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Short communication

Interleukin-4 contributes to the shift of balance of IgG subclasses toward IgG4 in IgG4-related disease

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ABSTRACT

IgG4-related disease (IgG4-RD) is a systemic disorder characterized by elevated serum IgG4 level, which is mediated by T follicular helper 2 (Tfh2) cell. However, the cytokines responsible for enhancing IgG4 production remain unclear in IgG4-RD. The aim of this study was to identify responsible Tfh2-related cytokines (interleukin (IL)-4 and IL-21) for enhancing IgG4 production in IgG4-RD. Peripheral blood mononuclear cells obtained from consecutive patients with active, untreated IgG4-RD and healthy controls were examined. The production of both IgG and IgG4 were significantly increased by stimulation with IL-4 alone as well as IL-21 alone compared to background stimulation with anti-CD40 antibody in IgG4-RD. On the other hand, the IgG4/IgG ratio was statistically higher by stimulation with IL-4 alone compared to the other Tfh2-related cytokines including IL-21 in IgG4-RD. IgG4 production was not increased by stimulation with IL-4 in healthy controls. These results suggest that IL-4 can contribute to the shift of balance of IgG subclasses toward IgG4 compared to the other Tfh2-related cytokines in IgG4-RD.

1. Introduction

IgG4-related disease (IgG4-RD) is a chronic inflammatory disorder characterized by elevated serum IgG4 levels and an increased number of circulating plasmablasts [1]. Recent studies have shown that circulating plasmablasts are oligoclonally expanded and exhibit somatic hypermutation, suggesting that plasmablasts are induced by interaction with T follicular helper (Tfh) cells in IgG4-RD [1,2].

Tfh cell is a distinct subset of $CD4^+$ T cell that acquired the ability to induce B-cell maturation, B-cell proliferation, somatic hypermutation, Ig class switching, and germinal center development [3]. These processes are primarily induced by cytokines and direct contact between Tfh cells and B cells via CD40-CD40L interaction [3]. Key histological features of IgG4-RD include a massive infiltration of $IgG4^+$ plasmablasts and plasma cells with expanded ectopic germinal centers [4,5], and the upregulation of Tfh cell-associated cytokines interleukin (IL)-4 and IL-21 at affected sites [5]. These findings suggest that Tfh cells play a role in IgG4 class switching and plasmablast and plasma cell differentiation in the pathogenesis of IgG4-RD [2].

Although it is a matter whether circulating cells reflect those events at affected sites, recent studies have revealed that circulating Tfh cells share phenotypic and functional features with bona fide Tfh cells [3,6]. Circulating Tfh cells comprise at least three major subsets (Tfh1, Tfh2 and Tfh17 cells), which are functionally distinct based on their ability to help B cells [6]. We previously reported that the number of circulating activated Tfh2 cells was markedly increased in IgG4-RD and correlated with disease activity [7–9]. Of note, IgG4 class switching is mediated by Tfh2 cells in IgG4-RD [9]. Given that Tfh2 cells secrete IL-4 and IL-21, which are overexpressed at affected sites in IgG4-RD [5,6,10], it is possible that these cytokines may be involved in IgG4 class switching. However, few studies conducted *in vitro* analyses, and the cytokines responsible for enhancing IgG4 production in IgG4-RD have not been identified to date.

The aim of this study was to identify responsible Tfh2-associated cytokines (IL-4 and IL-21) for enhancing IgG4 production in IgG4-RD.

2. Material and methods

2.1. Participants

Seven consecutive patients with active, untreated, biopsy-proven IgG4-RD (2 women and 5 men, mean age ± standard error of the mean (SEM) = 66.6 ± 4.9 years) who were referred to Keio University Hospital, and 6 healthy controls (HC; 1 woman and 5 men, mean age ± SEM = 37.0 ± 3.1 years) were enrolled. The diagnosis of IgG4-RD was based on the 2011 comprehensive IgG4-RD diagnostic criteria [11].

None of patients had received glucocorticoids at the time of blood

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sampling. The affected organs (some patients had more than one site of involvement) were the lacrimal glands (4 cases), submandibular glands (4 cases), orbits (2 cases), pancreas (2 cases), lymph nodes (2 cases), parotid gland (1 case), and lung (1 case). Mean concentrations of serum IgG and IgG4 were 1569 mg/dL (range 934–2111 mg/dL) and 386 mg/dL (range 201–572 mg/dL), respectively. This study was approved by the ethics committee of Keio University Hospital and informed consent was obtained from all the patients and HC.

