Expansion and surface makers of age-associated B cells in IgG4-related disease

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Abstract

Objective

To assess the expression of age-associated B cells (ABCs), and characterise the surface markers of ABCs in patients with IgG4-related disease (IgG4-RD).

Methods

Fifty-one newly diagnosed patients with IgG4-RD, 18 IgG4-RD patients with disease remission, 34 patients with other autoimmune diseases, and 61 age- and sex-matched healthy controls (HCs) were included. Circulating ABCs, as well as surface markers were detected by flow cytometry, and tissue infiltration of ABCs were assessed by immunofluorescence (IF). The expression of ABCs in the affected organs of Lat^{Y136F} knock-in (LAT) mouse models (IgG4-RD mouse model) were explored by flow cytometry and IF.

Results

The percentages and absolute numbers of ABCs (gated as CD21-T-bet+CD11c+) in CD19+ B cells raised remarkably in untreated IgG4-RD patients than HC, and reduced significantly after treatment. The percentage of CD27+ABCs, DN2 B cells and activated naive B cells was higher in patients with IgG4-RD than in HCs and patients with multiple autoimmune diseases, whereas the percentage was comparable with that in patients with systemic lupus erythematosus. Phenotypical analysis revealed upregulated levels of CD86, TACI, CD38, and downregulated level of CXCR3 in peripheral CD19+CD21-CD11c+ B cells of IgG4-RD patients compared with that of HC. In IgG4-RD patients, CD19+CD21- CD11c+ cells expressed higher levels of CD80, CXCR3, TACI, CD95, and BAFF-R, while lower levels of CD86, CD27, CD38, and CXCR5 compared with CD19+ CD21- CD11c- B cells. ABCs (CD11c+T-bet+ gated in B220+ cells) were increased significantly in lungs of LAT mice than that of wild type (WT) mice.

Conclusion

ABCs were expanded both in the peripheral blood and affected tissues of patients with IgG4-RD as well as in the lungs of LAT mice, indicating the potential roles of ABCs in IgG4-RD pathogenesis.

Key words

IgG4-related disease, age-associated B cells, surface markers, Lat^{Y136F} knock-in mice

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Introduction

Immunoglobulin (Ig) G4-related disease (IgG4-RD) is a fibroinflammatory disease, characterised by a striking heterogeneity of swollen affected organs, serum IgG4 elevation, and infiltration of IgG4-positive plasma cells in affected organs (1-5). The aetiology of IgG4-RD is unclear, while B cell abnormality plays a central role in the pathogenesis by augmenting presentation of antigens to pathogenic T cells, the excessive production of autoantibodies, cytokines and the virtue of contributing to fibrosis directly (6-9).

Identified in 2011, age-associated B cells (ABCs), a unique B cell population, have high expression of both CD11c and transcription factor (TF) T-bet and low expression of CD21 in aging and autoimmunity10. The ABC subset was first defined by Hao *et al*. (11) as B220+CD19+ splenic cells that lack CD21, CD23, and CD95 and Rubtsov *et al*. (12) as B220+CD19+ splenocytes with CD11c expression. Different from classical B cells, ABCs exhibit a distinctive phenotype and defined as CD11c+ T-bet+ B cells (13). Atypical memory B cells (AtMs), and T-bet+ B cells are different names describing what is likely a similar population, which have been emerged as one of important components of immune responses in autoimmune disorders and infections (10, 14).

Recent studies reported that ABCs played an essential role in lupus development, as the expression of ABCs was associated with high disease activity and disease-specific autoantibodies (15-17). In mice, ABCs (murine AtMs) were reported to arise from follicular B cells, enriched with antichromatin autoantibodies, and deletion of CD11c+ ABCs ameliorated the disease severity in lupus-prone mice (18). In addition, phenotypically similar B cells also expanded in other autoimmune diseases, including rheumatoid arthritis (RA), primary Sjögren's syndrome (pSS), systemic sclerosis, and infections (12, 19-21). These studies demonstrated a common pathogenic role of T-bet+CD11c+ B cells in inflammatory disease in both human and murine models.

It has been revealed that several factors such as Toll-like receptor (TLR) signalling, CD40L, MHC class II obtained by interactions with activated T cells in the germinal centre (12, 22-24) and cytokines of IL-21, IL-27, IFN-γ (22, 25) were implicated in the differentiation of ABCs, indicating that a chronic inflammatory environment promotes ABCs production.

IgG4-RD is a chronic inflammatory autoimmune disease, and no study has yet reported ABCs and their role in IgG4-RD. In this study, we investigated the expression of ABCs in both patients of IgG4-RD and mouse models of IgG4-RD, with a purpose to understand the role of ABCs in the pathogenesis of IgG4-RD.

Methods and materials

Patients and controls

A total of 51 newly diagnosed and untreated patients with IgG4-RD fulfilling 2011 comprehensive IgG4-RD diagnostic criteria were enrolled in this study (26). Patients with recurrent infections, malignancies, other autoimmune diseases, or conditions that could mimic IgG4-RD were excluded. Details regarding about patients' clinical and laboratory parameters were shown in Supplementary Table S1. All enrolled patients fulfilled the 2019 ACR/EULAR criteria (27), and 35 patients (68.6%) had biopsy proof of IgG4-RD and the other 16 patients (31.4%) were diagnosed by clinical surrogate and increased serum IgG4 levels. Thirty-five patients were classified to be definite IgG4-RD in the comprehensive diagnostic criteria (26). Besides, 61 age- and sex-matched healthy controls (HCs) and 18 IgG4-RD patients in remission were also included. Clinical parameters and treatment strategy of 18 patients were listed in Supplementary Table S2. Four submandibular glands (SMGs) from patients with IgG4-RD, one SMG from patients with chronic non-specific sialadenitis (control group) and two labial glands from patients with pSS (control group) were obtained to investigate the infiltration of CD20+Tbet+ cells in involved organs. Five paraffin-embedded biopsy-proven SMGs from patients with IgG4-RD, five labial gland tissue samples

from patients with pSS (control group) and one submandibular gland from patients with chronic non-specific sialadenitis were obtained for IF examination to define ABCs cells.

The definition of disease remission was defined as IgG4-RD responder index (RI) of every single organ ≤1 and glucocorticoid tapered to maintenance dosage (≤7.5 mg prednisone therapy) without relapse (28). All 18 patients after treatment achieved disease remission.

In order to demonstrate whether ABCs could be a biomarker of IgG4-RD, patients with multiple autoimmune diseases were also enrolled. Five types of patients with autoimmune diseases in active disease condition were included as controls to IgG4-RD. ABCs were detected in 4 patients with pSS, 6 patients with dermatomyositis (DM), 10 patients with RA, 7 patients with Castleman disease (CD) and 7 with systemic lupus erythematosus (SLE). All patients fulfilled the corresponding diagnostic criteria.

As double negative (DN) 2 B cells and activated naive B cells also express CD11c and T-bet (29), whether ABCs were switched CD27+ ABCs, DN2 B cells or activated naive B cells was assessed in another 14 untreated IgG4-RD, nine sex-age matched HCs and 27 patients with other autoimmune diseases (4 patients with pSS, 6 patients with DM, 10 patients with RA and 7 with SLE).

The study was approved by the ethnic committee of Peking Union Medical College Hospital (approval no. S-442). Written informed consent was obtained for patients and HCs.

Animal models

LAT mice were obtained from Institute of Laboratory Animal Sciences, Chinese Academy of Medical Science & Peking Union Medical College, according to the previous research (30). LAT mice and Wild type C57BL/6 (WT) mice were maintained in the specific pathogen free (SPF) conditions. LAT mouse was a model for IgG4-RD, which displayed increased production of IgG1 (a homologue of human IgG4) and developed multiple organ tissue lesions (characterised by inflammatory mononuclear cell infiltration and fibrosis in salivary glands, lung, pancreas, and kidneys) from 6-weeks old to 20-weeks old. WT mice were used as controls. LAT mice (n=5, male=3, female=2) and WT (n=5, male=3, female=2) mice were sacrificed at ages of 12 weeks. Lung, spleen, and liver were obtained. All animal experiments were approved by ethnic committee of Peking Union Medical College Hospital (approval no. XHDW-2020-027).

