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IgM$^+$CD27$^+$ B cells possessed regulatory function and represented the main source of B cell-derived IL-10 in the synovial fluid of osteoarthritis patients

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ABSTRACT

Synovial inflammation is observed in patients with osteoathritis (OA) and likely contributed to its exacerbation. Regulatory B (Breg) cells are shown to suppress inflammation in various diseases, including rheumatoid arthritis (RA). To examine whether Breg cells also participated in OA, we examined the synovial fluid from OA patients, and compared with that in RA patients. In OA synovial fluid, IL-10-producing B cells were present directly *ex vivo* and were increased upon stimulation, indicating that B cells were a source of IL-10 directly at the affected site of OA patients. Interestingly, the frequency of IL-10$^+$ cells in synovial B cells was higher in OA patients than in RA patients, but the total number of IL-10$^+$ B cells in OA was lower than that in RA, suggesting that OA patients presented lower B cell infiltration than RA patients. Phenotypical analysis demonstrated that the IL-10$^+$ B cells were IgM$^+$ and CD27$^+$, but not CD24^{hi} or CD38^{hi}. To allow functional analysis of IgM$^+$CD27$^+$ B cells, the IgM$^+$CD27$^+$ B cells in the blood of OA patients were examined. These blood IgM$^+$CD27$^+$ B cells expressed more IL-10, but less CD80 and CD86 than non-IgM$^+$CD27$^+$ B cells. Blood IgM$^+$CD27$^+$ B cells suppressed the proliferation and IFN-γ expression of autologous T cells, and this effect could be reverted if IL-10 was inhibited. Furthermore, we found that patients with more severe OA presented lower levels of IL-10$^+$ B cells in the synovial fluid. Together, our study described an IgM$^+$CD27$^+$ B cell subset in OA patients, which represented the major IL-10-secreting B cell type in the synovial fluid of OA patients and possessed regulatory function.

1. Introduction

Some B cell subsets possess the capacity to suppress inflammatory responses and are thus termed regulatory B (Breg) cells [1,2]. These Breg cells mediate suppression via both contact-independent mechanisms, such as the secretion of regulatory cytokines IL-10 and TGF-β [3–5], and contact-independent mechanisms, such as the upregulation of PD-L1 and FasL [6,7]. Currently, the IL-10-producing Breg cells are the best described, and many Breg subsets with distinctive sets of surface markers were found to share the IL-10 producing capacity, including the CD24^{hi}CD38^{hi}, CD1d^{hi}CD5$^+$, Tim-1$^+$, CD138$^+$, and the CD24$^+$CD27$^+$ subsets [5,8–10]. Through IL-10 and sometimes TGF-β, these Breg cells could suppress the activation of Th1, Th17, and CD8$^+$ T cells, mediate the regulatory differentiation of antigen-presenting dendritic cells and macrophages, and promote the formation of regulatory T (Treg) cells [3,11,12].

Many autoimmune diseases are associated with a dysfunction in Breg cells. Patients with systemic lupus erythematosus (SLE) displayed a reduction in the absolute number of Breg cells, and these Breg cells were unable to produce IL-10 upon CD40L engagement [8]. In rheumatoid arthritis (RA), Breg cells were not only reduced in frequency but also unable to suppress Th17 and support Treg development [11]. In addition, the frequency of Breg cells was inversely correlated with rheumatoid arthritis disease activity [13–15]. In patients with relapsing-remitting multiple sclerosis (RRMS), the patients in relapse phase presented significant downregulation in IL-10-producing Breg cells than healthy controls and patients in remission phase [16]. B cells from type 1 diabetes patients presented impaired capacity to produce IL-10 [17]. In animal models of these autoimmune diseases, adoptive transfer of Breg cells markedly suppressed the inflammation and reduced severity.

Osteoarthritis (OA) is traditionally considered a noninflammatory disease caused by mechanical wear and tear, as opposed to RA, which is

