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東岡, 和彦

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Generation of a novel CD30⁺ B cell subset producing GM-CSF and its possible link to the pathogenesis of systemic sclerosis

A short title: GM-CSF-producing B cells in SSc

Kazuhiko Higashioka¹, Yoshikane Kikushige¹, Masahiro Ayano¹, Yasutaka Kimoto², Hiroki Mitoma¹, Makoto Kikukawa³, Mitsuteru Akahoshi¹, Yojiro Arinobu¹, Takahiko Horiuchi², Koichi Akashi¹, Hiroaki Niiro³

¹Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka, 812-8582, Japan

²Department of Internal Medicine, Kyushu University Beppu Hospital, 4546 Tsurumihara, Beppu, Oita 874-0838, Japan

³Department of Medical Education, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka, 812-8582, Japan

Correspondence:

Hiroaki Niiro MD, PhD

Department of Medical Education, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka, 812-8582, Japan

Phone: +81-92-642-6678 Fax: +81-92-642-6188

E-mail: hniiro@med.kyushu-u.ac.jp

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GM-CSF, Th subsets, B cells, CD30, systemic sclerosis

List of abbreviations:

SSc: Systemic sclerosis; Th2: T helper type 2; IL: Interleukin; GM-CSF: granulocyte macrophage colony-stimulating factor; GM-Beffs: GM-CSF producing effector B cells; HCs: healthy controls; Tfh: T follicular helper; TGF: Transforming growth factor; JAK: Janus kinase; DC-SIGN: dendritic cell-specific ICAM-3 grabbing non-integrin, AID: autoimmune disease; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; Breg: regulatory B cells; RANKL: Receptor activator of nuclear factor κ B ligand; EAE: experimental autoimmune encephalomyelitis; CIA: collagen-induced arthritis; MS: Multiple sclerosis; ILD: interstitial lung disease; HRCT: high-resolution computed tomography; BCR: B cell receptor; CD40L: CD40 Ligand; IFN: Interferon; CXCR: CXC chemokine receptor; PBMCs: peripheral blood mononuclear cells; PCR: polymerase chain reaction; PMA: phorbol 12-myristate 13-acetate; ELISA: enzyme-linked immunosorbent assay; SEM: standard error of the mean; SD: significance of differences; TNF: Tumor necrosis factor; PB: plasmablast; IP: interstitial pneumonia; STAT: Signal transducer and activator of transcription; GC: Germinal center; cTfh: circulating Tfh; CTY: Cell Trace Yellow; DMSO: Dimethyl sulfoxide

Summary

Systemic sclerosis (SSc) is a T helper type 2 (Th2)-associated autoimmune disease characterized by vasculopathy and fibrosis. Efficacy of B-cell depletion therapy underscores antibody-independent functions of B cells in SSc. A recent study showed that the Th2 cytokine IL-4 induces granulocyte macrophage colony-stimulating factor (GM-CSF)-producing effector B cells (GM-Beffs) in humans. In this study we sought to elucidate the generation mechanism of GM-Beffs and also determine a role of this subset in SSc. Among Th-associated cytokines, IL-4 most significantly facilitated the generation of GM-Beffs within memory B cells in healthy controls (HCs). In addition, the profibrotic cytokine TGF- β further potentiated IL-4- and IL-13-induced GM-Beffs. Of note, tofacitinib, a JAK inhibitor, inhibited the expression of GM-CSF mRNA and protein in memory B cells induced by IL-4, but not by TGF- β . GM-Beffs were enriched within CD20⁺CD30⁺CD38^{-/low} cells, a distinct population from plasmablasts, suggesting that GM-Beffs exert antibody-independent functions. GM-Beffs were also enriched in CD30⁺ fraction of freshly isolated B cells. GM-Beffs generated under Th2 conditions facilitated the differentiation from CD14⁺ monocytes to DC-SIGN⁺CD1a⁺CD14⁻CD86⁺ cells, which significantly promoted the proliferation of naïve T cells. CD30⁺ GM-Beffs were more pronounced in patients with SSc than in HCs. A subpopulation of SSc patients with the diffuse type and concomitant interstitial lung disease exhibited high numbers of GM-Beffs. Together, these findings suggest that human GM-Beffs are enriched in a CD30⁺ B cell subset and play a role in the pathogenesis of SSc.

Introduction

Systemic sclerosis (SSc) is an intractable autoimmune disease (AID) characterized by vasculopathy, fibrosis and immune dysregulation [1]. Although the pathogenesis of SSc remains still largely uncharacterized, genome-wide association studies showed that a set of immune-related genes is closely associated with this disease [2, 3]. T helper type 2 (Th2) cytokines such as IL-4 and IL-13 exert profibrotic functions [4], however an impact of these cytokines on immune cells in SSc remains somewhat elusive. Due to the debilitating nature of the disease, there are still high unmet needs for novel therapeutic strategies.