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from IgG4-RD patients and HCs by standard Ficoll-Hypaque procedures. Samples were stained with the following monoclonal antibodies of human (mAbs): phycoerythrin (PE)-conjugated anti-human transmembrane activator and CAML interactor (TACI), PerCP-Cy5.5-conjugated anti-human CXCR5, Alexa Fluor® 647-conjugated anti-human T-bet, fluorescein isothiocyanate (FITC)-conjugated anti-human CD11c, PE-cyanine 7 (Cy7)-conjugated anti-human CD21, PE-conjugated anti-human CD19, PE-Cy7-conjugated anti-human CD19, allophycocyanin (APC)-conjugated anti-human CD80, PE-conjugated anti-human CD95, APC-conjugated anti-human CD40, PE-conjugated anti-human CD86, APC-conjugated anti-human CD38, APC-conjugated anti-human CXCR3, PerCP-Cy5.5-conjugated anti-human CD27, APC-conjugated anti-human HLA-DR, PE-Cy7-conjugated anti-human CD32, PE-conjugated anti-human BCMA, APC-conjugated anti-human/mouse CXCR7, PE-conjugated anti-human B cell activating factor receptor (BAFF-R), APC-conjugated anti-human IL-6, PerCP-Cy5.5-conjugated anti-human TNF-α.

BV421-anti-human CD19, Pecy7-conjugated anti-human CD21, FITC-conjugated anti-human CD11c, APC-conjugated anti-human T-bet, PerCP-Cy5.5-conjugated anti-human CD27; PE-conjugated anti-human IgD; BV650-anti-human CD38- and BV510-anti-human CD24 were stained to differentiate the subtype of ABCs. CD27+ ABCs were defined as CD19+CD21-CD11c+Tbet+CD27+, DN2 B cells were CD19+CD21-CD11c+Tbet+IgD-CD27-CD38-CD24- cells, and activated naive B cells were CD19+CD21-CD11c+Tbet+IgD+CD27-CD38-CD24- cells.

In order to obtain single cell suspensions from the tissues (lung, liver, and spleen) of mice, C Tubes (Miltenyi Biotec), dissociators, in combination with collagenase type I (Sigma-Aldrich) were used. Samples were stained with the following monoclonal antibodies (mAbs): FITC-conjugated anti-mouse B220, Percp-Cy5.5-conjugated anti-mouse CD19, APC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse T-bet.

The APC-conjugated anti-human CD38 mAB was obtained from BD Biosciences (San Jose, CA, USA), and the remaining mABs were purchased from biolegend (San Diego, CA, USA). For surface staining, cells were incubated for 30 minutes at 4°C, then were washed and resuspended in PBS. For intracellular staining, cells were surface stained firstly, and BD perm and fixation buffer were used according to manufactural instructions. All samples were measured by BD FACSAria II system (BD Biosciences). Data were analysed by Flowjo version X software (Flowjo, Ashland, OR, USA).

Prior to performing flow cytometry, freshly isolated PBMCs were preincubated with a dead cell removal kit (no. 130-090-101, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacture's procedures. To validate the efficacy of dead cell removal capacity, the PBMCs were stained with Annexin-V-PE and 7-AAD, and the lymphocytes were gated. The results showed that Annexin-V and 7-AAD double negative lymphocytes were >99% of total cells (Suppl. Fig. S1A).

Cell culture

For intracellular staining of IL-6 and TNF-α, PBMCs isolated from IgG4-RD patients and HCs, were activated by 2.5 μg/ml cytosine-phosphate-guanine oligodeoxynucleotide (CpG) (Invivogen, Carlsbad, CA, USA) in RPMI-1640 medium supplemented with 10% foetal bovine serum and antibiotics at 37°C. Two hours later, PMA,

ionomycin and golgi-stop were added to the culture medium. With another 4 hours, PBMCs were collected for intracellular staining.

Immunofluorescence

Formalin-fixed, paraffin-embedded samples (SMGs and labial glands from human) were cut into consecutive 3-um-thick sections. To define CD20+Tbet+ cells, rabbit anti-human T-bet (ab154200, Abcam) and rabbit anti-human CD20 (ab9475, Abcam) were used. Slides were stained with rabbit anti-human CD19 (clone 6OMP31, Invitrogen), mouse anti-human T-bet (clone eBio4B10, Invitrogen), and rabbit anti-human CD11c (clone D3V1E, Cell Signaling Technology).

Lung tissues from mice were fixed in 4% formalin and embedded in paraffin. Samples were sectioned with largest cut surface to obtain the entire field of view and prepared for haematoxylin-eosin (HE) staining. Slides were stained with goat anti-mouse IgG1 (abcam) and then sequentially incubated with biotinylated goat anti-rabbit IgG secondary antibodies and streptavidin-conjugated HRP according to conventional immunohistochemical methods. For immunofluorescence (IF), the primary antibodies were used: rabbit anti-mouse CD11b, rabbit anti-mouse B220 (CD45) from Servicebio.

DAPI was used as the nuclear counterstain. IFC images were acquired and merged by EVOS FL Auto 2 Imaging System (ThermoFisher Scientific). Positive cell counting was performed using ImageJ software using the 'analyse' function applied to rectangles of equal area.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v. 24.0 software (IBM, Armonk, NY, USA). Normally distributed data were presented as the mean ± SD and analysed using the Student's t-test, and continuous non-normally distributed data were presented as median (first quartile, third quartile) and assessed by Mann-Whitney U-test. Comparison of mean values between the groups was performed using one-way analysis of variance or Kruskal-Wallis test. Correlations between variables were determined using Pearson's Rank correlation test or Spearman's rank correlation test. A p-value <0.05 was considered significantly different.

Results

Aberrant expansion of ABCs in IgG4-RD

The representative gating strategies of T-bet, CD11c, CD21- in CD19+ B cells and ABCs in PBMCs isolated from HC and patients with IgG4-RD patients before and after treatment were shown in Figure 1A and Supplementary Figure S1B. We firstly assessed the expression level of T-bet on B cells, which showed that the percentages of T-bet+ cells in CD19+ B cells of newly diagnosed IgG4-RD patients were significantly higher than that of HC (9.59%±7.85% *vs*. 4.95%±3.96%, p<0.0001, Fig. 1B). Meanwhile, flow cytometry also revealed that the proportions of CD21- cells (17.3%±10.4% *vs*. 8.77%±4.01%, p<0.0001), as well as the percentages of CD11c+ cells (6.44%±4.51% *vs*. 3.87%±3.63%, p=0.007) in CD19+ B cells increased dramatically in untreated IgG4-RD patients compared with HC (Fig 1C-D). The percentage of CD21-CD11c+ cells in CD19+ B cells were increased in IgG4-RD before treatment (4.11%±3.70%) compared with that in HCs (1.86%±2.01%), p<0.001(Fig 1E). In addition, the proportions of CD21-T-bet+ cells in CD19+CD11c+ cells were significantly altered in untreated IgG4-RD compared with HC (53.0%±16.4% *vs*. 41.7%±18.2%, p=0.008, Fig. 1F). Finally, the percentages of ABCs (gated as CD21-T-bet+CD11c+) in CD19+ B cells raised remarkably in untreated IgG4-RD patients than that in HC (3.65%±3.20% *vs*. 1.38%±1.09%, p<0.0001, Fig 1G).

Then, we explored the clinical relevance of ABCs in IgG4-RD was explored. In the context of disease remission, the expression level of T-bet in CD19+ B cells (9.59%±7.85% *vs*. 4.94%±2.79%, p=0.017, Fig 1B), the percentages of CD21-T-bet+ cells in CD19+CD11c+ cells (53.0%±16.4% *vs*. 19.6%±14.6%, p<0.0001, Fig 1F), and the proportion of ABCs in CD19+B cells (3.65%±3.20% *vs*. 1.91%±1.74%, p=0.031, Fig 1G) reduced significantly after treatment, whereas the expression levels of CD21- and CD11c+ in CD19+ B cells and CD21-CD11c+ cells in CD19+ B cells were comparable before and after treatment (Fig. 1C, D and E).

