Title

The Role of CD19⁺CD24^{high}CD38^{high} and CD19⁺CD24^{high}CD27⁺ Regulatory B Cells in Patients with Type 1 Autoimmune Pancreatitis.

Authors

Kimi Sumimoto, Kazushige Uchida, Takeo Kusuda, Toshiyuki Mitsuyama, Yutaku Sakaguchi, Toshiro Fukui, Mitsunobu Matsushita, Makoto Takaoka, Akiyoshi Nishio, and Kazuichi Okazaki.

Institute

The Third Department of Internal Medicine, Division of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan.

Correspondence

Kazuichi Okazaki, M.D., Ph.D.

The Third Department of Internal Medicine, Division of Gastroenterology and Hepatology, Kansai Medical University
2-5-1 Shinmachi, Hirakata, Osaka 573-1191, Japan.

Phone: 81-72-804-0101 (ext 2520)

Fax : 81-72-804-2061

E-mail: okazaki@hirakata.kmu.ac.jp

Short title

The role of Bregs in type 1 AIP patients

Abstract

Background: Patients with type 1 autoimmune pancreatitis (AIP) have several immunologic and histologic abnormalities. It is known that depletion of B cells by rituximab is effective for treatment of IgG4-related disease (IgG4-RD) such as type 1 AIP, suggesting that B cells may be a key player in IgG4-RD. However, the role of regulatory B cells (Bregs) in type 1 AIP is unclear, and the objective of this paper is to clarify the role of Bregs in the pathophysiology of type 1 AIP by analyzing circulating Bregs.

Method: We recruited 21 patients with type 1 AIP as determined by the International Consensus Diagnostic Criteria for AIP (ICDC). No patients received corticosteroid treatments. For comparison, we recruited 14 patients with chronic pancreatitis (CP), 20 patients with pancreatic cancer, and 25 healthy subjects as controls. We analyzed Bregs as CD19⁺CD24^{high}CD38^{high} and CD19⁺CD24^{high}CD27⁺ from peripheral blood by flow cytometry.

Results: In peripheral blood, CD19⁺CD24^{high}CD38^{high} Bregs were significantly increased in type 1 AIP patients compared with CP, pancreatic cancer, and healthy controls. Although not significant different, CD19⁺CD24^{high}CD27⁺ Bregs of type 1 AIP were decreased compared to those of other groups. IL-10⁺ B cells were not significantly different from type 1 AIP patients and healthy controls. In untreated type 1 AIP patients, the number of CD19⁺CD24^{high}CD38^{high} Bregs and IgG4 were not correlated.

Conclusions: Our data suggested that CD19⁺CD24^{high}CD38^{high} Bregs seemed to increase reactively to suppress

the disease activity, and are consistent with the hypothesis that CD19⁺CD24^{high}CD27⁺ Bregs might be involved in the development of type 1 AIP, although it still remains unclear whether the decrease of CD19⁺CD24^{high}CD27⁺ cells is cause or effect of AIP.

Key words

type 1 autoimmune pancreatitis (AIP), regulatory B cells (Bregs), interleukin-10 (IL-10)

1. Introduction

Autoimmune pancreatitis (AIP) is a particular type of chronic pancreatitis due to autoimmune mechanism. In 1961, Sarles et al. [1] first observed a case of particular pancreatitis with hypergammaglobulinemia. In 1995, Yoshida et al. [2] proposed AIP as a new clinical entity. In 2001, Hamano et al. [3] reported that patients with AIP had high serum immunoglobulin G4 (IgG4) concentrations. In 2003, Kamisawa et al. [4] suggested that AIP was a systemic disease, based on the findings that the pancreas and other involved organs have abundant infiltration of IgG4-positive plasma cells. Many cases of AIP have been reported in Asia, Europe, and the United State [5-12]. There are several diagnostic criteria in each countries and areas. The diagnosis of AIP must be made on the presence of a unique set of characteristics [13-18].