Efficacy of B cell-targeting agents such as anti-CD20 in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and SSc highlights a pathogenic role of B cells in AIDs [5-9]. Notably, correlation is not always found between autoantibody titers and clinical symptoms, thus underscoring antibody-independent functions, particularly cytokine production, of B cells [5, 10]. Cytokine-producing B cells are functionally divided into two subsets, effector B cells (Beffs) and regulatory B cells (Bregs). In patients with RA, Bregs are less abundant and their suppressive function is impaired [11, 12]. We showed that synovial fluid of patients with RA includes abundant Receptor activator of nuclear factor κ B ligand (RANKL)-producing Beffs capable of inducing osteoclast differentiation [13]. In patients with SSc, although IL-6-producing Beffs are increased and Bregs are functionally defective [10, 14, 15], it remains to be elucidated whether other cytokine-producing Beffs are also involved.

Granulocyte macrophage colony-stimulating factor (GM-CSF) was originally

recognized as a hematopoietic growth factor that can induce proliferation and differentiation of granulocytes and macrophages. However, GM-CSF also exerts pro-inflammatory functions and plays critical roles in AIDs [16]. For instance, GM-CSF is involved in the differentiation of monocytes to myofibroblasts and macrophages that harbor the potential to promote fibrosis in the pathogenesis of SSc [17, 18]. It is well known that GM-CSF is abundantly produced by monocyte/macrophages and dendritic cells. Notably, T cells have recently been drawn attention as another source of GM-CSF. GM-CSF-producing CD4⁺ T cells are involved in inflammatory mouse models such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and interstitial lung disease in SKG mice [19-22]. Likewise, in humans, the significance of GM-CSF-producing CD4⁺ T cells is implicated in RA and multiple sclerosis (MS) [23, 24].

A recent study showed that GM-CSF-producing B cells (GM-Beffs) abundantly exist in patients with MS and IL-4 appears to be a potent inducer of GM-Beffs [25]. It, however, remains somewhat elusive about the generation mechanisms and the specific markers of GM-Beffs. In addition, given that Th2 cytokines are associated with SSc [4], it is of great interest to determine whether GM-Beffs are involved in the pathogenesis of this disease.

In this study we sought to demonstrate how GM-Beffs are induced by Th-associated cytokines, and to clarify what surface marker enriches for GM-Beffs in humans. In addition, we aimed to demonstrate whether GM-Beffs really exist and their functional roles in SSc.

Materials and methods

SSc patients and controls

We studied 44 Japanese patients with SSc who were treated at the Kyushu University hospital and 16 healthy controls (HCs). We included the patients who fulfilled the 1980 classification criteria of the American College of Rheumatology for SSc. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects (No 29-544). We obtained the information from the medical records of the patients, including demographic data, clinical manifestations, laboratory findings and medications. Patients with SSc were classified as having limited cutaneous or diffuse cutaneous disease according to the criteria of LeRoy et al [26]. The existence of SSc-related ILD was based on chest high-resolution computed tomography (HRCT).

Reagents

An affiniPure F (ab')₂ Fragment Goat Anti-Human IgA/ IgG/IgM (H+L) (anti-BCR, 10 µg/ml) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Recombinant human CD40 ligand (CD40L; 100 ng/ml) was from Biolegend (San Diego, CA, USA). Recombinant human cytokines (IFN- γ (20 ng/ml), IL-4 (20 ng/ml), IL-17 (100 ng/ml), IL-13 (20 ng/ml), IL-6 (50 ng/ml), IL-10 (10 ng/ml), IL-15 (10 ng/ml), IL-21 (50 ng/ml), TGF- β (50 ng/ml), recombinant human IL-6 Receptor (100 ng/ml) and a fully human mAb against GM-CSF (α GM-CSF, 1 µg/ml) were from R&D Systems (Minneapolis, MN, USA). Tofacitinib (CP-690550) was purchased from Selleckchem (Houston, TX, USA).

