INSIGHTS INTO REGULATORY B CELLS

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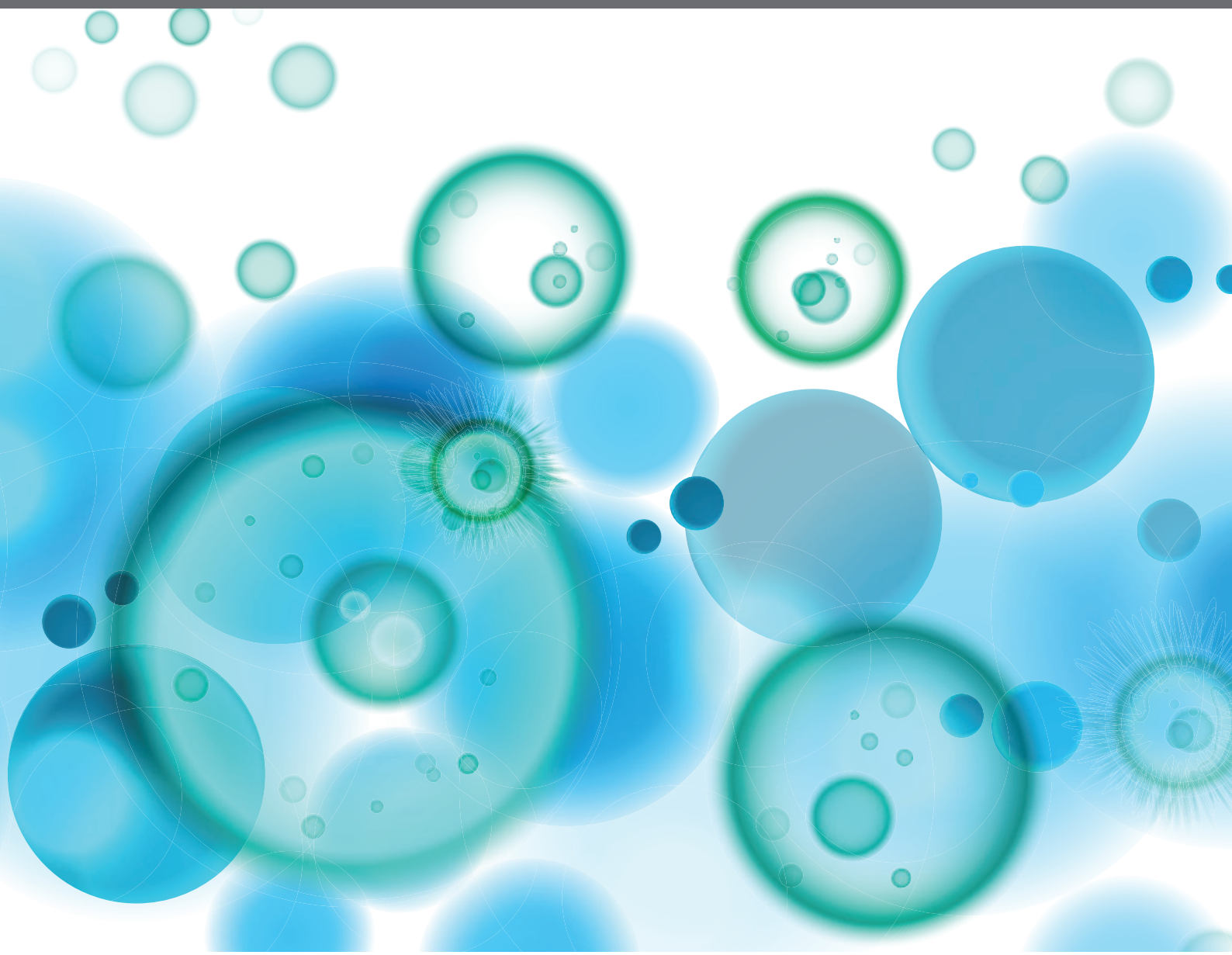

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INSIGHTS INTO REGULATORY B CELLS

Topic Editors:
Yiwei Chu, Fudan University, China
Damo Xu, Shenzhen University, China
Luman Wang, Fudan University, China

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Table of Contents

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EDITORIAL
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Editorial: Insights into Regulatory B Cells

Luman Wang[1], *Damo Xu*[2] *and Yiwei Chu*[1]*

[1] Department of Immunology, School of Basic Medical Sciences, and Institutes of Biomedical Sciences, Fudan University, Shanghai, China, [2] State Key Laboratory of Respiratory Disease for Allergy at Shenzhen University, Shenzhen University School of Medicine, Shenzhen, China

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Editorial on the Research Topic

Insights into Regulatory B Cells

B cells are typically characterized by their ability to produce antibodies, function as secondary antigen-presenting cells (APCs) and secrete various cytokines. Regulatory B cell (Breg), one of the B cell populations is now widely accepted as an important regulatory subset in the immune system. In this collection, we have received articles from all over the world, including the Dittel lab and the Chu lab who have been working in this field for many years. The published articles and reviews describe current understanding of Breg subsets, functions either in health or diseases, and their therapeutic potential.

Unlike Treg, there are no unique markers or released factors (i.e., specific cytokine profiles) to define Breg. Thus, the term Breg refers to B cells with regulatory functions. In the article of the collection, Fu et al. from Chu lab identified a specific Breg subgroup—CD11b$^+$B cells, which specifically secrete IgA in the mucosal immune response and maintain homeostasis. This regulatory function depends on TGF-β signaling pathway. Similarly, the review by Zhong et al. described the regulatory role of IgA$^+$B cells in tumor and other diseases. On the contrary, IgA$^+$B cells could be induced by tumor microenvironment. Mechanistically, the review by Ding et al. summarized the dysregulation of T follicular helper cells (Tfh)/T follicular regulatory cells (Tfr) in autoimmune diseases are closely associated with Breg under different environments through IL-21, PD-L1/PD-1 and other molecules.

With the aim of discovering the unique phenotypes of Breg, Yang et al. from Lian lab published the article "Organ-Specific Regulatory B Cells Using Single-Cell RNA Sequencing". They characterized 19 common genes significantly express in Breg cell, including *Fcrl5*, *Zbtb20*, *Ccdc28b*, *Cd9* and *Ptpn22*. Further, they detected the infiltrated Breg in seven tissue or organs and found Breg subgroups existed with different phenotypes. Moreover, they explored *Atf3* molecule as the potential transcription factor to modulate immune response. In addition, Michée-Cospolite et al. demonstrated the role of transcription factors STAT3 and c-MAF in controlling IL-10 production both in murine and human B cells, and they may be the key drivers to initiate the function of Breg. In the review by Neu and Dittel., they provided a complete and in-depth summary of the discovery history of Breg with phenotypic characteristics, functional modes and interaction with Treg.

With the in-depth research in the field of Breg, its applications in diseases have been studied increasingly. Du et al. from Lu lab, reviewed Breg with impaired immunosuppressive function is negatively correlated with Tfh cell in primary Sjögren's Syndrome (pSS) patients. Further studies have revealed a pivotal role of Breg in constraining Tfh response in autoimmune pathogenesis. This review provides an overview of recent advances in the identification of pathogenic B cell subsets and

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Harry W. Schroeder, University of Alabama at Birmingham, United States

***Correspondence:**
Yiwei Chu
yiweichu@fudan.edu.cn

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Breg cells, as well as new development of B-cell targeted therapies in pSS patients. Additionally, Cui et al. reported a retrospective analysis on positron-emission tomography/computed tomography (PET/CT) scans and Breg detection from patients with newly diagnosed multiple myeloma (NDMM) who received bortezomib plus dexamethasone-based (BD) therapy in southwest China. The baseline PET/CT in combination with Bregs can be used as prognosticators. In the article of "Altered Tim-1 and IL-10 Expression in Regulatory B Cell Subsets in Type 1 Diabetes", Liu et al. showed that altered Tim-1 and IL-10 expression on regulatory B cell in T1D patients. Tim-1 is associated with islet function and blood glucose levels. These findings indicate that Tim-1^+ and IL-10^+ Bregs were involved in the pathogenesis of T1D. In drug therapy, Ma et al. found that drugs for treating B cell mediated diseases like Chloroquine (CQ) had a suppressive effect on IL-10 regulatory B cells whereas Granzyme B (GrB) secreting B cells were less affected. Effector functions of B cells such as plasma blast formation and IgG secretion were potently inhibited by CQ. Effector B cells derived from renal transplant patients under immunosuppression circumstance could also be suppressed with diminished IgG secretion by CQ.

In conclusion, this special issue encompasses a collection of articles that highlight the phenotypes, functions, and applications of Breg in regulatory activity by a variety of mechanisms. B cells are no longer considered as effectors in immune response, but are active participants in immune regulation. This new perspective will likely lead to further investigations into regulatory roles for B cells in health and diseases, and to capitalize on the findings to harness B cells functions as therapies. We welcome more immunologists participate in the research of Breg and broaden the field.

AUTHOR CONTRIBUTIONS

LW, DX and YC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Frontiers of Autoantibodies in Autoimmune Disorders: Crosstalk Between Tfh/Tfr and Regulatory B Cells

*Tingting Ding[1], Rui Su[1], Ruihe Wu[1], Hongwei Xue[1], Yanyan Wang[1], Ronghui Su[1], Chong Gao[2], Xiaofeng Li[1] and Caihong Wang[1]**

[1] Department of Rheumatology, The Second Hospital of Shanxi Medical University, Taiyuan, China, [2] Pathology, Joint Program in Transfusion Medicine, Brigham and Women's Hospital/Children's Hospital and Harvard Medical School, Boston, MA, United States

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Edited by:
Luman Wang, Fudan University, China

Reviewed by:
Steven M. Kerfoot, Western University, Canada
Yuli Lin, Fudan University, China

***Correspondence:**
Caihong Wang
snwch@sina.com

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Balance of Tfh/Tfr cell is critically important for the maintenance of immune tolerance, as evidenced by the fact that T follicular helper (Tfh) cells are central to the autoantibodies generation through providing necessary help for germinal center (GC) B cells, whereas T follicular regulatory (Tfr) cells significantly inhibit autoimmune inflammation process through restraining Tfh cell responses. However, signals underlying the regulation of Tfh and Tfr cells are largely undefined. Regulatory B cells (Bregs) is a heterogeneous subpopulation of B cells with immunosuppressive function. Considerable advances have been made in their functions to produce anti-inflammatory cytokines and to regulate Th17, Th1, and Treg cells in autoimmune diseases. The recent identification of their correlations with dysregulated Tfr/Tfh cells and autoantibody production makes Bregs an important checkpoint in GC response. Bregs exert profound impacts on the differentiation, function, and distribution of Tfh and Tfr cells in the immune microenvironment. Thus, unraveling mechanistic information on Tfh-Breg and Tfr-Breg interactions will inspire novel implications for the establishment of homeostasis and prevention of autoantibodies in diverse diseases. This review summarizes the dysregulation of Tfh/Tfr cells in autoimmune diseases with a focus on the emerging role of Bregs in regulating the balance between Tfh and Tfr cells. The previously unsuspected crosstalk between Bregs and Tfh/Tfr cells will be beneficial to understand the cellular mechanisms of autoantibody production and evoke a revolution in immunotherapy for autoimmune diseases.

Keywords: B regulatory cells, autoantibody, T follicular helper cell, T follicular regulatory cell, germinal center, autoimmune diseases, immunotherapy

INTRODUCTION

Autoimmune disorders encompass a heterogeneous group of diseases in which immune tolerance is broken and the self-immune system mistakenly attack autologous tissues, leading to local and/or systemic damage. In the majority of these diseases, self-reactive lymphocytes and pathogenic autoantibodies are pivotal in inflammation-mediated tissue and organ damage.

The generation of autoantibodies is mainly related to lymphoid follicular germinal centers (GCs), which are microenvironment structures where antigen-specific B cell clones through a multistage differentiation process (1, 2). After priming by antigen, naive B cells undergo somatic hypermutation, affinity maturation, and class switch recombination in a T cell-dependent mechanism in the GCs, and further differentiate into antibody-producing plasma cells and memory B cells. Generally, the GC response is regulated and contributes to the production of antibodies specialized for preventing foreign pathogens. Multiple factors including follicular dendritic cells (FDCs) (3, 4), regulatory T cells (5), and microbiota (6) collaborate to regulate this response. Particularly, T follicular helper (Tfh) cells (7) and T follicular regulatory (Tfr) cells (8–10) are central to the production of antibody in GCs. Tfh cells are characterized by CXC chemokine receptor 5 (CXCR5) and lineage-defining transcription factor B-cell lymphoma 6 (Bcl-6) (11–13). These cells differentiate from naive CD4+T cells after priming by antigen-presenting cells and can access B cell follicles in a CXCR5-dependent manner. During the multistep process of GC reactions, Tfh cells emerge as superior helpers, providing proliferation, selection, and survival signals to cognate B cells, indicating their important role in antibody production (14). However, this help from Tfh cells must be restrained to prevent the production of autoantibodies which underlie unwanted reactions to self-antigens. In 2011, three separate studies initially found a specialized GC-located subset of regulatory T cells (Tregs) required for suppression of the GC response in mice and termed them as Tfr cells (8–10). Since then, Tfr cells in the regulation of immunity have received intense attention. Tfr cells constitutively expressed Foxp3 and CXCR5 and have a phenotype similar to both Treg and Tfh cells. A popular model of their function is to restrain the magnitude of the GC reaction by inhibiting Tfh cell proliferation and self-reactive B cell activation (8–10). Clinical studies have investigated Tfr and Tfh cells in multiple autoimmune diseases and have found a decreased frequency of circulating Tfr cells and an increased Tfh frequency as well as an aberrant Tfh/Tfr ratio in rheumatoid arthritis (RA) (15), systemic lupus erythematosus (SLE) (16) and myasthenia gravis (17). Importantly, the Tfh/Tfr ratios is positively correlated with serum anti-double stranded DNA (dsDNA) antibody level in SLE patients (16). Moreover, previous study transferred Tfr cells *in vivo* to demonstrate their function in restricting autoreactive GC formation and reducing autoantibody-producing B cells (18). Thus, dysregulation of Tfh and Tfr cells may contribute to the production of autoantibodies in autoimmune diseases.