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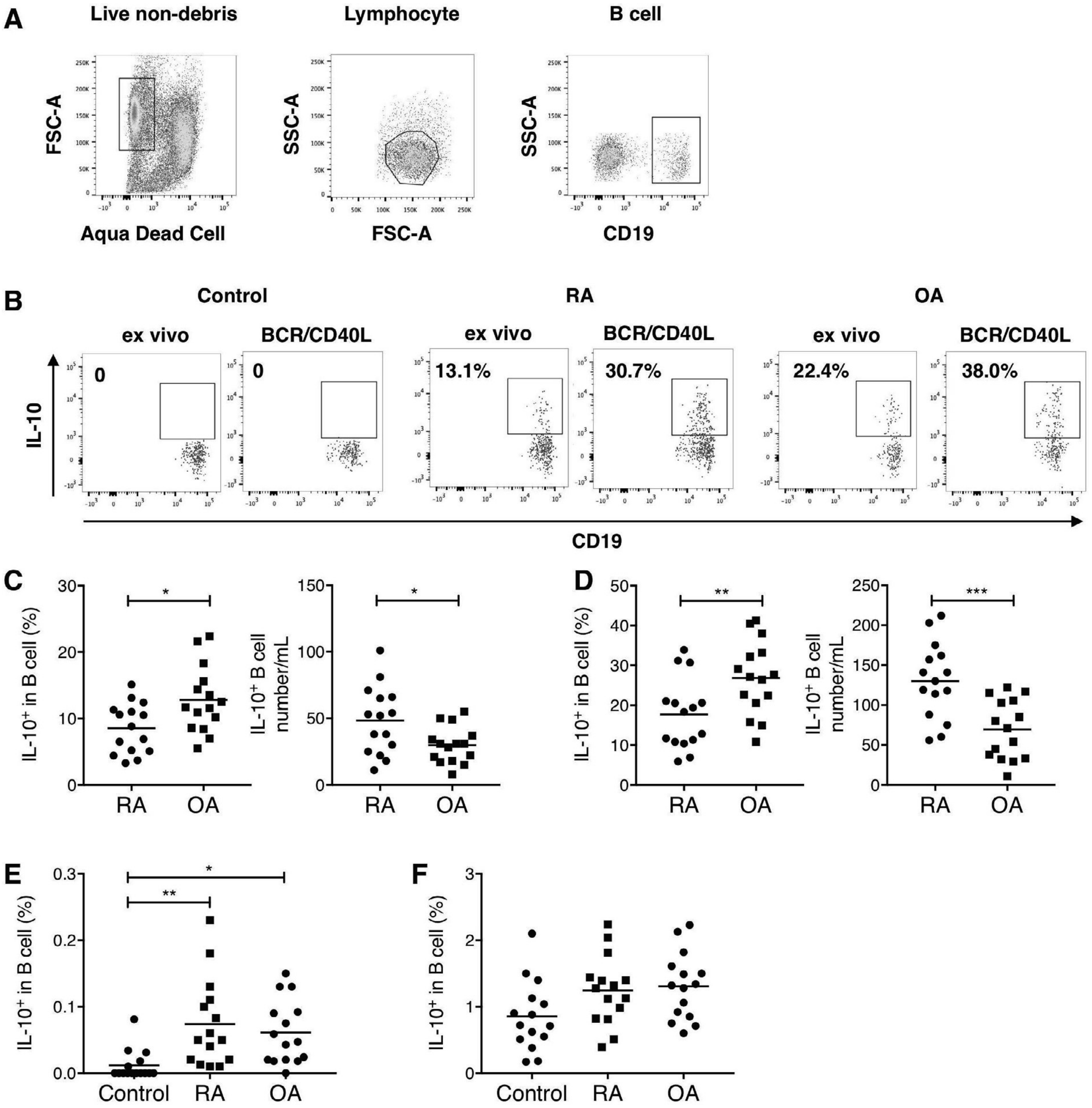

Fig. 1. The amount of IL-10-expressing B cells in synovial fluid. (A) Identification of synovial B cells in one representative OA subject. (B) Expression of IL-10 in synovial B cells from one representative RA patient and one representative OA patient. Negative IL-10 expression was gated using an isotype control antibody. (C) The frequency and number of synovial B cells expressing IL-10 *ex vivo* in RA patients and OA patients. (D) The frequency and number of synovial B cells expressing IL-10 after BCR/CD40L stimulation in RA patients and OA patients. Unpaired *t* test. (E) The frequency of IL-10^+ B cells in the peripheral blood of healthy, RA, and OA participants directly *ex vivo*. (F) The frequency of IL-10^+ B cells in the peripheral blood of healthy, RA, and OA participants following BCR/CD40L stimulation. One-way ANOVA followed by Tukey's test. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

driven by autoimmune responses. In recent years, accumulating evidence demonstrated that aberrant inflammation contributed to the progression of OA. Proinflammatory cytokines, including TNF-α, IL-1β and IL-6, promoted the expression of metalloproteinases, which catalyzed the degradation of cartilage [18]. The synovial tissue of OA patients is infiltrated by many types of immune cells, including macrophages, neutrophils, mast cells, T cells, B cells, and natural killer (NK) cells, to a degree that in general is lower than that in RA patients but higher than that in healthy controls. These cells orchestrated the inflammatory response within the synovial tissue and contributed to further cytokine production and tissue damage [19]. IL-17, a cytokine primarily produced by activated $CD4^+$ T cells, is shown to act on chondrocytes and inhibits proteoglycan synthesis, thus suppressing the repair of damaged cartilage [20].

The existence of aberrant inflammation in OA may have resulted from a reduction in Breg cells. In this study, we examined the characteristics of Breg responses in the synovial fluid and the circulating blood from OA patients.

2. Methods

2.1. Sample collection

OA was diagnosed at Qianfoshan Hospital via a combination of X-ray imaging, magnetic resonance imaging, and joint fluid analysis. A total of 15 patients, including 10 females and 5 males between 44 and 60 years of age were recruited. Staging of OA severity was based on the Kellgren and Lawrence system [21]. Fifteen RA patients, who fulfilled

