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Original Article

Disrupted Th1/Th2 balance in patients with rheumatoid arthritis (RA)

Chunyan He[1*], Ping Feng[1*], Honghong Su[1], Aiping Gu[1], Zhaowei Yan[2], Xueming Zhu[1]

[1]Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu, China; [2]Department of Clinical Pharmacology, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu, China. [*]Equal contributors.

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Abstract: The imbalance of Th1/Th2 polarization in patients with Rheumatoid Arthritis (RA) and the effect of Mps derived from plasma of RA patients were reported in this study. It was found that the percentage of IFN-γ$^+$CD4$^+$ T cell in peripheral blood and Th1/Th2 ratio were significantly increased in RA patients. Sera from patients with RA promoted Th1/Th2 balance biased towards Th1 by enhancing Th1-type cytokine (IFN-γ and TNF-α) expression and reducing Th2-type (IL-6 and IL10) cytokine expression, respectively, in a dose-dependent manner. Conversely, Mps derived from RA patients disrupted the balance of Th1/Th2 by decreasing TNF-α expression and increasing IL-6 and IL-10 expression. The results illustrated RA patients were characterized by a disruption of Th1/Th2 balance towards Th1. Sera from RA patients promoted a Th1/Th2 balance bias towards Th1, which could be counteracted by Mps derived from RA patients disrupting the balance of Th1/Th2 in an opposing manner.

Keywords: Cytokines, rheumatoid arthritis, microparticles

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease of unknown etiology. Emerging evidence has indicated that disturbed T-cell homeostasis plays a critical role in the development of RA. CD4$^+$ helper T cells are largely defined as Th1 or Th2-cells, characterized by differential expression of certain cytokines. The Th1 cytokines includes interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α which together are responsible for the activation of cellular immunity. Th2 cytokines, such as IL-4, IL-6 and IL-10, are responsible for the activation of humoral immunity and antibody production [1].

Previous studies have hypothesized that RA is a Th1-driven disease with abnormal T cell activation [2] resulting in a Th1/Th2 cytokine imbalance [3] that correlates with disease activity [4]. While intriguing, this hypothesis remains unproven. Recently, evidence has shown that activated whole blood cells can result in increased cytokine production of Th2 over Th1 in RA patients [5]. Moreover, decreased IFN-γ and increased IL-4 production correlated with disease activity in RA patients [6]. These discrepancies imply that the Th cytokine response in RA is more complex than originally anticipated and requires further investigation.

T cell immunoglobulin domain and mucin domain-containing molecule 3 (Tim-3) is a member of the Tim family, selectively expressed on Th1 cells but not Th2 cells. While Tim-3 downregulates Th1 immunity and tolerance *in vivo* [7-9], blockade of Tim-3 exacerbated EAE as well as disease in the NOD model of Type I diabetes [6]. More recent studies demonstrated that Tim-3 was specifically expressed on HIV-specific T cells undergoing "exhaustion" [10]. Right now, the role of Tim 3 in RA remains unexplored.

Microparticles (Mps) are small vesicles released from the plasma membrane of various cell types, which range in size from 0.1 to 1 μm. Mps include cytoplasmic components and cell surface-derived elements, such as membrane receptors that could be transferred between cells, carrying with them membrane proteins

Table 1. Characteristics of the studied subjects

Characteristics	RA patients (n=26)	Healthy individuals (n=26)
Female, n (%)	18 (69%)	18 (69%)
Age, years (range)	48 (26-69)	49 (22-67)
Anti-CCP positive, n (%)	26 (100%)	-
DAS28 (range)	5.8 (4.1-9.8)	-
Number of tender joints (range)	10 (0-36)	-
Number of swell joint (range)	9 (0-32)	-
RF (IU/ml)	507.4 (19-4110)	-
ESR (mm/h)	36.3 (3-111)	-
CRP (mg/l)	46.2 (11-105)	-

Anti-CCP, Anti cyclic citrullinated peptide antibody; CRP, C reactive protein; DAS, disease activity score; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; RF, rheumatoid factor.

[11-13]. Mps are defined by their size and the presence of negatively charged phospholipids like phosphatidylserine (PS) in the outer membrane leaflet [14]. As reported, Mps shed from platelet (PLT), polymorphonuclear (PMN) and erythrocytes (ERY) demonstrate immune suppressive properties related to a large extent to the expression of PS [23-28]. Little is known about whether Mps can impact T cell activation, including effector and regulatory cells. In the current study, we focused on the immunomodulatory effects of Mps, purified from sera of RA patients, on peripheral blood $CD4^+$ T cells.

In the present study, we tested the hypothesis that imbalance in Th1/Th2 cytokines occurs in patients with RA. Differential expression of cytokines IFN-γ and IL-4, respectively, were used to evaluate the distribution of Th1 and Th2 in peripheral blood from RA. Simultaneously, the sera and MPs from RA patients were added as stimuli to peripheral blood mononuclear cells (PBMC) derived from healthy volunteers. The levels of Th1/Th2 cytokines in culture supernatant was analyzed, while Tim-3 expression on resting and activated $CD4^+$ T cells was detected by flow cytometry (FCM). By further defining the molecular mechanisms regulating Th cells differentiation this work will provide not only new insight into the pathogenesis of RA but will also reveal novel targets for immunotherapy.