In addition to Tfh and Tfr cells, regulatory B cells (Bregs) exhibit potent regulatory function in autoantibody production through complex interactions with multiple lymphocytes involved in the GC response. Bregs represent a heterogeneous population of B cells possessing immunosuppressive functions through different mechanisms, and there is no lineage-specific transcription factor for the identification of these cells (19). Indeed, the well-established functions of B cells in immune responses are antibody production, pro-inflammatory cytokine secretion and antigen presentation. It was not until the 1970s, that the existence of suppressive subsets of B cells was first confirmed in delayed hypersensitivity reactions (20). In 2002, Mizoguchi et al. (21, 22) reported that interleukin 10 (IL-10)-producing B cells can suppress intestinal inflammation progression in mouse models and first described these cells as Bregs. Currently, various Bregs have been identified, such as $CD1d^{hi}CD5^{+}$Bregs (23), $CD25^{hi}FoxP3^{hi}$Bregs (24) and Tim-1^{+}Bregs (25). The potent regulatory functions of various Bregs have been identified in immune-related pathologies, including inflammation, autoimmunity, and transplantation (22, 23, 26). Several research groups have shown the association between numerically and/or functionally aberrant Bregs and autoimmune diseases such as SLE (27), RA (28), and multiple sclerosis (MS) (29).

There is increasing interest in exploring the regulatory mechanisms of Bregs, especially the interaction with multiple targets cells. $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs function to maintain the Th1/Th2 and Th17/Treg balance *via* IL-10 (28). Moreover, IL-10-producing Bregs inhibit the function of natural killer (NK) cells (30) and plasmacytoid dendritic cells (31). Recently, clinical studies have shown that the dysregulated Tfr and Tfh cells were correlated with impaired Bregs in many autoimmune disorders (32–34). Immunological advance further suggests the regulatory potential of Bregs on Tfr and Tfh cells in the germinal response (26, 33, 35, 36), providing new implications for understanding the production of autoantibodies in autoimmune diseases.

In this review, we briefly outline recent advances in the biology of Bregs and their involvement in autoimmune diseases. In particular, we focus on the interactions between Bregs and Tfh/Tfr cells and how such interactions regulate autoantibody production in autoimmune diseases. Finally, we discuss the therapeutic implications based on Breg-mediated regulation of Tfh/Tfr cells in autoimmune diseases and propose several problems that needed to be solved regarding this therapy. This will give rise to more effective therapies and monitors for autoimmune disorders.

PHENOTYPES OF BREGS

Currently, Bregs are primarily defined by their immunosuppressive function *in vitro* or *in vivo*, especially their capacity to produce the anti-inflammatory cytokine IL-10. Diverse B cell subsets with suppressive functions have been identified in experimental animal models and human, although the unique phenotypic markers for distinguishing Bregs from effector B cells have not been unified, due to the strong heterogeneity of Bregs. In mice, Mauri et al. (37) showed that $CD1d^{+}CD21^{+}CD23^{+}$transitional 2-MZ precursor (T2-MZP) B cells, which secrete IL-10 have immunosuppressive activity both *in vitro* or *in vivo*. Studies have also identified $CD21^{+}CD23^{-}CD24^{hi}$ MZ Bregs (38, 39) and $CD1d^{hi}CD5^{+}$ Bregs (40) with the capacity to

produce IL-10 in mice. In addition, IL-10-producing $CD19^+CD138^+$ plasmablasts have been found in experimental autoimmune encephalomyelitis (41). A study also indicated that the cluster of differentiation 9 (CD9) is a functional marker of IL-10-expressing Bregs in mice (42). In other studies, T cell immunoglobulin and mucin-domain-containing protein (Tim-1) is an important marker for murine IL-10-producing B cells (25, 43). In the study by Lino et al. (44), LAG-3^+CD138^{hi} plasma cells mediated immune regulation in mice. Similarly, $CD24^{hi}CD38^{hi}$ immature B cells (27), $CD24^{hi}$CD27+ B cells (45, 46), and $CD25^{hi}CD71^{hi}CD73^{lo}$ Br1 cells (47) have been reported in humans. Recently, human IL-10-producing IgA+ B cells induced by a proliferation-inducing ligand have been shown to inhibit T cell and macrophage responses (48).

Apart from the most investigated IL-10-producing Bregs, great efforts have been made to identify expanding Breg subsets independent of IL-10. For example, Liu et al. (49) found novel CD11b+ Bregs can suppress CD4+ T-cell responses in mice with experimental autoimmune hepatitis. Moreover, CD38+CD1d +IgM+CD147+granzymeB+ B cells (50) and IL-35-producing Bregs (51) have been identified in humans and mice, respectively. It has also been determined that murine PD-L1hi B cells can suppress humoral immunity (33). B cells expressing IgD at a low level have been identified as a novel population of Bregs in humans (52).

Collectively, the phenotypes of Bregs in different species, organs, and disease models are partially overlapping or distinct, which might due to their adaption to special immune environments (19). Additional studies are needed to determine whether the immunomodulatory function of Bregs relies on their phenotype.

ORIGIN AND INDUCTION OF BREGS

The inability to identify different Breg cell subsets in complex immune microenvironment makes it difficult to understand the generation and biological characteristics of Bregs more deeply. Up to now, the origin of Bregs remains elusive and controversial. B cells can be divided into two main populations according to their distinct origins, B1 and B2 B cells. B1 B cells develop in the fetal liver and mainly present in the peritoneal cavity. B2 B cells develop in the bone marrow and further differentiate into marginal-zone (MZ) B cells or follicular B cell after several transitional stages. Some researchers proposed that Bregs with various phenotypes rise from a special progenitor and lineage-defining transcription factors determine their immunosuppressive nature. Early studies have suggested the B1 lineage of IL-10 producing Bregs as B1 B cells are the main source of B cell-derived IL-10 (53). Bregs can also originate from B2 B cells. In one study, Gαi2-deficient mice developed a spontaneous colitis due to the absence of splenic T2-MZP and MZ B cells, although follicular B cells were not altered, suggesting the potential origin of Bregs from T2-MZP and MZ B cells (54). Subsequent studies also demonstrated that T2-MZP can convert into Bregs (37, 55, 56). Notably, the identification of $CD19^+CD5^+CD1d^{high}$ thymic B cells with suppressive ability suggest the thymic origin of partial Bregs (57). However, there is no lineage-specific markers have been identified in the gene arrays of Bregs (47, 51), which does not support the hypothesis that Bregs arise from a dedicated progenitor. Indeed, growing evidence has demonstrated that multiple B cells at different stages of development can acquire suppressive functions under special microenvironmental stimulations (27, 41, 58). Moreover, Maseda's group (59) reported that antigen-specific *in vivo* signals initiated genetic and phenotypic alternations in B10 cells and led to the conversion of these cells into antibody-producing plasmablasts. These facts strongly support the hypothesis that any B cell has the potential to convert into Bregs in response to appropriate stimuli and their regulatory capacity may change with the environmental alternations.

A complete understanding of the differentiation of diverse Breg is needed. Currently, great progresses have been made in understanding the induction of Bregs by microenvironmental molecules, especially inflammatory regulators. As extensively summarized elsewhere, B cell receptor (BCR) recognition, CD40, and Toll-like receptors (TLRs), contribute to the induction of IL-10-producing Bregs (60). Pro-inflammatory cytokines such as IL-6, IL-1β (61), IL-21 (62), interferon gamma alpha (IFN-α) (31), and B cell-activating factor (BAFF) (63) are also potent inducers of Bregs. Pro-inflammatory cytokines-mediated induction of Bregs can be explained as a feedback mechanism that suppresses the expansion of pro-inflammatory cells and restores immune homeostasis. However, it appears that not all pro-inflammatory cytokines play a role in the generation of Bregs. For example, the deficiency of IL-17, a pro-inflammatory cytokine, led to an increased number of $CD19^+IL\text{-}10^+$Breg in the spleen of a murine model of lupus (64). Of note, IL-35 can induce IL-35-producing Bregs as well (65), suggesting the potential role of anti-inflammatory cytokines in the differentiation of Bregs. Moreover, gut-microbiota-derived signals are important in the development of IL-10-producing Bregs and drive their differentiation by inducing the production of pro-inflammatory cytokines by DCs and macrophages (61). This was corroborated by study showing that the elimination of gut microbiota through antibiotic treatment led to reduced IL-10-expressing Breg frequency in mice compared to the controls (61). Subsequent studies suggested that intestinal microbiota drove B cells into IL-10-producing Bregs *via* TLR2/MyD88/PI3K signaling (66). Furthermore, stimulation from bacteria-derived oligodeoxynucleotides bas been shown to induce the generation of human $CD24^{hi}CD38^{hi}$ Breg-like cells *in vitro* (67). Similarly, the DNA of gut microbiota has been shown to mediate the expansion of Bregs in MRL/lpr mice (68). More studies are needed to determine the mechanism of underlying bacterial DNA-mediated induction of IL-10-producing Bregs.

A recent study identified IL-10^+LAG-3^+CD138^{hi} regulatory plasma cells in germ-free mice, suggesting that gut microbiota is not indispensable in the generation of all subsets of Bregs (44). The study also found that these regulatory plasma cells were naturally existed in the spleen and bone marrow of naive mice, rather than develop in response to stimulation. They originate from several B cell subsets including B1a, B1b, and B2 cells in a BCR-dependent manner and produce IL-10 after activation by TLR signals (44). Indeed, the existence of naturally occurring $CD19^+CD25^{high}CD27^{high}CD86^{high}CD1d^{high}IL\text{-}10^{high}$ Bregs in

humans has also been confirmed (69). A complete understanding of these natural Bregs and their relationship with inducible Bregs is needed. In addition, more studies are needed to explore the origin and development of various Bregs, which will provide novel insights into the therapeutic potential of Bregs in autoimmune disorders.

SUPPRESSIVE MECHANISMS AND EFFECTORS OF BREGS

The most investigated regulatory mechanism of Bregs centers around their production of IL-10. Mice with IL-10 knocked-out in B cells developed exacerbated arthritis accompanied by increased antibodies and inflammatory Th1 and Th17 cells, suggesting the important role of IL-10 in B cell-mediated immune regulation (70). Consistently, human $CD19^{+}CD25^{high}$ Bregs can inhibit the expansion and function of autologous CD4+ T cells, and promote the differentiation and activity of Tregs *via* secretion of IL-10 (71). Moreover, $CD1d^{hi}CD5^{+}$Breg-derived-IL-10 inhibits the activation of $IL\text{-}13^{+}$ type 2 innate lymphoid cells (ILC2s) and thereby suppressing the inflammation response in contact hypersensitivity (72). In experimental allergic encephalomyelitis (EAE), regulatory plasmablast-derived IL-10 also inhibits the production of IFN-α by DCs, a key cytokine in the expansion of pathogenic T cells (41).

The IL-10-independent regulatory mechanisms of Bregs are primarily mediated by IL-35 (51), transforming growth factor beta (TGF-β) (73), and granzyme B (50). These cytokines act by inducing Treg and inhibiting effector T cell differentiation (50, 51, 73). Moreover, programmed death-ligand 1 high (PD-L1hi) Bregs inhibit the expansion of Tfh cells and effector T cells through programmed cell death protein 1 (PD-1)/PD-L1 signaling (33). $CD1d^{+}$T2-MZP Bregs functioned to regulate the activity of invariant natural killer (iNKT) cells through CD1d–lipid presentation, thereby inhibiting excessive inflammation (74). Notably, specific IgG4 antibodies produced by human $CD73^{-}CD25^{+}CD71^{+}$ IL-10-producing regulatory B cells also play an important role in the suppression of antigen-specific CD4+ T cell proliferation (47). Other molecules such as glucocorticoid-induced tumor necrosis factor receptor ligand (52, 75), intercellular adhesion molecule 1, and fasciclin 1 also mediate the suppressive function of several populations of Bregs (76). Recently, the importance of microbiota-derived butyrate, a type of short-chain fatty acid (SCFA) in the function of IL-10-producing CD19+CD21hiCD24hiB cells has been identified. Butyrate activates transcriptional marker aryl-hydrocarbon receptor (AhR) in a manner dependent on 5-Hydroxyindole-3-acetic acid (5-HIAA) and therethrough indirectly supports $CD19^{+}CD21^{hi}CD24^{hi}$Breg cell function (77). Although butyrate supplementation can support suppressive function of Bregs and inhibits arthritis in mouse models, it is unclear which species of the gut microbiota are involved in SCFA-mediated regulation of Bregs. The efficacy of dietary invention with butyrate also remains to be confirmed in RA patients.

Importantly, several transcription factors important for the suppressive functions of Bregs have also been identified. A study from Florian et al. (78) on the transcriptomic meta-analysis of human Bregs identified two critical immune regulatory transcriptional signatures, *GZMB* and *IL10RA*, among 126 differentially expressed genes between Bregs and non-Bregs. Recent research also suggests an important role for transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) in the function of activated B10 cells. The authors found that Blimp-1 promoted the transcription of IL-10 when accompanied by phosphorylated signal transducer and activator of transcription 3 (STAT3) (79). Another transcription factor interferon regulatory factor 4, serves as a crucial modulator of TLR signaling, promoting IL-10 secretion by plasmablasts in the draining lymph nodes (LNs) (41). Studies have also identified the high expression of AhR in Bregs, which maintains the phenotype of splenic $CD19^{+}CD21^{hi}CD24^{hi}$Bregs by regulating IL-10 production and by restricting pro-inflammatory gene expression. This is supported by the fact that mice with AhR deletion developed exacerbated arthritis accompanied by reduced IL-10-producing $CD19^{+}CD21^{hi}CD24^{hi}$Bregs (80). Hypoxia-inducible factor-1α is also a critical transcription factor which regulate IL-10 expression in B cells (81). However, it is unclear whether these transcriptional determinants of Breg immunosuppressive functions are shared by different Breg subsets. To date, Breg-cell-specific transcription factors are largely undefined, and more studies are required in the future.

