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High levels of soluble GPR56/ADGRG1 are associated with positive rheumatoid factor and elevated tumor necrosis factor in patients with rheumatoid arthritis

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Abbreviations: AS, ankylosing spondylitis; ECD, extracellular domain; ELISA, the enzyme-linked immunosorbent assay; GPCR, G protein-coupled receptor; GPS, GPCR proteolysis site; MMP, matrix metalloproteinase; NSAIDs, non-steroid anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor; sGPR56, soluble GPR56 protein; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; TNF-α, tumor necrosis factor-α.

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KEYWORDS
Adhesion-GPCR;
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sGPR56;
Shedding;
RA

Abstract *Background:* GPR56/ADGRG1 is a member of the adhesion-class G protein-coupled receptor (aGPCR) family important in brain development, oncogenesis and tumor metastasis. Like other aGPCRs, GPR56 is cleaved at the GPCR proteolysis site (GPS) motif into an N-terminal fragment (NTF) and a C-terminal fragment (CTF). Existence of soluble GPR56 (sGPR56) has been shown *in vitro*, however the underlying mechanism and its pathophysiologic role remains undetermined.
Objective: To assess the presence of sGPR56 in human serum using ELISA assay and compare the serum sGPR56 levels among patients of various chronic inflammatory diseases and healthy subjects.
Patients and methods: In this study, serum samples from patients with systemic lupus erythematosus (SLE) (n = 57), rheumatoid arthritis (RA) (n = 95), Sjögren's syndrome (SS) (n = 29), ankylosing spondylitis (AS) (n = 51), and normal controls (n = 81) were analyzed using sGPR56-specific ELISA.
Result: We show that serum sGPR56 levels are increased in patients of RA, but not in those with SLE, SS and AS. Intriguingly, serum sGPR56 levels in RA patients correlated with positive rheumatoid factor, a marker of bone erosion and poor outcome. In addition, an elevated sGPR56 level is also noted in RA patients with higher tumor necrosis factor level.
Conclusion: we conclude that sGPR56 is present *in vivo* and sGPR56 level is elevated in certain chronic inflammatory diseases such as RA. Hence, sGPR56 might be considered a potential biomarker for RA disease progression.

Introduction

G protein-coupled receptor 56 (GPR56/ADGRG1) is a member of the adhesion-class G protein-coupled receptor (aGPCR) family, which is hallmarked by a seven transmembrane (7TM) core structure containing an extended extracellular domain (ECD).[1,2] GPR56 is highly expressed in various tissues/organs including thyroid, brain, and heart.[3] Increasing evidences have pointed to a critical role for GPR56 in many human physiologic and pathological processes. For example, genome-wide screening and analysis identified a direct link between GPR56 null mutations and a human brain cortical malformation called bilateral frontal parietal polymicrogyria (BFPP).[4]

In oncogenesis and tumor metastasis, both positive and negative roles have been reported for GPR56. Compared with normal adult brain tissue, higher expression levels of GPR56 were detected in glioblastoma/astrocytoma tumors.[5] Similarly, significant GPR56 expression was detected in acute myeloid leukemia (AML) cells with high ecotropic viral integration site-1 ($EVI1^{high}$) expression.[6] Likewise, the strong GPR56 expression detected in the parental HeLa cells was significantly reduced in a nontransformed HeLaHF subclone that has lost the capacity of anchorage-independent growth and tumorigenicity.[7] In contrast, highly metastatic human melanoma cell lines expressed little GPR56 in comparison to the weaker metastatic counterparts.[8] Forced overexpression of GPR56 in metastatic melanoma cells suppressed tumor growth and metastasis. In addition, down-regulated expression of GPR56 was also noted in human pancreatic cancer cell. These contradictory results suggest a cell type- and/or context-specific function for GPR56 in tumor development.[9]

In the immune system, GPR56 is restrictedly expressed in NK cells, cytotoxic effector/memory T and γδT cells.[10,11] In fact, GPR56 was identified recently by us as a novel maturation marker and inhibitory receptor of human NK cells. The expression of GPR56 in conjunction with CD81 reduces the cytotoxicity and cytokine production of NK cells.[12] In hematopoiesis, mouse GPR56 is critical for the maintenance of hematopoietic stem cells (HSCs) in the bone marrow and the *in vivo* repopulating ability of the HSCs.[6] A role of GPR56 in the formation of hematopoietic clusters during endothelial to hematopoietic cell transition was reported recently.[13]