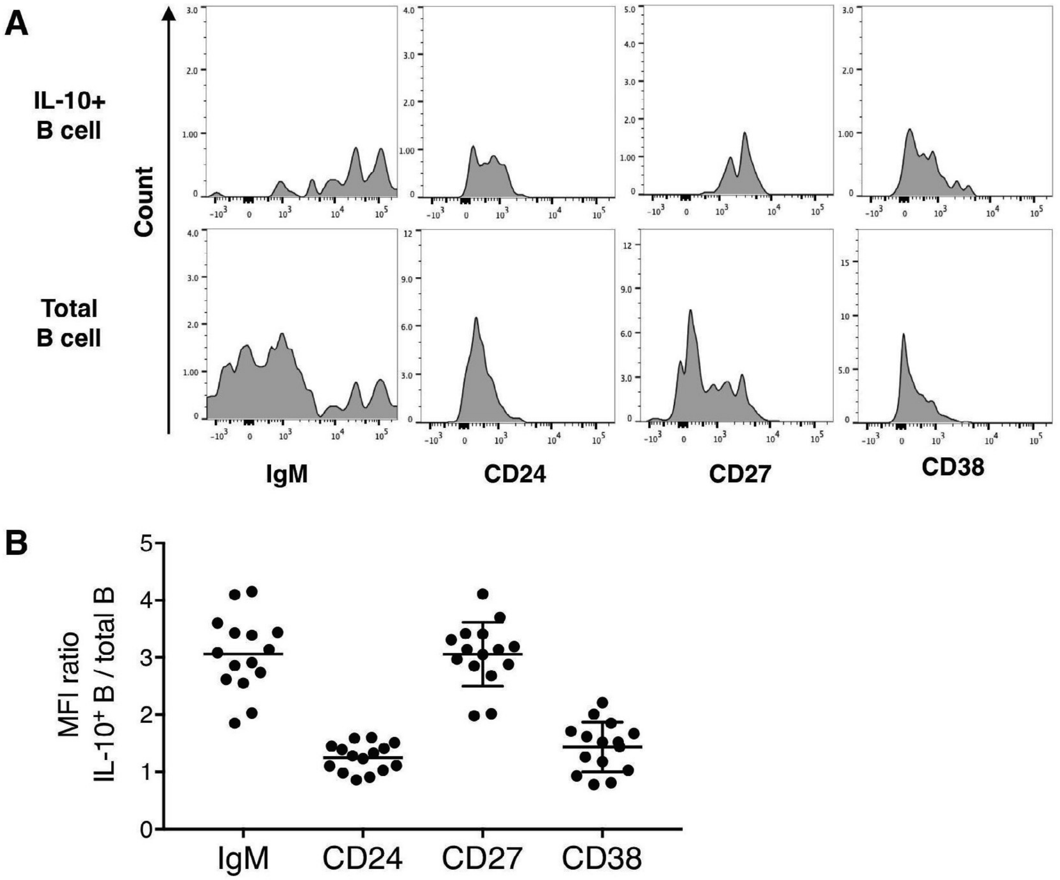

Fig. 2. Surface marker expression by IL-10 $^{\pm}$ synovial B cells from OA patients. (A) Expression of IgM, CD24, CD27, and CD38 by pre-gated IL-10 $^{+}$ synovial B cells, compared to that by total synovial B cells, in one representative OA patient. (B) The ratio of the IgM, CD24, CD27, and CD38 MFI in IL-10 $^{+}$ synovial B cells to the IgM, CD24, CD27, and CD38 MFI in total synovial B cells from OA patients.

the American College of Rheumatology criteria, were included as disease controls [22]. Healthy controls were recruited from 15 age- and sex-matched healthy individuals. Peripheral blood samples were obtained from all participants, and synovial fluid samples were obtained from all RA and OA patients. The peripheral blood mononuclear cells (PBMCs) and synovial mononuclear cells were harvested using standard Ficoll (GE Healthcare) centrifugation methods. At the time of sample collection, no nonsteroidal anti-inflammatory drugs were given. The Ethics Review Committee of Qianfoshan Hospital provided approval for this study. All patients provided written informed consent.

2.2. *Flow cytometry*

The following antibodies were used, including anti-human CD19, anti-human IgM, anti-human CD24, anti-human CD27, anti-human CD38, anti-human CD80, anti-human CD86, anti-human IL-10, and isotype control to anti-human IL-10 (BioLegend). For ex vivo IL-10 expression, synovial mononuclear cells or PBMCs were incubated with 5 µg/mL GolgiStop and 5 µg/mL GolgiPlug (BD Biosciences) for 5 h. For stimulated IL-10 expression, synovial mononuclear cells or PBMCs were incubated with 2 µg/mL of anti-BCR antibody (Goat anti-human IgA/G/M F(ab′)$_2$; Jackson ImmunoResearch) and 2 µg/mL recombinant human CD40L (BioLegend) for 5 h in the presence of 5 µg/mL GolgiStop and 5 µg/mL GolgiPlug. The cells were then washed and incubated with surface antibodies and Aqua Dead Cell Stain (Invitrogen) for 30 min in 4 °C fridge. Excess surface antibodies were removed by washing, and the cells were incubated with Perm Wash (BD Biosciences) for 15 min in 4 °C, followed by washing and incubation with anti-human IL-10 or its isotype control for 30 min in 4 °C. Excess antibody was removed by washing, and the cell samples were acquired using the FACSCanto system (BD Biosciences).