The potential role of Bregs in regulating Tfh and Tfr cells may also contribute to suppressing inflammation (33). However, this regulation appears more complex and will be discussed below. In summary, diverse mechanisms of Breg function are associated with the complex interactions between Bregs and other immune cells. Further studies are need to explore the transcriptional mechanisms underlying these interactions.

INVOLVEMENT OF TFH AND TFR IN AUTOIMMUNE DISEASES

Tfh and Tfr Control Autoantibody Production

Autoantibodies are serological markers and pathological contributors to many autoimmune diseases. Autoantibodies can lead to the deposition of immune complex in various organs, thereby activating the complement system and/or activating immune cells, resulting in severe inflammatory damage. Moreover, autoantibodies cause direct damage of target tissue *via* antibody-dependent cell-mediated cytotoxicity (82). Generally, high-affinity antibodies generated in the GC during humoral immunity are responsible for providing long-term protection against the multiple pathogens. Upon antigen recognition, mature naïve B access the GC and further interact with T cells to differentiate into antibody-producing plasmocyte (1). A central step of high-affinity antibody maturation is somatic hypermutation (SHM) of BCR genes. However, in addition to enhancing affinity, this process can give rise to self-reactive BCRs at the same time (83), which underlines autoantibodies production. Although multiple checkpoints exist, including central (84) and peripheral tolerance (85), elimination of massive autoreactive B cells, more direct regulations at the GC level are necessary to prevent autoantibody production.

Since the discovery of Tfh and Tfr cells, the immunological mechanism underlying autoantibody generation in autoimmune diseases gradually becomes clear. Tfh cells are specialized

CD4+ T lymphocytes required for the initiation of GC response in humoral immune responses. CXCR5, BCL6 and IL-21 are functional markers commonly used to define these cells. Within the follicle, Tfh cells provide signals for GC-B cell survival, affinity maturation, proliferation, and differentiation (86). These signals include cytokines such as IL-4 and IL-21 (14), and cellular interaction through surface molecules including CD40L (14, 87, 88), ICOSL (89) and PD-1 (90). The interaction between Tfh and GC-B cells is not well defined. It has been reported that the frequency and antigen affinity of B cells determine their capacity to receive help from T cells (91). Normally, only high-affinity B cells successfully compete for Tfh help and undergo rounds of selection to differentiate into antibody-producing plasma cells, whereas other B cells without this help may die rapidly. A series of studies have indicated that excessive Tfh cell response leads to the production of autoantibodies (92–94). It is possible that self-reactive B cells can receive help from excessive Tfh cell signals and escape from tolerance, leading to the generation of autoantibodies.

Conversely, Tfr cells act as negative regulators of autoantibody generation. Tfr cells are mainly differentiated from thymic forkhead box protein 3 $(Foxp3)^+$ Treg precursors and characteristically express Foxp3 and CXCR5. Moreover, other molecules such as Bcl-6 and PD-1 are also expressed in Tfr cells (8). Tfr cells are critically involved in regulating the GC response. On the one hand, Tfr cells inhibit the proliferation and function of Tfh and B cells in GC in a cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-dependent manner (95, 96). Deletion of CTLA-4 on Tfr cells results in impaired class-switch recombination to IgG1, as well as unrestrained proliferation of Tfh cells (97). On the other hand, Tfr cells act by secreting anti-inflammatory cytokines including IL-10 (98), TGF-β (10, 99), and granzyme B (10) (**Figure 1**). Additionally, Tfr cells seem to impair the B-cell response to Tfh cell stimulation by inhibiting B-cell metabolic pathways, thereby leading to decreased antibody production (96). Importantly, studies of infection models have indicated that Tfr cells eliminate autoreactive B cells in the GC response, suggesting the important role of these cells in maintaining B-cell tolerance (100). This is supported by the fact that ablation of Tfr cells promotes autoantibody production in house dust mite models (101). In another study, mice with impaired GC-Tfr cells due to conditional knockout of nuclear factor of activated T-cells 2 showed increased pathogenic anti-dsDNA and developed lupus-like disease after immunization with chromatin (102). Thus, it could be possible that abnormalities in Tfr cells numbers and functions lead to impaired negative selection of autoreactive B cell and enhanced Tfh activity, which ultimately promotes autoantibodies production in autoimmune diseases.

Given the opposing roles of Tfh and Tfr cells, a balance of them is indispensable for fine tuning the GC response. Multiple factors such as microRNAs (103), gut microbiota (104), and cytokines [especially IL-10 (26), IL-2 (100, 105) and IL-21 (18, 106)], are crucial in regulating the Tfr/Tfh balance. Indeed, the interactions of Tfh and Tfr cells are more complex than discussed above. Recent evidence indicates that Tfh cells can convert into Tfr cells in an IL-2 dependent manner, providing new insights into regulation of Tfr/Tfh balance (107). Moreover, in a breakthrough discovery, Wu et al. (108) found a distinct subset of Tfh cells that could promote Tfr cell differentiation by blocking the Wnt-β-catenin axis in a sclerostin domain-containing protein 1-dependent (SOSTDC-1) manner. Elucidating the cellular and molecular mechanisms underlying the activities of Tfh and Tfr cells in the GC response as well as predominant determinants of the Tfr/Tfh balance will contribute to the regulation of autoantibody production.

Dysregulated Tfh/Tfr in Autoimmune Diseases

Investigations of the function of Tfh and Tfr cells in regulating antibody production have promoted the discovery of detailed roles for these cells in the pathogenesis of autoimmune diseases. In various established mouse models of autoimmune diseases, both Tfh and Tfr cells are dysregulated. For example, the frequency of Tfr cells in the spleens of BXD2 mice is reduced, whereas Tfh frequency is significantly increased and positively correlated with the frequency of GC B cells (109). It has also been demonstrated that mice deficient in Tfr cells are more prone to developing experimental Sjögren's syndrome (ESS) than wild-type mice (110). By contrast, the transfer of Tfr cells into BXD2 mice suppressed GC development (18), indicating the crucial role of these cells in immune tolerance.

Studies on Tfh and Tfr cells in human autoimmune diseases are largely restricted to circulating Tfh (cTfh) and Tfr (cTfr) cells, given the difficulty obtaining human secondary lymphoid organs, although the GC response usually occurs in lymphoid organs. cTfh cells can home to LNs and have the superior capacity to provide help for B-cell activation, whereas cTfr cells have a much lower suppressive capacity than LN Tfr cells (98). Notably, these cells are derived from peripheral lymphoid tissues (111) and have comparable Foxp3 expression with tonsil-derived Tfr cells (112). After special activation, cTfr cells can be recruited to GCs to exert suppressive function (98). As a consequence, cTfh and cTfr cells are also closely related to the GC response. It is possible that cTfh cells and cTfr cells vary with alterations in the germinal center, making them indicators of humoral activity and disease severity (111).

Alterations in cTfr cells and cTfh cells occur in a variety of human autoimmune diseases. Patients with autoimmune diseases such as RA (15, 113), SLE (16, 114), myasthenia gravis (MG) (17), primary biliary cholangitis (PBC) (115), and antineutrophil cytoplasmic antibody-associated vasculitis (116) often have an uncontrolled expansion of Tfh cells and decreased Tfr cells in the blood. In MS patients, the functions of cTfr cells are significantly impaired (112) and the cTfr/cTfh ratio is conversely related to IgG production (117). More importantly, the ratio of cTfh/cTfr is inversely correlated with disease activity in many autoimmune diseases (15, 118, 119). Taken together, these findings raise the possibility that impairment of Tfr cells as well as excessive Tfh activity are implicated in the pathogenesis of autoimmunity.

Inconsistent with the above findings, recent studies have described an increase of cTfr cells in SLE (120), RA (121), Sjögren syndrome (111, 122), and AS (123), as well as unchanged frequency of total cTfh cells in muscle-specific kinase MG (MuSK-MG) patients (124). This can be explained by the heterogeneity of patients in different studies. Alternatively, the increase in Tfr cells can also be considered as feedback to enhance the Tfh response in these patients; however, this feedback might be insufficient. In a

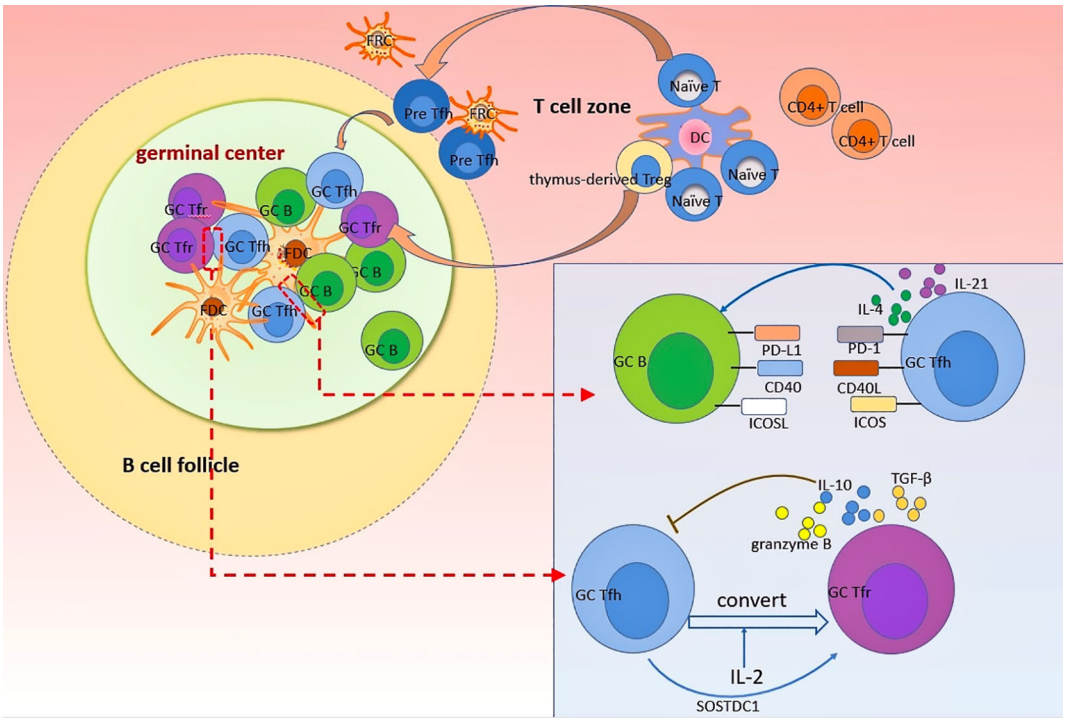

FIGURE 1 | Dynamics of Tfh and Tfr cells in GC response. Naïve T cells and thymus-derived Treg can differentiate into Tfr and Tfh cells, respectively, after the priming by dendritic cells. Differentiated Tfr and Tfh cells gradually migrate into follicles in a CXCR5-dependant manner to exert profound impacts on GC B cells. Follicular stromal cells such as FDC provide an important plat form for various cellular interaction. In GC, Tfh cells support B cells differentiation and antibodies production by providing essential signals to B cells through direct interactions mediated by PD-1, CD40L, and ICOS, as wells as cytokines such as IL-4 and IL-21. By contrast, Tfr cells uniquely inhibit the differentiation and function of Tfh cells through secreting several anti-inflammatory cytokines (including IL-10, TGF-β, granzyme B) to suppress the GC response. The crosstalk between Tfr and Tfh cells is complex. Tfh cells can convert into Tfr cells after stimulation by IL-2. Moreover, SOSTDC1-producing Tfh cells can serve as an inducer of Tfr cells.

study of RA, the Tfr/Tfh ratio decreased, despite increases in both cTfh and cTfr cells (118). Another explanation for cTfr increasing in some studies may be that the proportion of activated cTfr cell subsets is reduced, although overall cTfr cells are increased, ultimately leading to an excessive GC response. Consistently, studies of SLE patients have indicated that the active phenotypes of cTfh and cTfh cells are altered and might lead to autoimmunity, whereas overall cTfh and cTfr cells are not significantly different between patients and healthy controls (107). The recent description of Th2- and Th17-like cell subsets of cTfr cells representing ongoing humoral responses in pSS also supports the imbalance of cTfr subsets in autoimmune diseases to some extent (125). Moreover, observations from Li et al. (124) in MuSK-MG patients support a key role of Tfh17 cells in blood, but not total Tfh or activated Tfh cells, in the activation of B cells, which indicated cTfh subpopulation are also imbalanced in autoimmunity.

Taken together, besides the alternations in frequency and function, the imbalance of specific Tfr cell subsets and Tfh cells subsets in circulating also appears to account for the uncontrolled GC response in autoimmune diseases. The functional diversity of Tfh and Tfr cells in different complex immune compartments such as spleens, Peyer's patches, CLN, joints synovia of RA, and salivary glands of patients with SS are largely unknown. Although difficult, isolation of Tfh and Tfr cells from human tissue will greatly contribute to uncovering the function and underlying mechanism of these cells in autoimmune diseases in the future.