Anti-CD3 mAb (OKT3) was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich (St. Louis, MO, USA)

Isolation and cell sorting of B cell subsets

Peripheral blood mononuclear cells (PBMCs) were obtained using a density centrifugation with LSM (MP Biomedicals, LLC, Santa Ana, CA, USA). B cells were isolated by positive selection with CD19⁺ mAbs and a MACS magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated B cells exhibited greater than 99.5 % viability and more than 95 % purity, confirmed by flow cytometry. Cells were stained with mouse or rabbit mAbs against human CD19, CD20, CD27, CD30, CD38, CD124 (IL-4Ra) and CD183 (CXCR3). (all from BioLegend, San Diego, CA, USA). Memory (CD19⁺CD27⁺) B cell subsets were purified by flow cytometry. Isolated memory B cells exhibited more than 99 % purity (Supporting information, Fig. S1a).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from primary B cells using Isogen II reagent (Nippon Gene, Tokyo, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed in the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA). TaqMan target mixes for *CSF2* (Hs00929873_m1), *TNF* (Hs00174128_m1), *TNFRSF11* (Hs00243533_m1), *PRDM1* (Hs00153357_m1) and *IL6* (Hs00174131_m1) were all purchased from Applied Biosystems. 18S ribosomal RNA was separately amplified in the same plate as an internal control

for variation in the amount of cDNA in PCR. The collected data were analyzed using Sequence Detector software (Applied Biosystems). Data were expressed as the fold change in gene expression relative to the expression in control cells.

Intracellular staining of GM-CSF

Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Calbiochem, Nottingham, UK), ionomycin (1 μ M, Calbiochem) and Golgi Stop (Brefeldin-A, eBioscience, Carlsbad, CA, USA) were added 4 h before staining. Cell surface staining was performed before intracellular cytokine staining. After washing two times, fixation/permeabilization buffer (BD Biosciences) was added to fix the cells. Antibody to detect GM-CSF (BD Biosciences) was added to cell suspension and cells were analyzed by FACS AriaIII (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA)

Sorted memory B cells were stimulated for 48 h with Th-associated cytokines in the presence of anti-BCR and CD40L and supernatants were collected afterward. The concentration of supernatants was measured by using Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions.

Coculture experiments

Purified memory B cells were pre-stimulated with IL-4 and TGF- β in the presence of anti-BCR and CD40L for 48 h, washed thoroughly and cocultured with CD14⁺ monocytes for 72 h with anti-BCR and IL-4. CD14⁺ monocytes in a

12-well plate at a ratio of 1:10 monocytes: B cells (2×10^5 monocytes: 2×10^6 B cells/mL) were cultured. Cells were harvested and the expression of surface markers including CD1a, CD1c, CD14 and CD86 in DC-SIGN⁺CD19⁻ cells was analyzed by flow cytometry.

T cell proliferation assay

Naïve CD4⁺ T cells were labeled with Cell Trace Yellow (CTY) using Cell Trace Yellow Cell Proliferation kits according to the manufacturer's instructions (Thermo Fisher Scientific), then cultured alone or cocultured with sorted DC-SIGN⁺CD19⁻ cells differentiated from CD14⁺ monocytes in a manner described above for 5 days with OKT3 (1 µg/ml) and IL-2 (100 U/ml). DC-SIGN⁺CD19⁻ cells in a 96-well plate at a ratio of 1:2 DC-SIGN⁺CD19⁻ cells: naïve CD4⁺ T cells (1×10^5 DC-SIGN⁺CD19⁻ cells: 2×10^5 naïve CD4⁺ T cells/mL) were cultured. Cells were harvested and analyzed by flow cytometry.

Statistical analysis

Numerical data in the in vitro experiments were presented as mean of the different experiments and standard error of the mean (SEM). Multiple group comparisons were analyzed using the Kruskal-Wallis test. The significance of the differences was determined by Student's t-test for comparing differences between two groups. Numerical data in patient-sample analyses were presented as mean, and the significance of differences (SD) was determined by Student's t-test or nonparametric Mann-Whitney U-test according to distributions. For all tests, *P* values less than 0.05 were considered significant.

All analyses were performed using JMP statistical software (SAS Institute, Cary, NC).

Results

GM-Beffs are induced under Th2 and, to a lesser extent, Tfh2 conditions

We first sought to determine how GM-Beffs are induced in vitro. Given that Th-B interaction plays a critical role in cytokine production by B cells, identification of a major Th cell subset responsible for generation of GM-Beffs was of great interest. Since GM-CSF production is much higher in CD19⁺CD27⁺ memory B cells than in CD19⁺CD27⁻ B cells [25], we used memory B cells in this study. Purified human memory B cells were cultured with the cytokines including the Th1 cytokine IFN- γ (20 ng/ml), the Th2 cytokine IL-4 (20 ng/ml), the Th17 cytokine IL-17 (100 ng/ml) and the T follicular helper (Tfh) cytokine IL-21 (50 ng/ml) along with combination of anti-BCR (10 μ g/ml) and CD40L (1 μ g/ml). GM-CSF mRNA was remarkably up-regulated with IL-4, but not IFN- γ or IL-17, in memory B cells (Fig. 1a upper panel). IL-4 exerted such an effect upon BCR/CD40 stimulation, because IL-4R was remarkably induced only in stimulated B cells (Supporting information, Fig. S1b). The concentration of IL-4 in serum is within a range of 1 to 10 pg/ml in patients with SSc [27, 28], albeit may be much higher in tissues. The levels of GM-CSF mRNA were significantly up-regulated by IL-4 in a dose-dependent manner (Supporting information, Fig. S2a). Of note, IL-6 mRNA was also significantly up-regulated with IL-4 (Fig. 1a bottom panel). In contrast, as we previously reported [13], RANKL and TNF- α mRNAs were most up-regulated with IFN- γ (Fig. 1a middle 2 panels). Intriguingly, IL-21, involved in plasma cell differentiation [29], suppressed the generation of these cytokine-producing B cells including GM-Beffs (Fig. 1a). Consistent with transcript data, stimulation with IL-4 induced high levels of GM-

