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IL-10$^+$ T follicular regulatory cells are associated with the pathogenesis of IgG4-related disease

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ABSTRACT

IgG4-related disease (IgG4-RD) is a chronic fibroinflammatory disease characterized by elevation of serum IgG4 level as well as infiltration of IgG4$^+$ plasma cells in various affected organs. The etiology of IgG4-RD is still not fully understood. Since IgG4-RD is more prevalent in the elderly, aging in itself is considered to be an important risk factor of IgG4-RD. However, the relationship between the pathogenesis of IgG4-RD and immunosenescence remains unknown. To clarify age-related features underlying IgG4-RD, we focused on T follicular regulatory (Tfr) cells, which share forkhead box P3 with regulatory T cells, since the percentage of Tfr cells is known to depend on age. Studies of blood specimens from patients with IgG4-RD and from healthy volunteers demonstrated a marked elevation of circulating Tfr (cTfr) cells in patients with IgG4-RD. Moreover, the percentage of cTfr cells was significantly correlated with various clinical parameters including the level of serum IgG4 and the number of involved organs in IgG4-RD patients. The percentages of tonsillar and blood Tfr cells were increased with aging in healthy volunteers, whereas the suppressive effect of cTfr cells on B cell function in elderly subjects was impaired in comparison with that in young subjects due to a defect in the production of a regulatory cytokine, IL-10. Given that the number of IL-10-producing cTfr cells in IgG4-RD patients was markedly increased compared with that in healthy elderly subjects, these findings suggest that an abnormal aging process of Tfr cells may be related to the pathogenesis of IgG4-RD.

1. Introduction

IgG4-related disease (IgG4-RD) is characterized by elevation of serum IgG4 level and persistent fibroinflammation in an involved organ or involved organs such as the lacrimal glands, salivary glands, lymph nodes, pancreas, retroperitoneum, and lungs [1]. The main histopathological findings of the involved organs in IgG4-RD are marked infiltration of B cells and plasma cells producing IgG4, storiform fibrosis formed by spindle cells resembling fibroblasts, and obliterative phlebitis [1]. Additional pathological features of IgG4-RD are a dense lymphoplasmacytic infiltrate and the formation of ectopic germinal centers (GCs) where lymphoplasmacytes promote the production of IgG4 at an unusually high level. In general, systemic administration of glucocorticoids is the first line of therapy for IgG4-RD, though recent studies have shown that biologics such as rituximab, which is an anti-CD20 antibody that depletes B cells, and abatacept, which is an anti-CTLA-4 antibody that inactivates T cells, are quite effective even in recurrent cases as well as cases that are refractory to steroid therapy [2,3]. In addition, a recent study showed that abundant T follicular helper (Tfh) cells are localized in lesions of IgG4-RD [4]. These findings

Abbreviations: BCL6, B-cell lymphoma 6; cTfh, circulating Tfh; cTfr, circulating Tfr; cTreg, circulating Treg; Foxp3, forkhead box P3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, germinal center; GITR, glucocorticoid-induced TNFR-related protein; ICOS, inducible T-cell co-stimulator; IgG4-DS, IgG4-related dacryoadenitis and sialadenitis; IgG4-RD, IgG4-related disease; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death 1; sIL-2R, soluble IL-2 receptor; SMG, submandibular gland; Tfh, T follicular helper; Tfr, T follicular regulatory; Treg, regulatory T

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suggest that uncontrollable GC responses underlie the pathogenesis of IgG4-RD.

In collaboration with Tfh cells, T follicular regulatory (Tfr) cells, a specialized $CD4^+$ T cell subset, participate in the control of GC formation and class-switch recombination of B cells [5–7]. Tfr cells present CXCR5, which is also shared by B cells and Tfh cells. Moreover, they are regulated by B-cell lymphoma 6 (BCL6), programmed cell death 1 (PD-1), and inducible T-cell co-stimulator (ICOS) as well as forkhead box P3 (Foxp3), which is shared by regulatory T (Treg) cells [6,8]. To exert GC responses, Tfr cells produce IL-10 and TGF-β for the direct regulation of B cells and Tfh cells. Of note, IL-10 acts as a critical cytokine not only for a suppressive effect against immune cells but also for the class-switch recombination of IgG4 and for the promotion of GC responses [7,9,10]. However, the pathological significance of Tfr cells in IgG4-RD is not fully understood.

It is well recognized that circulating Tfh (cTfh) cells in elderly adults have a lower capacity to help B cells than those in young adults with accompanying increased expression of PD-1 and decreased expression of ICOS [11]. Other studies have further shown an increased level of $Foxp3^+$ Treg cells in aged humans and mice [12,13]. Therefore, the immune system undergoes striking aging-related changes, which eventually proceed to a state of so-called immunosenescence [14]. In aged individuals, immunosenescence preferentially leads to inability to protect against infections and also predisposes the individuals to autoimmune and allergic disorders and even to malignancies [14]. Since one of the common clinical characteristics of IgG4-RD is a higher prevalence in the elderly, a possible relationship between immunosenescence and the pathogenesis of IgG4-RD has been suggested. Nonetheless, aging-related physiological changes of Tfr cells in IgG4-RD remain elusive.

In the present study, we performed experiments using clinical specimens to address the question of whether Tfr cells have a role in the pathogenesis of IgG4-RD. The results showed that the numbers of Tfr cells in blood and submandibular glands (SMGs) from patients with IgG4-RD were significantly increased compared with those in peripheral blood and tonsils from healthy volunteers. The percentage of Tfr cells was positively correlated with clinical parameters including serum level of IgG4 and number of involved organs in IgG4-RD patients. We also found that, although tonsillar and circulating Tfr (cTfr) cells increased with aging, suppressive functions of Tfr cells from elderly subjects were impaired due to a decrease in their IL-10 production compared with the suppressive functions of Tfr cells from young subjects. Interestingly, the number of IL-10-producing cTfr cells in IgG4-RD patients was increased compared with that in healthy elderly subjects. Collectively, the findings provide a novel insight into the role of Tfr cells in the pathogenesis of an aging-related disease, IgG4-RD.

