Expression of Galectin-9 and correlation with disease activity and vascular endothelial growth factor in rheumatoid arthritis

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Abstract

Objective

Despite the important role of Galectin-9 (Gal-9) in inflammation and angiogenesis, only a few studies on Galectin-9 expression have been performed in rheumatoid arthritis (RA) patients. The present study aimed to identify the concentration of Galectin-9 in plasma, its expression in peripheral blood T cell subsets in RA patients, and the association between Galectin-9 and vascular endothelial growth factor (VEGF) in RA.

Methods

One hundred and five active RA patients and 52 age- and sex-matched healthy controls (HCs) were enrolled in the study. Gal-9, vascular endothelial growth factor (VEGF), and tumour necrosis factor (TNF)-α level in plasma were determined by ELISA. The intracellular positive expression rates and medium fluorescence intensity (MFI) of Gal-9 and VEGF in T cell subsets, were examined by flow cytometry.

Results

Plasma Gal-9, TNF-α, and VEGF levels were higher in the RA group than in the HCs group. The plasma Gal-9 level positively correlated with Gal-9 expression in the total lymphocyte and $CD3^+$ T cell, CRP, SDAI, and CDAI of RA patients. The Gal-9 expressions in the cytoplasm of $CD4^+$ T, $CD8^+$ T, Treg, and DNT cells positively correlated with plasma TNF-α levels in RA patients. The Gal-9 expressions in $CD4^+$ T and $CD8^+$ T subsets also positively correlated with plasma VEGF levels. The plasma VEGF levels and VEGF expressions in $CD4^+$ T, $CD8^+$ T, and Treg subsets positively correlated with SDAI and CRP in RA patients.

Conclusion

Gal-9 can be a potential biomarker for RA disease activity. Gal-9 is probably associated with angiogenesis processing in RA.

Key words

rheumatoid arthritis, Galectin-9, disease activity, inflammation, angiogenesis

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterised by synovial hyperplasia and angiogenesis (1). Multiple cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL) -1, IL-6 (2-4) and immune cells (5-8) contribute to the development of RA. Although current research provides insights into the pathogenesis of RA (9), the exact mechanism remains unknown. Meanwhile, targeting treatment on cytokines and anti-angiogenic therapy also have made great progress in RA (10-12). However, there are still quite a number of patients that may have an inadequate response to the current treatment regimens due to primary lack of response or secondary treatment failure due to resistance or intolerance (13-15). Finding more and more effective therapeutic targets is the focus of future research.

Galectins are a group of mammalian lectins with a high affinity for β-galactosides that share a highly conserved carbohydrate recognition domain (CRD) (16). Galectin-9 (Gal-9), a subfamily of galectins (17), can induce apoptosis of T helper type 1 (T_H1) cells (18), suppress generation of T_H17 cells, and promote induction of regulatory T (Treg) cells (19). Gal-9 is highly expressed in rheumatoid arthritis (RA) synovial fluid (SF) and synovial tissue (ST) (20) and plays an anti-inflammatory effect in collagen-induced arthritis (CIA) animal models (20, 21).

Galectin-9 decreased the levels of pro-inflammatory cytokines, IL-17, IL-12, and interferon (IFN)-γ in the joints of CIA mice (19). Macrophages from Gal-9-treated immune complex-induced arthritis mice produced fewer TNF-α and IL-1ß but more IL-10 than PBS-treated mice (22). On the other hand, TNF-α (23) and IFN-γ (24) can induce the expression of Gal-9 in cultured endothelial cells and human RA synovial fibroblasts (RASF) (25), and IL-4 did not affect the expression of Gal-9 (24). Moreover, Gal-9 acts as an important adhesion molecule for migrating lymphocytes and eosinophils (26) and is involved in cell matrix interaction and appears to regulate adhesion at multiple levels, both directly and indirectly (27). Gal-9 has integrin-mediated strong adhesion to laminin III and compared with control cells, Gal-9 knockout cells adhered less efficiently to laminin-III, collagen I, and Matrigel™, and Gal-9 was required for optimal long-term cell adhesion to both collagen I and laminin-III (28).

However, whether the Gal-9 plays a role in promoting inflammation and angiogenesis, has a negative immune regulation, or is a key factor in immune response and angiogenesis coupling in RA remains unclear. Therefore, in the present study we emphatically investigated the plasma level of Gal-9, the expression of Gal-9 on different T cell subsets, and the association between Gal-9 and disease activity, plasma pro-inflammatory cytokine, and various T cell subsets in RA patients; At the same time, based on the study of Gal-9 inducing human dermal microvascular endothelial cell (HMVEC) migration and tube formation on Matrigel, as well as in mouse vivo angiogenesis, and joint inflammation in mice in vitro and in animal models (23), the relationship between Gal-9 and VEGF was also preliminarily investigated, all of which contributed to our understanding of Gal-9 in the pathogenesis of RA.