REGULATION OF BREGS BY TFH AND TFR CELLS

The development and function of Bregs are likely to be regulated by Tfh cells. One of the early lines of evidence from MRL/lpr mice revealed that B10 cells in the spleens were expanded and the percentage of these cells had a strong positive correlation with Tfh percentage (126). In *vitro* data also showed that supernatants from cultured Tfh cells induced IL-10 production by B10 cells, and this impact could be eliminated by neutralization of IL-21 (126). In this regard, the author speculated that Tfh cell-derived cytokine IL-21 drove the differentiation and IL-10 production of B10 cells. Further data showed that this mechanism was related to the activation of phosphorylated -STAT3 by IL-21 (126). A similar study also revealed that cTfh cell from SLE patient contributed to the expansion of $CD19^{+}CD5^{+}CD1d^{hi}$Bregs (34). This finding

proposed that the induction of $CD19^+CD5^+CD1d^{hi}$Bregs by Tfh-derived IL-21 was regulatory feedback. Indeed, the importance of IL-21 in promoting $CD19^+CD5^+CD1d^{hi}$Bregs generation has already been identified in mice with MS (62). Moreover, Tfh cells as the predominant origin of IL-21 in GC have also been reported (127). This study on SLE linked IL-21 to Tfh-mediated regulation of B cells, providing important evidence that Tfh cells can induce $CD19^+CD5^+CD1d^{hi}$Bregs, possibly through IL-21 in autoimmune diseases. Most recently, it was found that CD25+ Foxp3+ Treg-like Tfh cells, a specific subset of Tfh cells with the capacity to produce IL-21 during chronic hepatitis B virus (HBV) infection, not only promote the differentiation of B cells into IL-$10^+CD10^-CD27^-CD19^+$ Bregs but also enhance the suppressive function of Breg (128). It is conceivable that excessive Tfh cell responses evoke reactive differentiation of Bregs both in autoimmunity and infection. The upregulation of Bregs by Tfh cells can be considered as a potential regulatory feedback mechanism to inhibit proinflammation response. We speculate the function of these Tfh cell-induced Bregs are likely impaired, which eventually led to autoimmunity.

Studies regarding the regulatory properties of Tfr cells on Bregs in autoimmune diseases are rare. However, in acute respiratory distress syndrome, expanded Tfr cells reportedly significantly increase IL-10+Breg cell frequency significantly *in vitro*, extending the understanding of Tfr cell function in immune suppression (129). A recent study in atherosclerosis also indicated that Tfr cells are able to promote $B220^+CD43^-CD1d^{hi}CD5^+$Bregs generation both *in vivo* and *in vitro*. In this study, transfer of Tfr cells into mice with atherosclerosis triggered a significant expansion of $B220^+CD43^-CD1d^{hi}ghCD5^+$Bregs (130). *In vitro* studies implicating the Tfr cell-mediated increase of $B220^+CD43^-CD1d^{hi}ghCD5^+$Bregs requires direct cellular contact and was in proportion to the number of Tfr cells (130). Besides, this effect relies on the presence of Tfh cells (130). It is possible that excessive Tfh activity initiate a series of Tfr-mediated anti-inflammatory responses, the expansion of $B220^+CD43^-CD1d^{hi}ghCD5^+$Bregs Bregs is one of them. Yet Breg expansion in this study may also due to the direct induction by Tfh cells. To date, similar findings in autoimmune diseases have been absent. Further studies are needed to determine how Tfr cell functions to regulate Bregs and whether this impaired regulation correlates with the development of autoimmune diseases.

NOVEL INSIGHTS INTO BREG-MEDIATED REGULATION OF TFH AND TFR IN AUTOANTIBODY PRODUCTION

Bregs Regulate the Differentiation and Function of Tfh and Tfr Cells

As a potent regulator, Bregs have extensive impacts on various immune cells in complex immune microenvironment (19). The interactions between Bregs and their target cells is a hot topic in immune regulation. Recent evidence suggests the emerging role of several Breg cell types in the regulation of Tfh cell differentiation and function. Tfh and B cells co-culture experiment has shown that the addition of $CD19^{hi}IgD^+CD38^{hi}CD24^{hi}CD40^{hi}$PD-$L1^+$IL-$21R^+$ human Bregs in the system significantly inhibit Tfh cell maturation, while Tfr cells proportion was increased significantly (35). These Bregs also preclude mature Tfh cells-mediated plasma cell survival and IgM, IgG, and IgA production, suggesting an important role of Breg in inhibiting Tfh cell function (35). Consistently, in a variety of diseases, the number and function of Bregs are decreased, and they are also strongly correlated with the frequency and function of Tfh cells. For example, the study conducted on pSS patients have described an inverse relationship between Tfh percentage and IL-$10^+CD19^+CD24^+CD38^{hi}$ Breg percentage in the circulating (32). The authors further confirmed that IL-10-producing ability of these Bregs in pSS patients was significantly impaired than that in healthy controls, and these Bregs could not effectively inhibit autologous Tfh cell expansion (32). Studies performed in mouse models of EAE (33) and atherosclerosis (36) have further confirmed that PD-$L1^+$ Bregs and MZB cells could inhibit the development of Tfh cells and Tfh cell-mediated proatherogenic response, eventually abrogating the acceleration of diseases. Thus, Bregs regulate Tfh cell response and this regulation are more likely to be impaired during various inflammatory diseases.

The detailed Breg regulatory mechanism of Tfh cells differentiation and function are largely undefined and are suggested to rely on direct Breg-Tfh cell contacts and several soluble factors (35). Particularly, Breg-derived IL-10 may play a central role in Tfh cells regulation. Lin et al. (32) found that $CD19^+CD1d^{hi}CD5^+$Breg from mice suppressed Tfh cells differentiation in an IL-10-dependent manner *in vitro*. Adoptive transfer of IL-10−/− B cells into ESS mice led to higher GC Tfh and plasma cell accumulation than control mice transferred with WT B cells. They also detected high expression of IL-10 receptor on Tfh cells in mice and man, suggesting the potential role of IL-10 in the regulation of Tfh (32). Importantly, IL-10 downregulated the expression of transcription factor achaete-scute homologue 2 (Ascl2) in Tfh cells (32). Ascl2 is a key regulator during Tfh cells development (131). Downregulation of Ascl2 is thought to inhibit CXCR5 expression, which results in impaired Tfh differentiation and function as CXCR5 is constitutively expressed in Tfh cells and is critical for the maturation and activity of these cells (86, 132, 133). Notably, p-STAT5 inhibition can eliminate IL-10-mediated suppression on Tfh cells (32). These findings suggest the important role of the IL-10-pSTAT5-Ascl2-CXCR5 axis in Tfh cell differentiation and function. Moreover, PD-1/PD-L1 signaling is also involved in Breg-mediated inhibition of Tfh cells. Splenic B cells show the enhanced capacity to promote functional transcription factor Bcl-6 expression in Tfh cells after blocking PD-L1 on B cells (134). PD-L1- abrogated MZB cells are unable to limit the proatherogenic Tfh response and caused severe atherosclerosis (36). The author further found that MZB cells suppress Tfh cell motility in a PD-L1-mediated manner, which may be associated with the impaired capacity of Tfh cells to provide help for B cells (36).This is in agreement with a previous study which indicated that PD-$L1^{hi}$ Bregs require the high expression of PD-L1 to repress Tfh expansion in EAE mouse

models (33). Notably, it is possible that Tfr cells participate in Breg-mediated regulation of Tfh cells, given that the differentiation and function of Tfh cells are restricted by Tfr cells. However, after the transfer of PD-L1hi Breg, the percentage and number of Tfh cells in mice were markedly decreased without an increase in Tfr cells, suggesting that this Breg cell type might act directly on Tfh cells rather than *via* Tfr cells (33). Additionally, MZB cells inhibit the accumulation of Tfh cells and GC B cells in the spleen to achieve atheroprotective effects, and the activating transcription factor 3 plays a central role in this effect (36). The expression of transcription factor CTLA-4 is downregulated in Tfh cells when MZB cells are deficient, making Tfh cells susceptible to being activated, suggesting the important role of MZB cells in suppressing the activation of Tfh cells (36). Notably, CTLA-4 expressed at a high level in Tfr cells is critically linked to the suppressive capacity of these cells (135). Considering the role of MZB cells in the maintenance of CTLA-4 in Tfh cells, it will be interesting to determine whether MZB cells affect the function of Tfr cells.

Notably, current studies indicate that Tfh cells contain three subsets with different expression of chemokine receptors, including Tfh1, Tfh2, and Tfh17 (136). Thus, studies on the impact of various Bregs on Tfh cells should evaluate the level of these cellular subsets. In patients with idiopathic pulmonary fibrosis, a decrease in circulating $CD3^-CD19^+CD24^{hi}CD27^+$Bregs has been observed, accompanied by the altered profile of Tfh-cell subsets, in which the proportion of Tfh2 cells and $PD\text{-}1^+ICOS^+$-activated Tfh cells are elevated while Tfh17 cells are decreased (137). Similarly, Tfh2 skewing and $CD19^+CD24^{hi}CD27^+$Bregs decrease also occurs in the peripheral blood of patients with allergic rhinitis (AR) and AR combined with Asthma (138, 139). The %Tfh2 cells per $\%CD19^+CD24^{hi}CD27^+$Bregs had a positive correlation with the levels of biomarkers of allergic airway inflammation, making it an exaggerating factor during AR progression to AR with asthma (138). Furthermore, the cTfh2/$CD19^+CD24^{hi}CD27^+$Bregs ratio correlates with plasma levels of CXCL13 in asthma (139). Studies in autoimmune diseases previously showed that CXCL13 indicated the disease activity (140–142). Thus, the cTfh2/$CD19^+CD24^{hi}CD27^+$cBreg ratio might represent a useful biomarker for diagnosis, severity, and treatment efficacy of autoimmune diseases. Taken together, these data raise a question about the impact of Bregs on different subsets of Tfh cells, especially Tfh17 and Tfh2, two subsets of Tfh cells reported to be correlated positively with antibody production in several autoimmune diseases such as myositis (136), vasculitis (143) and Sjögren's syndrome (144). It is possible that the functional and/or numerical deficit of Bregs contributes to the polarization of Tfh2 and underlies the development of autoimmune diseases. However, after asthma treatment, the symptoms and cTfh2 skewing were improved, while the percentage of $CD19^+CD24^{hi}CD27^+$Bregs was not significantly different (139). This finding indicates other mechanisms, independent of Bregs function, operate in the regulation of Tfh cell subsets, which require more in-depth studies.

Although Tfr cells have similar phenotypes to Tfh cells, studies regarding the role of Bregs in regulating Tfr cell generation are rare. IL-10 deficiency in B cells caused decreased Tfr cells in the B cell follicles (26). One *in vitro* assay cultured Tfh and $CD19^{hi}IgD^+CD38^{hi}CD24^{hi}CD40^{hi}PD\text{-}L1^+IL\text{-}21R^+$ Bregs sorted from humans showed that the proportion of Foxp3+ cells was increased, suggesting this type of Bregs can induce Tfr cells (35). The increased level of TGF-β in the cocultures and diminished frequencies of Foxp3+ T cells after anti-TGF-β blocking antibodies indicated the requirement for TGF-β in the induction of Tfr cells by these Bregs (35). Remarkably, the effects of several Bregs types on Tregs, a subset of T cells known to differentiate into Tfr cells (8, 10), have been extensively described. $CD19^+CD24^{hi}CD38^{hi}$ Bregs (28) and $CD19^+CD25^{hi}$ Bregs (71) from human as well as $IL\text{-}10^+CD1d^{hi}CD5^+$ Bregs (145) and $B220^+CD23^+$T2-MZP B cells (37) from mice all have the capacity to induce Tregs through IL-10 production. Thus, it is conceivable that Bregs promote the development of Tfr cells *via* inducing Treg cells. Studies have also shown that IL-10 deficiency in B cells leads to impaired Tfr cells differentiation and prevent tolerance to the allogeneic cardiac allograft, indicating an important role for IL-10 in B cells-mediated regulation of Tfr cells (26). Notably, IL-10 production is not unique to Bregs. Tfh and Treg cells reportedly produce IL-10 (146, 147). The possibility that IL-10 produced by these cells is also involved in the Tfr cells regulation cannot be excluded.

Interestingly, the ability of different subsets of Bregs to regulate Tfr cells appears to be distinct, due to their different functional markers. For example, human IL-10-producing $CD19^{hi}IgD^+CD38^{hi}CD24^{hi}CD40^{hi}PD\text{-}L1^+IL\text{-}21R^+$ Bregs expand Tfr cells (35), while $CD3^-CD19^+PD\text{-}L1^{hi}$ Bregs in mice exhibit a negative effect on the generation of Tfr cells (33). Considering that Tfr cells express large amounts of PD-1 which inhibit Tfr cell development (135), this difference might be attributed to the interactions between Tfr cells and $PD\text{-}L1^{hi}$ Bregs through PD-1/PD-L1. Certainly, species differences might also account for this difference, which needs further confirmation.

Collectively, all these data support the fact that multiple Bregs regulate the function and differentiation of Tfh cells and Tfr cells through complex mechanisms (See **Figure 2**). Possibly, Bregs balance Tfh and Tfr to ensure central tolerance and prevent autoantibodies production, and dysregulation of Tfh/Tfr cells in autoimmune diseases may due to defective Bregs regulation. Notably, different subsets of Breg and/or different contexts of diseases may cause distinct regulatory effects on Tfh and Tfr cells. The detailed mechanisms of underlying this regulation need further study.

