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ORIGINAL ARTICLE

The Role of Interferon Regulatory Factor 5 in Macrophage Inflammation During Osteoarthritis

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Abstract— Mounting evidence suggests that aberrant immune responses are involved in the pathogenesis of osteoarthritis (OA). Synovial macrophages are likely involved. In this study, we sought to investigate the role of interferon regulatory factor 5 (IRF5). *In vitro* M1-polarized macrophages presented significantly higher IRF5 expression than M2-polarized macrophages. Interestingly, IRF5 expression was observed in macrophages from the synovial fluid of OA patients, and the level of IRF expression was positively correlated with disease severity, such that stage 4 OA synovial macrophages presented significantly higher levels of IRF5 than stage 2 and stage 3 OA synovial macrophages. Circulating monocytes from OA patients, on the other hand, expressed little IRF5. However, synovial fluid from OA patients could significantly upregulate IRF5 expression in circulating monocytes. Synovial macrophages also expressed significantly higher IL-12 than circulating monocytes, and circulating monocytes conditioned in OA synovial fluid demonstrated significantly higher IL-12 expression. Direct IRF5 transfection could increase IL-12 expression in circulating monocytes. Interestingly, IRF5-transfected monocytes promoted the expression of Th1-associated genes in naive CD4 T cells *via* an IL-12-dependent mechanism. Overall, our study demonstrated that IRF5 expression was associated with OA severity and could contribute to the activation of the M1-Th1 axis.

KEY WORDS: interferon regulatory factor 5; macrophage; osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is a progressive disease characterized by the degeneration of joints, leading to pain and immobility in the affected individuals. Aging is considered the most prominent contributor to OA development, as the majority of symptomatic OA occurs in older adults [1]. Other factors, such as bone deformities, traumatic injuries of the joints, obesity, and genetic predisposition, also significantly increase the risk of OA [2].

In the past, OA was mainly regarded as a non-rheumatic condition that was caused by wear and tear of

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the cartilage through mechanical stress over time. Recently, mounting evidence suggests that a low-grade chronic inflammation exists locally in the synovial fluid of the affected joint and systemically in the peripheral blood of the OA patients. Studies have found numerous features of inflammation in OA patients, including elevated expression of proinflammatory mediators in the serum and the synovial fluid, inflammation-induced angiogenesis, elevated infiltration by immune cells, and dysfunction of regulatory T cells [3–6], thus demonstrating the involvement of immune responses in OA. For example, the serum concentration of IL-6, a proinflammatory cytokine linked with the development of rheumatoid arthritis [7], is increased with age and is significantly associated with OA development [8, 9]. Obesity, another low-grade inflammatory condition, is more common in older adults. Patients with obesity not only have higher weight burden on the joints, especially at the lower body, but also present higher infiltration of macrophages in the adipose tissue, which release proinflammatory cytokines and adipokines, and induce catabolic changes to the extracellular matrix and the cartilage of the affected joints [10, 11].

Macrophages are innate immune cells that reside in the cartilage-pannus junction of the joints [12]. Although normally quiescent, activated macrophages can express a variety of proinflammatory and antiinflammatory cytokines, depending on the activation signals and the direction of polarization [13]. Macrophages from OA synovial cell culture are shown to express TNF-α and IL-1β, which then promote the expression of cytokines and matrix metalloproteinases (MMPs) from synovial fibroblasts [14], and catalyze the destruction of soft tissues at the joint. TNF-α can also upregulate the expression of adhesion molecules, allow further infiltration of the synovial tissue by immune cells, and facilitate the activation and proliferation by T cells and macrophages [15]. Interferon regulatory factor 5 (IRF5) is a transcription factor encoded by the IRF5 gene. When expressed in macrophages, IRF5 promotes M1 polarization and enhances Th1 and Th17 differentiation [16]. Mutations at the IRF5 gene confer susceptibility to many inflammatory diseases, including rheumatoid arthritis [17]. In mice with IRF5-knockout, the inflammation at the joint is significantly milder than that in the wild-type control, together with a reduction in IL-1β [18].