Materials and methods

Study design and human subjects

One hundred and five active RA patients from the rheumatology clinic of Qilu Hospital of Shandong University were enrolled in the study between March 2015 and December 2017. RA was diagnosed according to the 1987 American College of Rheumatology (ACR) criteria (29). At the time of enrolment, all patients were assessed for disease activity, filled in a health assessment questionnaire (HAQ), and recorded their treatment regimen. Disease activity score 28-ESR (DAS28-ESR), clinical disease activity index (CDAI) and simplified disease activity index (SDAI) were selected as clinical disease activity evaluation indices (30, 31). Patients were divided into naïve to glucocorticoid and DMARDs group (RA naive) and DMARDs treatment group with inadequate response to DMARDs (RA-IR) according to wheth-

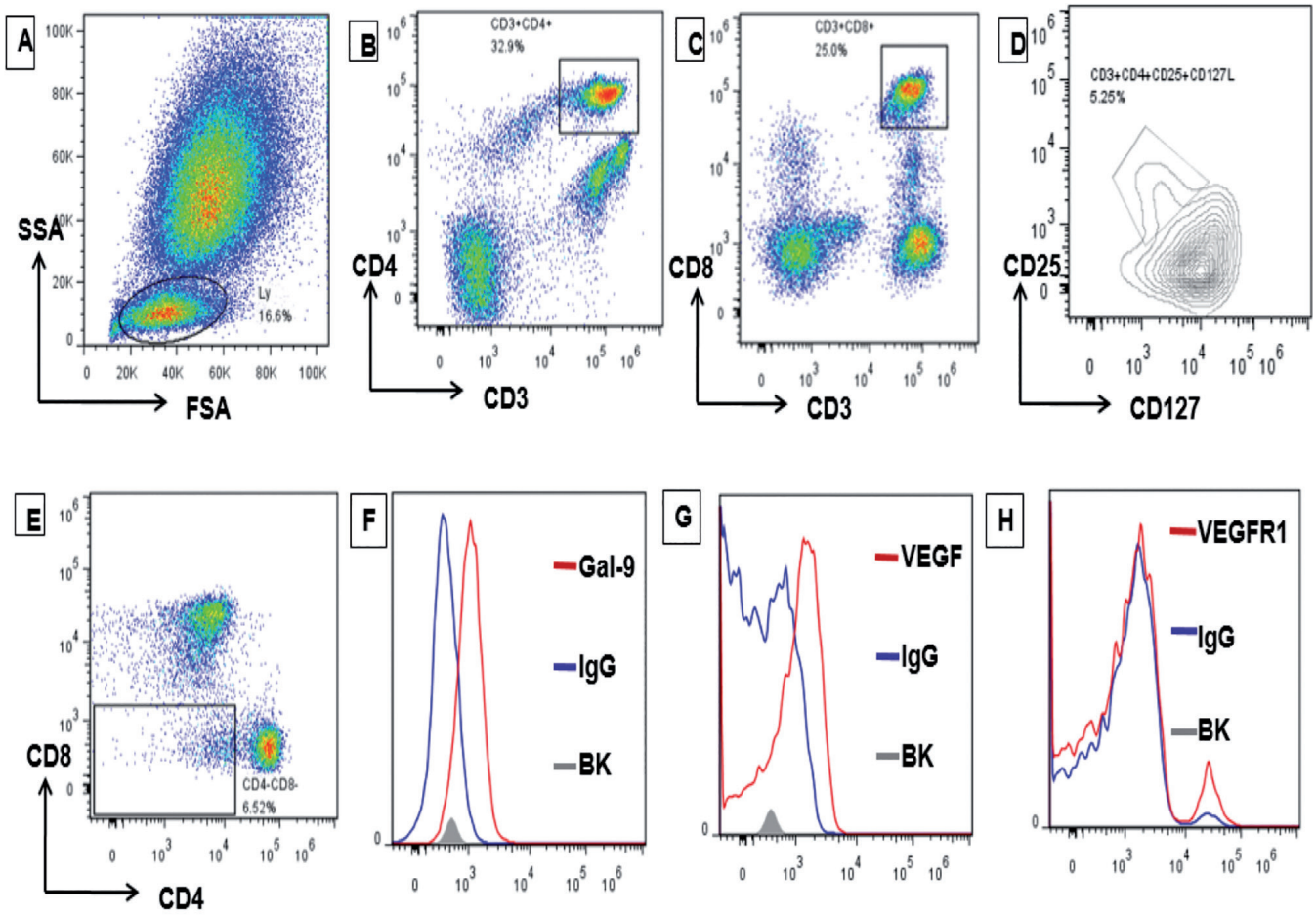

Fig. 1. Different cell subsets in peripheral blood mononuclear cells (PBMCs) and expression of Gal-9, VEGF, VEGFR1 in one RA patient.
A. Lymphocytes in ungated PBMCs; **B**. $CD3^+CD4^+$ T subsets in lymphocytes; **C**. $CD3^+CD8^+$ T subsets in lymphocytes; **D**. $CD25^+CD127^{Low}$ (Treg) in $CD4^+$ T subsets; **E**. $CD4^-CD8^-$ T subsets in $CD3^+$ T subsets. **F**. Gal-9 expression in $CD4^+$ T cells of one RA patient. BK: Gal-9 expression in blank control; IgG: PE mouse IgG1 isotype control of Gal-9: Gal-9: PE anti-human Gal-9. **G**. VEGF expression in $CD4^+$ T cells of one RA patient. BK: VEGF expression in blank control; IgG: APC rat IgG1 isotype control of VEGF; VEGF: APC anti-human VEGF. **H**. VEGFR1 expression in $CD4^+$ T cells of one RA patient. BK: VEGFR1 expression in blank control; IgG: APC rat IgG1 isotype control of VEGFR1; VEGFR1: APC anti-human of VEGFR1.

er the patients used glucocorticoid and DMARDs and their responses to these two kinds of drugs. Fifty-two age- and sex-matched healthy people were also enrolled as healthy controls (HCs). Patients and HCs who had other diseases or were pregnant were excluded. Written informed consents were obtained from all participants and this study had been approved by the Human Research Ethics Committees of Qilu Hospital of Shandong University (KYLL-2015-269) from January 2015 to December 2018.

Enzyme-linked immunoabsorbent assay (ELISA)

Gal-9 (Human Gal-9 ELISA Set, R&D, Minneapolis, MN, USA), VEGF (Human VEGF ELISA Set, eBioscience, San Diego, CA, USA) and TNF-α (BD OptEIA™ Human TNF ELISA Set, eBioscience, San Diego, CA, USA) in plasma of RA patients and HC were determined by ELISA according to the manufacturer's protocol.