The absolute number of B cells in treatment naive patients with IgG4-RD (184,381/mL ±119,246/mL) was lower than that in HCs (236,203/mL ±92,893/mL), but the number increased after treatment (242,115/mL±169,068/mL), p=0.01 and p=0.024, respectively (Suppl. Fig. S2A). CD19+T-bet+ cell numbers were increased in IgG4-RD before treatment (16,073/mL±16,520/mL) compared with those in HCs (10,476/mL±8,356/mL), p=0.023; but there was a decreasing trend after treatment (9,486/mL±6,010/mL), p=0.105 (Suppl. Fig. S2B). CD19+CD11c+ and CD19+CD21- cell numbers were higher in IgG4-RD before treatment (10,577/mL±9,034/mL, 29,452/mL±26,451/mL, respectively) than in HCs (7,266/mL±4,279/mL, 19,336/mL±9,111/mL, respectively), p=0.013 and p=0.006 respectively (Suppl. Fig. S2C-D). CD19+CD11c+ cells and CD19+CD21- cells were comparable in IgG4-RD before and after treatment, p=0.213 and p=0.601 respectively. Compared with those in HCs (2,801/mL±1,780/mL), CD19+CD21-CD11c+T-bet+ ABCs in IgG4-RD were increased before treatment (6,112/mL±7,479/mL) and decreased after treatment (2,746/mL±2,382/mL), p=0.001 and p=0.007, respectively (Suppl. Fig. S2E). From our present study, the changes of ABCs were comparable among IgG4-RD patients of different treatment regimens.

Of note, correlation analysis revealed that the percentages of ABCs correlated positively with serum IgG4 levels (r=0.467, p=0.007, Suppl. Fig. S3). These findings provided a role of ABCs in the pathogenesis and suggested ABCs as a potential biomarker of IgG4-RD.

Evaluation of ABCs, switched CD27+ ABCs, activated naive B cells and DN2 B cells in IgG4-RD and multiple autoimmune diseases

The gating strategy of switched CD27+ ABCs, activated naive B cells

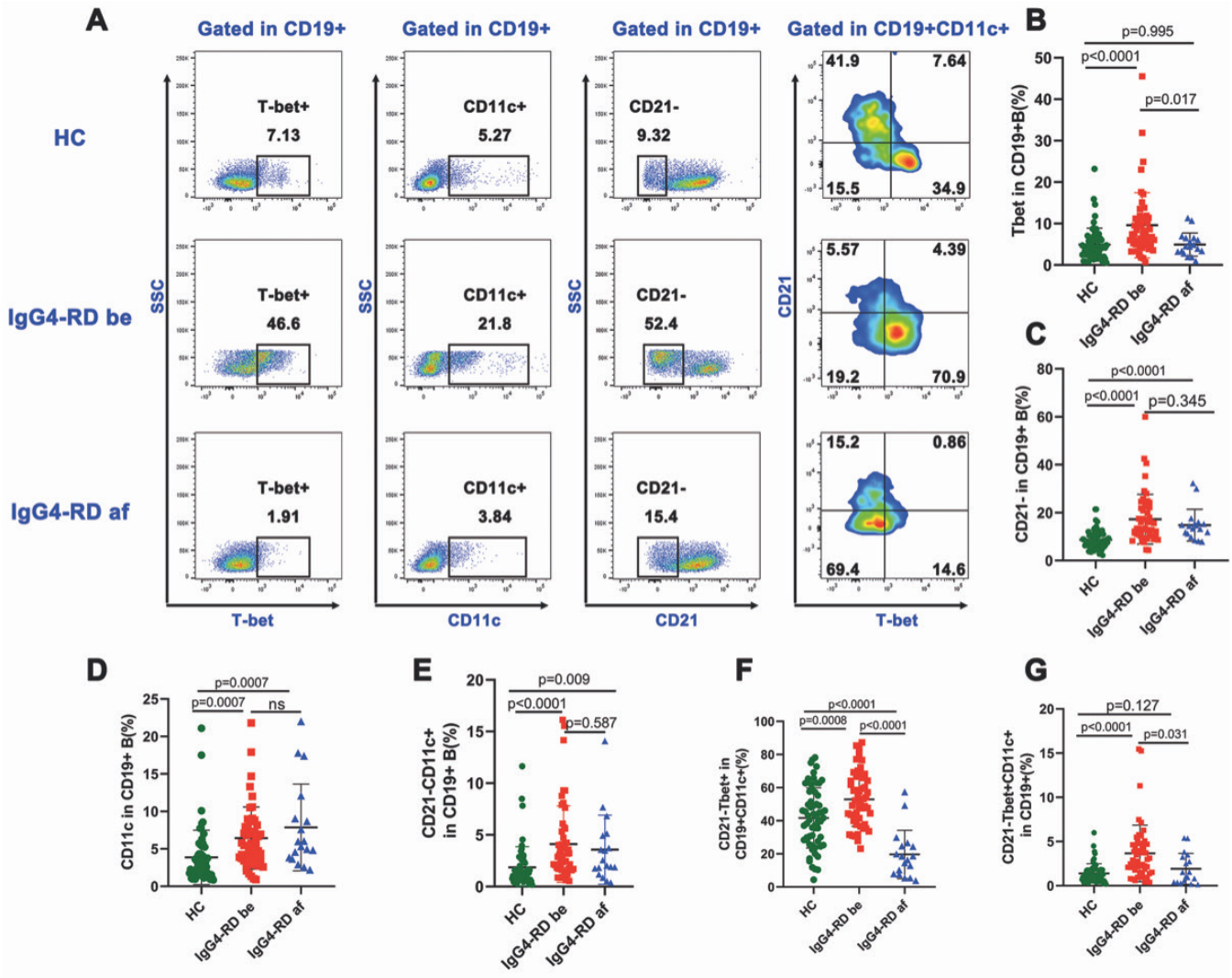

Fig. 1. Age associated B cells in peripheral blood mononuclear cells of patients with IgG4-RD.
A: Gating strategies of T-bet, CD11c in B cells and age associated B cells in PBMCs of HC, IgG4-RD patient before and after treatment.
B to **D**: T-bet, CD21- and CD11c+ cells in CD19+B cells respectively.
E: Percentage of CD21-CD11c+ cells in CD19+ B cells.
F: Percentage of CD21-Tbet+ in CD19+CD11c+ cells.
G: CD21-Tbet+CD11c+ cells in CD19+ B cells. HC, healthy controls; IgG4-RD be, IgG4-RD before treatment; IgG4-RD af, IgG4-RD after treatment; ns, not statistically significant.

and DN2 B cells were shown in Figure 2A. The results revealed that the percentage of CD21-T-bet+ cells in CD19+CD11c+ cells were higher in IgG4-RD (53.0%±16.4%) than in pSS (33.8%±9.1%), DM (30.1%±13.2%), RA (33.3%±10.7%) and CD (31.2%±19.8%) (Fig. 2B), p=0.025, p=0.002, p=0.001 and p=0.002, respectively (Fig. 2B). However, the percentage of CD21-T-bet+ cells in CD19+CD11c+ cells were comparable between IgG4-RD and SLE (57.0%±8.8%), p=0.477 (Fig. 2B). ABCs was higher in IgG4-RD than in multiple autoimmune diseases evaluated in the present study.