CSF proteins in memory B cells (Fig. 1b, 1c). Circulating T follicular helper type2 (Tfh2) cells produce IL-4 as well as IL-21 [30, 31]. GM-Beffs were thus induced, to some extent, under Tfh2 conditions. These results suggest that GM-Beffs are induced under Th2 and, to a lesser extent, Tfh2 conditions.

TGF- β enhances IL-4- and IL-13-mediated GM-Beff induction

GM-Beffs were induced under Th2 conditions (Fig. 1), however we further sought to identify optimal conditions of GM-Beff induction. In addition to IL-4, IL-13 (20 ng/ml), another Th2 cytokine [31], and the profibrotic cytokine TGF- β (50 ng/ml) were added to the culture. TGF- β increased BCR/CD40-induced GM-CSF mRNA but not IL-6 mRNA (Fig. 2a). IL-13, a cytokine capable of activating the type II IL-4R, upregulated GM-CSF and IL-6 mRNAs (Fig. 2a). The concentration of IL-13 and TGF- β in serum is within a range of 1 to 5 ng/ml and 10~50 ng/ml in patients with SSc [27, 28], albeit may be much higher in tissues. The levels of GM-CSF mRNA were significantly up-regulated by IL-13 and TGF- β in a dose-dependent manner (Supporting information, Fig. S2b, S2c). Consistent with transcript data, TGF- β enhanced IL-4- and IL-13-mediated GM-CSF protein production in memory B cells (Fig. 2b). Janus kinase (JAK) is a pivotal signaling molecule of IL-4 and IL-13 [32]. Tofacitinib, a JAK inhibitor, inhibited the expression of GM-CSF mRNA and protein in memory B cells induced by IL-4, but not by TGF- β (Fig. 2b lower panel, Fig. 2c). Tofacitinib did not affect the viability of stimulated B cells (data not shown). These results suggest that GM-Beffs are most efficiently induced under Th2 conditions along with TGF- β and this process is partially abrogated by JAK inhibition.

GM-Beffs are enriched within CD20⁺CD30⁺CD38^{-low} cells, a distinct population from plasmablast (PB)

To enrich human GM-Beffs prospectively, we next focused on CD30, previously described as a Th2 specific marker [33, 34]. We checked CXCR3, CD20 and CD30 expressions on human memory B cells cultured with several cytokines including IFN- γ , IL-4, IL-21 and TGF- β along with anti-BCR and CD40L for 4 days (Fig. 3a). CD30 expression was most up-regulated with IL-4 in combination with TGF- β , whereas CXCR3 expression was most up-regulated with IFN- γ and CD20 expression was most down-regulated with IL-21 (Fig. 3a). Notably, IL-4 in combination with TGF- β was most potent for the generation of CD20⁺CD30⁺ B cells (Fig. 3b). To test whether GM-CSF production is derived from CD30⁺ B cells induced by IL-4 and TGF- β , we co-stained CD30 and GM-CSF on memory B cells. GM-CSF expression was mainly confined to CD30⁺ B cells (Fig. 3c). We also tested whether the same is true in freshly isolated B cells. GM-Beffs were again enriched in CD30⁺ B cells in this condition (Fig. 3d). Acquisition of CD30 can distinguish activated B cells from pre-PB stage and loss of CD30 along with strong expression of CD38 characterizes PB stage [35]. Given that IL-21 suppressed the induction of GM-Beffs (Fig. 1), we hypothesized that GM-Beffs were distinct cellular populations from PB. To test this, we induced the differentiation of human memory B cells into PB using a combination of anti-BCR, CD40L, IL-21, IL-15 (10 ng/ml) and IL-6 (50 ng/ml) for 6 days. IL-10 (50 ng/ml) and IL-4 were added for the last 24 h. Representative flow cytometry plots of CD30 and CD38 are shown (Fig. 3e left panel). As