Patients and methods

Study population

Blood samples from patients (n=26) with RA fulfilling American College of Rheumatology criteria [15] in the Department of Rheumatology of the Second Affiliated Hospital of Soochow University were obtained. Twenty six (n=26) healthy individuals who presented with no inflammatory, neoplastic, and autoimmune or metabolic diseases was used as a control group (HC).

Fresh peripheral blood mononuclear cells (PBMC) from RA patients and controls were collected for FCM. Sera from 18 RA patients was used for co-culturing with PBMC and for evaluating Th1/Th2 cytokines production. The clinical characteristics of RA patients and HCs are summarized in **Table 1**. Disease activity was measured by the Disease Activity Score for 28 joints (DAS28) on the day of blood testing for RA patients. No subjects received immunomodulatory treatment during a 2-monthes period prior to sampling. This study was approved by the Institutional Research Ethics Committee of the Second Affiliated Hospital of Soochow University for Clinical Investigation. Written informed consent was obtained from all study participants before enrollment.

Intracellular staining of IFN-γ and IL-4

For Th1 and Th2 cytokine analysis, cells were stimulated for 6 h with a cell stimulation cocktail, according to the manufacturer's recommendation. The cocktail numerous protein transport inhibitors (Phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A and monensin, BD Pharmingen). Cells were stained with anti-human CD4 antibody and consecutively fixed and permeabilized (Fix-Perm-Solutions A and B, Life Technologies, Darmstadt, Germany) for staining with antihuman IFN-γ (detection of Th1 cells) and IL-4 (detection of Th2 cells, all BD Pharmingen). $CD4^+$ T cell distribution was measured by FACS analysis using a FACSCalibur instrument (BD Biosciences). Isotype controls (BD Pharmingen) were given for compensation and confirmation of antibody specificity.

Tim-3 expression detection

Fifty microliters (50 μl) of fresh peripheral blood was incubated for 15 min in 25°C in dark with monoclonal antibodies or isotype matched control. FITC-conjugated anti-human CD4 (BD