The percentage of CD27+ ABCs was higher in patients with untreated IgG4-RD (2.92%±2.04%) compared with HCs (0.86%±0.51%), pSS (0.83%±0.43%), DM (0.28%±0.25%) and RA (0.75%±0.44%), p=0.008, p=0.033, p=0.006 and p=0.003, respectively (Fig. 2C). There was no statistical difference of CD27+ ABCs in IgG4-RD and SLE (1.33%±0.57%), p=0.061. The percentage of activated naive B cells was higher in untreated IgG4-RD (0.67%±0.48%) than that in DM (0.07%±0.10%) and RA (0.27%±0.24%), p=0.007 and p=0.02, respectively, and a trend to be higher than HCs (0.34%±0.29%) and pSS (0.29%±0.15%), p=0.07 and p=0.143 (Fig. 2D). The percentage of activate naive B cells was comparable in SLE (0.51%±0.37%) and IgG4-RD, p=0.441. Besides, the percentage of DN2 cells was higher in IgG4-RD (2.05%±2.25%) compared with HCs (0.52%±0.34%), DM (0.24%±0.20%) and RA (0.69%±0.43%), p=0.028, p=0.001 and p=0.022, respectively (Fig. 2E). Compared with pSS (0.73%±0.55%) and SLE (1.62%±1.28%), the level of DN2+ B cells was not significantly different in IgG4-RD, p=0.277 and p=0.660, respectively.

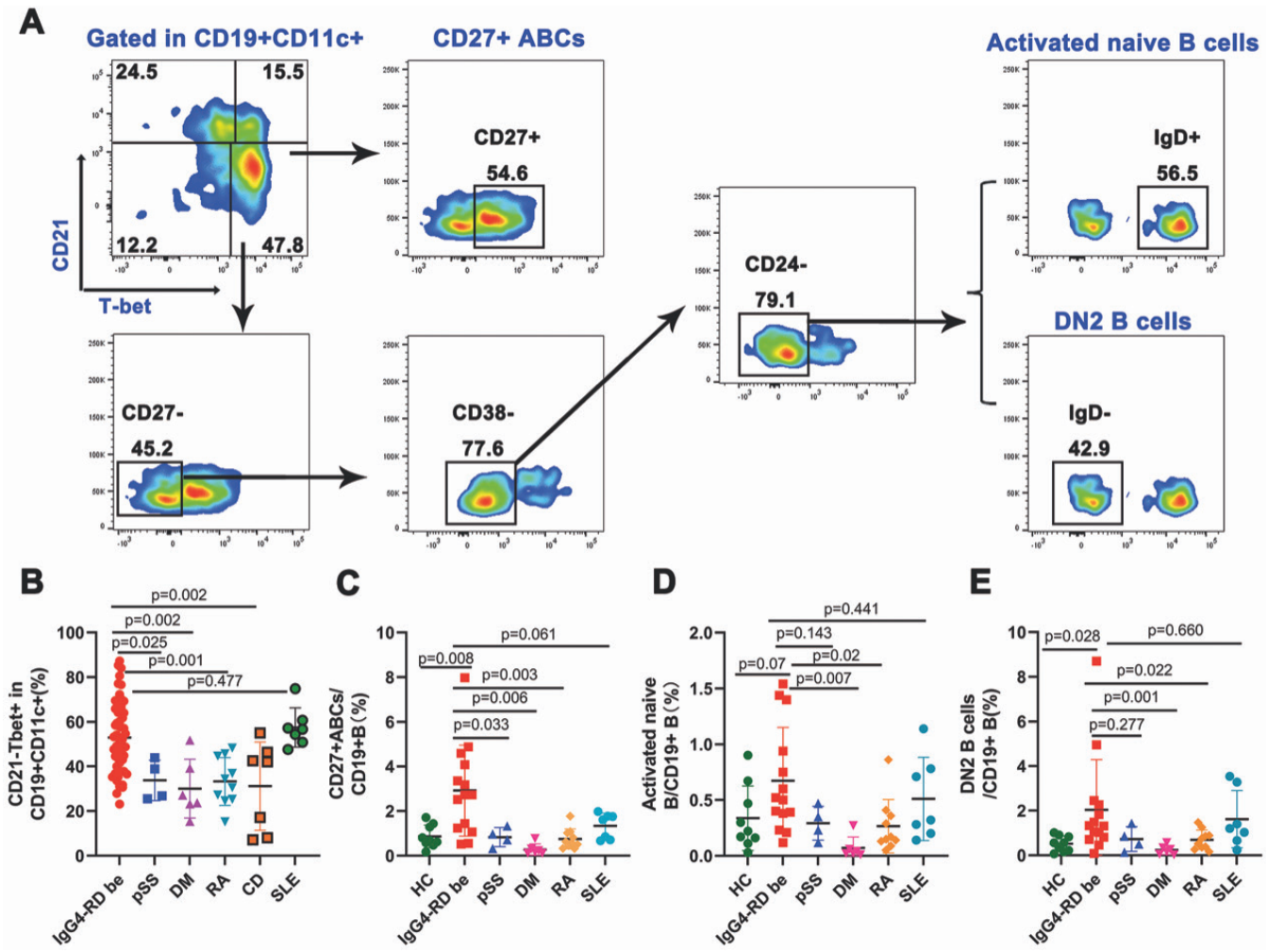

Fig. 2. Comparison of ABCs, CD27+ ABCs, DN2 B cells and activated naive B cells in IgG4-RD and other autoimmune diseases.
A: Gating strategy of CD27+ ABCs, activated naive B cells and DN2 B cells.
B: ABCs in patients with IgG4-RD (n=14), pSS, DM, RA, CD and SLE.
C to **E**: CD27+ ABCs, activated naive B cells and DN2 B cells in IgG4-RD, pSS, DM, RA and SLE. IgG4-RD be, IgG4-RD before treatment; IgG4-RD af, IgG4-RD after treatment.

Expression of surface markers and intracellular cytokines on CD19+CD21-CD11c ± cells

Wang *et al*. revealed that CD11chi cells gated in CD19+CD21- cells are mainly T-bet+ cells (17). The result of our present study was consistent with the Wang *et al*. study (Suppl. Fig. S5A). Thus, we focused on disparities of cell surface markers expressed in CD19+CD21-CD11c+ cells and CD19+CD21-CD11c- cells (gating strategy is shown in Suppl. Fig. S1C and Fig. 3A). In order to explore the characteristics of ABCs in IgG4-RD patients, we elucidated the expression of conventional costimulatory factors and chemokine receptors between CD11c+ B cells and CD11c- B cells in CD19+CD21- B cells of newly diagnosed treatment naive IgG4-RD patients (Fig. 3). Particularly, phenotype analysis revealed that higher levels of CD80 (Median fluorescence intensity 1800.1±1057.2 *vs*. 1258.9±393.4, p=0.032), CXCR3 (37.4%±11.8% *vs*. 23.4%±12.0%, p=0.009), TACI (11.2%±3.03% *vs*. 6.92%±4.99%, p=0.014), CD95 (81.5%±8.80% *vs*. 61.0%±15.9%, p=0.011) and BAFF-R (88.4%±7.78% *vs*. 56.0%±11.0%, p<0.0001) were expressed in CD11c+ cells than that in CD11c- cells of IgG4-RD patients. Whereas, lower levels of CD86 (19.0%±8.18% *vs*. 42.6%±20.8%, p=0.0014), CD27 (25.3%±8.92% *vs*. 36.5%±13.5%, p=0.025), CD38 (23.6%±5.36% *vs*. 55.4%±13.4%, p<0.0001) and CXCR5 (14.0%±7.75% *vs*. 36.2%±15.9%, p=0.0003) was observed in CD11c+ cells than in CD11c- B cells. While, the percentages of BCAM, HLA-DR, and CD32 were comparable between the two groups. The above results revealed the unique characteristics of CD11c+ B cells in CD19+CD21- B cells of patients with IgG4-RD.

