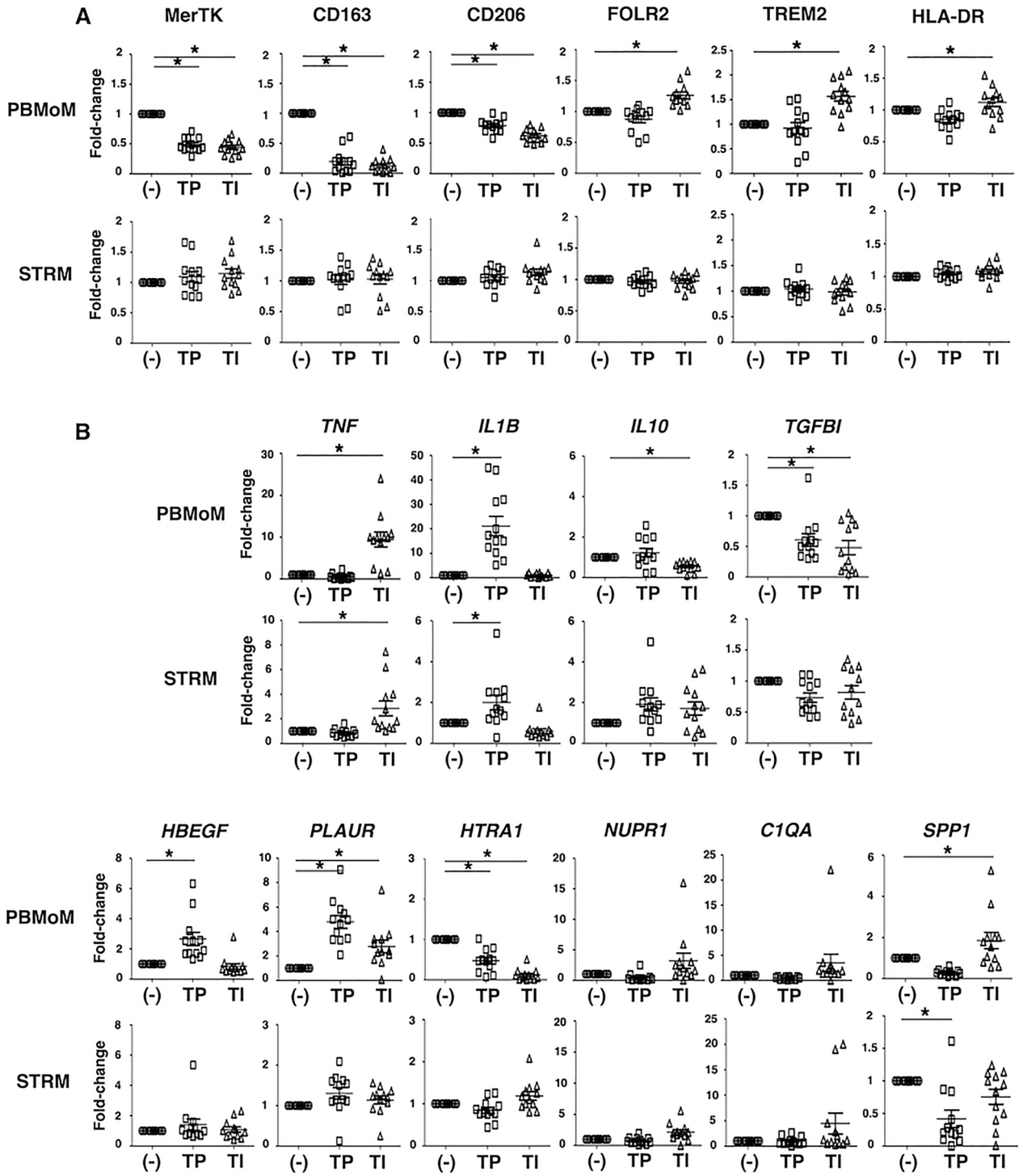

Figure 4. Changes in the phenotypes and functions of STRMs in response to inflammatory mediator stimulation. (A) Changes in the expression of surface molecules on PBMoM (upper panels) and STRMs (lower panels) after stimulation with TNF-α and PGE2 (T + P) or TNF-α and IFN γ (T + I) or without stimulation (–) ($n = 12$ per group). (B) Changes in the expression levels of mRNA for cytokines or cluster markers in PBMoMs (upper panels) and STRMs (lower panels) after stimulation with TNF-α and PGE2 (T + P) or TNF-α and IFN-γ (T + I) or without stimulation (–) ($n = 12$ per group). *$p < 0.05$.

convert STRMs to more proinflammatory ones. Since PBMoMs are critical cellular source of inflammatory mediators including TNF-a [5,6], an important question is whether STRMs interact with PBMoMs in RA joint. The separated distribution of STRMs and PBMoMs, mainly synovial lining and sublining, respectively, makes it unlikely. However, they might be more closely located at an early phase of disease. Moreover, the abundance of secreted inflammatory mediators in the synovium, as well as in the synovial fluid, might overcomes the distance.

Another important finding of our study is that the expression of surface molecules as well as marker genes of synovial macrophage clusters in STRMs was

hardly affected by external stimuli, either IgG or inflammatory mediators, *in vitro*. If this also applies *in vivo*, the macrophage clusters identified by the cross-sectional, comprehensive gene expression analysis are stable throughout the course of RA from healthy joints. This might not necessarily conflict with the observed functional shift of STRMs by the inflammatory stimuli, because it was shown that synovial macrophages of MerTK + clusters, which likely originated from STRMs, exhibited slightly altered gene expression depending on the disease status of RA, namely healthy, active, or in remission [15]. In fact, MerTK + macrophages purified from active, but not remission, RA induced partial activation of SFs [15], supporting our results that *in vitro* stimulated STRMs, which maintain MerTK expression, are exerted proinflammatory functions.

The limitations of this study might include the use of OA synovium as the source of STRMs instead of normal synovium due to limited cell number. Although some inflammatory mediators are implicated in the pathology of OA [18], there are large differences in the immune pathology of RA and OA, which is evident from the distinct clinical efficacy of targeting inflammatory cytokines, including TNF-α and IL-6. Furthermore, we only used samples that histologically displayed no signs of inflammation and confirmed the macrophages similar to those in healthy joint, as mentioned above. There is also another limitation relating to the limited cell number. Although we analyzed expression of selected genes using conventional RT-PCR, more comprehensive analysis, especially single-cell RNA sequencing, might provide us more detailed information. It is also an obstacle to the epigenetic analysis, which is useful for elucidating the mechanism of the relative hypo-responsiveness of STRMs to the inflammatory stimuli. In addition, our experimental system using *in vitro*-induced macrophages is not suitable for examining whether the STRM-derived macrophages are as proinflammatory as PBMoMs-derived ones in RA joints. These should be addressed in the future for further understanding the biology of STRMs.

In summary, our data implies that STRMs play proinflammatory roles during the inflammatory process of RA and might also be the therapeutic target, in addition to PBMoMs. Therefore, pan-macrophage reagents can be used for the treatment of RA. In support of this, depleting joint macrophages by injecting clodronate-laden liposomes suppressed a murine model of arthritis [20]. However, it should be noted that anti-inflammatory, tissue-remodeling macrophages, which are also likely of STRM origin, appear RA in remission [15]. Further understanding the biology of STRMs might pave the way for novel and safe treatment strategy of disturbed joint homeostasis, including RA.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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