In this study, the synovial fluid, synovial lymphocytes, and peripheral blood mononuclear cells (PBMCs) were obtained from OA patients. The effects of IRF5 expression by synovial macrophages and circulating monocytes were then investigated.

METHODS

Ethical Statement

This study was approved by the Ethics Committee of 903 Hospital of PLA, and performed according to the Declarations of Helsinki. All participants provided written informed consent.

Sample Collection

The characteristics of study participants were summarized in Table 1. All patients were untreated at sample collection. The grading of OA severity was performed according to the Kellgren and Lawrence system [19]. Five patients took supplements such as *Eucommia ulmoides*. OA patients and healthy controls with familial history of inflammatory arthritis, such as rheumatoid arthritis and psoriatic arthropathy, were excluded from the study.

Peripheral blood was mixed with citrate to prevent coagulation. The blood was layered with Ficoll (GE Healthcare) and centrifuged at 350*g* for 30 min to separate peripheral blood mononuclear cells (PBMCs). Synovial fluid was centrifuged at 350*g* for 10 min to separate the cell-free supernatant and the cells. The cell-containing

Table 1. Characteristics of Study Participants

	Stage 2	Stage 3	Stage 4	Healthy	*p*
N	10	15	15	5	
Age (years)	58.4 ± 5.1	61.6 ± 4.1	60.2 ± 6.1	60.1 ± 5.1	> 0.05
BMI (kg/m^2)	27.8 ± 3.1	27.1 ± 3.5	26.8 ± 3.7	27.3 ± 2.5	> 0.05
Gender (F/M)	5/5	8/7	7/8	3/2	> 0.05
Smoking (Y/N)	4/6	5/10	6/9	2/3	> 0.05
Alcohol abuse (Y/N)	2/8	1/14	2/13	0/5	> 0.05
Eucommia ulmoides (Y/N)	0/5	2/13	3/12	0/5	> 0.05

pellet was then resuspended in sterile phosphate-buffered saline (PBS).

Monocyte Polarization

Circulating monocytes and synovial macrophages were isolated using Monocyte Isolation Kit II, human (Miltenyi Biotec), following the manufacturer's protocol, and rested in tissue culture plates for 2 h at 37 °C. The non-adherent cells were rinsed off, and the remaining cells were incubated in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1× L-glutamine, and 1× penicillin-streptomycine (Thermo Fisher Scientific). 50 ng/mL GM-CSF or 50 ng/mL M-CSF (Miltenyi Biotec) were supplemented for the polarization of M1 or M2 phenotypes, respectively.

Reverse Transcription and Real-Time PCR

Total RNA was obtained using RNeasy Mini Kit (Qiagen), and the mRNA was reverse transcribed into cDNA using SuperScript IV Reverse Transcription Kit (Invitrogen). The gene expression levels were then measured in an ABI PRISM 7900 system (Applied Biosystems) using the change-in-threshold (ΔΔCT) method. TaqMan primer sets for NOS2, ARG1, IRF5, IL-12p35, IL-12p40, IFN-γ, IL-17, TBX21, RORC, and ACTB (reference gene) were obtained from Thermo Fisher Scientific.

IRF5 Transfection

IRF5 was placed under CMV promoter using pcDNA3.3-TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. The IRF5-pcDNA3.3 plasmid or empty plasmid was then transfected into circulating monocytes using the Lipofectamine® 3000 Reagent (Invitrogen), following the manufacturer's protocol. IRF5-transfected cells were selected using G-418 (Gibco) for 6 days, and the expression of IRF5 was confirmed using real-time PCR.