expected, CD30⁻CD38^{high} B cells down-regulated CD20 expression, whereas CD30⁺CD38^{-/low} B cells retained its expression (Fig. 3e second from left panel). In addition, GM-CSF mRNA in CD30⁺CD38^{-/low} cells was significantly up-regulated than that in CD30⁻CD38^{high} cells (Fig. 3e second from right panel). The same was true at the protein level (Fig. 3e right panel). Conversely, Blimp-1 mRNA in CD30⁻CD38^{high} cells was significantly up-regulated than that in CD30⁺CD38^{-/low} cells (Fig. 3e second from right panel). These results strongly suggest that GM-Beffs are enriched in CD20⁺CD30⁺CD38^{-/low} cells which are distinct from cells of the PB stage.

GM-Beffs induce differentiation to monocyte-derived dendritic cells (MoDCs)

Given that combination of GM-CSF and IL-4 induces differentiation of monocytes to DCs [36], we reasoned that GM-Beffs cause such differentiation. To test this, purified CD14⁺ monocytes from HCs were cultured alone or co-cultured with memory B cells stimulated with anti-BCR and IL-4 for 3 days with subsequent analysis of surface markers including CD1a, CD1c, CD14, CD86 and DC-SIGN (DC-specific intercellular adhesion molecule-grabbing nonintegrin) [37], in CD19⁻ cells by flow cytometry. Since CD14 is down-regulated as monocytes differentiate into DCs which express CD1, DC-SIGN and co-stimulatory molecules including CD86 [36, 37], we focused on the populations of CD14⁻CD1a⁺, CD14⁻CD1c⁺ and CD14⁻CD86⁺ cells among DC-SIGN⁺CD19⁻ cells. Intriguingly, GM-Beffs significantly increased the populations of CD14⁻CD1a⁺, CD14⁻CD1c⁺ and CD14⁻CD86⁺ cells among DC-SIGN⁺CD19⁻

cells and this process was significantly suppressed by anti-GM-CSF, indicating that GM-Beffs significantly enhanced differentiation of CD14⁺ monocytes into DCs via GM-CSF secretion (Fig. 4a, 4b). To further validate the function of GM-Beff-induced DCs, naïve CD4⁺ T cells were cultured alone or cocultured with sorted DC-SIGN⁺ CD19⁻ cells along with anti-CD3 mAb and IL-2, then analyzed for their proliferation by flow cytometry. DC-SIGN⁺ CD19⁻ cells clearly promoted the proliferation of naïve CD4⁺ T cells (Fig. 4c). These results suggest that GM-Beffs have the potential to differentiate of CD14⁺ monocytes to functional DCs in vitro.

CD30⁺ GM-Beffs increase in SSc patients, especially in the subpopulations with the diffuse type and concomitant ILD

Given a pathological relevance of IL-4 and TGF- β in SSc [1, 4], we investigated the relevance of GM-Beffs in patients with SSc. We thus evaluated GM-CSF production from circulating B cells in patients with SSc and age- and sex-matched HCs (all women; 57.9 ± 15.9 years old) by flow cytometry. The clinical characteristics of SSc patients are shown in Table S1 (Supporting information). The number of circulating GM-Beffs was significantly increased in patients with SSc (114.6 ± 193.5 cells/100 μ l; N=44) as compared with that in HCs (47.8 ± 28.8 cells/100 μ l; N=16 $p=0.043$) (Fig. 5a). The number of CD30⁺ memory B cells in patients with SSc was also significantly increased as compared with that in HCs (262.5 ± 495.0 cells/100 μ l vs 58.6 ± 36.3 cells/100 μ l, respectively, $p=0.042$) (Fig. 5b). Moreover, the number of GM-Beffs with diffuse type, limited type, with and without concomitant interstitial lung disease (ILD) were $164.2 \pm$

257.0, 54.0 ± 47.0 , 150.6 ± 245.4 and 55.8 ± 44.3 cells/100 μ l, respectively ($p>0.05$ for diffuse type versus limited type; $p>0.05$ for interstitial pneumonia [IP]⁺ versus IP⁻) (Fig. 5c). Intriguingly, the number of a subpopulation of SSc patients with the diffuse type and concomitant ILD was significantly increased than that of other patients except this characteristic (168.1 ± 248.0 versus 52.1 ± 45.3 cells/100 μ l, $p=0.026$) (Fig. 5c). Since SSc patients with the diffuse type and concomitant ILD have a poor prognosis [40], these results suggest that GM-Beffs are involved in the pathogenesis and severity of SSc.