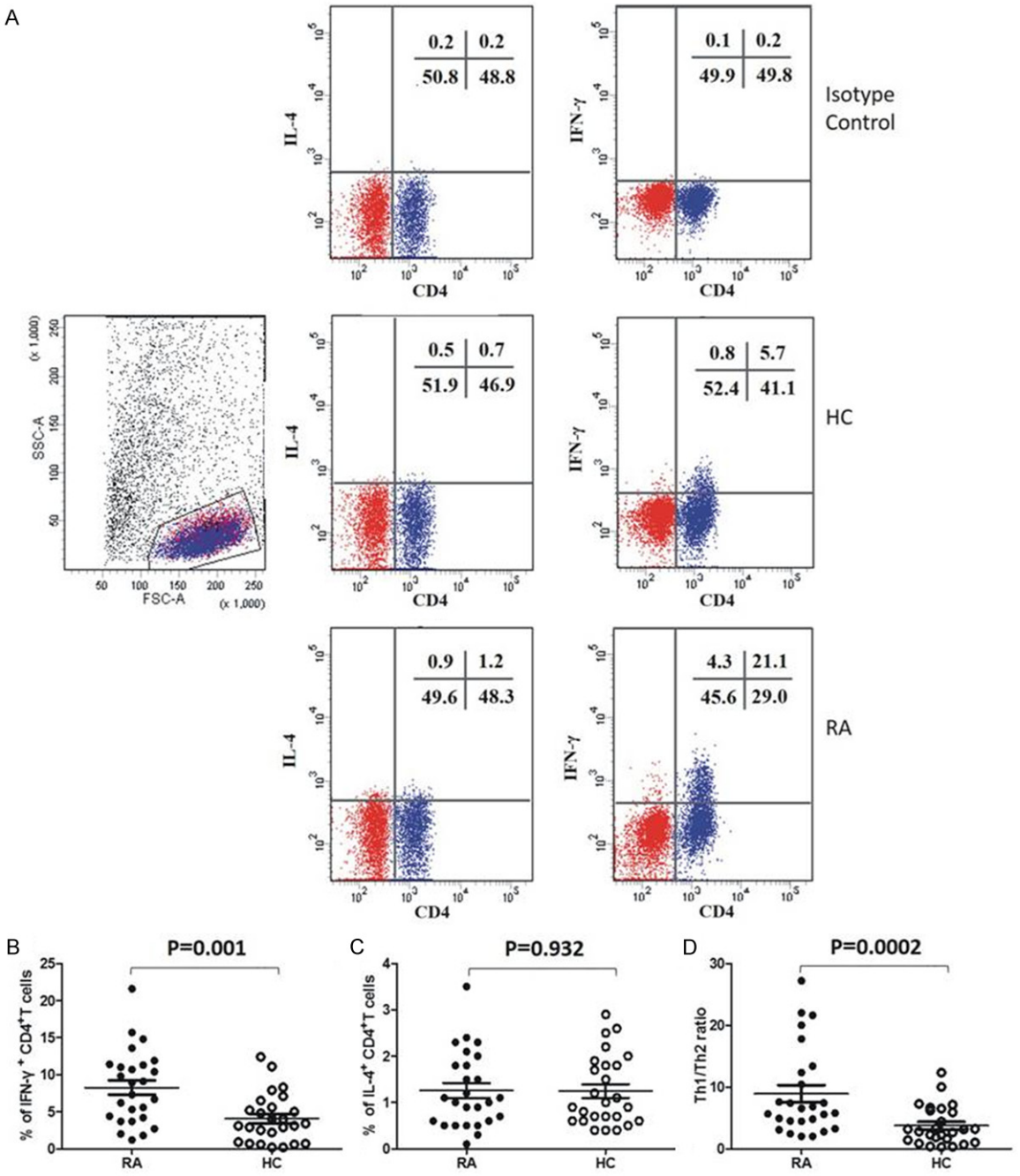

Figure 1. Flow cytometric analysis of IFN-γ and IL-4 expression in $CD4^+$ T-cells from RA patients and healthy controls (HC). Whole blood was stimulated with PMA/Ionomycin and analyzed for intracellular cytokine expression of IFN-γ and IL-4 among $CD4^+$ T-cells. A: Representative dot plots of IFN-γ and IL-4 expressing $CD4^+$ T-cells from RA patient and HC upon stimulation are shown. B: The percentage of Th1 cells is shown. C: The percentage of Th2 cells is shown. D: The ratio of Th1/Th2 cells is shown. Horizontal lines represent mean ± SEM (n=26). *P*-values were calculated using the nonparametric Mann-Whitney U-test.

Pharmingen), monoclonal APC-conjugated anti-human Tim-3 and APC-conjugated rat IgG2a isotype control (all from eBioscience) were used for flow cytometric analysis. At least 10,000 cells were analyzed using a FACSCalibur instrument (BD Biosciences).