Flow cytometry

Flow cytometry was used to detect the percentage of different T cell subsets in peripheral blood of patients with RA and HCs, as well as the intracellular positive expression rates and median fluorescence intensity (MFI) of Gal-9 and VEGF, and membranous positive VEGFR1 expression in above T cell subsets.

- Extracellular staining

150ul whole blood of EDTA anticoagulant was placed in BD flow tube, and the following fluorochrome-conjugated monoclonal antibodies were added: CD3 APC-eFluor780 (eBioscience, San Diego, CA, USA), CD4 Alexa Fluor 700 (BioLegend, San Diego, CA, USA), CD8 FITC (eBioscience, San Diego, CA, USA), anti-human CD25 PE/Dazzle 594 (BioLegend, San Diego, CA, USA), CD127 PE-Cyanine7 (eBioscience, San Diego, CA, USA), VEGFR1 APC (R&D, Minneapolis, MN, USA) or isotype-matched controls (eBioscience, San Diego, CA, USA). After staining at room temperature against the light for 20min, 2ml of 1:10 diluted RBC lysis buffer (BD Biosciences, Franklin Lakes, NJ, USA) was added. After cracking at room temperature against the light for 10min, cleaning and resuspension were performed. Data were obtained from multicolour analysis using a FACS Calibur flow cytom-

Table I. The demographic and clinical characteristics of the study cohort.

Items	RA n=105	RA naïve n=48	RA-IR n=57	HCs n=52
Age (year)	50.48 ± 13.93	51.56 ± 13.84	49.56 ± 14.05	47.44 ± 9.19
Female, n (%)	81 (77.14)	35 (72.92)	46 (80.70)	41 (78.85)
Male, n (%)	24 (22.86)	13 (27.08)	11 (19.30)	11 (21.15)
DAS28	4.69 ± 1.02	4.91 ± 0.89	4.51 ± 1.09	-
SDAI	63.64 ± 44.48	67.05 ± 41.48	60.76 ± 47.03	-
CDAI	23.44 ± 12.10	25.46 ± 13.54	21.73 ± 10.56	-
CRP (mg/L)	40.48 ± 37.54	42.22 ± 33.39	39.02 ± 40.94	-
ESR (mm/h)	53.60 ± 26.67	55.60 ± 1.81	51.91 ± 30.25	-
HAQ	1.02 ± 0.56	1.09 ± 0.55	0.95 ± 0.56	-

RA: rheumatoid arthritis patients; HCs: healthy controls; DAS28: Disease Activity Score-28; SDAI: Simplified Disease Activity Index; CDAI: Clinical Disease Activity Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HAQ: Health Assessment Questionnaire; RA naïve: naïve to glucocorticoid and DMARDs; RA-IR: inadequate response to DMARDs.
Values are shown as mean ± SD unless otherwise noted.

eter (BD Biosciences, Franklin Lakes, NJ, USA) and were analysed using FlowJo software 7.6.2 (Tree Star, Ashland, OR, USA). The specific analysis methods are shown in Figure 1.