As with most aGPCRs, GPR56 undergoes a unique proteolytic reaction at the GPCR proteolysis site (GPS) motif and is split into an N-terminal fragment (NTF, the ECD-subunit) and a C-terminal fragment (CTF, the 7TM-subunit).[2] The NTF is usually associated non-covalently with the respective CTF on the cell membrane. Nevertheless, several recent studies reported the presence of soluble GPR56 (sGPR56) in the conditioned medium (CM) of transfected cells, suggesting constitutive shedding of GPR56 *in vitro*.[14,15] However, the underlying mechanism and physiopathological role of GPR56 shedding are still elusive and the production of sGPR56 *in vivo* remains undetermined. More interestingly, recent advances in understanding the activation of aGPCRs reveal a novel mechanism whereby the exposure of a tethered agonistic peptide on the CTF, upon the shedding or removal of the NTF, presumably binds and activates the 7TM region.[16,17] Furthermore, aGPCR activation can be achieved directly by the addition of exogenous agonistic peptides, suggesting that the tethered peptide is a true receptor agonist. This novel "tethered agonism" of aGPCR activation implies that ectodomain shedding is likely an important regulatory mechanism for aGPCR activation.[18]

In this paper we assess the presence of sGPR56 in human serum using a previously described GPR56-specific ELISA assay[19] and compare the serum sGPR56 levels among patients of various chronic inflammatory diseases and healthy subjects. Our results show that the serum sGPR56 level in rheumatoid arthritis (RA) patients is significantly elevated. Interestingly, elevated serum sGPR56 levels of RA patients

are correlated with the presence of rheumatoid factor (RF), an important diagnostic factor[20] and bone erosion predictor in RA patients.[21] Moreover, we found that patients with higher levels of serum TNF-α ($\geq$8.1 pg/ml) have more elevated serum sGPR56 levels. Hence, we conclude that the serum sGPR56 level is significantly up-regulated in RA patients, particularly in those with markers of disease progression and poor prognosis. The elevated serum sGPR56 level therefore can be considered as a potential marker of cell activation status in certain chronic inflammatory diseases such as RA.

Materials and methods

Patients

This study was approved by the Chang Gung Memorial Hospital Ethics Committee (CGMH IRB No: 97-1457B and 102-4095C) and all procedures were performed according to the guideline set by the Committee. The number of patients recruited for this study include: systemic lupus erythematosus (SLE), 57; rheumatoid arthritis (RA), 95; Sjögren's syndrome (SS), 29, and ankylosing spondylitis (AS), 51. A total of eighty-one healthy volunteers are also included. All participants were recruited from the outpatient clinics of Chang Gung Memorial Hospital (Taiwan). Patients were screened to meet the criteria set by American College of Rheumatology for the diagnosis of SLE and RA, American-European Consensus criteria for Sjögren's syndrome, and the modified New York criteria for AS.

Patients were evaluated by specific validated disease activity indexes such as Systemic Lupus Erythematosus Activity Index (SLEDAI) (median: 2, interquartile range: 2—6), DAS28 (median: 3.89, interquartile range: 3.13—4.87), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (median: 3.2, interquartile range: 2.15—4.9). Almost all SLE patients were treated with hydroxychloroquine and prednisolone (5—25 mg/day) and twelve of them received azathioprine (Imuran) at inclusion. Nearly all RA patients were already receiving at least one disease modifying antirheumatic drug and corticosteroids at inclusion (50 patients with MTX 5 mg—15 mg/week, 5 patients with leflunomide 10—20 mg/day, 38 patients with sulfasalazine 0.5—2 gm/day, 54 patients with hydroxychloroquine 200—400 mg/day and 56 patients with prednisolone 2.5—15 mg/day) and part of them also receiving biologic agents (5 patients with tumor necrosis factor blockers). Almost all AS patients were treated with non-steroid anti-inflammatory drugs (NSAIDs) and some of them were treated with sulfasalazine (23 patients with sulfasalazine 0.5—2 gm/day) and tumor necrosis factor blockers (3 patients) at inclusion. Patients and healthy volunteers were informed about the nature of the experimental procedures and all have given informed consent. Whole blood ($\sim$10 ml) were taken from patients, serum was isolated and stored at -80 °C until use.