Discussion

In this study, we demonstrate that GM-Beffs were most efficiently induced under Th2 and, to a lesser extent, Tfh2 conditions. Generation of GM-Beffs was synergistically accentuated by TGF- β and conversely blocked by a JAK inhibitor. The phenotype of GM-Beffs was CD20⁺CD30⁺CD38^{-/low} cells and they cooperated with IL-4 to facilitate the differentiation from CD14⁺ monocyte to DC-SIGN⁺CD1a⁺CD1c⁺CD14⁻CD86⁺ DC-like cells, which significantly promoted the proliferation of naïve T cells. CD30⁺ GM-Beffs were abundant in patients with SSc. A subpopulation of patients with the diffuse type and concomitant ILD, in particular, exhibited high levels of GM-CSF production.

Th-derived cytokines play a critical role in the generation of cytokine-producing B cells. As we showed previously [13], the Th1 cytokine IFN- γ effectively generates RANKL-producing Beffs which are involved in the pathogenesis of RA. In SSc Th2 cytokines exert profibrotic functions [4], however their impact on cytokine-producing Beffs has not thus far been addressed. We here clearly show that IL-4 and IL-13 generate a novel B cell subset (GM-Beffs) which was increased in patients with SSc. It should be noted that romilkimab, a humanized bispecific IgG4 antibody that binds and neutralizes both IL-4/IL-13, was effective for diffuse cutaneous SSc in a phase II a randomized, double-blind, placebo-controlled trial (NCT02921971) [41]. This treatment thus would in theory selectively suppress the induction of GM-Beffs.

Notably we found that GM-Beffs were also induced, to a lesser extent, under Tfh2 conditions (Fig. 1). Tfh cells, defined as CXCR5⁺CD4⁺T cells, mainly reside

in the germinal center (GC) and produce IL-21 that helps the generation of memory B cells and plasma cells [29]. Of note, humans have peripheral blood counterpart of Tfh cells and termed circulating Tfh (cTfh). cTfh cells can be further divided into three subsets by additional surface markers, namely cTfh1, cTfh2, and cTfh17 [30]. Intriguingly, IL-21 alone exerted a suppressive effect on the induction of GM-Beffs (Fig. 1). IL-4, however, partially abrogated IL-21-induced suppression of GM-Beff induction (Fig. 1), underscoring a critical role of IL-4 in the generation of GM-Beffs. Recent studies showed that Tfh cells not only induce plasma cell differentiation but also are directly associated with skin fibrosis in SSc [42, 43]. Based on our current results that GM-Beffs are generated by a mechanism distinct from plasma cell differentiation (Fig. 3e), Tfh-induced GM-Beffs might be also involved in skin fibrosis in SSc. Further work is needed to address this provocative issue.

CD30 is one of TNF receptor superfamily molecules and it is expressed on activated lymphocytes [44]. It is preferentially expressed on both CD4⁺ and CD8⁺ T cells which produce Th2 cytokines [45]. Indeed, CD30⁺ T cells are seen in several diseases including atopic dermatitis and SSc in which Th2 cytokines play a pathogenic role [33, 34]. We here clearly show that human memory B cells expressed CD30 in response to IL-4, but not IFN- γ or IL-21 (Fig. 3a). This is in line with a recent paper showing that CD30 is a critical molecule downstream of the IL-4-STAT6 pathway in mouse B cells [46]. In addition, TGF- β , another profibrotic cytokine involved in the pathogenesis of SSc, synergistically accentuated the expression of CD30 (Fig. 3a). It should be noted that the clinical trial to assess the therapeutic efficacy of brentuximab vedotin, a

CD30-directed antibody-drug conjugate, in active diffuse cutaneous SSc is now ongoing (NCT03198689, NCT03222492). This treatment, thus, in theory would deplete preferentially GM-Beffs as well as CD30⁺ T cells in patients with SSc. It is thus of great interest to await the results in the future.

What, then, is a role of GM-Beffs in the pathogenesis of SSc? Given that GM-CSF and IL-4 are critical cytokines for generation of inflammatory DC from monocytes [36], we hypothesized that GM-Beffs induced under Th2 conditions promote this process in concert with IL-4. As we expected, GM-Beffs cooperated with IL-4 to facilitate the differentiation of CD14⁺ monocytes into DC-SIGN⁺CD1a⁺CD1c⁺CD14⁺CD86⁺ DCs (Fig. 4), a DC subset previously reported in skin of SSc patients and SSc model mice [47, 48, 49]. DCs play a role in the development of fibrosis in SSc by producing inflammatory cytokines including IL-6 and TNF- α . These cytokines then lead to the transformation of fibroblasts into myofibroblasts that contribute to overproduction of extracellular matrix [50]. Aside from IL-4, GM-CSF alone induce the differentiation from CD14⁺ monocytes to CD1c⁺DCs in patients with rheumatoid arthritis [21]. Similar situations thus might be also true in the case of SSc. In addition to their role in skin, B cells infiltrate in the pulmonary inflammatory lesions of SSc patients with ILD and B cell-depleting therapy has a beneficial effect on pulmonary function, suggesting that B cells also contribute to the pathogenesis of SSc-associated ILD [7, 8, 51]. Based on these observations, we reason that GM-Beffs are involved in the pathogenesis of SSc by enhancing the fibrosis through the induction of DCs.