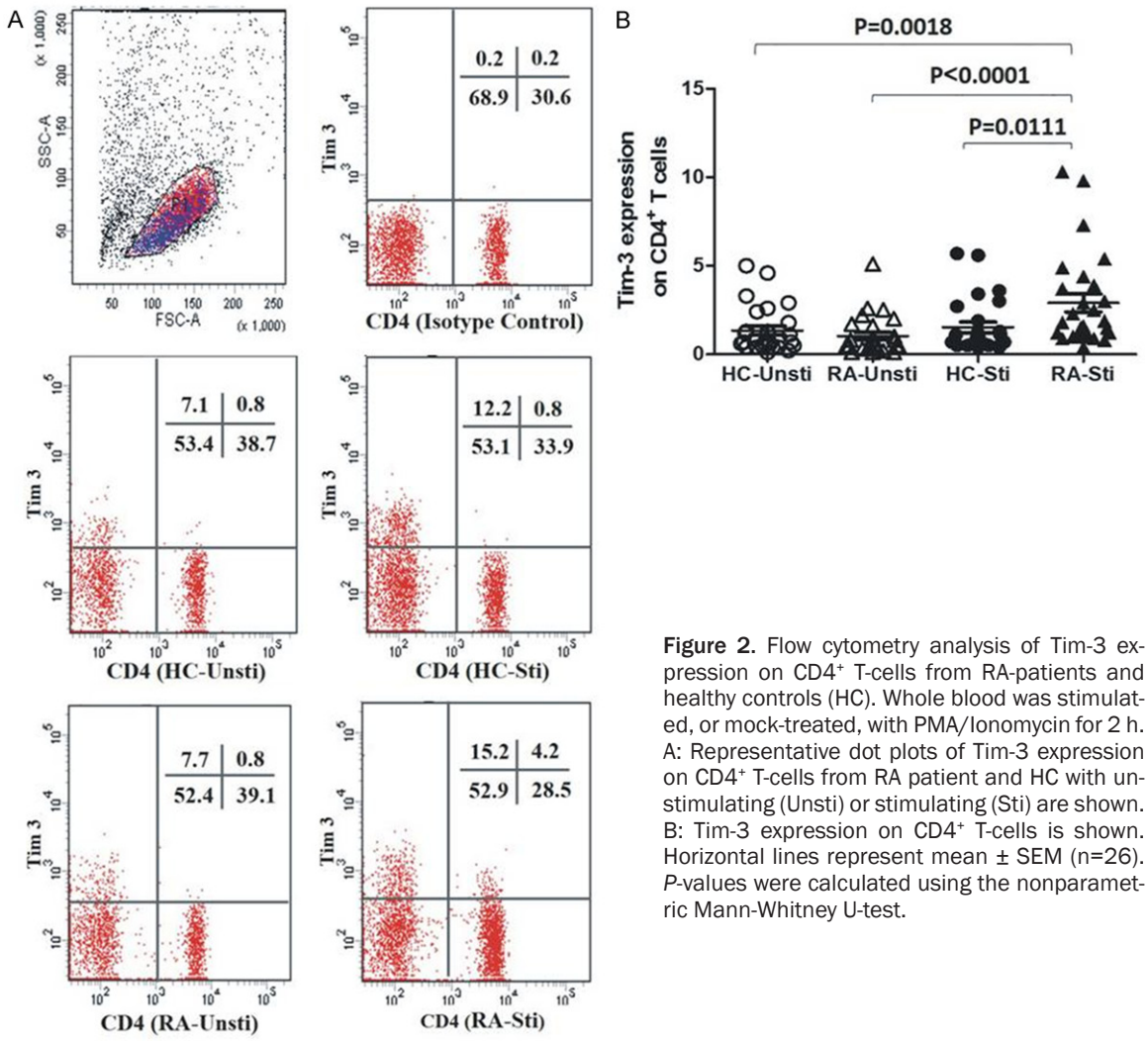

Figure 2. Flow cytometry analysis of Tim-3 expression on $CD4^+$ T-cells from RA-patients and healthy controls (HC). Whole blood was stimulated, or mock-treated, with PMA/Ionomycin for 2 h. A: Representative dot plots of Tim-3 expression on $CD4^+$ T-cells from RA patient and HC with unstimulating (Unsti) or stimulating (Sti) are shown. B: Tim-3 expression on $CD4^+$ T-cells is shown. Horizontal lines represent mean ± SEM (n=26). *P*-values were calculated using the nonparametric Mann-Whitney U-test.

Isolation and culture of peripheral blood mononuclear cells (PBMC)

Human PBMCs were isolated and counted from heparinized blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Isolated PBMCs were cultured in RPMI 1640 containing 2 mM glutamine, 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies), and 10% heat-inactivated fetal calf serum (Gibco Life Technologies). Cells were seeded at 5×10^5 cells/ml in 96-well plates and incubated (5-48) h at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were either left unstimulated or stimulated with *E. coli* LPS (Lipopolysaccharide) (1 μg/ml) (Invitrogen Life Technologies). After incubation, plates were centrifuged and supernatants were harvested and stored at -80°C.

Measurements of cytokines

The levels of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ in culture supernatant from PBMC were measured by FCM using a CBA cytokine kit (BD Cytometric Bead Array, USA) according to the manufacturer's instructions.

Isolation, labeling and analysis of Mps by flow cytometry

Mps were isolated from platelet-poor plasma as described by Christoffer *et al.,* [16], with minor modifications of the platelet-poor plasma isolation protocol (1,800 g for 10 minutes and 3,000 g for 10 minutes at 21°C). Aliquots of platelet poor plasma (500 μl) were thawed on ice and centrifuged at 18,000 g for 1 h at 4°C. Four-hundred fifty (450 μl) microliters of super-