2.3. *Il-10 ELISA*

B cells were negatively isolated using EasySep Human B Cell Enrichment kit (Stemcell Technologies) from total PBMCs. IgM^{+}CD27^{+} B cells and non-IgM^{+}CD27^{+} B cells were then separated using fluorescence-activated cell sorting in the FACSAria system (BD Biosciences), and incubated at 5×10^4 cells per 200 µL media each well in a 96-well plate for 12 h. For stimulation, 2 µg/mL of anti-BCR antibody and 2 µg/mL recombinant human CD40L were added. The supernatant was then collected and the secretion of IL-10 was examined using Human IL-10 ELISA kit (Abcam).

2.4. *Tritiated thymidine incorporation assay*

T cells were negatively isolated using EasySep Human T Cell Enrichment kit (Stemcell Technologies) from total PBMCs, and were incubated with the same number of autologous IgM^{+}CD27^{+} B cells or non-IgM^{+}CD27^{+} B cells, together with 2 µg/mL anti-CD3 antibody (OKT3; BioLegend), 2 µg/mL anti-BCR antibody, and 2 µg/mL CD40L. In select experiment, 10 µg/mL recombinant human IL-10R alpha subset (R&D Systems) or 1 µg/mL anti-human CD28 antibody (BioLegend) was added. After 72 h, T cells were isolated using EasySep Human T Cell Enrichment kit, and were pulsed with tritiated thymidine at 0.1 µCi/mL for 6 h. The cells were then harvested and the radioactivity was counted in a direct beta-counter.

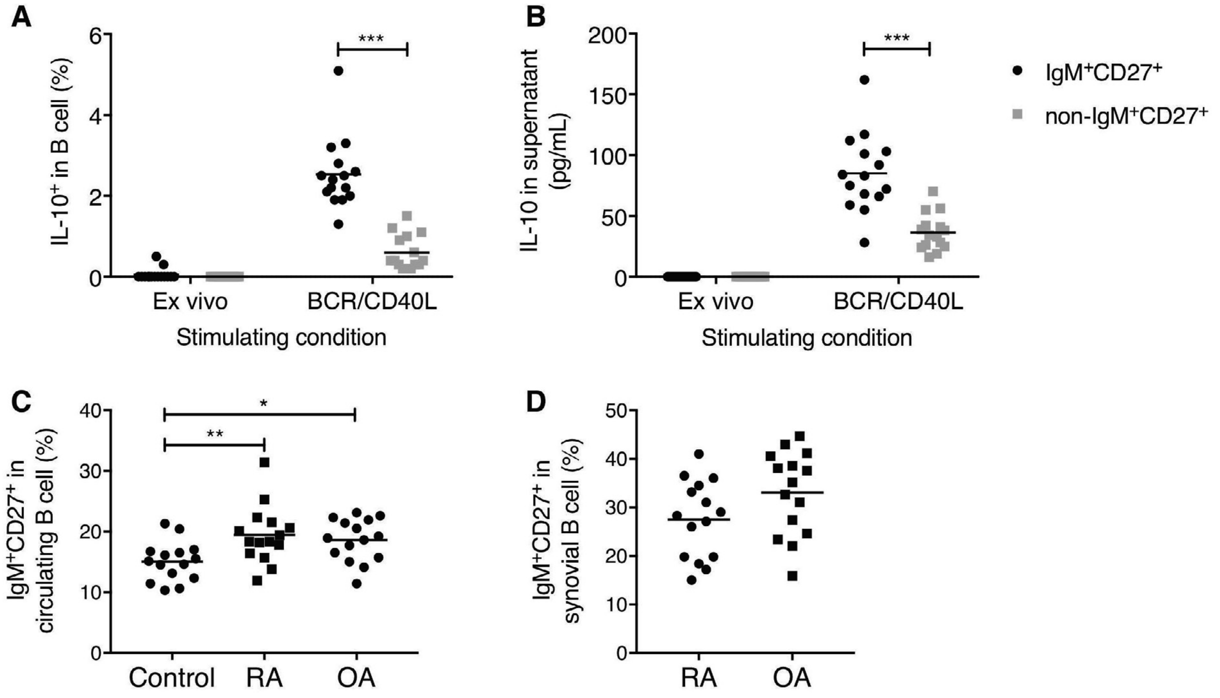

Fig. 3. Expression of IL-10 by peripheral blood B cells. (A) The frequency of IL-10^+ cells in blood IgM$^+$CD27$^+$ B cells and non-IgM$^+$CD27$^+$ B cells from OA patients, directly *ex vivo* or with anti-BCR/CD40L stimulation (B) IgM$^+$CD27$^+$ B cells and non-IgM$^+$CD27$^+$ B cells were sorted from the peripheral blood of OA patients. The IL-10 secretion *ex vivo* or with anti-BCR/CD40L stimulation. Two-way ANOVA followed by Sidak's test. (C) The frequency of IgM$^+$CD27$^+$ cells in circulating B cells from control, RA, and OA subjects. One-way ANOVA followed by Tukey's test. (D) The frequency of IgM$^+$CD27$^+$ cells in synovial B cells from RA and OA subjects. Unpaired *t* test. $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