Soluble GPR56 protein purification by the Ab-conjugated affinity column chromatography

A GPR56-specific CG2 mAb-conjugated affinity column was prepared as described previously.[19] In brief, sGPR56 protein was purified by passing the serum-free CM of stable A375 melanoma cells over-expressing GPR56-ECD protein through the affinity column, followed by extensive washes with 1X PBS. Purified sGPR56 protein was quantified by BCA protein assay (Thermo Fisher Scientific Inc. Rockford, IL) and used as a standard for the GPR56-specific sandwich ELISA assay.

GPR56 sandwich ELISA assay

The quantitative detection of sGPR56 protein was done using an in-house established sandwich ELISA assay described previously.[19] Briefly, CG2 mAb (3 μg/ml in PBS, 100 μl/well) was coated onto Costar EIA 96-well plates at room temperature (RT) overnight. After washes with washing buffer (0.1% Tween-20 in PBS), the wells were blocked with blocking buffer (2% BSA in PBS) at RT for 1 h. Serum samples (100 μl/well) were added to wells. Plates were incubated at RT for at least 2 h or at 4 °C overnight. Afterward, wells were washed and incubated with the detection antibody (CG3-biotin, 0.25 μg/ml) at RT for 1 h. Following extensive washes, streptavidin-HRP (1:1000) was added and incubated for 30 min. Wells were washed before adding TMB (tetramethylbenzidine) substrate solution for color development. Results were immediately detected by ELISA reader at OD_{450}.

Statistical analysis

Statistical analysis was done using GraphPad Prism 6 software. The ELISA data of serum samples of patients did not fit a Gaussian distribution despite attempts at log transformation. Non-parametric Kruskal—Wallis test was therefore used to analyze these data to determine if there was significant variation in the medians of the groups analyzed. If 95% significance was achieved, Dunn's multiple comparison post-test was then used to compare the assay results of one group with another. Correlations between sGPR56, age, gender, rheumatoid factor (RF) and tumor necrosis factor (TNF) were analyzed by Mann—Whitney *U* test. p-Values are as follows: $^{*}p < 0.05$.

Results and discussion

Previous studies have demonstrated the ectodomain shedding of human aGPCR GPR56/ADGRG1 *in vitro*. Indeed, GPR56 shedding was shown to occur constitutively and was further increased in activated melanoma cells that express endogenous GPR56.[14,19,22] However, the existence of sGPR56 *in vivo* is still elusive. In an attempt to evaluate the production and functional implication of sGPR56 *in vivo*, we first assessed the presence of sGPR56 in serum of healthy subjects using the GPR56-specific ELISA assay established recently.[19] A total of 81 healthy volunteers (9 males, 72 females) of mean age of 53 $\pm$ 13.3 (range from 20 to 80) years were enrolled in this study. The median values of serum sGPR56 levels are shown in Table 1. The potential influence of gender and age on serum sGPR56 level was assessed. As shown in Fig. 1, no significant differences between males and females were observed, nevertheless sGPR56 levels are significantly higher in the elder group

Table 1 Clinical characteristics and sGPR56 levels of normal donors and subjects with SLE, RA, SS and AS.

Variable	Normal donor (N = 81)	SLE (N = 57)	RA (N = 95)	SS (N = 29)	AS (N = 51)
Age (y/o)	52.94 ± 13.30	44.19 ± 12.53	58.9 ± 13.46	54.28 ± 14.23	42.62 ± 13.11
Male sex no. (%)	9 (11.11)	8 (14.03)	17 (17.89)	6 (20.69)	39 (76.47)
Serum sGPR56 (pg/ml) median (IQR 25th–75th)	12.95 (5.77–33.62)	11.13 (5.199–23.32)	21,95 (10.28–47.52)	11.95 (6.05–28.58)	14.20 (4.53–25.03)

Data are expressed as means ± SD and median (Interquartile range, 25th–75th).
SLE, systemic lupus erythematosus; RA, Rheumatoid arthritis; SS, Sjögren's syndrome; AS, Ankylosing spondylitis.