Bregs Appear to Be Critical to the Distribution of Tfh and Tfr Cells

In addition to the function and frequency, the spatial distribution of Tfr cells in the immune environment also appears to be critical to the induction of immune tolerance (99). In particular, Tfr cells at the T-B border and within the follicle, but not in the GC, have the most efficient ability to mediate immune suppression (99). Studies using tolerogen-treated mice have shown that selective deletion of IL-10 in B cells results in reduced localization of both Tfr and Tfh cells in B cell follicles, and, in contrast, increased Th17 cells in the GCs of lymph node (LN) and the spleen (26). This distribution of Tfr and Tfh cells can be restored by adoptive

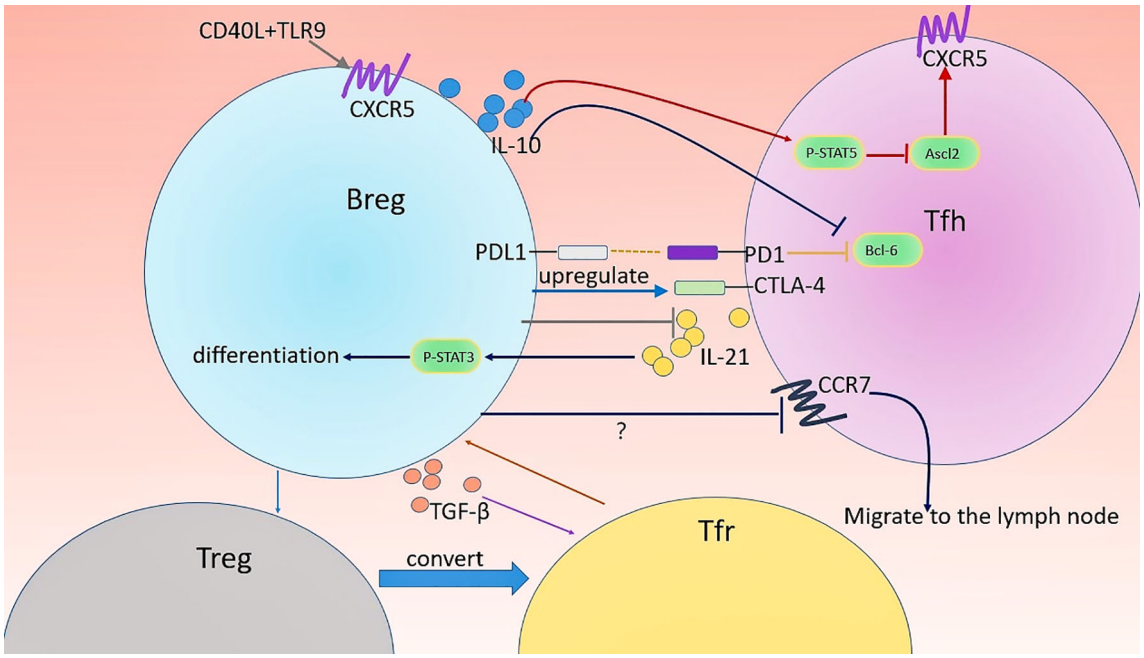

FIGURE 2 | Regulation of Tfh and Tfr cells by Bregs. Several types of Breg are involved in the regulation of the differentiation, function, and distribution of Tfh and Tfr cells. Firstly, CD40 and TLR9 might favor the migration of $CD19^{hi}IgD^{+}CD38^{hi}CD24^{hi}CD40^{hi}PD\text{-}L1^{+}IL\text{-}21R^{+}$ human Bregs into GC by promoting CXCR5 expression. $CD19^{+}CD1d^{hi}CD5^{+}$Bregs-derived IL-10 downregulates the expression of CXCR5 in Tfh cells by inhibiting Ascl2, the positive regulator of CXCR5, leading to impaired maturation and Il-21 production of Tfh cell. Moreover, MZB cells play a crucial role in the maintenance of CTLA-4 in Tfh cells. The location of Tfh cells can affect their function. $IL\text{-}10^{+}$ MZP B cells direct this migration of Tfh cells out of follicles possibly through altering the expression of CCR7 (crucial chemokine receptors required for the localization of T cells in the LN) in Tfh cells. In turn, Tfh cell activates p-STAT3 in an IL-21-depadent manner therethrough driving the differentiation of $CD19^{+}CD5^{+}CD1d^{hi}$Bregs. $CD19^{hi}IgD^{+}CD38^{hi}CD24^{hi}CD40^{hi}PD\text{-}L1^{+}IL\text{-}21R^{+}$ human Bregs can promote Tfr cell differentiation and TGF-β play an important role in this process. Possibly, these Bregs induce Treg through IL-10 production and thereby increase Treg conversion into Tfr cells.

transfer of $IL\text{-}10^{+}$ MZP B cells (26). This study showed that the distribution of Tfh, Tfr, and Th17 cells in secondary lymphoid organs was at least partly controlled by Bregs and this function might be mediated by MZP B cells-derived IL-10, indicating a novel regulatory mechanism for T cell distribution in the GCs. However, it remains largely unknown whether a similar migratory behavior is performed in human. Moreover, the question of what detailed mechanisms are involved in this migration remains. CCR7 are crucial chemokine receptors directing T cells residing in the T zone and down-regulation of CCR7 is indispensable for the follicular positioning of Tfh cells (133). It is possible that MZP B cells downregulate the expression of CCR7 so that Tfh cells can migrate into B cell follicles. However, B cell depletion leads to reduced CCR7 in Tfh cells accompanied by decreased migration of Tfh cells into B cell follicles (26). Thus, the function of MZP B cells in supporting the migration of Tfh cells through CCR7 needs further confirmation. Notably, cTfh cells with low CCR7 expression are increased and have been described as an indicator in autoimmune diseases including AIH (148) and pSS (125), as well as chronic HBV infection (149). $CCR7^{int}$Tfh cells in the blood are closest to tonsillar Tfh lineage cells and exhibit a more potent capacity to induce memory B cells to differentiate into antibody-producing cells than $CCR7^{high}$Tfh cells (150). Therefore, downregulating CCR7 may be important for Tfh cells in promoting antibody production. The effective regulation of GC response may be achieved by altering Tfh cell distribution in the future.

Indeed, the migration of Tfh and Tfr cells into the GC is a complex mechanism with multiple factors involved. Besides CCR7, other elements are also being elucidated. CXCR5 is one of the important molecules guiding the residence in GCs (10, 86, 133). Multiple types of stromal cells in secondary lymphoid organs, including FDCs and fibroblastic reticular cells, which expresses directing signals and provide an important platform for cellular interactions, are also critically determine the GC localization of Tfh and Tfr cells (151). Importantly, activated FDCs express amounts of CXCL13, the ligand for CXCR5, inducing the migration of CXCR5-expressing T and B cell into follicles (132, 152). Whether Bregs change the expression of CXCRL13 in FDCs to mediate the migration of Tfh and Tfr cells warrants further study (26). Notably, positive regulator sphingosine-1-phosphate receptor 2 (153) and negative regulator ephrin-B1 (154) are also involved in the retention of Tfh cells in the GC microenvironment. Thus, the mechanism by which Bregs to regulate the distribution of Tfr and Tfh cells may not be confined to CXCR5. The presence of CXCR5-deficient Tfr cells in the GC also suggest that CXCR5-independent mechanisms operate in their location in the GC (155).

Whether abnormal distribution impacts on the effector functions of Tfh cells and Tfr cells remains unclear. A recent

study conducted in fresh human adenoids showed that Tfh cells in distinct locations differed in their motility and functions (156). In outer zones of GC, Tfr cells are fast-migrating and exhibit brief cellular interaction while the majority of Tfr cells in the central GC zone are static with long-lasting capacity to interact with each other (156). Thus, it is possible that the altered location of Tfh and Tfr cells in the GC of secondary lymphoid organs may lead to their changes in function, in parallel. Notably, studies conducted in sarcoidosis have indicated that cTfh cells can migrate into granulomas to play an inflammatory role (157). Thus, the migration of Tfh cells not only exists in the GC microenvironment in one lymphoid tissue or organ but also occurs between different secondary lymphoid organs. Whether Bregs also play a role in directing this distribution between different lymphoid tissues or organs is unknown. Moreover, the impact of Bregs on the migration of Tfh and Tfr cells in the blood and their counterparts in non-lymphoid tissue such as joint synovia of RA patients and salivary glands of patients with pSS warrant further study.

THERAPEUTIC PROSPECTS OF BREGS-MEDIATED TFR/TFH REGULATION

Given the considerable role of autoantibodies in the development of autoimmune diseases, current therapies focus on eliminating autoantibodies and/or blocking their function in a more precise manner. Particularly, targeting key cells involved in autoantibodies production is more popular due to the high efficacy and less adverse effects. In this regard, Tfr and Tfh cells modulation strategies have potent potential, for the contrasting roles of Tfr and Tfh in the regulation of GC response (158). However, therapies targeting Tfr and Tfh cells directly are extremely limited, due to a poor understanding of their development and function.

Strikingly, specific subsets of Bregs hold considerable promise to regulate Tfr/Tfh balance (26, 32, 33, 35, 36). As mentioned above, defective Bregs may lead to aberrant differentiation, function and distribution of Tfr and Tfh cells in diseases. Indeed, Breg number is decreased and the cross-talks between Breg and their target cells are also compromised in multiple autoimmune diseases (27, 159–161). For instance, in SLE, $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs were unable to restrain IFN-α production by pDCs. pDCs also failed to drive these Bregs differentiation (31). In pSS, CD19+CD24+CD38hi Breg were defective in suppressing the expansion of Tfh cells (32). $IL\text{-}10^{+}CD19^{+}CD24^{hi}CD38^{hi}$ Bregs from thyroid associated ophthalmopathy patients also failed to activate IFN-γ+ and IL-17+ T cells (162). Accordingly, therapies of selectively transferring and/or inducing Bregs, such as $CD1d^{hi}CD5^{+}$Breg and $CD19^{+}CD25^{+}CD1d^{hi}IgM^{hi}$Breg, have been developed to restore homeostasis and shown some success in mice disease models, which provide evidence for the application of Bregs in the treatment (40, 55, 163, 164). Importantly, in mice models of experimental Sjögren's syndrome (ESS), adoptive transfer of $CD19^{+}CD1d^{hi}CD5^{+}$ Bregs effectively suppressed the Tfh cell response, leading to the amelioration of diseases progression (32).

It is noteworthy that multiple strategies to induce Breg cell through key molecules, such as anti-CD40 mAb (163), BAFF (63), enteric microbiota (61, 66), and bacteria-derived oligodeoxynucleotides (CpG-ODN) (67), may also indirectly affect the balance of Tfh/Tfr cells; however, studies in this regard are scarce. Furthermore, as discussed above, the immunosuppressive function of Breg is mostly related to IL-10 secretion. IL-10 would be promising to regulate the differentiation, function and migration of Tfr and Tfh cells. However, IL-10 also acts as a B cell growth factor and can promote autoantibody production (165). The application of IL-10 into Tfr and Tfh cells regulation may cause unwanted pro-inflammatory effects. In addition to treatment, Bregs may be an excellent indicator for evaluating the efficacy and prognosis of diseases. A higher ratio of pre-transplant cTfh/$IL10^{+}CD19^{+}CD24^{+}CD38^{+}$ Bregs is reportedly correlated with graft rejection (166). It may be worth to determine whether this ratio contributes to assessing disease activity and correlates with the level of autoantibody in autoimmune diseases.

However, considering the exact mechanisms underlying the interplays of Breg, Tfh cells, and Tfr cells in health and patients are largely unknown, it is still of great challenge to achieve precise regulation of Tfh and Tfr cells through Bregs. For example, selectively induction of immunosuppressive B cells without activating effector B cells *in vivo* is still difficult, although the great plasticity of Bregs makes it possible to induce these cells in the various microenvironment. Meanwhile, it is hard to know how to make an adequate induction of Bregs. Excessive or insufficient induction of Breg may lead to various dysregulation of the immune system. The safety and efficacy also needed to be determined in more animal and clinical trials in the future. Moreover, Bregs have vast plasticity in disease microenvironments and can differentiate into other kinds of B cells subset including effector B cells. The maintenance of Bregs immunosuppressive function *in vivo* after transferring and/or inducing these cells in patients must be taken into consideration. Another limiting factor for Breg immunotherapy is the identification of multiple immunosuppressive Bregs types in humans. Bregs are extremely heterogeneous and not all types of Bregs equally regulate Tfh and Tfr cells.

Taken together, Breg-mediated Tfr/Tfh regulation provides novel insights in limiting the exaggerated autoantibody production without broad immunosuppression in autoimmune diseases.

CONCLUSIONS

As demonstrated in numerous reports within the literature, Tfh/Tfr balance is necessary to maintain proper antibody production, and this balance has strong links with several types of Breg, such as $PD\text{-}L1^{hi}$B cells, $IL\text{-}10^{+}CD19^{+}CD24^{+}CD38^{hi}$ Bregs, and MZB cells. These Bregs not only regulate the differentiation and function of Tfr and Tfh cells but also appears to affect their distribution in the immune microenvironment. More importantly, Bregs may achieve the fine tune of Tfh and Tfr cells in the level of subpopulations, which contribute to restore the balance of Tfr and Tfh subpopulation in autoimmune diseases. Thus, Breg-based therapy has theoretical feasibility and clinical application prospect to regulate the activity of Tfh and Tfr cells and autoantibody production in autoimmune

diseases. However, A number of questions on the development, phenotype, and function of Bregs remain to be answered, which restrain the translation of Bregs into clinical application. A complete understanding of Breg-Tfh cell and Breg-Tfr cell cross-talks in health and autoimmune diseases is needed, which will provide the rationale for designing more effective immunotherapy in autoimmune disorders.

AUTHOR CONTRIBUTIONS

TD drafted the manuscript, prepared illustrations, and discussed the content with the other authors. CW conceived the topic and revised the content of the manuscript. RS, RW, HX, YW, and RHS revised the manuscript. CG, and XL also critically revised the content of the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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The Multiple Roles of B Cells in the Pathogenesis of Sjögren's Syndrome

Wenhan Du[1†], Man Han[2†], Xiaoxia Zhu[3], Fan Xiao[1,4], Enyu Huang[1,4], Nan Che[5], Xiaopo Tang[2], Hejian Zou[3], Quan Jiang[2] and Liwei Lu[1,4*]*

[1] Department of Pathology and Shenzhen Institute of Research and Innovation, The University of Hong Kong, Hong Kong, China, [2] Division of Rheumatology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing, China, [3] Department of Rheumatology, Huashan Hospital and Fudan University, Shanghai, China, [4] Chongqing International Institute for Immunology, Chongqing, China, [5] Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University, Jiangsu, China

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Damo Xu,
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National and Kapodistrian University of Athens, Greece

***Correspondence:**
Liwei Lu
liweilu@hku.hk
Quan Jiang
drjiang111@126.com

[†]These authors have contributed equally to this work

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Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease characterized by lymphocytic infiltration and tissue destruction of exocrine glands such as salivary glands. Although the formation of ectopic lymphoid tissue in exocrine glands and overproduction of autoantibodies by autoreactive B cells highlight the critical involvement of B cells in disease development, the precise roles of various B cell subsets in pSS pathogenesis remain partially understood. Current studies have identified several novel B cell subsets with multiple functions in pSS, among which autoreactive age-associated B cells, and plasma cells with augmented autoantibody production contribute to the disease progression. In addition, tissue-resident Fc Receptor-Like 4 $(FcRL4)^+$ B cell subset with enhanced pro-inflammatory cytokine production serves as a key driver in pSS patients with mucosa-associated lymphoid tissue (MALT)-lymphomas. Recently, regulatory B (Breg) cells with impaired immunosuppressive functions are found negatively correlated with T follicular helper (Tfh) cells in pSS patients. Further studies have revealed a pivotal role of Breg cells in constraining Tfh response in autoimmune pathogenesis. This review provides an overview of recent advances in the identification of pathogenic B cell subsets and Breg cells, as well as new development of B-cell targeted therapies in pSS patients.