- Intracellular staining

100ul whole blood of EDTA anticoagulant was placed in BD flow tube, and the following fluorochrome-conjugated monoclonal antibodies were added: CD3 APC-eFluor780, CD4 Alexa Fluor 700, CD8 FITC, anti-human CD25 PE/Dazzle 594, CD127 PE-Cyanine7 or isotype-matched controls (same as **t**he way in extracellular staining). After staining at room temperature against the light for 20min, 100ul of IC Fixation Buffer (eBioscience, San Diego, CA, USA) was added. Fix at room temperature and away from light for 20min before cleaning, 100ul of Permeabilization Wash Buffer (eBioscience, San Diego, CA, USA) was added. Then Gal-9 PE (BioLegend, San Diego, CA, USA) and VEGF APC (R&D, Minneapolis, MN, USA) were added in turn (The specific antibody sampling method is shown in Table II). Incubate in the dark at room temperature for 20min before cleaning and resuspension. Data obtained and analysed were the same as **t**he way described in extracellular staining.

Statistical analysis

GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) was used for all analyses. All the data were analysed with the homogeneity test. All values were shown as mean ± SD or median (range) unless otherwise stated. Inter-group differences were assessed for significance using the independent-samples t-test or Mann-Whitney U-test. Correlations between variables were assessed by Pearson or Spearman's rank correlation coefficient. A probability value, $p<0.05$ was considered statistically significant.

Results

Descriptive characteristics of the study population

A total of 105 patients with RA were enrolled in this study, among which 48 patients were classified as RA naïve group and 57 patients were classified as RA-IR group. As a reference group, 52 age- and sex-matched HCs were enrolled. The demographic characteristics, clinical manifestations, and laboratory measurements were shown in Table I. As shown in Figure 2, the white blood counts and proportion of $CD4^+CD25^+CD127^{low}$ regulatory T (Treg) cell were higher in RA group than in HCs group ($p<0.05$), while the proportions of lymphocyte and $CD3^+CD4^-CD8^-$ double negative T (DNT) cell were lower ($p<0.05$). Compared to the HCs group, RA-IR group had higher white blood counts (7.43±2.46 *vs*. 5.94±1.03, $p<0.01$) and lower lymphocyte percentages (18.34±7.11 *vs*. 22.70±7.09, $p=0.02$). There were no significantly statistic differences for $CD8^+$ T cells percentage (21.69±10.53 *vs*. 25.39±8.89, $p=0.49$) and DNT cell percentage (4.60±3.96 *vs*. 6.53±4.90, $p=0.42$) between RA-IR group and HCs group.

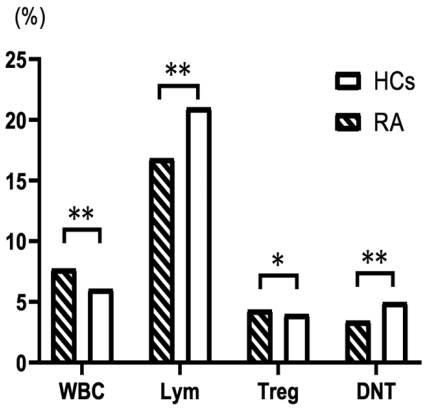

Fig. 2. Different cells proportion between RA patients and HCs.
The white blood counts and the proportion of Treg are higher, while the proportion of lymphocytes and DNT are lower in RA patients than in HCs. The differences between the 2 groups were analysed by Mann-Whitney U-test. WBC: white blood counts; Lym: lymphocytes, the percentage of lymphocytes in peripheral blood; Treg: regulatory T cells, the percentage of regulatory T cells in $CD4^+$ T cells; DNT: ($CD3^+CD4^-CD8^-$) double negative T cells, the percentage of double negative T cells in $CD3^+$ T cells. *$p<0.05$, **$p<0.01$.

Plasma cytokines levels in the RA group and HC group

Compared to the HC group, the plasma levels of Gal-9 (Fig. 3A), VEGF (Fig. 3B) and TNF-α (Fig. 3C) in both the RA group and RA-IR group were significantly increased. Moreover, the RA naïve group had higher plasma levels of Gal-9 and VEGF than the HC group (Fig. 3A-B).