2. Materials and methods

2.1. Study populations and clinical specimens

The characteristics of patients with IgG4-RD (n = 49) and age-matched healthy volunteers (for Figs. 1 and 2, n = 53) are summarized in Supplementary Table S2. Diagnosis of IgG4-RD was performed according to the 2011 comprehensive IgG4-RD diagnostic criteria [15]. Blood specimens were collected from patients with IgG4-RD and analyzed prior to the entrance to treatment protocols as previously described [16]. Tissues of the SMGs and palatine tonsils were obtained from 21 patients with IgG4-RD (age of donors ranging from 10 to 66 years old) and from 41 patients with tonsillar hypertrophy (n = 14, age of donors ranging from 3 to 46 years old) or recurrent tonsillitis (n = 27, age of donors ranging from 10 to 66 years old) for diagnosis or treatment at Sapporo Medical University Hospital. To examine Tfr cells and Treg cells in different age groups of healthy volunteers, blood specimens were obtained from 53 subjects (for Figs. 3 and 4, age of healthy donors ranging from 4 to 89 years old). None of the healthy volunteers had abnormal physical or chest X-ray findings, and results of all blood tests were normal. Written informed consent was obtained in all cases according to the Declaration of Helsinki. All of the protocols were approved by the institutional review boards of Sapporo Medical University Hospital.

2.2. Reagents

Anti-human monoclonal antibodies (mAbs) including anti-CD3-APC (UCHT1), anti-CD4-APC-Cy7 (RPA-T4), anti-PD-1-PE (EH12.1), anti-PD-1-PE-Cy7 (EH12.1), anti-PD-1-BV421 (EH12.1), anti-CXCR5-PerCP-Cy5.5 (RF8B2), anti-CD25-FITC (M-A251), anti-CD127-PE-Cy7 (HIL-7R-M21), anti-CD127-BV421 (HIL-7R-M21), and anti-ICOS-BV421 (DX29) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). An anti-Foxp3-eFluor 450 mAb (PCH101) was obtained from eBioscience (San Diego, CA, USA). Anti-CD3 (OKT3) and anti-CD28 (15E8) mAbs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). An anti-IL-10 (23738) mAb was obtained from R&D Systems (Minneapolis, MN, USA).

2.3. Flow cytometric analysis and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from blood specimens by centrifugation over a discontinuous density gradient (Lympholyte-H, Cedarlane; Burlington, ON, Canada). Tissue samples of tonsils and SMGs were mechanically disrupted into single lymphocyte suspensions for flow cytometry and cell sorting. Cell staining using cell surface markers was performed as previously described [4]. Intracellular staining for Foxp3 was performed with Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific; Waltham, MA, USA) as described in the protocol of the manufacturer. Samples were then analyzed using FACSCanto II (BD Biosciences). All data were analyzed using FACSDiva software (BD Biosciences) and FlowJo software (BD Biosciences). To isolate human Tfh cells and Tfr cells from tonsils or PBMCs, $CD4^+$ T cells were enriched by using a MagniSort human CD4 T cell enrichment kit (Thermo Fisher Scientific). $CD4^+$ T cells were stained with surface markers (CD3, CD4, CD25, CD127, CXCR5, and PD-1) for their detection. $CD3^+CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ and $CD3^+CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ cells were defined as Tfh and Tfr cells, respectively [17], and were sorted with FACSAria II (BD Biosciences). The purity of FACS-sorted cells was more than 95% after validation with reanalysis using FACSCanto II. B cells were isolated from tonsils or PBMCs using a MagniSort human B cell enrichment kit (Thermo Fisher Scientific). The purity of B cells was more than 90%.

2.4. T cell and B cell coculture assays

Sorted Tfh cells ($CD3^+CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ cells) in the presence or absence of Tfr cells ($CD3^+CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ cells) from tonsils were plated with autologous or allogeneic blood B cells ($CD3^-CD19^+$ cells) from healthy volunteers in a 1:1:1 ratio (1×10^5 cells each) and co-cultured for 10 days in AIM-V serum-free medium (Thermo Fisher Scientific) supplied with 10 μg/ml anti-CD3 Ab and 10 μg/ml anti-CD28 Ab (Miltenyi Biotec). Then culture supernatants were analyzed in triplicate by ELISA. For a suppression assay, 5 ug/ml anti-IL-10 mAb was added to the coculture medium.

2.5. Measurement of immunoglobulins

Concentrations of IgG in cell-free culture supernatants were analyzed in triplicate with ELISAs as described in the manufacturer's protocol (Bethyl Laboratories; Montgomery, TX, USA). The detection limit of IgG is 7.8 ng/ml.

2.6. RNA isolation and quantitative real-time PCR

For quantitative real-time PCR analysis, total RNA extracted by TRIzol reagent (Thermo Fisher Scientific) was reverse-transcribed using a MultiScribe MuLV (Thermo Fisher Scientific). Quantitative real-time PCR was performed with a LightCycler real-time PCR system (F. Hoffmann-La Roche; Basel, Switzerland) using TaqMan gene expression assay (Thermo Fisher Scientific) and SYBR Green (Takara Bio Inc.; Shiga, Japan). For the TaqMan-based detection system, the amount of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was used to standardize the amount of *IL10* (Hs00174086) and *TGFB1* (Hs00171257) mRNAs. Sequences of the primer set for SYBR Green-based detection were designed and used in this study (Supplementary Table S3). The ΔΔCT method was used to calculate the relative mRNA expression of triplicate specimens.

2.7. IL-10 secretion assay

To detect IL-10 production, lymphocytes from tonsils and blood specimens were assessed using an IL-10 secretion assay kit (Miltenyi Biotec) following the manufacturer's protocol. IL-10-producing cells were analyzed by FACSCanto II.

2.8. Statistical analysis

All data are shown as mean ± SD. Significant differences between any two groups were determined by using the Mann-Whitney *U* test. Multiple group comparisons were analyzed with the Kruskal-Wallis test. Correlations were determined by Spearman's correlation coefficients. Probability values less than 0.05 were considered significant.