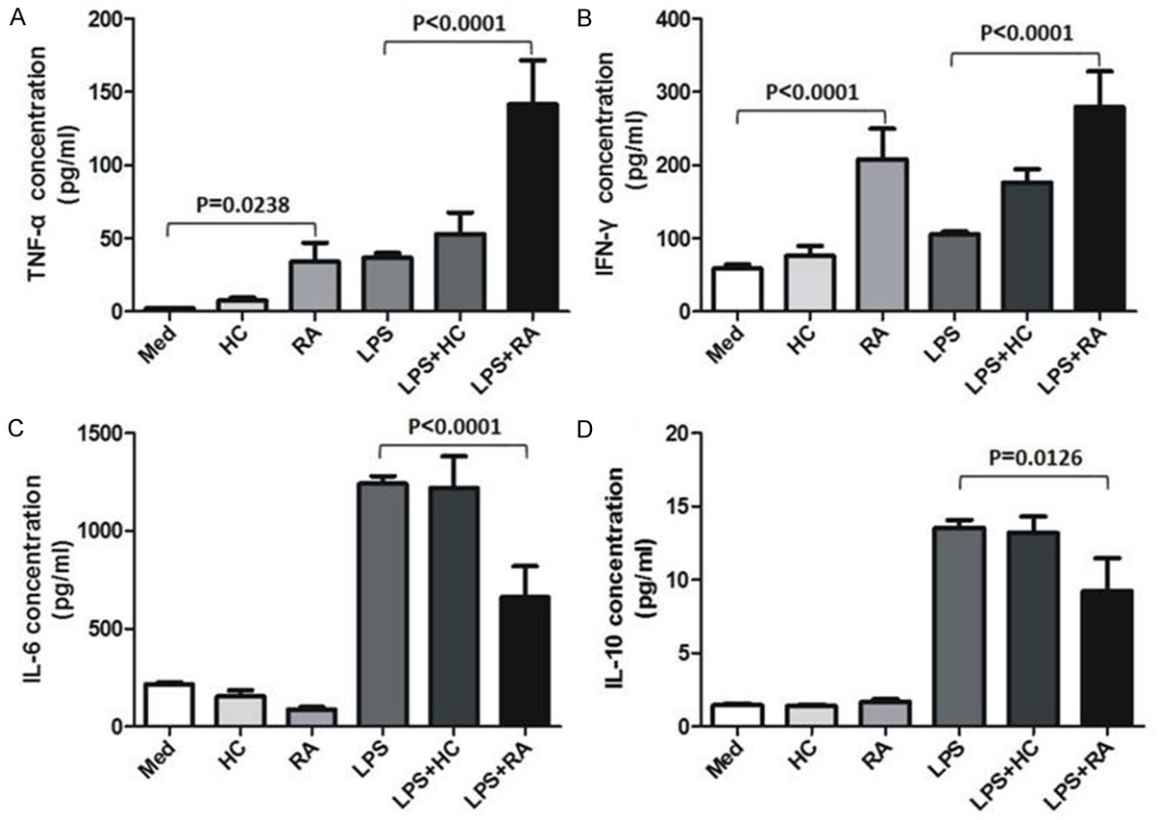

Figure 3. Sera from RA patients induced Th1-type cytokine secretion and inhibited Th2-type cytokine secretion. 10 μl sera and/or LPS (1 μg/ml) were co-cultured with PBMC for 5 h (IL-6 and TNF-α release) or 24 h (IL-10 and IFN-γ release). Cytokines in the supernatants were detected by CBA. A: TNF-α production by PBMC. B: IFN-γ production by PBMC. C: IL-6 production by PBMC. D. IL-10 production by PBMC. Data is presented as mean ± SEM (n=6), *P*-values were calculated using the nonparametric Mann-Whitney U-test. Similar results were observed in three separated experiments.

natant were replaced by 450 μl of PBS containing 10.5 mM trisodium citrate (PBS-citrate). After re-suspending Mps, the centrifugation step was repeated, and 450 μl of the supernatant was removed. The remaining Mps were re-suspended in 150 μl of PBS-citrate for a final purified volume of 200 μl. The Mps suspension was labeled with FITC-AnxV (final dilution 10 μg/ml, BD). For flow cytometric analysis, 5 μl of the Mps suspension was diluted in 45 μl loading buffer (PBS-Ca, 2.5 mM $CaCl_2$). The samples were analyzed within 1 h using a FACSCalibur flow cytometer (BD) controlled by CellQuest software version 5.1.1 in the "high" flow rate mode. All channels were recorded with logarithmic gain. Acquisition time was 60 seconds. Mps gating was accomplished using 1 μm beads (20). The gated microparticles were then plotted based on their binding of AnxV.

Statistical analysis

Data are presented as mean ± SD unless stated otherwise. The nonparametric Mann-Whitney U-test was used to compare data from RA patients with that of healthy controls. All analyses were performed using GraphPad Prism 5.0, and differences were considered statistically significant at two sided *p*-values less than 0.05.

Results

$CD4^+$ T cells from RA patients presented high value of Th1/Th2 ratio

To analyze $CD4^+$ Th cell subsets, we measured the intracellular expression of the cytokines IFN-γ (detection of Th1 cells) and IL-4 (detection of Th2 cells) in RA patients and HCs. Spontaneous expression of IFN-γ and IL-4 in $CD4^+$ T cells was low and did not differ between