Gal-9 expressions in T cell subsets of RA patients and HCs group

The Gal-9 expressions in T cell subsets in RA and HCs were shown in Table II. The differences of Gal-9 expression in $CD3^+$T cells, $CD4^+$T cells, Treg cells, and DNT cells were not statistically significant in the RA group and HC group. However, compared to the HC group, RA-IR group higher Gal-9 expression in lymphocyte (Table II). At the same time, we examined the MFIs of Gal-9 expression in the above cells (Fig. 4). There were no statistically significant differences in the MFI of Gal-9 expression in all T subset cells except for in total lymphocyte between the RA group and the HC group. Compared to HCs group, RA-IR group had statistically higher MFI of Gal-9 expression in total lymphocyte and DNT cells (Fig. 4A and F).

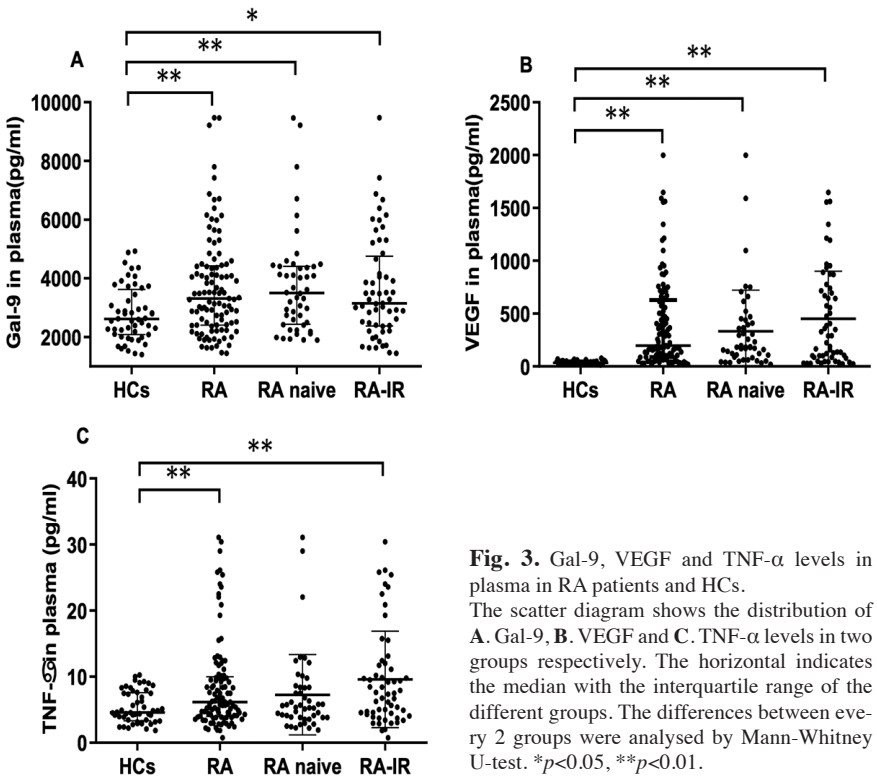

Fig. 3. Gal-9, VEGF and TNF-α levels in plasma in RA patients and HCs.
The scatter diagram shows the distribution of **A**. Gal-9, **B**. VEGF and **C**. TNF-α levels in two groups respectively. The horizontal indicates the median with the interquartile range of the different groups. The differences between every 2 groups were analysed by Mann-Whitney U-test. *p<0.05, **p<0.01.

Correlations between plasma Gal-9 levels and disease activity in RA patients

There was no statistical difference in the correlation between DAS28 and Plasma Gal-9 level (r=0.1893, p=0.0531). However, plasma Gal-9 levels positively correlated with disease activity indexes including SDAI (r=0.2686, p=0.0056), CDAI (r=0.2243, p=0.0215) and CRP (r=0.2596, p=0.0075).

Correlations between Gal-9 expression in T subsets and disease activity in RA patients

The MFI of Gal-9 expression in total lymphocyte, Gal-9$^+$CD3$^+$ T cells, Gal-9$^+$CD4$^+$ T cells, Gal-9$^+$CD8$^+$ T cells, Gal-9$^+$Treg cells, and Gal-9$^+$DNT cells positively correlated with multiple activity indexes including DAS28, CRP, SDAI and CDAI in RA patients (Table III); Different from the above results, only the proportion of Gal-9 expression in CD4$^+$ T cells was correlated with CRP (r=-0.2980, p<0.05) and SDAI (r=-0.3011, p<0.05) in the RA naive group, and only the proportion of Gal-9 expression in total lymphocytes was negatively correlated with DAS28-ESR (r=-0.2833, p<0.05), CRP (r=-0.3344, p<0.05), CDAI (r=-0.3129, p<0.05), and SDAI (r=-0.3735, p<0.01) in the RA-IR group.