3. Results

3.1. Blood and tissue Tfr cells are increased in IgG4-RD

Previous studies have shown that activated Tfh cells were increased in peripheral blood from patients with IgG4-RD and that their percentage was significantly correlated with clinical parameters [4,18]. To address the role of Tfr cells in IgG4-RD, we examined cTfr cells and cTfh cells as well as other immune cells in blood from patients with IgG4-RD. Initially, we analyzed $CD3^+CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$ cells and $CD3^+CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$ cells as Tfr cells ($CD25^+CD127^-$ Tfr cells) and Tfh cells ($CD25^-CD127^+$ Tfh cells), respectively [17]. As shown in Fig. 1A–C and Supplementary Table S4, the percentages and numbers of $CD25^+CD127^-$ cTfr cells and $CD25^-CD127^+$ cTfh cells were significantly increased in blood from patients with IgG4-RD compared with those in blood from age-matched healthy volunteers. To know the specificity of such cell surface markers to detect Tfr cells, we also analyzed $CD3^+CD4^+CD25^+CXCR5^+PD\text{-}1^+Foxp3^+$ cells ($Foxp3^+$ cTfr cells) and examined the correlation between the absolute numbers of $CD25^+CD127^-$ cTfr cells and $Foxp3^+$ cTfr cells. As expected, we found a clear correlation ($r = 0.8061$ and $p = 0.0072$) between the two phenotypes of cTfr cells (Fig. 1D). Indeed, the percentage and number of $Foxp3^+$ cTfr cells were significantly increased in blood from patients with IgG4-RD (Fig. 1E and Supplementary Table S5). In addition, we found that the percentages of $CD25^+CD127^-$ Tfr cells and $Foxp3^+$ Tfr cells were significantly increased in affected tissues of SMGs from patients with IgG4-related dacryoadenitis and sialadenitis (IgG4-DS) compared with those in tonsils from patients with tonsillar hypertrophy as controls (Fig. 1F). Taken together, the results showed that Tfr cells were markedly increased in both peripheral blood and involved tissues in IgG4-RD patients.

3.2. Circulating Tfr cells are positively correlated with clinical parameters in IgG4-RD

We next studied the relationships between the percentage of cTfr cells and various clinical parameters in IgG4-RD. Interestingly, the percentage of cTfr cells was significantly correlated with serum levels of IgG4 ($r = 0.5008$ and $p = 0.0176$) and IgG4/IgG ($r = 0.5246$ and $p = 0.0122$) but not with total IgG ($r = 0.3597$ and $p = 0.1001$), IgM ($r = -0.0745$ and $p = 0.7549$), IgA ($r = 0.0451$ and $p = 0.8502$), or IgE ($r = -0.0677$ and $p = 0.7768$) (Fig. 2A). We further analyzed the relationships of the percentage of cTfr cells with the number of involved organs, serum level of soluble IL-2 receptor (sIL-2R) as an activation marker of T cells, and serum levels of complement components (C3 and C4) that are involved in autoimmune diseases such as systemic lupus erythematosus [19]. The percentage of cTfr cells was also significantly correlated with the number of organs involved ($r = 0.4510$ and $p = 0.0459$) and with the serum level of soluble IL-2 receptor ($r = 0.6130$ and $p = 0.0031$), but we failed to find any significant correlation of cTfr cells with C3 ($r = -0.1997$ and $p = 0.4123$) and C4 ($r = -0.2491$ and $p = 0.3037$) (Fig. 2B and Supplementary Fig. S1). Therefore, these findings suggest that the elevated level of cTfr cells is clearly related to the levels of factors defining the clinical status of IgG4-RD.

It is well known that Tfr cells generally have a high capacity to produce IL-10 [6] and that the expression level of IL-10 in affected tissues as well as the serum level of IL-10 are profoundly increased in IgG4-RD [7,20,21]. Moreover, accumulating evidence suggests that IL-10 facilitates class-switch recombination of IgG4 in B cells [9,10]. Based on such reported evidence about IL-10, we further investigated the functional capacity of cTfr cells to produce IL-10 in IgG4-RD. Although the IL-10-producing capacity of cTfr cells in IgG4-RD patients was comparable to that of cTfr cells in age-matched healthy volunteers, the absolute number of IL-10-producing Tfr cells in IgG4-RD patients was significantly increased compared with that in age-matched healthy volunteers (Fig. 2C). These results suggest that IL-10 produced by Tfr cells, which are increased in blood of patients with IgG4-RD, possibly influences IgG4-specific class-switch recombination of B cells in the involved lesions.

3.3. Tfr cells increase with aging

Our findings indicated that the increased level of Tfr cells, which could normally produce IL-10, might be related to the pathogenesis of IgG4-RD. IgG4-RD is well recognized as a prevalent disorder that frequently occurs in elderly persons. Therefore, we were interested in an age-associated natural course of Tfr cells. To know this, characteristic alterations of $Foxp3^+$ Tfr cells ($CD3^+CD4^+CD25^+CXCR5^+PD\text{-}1^+Foxp3^+$ cells) and Treg cells ($CD3^+CD4^+CD25^+Foxp3^+$ cells) were investigated in peripheral blood of healthy volunteers of various ages. It was found that the percentages of cTfr cells and cTreg cells increased with advance of age, especially at ages with a high prevalence of IgG4-RD (Fig. 3A and B). In accordance with the evidence that Tfr cells originate from Treg cells, the percentage of cTfr cells is likely to be in parallel with the percentage of cTreg cells throughout life [22,23]. We next examined Tfr cells and Treg cells residing in tonsils from patients who underwent tonsillectomy due to simple hypertrophy. In accordance with the results obtained from blood specimens, it was found that Tfr cells and Treg cells in tonsils increased with aging (Fig. 3C and D). Collectively, the results suggest that Tfr cells tend to increase in blood and peripheral tissues of aged individuals as a natural property. In contrast, the percentage of activated Tfh cells in tonsils decreased with advance of age (Supplementary Fig. S2), while total Tfh cells in blood have been shown to gradually increase with aging [24].