Naive CD4 T Cell-Monocyte Coculture

Naive CD4 T cells were isolated from PBMCs using Naive $CD4^+$ T Cell Isolation Kit II, human (Miltenyi Biotec), following the manufacturer's protocol. The purity was greater than 96% overall, as identified using flow cytometry staining. The naive CD4 T cells were then co-incubated with autologous IRF5-transfected or mock-transfected monocytes, together with ImmunoCult Human CD3/CD28 T Cell Activator (Stemcell) for 72 h. When indicated, anti-human IL-12p70 monoclonal mouse IgG1 clone 24910, or isotype control, was added at 10 μg/mL. To examine the expression of Th1- and Th17-associated genes in CD4 T cells, the CD4 T cells were re-isolated using $CD4^+$ T cell Isolation Kit, human (Miltenyi Biotec).

Statistical Analysis

Line and error bars represent mean ± SD. $P < 0.05$ was considered significant. All tests were two-tailed. The test for each assay was specified in the figure legend. Prism version 7.0 software was used for performing statistical tests and drawing figures.

RESULTS

Synovial Macrophages Highly Expressed IRF5 in a Severity-Dependent Manner

To investigate whether IRF5 is involved in macrophage inflammation in OA, we collected synovial fluid from OA patients at various stages of OA, including 10 patients at stage 2, 15 patients at stage 3, and 15 patients at stage 4. The synovial macrophages were harvested *via* magnetic negative selection. The expression level of IRF5 was then examined in these synovial macrophages. To establish reference points for IRF5 expression, monocytes were collected from the peripheral blood of 5 non-OA healthy participants and were polarized *in vitro* into M1 and M2 macrophages using GM-CSF and M-CSF, respectively. The monocyte isolation efficiency was confirmed by flow cytometry (Supplementary Figure 1A), and the M1 and M2 identity was confirmed by mRNA expression assay of signature genes (Supplementary Figure 1B). We found that the M1-polarized macrophages expressed significantly higher levels of IRF5 than the M2-polarized macrophages (Fig. 1a). The level of IRF5 expression in OA synovial macrophages was, in general, intermediate between the M1-polarized and M2-polarized macrophages, with a few exceptions. The synovial macrophage from stage 4 OA patients presented significantly higher IRF5 expression than the synovial macrophages from stage 3 and stage 2 OA patients, suggesting that the level of IRF5 expression increased with disease severity.

To verify whether IRF5 protein expression followed the same trend with IRF5 mRNA, we performed intracellular staining of IRF5 in M1- and M2-polarized macrophages and in synovial macrophages from OA patients (Fig. 1b). The mean fluorescence intensity

(MFI) was then examined in each sample. Similar to the mRNA results, the IRF5 MFI was significantly higher in M1-polarized macrophages than in M2-polarized macrophages, and the IRF5 MFI in OA synovial macrophages was at an intermediate level between the two polar extremes (Fig. 1c). The IRF5 MFI in synovial macrophage from stage 4 OA patients was significantly higher than that in synovial macrophages from stage 3 and stage 2 OA patients.

Conditioning with OA Synovial Fluid Resulted in IRF5 Upregulation in Circulating Monocytes

Next, we examined IRF5 expression in circulating monocytes from healthy controls and OA patients. Compared to the IRF5 level in monocytes from healthy participants, the IRF5 levels in monocytes from OA patients were not significantly different (Fig. 2a). Also, for all participants, the level of IRF5 mRNA expression was markedly lower in circulating monocytes than in synovial fluid macrophages ($p < 0.001$ for all comparisons between corresponding populations).

In OA patients with a high volume of synovial fluid, including five stage 2 patients, seven stage 3 patients, and eight stage 4 patients, we performed additional experiments using synovial fluid conditioning. We incubated monocytes in 100% culture media, 50% autologous synovial fluid plus 50% culture media, or 100% synovial fluid, for 24 h. The IRF5 mRNA expression was then examined (Fig. 2b). Compared to monocytes that were incubated in pure media, the monocytes that were incubated in synovial fluid presented significantly higher IRF5 expression, in a manner that was dependent on the concentration of the synovial fluid.