Correlations between Gal-9 expression in T subsets and plasma Gal-9 level of RA patients

The plasma Gal-9 level positively correlated with Gal-9 expression in total lymphocyte (r=0.2838, p=0.0038) and CD3$^+$ T cell (r=0.2623, p=0.0115). However, there were no statistical correlations between plasma Gal-9 and Gal-9 expression in CD4$^+$ T cells (r=0.0443, p=0.6585), CD8$^+$ T cells (r=0.0648, p=0.5197), Treg cells (r=0.0394, p=0.6941), and DNT cells (r=0.0734, p=0.4992).

Correlations between Gal-9 expression in T subsets and plasma cytokines level of RA patients

Plasma TNF-α levels were positively correlated with Gal-9 expressions in the cytoplasm of CD4$^+$ T (r=0.323, p=0.001), CD8$^+$ T (r=0.284, p=0.004), Treg (r=0.428, p<0.001), and DNT (r=0.323, p=0.002) subsets in the RA group. Moreover, plasma VEGF levels were also positively correlated with Gal-9 expressions in the cytoplasm of CD4$^+$ T (r=0.1962, p=0.0482) and CD8$^+$ T (r=0.3055, p=0.0018) subsets in RA patients.

Correlations between VEGF and disease activity in RA patients

The plasma VEGF levels were positively correlated with SDAI (r=0.224, p=0.022) and CRP (r=0.197, p=0.044) in RA patients. The VEGF expressions in CD4$^+$ T, CD8$^+$ T, and Treg subsets positively correlated with both SDAI (r=0.3003, p=0.008; r=0.2680, p=0.0184; r=0.3375, p=0.0027, respectively) and CRP (r=0.3075, p=0.0069; r=0.2396, p=0.0371; r=0.2905, p=0.0109, respectively).

Table II. The Gal-9 expression percentage in each T subgroup measured by a flow meter in RA, RA naïve, RA-IR and HCs groups.

Items	RA n=105	RA naïve n=48	RA-IR n=57	HCs n=52
LyGal-9$^+$	8.00 (4.44, 23.55)	8.54 (5.61, 21.9)	7.30 (2.40, 28.90)*	11.85 (6.58, 24.28)
CD3$^+$Gal-9$^+$	9.21 (2.94, 21.98)	9.32 (4.30, 19.6)	9.09 (2.04, 22.80)	10.65 (4.73, 24.50)
CD4$^+$Gal-9$^+$	22.10 (15.75, 31.98)	22.10 (15.15, 30.65)	22.10 (16.6, 35.90)	21.40 (15.5, 29.93)
CD8$^+$Gal-9$^+$	24.65 (15.88, 35.13)	25.8 (17.60, 40.20)	24.20 (14.60, 31.80)	23.50 (19.33, 31.53)
TregGal-9$^+$	18.95 (12.10, 26.10)	18.70 (11.60, 29.70)	19.50 (12.35, 24.00)	14.30 (9.87, 23.10)
DNTGal-9$^+$	23.30 (15.40, 31.50)	22.80 (15.33, 31.68)	23.30 (15.25, 32.30)	23.40 (15.65, 34.00)

RA: rheumatoid arthritis patients; HCs: healthy controls; Ly: lymphocyte; Gal-9: galectin-9; Treg: regulatory T cell; DNT: double negative T cells.
*p<0.05 compared to healthy controls.
Values are shown as median with interquartile range unless otherwise noted.

Table III. The correlations of disease activity parameters and MFI of Gal-9 expression in lymphocyte, $CD3^+$T, $CD4^+$T, $CD8^+$T, Treg and DNT cells in RA patients.

	DAS28-ESR	CRP	SDAI	CDAI
Ly	0.177	0.200*	0.225*	0.204*
$CD3^+$T	0.137	0.178	0.223*	0.205*
$CD4^+$T	0.217*	0.270**	0.281**	0.222*
$CD8^+$T	0.325**	0.200*	0.279**	0.381**
Treg	0.202*	0.285**	0.268**	0.157

Ly: lymphocyte; DAS28: Disease Activity Score-28; CRP: C -reactive protein; SDAI: Simplified Disease Activity Index; CDAI: Clinical Disease Activity Index; Treg: regulatory T cell; DNT: double negative T cells.
The correlations between the two factors were analysed by the Spearman correlation test.
A *p*-value <0.05 was considered to be statistically significant.
*p<0.05; **p<0.01.