Furthermore, we compared the molecular features of CD19+CD21-CD11c+ B cells were compared between HC and patients with IgG4-RD (Fig. 4). Flow cytometry analysis showed upregulated levels of CD86 (19.0%±8.18% *vs*. 12.2%±3.28%, p=0.01), TACI (11.2%±3.03% *vs*. 8.63%±2.82%, p=0.045), and CD38 (23.6%±5.36% *vs*. 12.1%±2.91%, p<0.0001) and downregulated levels of CXCR3 (37.4%±11.8% *vs*. 48.8%±11.6%, p=0.02) in circulating CD19+CD21-

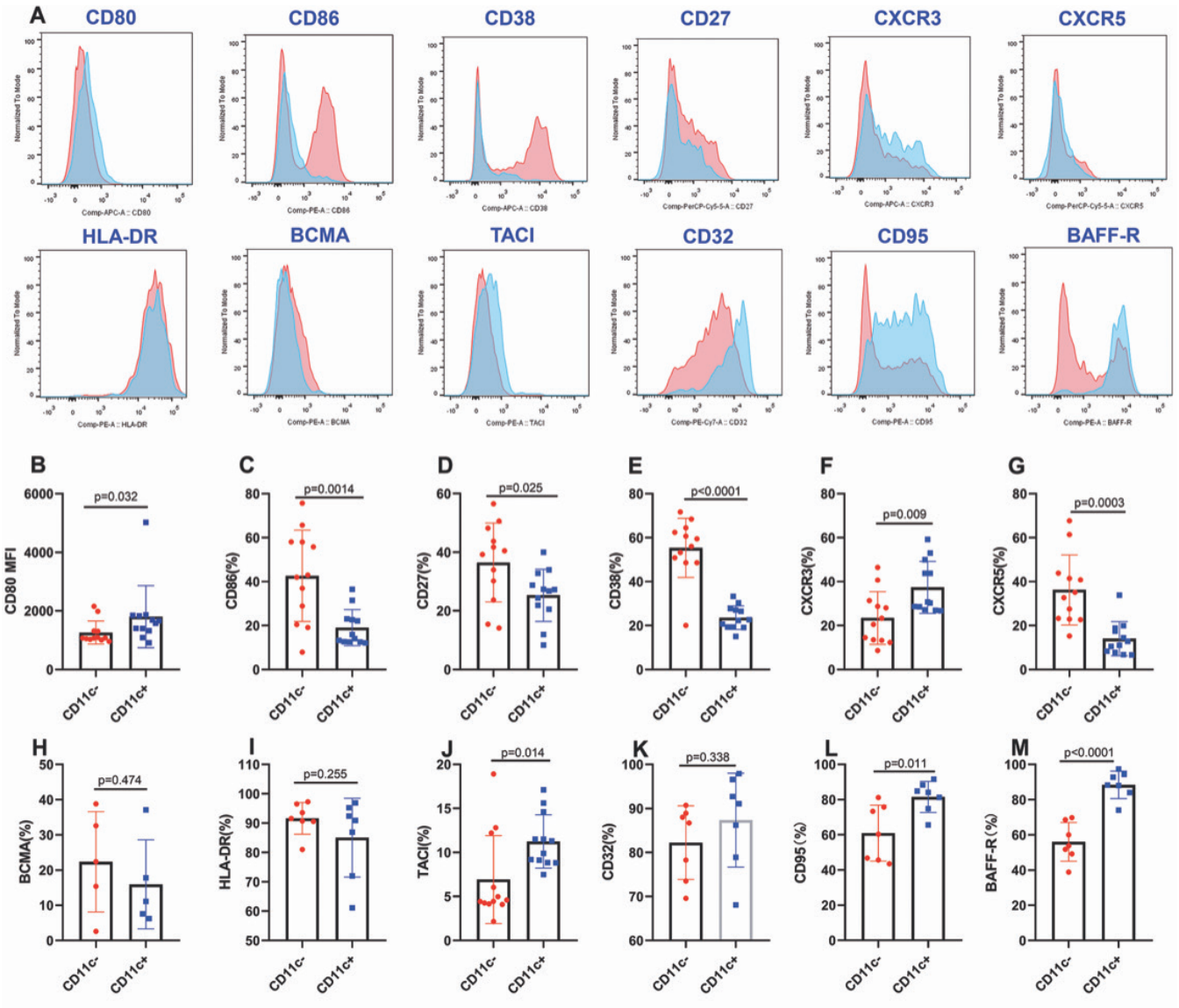

Fig. 3. Surface markers expressed in CD19+CD21-CD11c- and CD19+CD21-CD11c+ cells of PBMCs in patients with IgG4-RD.
A: Representative expression of surface makers in CD19+CD21-CD11c- (red) and CD19+CD21-CD11c+ cells (blue) in IgG4-RD patients.
B to **M**: The comparisons of CD80 MFI, percentages of CD86, CD27, CD38, CXCR3, CXCR5, BCMA, HLA-DR, TACI, CD32, CD95 and BAFF-R+ cells between CD19+CD21-CD11c- and CD19+CD21-CD11c+ cells.

CD11c+ B cells in IgG4-RD compared with HC. By contrast, the expression levels of CD27, CD40, CD95, CD32, CXCR7, BAFF-R and the intracellular cytokines, IL-6 and TNF-α in CD19+CD21-CD11c+ B cells were comparable between IgG4-RD patients and HCs (Suppl. Fig. S4).

Surface markers expression on ABCs of IgG4-RD and HCs

CD38 expression was higher in CD19+CD21-CD11c+T-bet+ ABCs of patients with IgG4-RD (29.28%±12.88%) than of HCs (14.28%±9.20%), p=0.007; whereas IgD expression was lower on the ABCs of patients with IgG4-RD (27.03%±13.50%) compared with HCs (46.46%±9.18%) (Suppl. Fig. S5B-C), p=0.001. The expression of CD24 and CD27 was comparable in IgG4-RD and HCs (Suppl. Fig. S5D-E).

ABCs in affected organs of IgG4-RD patients and LAT mouse models

In parallel, flow cytometry was performed to assess the expression of ABCs in the affected organs of LAT mice. The representative gating image was shown in Figure 5A and Supplementary Fig. S1D. Briefly, the percentages of T-bet+ cells in B220+ cells dramatically increased both in lung (7.03%±1.55% *vs*. 2.17%±1.05%, p=0.001) and liver (11.7%±3.15% *vs*. 4.52%±1.59%, p=0.002) tissues, while there was no difference in the percentage of cells in the spleen (6.07%±4.09% *vs*. 2.0%±0.73%, p=0.056, Fig. 5B) of LAT mice compared with WT mice. Besides, the percentages of CD11c+ B cells (7.03%±1.55% *vs*. 2.17%±1.05%, p=0.0004) and CD11c+T-bet+ B cells (2.49%±0.33% *vs*. 0.61%±0.46%, p<0.0001) in B220+ cells significantly raised in the lung tissues of LAT mice compared with WT mice (Fig. 5C-D). By contrast, there was no sig-