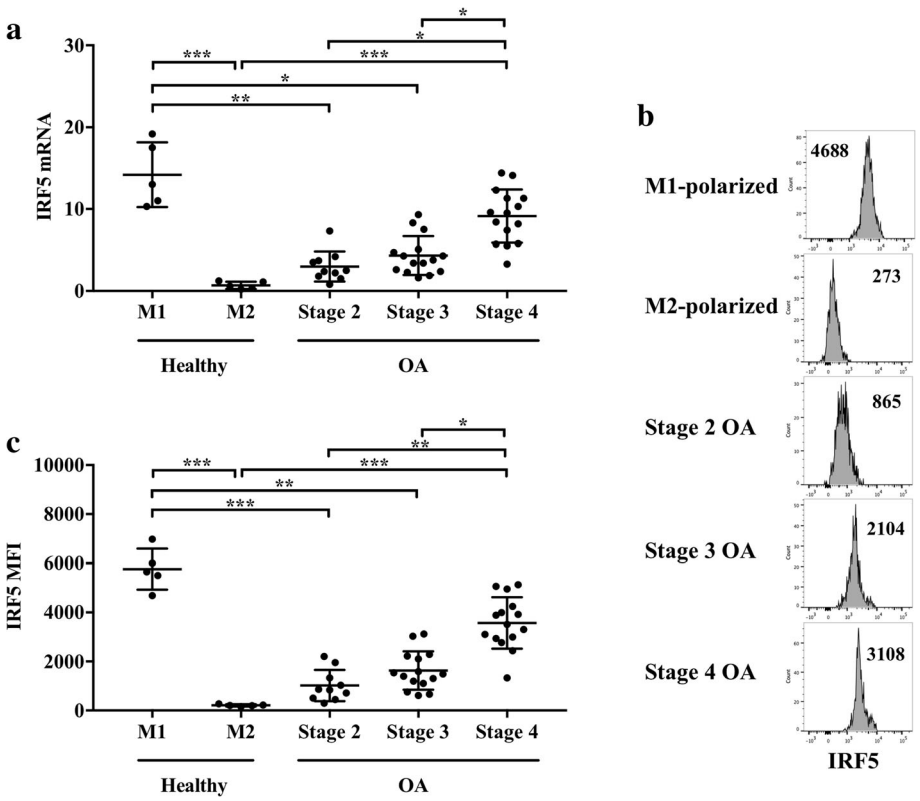

Fig. 1. IRF5 expression in synovial macrophages. Synovial macrophages were examined directly after isolation from synovial fluid, and the M1- and M2-polarized macrophages were from circulating monocytes of healthy participants polarized *in vitro*. **a** IRF5 mRNA expression in synovial macrophages from OA patients and M1- or M2-polarized macrophages from healthy participants. **b** MFI of IRF5 in pre-gated macrophages, one representative each group. **c** IRF5 MFI in synovial macrophages from OA patients and M1- or M2-polarized macrophages from healthy participants. **a**, **c** Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