2.2. Cell culture

Peripheral blood mononuclear cells (PBMCs) from active, untreated patients with IgG4-RD and HC were separated by gradient centrifugation using Lymphoprep (Axis-Shield; Oslo, Norway) according to the manufacturer's instructions. PBMCs (2×10^5/200 μL/well) were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a round-bottom 96-well plate in humidified atmosphere at 37 °C with 5% CO_2 for 7 days. PBMCs were stimulated with IL-4 (100 ng/mL; PeproTech, USA) or with IL-21 (100 ng/mL; PeproTech), upon stimulation with an anti-CD40 antibody (1.0 μg/mL; R&D Systems, Inc, USA). In some experiments, IL-13 (100 ng/mL; PeproTech) and IL-10 (100 ng/mL; PeproTech) were also used.

2.3. Measurement of IgG and IgG4 concentrations in culture supernatant

The concentrations of IgG and IgG4 in culture supernatants were measured by cytometric bead array (BD Biosciences, USA), according to the manufacturer's instruction, using a flow cytometer (LSRFortessa X-20; BD Biosciences).

2.4. Detection of plasmablasts and plasma cells

Immunophenotyping analysis of lymphocytes was performed using a LSRFortessa X-20. PBMCs were cultured in a 96-well plate and collected on day 7. After treatment with a human FcR-blocking reagent (Miltenyi Biotec) according to the manufacturer's instructions, PBMCs were incubated with the following antibodies: CD3-FITC (SK7, BioLegend, USA), CD19-BV421 (HIB19, BioLegend), IgD-PE (IA6-2, BD Biosciences), CD38-APC (HIT2, BD Biosciences), and CD138-Brilliant Violet (MI15, BioLegend). Non-viable cells were labeled with 7-amino-actinomycin D (BD Biosciences). $CD3^-CD19^+CD20^-CD27^+CD38^+CD138^-$ cells and $CD3^-CD19^+CD20^-CD27^+CD38^+CD138^+$ cells were defined as plasmablasts and plasma cells, respectively. The proportion of plasmablasts and plasma cells were analyzed by FlowJo v.7.6.4 software (Tree Star, Stanford University, CA, USA).

2.5. Statistical analysis

Continuous variables are reported as mean ± SEM. The Wilcoxon rank sum test was used for comparisons between two groups. Statistical significance was assessed by two-tailed analysis using GraphPad Prism software V.6.0 (GraphPad software; San Diego, CA, USA), and a *P*-value of less than 0.05 was considered as statistically significant.

3. Results

We first examined the effect of Tfh2-related cytokines on the maturation of B cells into plasmablasts and plasma cells. As shown in Fig. 1A, both IL-4 and IL-21 had an additive effect for differentiation into plasmablasts in IgG4-RD. Also, the same effect of IL-4 and IL-21 on plasmablast differentiation was observed in HC (Fig. 1A). With regard to the role of these cytokines in the differentiation into plasma cells (Fig. 1B), the similar effect of Tfh2-related cytokines was observed in IgG4-RD, whereas the additive effect of IL-4 and IL-21 was not observed in the samples from HC.

Then, we next examined the effect of Tfh2-related cytokines on immunoglobulin production. As shown in Fig. 2A, total IgG production was dominantly induced by IL-21 and no additive effect of IL-21 and IL-4 was observed both in IgG4-RD and HC. Total IgG was significantly upregulated by IL-4 in IgG4-RD, however, the impact on the net production of IgG was limited. As for the production of IgG4, the similar trend of the induction of total IgG was observed in IgG4-RD (Fig. 2B). But the effect of IL-4 on production of IgG4 was substantial, which was compared with the background stimulation with anti-CD40 antibody ($P = 0.0156$), and this effect was not observed in HC. Also, the impact of IL-4 on the net production of IgG4 was substantial because the amount of IgG4 induced by IL-4 was more than half of that induced by IL-21 stimulation. Moreover, as shown in Fig. 2C, when we examined the ratio of IgG4 to IgG, the preferential induction of IgG4 production was observed by IL-4 stimulation compared to the other Tfh2-related cytokines including IL-21 in IgG4-RD. This trend was not observed in HC. We also examined the effect of IL-10 and IL-13, but did not see any contribution to IgG4 production compared with the background stimulation with anti-CD40 antibody both in IgG4-RD and HC.