Keywords: primary Sjögren's syndrome, B cells, pathogenesis, treatment, regulatory functions

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a common systemic autoimmune disease, which mainly affects the lacrimal and salivary glands, resulting in dry eyes and dry mouth. However, the extra-glandular manifestations occur in 30 to 40% of pSS patients, involving lung, heart, kidney, nervous system and lymphoproliferative disorders (1, 2). Although the etiology of pSS remains unclear, numerous studies have demonstrated that both T and B cells are the major populations for pro-inflammatory cytokine production and autoantibody secretion with critical involvement in pSS pathology (3). A recent multiple-centre study reported that pSS patients in China had higher positive rates of anti-nuclear antibody (ANA) and anti-Sjögren's syndrome-related antigen A (anti-SSA) antibodies than those of patients in Europe and America, indicating that disease heterogeneity among pSS patients

in different regions (4). Growing evidence indicates that B cells play predominant roles in the pathogenesis of pSS patients with ectopic germinal center-like structures in the exocrine glands and systemic extra-glandular manifestations (5, 6). It has been well recognized that autoreactive B cells and plasma cells contribute to the development of pSS by producing various autoantibodies, including ANA, anti-SSA and anti-Sjögren's syndrome type B (anti-SSB) antibody (7, 8). Recent studies have revealed other functions of B cells such as cytokine production (9, 10) and antigen presentation (11) in the pathogenesis of pSS. Increasing evidence indicates the functional diversities of B cell subsets in both immunity and autoimmune pathogenesis (12). In particular, Breg cells with different phenotypes have been reported to be involved in the development of pSS (13–16). Breg cells exerted their regulatory functions by producing diverse regulatory cytokines and effector molecules, such as IL-10, IL-35 and Granzyme B (GrB). Although B cell targeted therapy is among the most promising therapeutic approaches to various autoimmune diseases, its efficacy in treating pSS patients remains to be further validated. In this review, we discuss the multiple functions of B cell subsets in pSS development and emerging B cell-targeted therapies.

PATHOGENIC B CELL SUBSETS

Fc Receptor-Like 4 (FcRL4)$^+$ B Cells

FcRL4$^+$ B cells are tissue-resident memory B cells that express Fc Receptor-Like 4 (FcRL4). As a member of Fc Receptor-Like proteins family, FcRL4 is mainly expressed by the B-cell lineage (17). FcRL4 is found to dampen B cell receptor-mediated signaling and proliferation, which plays an essential role in regulating B cell activation and differentiation. In 2005, Max Cooper and colleagues first described these B cells in human tonsils (18). Subsequent studies confirmed that FcRL4$^+$ B cells were mainly localized in the sub-epithelial region of lymphoid tissues but rarely in spleen and lymph nodes (19, 20). FcRL4$^+$ B cells are expanded in the inflamed tissues of patients with pSS (21, 22). Moreover, enriched FcRL4$^+$ B cells are detected in salivary glands of pSS patients (21). Further studies on the comparison of FcRL4$^+$ B cells with chemokine receptor CCR5 expression profiles from the parotid glands of pSS patients provide a perspective for understanding their migratory ability to the corresponding chemokines CCL3 and CCL5 produced by ductal epithelial cells and their infiltration in the inflamed glands (22, 23). In addition, studies with gene transcription analysis suggest that parotid FcRL4$^+$ B cells with upregulated *CXCR3* expression may enhance their migration (22). Notably, compared with patients without lymphoma, the percentages of FcRL4$^+$ B cells are significantly increased in pSS patients with MALT-lymphomas, indicating that these B cells may contribute to the development of B cell lymphoma in pSS patients (21).

Lines of evidences suggest that FcRL4$^+$ B cells play a pathogenic role in the development of pSS (21, 22) (**Table 1**). The percentages of parotid FcRL4$^+$ B cells are positively correlated with the numbers of lymphoepithelial lesions in pSS patients (21). Additionally, FcRL4$^+$ B cells produce multiple pro-inflammatory cytokines. *IL-6* gene is significantly upregulated in FcRL4$^+$ B cells from pSS patients, suggesting that FcRL4$^+$ B cell is a pro-inflammatory B cell subset in autoimmune diseases (22) (**Figure 1**). Interestingly, a recent study with transcription analysis of gene expression has revealed that FcRL4$^+$ B cells highly express *ITGAX* (CD11c) and *TBX21* (T-bet) genes (22), similar to the gene expression pattern detected in CD11c$^+$T-bet$^+$ B cells (24, 25). Although both B cell subsets displayed similar patterns of downregulated BCR signaling and enhanced TLR signaling, they may possess different capacities in differentiating into antibody-secreting cells (ASCs) since the lack of transcription factors Blimp1 and IRF-4 in FcRL4$^+$ B cells may reduce their ability to differentiate into ASCs (21). It has been reported that the treatment with Rituximab reduces the number of parotid gland FcRL4$^+$ B cells and restores the glandular epithelium in pSS patients (21). As the first and most widely studied B cell-targeted therapeutic agent, Rituximab depletes mature B cells effectively, lasting four to twelve months (47). In addition to FcRL4$^+$ B cells, circulating Tfh cells and Th17 cells are also reduced by Rituximab accompanied by decreased serum IL-21 and IL-17 levels (48). Thus, further elucidation of the pathogenic mechanisms of FcRL4$^+$ B cells may facilitate the development of novel therapeutic strategies for targeting this B cell subset in pSS.

CD11c$^+$Age-Associated B Cells

CD11c$^+$Age-associated B cells (ABCs) were firstly reported by two independent research groups in 2011 (24, 25). CD11c$^+$ABCs

TABLE 1 | Pathogenic and regulatory B cell subsets in pSS.

B cell subset	Markers	Functions	Potential therapy
FcRL4$^+$ B cell	FcRL4, CCR5, *CXCR3, ITGAX*, *TBX21* (22, 23)	Pathogenic	Rituximab (21)
Age-Associated B cell	CD11c, T-bet, CXCR5, CD21, CD23 (24–26)	Pathogenic	Belimumab (27) Telitacicept (28) Remibrutinib (29, 30) Iscalimab (31), Abatacept (32)
Transitional B cell	CD21, IgD, IgM (33)	Pathogenic	
Marginal Zone B cell	CD21, CD23, IgD (34)	Pathogenic	Rituximab
Memory B cell	CD27, CXCR4, CXCR5 (35)	Pathogenic	
Plasma cell	CD138, CD27, CD38, Bcl-2 (36–39)	Pathogenic	Bortezomib (40)
IL-10$^+$ Breg	CD24, CD38, CD1d, CD5, IL-10 (41, 42)	Protective	
GrB$^+$ Breg	CD5, GrB (43)	Protective	
IL-35$^+$ Breg	CD138, TACI, CXCR4, IL-35 (44, 45)	Protective	
Regulatory Plasma Cell	LAG-3, CD138 (46)	Protective	

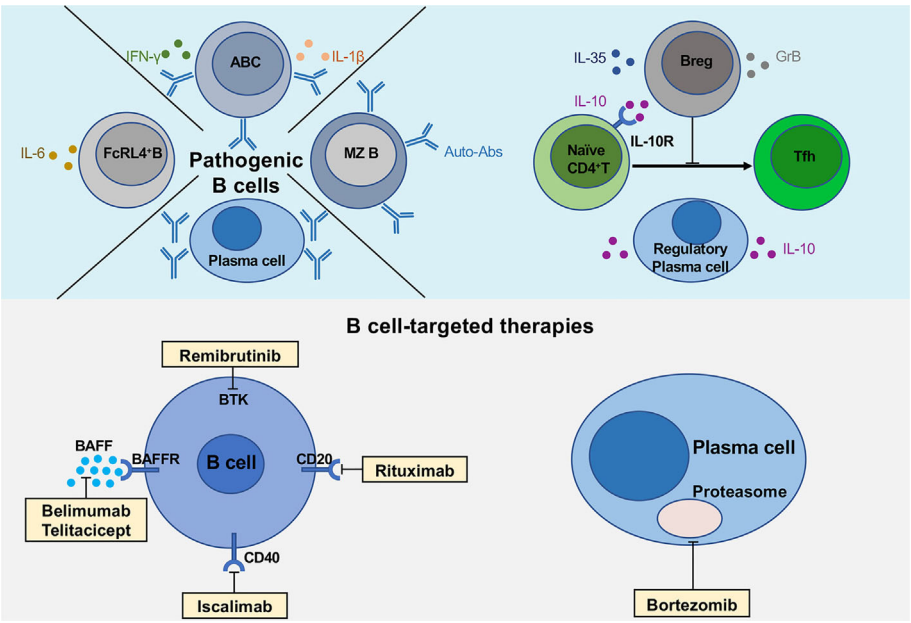

FIGURE 1 | Multiple functions of B cells and novel B cell-targeted therapies in pSS. The upper part of the figure shows the pathogenic B cell subsets (FcRL4$^+$ B cells, age-associated B cells and plasma cells), regulatory B cells and plasma cells secreting various cytokines, IL-10, IL-35 and GrB. The lower part describes the multiple B cell-targeted therapies, consisting of direct and indirect B cell depletion, plasma cell depletion.

are characterized by phenotypic markers including CD11c$^+$, CD11b$^+$, CD21$^{-/low}$, CD23^{low} (24, 25), which are notably expanded in the peripheral blood of patients with several autoimmune diseases, including systemic lupus erythematosus (SLE) (49, 50), RA (25) and multiple sclerosis (MS) (51). In pSS patients, CD11c$^+$ABCs infiltrated in parotid glands have been identified with double staining for CD11c and Pax5 (22). CD11c$^+$ABCs express high levels of the transcription factor T-bet, exhibiting a distinct phenotype from other B cell subsets (24–26). T-bet is a transcription factor that has been considered as a hallmark of Th1 cells. However, recent studies have revealed that T-bet is also expressed in certain B cells with co-expression of CD80 and CD86, suggesting that these B cells are potent antigen-presenting cells (52) (**Table 1**). CD11c$^+$ABCs appear to be recruited into inflamed tissues *via* specific chemokine-chemokine receptor axis. CD11c$^+$ABCs with low levels of CXCR5 and CCR7 expression are localized outside the B cell follicles (53). Experiments with adoptive transfer of CD11c$^+$ABCs have confirmed their location at the boundary region between T cell and B cell zones, which is very similar to the location of interfollicular large B cells that are found in the T cell-rich area of secondary lymphoid organs and ectopic lymphoid structures in pSS patients (54, 55). The interfollicular large B cells express high levels of Activation-induced cytidine deaminase and are associated with B cell lymphoma in patients with pSS (56). CD11c$^+$ABCs can secrete various cytokines, including IL-1β, IL-6, IFN-γ and IL-10 (**Figure 1**).

Available data suggest that CD11c$^+$ABCs may be derived from different cell compartments in healthy people and SLE patients. It has been reported that CD11c$^+$ABCs are mainly CD27$^+$IgD$^-$ switched memory B cells in healthy people (57). However, CD11c$^+$ ABCs in SLE patients are enriched in CD27$^-$IgD$^-$ double negative (DN) B cell population that can be further divided into two subsets, DN1 and DN2, according to the chemokine receptor CXCR5 expression (49). IFN-γ can trigger naïve B cell precursors to become activated naïve or DN1 B cells with high levels of T-bet expression (58). CD27$^-$IgD$^-$CXCR5$^-$ (DN2) B cells are capable of differentiating into plasma cells induced by IL-21 in lupus patients. Frequencies of DN2 B cell are associated with lupus-related autoantibodies, including antibodies to dsDNA, nucleosome, histones and chromatin. Thus, autoreactive CD11c$^+$ABCs may participate in the pathogenesis of lupus by secreting high titers of autoantibodies derived from an extrafollicular response. Moreover, type I interferon produced by plasmacytoid dendritic cells can promote plasmablasts to secrete anti-dsDNA antibodies from extrafollicular responses (59). It has been shown that depletion of CD11c$^+$ABCs greatly reduces autoantibody levels and disease manifestations in lupus mice, which further confirms that CD11c$^+$ABCs play a pathogenic role in the development of lupus (25). In pSS patients, the functional features of CD11c$^+$ABCs remain largely unclear. A recent study has demonstrated that upregulated IL-21 signaling pathway in salivary glands of pSS patients is associated with enriched B cells and increased disease activity (60). Moreover, expanded IL-21$^+$ Tfh cells in SS patients are associated with ectopic lymphoid structures and MALT-lymphomas (61). Given the accumulating evidence on a vital role of IL-21 in regulating CD11c$^+$ABCs in

lupus patients, it is likely that enhanced IL-21 signaling in salivary gland may promote CD11c$^+$ABCs functions in the pathogenesis of pSS.