IgG4-related AIP is characterized by irregular narrowing of the main pancreatic duct, enlargement of the pancreas, high serum levels of IgG4, other organ involvement, and steroid responsiveness. Recent studies suggested the existence of two subtypes of AIP; (a) type 1 AIP related with IgG4 showing lymphoplasmacytic sclerosing pancreatitis (LPSP) [19], and (b) type 2 AIP related with a granulocytic epithelial lesion (GEL) [20-22] showing idiopathic duct-centric chronic pancreatitis (IDCP) [23-25]. In 2011, the International Association of Pancreatology (IAP) proposed the International Consensus Diagnostic Criteria (ICDC) for AIP [26]. In the ICDC, AIP was classified into type 1 and type 2. Most of the Japanese AIP cases are type 1,

whereas those concerning type 2 are very few. Japanese consensus clinical guidelines have focused on type 1 AIP [27].

We previously reported that induced CD4⁺CD25^{high} regulatory T cells (Tregs) were significantly increased and naïve Tregs were significantly decreased in the peripheral blood of the patients with type 1 AIP [28]. We suggest that decreased naïve Tregs may be pathogenetic. We also previously reported that Tregs were increased in the pancreas with type 1 AIP compared with control [28]. In the patients with type 1 AIP, the numbers of infiltrated Tregs were significantly positively correlated with IgG4-positive plasma cells. In type 1 AIP, inducible co-stimulatory molecule (ICOS)⁺ and interleukin-10 (IL-10)⁺ Tregs significantly increased compared with control groups [29]. The ratio of Foxp3-positive cells to infiltrated mononuclear cells and IgG4-positive cells to infiltrated mononuclear cells were positively correlated. We suggest that increased quantities of ICOS⁺ Tregs may influence IgG4 production via IL-10 in type 1 AIP.

Recently, it has been reported that IL-10-producing B cell [30], T cell [28], dendritic cells (DCs) [31] and macrophage [32] are regulatory cells. In our previous studies, Miyoshi et al. [28] reported IL-10-producing Tregs in type 1 AIP and Hoshino et al. [33] reported IL-10-producing DCs in colitis models. Regarding B cells, great attention has been focused on relations between various diseases and IL-10 producing B cells, originally termed as regulatory B cells (Bregs) in mice by Mizoguchi et al. [30, 34-38]. B cells terminally differentiate into plasma cells that are long-lived and secrete pathogenic antibody, promote immune responses by presenting

antigen and providing costimulatory signals, and suppress immune responses through the production of IL-10 [39]. Recent studies suggest that human Bregs, in addition to mice Bregs, may be involved in the development of various autoimmune diseases such as systemic lupus erythematosus (SLE) [38], immune thrombocytopenia (ITP) [35], and sarcoidosis [36]. Recently, two novel phenotypes of human Bregs, CD19⁺CD24^{high}CD38^{high} [38] and CD19⁺CD24^{high}CD27⁺ [40], both of which can secrete IL-10 depending on various stimulations in vitro, have been identified. In IgG4-related disease (IgG4-RD), depletion of B cells by rituximab is effective for treatment [41]. However, it is unclear if Bregs are involved in the development of type 1 AIP. In the present study, to clarify the role of Bregs in the pathophysiology of type 1 AIP, we analyzed circulating Bregs.

2. Methods

2.1. Patients and controls

Peripheral blood was obtained from 21 untreated patients with type 1 AIP, diagnosed according to the ICDC for AIP proposed by the IAP [26], at Kansai Medical University and its affiliated hospitals (7 women and 14 men; mean age 67.0 years; range, 40-78 years). Twenty of the 21 patients were diagnosed as definitive