3.4. Suppressive function of Tfr cells is impaired in elderly subjects

We further investigated a possible functional alteration of Tfr cells

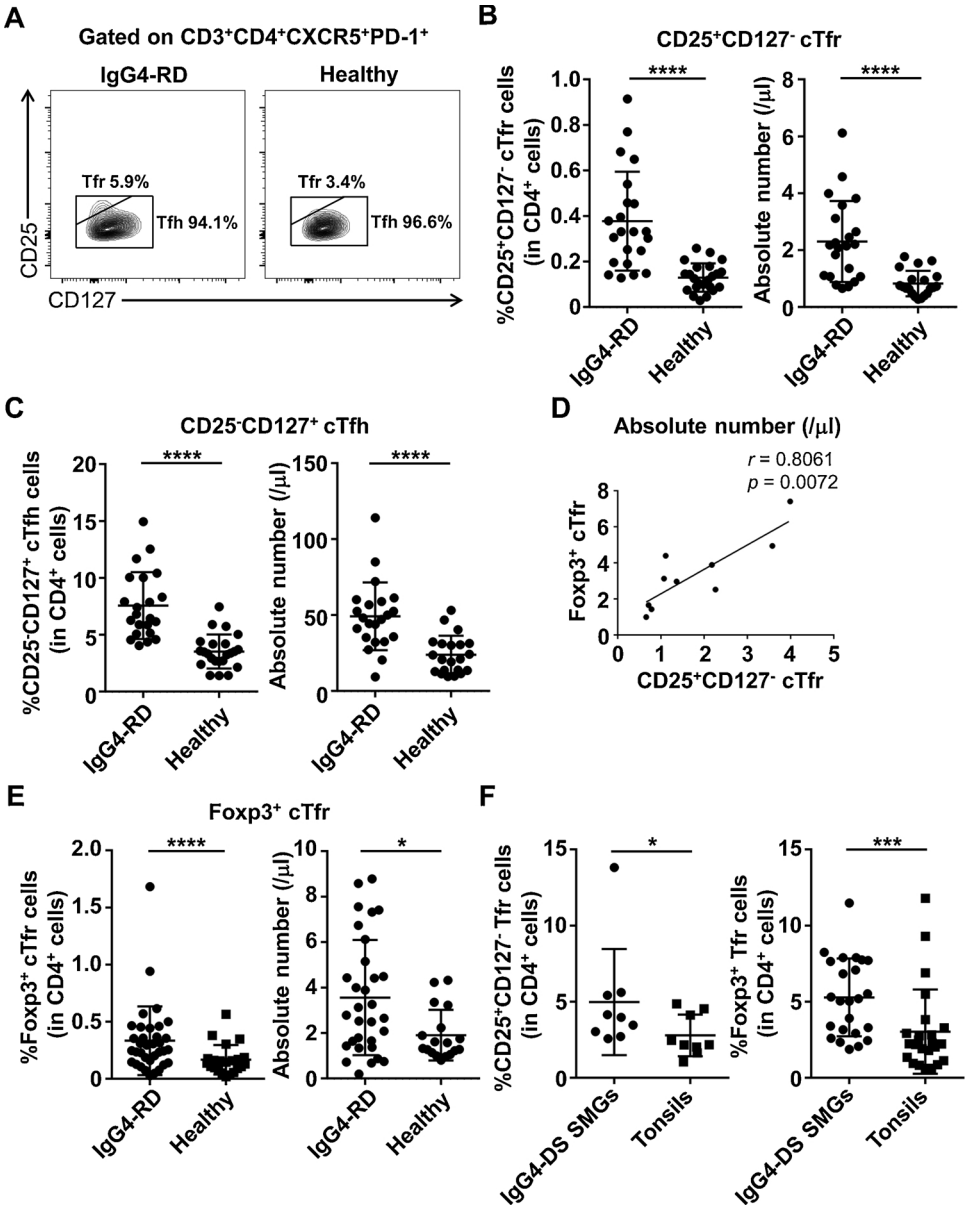

Fig. 1. Blood and tissue Tfr cells are increased in IgG4-RD.

(A) Representative FACS profiles indicating $CD25^+CD127^-$ Tfr cells and $CD25^-CD127^+$ Tfh cells in IgG4-RD patients and healthy volunteers. Plots were pregated on $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ cells and examined by the levels of CD25 and CD127. Numbers indicate percentage of cells in the gate.

(B) Percentages of circulating $CD25^+CD127^-$ Tfr ($CD25^+CD127^-$ cTfr; $CD3^+CD4^+CD25^+CD127^-CXCR5^+PD\text{-}1^+$) cells in $CD4^+$ cells (left) and absolute numbers of $CD25^+CD127^-$ cTfr cells (right) from patients with IgG4-RD (n = 22) and healthy volunteers (n = 23) are shown.

(C) Percentages of circulating $CD25^-CD127^+$ Tfh ($CD25^-CD127^+$ cTfh; $CD3^+CD4^+CD25^-CD127^+CXCR5^+PD\text{-}1^+$) cells in $CD4^+$ cells (left) and absolute numbers of $CD25^-CD127^+$ cTfh cells (right) from patients with IgG4-RD (n = 22) and healthy volunteers (n = 23) are shown.

(D) Scatter plots demonstrating the relationships between absolute number of $CD25^+CD127^-$ cTfr cells and absolute number of $Foxp3^+$ cTfr cells in IgG4-RD cases (n = 10).

(E) Percentages of $Foxp3^+$ cTfr cells ($CD3^+CD4^+CD25^+CXCR5^+PD\text{-}1^+Foxp3^+$ cells) in $CD4^+$ cells (left) and absolute numbers of $Foxp3^+$ cTfr cells (right) from patients with IgG4-RD (n = 35) and healthy volunteers (n = 22) are shown.