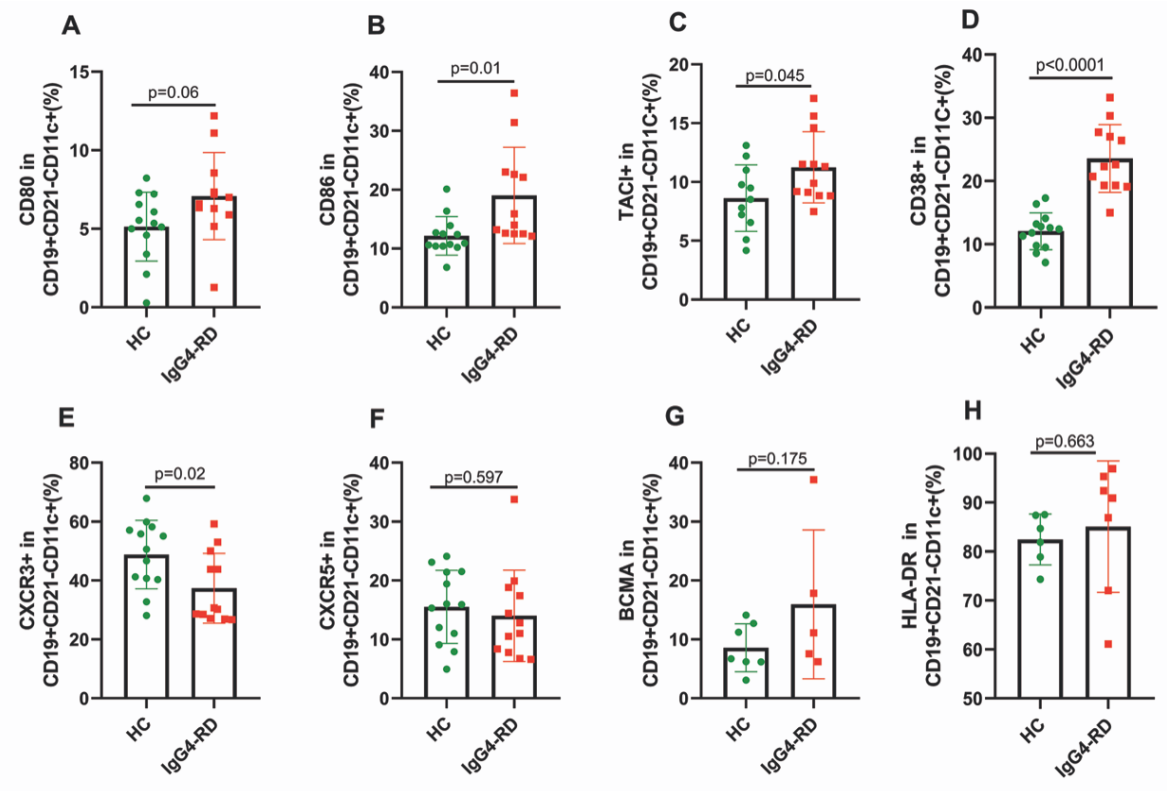

Fig. 4. Surface markers expressed in CD19+CD21-CD11c+ cells of PBMCs in IgG4-RD and HCs.
A to **H**: Percentages of CD80, CD86, TACI, CD38, CXCR3, CXCR5, BCMA and HLA-DR+ cells in CD19+CD21-CD11c+ cells of IgG4-RD patients and HCs respectively.

nificant difference in the proportions of CD11c+ B cells and CD11c+T-bet+ B cells in B220+ cells in liver and spleen between WT and LAT mice.

In triple-fluorescence immunostaining, CD20 (blue staining) and T-bet (red staining) were used to define CD20+Tbet+ cells. CD20+Tbet+ cells in IgG4-RD were remarkably higher than that in control group (20.85%± 8.08% *vs*. 0.0%± 0.0%, *p*=0.007) (Suppl. Fig. S6 and S7A). In tetra-fluorescence immunostaining, the markers CD11c, T-bet, and CD19 were used to characterise the localisation of ABCs in the submandibular glands of patients with IgG4-RD (green, pink and red immunostaining, respectively, Fig 6). DAPI was used to mark the cell nucleus (blue immunostaining). Labial glands derived from patients with pSS were used as controls. The results revealed that CD19+CD11c+T-bet+ cells were much more frequent in the affected organs of patients with IgG4-RD than that of patients with pSS (no ABCs found in pSS), 0.77%± 0.75% *vs*. 0.0%± 0.0%, *p*=0.049 (Suppl. Fig. S7B).

The lung tissues from LAT mice were examined histopathology and immunohistochemistry (IHC), which displayed massive inflammatory cells distributions and numerous IgG1 positive cells compared with WT mice, respectively (Fig. 7A-D). Notably, multiplex immunofluorescence microscopy revealed the colocalisation of CD11b and B220 (markers of ABCs) were much more frequent in the lungs of LAT mice than WT mice (Fig. 7E-L).

Discussion

Collectively, here we first described a population of ABCs presented in both patients with IgG4-RD and mouse models. The current study advanced our understanding of B cell phenotype, and exhibited a potential contribution of ABCs to the pathogenesis of IgG4-RD.

ABCs were generally considered as a population that uniquely originated from self-reactive naive B cells, and was independent of germinal centre origin (15, 31). In the context of chronic stimulation, ABCs could abundantly expand, efficiently mobilise into plasma cells, highly produce pathogenic autoantibodies, and form spontaneous germinal centres (16, 32). Meanwhile, ABCs expansion was widely observed in autoimmune disease, and was suggested to be significantly correlated with anti-dsDNA levels, as well as low complement in SLE (15). Consistently, our results also revealed that the proportion of ABCs increased in active patients but decreased in patients with disease remission, and was positively correlated with serum IgG4 level. Although, there was no correlation between the percentage of ABCs and IgG4-RD RI, we reasonably speculated the pathogenic role of ABCs in IgG4-RD by contributing to disease activity and participating in the process of autoreactive antibodies secretion.

As extrafollicular antibody secreting cell precursors (29), DN2 cells were derive from naive cells and were poised to generate plasmablasts. DN2 cells, which express CD11c, T-bet and hyper-responsive to TLR7, had been shown to predominate in SLE patients with active nephritis (33). Activated na-

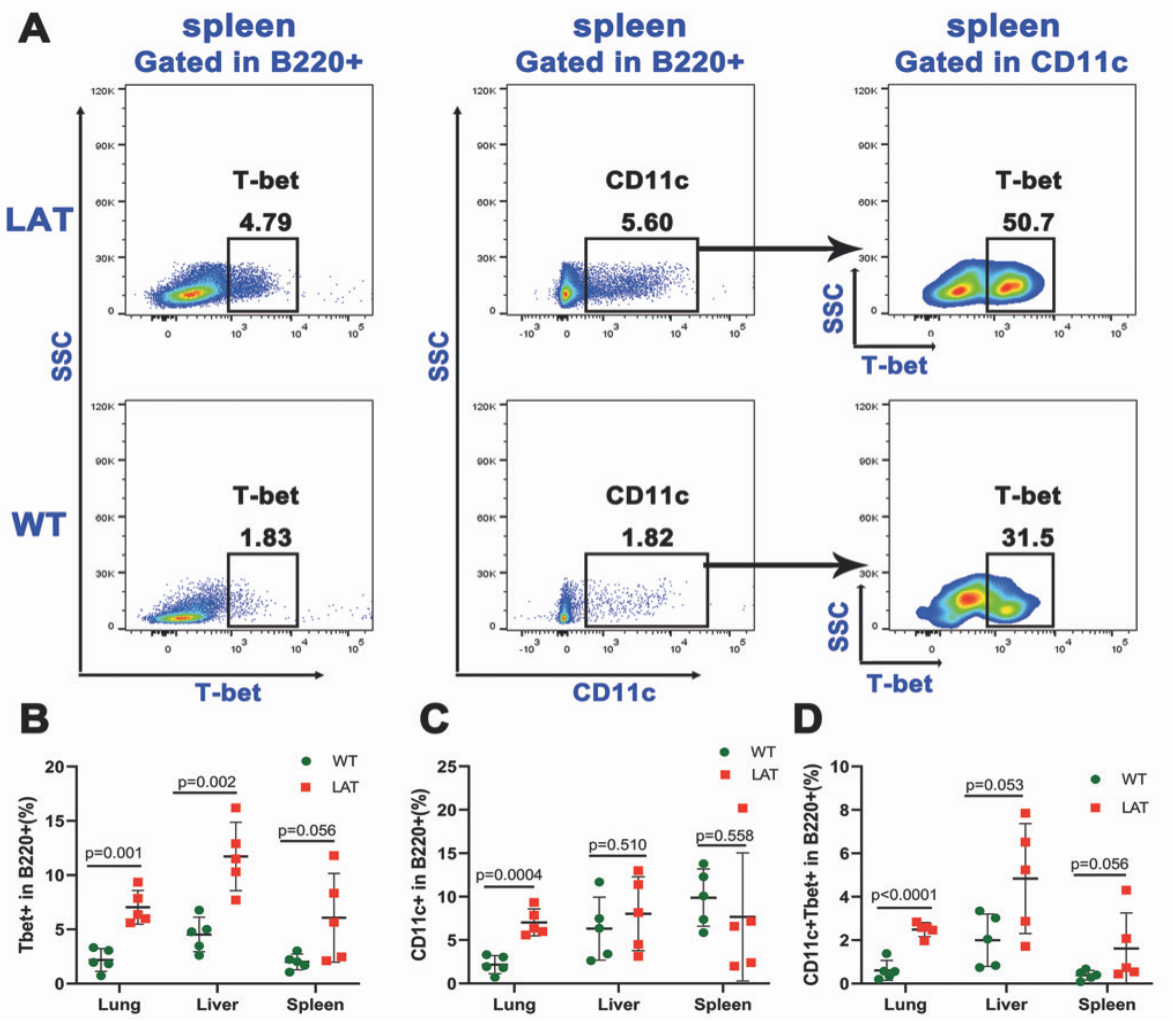

Fig. 5. Age associated B cells in affected organs of LAT mouse models.
A: Gating strategies of T-bet+ cells and CD11c+ T-bet+ cells of B220+ cells in spleen of LAT mouse and WT mouse respectively.
B to **D**: T-bet+ cells, CD11c+ cells and CD11c+T-bet+ cells in B220+ cells of lung, liver, and spleen in WT and LAT mouse models respectively.