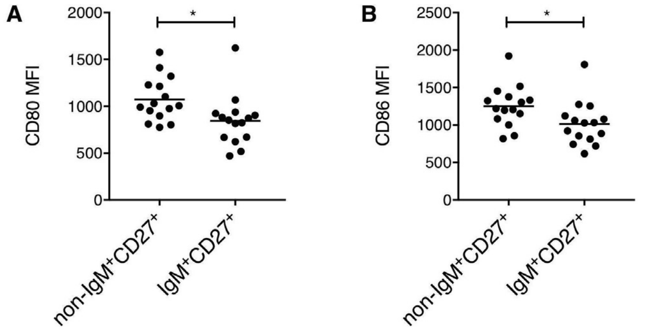

Fig. 4. CD80 and CD86 expression by IgM$^{\pm}$CD27$^{\pm}$ B cells and non-IgM$^{\pm}$CD27$^{\pm}$ B cells from OA patients. (A) The CD80 MFI in blood IgM$^+$CD27$^+$ B cells and non-IgM$^+$CD27$^+$ B cells from OA patients. (B) The CD86 MFI in blood IgM$^+$CD27$^+$ B cells and non-IgM$^+$CD27$^+$ B cells from OA patients. Unpaired *t* test. $^*P < 0.05$.

2.5. Statistics

All tests were two-tailed. Unpaired *t* test was used to compare differences between two groups. Two-way ANOVA followed by Sidak's test or one-way ANOVA followed by Tukey's test was used to compare differences among more than two groups. The specific test for each experiment is indicated in the figure legend. $P < 0.05$ was considered significant.

3. Results

3.1. IL-10-producing B cells were present in the synovial fluid of OA patients at higher frequencies but lower numbers than that of RA patients

To investigate whether IL-10-producing B cells participated in OA, we examined the synovial fluid from affected knee joints of OA patients, and compared with that of RA patients (Fig. 1A). Synovial fluid lymphocytes were analyzed *ex vivo* and after stimulation with anti-BCR antibody and CD40L (Fig. 1B). Directly *ex vivo*, a clear population of IL-10^+ B cells could be observed in both RA patients and OA patients (Fig. 1B), and the frequency of IL-10^+ cells in total B cells was significantly higher in OA patients than in RA patients, but the number of IL-10^+ B cells was higher in RA patients than in OA patients (Fig. 1C), reflecting the fact that RA synovial fluid contained higher numbers of infiltrating B cells. BCR/CD40L stimulation roughly doubled the frequency of IL-10^+ B cells in RA patients and OA patients (Fig. 1B and D). Again, the frequency of IL-10^+ cells in total B cells was significantly higher in OA patients than in RA patients, but the number of IL-10^+ B cells was higher in RA patients than in OA patients (Fig. 1D).

We also examined the frequency of IL-10^+ cells in blood B cells. Directly *ex vivo*, the frequencies of IL-10^+ cells in B cells were below 0.3% in all study participants (Fig. 1E). RA and OA patients presented significantly higher frequencies of IL-10^+ B cells than healthy controls. Following BCR/CD40L stimulation, the frequencies of IL-10^+ cells in B cells were significantly elevated but was still below 2.5%, and were comparable among the disease and control groups (Fig. 1F).

3.2. Phenotypical analysis of IL-10-producing B cells in the synovial fluid of OA patients

In RA, both the CD24^{hi}CD38^{hi} Breg subset and the CD19$^+$CD27$^+$ memory B cell subset have been described as the major source of B cell-derived IL-10 [23]. To determine whether the IL-10-producing B cells in OA synovial fluid belonged to one of those populations, we examined the expression of CD24, CD27, and CD38 on the IL-10^+ synovial B cells, and compared with that in total synovial B cells (Fig. 2A). To demonstrate a quantitative difference between IL-10^+ synovial B cells and total synovial B cells, the mean fluorescence intensity (MFI) of each of the surface antigen was examined for IL-10^+ synovial B cells and total synovial B cells, and the ratio of the former to the latter was