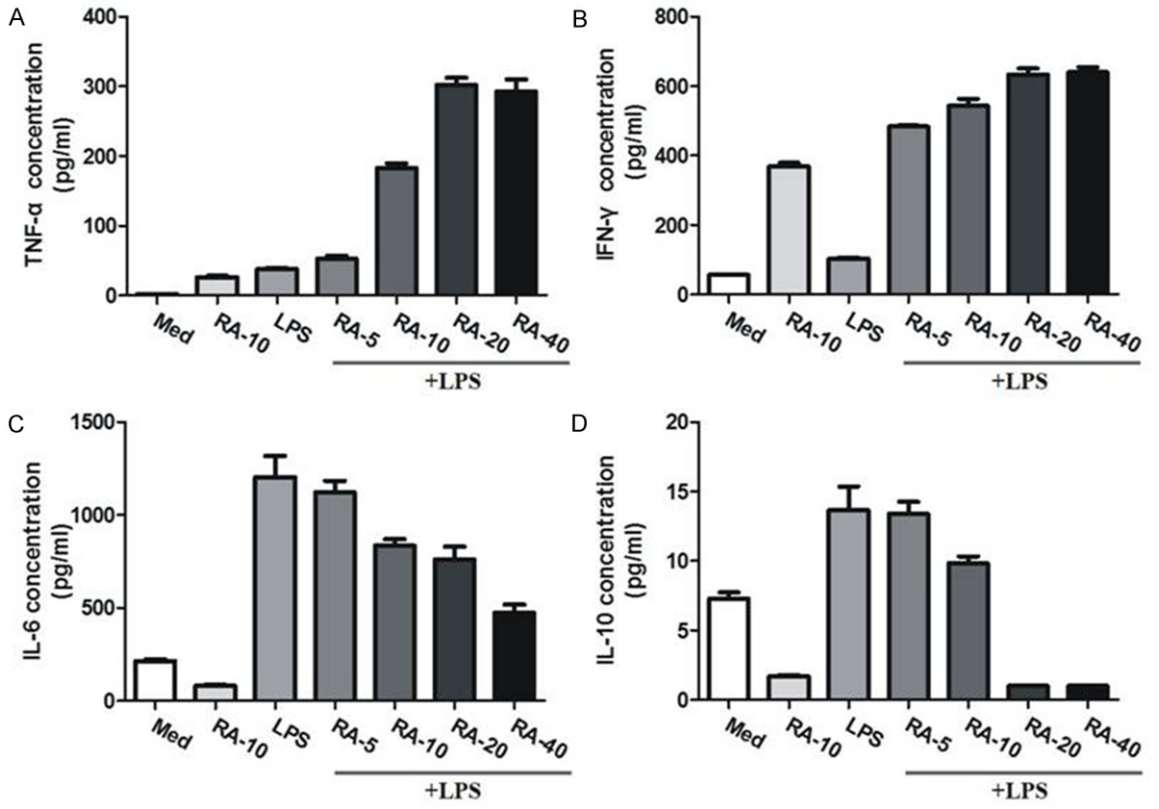

Figure 4. Th1/Th2 cytokine expression was affected by sera from RA patients in a dose-dependent manner. A serial dilution series of serum concentrations (5 μl-40 μl) and/or LPS (1 μg/ml) were co-cultured with PBMC for 5 h (IL-6 and TNF-α release) or 24 h (IL-10 and IFN-γ release). Cytokines in the supernatants were detected by CBA. A: TNF-α production by PBMC. B: IFN-γ production by PBMC. C: IL-6 production by PBMC. D: IL-10 production by PBMC. Data were presented as mean ± SEM (n=3). Similar results were observed in three separated experiments.

patients and HCs (data not shown). Upon stimulation by PMA/Ionomycin in the presence of Brefeldin A and Monensin for 5 h, the percentage of IFN-γ$^+$CD4$^+$ T-cells was significantly higher in RA patients as compared to HCs (median: 17.5% (range: 2.9-36.6%) vs. 33.5% (14.8-54.8%), P=0.0004) (**Figure 1A** and **1B**). No difference in the percentage of IL-4$^+$ was observed between RA patients and HCs (**Figure 1C**). In keeping with these findings, the ratio of Th1/Th2 was significantly higher in RA patients in comparison to HCs. (0.21 (0.04-0.96) vs. 0.12 (0.05-0.25), P=0.01) (**Figure 1D**).

Tim-3 expression on activated CD4$^+$ T cells was increased in RA patients

Tim-3 was previously identified as an antigen expressed on Th1 cells that plays a role in regulating Th1 response. Tim-3 expression on resting and activated CD4$^+$ T cells from RA patients were detected by FCM in this study. As showed in **Figure 2**, Tim-3 expression was down-regulated on resting CD4$^+$ T cells from RA patients and HCs. However, *in vitro* stimulation by PMA/Ionomycin in the presence of Brefeldin A and Monensin resulted in induction of Tim-3 expression on CD4$^+$ T cells from RA patients but not HCs (P<0.05).

Sera from RA patients promoted Th1/Th2 balance biased towards Th1

As previously described, IFN-γ$^+$CD4$^+$ Th1 cells from RA patients were obviously polarized in this study. Cytokines produced by Th1/Th2 cells were secreted into body fluid such as serum and as such, plasma concentrations of Th1/Th2 cytokines of RA patients were detected. The expressions of Th1/Th2 cytokines in sera were low and there were no difference between patients and HCs (data not shown). To enhance the effect of trace cytokines, sera from RA patients and HCs were co-cultured with PBMC which were collected from healthy donor. The expressions of Th1/Th2 cytokines in