ive B cells, recognised as precursor of short-lived plasmablast, participated in germinal centre reactions (29). In SLE, activated naive B cells were also elevated (34). A previous study revealed that CD19+CD24-CD38hi plasmablast/plasma cells, which participated in IgG4 production, were increased in IgG4-RD (8). In this study, except for ABCs elevation, DN2 B cells and activated naive B cells were also elevated, indicating that those autoreactive B cells involved in autoreactive antibody production in IgG4-RD. TLR7, IL-21 and IFN-γ could promote the formation of autoreactive germinal centres and the development of ABCs/DN2 B cell subset (35). The underling mechanism of ABCs development in IgG4-RD needs further investigation. Out study revealed that a proportion of CD19+CD21-T-bet+CD11c+ ABCs include DN2 B cells and activated naive B cells, which consistent with the existing evidence that T-bet and CD11c upregulation and CD21 downregulation occur in multiple B cell subsets (29, 36). Cells defined as ABCs probably consist several B cell types rather than being a uniform subset, which reflects further studies about definitions of ABCs and their markers.

We further demonstrated the functional markers specially expressed on ABCs in IgG4-RD, and found that the peripheral CD19+CD21-CD11c+ B cells expressed higher levels of CD95, and BAFF-R compared with CD19+CD21-CD11c- B cells in our study. This phenomenon was in accordance with the phenotype of CD11c hi B cells described previously in SLE patients and aged mice (11), indicating the unique characteristics and function of this cell type. In particular, TACI was one of the receptors of BAFF and was a proliferation-inducing ligand (APRIL), which regulated the expressions of crucial molecules, like Blimp-1 and ICOSL, inhibited B cell expansion, promoted the differentiation and survival of plasma cells, and induced IgG and IgA class switch recombination (37, 38). Mutations in TACI alleles were reported to increase susceptibility to autoimmune diseases and overly strong or very weak TACI signal in mice could disrupt the balance of immune system and promote the development of SLE (39, 40). In terms of CD27, it was a differentiation antigen, was induced after the activation of naive B cells and upregulated in the process of memory B cells differentiating into plasma cells. Interactions

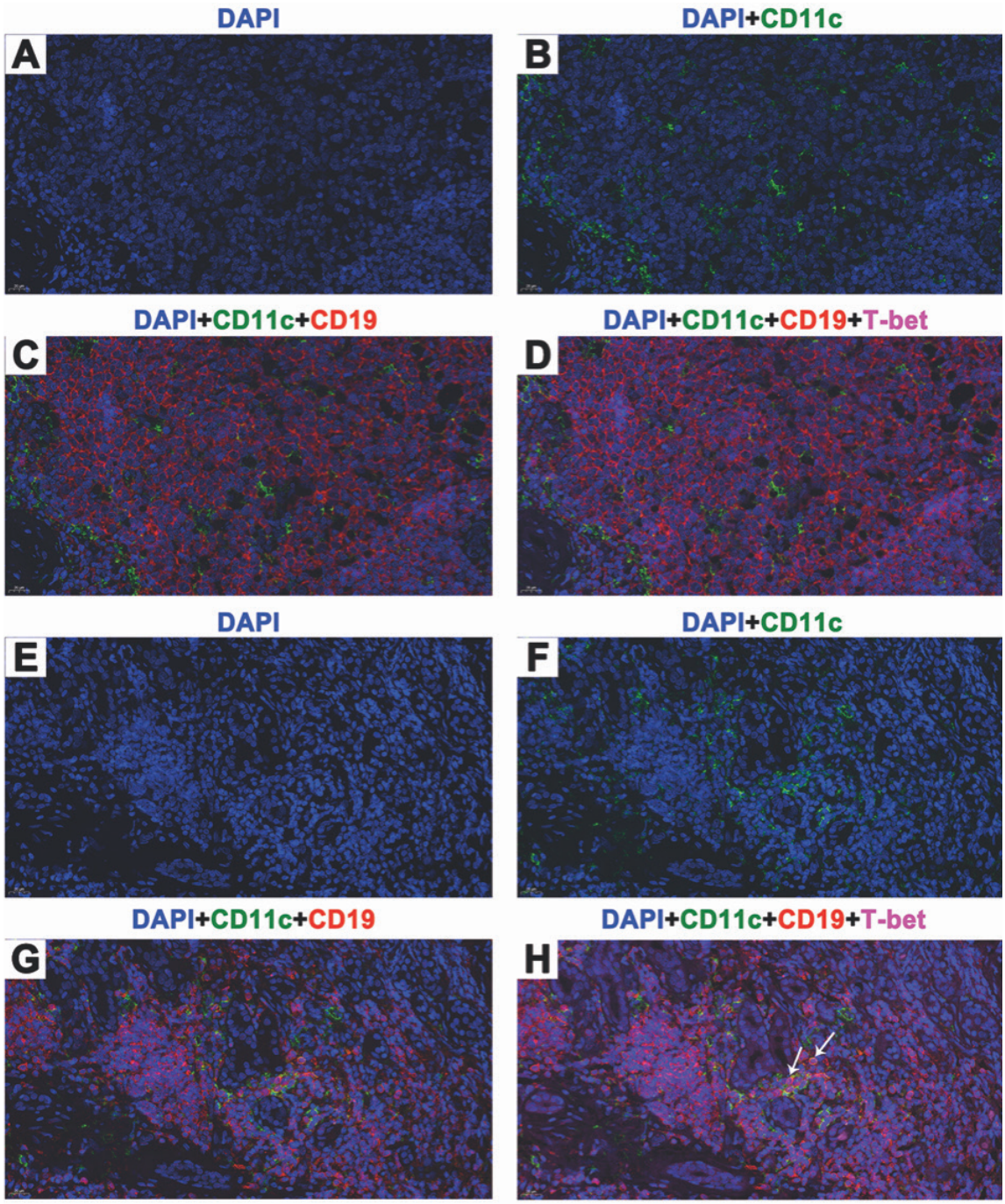

Fig. 6. Histological staining of CD20+T-bet+ cells in affected organs of patient with IgG4-RD and controls.
A to **D**: DAPI, DAPI+CD11c, DAPI+CD11c+CD19 and DAPI+CD11c+CD19+T-bet in a patient with pSS.
E to **H**: DAPI, DAPI+CD11c, DAPI+CD11c+CD19 and DAPI+CD11c+CD19+T-bet in a patient with IgG4-RD. DAPI was shown in blue, CD11c in green, CD19 in red and T-bet in pink.

between CD27 on B cells and CD70 on activated T cells regulated B cell activation, differentiation as well as antibody production, while downregulated CD27 expression on B cells (17).