(F) Percentages of $CD25^+CD127^-$ Tfr cells in $CD4^+$ cells (n = 9 in each group, left) and $Foxp3^+$ Tfr cells (n = 24 in each group, right) in submandibular glands (SMGs) from patients with IgG4-related dacryoadenitis and sialadenitis (IgG4-DS) and in tonsils are shown. Horizontal lines and error bars indicate mean and SD, respectively. *; $p < 0.05$, ***; $p < 0.001$, ****; $p < 0.0001$ by the Mann-Whitney U test.

during senescence. To determine age-related functional differences in Tfr cells, we analyzed two age groups of healthy individuals: a young group (less than 19 years old) and an elderly group (over 40 years old). Prior to investigation of Tfr cells, we confirmed a high level of expression of *FOXP3* transcripts in sorted Tfr cells ($CD25^+CD127^-$ Tfr cells) compared with that in sorted Tfh cells ($CD25^-CD127^+$ Tfh cells; Supplementary Fig. S3). When tonsillar lymphocytes were examined, the percentage of $CD25^+CD127^-$ Tfr cells in elderly subjects was increased compared with that in young subjects (Fig. 4A and B). To analyze the suppressive function of such Tfr cells, we performed a functional assay of Tfr cells using a coculture system in which autologous lymphocytes including Tfh cells and B cells were cultured in the presence or absence of Tfr cells for 10 days and then the concentrations of IgG in the supernatants were measured by ELISA (Fig. 4C). The concentrations of IgG in the supernatants without Tfr cells of young subjects were comparable to those of elderly subjects, whereas the concentrations of IgG with Tfr cells of young subjects were significantly lower than those of elderly subjects (Fig. 4D and E). These results suggest that Tfr cells from young subjects have a high suppressive potential in terms of IgG production compared with Tfr cells from elderly subjects. Results similar to those of the coculture experiments were obtained by using tonsillar lymphocytes including autologous Tfr cells and Tfh cells from young and elderly subjects in combination with allogeneic B cells (data not shown). Collectively, the results indicate that Tfr cells with a defective suppressive function increase with advance of age.

3.5. Decreased IL-10 production is responsible for dysfunction of Tfr cells with aging

To determine the mechanism by which the suppressive function of Tfr cells in elderly subjects is attenuated, we examined the expression levels of gene transcripts related to immune suppression. Quantitative real-time PCR analyses showed that Tfr cells in tonsils from elderly subjects had lower levels of transcripts encoding *IL10* than did those in tonsils from young subjects (Fig. 5A), while the amounts of transcripts encoding *TGFB1*, *CTLA4*, and glucocorticoid-induced TNFR-related protein (GITR, *TNFRSF18*) were comparable to those in tonsils from young subjects (Figs. 5B–D). We further determined the percentage of IL-10-producing Tfr cells by an IL-10 secretion assay. As shown in Fig. 5E and F, the percentages of IL-10-producing Tfr cells were significantly decreased in tonsils and peripheral blood from elderly

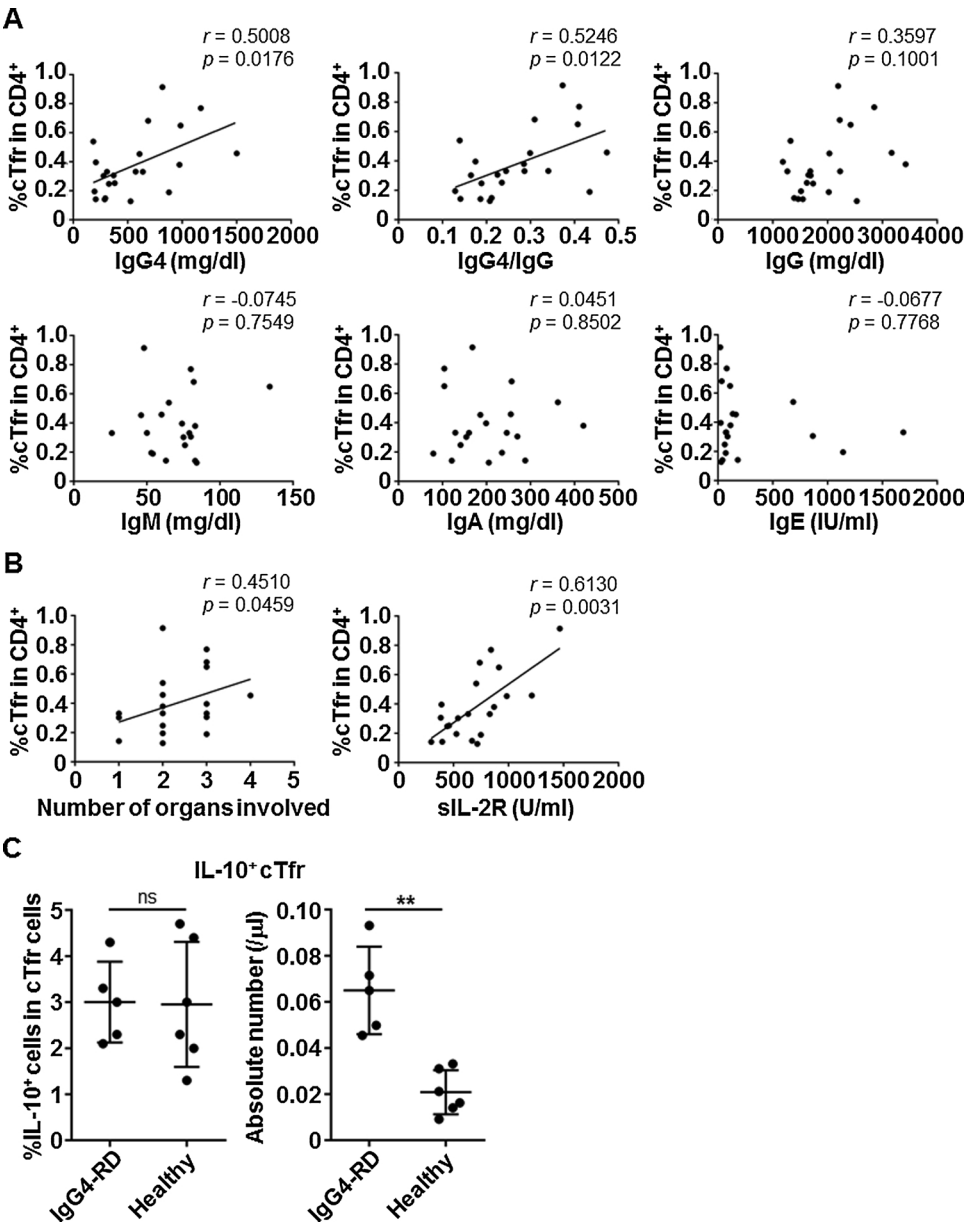

Fig. 2. Increased blood Tfr cells in IgG4-RD are positively correlated with clinical parameters.