Although clinical studies show the beneficial effect of rituximab, a mAb

against human CD20, on skin sclerosis and pulmonary fibrosis of SSc, it remains somewhat elusive how B cells deteriorate the pathogenesis of SSc [7, 8, 9]. Recent studies show that IL-6/TGF- β -producing B cells play a role in the pathogenesis of SSc [10, 14, 15]. Notably, the frequency of activated B cells harboring the potential to produce IL-6/TGF- β is increased in the diffuse type SSc patients with concomitant ILD [10, 14]. Based on our current findings that Th2 conditions significantly induce IL-6 as well as GM-CSF (Fig. 1a, 2a), whether IL-6/TGF- β -producing Beffs and GM-Beffs are identical or not needs to be further determined. Given that the clinical trial of IL-6 blockade has provided promising results in patients with SSc [52], it is of great importance to address these issues.

Based on our findings above, it is suggested that GM-Beffs play a role in the pathogenesis of SSc and they are an ideal target for the treatment of this disease. We show that tofacitinib, a JAK inhibitor, abrogated the generation of GM-Beffs induced by IL-4 which activates JAK1/3 (Fig. 2b, 2c). JAK-STAT signature is noted in skin and ILD of patients with SSc [53], indicating the possibility that tofacitinib is an effective treatment for SSc. Indeed, the clinical trial to assess the therapeutic efficacy of tofacitinib in early diffuse cutaneous SSc (TOFA-SSc) is now ongoing (NCT03274076). Tofacitinib, however, suppresses the signaling of several other cytokines including IL-2, IL-7, IL-15 and IL-21 that are crucial for lymphocytes activation, proliferation and function [54]. Infectious diseases such as viral infection induced by tofacitinib were reported [55]. The anti-CD20 therapy non-selectively depletes Bregs as well as pathogenic Beffs. There are thus still unmet needs for more selective targeting

strategy for Beffs.

The number of CD20⁺ B cells is much lower than that of CD3⁺T cells and CD68⁺ macrophages in skin samples from patient with SSc [56]. However, all patients who experienced more than 20% worsening in the skin score have the infiltration of CD20⁺ B cells in the skin biopsy specimens [56], suggesting that B cells play a pivotal role in the pathogenesis and development of SSc. Although the current study clearly demonstrates the generation mechanism and the existence of GM-Beffs in human blood, the main limitations are the lack of evidence for this novel subset in the involved tissues. Together with the sensitive methods, further studies with more SSc patients along with their tissue samples are required to clarify the role of GM-Beffs in the pathogenesis of this devastating disease.

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KH performed the experiments, statistical analysis, and drafted the manuscript. YK, MA, YK, HM, MK, MA, YA, TH, KA and HN designed the study and helped to draft the manuscript. YK and HN contributed to data analysis and interpretation. All authors read and approved the final manuscript. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology in Japan (HN: grant number 18K08410).

Disclosures

The authors declare no financial or commercial conflicts of interest.

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Figure legends

Fig. 1. Cytokine expression by human memory B cells under T cell-dependent conditions. **(a)** Purified human memory B cells (CD19⁺CD27⁺ cells) stimulated with several cytokines including IFN- γ , IL-4, IL-17 and IL-21 in combination with anti-BCR and CD40L were cultured for 24 h. Cells were harvested, then the transcriptions of several cytokines including GM-CSF, RANKL, TNF- α and IL-6 were evaluated by quantitative PCR (qPCR). **(b)** Memory B cells stimulated under the same conditions as **(a)** were cultured for 48 h, then culture supernatants were collected, tested for GM-CSF by ELISA (N=3). **(c)** The top panel depicts representative GM-CSF expression in memory B cells stimulated under designated conditions for 48 h (added PMA and Ionomycin for the last 4 h) and the bottom panel summarizes the results (N=4). * indicates $p<0.05$, ** indicates $p<0.01$, un: undetected. ns: no stimulation.