Although it is currently unclear whether CD11c$^+$ABCs can be depleted by anti-CD20 or anti-CD19 therapies, targeting CD11c$^+$ABCs is an appealing approach for the treatment of patients with autoimmune diseases. Previous studies reported elevated levels of both B-cell activating factor (BAFF) and A proliferation-inducing ligand (APRIL) in serum and saliva of pSS patients compared with healthy control (62, 63). Further immunohistochemical staining and transcript analysis reveal that the primary sources of BAFF are infiltrated T and B cells as well as ductal epithelial cells (64). Although the survival factors for CD11c$^+$ABCs remain to be determined, CD11c$^+$ABCs express high levels of BAFF receptor (BAFFR), intermediate densities of Transmembrane activator and CAML interactor (TACI) and minimal B-cell maturation antigen (BCMA) in lupus patients (50). A recent study has shown that anti-BAFF treatment with Belimumab reduces circulating CD11c$^+$ABCs in SLE patients (27). Hence, these findings suggest that BAFF may regulate the survival of CD11c$^+$ABCs in the local tissues of pSS patients. Recently, Belimumab has been evaluated in an open-label trial of 30 patients with pSS (65). The results showed that sixty percent of pSS patients achieved the primary endpoint whereas the disease activity index was significantly decreased at week 28. Considering that CD11c$^+$ABCs may participate in the pathogenesis of pSS, it is possible that Belimumab can ameliorate the disease progression by depleting ABCs in pSS. Another promising therapy for targeting CD11c$^+$ABCs is Telitacicept, which is a novel TACI-Fc fusion protein that binds to BAFF and APRIL (28). A phase 2b clinical trial of Telitacicept has reported a statistically significant difference in the clinical response rate between Telitacicept group (79.2%) and the placebo group (32%) (66). Currently, a phase 2 clinical study to evaluate Telitacicept effects on pSS patients is underway (Clinical Trials: NCT04078386). Furthermore, the blockade of cytokines such as IFN-γ, type I interferon or IL-21 may be promising strategies to target CD11c$^+$ABCs since these cytokines are critically involved in triggering B cell activation and differentiation (50, 58). Emerging evidence reveals that enhanced activity of Bruton's tyrosine kinase (Btk) in peripheral blood B cells promotes IL-21-mediated signaling pathway by inducing nuclear phosphorylated STAT1 levels in patients with autoimmune disease (29, 30). Hence, blockade of Btk (Remibrutinib) may possibly inhibit the pathogenic function of CD11c$^+$ABCs. It has been recently reported that both frequencies and numbers of ABC with high levels of CD11c and T-bet expression are significantly reduced in aged CD154 (CD40L)-deficient mice compared to controls, indicating that CD40-CD40L interaction is essential for ABC generation (31). Thus, the blockade of CD40-CD40L interaction with Iscalimab may also represent a potential therapeutic strategy for targeting CD11c$^+$ABCs in the treatment of pSS. CD21$^{-/low}$ B cells, a subset of CD11c$^+$ABCs, are found to be associated with lymphoproliferation in pSS patients (67), in which both percentages and absolute numbers in pSS patients are significantly increased when compared with healthy controls. Moreover, CD21$^{-/low}$ B cells are also expanded in other autoimmune diseases, such as SLE (68) and RA (69). Similar to FcRL4$^+$ B cells, CD21$^{-/low}$ B cells highly express CD11c and FcRLs, including FcRL2 and FcRL3. These B cells exhibit profound activation defects when they are triggered by BCR and CD40 but could be activated by the stimulation of TLRs (TLR3, TLR7 and TLR9) (67). Furthermore, CD21$^{-/low}$ B cells express high levels of co-stimulatory molecules CD80 and CD86, which enable them to act as antigen-presenting cells for T cell cognate interaction (70). CD21$^{-/low}$ B cells promote the progression of pSS by secreting high-affinity autoantibodies, including anti-cytoplasmic and anti-nuclear autoantibodies (67). As a selective co-stimulation modulator, Abatacept directly binds to CD80 and CD86, which may reduce the antigen-presenting function of CD21$^{-/low}$ B cells (32).

Transitional B Cells

Transitional B cells are the B cell subset newly emigrated from the bone marrow to the secondary lymphoid organs. Upon activation by cognate antigens, CD21$^{+/-}$IgD$^{+/-}$IgM$^{+/-}$ transitional type-1 B cells can differentiate into CD21$^+$IgD$^+$IgM$^+$ type-2 B cells. It has been recently reported that new transitional CD21^{low}CD10$^+$IgM^{hi}CD27$^-$ B cells expressing polyreactive antibodies are increased in the peripheral blood of pSS patients (33) (**Table 1**). This observed increase in transitional B cells may reflect the defective central B cell tolerance in pSS patients. Moreover, transitional type-2 B cells are expanded in the ectopic germinal center-like structures of salivary glands in pSS patients (71). The available findings suggest that transitional B cells may drive the local tissue functional impairment and inflammation by producing antibodies.

Marginal Zone B Cells

Recent studies have reported increased marginal zone B cells (MZ B) in patients with pSS (71) and mice with SS-like symptoms (8, 72). Phenotypic analysis shows that MZ B cells express high levels of CD21, but low levels of CD23 and IgD (34) (**Table 1**). In pSS patients, MZ B cells are found to be accumulated in the salivary glands and contribute to the glandular destruction by producing autoantibodies (71). In BAFF transgenic mice, expansion of MZ B cells is observed in both spleen and salivary glands while depletion of MZ B cells significantly reduces the infiltrations in the salivary glands (72). In another SS mouse model, IL-14 alpha transgenic mice with specific elimination of MZ B cells exhibit normal saliva secretions and histology of salivary glands, suggesting that MZ B cells play an indispensable role in the pathogenesis of SS (8). Moreover, MZ B cells are closely involved in the development of non-Hodgkin's B-cell lymphoma, one of the most severe complications, in pSS patients (73). In a clinical study, Rituximab has been used to treat pSS patients with marginal zone lymphomas, which represents a promising therapeutic option (74).

Memory B Cells

Recent studies have suggested that memory B cells are also involved in the pathogenesis of pSS. Although CD27$^+$ memory

B cells are reduced in the peripheral blood, memory B cells are accumulated in the salivary glands of pSS patients (75) (**Table 1**). Notably, $CD27^+$ memory B cells highly express chemokine receptors CXCR4 and CXCR5, which may facilitate the infiltration of memory B cells into the inflamed glands by the chemokines CXCL12 and CXCL13 derived from epithelial cells (35). Similar to transitional B cells, $CD27^+$ memory B cells appear to promote the formation of ectopic germinal center-like structures in the exocrine glands of pSS patients (76).

Plasma Cells

Compelling evidence indicates that plasma cells (PCs) contribute to the autoimmune pathogenesis by producing large amounts of autoantibodies (**Table 1**). It has been reported that the numbers of IgG but not IgA expressing $CD138^+$ PCs in salivary glands are correlated with focus scores of lymphocytic infiltrations in pSS patients (36). A recent study has also reported that $CD19^+CD27^{hi}$ plasma cells in salivary glands are positively correlated with serum ANA titers in pSS patients (37). Consistently, Szyszko et al. have observed increased $CD38^+CD138^+$ plasma cells in pSS patients (38). Notably, certain infiltrated PCs show phenotypic characteristics of the long-lived plasma cells (LLPCs), which highly express Bcl-2 but not Ki67. In a spontaneous SS mouse model, PCs detected in the submandibular glands are mostly $BrdU^-$ in 40-week-old mice, showing the key features of LLPCs (39). It has been suggested that salivary glands provide a unique microenvironment for the survival and maintenance of LLPCs. The salivary gland epithelial cells are found to produce IL-6, a pivotal cytokine that support the survival of LLPCs. Our recent studies have identified a novel function of IL-17 in maintaining the survival of LLPCs *via* p38-mediated Bcl-xL RNA stability in murine lupus (77). Interestingly, epithelial cells in the salivary gland also secrete BAFF and APRIL, which may promote the survival of LLPCs (78, 79). Thus, these key cytokines and other factors including CXCL12 and CD44 provide the survival niche for LLPCs and promote persistent antibody production in salivary gland during pSS development.

Bortezomib (BTZ), a proteasome inhibitor that induces plasma cell apoptosis, has been found to be effective in treating various autoantibody-mediated autoimmune diseases in mice (80–82). BTZ can eliminate both short- and long-lived plasma cells and ameliorate lupus nephritis in mice (83). Our recent studies have also shown that BTZ can suppress Th17 response and autoantibody production in mice with experimental Sjögren's syndrome (ESS) (40) (**Figure 1**). Several clinical studies have reported that BTZ treatment significantly reduces the levels of autoantibodies and improves the symptoms in patients with autoimmune disease, including refractory pSS, refractory SLE and thrombotic thrombocytopenic purpura (84–91). Thus, further investigation on the functional characteristics of LLPCs in pSS will facilitate the identification of therapeutic candidates for targeting these B cells.

Recently, two randomized controlled trials using Rituximab for treating pSS patients have failed to achieve their primary endpoints (92, 93). Several possible reasons may explain the failure of these clinical trials. As an anti-CD20 monoclonal antibody, Rituximab may deplete B cells by antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and, to a lesser extent, direct signaling through CD20. However, the inhibitory FcγRIIb expression on target B cells may promote Rituximab internalization and contribute to drug resistance (94). Another potential mechanism for Rituximab resistance is CD46-mediated inhibition of complement activation as increased serum CD46 levels are detected in pSS patients (95, 96). Thirdly, long-lived plasma cells express no or very low levels of CD20 and produce large amounts of autoantibodies in pSS patients, for which Rituximab treatment fails to target. In pSS patients, infiltrated B cells and locally differentiated plasma cells reside in the salivary glands while salivary gland epithelial cells produce large amounts of cytokines or pro-inflammatory factors for B cell survival such as BAFF (78). A clinical case report has recently shown that anti-CD20 treatment followed by Belimumab exhibits a synergistic effect with dramatically reduced EULAR Sjögren's syndrome disease activity index (ESSDAI) in pSS patients with refractory cryoglobulinemic vasculitis (97). Thus, further large clinical trials are needed to validate the efficacy of this sequential therapy in the treatment of pSS.

REGULATORY B CELL SUBSETS

Previous studies have demonstrated that Breg cells are a subset of B cells that can negatively regulate immune response and autoimmune inflammation (98–100). Accumulated data suggest that different Breg subsets may share overlapping surface markers. In 2010, an elegant study by Mauri and colleagues identified human Breg cells with a phenotype of $CD19^+CD24^{hi}CD38^{hi}$ B cells (41). Other investigations (101) also show that IL-10-producing B cells in human blood are enriched within $CD24^{hi}CD27^+$ B cell population. Moreover, IL-10-producing B cells are identified among $CD27^{int}CD38^{hi}$ plasmablasts and exert their regulatory function during autoimmune pathogenesis in humans and mice (102). Up to date, many studies have demonstrated that Breg cells exert their inhibitory functions *via* various effector mechanisms in autoimmune diseases (103–105) (**Table 1**).

IL-10-Producing Breg Cells

In the pathogenesis of pSS, both T and B cells are prominently involved in lymphocytic infiltration and tissue inflammation in salivary glands. In ESS mice induced by immunization with salivary gland protein, Th17 cells have been shown to play a key role in initiating autoimmune inflammation and disease progression (106). Moreover, studies by Fu et al. have revealed that the deficiency of Tfh cells attenuates autoantibody production and disease progression during ESS induction in $Bcl6^{fl/fl}Cd4^{Cre}$ mice, highlighting a crucial role of Tfh cells in driving autoantibody responses and ESS progression (107). Our early studies have indicated that IL-10-producing Breg cells can potently suppress Th17 response and ameliorate collagen-induced arthritis (108). Recently, we have observed negative correlations between IL-10-producing Breg cells and Tfh cell response in both pSS patients and ESS mice (42). During pSS

progression, gradually reduced Breg cell frequencies are accompanied with expanded Tfh cells along with increased disease activities. In culture, Breg cells suppressed human and murine Tfh cell differentiation by promoting STAT5 phosphorylation in IL-10-dependent manner (42). Together, these findings indicate that IL-10-producing Breg cells can restrain Tfh cell response during pSS development (**Figure 1**). Notably, adoptive transfer of Breg cells markedly suppressed Tfh cell response and alleviated disease progression in ESS mice, indicating the potential application of Breg cell transfer as cell therapy for pSS (42). Our previous studies have demonstrated that low levels of BAFF can induce the differentiation of IL-10-producing Breg cells (109). Further studies have revealed that BAFF can induce IL-10 production *via* activating TACI-mediated signal transduction in normal and chronic lymphocytic leukemia B cells (110). Thus, it remains to be investigated whether and how the blockade of BAFF with Belimumab treatment may affect the generation or maintenance of Breg cells in patients with pSS or other autoimmune diseases.

In pSS patients (16), multiple subtypes of IL-10-producing Breg cells with different markers including IgA, IgG and IgM are identified. Interestingly, percentages of IgA-expressing Breg cells are higher in pSS patients than that of healthy individuals. However, frequencies of IgG-expressing Breg cells are reduced in pSS patients while IgM-expressing Breg cells are similar between pSS patients and controls. It has been reported that APRIL can induce naïve human B cells to IgA^+ IL-10-producing Breg cells (111). Hence, elevated APRIL levels in SS patients may promote the formation of IgA-expressing Breg cells. Further characterization of Ig-expressing Breg cells will provide new insight in understanding their functional implications in the pathogenesis of pSS.

GrB-Producing Breg Cells

In addition to IL-10, other effector molecules or cytokines are also involved in the regulatory functions of B cells. GrB belongs to the serine protease family that triggers target cell apoptosis with perforin. An early study revealed that GrB inhibits $CD4^+$ T cell proliferation *via* a perforin-independent manner (112). The frequencies of GrB-producing Breg cells may vary among different autoimmune diseases. In pSS patients, GrB-producing $CD19^+CD5^+$ B cells with higher IL-21 receptor (IL-21R) expression are increased in the peripheral blood, along with expanded IL-21-producing invariant NKT cells (43). However, reduced GrB-producing Breg cells with lower IL-21R are observed in RA patients and negatively correlated with disease activity (113). Similarly, the frequencies of GrB-secreting Breg cells are decreased in SLE patients, especially in patients with lupus nephritis (114). Thus, further studies on the functional implication of GrB-producing Breg cells in the pathogenesis of pSS will provide new insight in understanding their target cells and immunopathology of pSS.