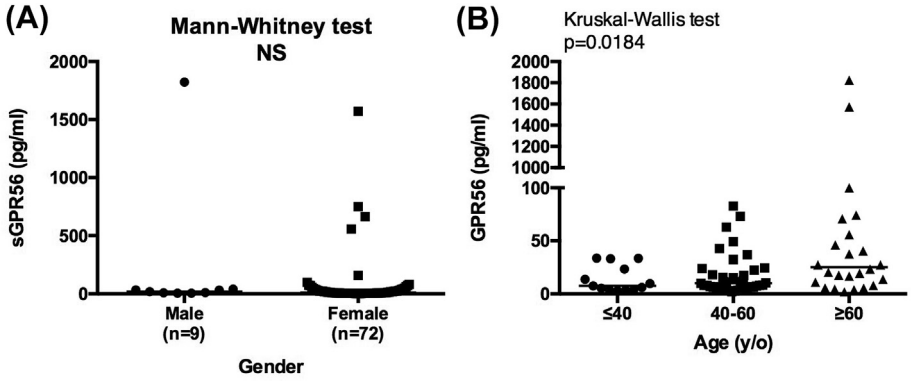

Figure 1. Comparison of the serum sGPR56 levels of healthy individuals in different age and gender groups. (A) Shown is the value of serum sGPR56 level in the male and female groups. Mann–Whitney test across both two groups shows no significant difference. (B) The value of serum sGPR56 level in the young (≤40 y/o), middle (40–60 y/o) and older (≥60 y/o) age groups. Kruskal–Wallis test across all three age groups shows $p = 0.0184$. Only significant Dunn's multiple comparisons post-test p values between groups are shown. (*$p < 0.05$).

(≥60 y/o, median 25.315 pg/ml) compared with the young group (≤40 y/o, median 7.624 pg/ml). These results confirm that sGPR56 is normally present in the serum of healthy individuals and its level seems to increase in aged individuals.

In the human immune system, GPR56 expression is restricted to cytotoxic T cell subsets and NK cells.[10,11] Indeed, Della Chiesa et al. have identified GPR56 as a cell surface marker of the $CD56^{dull}CD16^{+}$ NK cells, which can be recruited from blood to inflamed tissues.[11] In addition, we have recently shown that GPR56 is an inhibitory receptor of human NK cell that negatively modulates NK cell cytotoxicity and cytokine production.[12] Interestingly, qualitative and quantitative variations of cytotoxic T cell and NK cell subsets have been reported in many human autoimmune/autoinflammatory diseases, such as SLE, SS, RA and spondyloarthropathies, which are characterized by localized chronic inflammation and leukocyte recruitment and activation.[23,24] Furthermore, our recent study showed that the ectodomain cleavage of GPR56 is likely mediated in part by the matrix metalloproteinases (MMPs), which are often enriched/activated in the inflamed tissues of the autoimmune/autoinflammatory diseases mentioned above.[14,25–27] To assess the presence of sGPR56 in the serum of patients with autoimmune/autoinflammatory diseases and to determine whether the serum sGPR56 level is relevant to the disease progression/characteristics, we analyzed the sera of SLE, RA, SS, and AS patients by the same ELISA assay as described for the 81 normal subjects (Table 1 and Fig. 2A). The results indicated that RA patients had significantly higher sGPR56 concentrations than the normal subjects. Conversely, serum sGPR56 levels of SLE, SS, and AS patients were not different from those of the normal control individuals.

To determine if there is a correlation between the serum sGPR56 level and disease activity of RA, we divided RA patients into the low- (DAS < 3.2), moderate- ($3.2 \leq$ DAS ≤ 5.1), and high-disease activity groups (DAS > 5.1). No significant difference of serum sGPR56 level was noted in the three disease activity groups (Table 2 and Fig. 2B). We further assessed the correlation between serum sGPR56 level and certain specific biomarkers of RA. Rheumatoid factor (RF), an autoantibody against the Fc portion of IgG, is widely used as an important classification marker of RA.[28] Positive RF is usually associated with bone erosion, extra-articular manifestation and poor outcome.[29,30] Interestingly, higher serum sGPR56 levels were noted in the RF (+) RA patient group in comparison with the RF (−) RA patient group (Fig. 3A).