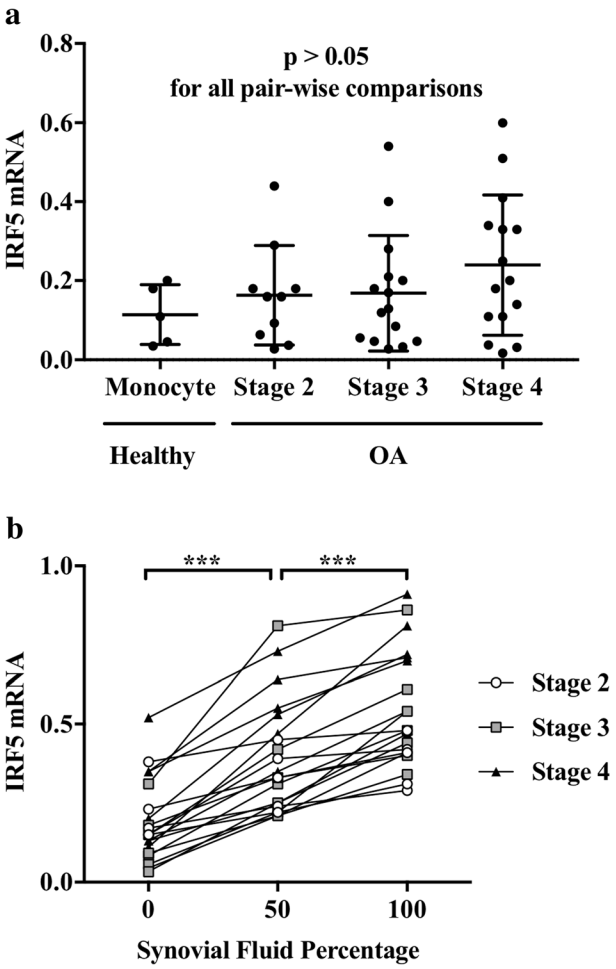

Fig. 2. IRF5 expression in circulating monocytes. **a** IRF5 mRNA expression in circulating monocytes from healthy participants and OA patients, examined directly after isolation. Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test. **b** IRF5 mRNA expression in circulating monocytes from OA patients, following incubation with various levels of synovial fluid. OA patients at various stages were treated as the same group. Friedman test followed by Dunn's multiple comparisons test. ***$p < 0.001$.

Synovial Fluid Conditioning Significantly Increased IL-12 Expression in Circulating Monocytes

M1-polarized macrophages express high level of IL-12, which supports the differentiation of Th1 cells [20]. Since IRF5 is critical for M1 polarization, we examined the expression of IL-12 in synovial macrophages and circulating monocytes from OA patients. In all OA groups, the synovial macrophages presented significantly higher IL-12p35 and higher IL-12p40 than the corresponding circulating monocytes (Fig. 3A, B). In addition, the IL-12p35 and IL-12p40 expression levels in stage 4 OA synovial macrophages were significantly higher than those in stage 2 and stage 3 OA synovial macrophages. The expression of IL-12p35 and IL-12p40 was then examined in circulating monocytes that were incubated in 100% culture media or 50% autologous synovial fluid plus 50% culture media. Synovial fluid conditioning significantly increased the expression of IL-12p35 and IL-12p40 in circulating monocytes (Fig. 3C, D).

IRF5 Transfection Significantly Elevated IL-12 Expression in Circulating Monocytes

To investigate the effect of IRF5 on the expression of IL-12, we transfected circulating monocytes from OA patients with IRF5-expression plasmid or mock plasmid. The IRF5 expression level in IRF5-transfected circulating monocytes was significantly higher than that in mock-transfected circulating monocytes (Fig. 4A), thus demonstrating the efficacy of IRF5 transfection. The expression of IL-12p35 and IL-12p40 was then investigated. IRF5-transfected circulating monocytes presented significantly higher expression of both IL-12p35 and IL-12p40 (Fig. 4B, C). No significant differences between stage 2, stage 3, and stage 4 patients were observed.

IRF5-Transfected Monocytes Promoted the Expression of Th1-Associated, but Not Th17-Associated, Gene in Naive CD4 T Cells *via* an IL-12-Dependent Mechanism

Since macrophage polarization could enhance or suppress the differentiation of certain Th subsets and modulate the CD4 T cell responses overall, we investigated the effect of IRF5-transfected or mock-transfected circulating monocytes on autologous CD4 T cells. Naive CD4 T cells were sorted from blood *via* magnetic negative selection, with purity greater than 96% (Supplementary Figure 2). Purified naive CD4 T cells were then co-incubated with mock-transfected or IRF5-transfected circulating monocytes. In preliminary investigations, the expression of IFN-γ by CD4 T cells after co-incubation with monocytes was investigated every 24 h for a total of 6 days, and we found that from 0 to 72 h, the IFN-γ level was marked increased, while from 72 h onward, only limited further increases were observed (Supplementary Figure 3). Hence, in the coculture of naive CD4 T cells and transfected monocytes, the CD4 T cells were re-isolated after 72-h co-incubation using magnetic negative selection, and the expression levels of Th1 signature cytokine IFN-γ and Th1-specific transcription factor TBX21 were investigated. CD4 T cells presented significantly higher IFN-γ and TBX21