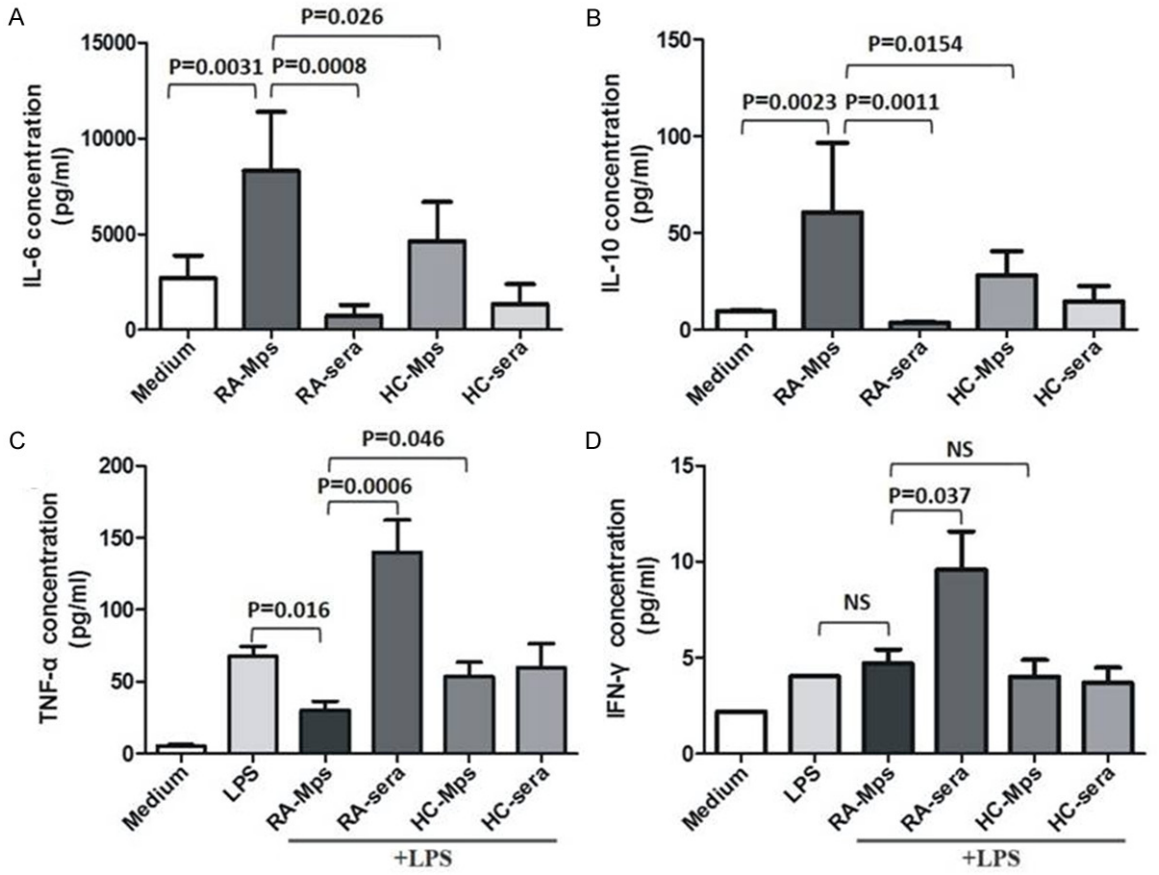

Figure 5. The effects of Mps from RA patients on Th1/Th2 cytokine production. Sera (10 μl), Mps (30 μg/ml) and/or LPS (1 μg/ml) were co-cultured with PBMC for 5 h (IL-6 and TNF-α release) or 24 h (IL-10 and IFN-γ release). Cytokines in the supernatants were detected by CBA. A: IL-6 production by PBMC. B: IL-10 production by PBMC. C: TNF-α production by PBMC. D: IFN-γ production by PBMC. Data were presented as mean ± SEM (n=6), *P*-values were calculated using the nonparametric Mann-Whitney U-test.

supernatants were detected by CBA. The results showed that PBMC produced high levels of Th1 cytokines (TNF-α and IFN-γ). Conversely, Th2 cytokines (IL-6 and IL-10) produced by PBMC were decreased following co-culturing with sera from RA patients (**Figure 3**). Furthermore, a serial dilution of serum concentrations were added with PBMC, and the concentrations of Th1/Th2 cytokines in supernatant were detected. The results demonstrated that sera from RA patients could markedly promote Th1 cytokines (TNF-α and IFN-γ) production and inhibit Th2 cytokines (IL-6 and IL-10) production in a dose-dependent manner (**Figure 4**).

MPs from RA patients mining the imbalance of Th1/Th2

Given the complexity of serum components, improved understanding of the specific activity factors in serum remains a subject of great importance. In previous study, we found sera from RA patients were filtered through 0.22 μm sterile filter or heated in 56°C water for 1 h or ultra-centrifuged (18000 g for 1 h) to remove Mps. Processed sera were co-cultured with PBMC and the levels of Th1/Th2 cytokines in supernatants were measured. The results demonstrated that the activity of sera persisted after filtering, heating or ultra-centrifuging. Surprisingly, while Mps purified from RA patient sera had strong activation on PBMC, the opposite effect was observed when Mps purified from HCs were used. As shown in **Figure 5**, after co-culturing with Mps, PBMC purified from healthy donors released increased IL-6 and IL-10 but resulted in decreased TNF-α in supernatants. As such, Mps could induce reduced IFN-γ expression, compared with sera from RA patients ($P<0.05$). However, no significant dif-

ference was observed between Mps purified from RA patients and Mps purified from HCs. These results suggest that some soluble components and Mps in RA patient sera played strong immune activity on T cells.

Discussion

In the present study we analyzed the phenotypes of effector Th-cells that express Th1 (IFN-γ) and Th2 (IL-4) cytokines in patients with RA and observed an altered balance of Th1/Th2 cells with increased Th1 cells and decreased Th2 cells. Furthermore, we found the sera from patients with RA could induce the imbalance of Th1/Th2 towards Th1. In contrast with these results, Mps derived from RA patients could mimic the disrupted balance of Th1/Th2.