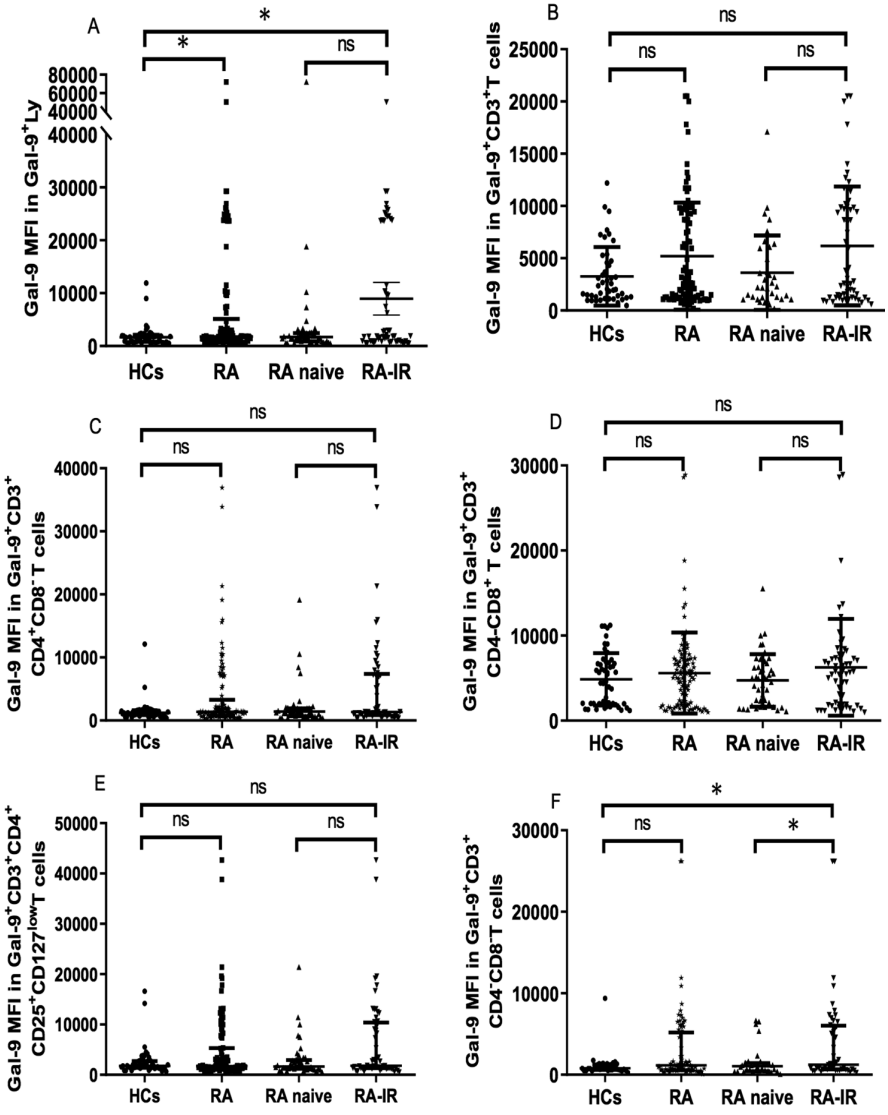

Fig. 4. The MFI of Gal-9 expression in lymphocyte, $CD3^+$T, $CD4^+$T, $CD8^+$T, Treg and DNT cells in RA patients and HCs.
A: Gal-9 MFI in Gal-9^+Ly, **B**: Gal-9 MFI in Gal-9^+CD3^+T cells, **C**: Gal-9 MFI in Gal-$9^+CD3^+CD4^+$T cells, **D**: Gal-9 MFI in Gal-$9^+CD3^+CD4^+$T cells, **E**: Gal-9 MFI in Gal-$9^+CD3^+CD4^+CD25^+CD127^{low}$T cells, **F**: Gal-9 MFI in Gal-$9^+CD3^+CD4^-CD8^-$T cells.
The horizontal indicates the median with the interquartile range of the different groups.
The differences between the 2 groups were analysed by Mann-Whitney U-test. *p<0.05.

Expression of VEGR1 on various T cell subsets

The differences of VEGFR1 expression on $CD3^+$T cells, $CD4^+$T cells, Treg cells, and DNT cells were not statistically significant (data not shown).

Discussion

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease attributed to both genetic and environmental factors (9). Early diagnosis and resulting in early DMARDs treatment can improve the prognosis of RA (32). Regular follow-up and monitoring of patient disease activity are essential to guide treatment and achieve RA remission or low disease activity state (33). Because of its convenience and high cost-effectiveness ratio, ESR (34) and CRP (35) are still the most commonly used laboratory indexes to monitor the activity of RA in clinical practice. Both ESR and CRP in patients with active RA increase the yield of identifying an elevated acute phase reactant (APR) level. However, APR levels do not always reflect disease activity, as measured by joint counts and global assessments (36). At present, biomarkers are playing a more and more important role in confirming the diagnosis, predicting outcome and suggesting specific treatments (37). Therefore, finding more effective and convenient indexes is beneficial in monitoring the changes of disease and guiding the clinical use of the drug.