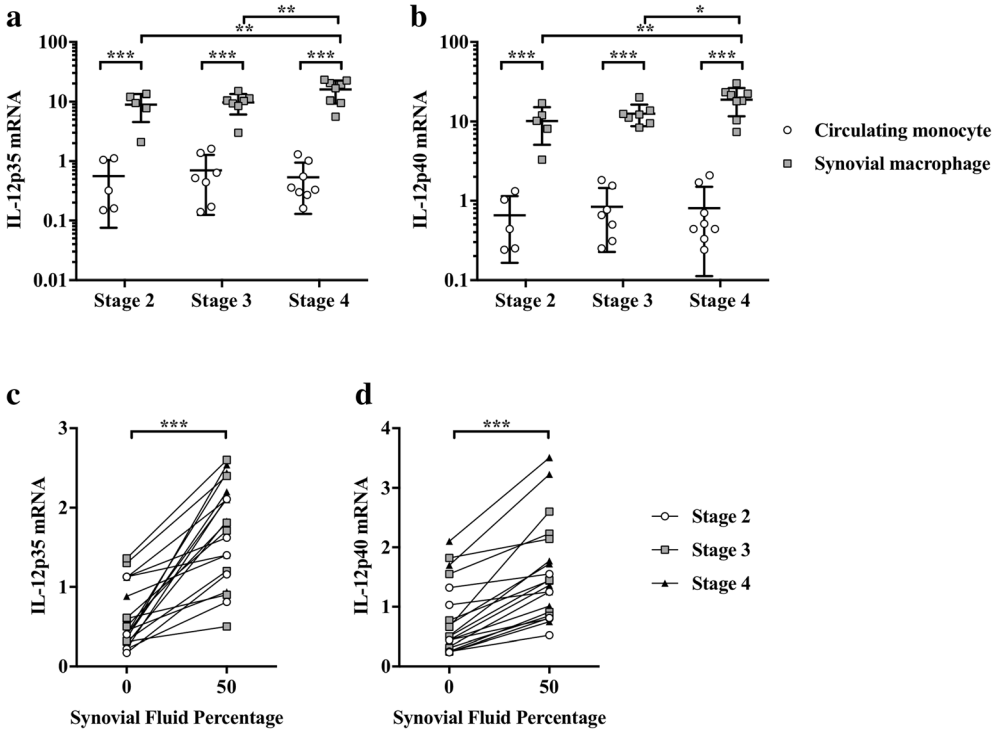

Fig. 3. IL-12 expression in synovial macrophages and circulating monocytes. (A) IL-12p35 mRNA expression in synovial macrophages and corresponding circulating monocytes, immediately following isolation. (B) IL-12p40 mRNA expression in synovial macrophages and corresponding circulating monocytes, immediately following isolation. (C) IL-12p35 mRNA expression in circulating monocytes from OA patients, following incubation with 0 or 50% synovial fluid. (D) IL-12p40 mRNA expression in circulating monocytes from OA patients, following incubation with 0 or 50% synovial fluid. (A and B) Repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test. (C and D) Wilcoxon matched pairs test. OA patients at various stages were treated as the same group. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

expression following co-incubation with IRF5-transfected monocytes (Fig. 5a). On the other hand, the expression levels of Th17 signature cytokine IL-17 and Th17-specific transcription factor RORC were not significantly altered by IRF5 transfection (Fig. 5a). Given that IRF5 transfection resulted in higher IL-12 expression, we examined the effect of IL-12 inhibition by adding IL-12 neutralizing antibody in the coculture of CD4 T cells and IRF5-transfected