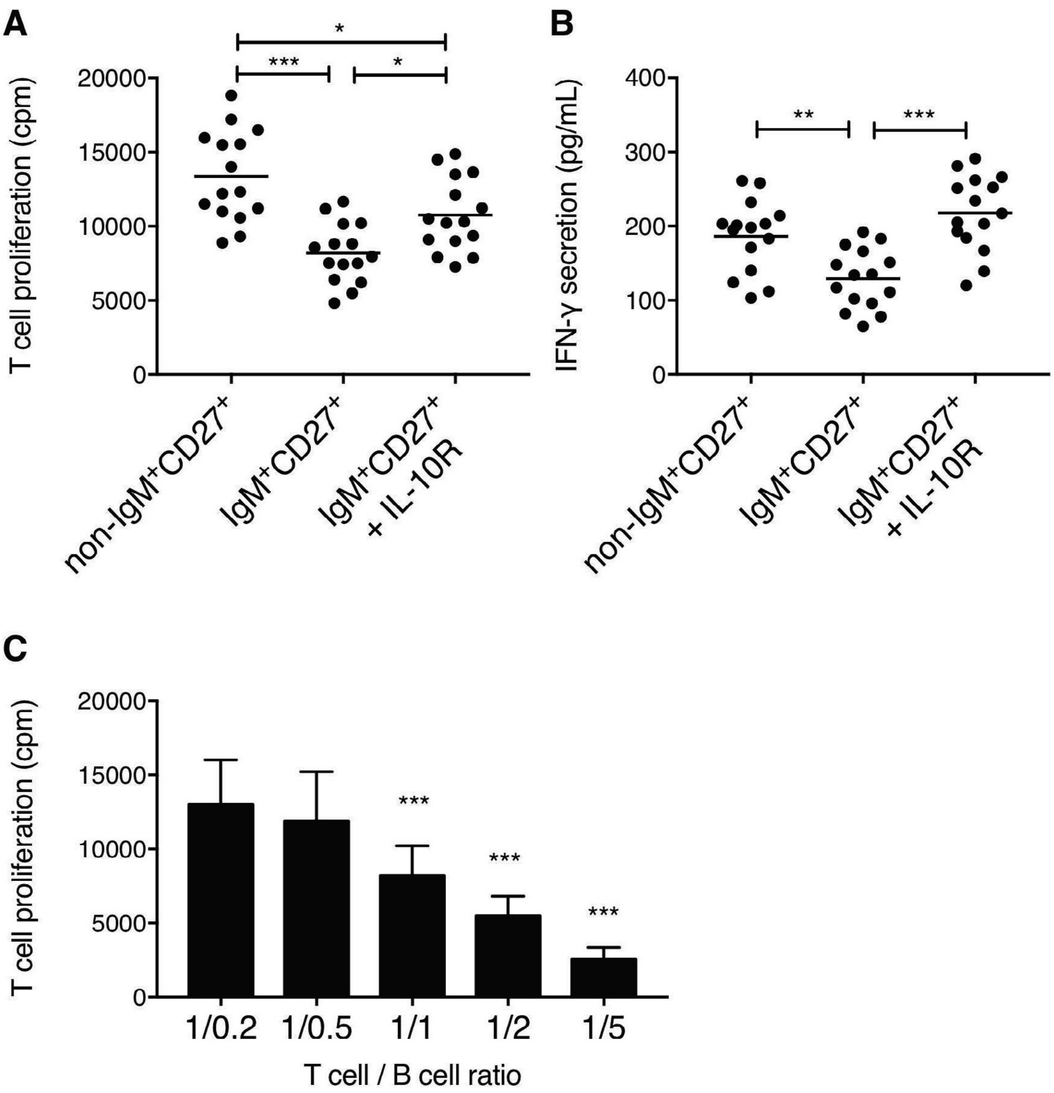

Fig. 5. T cell proliferation in coculture with IgM$^{\pm}$CD27$^{\pm}$ B cells and non– IgM$^{\pm}$CD27$^{\pm}$ B cells. CD3^{+} T cells (5×10^4) were coincubated with IgM^{+}CD27^{+} B cells or non-IgM^{+}CD27^{+} B cells, together with 2 µg/mL anti-CD3 antibody, 2 µg/mL of anti-BCR antibody, and 2 µg/mL CD40L. When indicated, 10 µg/mL IL-10R and 1 µg/mL anti-CD28 antibody was added. Proliferation was measured using a tritiated thymidine incorporation assay. (A) The proliferation of T cells in the presence of equal numbers of non-IgM^{+}CD27^{+} B cells, IgM^{+}CD27^{+} B cells, and IgM^{+}CD27^{+} B cells + IL-10R. (B) The supernatant IFN-γ concentration with equal numbers of non-IgM^{+}CD27^{+} B cells, IgM^{+}CD27^{+} B cells, and IgM^{+}CD27^{+} B cells + IL-10R. (C) The proliferation of T cells (mean ± SD; N = 15) in the presence of varying amounts of autologous IgM^{+}CD27^{+} B cells. One-way ANOVA followed by Tukey's test. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

demonstrated (Fig. 2B). Compared to total synovial B cells, the IL-10^{+} synovial B cells did not express marked higher CD24 or CD38, but expressed markedly higher CD27 (Fig. 2B). Also, the IL-10^{+} synovial B cells were mostly composed of IgM-expressing cells (Fig. 2A and B). Hence, it appears that the IL-10^{+} synovial B cells in OA patients did not belong to the CD24^{hi}CD38^{hi} Breg subset, but more closely resembled the CD19^{+}CD27^{+} memory B cell subset with IgM expression.

3.3. IL-10 production by peripheral blood B cells in OA patients

To enable functional analysis of IgM^{+}CD27^{+} B cells, we examined whether IL-10 production could be found in the IgM^{+}CD27^{+} B cells from peripheral blood, which is a more readily available source than synovial fluid B cells. Directly *ex vivo*, very few blood IgM^{+}CD27^{+} B cells expressed IL-10 (Fig. 3A). IL-10^{+} cells were not detected in blood non-IgM^{+}CD27^{+} B cells *ex vivo*. Following anti-BCR/CD40L stimulation, the frequency of IL-10^{+} cells was upregulated in both IgM^{+}CD27^{+} B cells and non-IgM^{+}CD27^{+} B cells, and was significantly higher in the former than in the latter (Fig. 3A). The amount of IL-10 release in the supernatant was then detected. Under *ex vivo* condition, the IL-10 secretion was below detection limit (Fig. 3B). After anti-BCR/CD40L stimulation, IgM^{+}CD27^{+} B cells produced significantly higher IL-10 than non-IgM^{+}CD27^{+} B cells (Fig. 3B). The frequency of IgM^{+}CD27^{+} cells in peripheral blood B cells was, on average, approximately 15% in healthy controls, and significantly higher at 20% in RA patients and OA patients (Fig. 3C). In synovial fluid B cells, the frequency of IgM^{+}CD27^{+} cells was approximately 30% on average, with no significant difference between the RA and the OA patients (Fig. 3D).