4. Discussion

We demonstrated that IL-4 contributed to the shift of balance of IgG subclasses toward IgG4 through enhancing IgG4 production in PBMCs from patients with active, untreated IgG4-RD but not in HC.

It is well established that cytokines are major regulators of IgG-subclass responses. A skewed response toward IgG4 is a characteristic feature of IgG4-RD, and although Tfh2 cell-associated cytokines (IL-4 and IL-21) have been suggested as candidates for inducing this IgG4 class switching [12], the exact cytokines responsible for driving IgG4 production remains unclear. We previously reported that Tfh2 cells that are co-cultured with naïve B cells from IgG4-RD patients induce IgG4 class switching [9]. In addition, IL-4 is exclusively secreted by Tfh2 cells among the Tfh-cell subsets [6] and serum IL-4, but not IL-10 or IL-21, levels are positively correlated with serum IgG4 levels and the number of circulating Tfh2 cells in patients with IgG4-RD [7]. Moreover, expression of IL-4 mRNA is positively correlated with the ratio of IgG4 to IgG mRNA and the number of $IgG4^+$ plasma cells at affected sites [5]. In the present study, we demonstrated that both IL-4 and IL-21 induced production of IgG4, however IL-4 preferentially enhanced IgG4 production in PBMCs from patients with IgG4-RD. Although other cytokines, IL-10 and IL-13, have also been suggested as candidates for inducing IgG4 class switching [12], IL-10 and IL-13 did not affect IgG4 production in IgG4-RD. These results suggest that IL-4 plays an important role in enhancing IgG4 production in IgG4-RD.

Interestingly, we found that the preferential expression of IgG4 enhanced by IL-4 was observed in IgG4-RD patients but not in HC. This may be due to the differences of the proportions of lymphocytes (both T and B cells) between the patients' PBMCs and the controls' PBMCs. It is possible that the patients' PBMCs contain more plasmablasts and plasma cells that are already primed or class-switched to IgG4-subclass before the blood sampling, resulting in being more prone to induce production of IgG4 by IL-4 stimulation in the patients' PBMCs than controls' PBMCs. Another possibility is a differential capacity of IL-4 to induce IgG4 class switching of PBMCs in IgG4-RD patients compared to HC. Prominent IgG4 responses have been observed in the sequential analysis of sera from novice beekeepers and during allergen-specific immunotherapy through chronic antigen stimulation [12]. Further, differential effects of IL-4 on IgG4 production have been shown to be dependent on the expression levels of high-affinity IL-4 receptors [13]. These observations suggest that the differential effects of IL-4 on IgG4 production may be due to a priming of B cells in IgG4-RD patients by chronic antigen stimulation, exposure to various cytokines or the presence of more high-affinity IL-4 receptors on activated B cells among PBMCs from IgG4-RD patients than HC. Further studies are required to determine the exact mechanism underlying this difference.

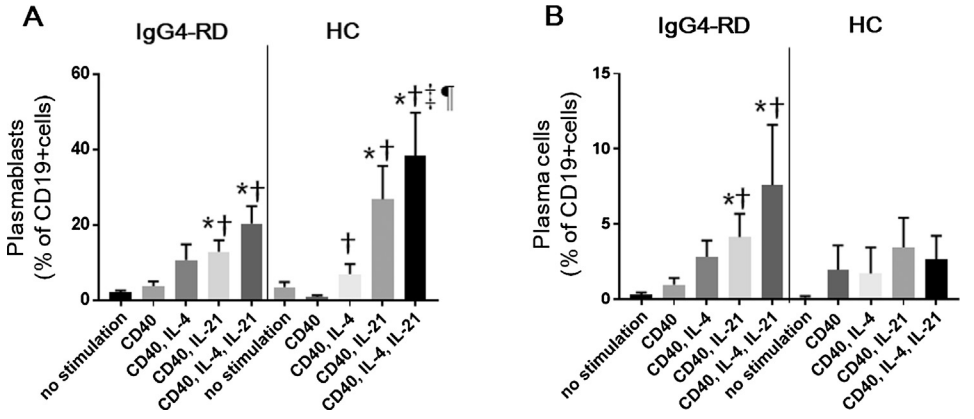
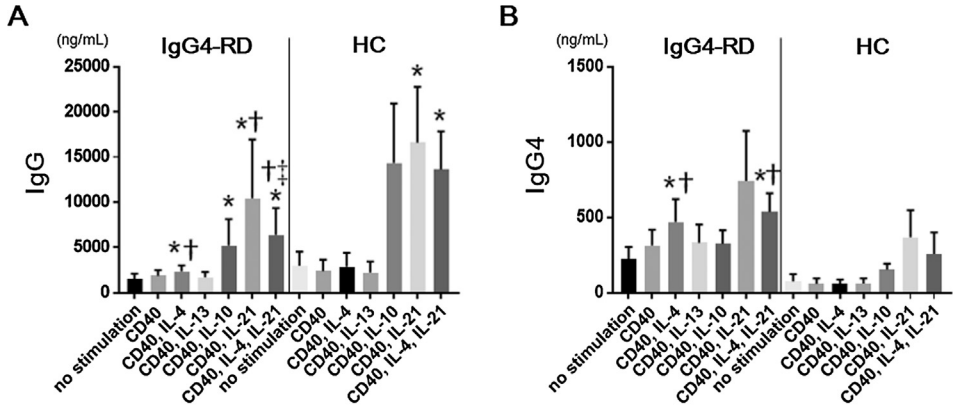