TNF-α is a proinflammatory cytokine critically involved in the pathogenesis of RA.[31] Importantly, anti-TNF therapy achieves dramatic improvement of clinical symptoms. NK cells, which express GPR56, have been suggested to play a disease-promoting role in RA as activated NK cells were more abundant in the synovium of RA patients.[32,33] These NK cells have the better ability to induce DC differentiation

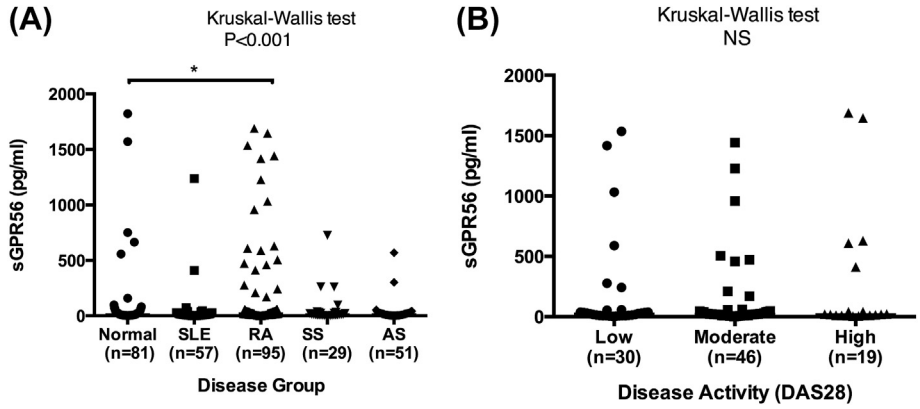

Figure 2. (A) Comparison of serum sGPR56 levels in different subject groups, including normal healthy volunteers and patients with SLE, RA, SS, and AS. Data are shown as dot plots. Kruskal–Wallis test across all five groups shows $p < 0.001$. Only significant Dunn's multiple comparisons post-test p values between groups are shown. (B) Comparison of serum sGPR56 levels in RA patients divided into the low-disease, moderate-disease, and high-disease activity groups as classified by DAS28. Data are shown as dot plots. A Kruskal–Wallis test across all three groups shows no significant difference. (*$p < 0.05$).

Table 2 Clinical characteristics and the sGPR56 levels of RA patients with low, moderate and high disease activity.

	Low disease activity (N = 30)	Moderate disease activity (N = 46)	High disease activity (N = 19)
	DAS28 < 3.2	3.2 ≤ DAS28 ≤ 5.1	DAS28 > 5.1
Age (y/o)	55.57 ± 15.35	61 ± 13.63	59.11 ± 9.09
Male sex n (%)	6 (20)	7 (15.22)	4 (21.05)
Disease duration ≥ 10 yrs n (%)	10 (33.33)	18 (39.13)	7 (36.84)
ESR	14.37 ± 10.53	23.35 ± 15.62	31.42 ± 25.52
RF positive n (%)	23 (76.67)	33 (71.73)	13 (68.42)
Treatment of methotrexate n (%)	16 (53.33)	24 (52.17)	10 (52.63)
Treatment of DMARDs n (%)	26 (86.67)	43 (93.48)	18 (94.74)
Treatment of steroids n (%)	19 (63.33)	27 (47.37)	11 (57.89)
Treatment of NSAIDs n (%)	18 (60)	30 (65.22)	15 (78.95)
Treatment of TNF inhibitors n (%)	3 (10)	2 (4.35)	0 (0)
Serum sGPR56 (pg/ml) median (IQR 25th–75th)	26.17 (9.79–55.04)	22.03 (10.11–46.58)	19.86 (11.59–412.5)

Data are expressed as means ± SD, n (%), and median (Interquartile range, 25th–75th).
DAS, 28-joint count Disease Activity Score; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; DMARDs, disease-modifying anti-rheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; TNF, tumor necrosis factor.

and maturation from monocytes.[34] Furthermore, the crosstalk between NK cells and myeloid DCs, in part through communication by various cytokines and chemokines, lead to NK cell activation in the inflamed joints.[34] Indeed, it is well known that activated NK cells secreted a diverse array of inflammatory cytokines including TNF-α.[35] Hence, we further investigated the correlation of TNF-α with serum sGPR56 levels in RA patients. We found that the serum sGPR56 level in the group of RA patients with elevated TNF-α levels (≥8.1 pg/ml) is higher than the patients with normal TNF-α levels (<8.1 pg/ml) (Fig. 3B). Therefore, we conclude there is a positive relationship between the TNF-α and sGPR56 levels in the sera of RA patients.