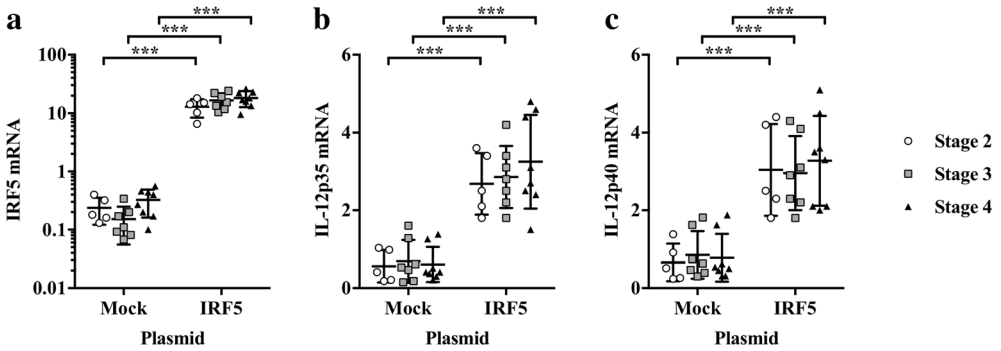

Fig. 4. IRF5 transfection in circulating monocytes. (A) IRF5 expression levels in IRF5 plasmid-transfected or mock plasmid-transfected circulating monocytes. (B) IL-12p35 expression levels in IRF5 plasmid-transfected or mock plasmid-transfected circulating monocytes. (C) IL-12p40 expression levels in IRF5 plasmid-transfected or mock plasmid-transfected circulating monocytes. Repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test. ***$p < 0.001$.