Fig. 2. GM-Beffs are most efficiently induced under Th2 conditions along with TGF- β . **(a)** Purified memory B cells stimulated with several cytokines including IL-4, IL-13 and TGF- β in combination with anti-BCR and CD40L were cultured for 24 h. Cells were harvested, then the transcriptions of GM-CSF and IL-6 were evaluated by qPCR. **(b)** Stimulated memory B cells were cultured for 48 h, then culture supernatants were collected, tested for GM-CSF by ELISA (top panel). In addition, tofacitinib or vehicle control (0.1% DMSO) was added and culture supernatants were tested for GM-CSF by ELISA (bottom panel). **(c)** The top panel depicts representative GM-CSF expression in memory B cells stimulated

under designated conditions for 48 h (added PMA and Ionomycin for the last 4 h) and the bottom panel summarizes the results (N=4). * indicates $p<0.05$, ** indicates $p<0.01$, un: undetected, ns: non-significant.

Fig.3. GM-Beffs are enriched within CD30⁺ memory B cells. **(a)** The histogram panels show the expression of several surface molecules including CD20, CD30 and CXCR3 on memory B cells stimulated for 96 h. **(b)** These panels show expression of CD20 and CD30 on stimulated memory B cells with several cytokines including IL-4, IL-21 and TGF- β . **(c)** The panels show expression of CD30 and GM-CSF. **(d)** The panels show GM-CSF expression in freshly isolated CD30⁻ and CD30⁺ B cells. **(e)** The left panel shows expressions of CD30 and CD38 (CD30⁺CD38^{-/low} and CD30⁻CD38⁺ gates surrounded by heavy lines). The second from left panel shows the expression level of CD20⁺ cells. The second from right panel shows the transcription levels of GM-CSF and Blimp-1 in CD30⁺CD38^{-/low} and CD30⁻CD38⁺ cells. The right panel shows GM-CSF production by flow cytometry. ** indicates $p<0.01$.

Fig.4. Differentiation to DCs from monocytes induced by GM-Beffs. Purified memory B cells were pre-exposed with several cytokines including IL-4 and TGF- β in concert with anti-BCR and CD40L for 48 h, then were thoroughly washed and cultured in fresh medium with purified CD14⁺ monocytes from HCs with BCR and IL-4, added either anti-GM-CSF or isotype control, for an additional 72 h. After co-culture, surface markers including CD1a, CD1c, CD86 and CD14 on DC-SIGN⁺ CD19⁻ cells were assessed by flow cytometry. **(a)** The

representative data are shown. **(b)** The bar charts summarize the results (N=4). ** indicates $p<0.01$. **(c)** Monocyte-derived DCs promote the proliferation of naïve CD4⁺ T cells. Purified memory B cells were pre-exposed with IL-4 and TGF- β in concert with anti-BCR and CD40L for 48 h, then thoroughly washed and cultured with CD14⁺ monocytes from HCs with anti-BCR and IL-4 for an additional 72 h, followed by sorting DC-SIGN⁺CD19⁻ cells. CTY-labeled naïve CD4⁺ T cells were cultured alone or co-cultured with the above DC-SIGN⁺CD19⁻ cells with anti-CD3 mAb and IL-2 for 5 days, then cell proliferation of naïve CD4⁺ T cells was analyzed by flow cytometry. Histogram shows CTY fluorescence of naïve CD4⁺ T cells cultured alone (top panel) and co-cultured with DC-SIGN⁺CD19⁻ cells (bottom panel).

Fig.5. CD30⁺GM-Beffs in HCs and patients with SSc. **(a)** The top panels show the representative datum regarding GM-CSF⁺ memory B cells from HCs and patients with SSc. The bottom panel summarizes the results. **(b)** The top panels show the representative datum regarding CD30⁺ memory B cells from HCs and patients with SSc. The bottom panel summarizes the results. **(c)** These bar charts summarize the number of GM-CSF⁺ memory B cells in each clinical feature of patients with SSc. * indicates $p<0.05$, ** indicates $p<0.01$.

Supporting information

Fig. S1 (a) The purity of memory B cells isolated by flow cytometry. The left panel shows expression of CD19 and CD27 on B cells before sorting. The right panel shows expression of CD19 and CD27 on B cells after sorting. **(b)** The histogram of IL-4R in resting and BCR/CD40-stimulated B cells. Shadow: isotype control; Solid line: IL-4R in BCR/CD40-stimulated memory B cells; Dotted line: IL-4R in resting memory B cells.

Fig. S2 Dose titration of IL-4, IL-13 and TGF- β . Purified memory B cells stimulated with the designated dose of IL-4 **(a)**, IL-13 **(b)**, or TGF- β **(c)** in combination with anti-BCR and CD40L were cultured for 24 h. Cells were harvested, then GM-CSF mRNA was evaluated by qPCR. ** indicates $p < 0.01$, un: undetected.