The presence of CD19+CD11c+T-bet+ cells in the affected organs of IgG4-RD indicated that this population of cells not only existed in the circulation, but also had the capacity to migrate into target tissues, and promoted us to focus on chemokine receptors and migration capabilities. Our results showed that CXCR3 was upregulated but CXCR5 was downregulated in CD11c+ B cells compared with CD11c- B cells in CD19+CD21- B cells of IgG4-RD. CXCR3 was reported to be associated with plasmablasts and IgA antibody secreting cells that migrating to the centre of inflammation and mucosa (41), the higher expression level of CXCR3 might be conductive to the recruitment of ABCs in inflamed tissues. Conversely, CXCR5 was the maker for cells to migrate into lymphoid organs, and the lower expression level of CXCR5 sug-

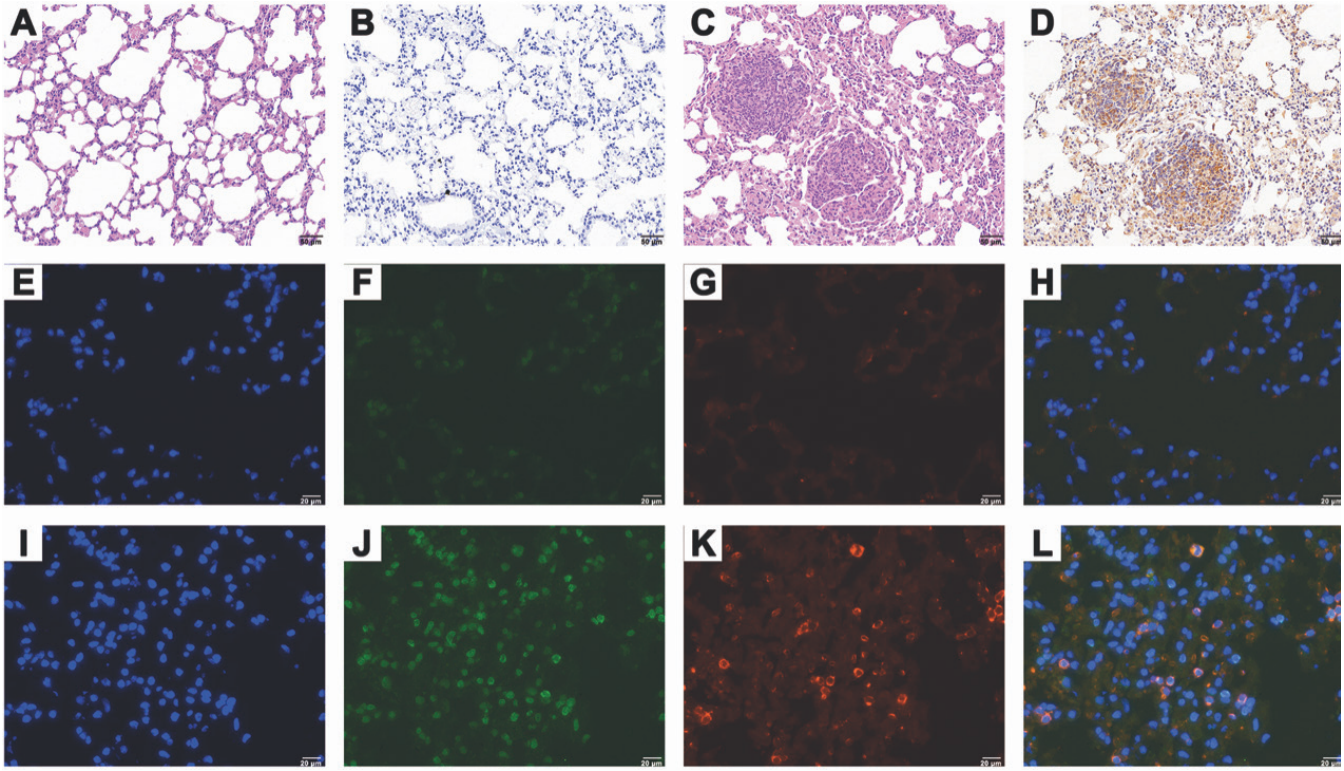

Fig. 7. Histological impairment and infiltration of CD11b+ B220+ age-associated B cells in lungs of WT and LAT mice models.
A and **C**: HE staining in lungs of WT and LAT mice respectively.
B and **D**: IgG1 staining in lungs of WT and LAT mice respectively.
E to **H**: DAPI, CD11b, B220 and merged pictures in lungs of WT mice.
I to **L**: DAPI (blue), CD11b (green), B220 (red) and merged pictures in lungs of LAT mice.

gests that this population was of extrafollicular B cells independent of germinal centre origin. In SLE patients, it was also observed that CD11c+ B cells manifested as a pattern of higher expression of CXCR3 and lower expression of CXCR5, and presented with the infiltration of CD20+CD11c+ B cells in nephritic kidney (15, 17, 42).

Taken together, the above evidence revealed that the CD11c+ B cells expressed lower levels of memory makers, higher levels of costimulatory molecules, and BAFF receptors compared with CD11c- B cells, which suggested that this phenotype may initiate or enhance autoreactivity not simply by secreting autoantibody but also presenting self-antigens and interacting with T cells. Therefore, we speculated that ABCs might be a useful target for therapeutic strategies in IgG4-RD.

LAT mice were established by replacement of the tyrosine residue at position 136 with phenylalanine, resulting the loss of binding capacity to phospholipase C-γ1 (PLC- γ1), This led to the development lymphoproliferative disorder characterised by expansion of CD4+ T cells and Th2 cytokine production (IL-4, IL-5, and IL-13) (43, 44). Besides, LAT mice displayed with massive activation of B cells as well as elevated serum levels of IgG1 (considered to be a homologue of human IgG4) and IgE levels (45). Previous studies reported that ABCs, were observed in the skin biopsies, kidney tissues, and peripheral blood of patients with SLE, as well as the affected tissues of lupus-prone mice such as NZB/WF1, Mer$^{-/-}$, BXSB and MRL/lpr mice (11, 12, 46, 47). In addition, depletion of ABCs from Mer$^{-/-}$ mice and cGVHD mice resulted in a dramatic reduction of autoantibodies, providing potential pathogenic clues and opening avenues for different therapies (16, 48). To evaluate the promising role of ABCs in IgG4-RD mouse models, we firstly detected the expression level of ABCs in LAT mice. As expected, our results showed that ABCs was upregulated in various affected organs, especially widely infiltrated in lung, indicating ABCs participated in the tissue injury of IgG4-RD mouse models.

LAT mice are now recognised as a mice model for IgG4-RD which provides insights in understanding the pathogenesis of IgG4-RD, we detected the expression level of ABCs in LAT mice. As expected, the results showed that ABCs was upregulated in various affected organs, especially widely infiltrated in lung, indicating ABCs participated in the tissue injury of IgG4-RD mouse models. Although LAT mice are not the ideal animal model for IgG4-RD in the following aspects: involved organs not including retroperitoneum/periaorta and bile duct (30), etc.; absence of typical storiform fibrosis and obliterative phlebitis (22), as well as regulatory T cells were absent in LAT mouse models (49).

This study has several limitations. The low level of ABCs made it difficult to isolate them and perform cell culture experiments. The percentage of ABCs in pSS, dermatomyositis and RA were

comparable with HCs in our study may due to the low sample numbers and immunosuppressive treatment previously of some patients recruited in our study. In addition, the number of IgG4-RD patients after treatment was relatively small. Although CD19+CD21-CD11c+ cells were mainly T-bet+ cells, it was still one of our limitations that T-bet was not stained when detecting the expression of surface markers. In mouse tissues, ABCs was stained by the markers of B220 and CD11b according to the previous study (50), and T-bet, another important marker of ABCs was not stained, which was also one of limitations of this study. Further research was required to assess the specific mechanism of ABCs regulation, autoantibodies and cytokines release. Moreover, whether the accumulation of ABCs was pathogenic in IgG4-RD mouse models remains unclear.

Finally, analysing this population and observing pathophysiological changes in mouse models are worth exploring.

Conclusion

In summary, the present study provides new perspectives of molecular and functional features of ABCs in IgG4-RD, indicating the potential roles of ABCs in IgG4-RD pathogenesis.

Key messages

- What is already known on this topic?
  B cell abnormality plays a central role in the pathogenesis of IgG4-related disease (IgG4-RD).
- What does this study add?
  Age-associated B cells (ABCs) were expanded in patients with IgG4-RD, and the percentages of ABCs correlated positively with serum IgG4 levels.
- How might this study affect research, practice or policy?
  ABCs might be a useful biomarker predicting disease activity and a potential therapeutic target in IgG4-RD.

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