Galectin-9 is an easily measured biomarker for the interferon signature in systemic lupus erythematosus and antiphospholipid syndrome (38). In our present study, we found that the plasma Gal-9 levels were significantly higher in RA patients compared with the HCs group and positively correlated with RA disease activity indexes such as SDAI, CDAI and CRP, which means that plasma Gal-9 may be useful as an index of disease activity in RA. Lee *et al*. reported that Gal-9 mRNA level in PBMCs of RA patients was higher than in healthy controls, and was significantly higher in patients with low disease activity compared to those with moderate to high disease activity (39). Therefore, increased plasma Gal-9 level and

increased Gal-9 mRNA level in PBMCs but not Gal-9 expression in total lymphocyte of RA patients suggested that plasma Gal-9 level probably was affected by other cells except for T cells.

Although the treatment of rheumatoid arthritis has achieved a landmark breakthrough, the search for new therapeutic targets has been the relentless pursuit of clinicians (12). Galectin-9 has displayed a variety of physiological and pathological functions, such as cell differentiation, adhesion, aggregation and cell death (40-42). Masako-Seki *et al*. have reported that Gal-9 suppresses RA at 2 different levels: by inducing cell death of pathogenic Th1 and Th17 cells via interactions between Gal-9 and TIM-3, and by inducing cell death of proliferating RA FLS (20). Unlike exogenous Gal-9, which has anti-inflammatory properties, endogenous Gal-9 is protective against apoptosis and enhances synovial fibroblast viability suggesting that its role in RA is both pathogenic and pro-inflammatory (25). Taken together, Gal-9 is a negative regulator of arthritis and can induce apoptosis of FLS which may prevent synoviocyte hyperproliferation in RA joints (43). In the present study, we found that Gal-9 expression in total lymphocytes of RA was decreased compared to HCs though the difference was not statistically significant. Therefore, further up-regulating Gal-9 is a promising strategy for the treatment of RA.

Our recent study found that Gal-9 M induces human dermal microvascular endothelial cell (HMVEC) migration and tube formation on Matrigel, as well as *in vivo* angiogenesis (23), which means that Gal-9 might be involved in the angiogenesis in RA patients. Based on this study, we tried to investigate the association between Gal-9 and VEGF, which plays a central role in physiological and pathological angiogenesis (44, 45). Our present study displayed that the expression rates of Gal-9 in $CD4^+$ T and $CD8^+$ T cells were positively correlated with plasma VEGF levels. However, no correlations between Gal-9 expression and VEGF expression in $CD3^+$T cells, $CD4^+$T cells, $CD8^+$T cells, Treg cells, and DNT cells were observed. A recent study suggests that a novel T cell population, termed angiogenic T cells (Tang), may promote the formation of new blood vessels and enhance the repair of damaged endothelium (46). It has been reported that Tang cells are implicated in multiple autoimmune diseases including RA (47, 48), systemic lupus erythematosus (49), systemic sclerosis (50) and primary Sjögren syndrome (51). Further exploration of the expression of Gal-9 and VEGF in Tang cells, as well as the relationship between the three, will help clarify the effect of Gal-9 on angiogenesis in RA patients.

One notable result was that the proportion of DNT was lower and the MFI of Gal-9 expression in DNT cells was higher in the RA-IR group than in the RA naïve group and HCs. Zhang *et al*. firstly identified and characterised the immunoregulatory function of DN T cells (52). Further studies found that DNT cells were highly potent suppressors of both $CD4^+$ and $CD8^+$ T-cell responses and could induce $CD4^+$ T cell apoptosis by Fas/FasL pathway (53, 54). However, DNT cells were significantly increased in peripheral blood in multiple autoimmune diseases such as Sjögren syndrome (55) and systemic lupus erythematosus (56), which are inconsistent with our present study results in RA. The exact role of DNT cells in RA and the relationship between DNT cells and angiogenesis as well as Gal-9 remains unclear. The decrease of DNT cells in RA patient probably means a reduced immunosuppressive effect on both $CD4^+$ and $CD8^+$ T-cell responses, leading to immune imbalance, which contributes to the occurrence of RA.

The limitation of this study is that it is a cross-sectional study. The results showed that plasma levels and intracellular expression of Gal-9 in RA patients were correlated with disease activity, pro-inflammatory cytokines, and VEGF, however, the present study is descriptive and the present data cannot definitely prove that Gal-9 is important in driving RA-related angiogenesis. In addition, it remains unclear whether the Gal-9 expression is affected by the DMARDs treatment and how it changes in patients who respond well to treatment. To clarify the changes of the Gal-9 expression before and after DMARDs treatment and the exact effects of Gal-9 on inflammation and angiogenesis in RA will be the focus of the next step.

Conclusions

Taken as a whole, our results demonstrate that Gal-9 can be used as an evaluation index for disease activity of RA. Gal-9 is probably associated with angiogenesis and is a potential treatment target in RA.

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