3.4. IgM$^{\pm}$CD27$^{\pm}$ B cells presented lower CD80/CD86 expression than non-IgM$^{\pm}$CD27$^{\pm}$ B cells

B cells could act as antigen-presenting cells by expressing major histocompatibility molecules (MHC) class I and class II and costimulatory molecules CD80 and CD86. It was shown that CD80/CD86 were used by CD24^{hi}CD38^{hi} Breg cells to suppress T cell activation [8]. Here, we examined the level of CD80 and CD86 expression by blood

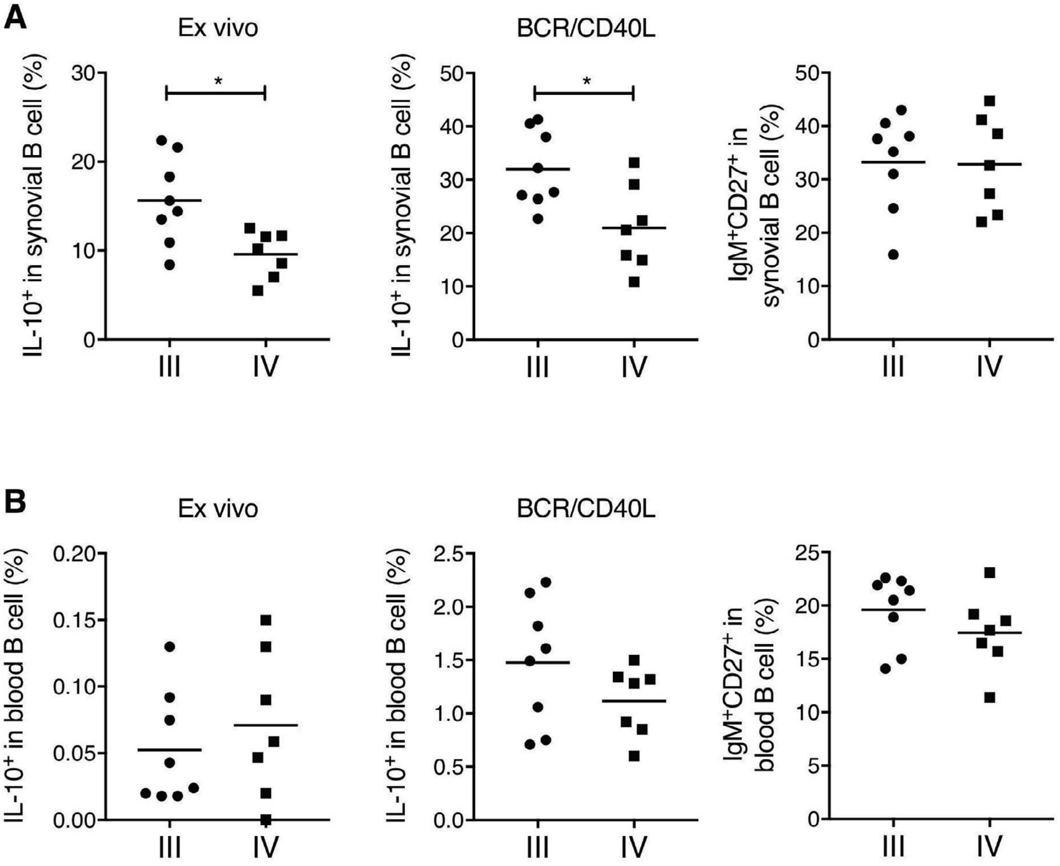

Fig. 6. Frequencies of IL-10$^{\pm}$ B cells and IgM$^{\pm}$CD27$^{\pm}$ B cells in stage III vs. stage IV OA patients. (A) The frequencies of IL-10$^{\pm}$ B cells directly ex vivo and after BCR/CD40L stimulation and the frequencies of IgM$^{\pm}$CD27$^{\pm}$ B cells in synovial fluid from stage III and stage IV OA patients. (B) The frequencies of IL-10$^{\pm}$ B cells directly ex vivo and after BCR/CD40L stimulation and the frequencies of IgM$^{\pm}$CD27$^{\pm}$ B cells in peripheral blood from stage III and stage IV OA patients. Unpaired *t* test. $^{*}P < 0.05$.

IgM^{+}CD27^{+} B cells and non-IgM^{+}CD27^{+} B cells in OA patients. The IgM^{+}CD27^{+} B cells presented significantly lower levels of CD80 and CD86 than non-IgM^{+}CD27^{+} B cells (Fig. 4A and B).