type 1 AIP, and one patient was diagnosed as probable type 1 AIP. Fourteen patients with alcoholic or idiopathic pancreatitis (8 women, 6 men; mean age, 65.9 years; range, 48-79 years), 20 patients with pancreatic cancer (7 women, 13 men; mean age, 71.3 years; range, 59-86 years), and 25 healthy volunteers (7 women, 18 men; mean age, 65.6 years; range, 39-81 years) served as controls (Table 1). There was no difference in age and sex between type 1 AIP, chronic pancreatitis (CP), pancreatic cancer, and healthy controls (Table 1). IL-10 and each phenotype of Bregs were examined in some of the enrolled subjects; 16 out of 21 type 1 AIP patients and 18 out of 25 healthy volunteers for CD19⁺CD24^{high}CD38^{high} Bregs group, and 12 out of 13 type 1 AIP patients and 10 healthy volunteers for CD19⁺CD24^{high}CD27⁺ Bregs group. All the patients with CP were diagnosed according to the clinical diagnosis criteria of the Japan Pancreatic Society (JPS) [42]. Fifteen patients were histologically confirmed as having adenocarcinoma of the pancreas during surgery or by endoscopic ultrasonography-guided fine-needle aspiration (EUS-FNA), cytology of pancreatic juice, brushing cytology of pancreatic duct, bile cytology, or bile duct biopsy. One patient with peritoneal dissemination of pancreatic cancer and 3 patients with metastatic liver cancer were diagnosed as pancreatic cancer by the ascites cytology and liver biopsy. One patient was diagnosed as pancreatic cancer based on the clinical course. This study was approved by the Kansai Medical University's ethics committee, and all patients and healthy volunteers gave informed consents.

Serum IgG4 were significantly increased in type 1 AIP patients (mean 391.8 mg/dl, range 93.1-1010

mg/dl) compared with CP (mean 62.0 mg/dl, range 6.4-147 mg/dl), pancreatic cancer (mean 55.1 mg/dl, range 10.5-244 mg/dl) (Table 1). In this study, one group was studied CD19⁺CD24^{high}CD38^{high} Bregs phenotype and another group was studied CD19⁺CD24^{high}CD27⁺ Bregs phenotype, independently. There was no difference of age, sex, count of B cells, and serum IgG4 in each group of type 1 AIP, CP, pancreatic cancer, and healthy controls between CD19⁺CD24^{high}CD38^{high} Bregs group and CD19⁺CD24^{high}CD27⁺ Bregs group (Table 2).

2.2. Flow Cytometry of Bregs in Peripheral Blood Cells

Flow cytometry was performed according to the method described previously [28, 29, 43]. Briefly, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD38 (eBioscience, San Diego, CA), FITC-conjugated anti-CD27 (eBioscience), phycoerythrin (PE)-conjugated anti-CD24 (eBioscience), and peridinin chlorophyll protein (PerCP)-cyanine (Cy)5.5-conjugated anti-CD19 (eBioscience) at 4C° for 30 minutes. After staining, the cells were fixed in 1% paraformaldehyde, and 3 color flow cytometric analyses were performed using FACS-Caliber (BD Biosciences, Franklin Lakes, NJ).

2.3. Flow Cytometry of Intracellular IL-10

Intracellular staining of IL-10 was performed according to the manufacture instruction and our previous study [29]. The cells were then washed with phosphate-buffered saline before analysis. For

intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate, ionomycin for 4 hours before adding Golgi stop/Golgi plug solution (BD Biosciences). The cells were then stained with FITC-conjugated anti-CD38 (eBioscience), PE-conjugated anti-CD24 (eBioscience), and PerCP-Cy5.5-conjugated anti-CD19 (eBioscience). The cells were then stained with FITC-conjugated anti-CD27 (eBioscience), PE-conjugated anti-CD24 (eBioscience), and PerCP-Cy5.5-conjugated anti-CD19 (eBioscience). After fixation with fixation buffer (eBioscience) for 20 minutes at 4C°, the cells were permeabilized with permeabilization buffer (eBioscience), counterstained with allophycocyanin (APC)-conjugated anti-IL-10 (BD Biosciences) for 30 minutes. Stained cells were analyzed using FACS-Caliber (BD Biosciences).

2.4. Statistical Analysis

For all studies, data are expressed as mean $\pm$ standard deviation (SD); differences were analyzed using Fisher's Protected Least Significant Difference (PLSD), where $P < 0.05$ was considered significant.