(A, B) Scatter plots demonstrating the relationships of circulating $CD25^+CD127^-$ Tfr cells ($CD25^+CD127^-$ cTfr cells) in $CD4^+$ cells with serum IgG4, IgG4/IgG ratio, IgG, IgM, IgA, IgE (A), number of involved organs, and serum soluble IL-2 receptor (sIL-2R) (B) in IgG4-RD cases (n = 20).

(C) Graphs showing percentages (left) and absolute numbers (right) of IL-10-producing Tfr cells in blood from patients with IgG4-RD (n = 5) and healthy volunteers (n = 6). Horizontal lines and error bars indicate mean and SD, respectively. **; $p <$ 0.01, ns indicating not significant by the Mann-Whitney *U* test.

subjects compared with those in tonsils and peripheral blood from young subjects. To clarify the role of IL-10 produced by Tfr cells, we performed coculture experiments and examined IgG production in the presence of a blocking antibody specific to IL-10 (Fig. 5G). As shown in Fig. 5H, blocking with an anti-IL-10 antibody restored IgG production by Tfr cells from young subjects. However, blockade of IL-10 production did not affect the regulation of IgG production by Tfr cells from elderly subjects (Fig. 5I). These results suggest that IL-10 is one of the essential factors that exert the suppressive activity of Tfr cells in young individuals and that secretion of IL-10 by Tfr cells decreases with advance of age.

4. Discussion

In this study, we performed a comprehensive analysis of circulating $CD4^+$ T cells and for the first time showed the possible involvement of Tfr cells in the pathogenesis of IgG4-RD. Tfr cells have a critical function to control the magnitude of GC responses by modulating Tfh cells and B cells [6]. Moreover, recent studies have revealed that IL-10 produced from Tfr cells promotes the germinal center response [7], which means that Tfr cells have not only a suppressive function but also a stimulatory function. Although the mechanism by which IgG4 production from B cells is morbidly enhanced in lesions of IgG4-RD remains unclear, our findings suggest that an increase in Tfr cells in blood and lesions of IgG4-RD is associated with the immunological mechanisms of IgG4-RD.

As shown in Fig. 2A, the percentage of Tfr cells in IgG4-RD was significantly correlated with the serum level of IgG4 but not with the levels of other immunoglobulin subclasses. In addition, the absolute number of IL-10-producing Tfr cells in IgG4-RD patients was significantly increased compared with that in age-matched healthy volunteers (Fig. 2C). We and another group recently reported that activated Tfh cells expressing high levels of BCL6 and PD-1 are remarkably accumulated in the lesions of IgG4-RD and that Tfh cells in patients with IgG4-RD more efficiently facilitated B cell proliferation as well as differentiation of naive B cells into plasmablasts/plasma cells, resulting in increased secretion of IgG4 [4,25]. Other studies showed that Tfr cells have the capacity to control the proliferation of Tfh cells [26] and that the proliferative activity of GC B cells depends on IL-10 secreted by Tfr cells [7]. Moreover, IL-10 facilitates class-switch recombination of

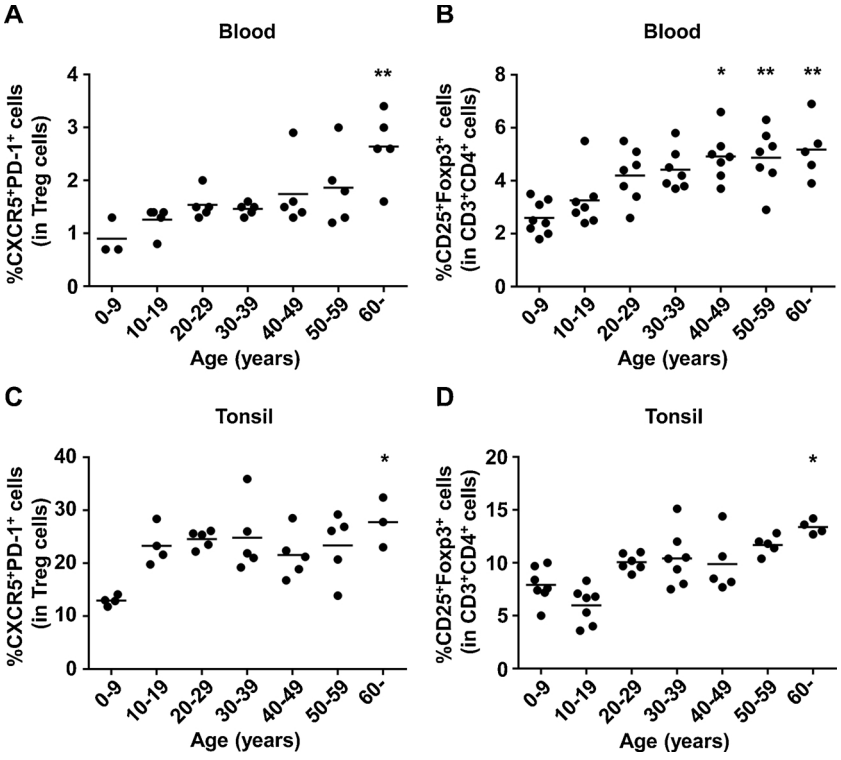

Fig. 3. Percentages of Tfr cells and Treg cells increase with advance of age.

(A, B) Percentage of $CXCR5^+PD\text{-}1^+$ cells in regulatory T (Treg; $CD3^+CD4^+CD25^+Foxp3^+$) cells (A) and percentage of $CD25^+Foxp3^+$ cells in $CD3^+CD4^+$ cells (B) in blood from healthy volunteers are shown (n = 3–8 in each group).

(C, D) Percentage of $CXCR5^+PD\text{-}1^+$ cells in Treg cells (C) and percentage of Treg cells in $CD3^+CD4^+$ cells (D) in tonsils are shown (n = 3–7 in each group).

(A–D) Horizontal lines indicate mean, and statistical significance was determined using the Kruskal-Wallis test with 0–9-years-old age group v.s. each age group. *; $p < 0.05$, **; $p < 0.01$.