3.5. IgM$^{\pm}$CD27$^{\pm}$ B cells suppressed T cell proliferation via IL-10-mediated effects

To examine whether IgM^{+}CD27^{+} B cells possessed T cell inhibiting capacity, CD3^{+} T cells were coincubated with autologous IgM^{+}CD27^{+} B cells and non-IgM^{+}CD27^{+} B cells, in the presence of anti-CD3 stimulation for T cells, and anti-BCR/CD40L stimulation for B cells. T cells in the presence of non-IgM^{+}CD27^{+} B cells presented significantly higher levels of proliferation than T cells in the presence of IgM^{+}CD27^{+} B cells (Fig. 5A). To eliminate IL-10 in the supernatant, recombinant human IL-10 receptor (IL-10R) was added in the T cell-IgM^{+}CD27^{+} B cell coculture. This intervention partially increased the proliferation of T cells (Fig. 5A). The IFN-γ concentration was then examined in the supernatant from each experiment. T cells in the presence of IgM^{+}CD27^{+} B cells presented significantly lower IFN-γ secretion than in the presence of non-IgM^{+}CD27^{+} B cells (Fig. 5B). The addition of IL-10R reverted this effect (Fig. 5B). In addition, the proliferation of T cells was incrementally decreased with increasing levels of IgM^{+}CD27^{+} B cells (Fig. 5C).

3.6. The frequency of IL-10$^{\pm}$ B cells in the synovial fluid was reduced in more severe OA

Subsequently, we divided the total OA patients into stage III and stage IV patients based on severity. The frequencies of IL-10^{+} B cells and IgM^{+}CD27^{+} B cells were then compared between these two groups. Stage IV patients presented significantly lower levels of IL-10^{+} B cells in the synovial fluid than stage III patients, both directly *ex vivo* and after BCR/CD40L stimulation (Fig. 6A). The frequency of IgM^{+}CD27^{+} B cells in synovial B cells, and the frequencies of IL-10^{+} B cells and IgM^{+}CD27^{+} B cells in peripheral blood, were not associated with OA severity (Fig. 6A and B).

4. Discussion

OA is the most common form of arthritis worldwide and is causing debilitating symptoms in millions of people worldwide. While the symptoms can be managed to alleviate pain, OA remains degenerative with no cure. The discovery that the immune system is involved in OA has allowed better understanding of the pathogenesis of OA. In the future, the immune system might be modulated to alleviate the clinical symptoms and revert some of the degenerative processes, leading to a potential cure. More detailed investigation into the various immune cells should be performed.

Breg-mediated regulation is critical in the prevention of autoimmune diseases, including RA. Since inflammatory features are also present in OA, this study investigated the characteristics of the Breg responses. In the synovial fluid, IL-10-producing B cells were present directly ex vivo, and were increased in frequency upon stimulation, indicating that B cells were a source of IL-10 directly at the OA affected site. Interestingly, the frequency of IL-10^{+} cells in synovial B cells was higher in OA patients than in RA patients, but the total number of IL-10^{+} B cells present in OA was lower than that present in RA, suggesting that the level of B cell infiltration into the synovial fluid was reduced in

OA compared to RA. Phenotypical analysis demonstrated that, unlike other autoimmune diseases, the main source of IL-10 was IgM^+CD27^+ B cells but not $CD24^{hi}CD38^{hi}$ B cells. We further found that in circulating blood of OA patients, IgM^+CD27^+ B cells expressed more IL-10 than non-IgM^+CD27^+ B cells. To investigate the antiinflammatory potential of IgM^+CD27^+ B cells, we coincubated IgM^+CD27^+ B cells or non-IgM^+CD27^+ B cells with autologous T cells. IgM^+CD27^+ B cells suppressed the proliferation of T cells. This effect could be reverted if IL-10 was inhibited. Blair et al. reported that the $CD24^{hi}CD38^{hi}$ Breg cells inhibited autologous T cells in part via CD80 and CD86, as inhibition of CD80 and CD86 also reduced the $CD24^{hi}CD38^{hi}$ Breg cell-mediated suppression. Here, we found that the IgM^+CD27^+ B cells presented lower CD80 and CD86 than the non-IgM^+CD27^+ B cells. Whether CD80 and CD86 participated in IgM^+CD27^+ B cell-mediated suppression still require further investigations. Together, our study described an IgM^+CD27^+ B cell subset, which represented the major IL-10-secreting B cell type in the synovial fluid of OA patients and possessed regulatory function.

The canonical memory B cells are characterized by class-switched hypermutated IgG expression and $CD27^+$ phenotype. However, in humans, a subset of $IgM^+(IgD^+)CD27^+$ memory B cells is found and represents approximately 20% of total B cells in the peripheral blood[24]. These B cells present features of canonical memory B cells, including somatically hypermutated IgV and rapid response to *in vitro* activation signals, but also have phenotypical and functional features of marginal zone B cells [25,26]. The origin and function of this particular B cell subset is still debated. Our study here suggests that these cells represent a Breg subset. However, we have yet to demonstrate that the synovial IgM^+CD27^+ B cells and peripheral blood IgM^+CD27^+ B cells represent the same subset, the proof of which requires further investigations into the phenotypical marker expression and gene transcription in these two subsets.

As important regulators of immune responses, Breg cells may be used to treat inflammation-mediated diseases. Ex vivo or in vitro expansion, followed by adoptive transfer, of Breg cells could reduce inflammation-caused symptoms and help reestablish tolerance, thus acting as a potential therapeutic option. For OA patients, the IgM^+CD27^+ B cells might have therapeutic potential. Future investigations should examine the optimal stimulating agent and stability/plasticity of this B cell subset.

Conflict of interest

None.

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