3. Results

3.1. The Rate of CD19⁺ B cells in Peripheral Blood

CD19⁺ B cells appear as a subpopulation to upper the right of the major population (Fig. 1a). In peripheral blood, CD19⁺ B cells of type 1 AIP ($14.9\% \pm 6.17\%$) was decreased compared to that of CP ($18.0\% \pm 8.38\%$, $P = 0.07$), but not significantly (Fig. 2a). CD19⁺ B cells were significantly decreased in type 1 AIP patients ($14.9\% \pm 6.17\%$) compared with healthy controls ($18.7\% \pm 6.83\%$, $P < 0.05$), and were significantly increased in type 1 AIP patients ($14.9\% \pm 6.17\%$) compared with pancreatic cancer ($10.8\% \pm 5.29\%$, $P < 0.05$).

3.2. CD19⁺CD24^{high}CD38^{high} Bregs in Peripheral Blood

CD19⁺CD24^{high}CD38^{high} Bregs appear as a subpopulation to the upper right of the major population (Fig. 1b). We measured CD19⁺CD24^{high}CD38^{high} Bregs using the gating illustrated in Fig. 1b, as described in [38]. In peripheral blood, CD19⁺CD24^{high}CD38^{high} Bregs were significantly increased in type 1 AIP patients ($6.44\% \pm 3.19\%$) compared with CP ($4.73\% \pm 2.13\%$, $P < 0.05$), pancreatic cancer ($3.98\% \pm 1.88\%$, $P < 0.01$), and healthy controls ($4.84\% \pm 2.20\%$, $P < 0.05$) (Fig. 2b).

3.3. CD19⁺CD24^{high}CD27⁺ Bregs in Peripheral Blood

CD19⁺CD24^{high}CD27⁺ Bregs appear as a subpopulation to the upper right of the major population (Fig. 1c). We measured CD19⁺CD24^{high}CD27⁺ Bregs using the gating illustrated in Fig. 1c, as described in [40]. Although not significant different, CD19⁺CD24^{high}CD27⁺ Bregs of type 1 AIP ($8.95\% \pm 4.00\%$) were decreased

compared to those of CP ($12.7\% \pm 6.57\%$, $P = 0.10$), pancreatic cancer ($12.5\% \pm 5.96\%$, $P = 0.13$), and healthy controls ($13.3\% \pm 5.47\%$, $P = 0.06$) (Fig. 2c).

The absolute numbers were also evaluated and the results were similar, respectively (Fig. 2).

3.4. *IL-10⁺ B cells in Peripheral Blood*

The population of IL-10⁺ B cells was not significantly different between type 1 AIP ($1.61\% \pm 1.06\%$) and healthy control ($2.09\% \pm 1.43\%$) (Fig. 3a); this suggested that the function of B cells to produce IL-10 was not impaired. CD19⁺CD24^{high}CD38^{high} IL-10⁺ cells in type 1 AIP ($0.036\% \pm 0.027\%$) were not significantly different from those in healthy control ($0.039\% \pm 0.025\%$) (Fig. 3b). CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells in type 1 AIP ($0.29\% \pm 0.19\%$) were not different from those in healthy control ($0.35\% \pm 0.25\%$) (Fig. 3c).

3.5. *Correlation between CD19⁺CD24^{high}CD38^{high} Bregs or CD19⁺CD24^{high}CD27⁺ Bregs and serum IgG4*

In untreated type 1 AIP patients, the numbers of CD19⁺CD24^{high}CD38^{high} Bregs and serum IgG4 were not correlated ($R = 0.39$, $P = 0.09$) (Fig. 4). In the untreated type 1 AIP patients, the numbers of CD19⁺CD24^{high}CD38^{high} IL-10⁺ cells or CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells and serum IgG4 were not correlated (data not shown). CD19⁺CD24^{high}CD38^{high} Bregs or CD19⁺CD24^{high}CD27⁺ Bregs, and serum IgG4 were not correlated in patients with CP and pancreatic cancer (data not shown).