IgG4 in B cells [9,10]. From the results of those previous studies and results of our study shown in Figs. 1 and 2, an increased number of Tfr cells might lead to the activation of Tfh cells and B cells, and an increased number of IL-10-producing Tfr cells is potentially involved in IgG4-specific class-switch recombination in a lesion of IgG4-RD. The precise mechanisms by which Tfr cells induce proliferation of activated Tfh cells and the mechanism of IgG4-specific class-switch recombination in a lesion of IgG4-RD are still unclear but warrant examination. Moreover, although the reason why Tfr cells in blood from patients with IgG4-RD were increased compared with those in age-matched healthy

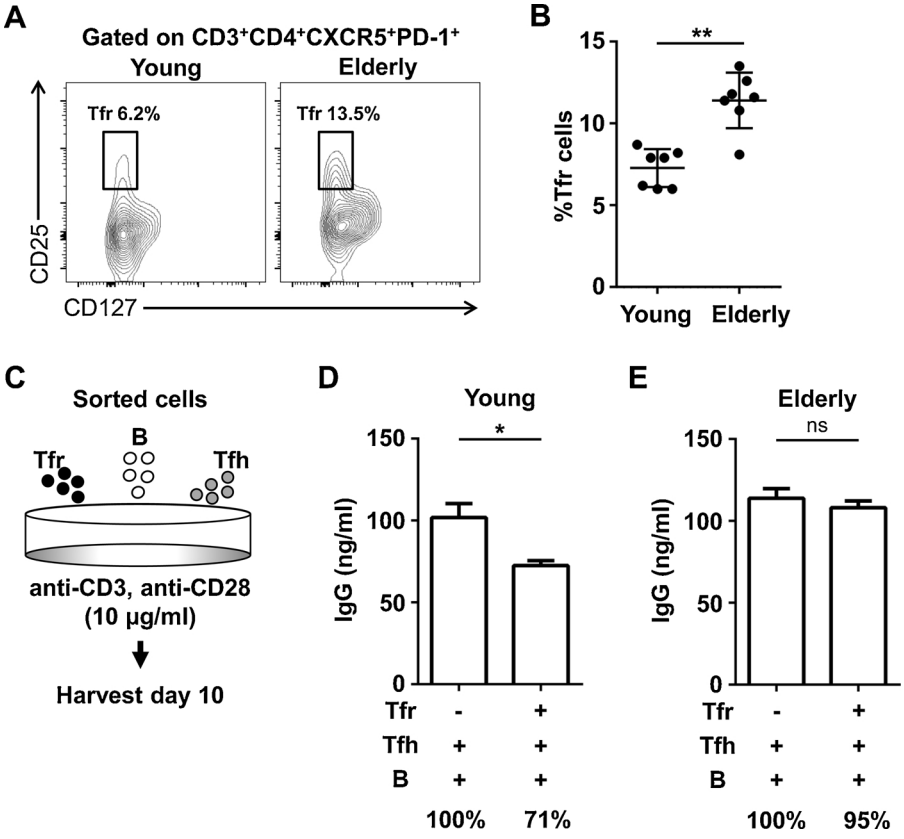

Fig. 4. Suppressive function of Tfr cells is impaired in elderly subjects.

(A) Representative FACS profiles indicating $CD25^+CD127^-$ Tfr cells in young and elderly subjects. Plots were pregated on $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ cells and examined by the levels of CD25 and CD127. Numbers indicate percentage of cells in the gate.

(B) Percentages of $CD25^+CD127^-$ Tfr cells in $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ cells in tonsils from young and elderly subjects are shown (n = 7 in each group).

(C) Schematic representation of the suppression assay. Sorted $CD25^-CD127^+$ Tfh cells (1×10^5) and B cells (1×10^5) in tonsils from young and elderly subjects were cocultured with/without 1×10^5 autologous $CD25^+CD127^-$ Tfr cells with stimulation by 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28 antibodies for 10 days.

(D, E) ELISA determination of IgG (ng/ml) in supernatants after 10 days of in vitro coculture (D: young, E: elderly) performed as described in (C). Numbers indicate the ratio of IgG concentration in the supernatant from coculture of Tfr, Tfh, and B cells to that in the supernatant from coculture of Tfh and B cells. Representative data of three independent experiments are shown.

(B, D, E) Horizontal lines and error bars indicate mean and SD, respectively. *; $p < 0.05$, **; $p < 0.01$, ns indicating not significant by the Mann-Whitney *U* test.