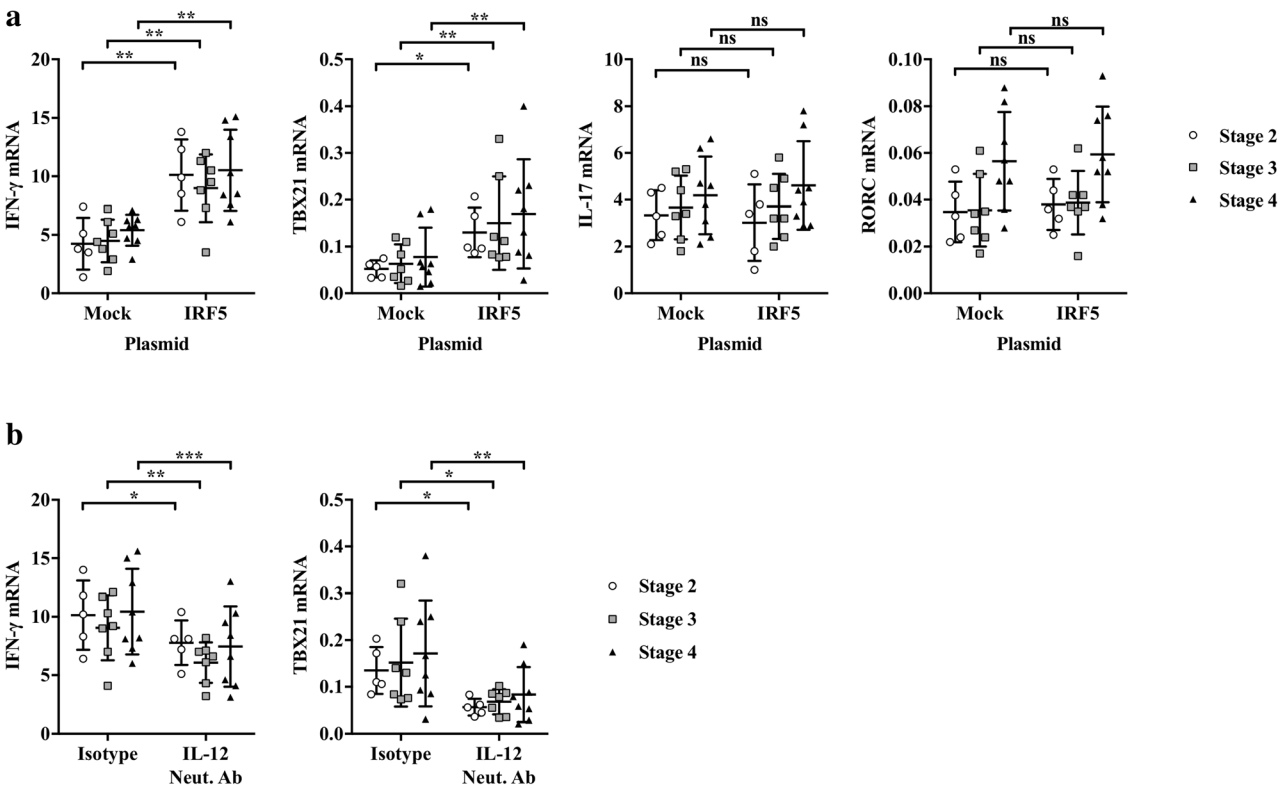

Fig. 5. Th1 and Th17 signature gene expression in CD4 T cells following co-incubation with circulating monocytes. Naive CD4 T cells were isolated *via* magnetic negative selection, and then co-incubated with autologous mock-transfected or IRF5-transfected circulating monocytes at 1/1 ratio. **a** IFN-γ, TBX21, IL-17, and RORC mRNA expression levels in CD4 T cells after co-incubation with monocytes. **b** IFN-γ and TBX21 mRNA expression in CD4 T cells in the presence of IL-12 neutralization antibody or isotype control, during co-incubation with monocytes. Repeated measures two-way ANOVA followed by Bonferroni's multiple comparisons test. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

monocytes. Compared to isotype control antibody, IL-12 neutralizing antibody significantly lowered the expression levels of IFN-γ and TBX21 in CD4 T cells (Fig. 5b). No significant differences between OA patients at different stages were observed.

DISCUSSION

Macrophages are composed of phenotypically and functionally diverse subsets, with critical roles in shaping and modulating the adaptive immune responses. In this study, we identified that the synovial macrophages from OA patients demonstrated high IRF5 expression, which was previously shown to promote M1 polarization and suppress M2 polarization in macrophages [16]. The circulating monocytes, from OA patients, on the other hand, presented low IRF5 expression, indicating that the IRF5 upregulation was restricted to the local arthritis site and was not present systemically. IL-12p35 and IL-12p40 expression levels were also significantly higher in synovial macrophages from OA patients than in autologous circulating monocytes. Given that high IL-12 expression was a hallmark of M1-polarized macrophages [20], this discovery further demonstrated that the synovial macrophages presented strong M1-like features.

Interestingly, we also observed that the expression of IRF5, IL-12p35, and IL-12p40 by synovial macrophages was significantly higher in patients with more advanced OA. It should be addressed in future studies whether high M1 polarization directly contributes to the degeneration of OA-affected joints. Recent studies in rheumatoid arthritis patients have demonstrated that the M1/M2 monocyte ratio is positively associated with the number of differentiated

osteoclasts, which mediate catabolic changes of the bone and contribute to bone erosion [21]. Whether a similar effect exists in OA patients should be further investigated.

Additionally, we observed that the transfection of IRF5 in circulating monocytes directly resulted in the upregulation of IL-12p35 and IL-12p40. Furthermore, IRF5-transfected monocytes had gained the ability to induce the expression of the Th1 signature cytokine IFN-γ and the Th1-specific transcription factor TBX21 in activated naive CD4 T cells in an IL-12-dependent manner [22]. Interestingly, in the synovia of OA patients, strong infiltration by CD4 T cells that were biased toward the Th1 phenotype was observed [22]. Results from this study suggest that the IRF5 overexpression in macrophages might be involved to the enrichment of Th1 in the synovial fluid of OA patients.

Aside from direct IRF5 transfection, we also observed that synovial fluid from OA patients could significantly elevate the expression of IRF5 in circulating monocytes. IRF5 is a p53 target gene that could be upregulated upon DNA damage [23, 24]. Signaling from toll-like receptors could also increase IRF5 expression [25]. In this study, it appeared that the synovial fluid contained soluble factors that were involved in the IRF5 upregulation. Further studies are required to investigate the origin and identity of these soluble factors.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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