4. Discussion

B cells participate in immune responses through production of antibodies, antigen presentation, cytokine secretion, and T cell activation. IL-10-producing B cells with suppressive functions were found and named Bregs [44]. In 1974, it was reported that B cells suppressed delayed-type hypersensitivity skin responses in guinea pigs; that study showed that B cells had suppressive ability [45]. B cell-deficient mice suffered an unusually severe and chronic experimental autoimmune encephalomyelitis (EAE) [46]. B cells regulate autoimmunity by provision of IL-10 [47]. In mice, Bregs subset was reported as CD1d^{high}CD5⁺CD19⁺ [48]. In humans, IL-10 producing B cells have been studied in multiple sclerosis (MS) [49]. It has been reported CD19⁺CD24^{high}CD38^{high} [38] and CD19⁺CD24^{high}CD27⁺ [40]. It is, however, little known the role of human Bregs in healthy individuals and several diseases.

In the patients with type 1 AIP, we demonstrated a significant decrease and tendency of decrease in CD19⁺ B cells compared with the healthy controls and CP, respectively. Moreover, the patients with pancreatic cancer showed the most decreased CD19⁺ B cells among the other groups. As B cell development in vivo is down-regulated by transforming growth factor (TGF) - β 1, the patients showing increased TGF- β may have

decreased B cells [50]. Indeed, in our previous studies, patients with AIP showed increased subtypes of Tregs, ICOS⁺ Tregs secreting IL-10, and ICOS⁻ Tregs secreting TGF- β [29, 51].

In the present study, CD19⁺CD24^{high}CD38^{high} Bregs were increased in peripheral blood of type 1 AIP patients compared with healthy controls and patients with CP and pancreatic cancer (Fig. 2b). On the other hand, CD19⁺CD24^{high}CD27⁺ Bregs of type 1 AIP tended to be decreased in comparison with those of CP, pancreatic cancer, and healthy control (Fig. 2c). Similarly, an increased ratio of circulating CD19⁺CD24^{high}CD38^{high} Bregs was observed in the active sarcoidosis patients compared to healthy controls and inactive sarcoidosis patients [36], and SLE patients compared to healthy controls or to other arthritic diseases patients [38]. We posit that CD19⁺CD24^{high}CD38^{high} Bregs may have no effect on the initiation of inflammation but may serve as a “rheostat” to fight against inflammation once established. On the other hand, the frequency of circulating CD19⁺CD24^{high}CD38^{high} Bregs was decreased in nonsplenectomized ITP patients with low platelet counts compared to healthy controls [35], suggesting that the role of the Bregs may be different for each diseases. We suggest that the decrease of CD19⁺CD24^{high}CD27⁺ Bregs may be involved in the development of type 1 AIP. IL-10-producing ability of CD19⁺CD24^{high}CD38^{high} Bregs in type 1 AIP and healthy control are equivalent (Fig. 4), so we suggest their function is normal. Although they increase in number, they cannot suppress the disease completely.

IL-10 is an immune regulatory cytokine that has pleiotropic functions, including restraining

inflammation [52]. We focus on IL-10-producing cells as regulatory cells. IL-10 is expressed by many cells of the adaptive immune system; including T helper (Th) 1, Th2 and Th17 subsets, Treg, CD8⁺ T cells and B cells, and by those of the innate immune system; including DCs, macrophages, mast cells, natural killer cells, eosinophils and neutrophils [52-58]. Studies in mice have demonstrated that Bregs play important roles in the suppression of autoimmune diseases such as EAE, but relatively little is known about human Breg cells in healthy individuals and patients [59, 60]. In the present study, we found that IL-10⁺ B cells, CD19⁺CD24^{high}CD38^{high} IL-10⁺ cells, and CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells were not significantly different between type 1 AIP and healthy control. These findings suggest IL-10-producing ability of the patients with type 1 AIP and healthy control are equivalent in B cells, CD19⁺CD24^{high}CD38^{high} Bregs, and CD19⁺CD24^{high}CD27⁺ Bregs. An increased ratio of circulating IL-10⁺ B cells was observed in the active sarcoidosis patients [36]. On the other hand, IL-10⁺ B cells were significantly less in ITP patients with low platelet counts than in healthy controls [35]. An increased ratio of circulating CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells was observed in the autoimmune diseases including SLE, rheumatoid arthritis, Sjögren syndrome, blistering skin diseases, MS patients [40]. Thus, something other than IL-10 that Bregs produce may be involved in mechanism to suppress the diseases. It has been reported that TGF- β -producing B cells [61] and B cells which directly interacted with pathogenic T cells to dampen harmful immune responses were found [62]. We suspect that B cells with immunosuppressive functions consist of some populations.