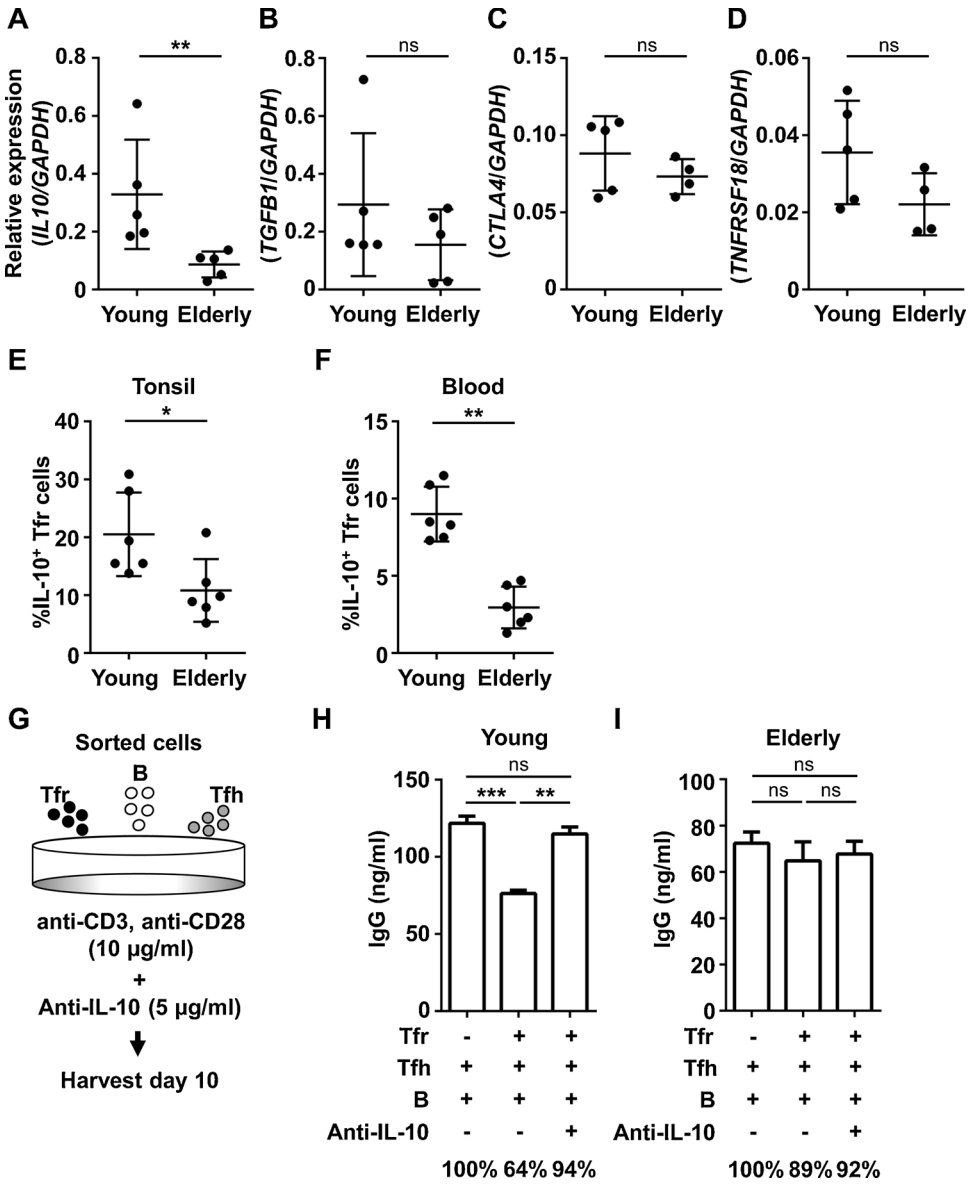

Fig. 5. Decreased IL-10 production is responsible for dysfunction of Tfr cells with aging.

(A–D) Graphs showing expression levels of *IL10* (A), *TGFB1* (B), *CTLA4* (C), and *TNFRSF18* (D) mRNA in tonsillar $CD25^+CD127^-$ Tfr cells from young and elderly subjects (n = 4–5).

(E, F) Graphs showing percentages of IL-10-producing cells in tonsillar (E) and blood (F) $CD25^+CD127^-$ Tfr cells from young and elderly subjects (n = 6).

(G) Schematic representation of the suppression assay. Sorted $CD25^-CD127^+$ Tfh cells (1×10^5) and B cells (1×10^5) in tonsils from young and elderly subjects were cocultured with/without 1×10^5 autologous $CD25^+CD127^-$ Tfr cells with stimulation by 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28 antibodies and in the presence or absence of 5 μg/ml anti-IL-10 blocking antibody for 10 days.

(H, I) ELISA determination of IgG (ng/ml) in supernatants after 10 days of in vitro coculture (D: young, E: elderly) performed as described in (G). Numbers indicate the ratio of IgG concentration in the supernatant from coculture of Tfr, Tfh, and B cells to that in the supernatant from coculture of Tfh and B cells. Representative data of three independent experiments are shown.

(A–F, H, I) Horizontal lines and error bars indicate mean and SD, respectively. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, ns indicating not significant by the Mann-Whitney *U* test.

volunteers (Fig. 1) is not known, it is possible that the increased numbers of circulating and lesional Tfr cells in IgG4-RD reflect a compensatory change so as to suppress abnormal immune responses. Further studies are needed in order to clarify the mechanisms of accumulation of Tfr cells in IgG4-RD. Collectively, the results support the unique immunological environment in IgG4-RD, in which there is an increased number of Tfr cells and activated Tfh cells, probably underlying the pathogenesis of IgG4-RD.

Patients with autoimmune diseases such as systemic lupus erythematous and primary biliary cirrhosis show a lower Tfr/Tfh ratio in blood lymphocytes, which is probably associated with the production of self-specific antibodies [27,28]. Thus, it would be worthwhile to focus on the relationship between Tfr cells and Tfh cells as a new clue to elucidate the pathogenesis of immune disorders. Our results showed that there was no significant difference in the Tfr/Tfh ratio in blood lymphocytes between healthy subjects and IgG4-RD patients (Supplementary Fig. S4). A more recent study on autoimmune pancreatitis of IgG4-RD revealed for the first time an autoantibody against one of the extracellular matrix proteins [29], suggesting a certain autoimmune mechanism underlying IgG4-RD. The manner in which B cells are made to produce autoantibodies in IgG4-RD may be unique because the characteristics of Tfr cells and Tfh cells and the relationship between them in IgG4-RD are likely to be different from those in typical autoimmune diseases.

We showed that the percentages of tonsillar and blood Tfr cells increased with aging in healthy volunteers, whereas the suppressive effect of blood Tfr cells on B cell function in elderly subjects was impaired in comparison with that in young subjects due to a defect in the production of IL-10 (Figs. 4 and 5). On the other hand, the percentage of activated Tfh cells also decreased with advance of age (Supplementary Fig. S2). A recent study in mice showing that an increase in suppressive Tfr cells combined with impaired function of aged Tfh cells resulted in reduced T-cell-dependent antibody responses in aged mice [30] supports our findings. Regarding change in suppressive function of Tfr cells with aging we have to consider not only functional defects of Tfr cells, but also those of target cells such as activated Tfh cells and B cells. Little has been reported regarding aging-related physiological changes of Tfr cell function in humans. Therefore, this study provides important information on such changes.

In conclusion, this study provides novel and important information on the functional significance of Tfr cells and IL-10 produced by Tfr cells in the pathogenesis of IgG4-RD. Focusing on the regulation of Tfr cells and activated Tfh cells may be an effective strategy for preventing the development of IgG4-RD. In this study, we showed stimulatory

(Figs. 1 and 2) and suppressive functions (Figs. 4 and 5) of Tfr cells. Owing to the limitation of clinical specimens, we could not examine the suppressive function of Tfr cells in IgG4-RD and the stimulatory function of Tfr cells in healthy volunteers. They need to be examined in a future study.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.01.008.

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