Fig. 1. The proportion of plasmablasts and plasma cells induced by stimulation with cytokines and CD40 in IgG4-related disease (IgG4-RD) patients and healthy controls (HC). The proportion of (A) plasmablasts and (B) plasma cells in 7 patients with IgG4-RD and 6 HC. *$P < 0.05$ when compared with no stimulation. †$P < 0.05$ when compared with anti-CD40 antibody alone. ‡$P < 0.05$ when compared with IL-4 alone. ¶$P < 0.05$ when compared with IL-21 alone.



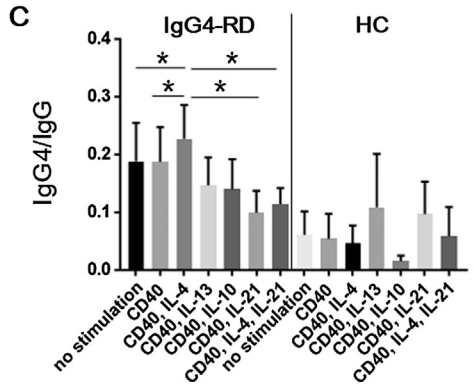


Fig. 2. Effects of stimulation with cytokines and CD40 on IgG and IgG4 production by peripheral blood mononuclear cells (PBMCs) from 7 IgG4-related disease (IgG4-RD) patients and 6 healthy controls (HC). (A) IgG production, (B) IgG4 production, and (C) IgG4 to IgG ratio in the culture supernatants detected by cytometric bead array. The number of examined samples for IL-13 and IL-10 is 6. *$P < 0.05$ when compared with no stimulation. †$P < 0.05$ when compared with anti-CD40 antibody alone. ‡$P < 0.05$ when compared with IL-4 alone.

It should be noted that the increase of IgG4 production seemed to occur in controls' PBMCs stimulated with IL-4, IL-21 and anti-CD40 antibody even if there was no statistically significant difference (Fig. 2B). Importantly, IgG4/IgG ratio was not increased by stimulation with IL-4 plus IL-21 compared to no stimulation and anti-CD40 antibody alone in controls' PBMCs. Therefore, the increase of IgG4 production by stimulation with IL-4 and IL-21 in controls' PBMCs reflected the polyclonal elevation. In IgG4-RD, no additive effect of IL-4 and IL-21 on IgG4 production was observed (Fig. 2B). Thus, IL-21 and IL-4 have an independent effect in IgG4 class-switching. We recognized that both IL-21 and IL-4 have a substantial role in the production of IgG4, however, IL-4 preferentially induce IgG4 compared with the other Tfh2-related cytokines as shown in Fig. 2C, suggesting that IL-4 can contribute to the shift of IgG subclasses toward IgG4 in IgG4-RD.

Our study has several limitations. For example, since PBMCs contain various types of cells, such as IgG4-class-switching plasmablasts and plasma cells, the experiments using naïve B cells are essential. In addition, to examine inhibiting IL-4 or knockdown of IL-4 is important to more clearly elucidate the role of IL-4 in IgG4 class-switching. Also, the limited number of the samples of active and untreated IgG4-RD patients is an important limitation of this study. Further studies are needed to confirm our results in a large number of the samples.

5. Conclusions

We showed that IL-4 plays a role in shifting IgG4 production by PBMCs from patients with active, untreated IgG4-RD. Our results may provide a foundation for the development of novel therapies targeting cytokines for the treatment of IgG4-RD.

Disclosure statement

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