It is well known that levels of serum IgG4 in type 1 AIP patients are higher than those in patients with other pancreatic diseases or healthy control [3, 4]. In our study, levels of serum IgG4 in type 1 AIP patients are higher than those in patients with CP and pancreatic cancer. In untreated type 1 AIP patients, the number of CD19⁺CD24^{high}CD27⁺ Bregs and IgG4 were positively correlated ($R = 0.63$, $P < 0.05$), however the number of CD19⁺CD24^{high}CD38^{high} Bregs and IgG4 were not correlated ($R = 0.39$, $P = 0.09$). Our results showed that CD19⁺CD24^{high}CD38^{high} Bregs might not mainly influence IgG4 production via IL-10 in type 1 AIP, while CD19⁺CD24^{high}CD27⁺ Bregs might affect IgG4 production. Previously, we reported that ICOS⁺ Tregs might influence IgG4 production in type 1 AIP. On the other hand, Watanabe et al. [63, 64] reported that B cell activating factor (BAFF)-mediated signaling pathways induce T cell-independent IgG4 production either by activation of Toll-like receptors (TLRs) in basophils or by that of nucleotide-binding oligomerization domain 2 (NOD2) in monocytes. These findings lead us to speculate that there are several mechanisms for IgG4 production, although further studies are necessary.

We reported that the MRL/Mp mice was used as AIP model mice, ligation- and ligation+cerulein-treated mice was caused due to inflammation without autoimmune mechanisms, and the Foxp3/mono ration in poli I:C-treated MRL/Mp mice was significantly increased compared with ligation- and ligation+cerulein-treated mice [65, 66]. It was thought that Tregs were possibly involved in the development of immune-mediated pancreatitis in MRL/Mp mice. These findings are similarly observed in the patients with

type1 AIP [67]. As well as Tregs, we think that Bregs may be related to the development of immune-mediated pancreatitis. Therefore, we suggest that CD19⁺CD24^{high}CD27⁺ Bregs might be decreased in relation to the immune mechanism.

We previously proposed a hypothesis of the pathogenesis in type 1 AIP [28, 29, 68, 69], in which the production of IgG4 may be regulated by increased IL-10 secreted from ICOS⁺ effector Tregs, and fibrosis may be regulated by TGF- β secreted from ICOS⁻ Tregs. Taken together, our present study suggested the two subtypes of CD19⁺CD24^{high}CD38^{high} Bregs and CD19⁺CD24^{high}CD27⁺ Bregs showed different roles in the development of type 1 AIP. CD19⁺CD24^{high}CD38^{high} Bregs may be reactively increased like ICOS⁺ Tregs to suppress the disease activity. On the other hand, CD19⁺CD24^{high}CD27⁺ Bregs might be involved in the development of type 1 AIP, although it still remains unclear whether the decrease of CD19⁺CD24^{high}CD27⁺ cells is cause or effect of type 1 AIP (Fig. 5). Further studies are necessary to clarify the role of Bregs in type 1 AIP including the mechanism for IgG4 production on CD19⁺CD24^{high}CD27⁺ Bregs and functions of locally infiltrating Bregs.