In accordance with numerous studies, our findings illustrated RA is a Th1-drived disease, however some inconsistent results remain to be clarified. Talaat RM *et al.* reported a higher levels of plasma IL-4 and lower levels of plasma IFN-γ in RA patients [17]. Gerli R *et al.* found that patients with active RA had strong predominance of Th1 activity while those patients with early onset RA primarily expressed IL-4, indicative of Th2 activity, while patients not yet being treated manifested IL-10 expression. Taken together, those results indicated RA is a Th1-driven condition, however, a Th2 response is evident early in the disease process [18]. These studies yield inconsistent and even conflicting findings. The reasons for this discrepancy are two-fold: (1) most of studies examined patients with varying degrees of disease activity and (2) the shift of Th1/Th2 response may alter in RA patients with heterogeneous disease activity [2]. In addition, corticosteroids or hydroxychloroquine was commonly used to relieve clinical symptoms. Both drugs affect cytokines secretion by PBMC. Moreover, in mice two stages of T cell activation and cytokine secretion have been observed with expression of Th1-type cytokines first, followed later by induction of Th2-type cytokines [19]. The outcome of the cross-talk between Th1 and Th2 cytokines is complex. These cytokines may act in synergy or oppose each other in promoting lymphocyte proliferation and differentiation on the basis the timing of their secretion and their relative concentrations. Although these inconsistent reports exist, it has been suggested that both Th1 and Th2 responses play prominent roles in the pathogenesis of arthritis-associated tissue injury.

Tim-3 is selectively expressed on activated Th1 cells but not Th2 cells. Numerous studies have demonstrated that Tim-3 functions as a powerful regulator on both adaptive and innate immunity. Upon engagement with its ligand, Tim-3 negatively regulates IFN-γ secretion and influences the ability to induce T cell tolerance in both mice and human [20, 21]. The expression on resting and activated $CD4^+$ T cells and the relationship between Tim-3 expression and IFN-γ secretion were investigated in this study. Consistent with findings from Hastings WD *et al.*, Tim-3 was largely absent from resting $CD4^+$ cells isolated from peripheral blood [22]. In addition, we found activation with PMA/Ionomycin in vitro resulted in induction of Tim-3 expression. Compared with healthy subjects, the expression level of Tim-3, obviously increased after stimulation in RA patients. However, Tim-3 did not negatively regulate IFN-γ production by *ex vivo* $CD4^+$ T cells in our study. In fact, we found that Th1 polarization enhanced Tim-3 expression. This is not surprising, as reported that Tim-3^+ Th1-polarized cells were actively secreting IFN-γ detected by intracellular staining while Th2 polarized cells showed reduced Tim 3 expression.

Many studies suggest that microparticles shed from the cell surface of polymorphonuclear, erythrocytes and platelets have been shown to inhibit cytokine release by activated macrophages and to block dendritic cell maturation. MPs released by PMNs could modify human monocyte-derived dendritic cells maturation and function by reducing expression of cell surface markers (CD40, CD80, CD83, CD86, and HLA-DP DQ DR), inhibiting of cytokine-release (IL-8, IL-10, IL-12, and TNF-α), and reducing the capacity to induce T cell proliferation [23]. MPs shed from erythrocytes showed several down-modulatory activities on human macrophages by exhibiting the release of TNF-α and IL-8 secreted by macrophages activated by LPS or zymosan A [24]. MPs derived from platelets could diminish the release of inflammatory cytokines by activated $CD4^+$ T cells and promote their differentiation into functional Tregs, in a TGF-β1-dependent manner [25].

Our results demonstrated that Mps purified from sera could mimic the imbalance of Th1/

Th2 in RA patients by boosting Th2-type cytokine expression (IL-6 and IL-10) and suppressing Th1-type cytokine release (TNF-α). The immunosuppressive properties of Mps might be related to the expression of PS. PS is an anionic amino phospholipid restricted mostly to the inner leaflet of plasma membrane in live cells [26] and it is commonly expressed by apoptotic cells and MPs released by ERY, PMN and PLT. Conventionally, PS plays a central role in the pro-coagulant properties of many different cells and vesicles, which are in contact with blood. However, PS expression induces other reactions as well. On apoptotic cells, the exposure of PS on the outer leaflet of the cell plays a major role in down-regulating immunity and inflammation [27]. On MPs, the expression of PS has been shown to be responsible for suppression on macrophage and dendritic cells [23-25]. More directly, PS liposomes inhibited the production of IL-12p70 by human DCs in response to LPS. DCs exposed to PS had diminished capacity to stimulate allogeneic T cell proliferation and to activate IFN-γ-producing $CD4^+$ T cells [28]. These observations corresponded to the known biological effects of PS expression on apoptotic cells and MPs, which are not only "eaten in silence", but also inhibit the phagocytes that have ingested them [29, 30].

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Zhaowei Yan, Department of Clinical Pharmacology, The First Affiliated Hospital of Soochow University, Suzhou, China. Tel: 86-512-67780467; Fax: 86-512-67780467; E-mail: 1983joyjoy@163.com; Xueming Zhu, Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou, China. Tel: 86-512-67783432; E-mail: doodou2007@163.com

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