Our present report is the first systematic analysis of the presence of sGPR56 *in vivo* and demonstrates a positive link between the serum sGPR56 levels and specific disease markers (RF and TNF-α) in RA patients. In sum, the serum sGPR56 level is shown as a potential novel marker associated with enhanced cell activation status in patients with RA. Despite these interesting results, several questions remain unanswered. First, the cellular source of elevated sGPR56 of RA patients is not defined. While GPR56 is expressed in NK and certain T cell subsets that may be enriched and activated in the joints of RA patients, other cell types such as neurons and muscle cells as well as HSCs also express GPR56. Identification of the major cell type that shed sGPR56 will help dissect the significance of elevated serum sGPR56 in RA patients. Another intriguing question is why the serum sGPR56 level is not elevated in other autoimmune/autoinflammatory diseases such as SLE, SS and AS.

Finally, the mechanism(s) of sGPR56 production in RA patients is not delineated. Due to the novel non-covalent NTF-CTF complex of GPR56, there are two most possible scenarios for GPR56 shedding. One is the dissociation of NTF from the membrane-bound CTF. It has been shown

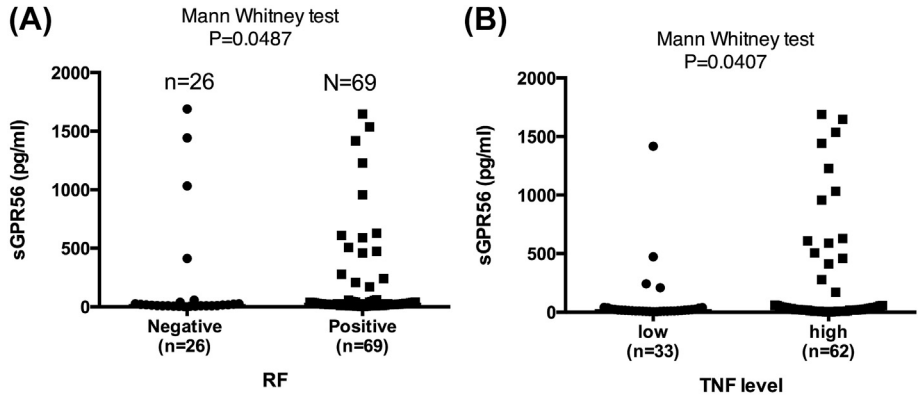

Figure 3. Investigation of the correlation of sGPR56, RF, and TNF-α levels in RA patients. (A) Shown is the value of serum sGPR56 level in the RF (+) and RF (−) groups of RA patients. (B) Shown is the value of serum sGPR56 level in the low TNF-α (<8.1 pg/ml) and high TNF-α (≥8.1 pg/ml) groups of RA patients. The difference between groups was compared by Mann−Whitney test.

recently that aGPCRs are able to be activated by NTF dissociation and the subsequent exposure of a N-terminal tethered peptide located at the CTF.[16,17] Signals for NTF dissociation may include the binding of specific matricellular ligand(s) and/or mechanical stimuli such as cellular stretch, fluid shear stress and membrane viscosity.[36,37] The other potential NTF shedding mechanism is mediated by cellular protease(s) such as proprotein convertase(s) and/or MMPs. Indeed, several aGPCRs have been shown to be processed additionally by MMPs, generating multiple ECD fragments.[38−40] We have shown previously that MMPs might be involved in GPR56 shedding as it was attenuated by a pan-MMP inhibitor.[14] As TNF-α is a well-known cytokine able to induce the expression and activity of various MMPs,[41,42] it is tempting to think that the elevated serum sGPR56 levels in RA patients are due to the TNF-induced MMPs. We have shown recently that GPR56 is an inhibitory receptor on human NK cell and its expression/activity can be modulated by receptor shedding and internalization. The detection of higher serum sGPR56 levels may suggest the presence of an activated NK cell population in the RA patients. Alternatively, the production of sGPR56 might simply reflect a higher overall activities of MMPs in the RA patients. As GPR56 was also shown to be involved in the maintenance of bone marrow HSCs, the elevated sGPR56 levels may contribute to accelerated aging and defective proliferative capacity of HSCs in RA.[43] Determining the exact GPR56 shedding mechanism in RA patients will be of importance to understand more about the potential role of GPR56 in the pathogenesis of RA.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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