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Fig. 1. Phenotypic analysis of peripheral B cell subpopulations. Peripheral blood were stained with CD19, CD24, CD38, and CD27, and gated according to the methods described by Blair PA *et al.* [38, 40] and Iwata Y *et al.* [40]. **(a)** CD19⁺ B cells appear as a subpopulation to upper the right of the major population. **(b)** CD19⁺CD24^{high}CD38^{high} Bregs appear as a subpopulation to the upper right of the major population. We measured CD19⁺CD24^{high}CD38^{high} Bregs using the gating illustrated. **(c)** CD19⁺CD24^{high}CD27⁺ Bregs appear as a subpopulation to the upper right of the major population. We also measured CD19⁺CD24^{high}CD27⁺ Bregs using the gating illustrated.

Fig. 2. The Ratio of CD19⁺ B cells, CD19⁺CD24^{high}CD38^{high} Bregs, and CD19⁺CD24^{high}CD27⁺ Bregs in peripheral blood. **(a)** CD19⁺ B cells of type 1 AIP ($14.9\% \pm 6.17\%$) was decreased compared to that of CP ($18.0\% \pm 8.39\%$, $P = 0.07$), but not significantly. CD19⁺ B cells were significantly decreased in type 1 AIP patients ($14.9\% \pm 6.17\%$) compared with healthy controls ($18.7\% \pm 6.83\%$, $P < 0.05$), and were significantly increased in type 1 AIP patients ($14.9\% \pm 6.17\%$) compared with pancreatic cancer ($10.8\% \pm 5.29\%$, $P < 0.05$). **(b)** CD19⁺CD24^{high}CD38^{high} Bregs were significantly increased in type 1 AIP patients ($6.44\% \pm 3.19\%$) compared with CP ($4.73\% \pm 2.13\%$, $P < 0.05$), pancreatic cancer ($3.98\% \pm 1.88\%$, $P < 0.01$), and healthy control ($4.84\% \pm 2.20\%$, $P < 0.05$). **(c)** There was no significant difference of CD19⁺CD24^{high}CD27⁺ Bregs between type 1 AIP patients ($8.95\% \pm 4.00\%$), CP ($12.7\% \pm 6.57\%$, $P = 0.10$), pancreatic cancer ($12.5\% \pm 5.96\%$, $P = 0.13$), and healthy control ($13.3\% \pm 5.47\%$, $P = 0.06$). Although not significant different,

CD19⁺CD24^{high}CD27⁺ Bregs of type 1 AIP were decreased compared to those of CP, pancreatic cancer, and healthy control. The absolute numbers were also evaluated and the results were similar, respectively.

Fig. 3. The ratio of IL-10⁺ B cells, CD19⁺CD24^{high}CD38^{high} IL-10⁺ cells, and CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells in peripheral blood. **(a)** The population of IL-10⁺ B cells was not significantly different from type 1 AIP (1.61% ± 1.06%) and healthy control (2.09% ± 1.43%). **(b)** The population of CD19⁺CD24^{high}CD38^{high} IL-10⁺ cells was not significantly different from type 1 AIP (0.036% ± 0.027%) and healthy control (0.039% ± 0.025%). **(c)** The population of CD19⁺CD24^{high}CD27⁺ IL-10⁺ cells was not significantly different from type 1 AIP (0.29% ± 0.19%) and healthy control (0.35% ± 0.25%).

Fig. 4. Correlation between CD19⁺CD24^{high}CD38^{high} Bregs and serum IgG4.

In untreated type 1 AIP patients, the number of CD19⁺CD24^{high}CD38^{high} Bregs and serum IgG4 were not correlated ($R = 0.39$, $P = 0.09$).

Fig. 5. Our hypothesis for the pathogenesis of type 1 AIP.

CD19⁺CD24^{high}CD27⁺ Bregs might be involved in the pathogenesis of type 1 AIP. On the other hand, CD19⁺CD24^{high}CD38^{high} Bregs might be involved in the pathophysiology of type 1 AIP but not pathogenic. CD19⁺CD24^{high}CD38^{high} Bregs might not mainly influence IgG4 production via IL-10 in type 1 AIP. Increased numbers of CD4⁺CD25^{high} Tregs may influence IgG4 production in type 1 AIP, whereas decreased numbers of CD4⁺CD25⁺CD45RA⁺ (naïve) Tregs may be involved in the pathogenesis of type 1 AIP [28]. This figure

modified from Okazaki et al. [69]. T_E: effector T cell, nTreg: natural Tregs, NODR: nucleotide-binding oligomerization domain receptor, TLR: Toll-like receptor, Mo: monocytes, Baso: basophils, LF: lactoferrin, CA-II: carbonic anhydrase II, PSTI: pancreatic secretory trypsin inhibitor, PBP: plasminogen binding protein, IFN- γ : interferon- γ , TNF- α : tumor necrosis factor- α , P: pancreas.

Table 1. Clinical profiles and characteristics of the patients and control groups

	Type 1 AIP	CP	Pancreatic cancer	Healthy controls
N	21	14	20	25
Age (years) ^a	67.0 ± 10.6 (40-78)	65.9 ± 9.07 (48-79)	71.3 ± 7.34 (59-86)	65.6 ± 11.0 (39-81)
Sex (Female/Male)	7/14	8/6	7/13	7/18
Serum IgG4 (mg/dl) ^a	391.8 ± 281.9 (93.1-1010)	62.0 ± 43.1* (6.4-147)	55.1 ± 55.7* (10.5-244)	N.D.

^a Values are given as the mean ± standard deviation (SD), with the range in parenthesis

Serum IgG4 were significantly increased in patients with type 1 AIP compared with CP and pancreatic cancer. * $P < 0.01$

There was no difference of age and sex between type 1 AIP, CP, pancreatic cancer, and healthy controls.

AIP: autoimmune pancreatitis, CP: chronic pancreatitis

Table 2. Patients' profiles of CD19⁺CD24^{high}CD38^{high} Bregs groups and CD19⁺CD24^{high}CD27⁺ Bregs groups

	CD19 ⁺ CD24 ^{high} CD38 ^{high} Bregs				CD19 ⁺ CD24 ^{high} CD27 ⁺ Bregs			
	Type 1 AIP	CP	Pancreatic cancer	Healthy controls	Type 1 AIP	CP	Pancreatic cancer	Healthy controls
N	21	14	20	25	13	11	10	10
Age (years) ^a	67.0 ± 10.2 (40-78)	65.9 ± 9.07 (48-79)	71.3 ± 7.54 (59-86)	65.6 ± 11.0 (39-81)	68.4 ± 9.05 (50-78)	65.2 ± 9.87 (48-79)	69.0 ± 7.50 (60-86)	65.7 ± 7.99 (51-76)
Sex (Female/Male)	7/14	8/6	7/13	7/18	5/8	7/4	4/6	3/7
Count of B cells (/μl) ^a	4296 ± 1886 (1514-7794)	5313 ± 2501 (1947-10713)	3438 ± 1688 (875-7483)	5373 ± 2057 (2513-10356)	4766 ± 1799 (2121-7558)	5512 ± 2649 (1988-10698)	2871 ± 1350 (773-4908)	6295 ± 2022 (3467-10132)
Serum IgG4 (mg/dl) ^a	391.8 ± 281.9 (93.1-1010)	62.0 ± 43.1 (6.4-147)	55.1 ± 55.7 (10.5-244)	N.D.	379.0 ± 318.3 (93.1-1010)	56.1 ± 37.4 (6.4-122)	45.8 ± 25.7 (12.7-94.3)	N.D.

There was no difference of age, sex, count of B cells, and serum IgG4 in each of type 1 autoimmune pancreatitis (AIP), chronic pancreatitis (CP), pancreatic cancer, and healthy controls between CD19⁺CD24^{high}CD38^{high} Bregs groups and CD19⁺CD24^{high}CD27⁺ Bregs groups.

^a Values are given as the mean ± SD, with the range in parenthesis

Fig 1

Fig 2

Fig 3

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Fig 5