IL-35-Producing Breg Cells

As a novel cytokine, IL-35 consists of p35 and EBI3, which is involved in mediating the regulatory functions of B cells. In mice with B cell-specific p35 ($p35^{-/-}$) or EBI3 ($Ebi3^{-/-}$) deficiency, exacerbated experimental autoimmune encephalomyelitis (EAE) was developed (44), indicating that IL-35-producing Breg cells restrain the pathogenesis of EAE. Moreover, IL-35-producing Breg cells have been shown to suppress Th1 and Th17 cells but induce Treg cell proliferation in the murine uveitis model (45). A recent study revealed higher levels of IL-35 expression in peripheral blood from SLE patients than healthy controls (115). Further investigations (116) have found that serum IL-35 concentrations are reduced in patients with lupus nephritis (LN) compared to those without LN and negatively correlated with disease activity, suggesting that IL-35 may take part in the development of lupus nephritis. Treatment of MRL/Lpr mice with IL-35 promotes the expansion of IL-10-producing Breg cells and ameliorates the disease progression (117). Although serum levels of IL-35 are reduced in pSS patients compared with healthy controls (13), increased $EBI3^+$ B cells are observed, indicating that IL-35-producing Breg cells may play a role in the pathogenesis of pSS.

Regulatory Plasma Cells

Recent studies have identified a novel subset of plasma cells expressing the inhibitory receptor LAG-3, which exert regulatory functions by secreting IL-10 in mice (46, 118). It has been shown that B cell receptor signaling is essential for the differentiation of $LAG\text{-}3^+CD138^{hi}$ regulatory plasma cells, since these plasma cells are not present in mice deficient for Btk. Moreover, Toll-like receptor signaling is critically involved in controlling IL-10 production in regulatory plasma cells (46). In addition, plasmablasts have also been reported to exert the regulatory function in an EAE mouse model, in which plasmablasts in the draining lymph nodes produce IL-10 to suppress autoimmune pathogenesis (102). Together, these studies provide further evidence on the expanding functional diversity of plasma cells in immunity and inflammation.

INTERACTIONS BETWEEN SALIVARY GLAND EPITHELIAL CELLS AND B CELLS

Lines of evidence suggest that the interactions between salivary gland epithelial cells (SGECs) and B cells may promote SS pathogenesis *via* multiple effector mechanisms (119). It has been shown that SGECs can drive B cell activation, differentiation and survival through the direct interaction and cytokine production (120, 121). In culture, SGECs from pSS patients promote B cell differentiation into mature B cell phenotypes (120). Moreover, the survival rates of B cells are also increased when cultured with SGECs from pSS patients (121). There is increasing evidence that SGECs may induce B cell differentiation in an indirect manner. It has been reported that SGECs can promote T follicular helper cell differentiation and IL-21 production (122), which may further enhance B cell hyperactivity in the salivary gland of pSS patients. Accordingly, the crosstalk between SGECs and B cells highlights a critical role of SG epithelial cells in SS pathogenesis.

CONCLUSION

Available studies have indicated that various pathogenic B cell subsets contribute to the disease progression of pSS, while Breg cells alleviate the disease activities. However, there is evidence that B cell predominant phenotype does not involve all pSS patients, which warrants further studies on the pivotal roles of T cells and innate immune cell types in the pathogenesis of pSS (3, 123). Recent findings have provided new insight in understanding the pathogenic mechanisms of pSS and validated B cell-targeted therapy as future therapeutic options for patients with pSS (**Figure 1**). The major challenges in B cell-targeted therapy include the specific reduction of disease-related B cell subsets, instead of the complete depletion of the broader B cell population. In addition, Breg cell-based therapy may represent a promising clinical application for the therapeutic intervention. Further studies on the identification and functional characterization of novel B cell subsets in the pathogenesis of pSS will facilitate the development of new therapeutic strategies for Sjögren's syndrome and other autoimmune diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Predictive Values of PET/CT in Combination With Regulatory B Cells for Therapeutic Response and Survival in Contemporary Patients With Newly Diagnosed Multiple Myeloma

Jian Cui[1,2†], Zhongqing Zou[1,3†], Jiayu Duan[1,2], Wenjiao Tang[1], Yan Li[1], Li Zhang[1], Ling Pan[1] and Ting Niu[1]*

[1] Department of Hematology, West China Hospital, Sichuan University, Chengdu, China, [2] West China School of Medicine, Sichuan University, Chengdu, China, [3] Department of Hematology, Clinical Medical College & Affiliated Hospital of Chengdu University, Chengdu University, Chengdu, China

To assess patients with multiple myeloma (MM), the whole-body positron-emission tomography/computed tomography (PET/CT) occupies a pivotal position for diagnostic stratification, response evaluation, and survival prediction, while important limitations are recognized as incapable of representing tumor microenvironment. Regulatory B cells (Bregs) have been reported to have an inhibitory immune function, contributing to bone marrow (BM)-immunosuppressive microenvironment for MM. Therefore, to investigate the role of PET/CT in combination with Bregs' ratios to predict therapeutic response and survival, we sequentially enrolled 120 patients with newly diagnosed MM (NDMM) who were treated with novel agents in our center, while conventional PET/CT parameters including maximum standard uptake value (SUVmax), ratios of BM-derived Bregs within $CD19^+$ B cells, and patients' clinical characteristics were collected. After a median follow-up of 28.20 months (range 7.00–46.93 months), SUVmax > 4.2 at onset, accounting for 53.2% of NDMM, was uncovered to predict inferior progression-free survival (PFS) as well as overall survival (OS). With regard to the ratios of BM-derived Bregs within $CD19^+$ B cells, the cohort with the Bregs' proportions lower than 10%, accounting for 46.2%, exerted poorer OS. Additionally, the patients with both SUVmax > 4.2 and Bregs' ratios < 10%, accounting for 31.7%, yielded compromised therapeutic response and long-term survival. Collectively, this study may draw attention on the prognostic value of combination of PET/CT and Bregs' ratios when clinical decisions are made for MM in the era of novel agents.

Keywords: myeloma, PET/CT, regulatory B cells, prognosis, tumor microenvironment

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Ding Chong Yang,
The First Affiliated Hospital of Nanjing Medical University, China

***Correspondence:**
Li Zhang
drzhangli2014@sina.com

†These authors have contributed equally to this work

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INTRODUCTION

Risk stratifications of newly diagnosed multiple myeloma (NDMM) have been assessed by a number of prognostic variables. The International Staging System (ISS) and the revised ISS (R-ISS), which combine biochemical indicators and cytogenetic abnormalities (1, 2), are the most widely used. However, neither ISS nor R-ISS can reflect tumor biology determined by bone destruction and abnormalities of tumor microenvironment (TME), which are important pathogenic factors in MM (3, 4). Additionally, the reliability of cytogenetic results is unavoidably influenced by the uncertainty of collecting clonal plasma cells due to local tumor infiltration in MM's bone marrow (BM).

Positron-emission tomography/computed tomography (PET/CT) with fluorine-18 fluorodeoxyglucose (^{18}F-FDG) has been applied for the assessment of systemic tumor burden, response evaluation, and survival prediction in MM (5–9), for it provides 'functional' information regarding lesions as well as metabolic and anatomic information. Compared with traditional X-rays, systemic CT is a sensitive technique to detect the presence of bone lesions and/or BM involvement at the onset of MM (7). Moreover, ^{18}F-FDG uptake in bone lesions represents the tumor metabolic activity, and maximum standard uptake value (SUVmax) is considered to have prognostic value in NDMM (6, 10–12).

In addition to systemic tumor burden, the BM immune TME, including T cells (13), osteoclasts (14) and extracellular matrix (15), are also involved in the occurrence, disease progression, and drug resistance of MM. Regulatory B cells (Bregs) are currently uncovered to be immunosuppressive cells that exist in the BM immune TME of MM (16). In previous studies, we first characterized $CD19^+CD24^{hi}CD38^{hi}$ Bregs in BM samples from MM patients by flow cytometry (FCM) (17–19). We also explained the relationship between Bregs and disease status in MM that ratios of BM-derived Bregs within $CD19^+$ B cells were significantly higher in patients with NDMM than in those on maintenance therapy after response (17). Bregs were also reported to predict progression-free survival (PFS) for patients with relapsed or refractory MM (20). However, prognostic impact of Bregs for NDMM patients has been still unclear.

To further uncover prognostic roles of TME-derived Bregs and systemic PET/CT, in this study, we herein report the results of a retrospective analysis collecting PET/CT scan and Bregs detection performed at baseline for patients with NDMM who received bortezomib plus dexamethasone-based (BD) therapy in southwest China.

METHODS

Patients and Treatment Protocols

NDMM patients were sequentially enrolled from April 2017 to July 2020 at West China Hospital if they were over the age of 18 years, received PET/CT scan or FCM detection of Bregs at the onset of disease, and newly diagnosed with symptomatic MM based on the International Myeloma Working Group (IMWG) (21) diagnostic criteria. The final follow-up ended in May 31, 2021. Different risk groups were classified using Durie and Salmon staging system (DS) (22), ISS (1), or R-ISS (2). Responses were assessed according to IMWG 2016 consensus criteria (23). Best response was defined as the maximal response during treatment. In treatment decision making, BD regimen has become a backbone to which several other agents have been integrated. BD regimen was administered every 4 weeks or 1 natural month, and 1.3 mg/m^2 of bortezomib was administered subcutaneously weekly on days 1, 8, 15, and 22 with weekly dexamethasone 20 mg/m^2 on the day of and the day after bortezomib administration. The third agent was added or omitted under certain conditions. Specifically, for patients with good tolerability to BD regimen, specific indications for initiation of the other therapy for MM existed. For patients with renal impairment and/or amyloidosis attributable to MM, cyclophosphamide was in combination with BD regimens using a 300 mg/m^2 dosing on days 1, 8, 15, and 22 per cycle. Patients with extramedullary involvement received 40 mg of pegylated liposomal doxorubicin (in combination with BD backbone) on days 1 of each cycle. Following the BD regimen induction, stem cell harvest is all recommended for patients who met eligibility criteria (i.e., ≤65 years and ≥very good partial response (VGPR) in first remission). Upfront autologous stem cell transplantation was performed for patients from whose consent was obtained. For transplant ineligible patients, maintenance therapy was given after nine cycles of induction or treatment response was stable for three cycles of VGPR or above. All patients were given routine maintenance for at least 2 years with either lenalidomide or bortezomib, given both patients' intent and risk stratification. This study was approved by our hospital's institutional review board/research ethics board. All subjects provided written informed consent authorizing the use of their data for research purposes.

Imaging Studying

FDG-PET/CT scan was performed according to the European Association of Nuclear Medicine guidelines version 1.0 and, from February 2015, version 2.0. Steps of PET/CT scan were reported previously (24). Simple, circular regions-of-interest (ROIs) were drawn by hand on axial, coronal, or sagittal co-registered PET/CT slices. SUVmax was obtained and corrected for body weight using the standard formula: mean ROI activity (MBq/ml)/[injected dose (MBq)/body weight (kg)] (25). ROIs were placed manually over all lesions, and the SUVmax was recorded for every lesion. Also, the highest SUVmax for every PET/CT scan was recorded, and these lesions were identified as indicator lesions. In an effort to standardize the interpretation of the baseline PET/CT scans, the criteria in three previous papers of the groups of Bologna and Udine were adopted in this study (6, 10, 26). Briefly, positive PET/CT findings were defined either by the presence of focal areas of increased tracer uptake within the bones, with or without any underlying lesions identified on CT presented on at least two consecutive slices, or by a SUVmax ≥ 2.5 within the osteolytic CT areas > 1.0 cm in size or a SUVmax ≥ 1.5 within the osteolytic CT areas ≤ 1.0 cm. The number, size, and location of hypermetabolic

focal lesions (FLs) were recorded, and FDG-avid tissue that was not contiguous to bone and arose in soft tissue according to CT examination was defined as extramedullary disease (EMD) tissue. SUVmax > 4.2 was continued to be considered an unfavorable cutoff value for therapeutic response and survival in this study, which has been confirmed in previous studies (6, 10, 26).

For PET/CT is subject to the constraints of interobserver reproducibility and an imperfect systemic description when using only SUVmax (27), the study compared the predictive ability of SUVmax and the ratios between SUVmax of the tumor lesions to liver (rPET) (27).

Laboratory Investigations

Bregs were characterized as $CD19^{+}CD24^{hi}CD38^{hi}$ in BM samples from NDMM patients by FCM as previously described (17, 18). Heparinized BM was obtained from NDMM patients prior to treatment. Briefly, BM mononuclear cells (BMMNCs) were isolated and washed twice in PBS. After discarding the supernatant, BMMNCs were incubated with antibodies against CD38 (PE-cy7), CD19 (FITC), and CD24 (APC) (BioLegend) for 15 min. Excess (unbound) antibodies were removed by washing with PBS, and cells were resuspended in 0.2 ml PBS for FCM detection (Beckman).

Statistical Analysis

Distributions of PFS and overall survival (OS) were calculated using the Kaplan-Meier method, and differences among survival curves were analyzed by the log-rank test. PFS was defined as the time from diagnosis to progression or death from any cause. OS was defined as the time from diagnosis to death from any cause. Significant risk factors for both PFS and OS that showed a $P < 0.10$ on univariate analysis were further tested in the multivariate Cox proportional hazards regression analysis. The cutoffs for SUVmax, rPET, and Bregs' ratios were identified after applying sequential log-rank tests and selecting the most powerful values for discriminating the outcomes. The chi square test and the Fisher's exact test were used to test for the independence of categories. Statistical significance was defined when $P < 0.05$. SPSS 25.0 software was used to process all collected data.

RESULTS

Patient Characteristics

A cohort of 120 NDMM patients was enrolled with median follow-up of 28.20 months (range 7.00–46.93 months), and their baseline characteristics were shown in **Table 1**. A total of 12 (10.0%) deaths and 32 (26.7%) cases with disease progression occurred. The overall response rate (ORR: [complete response (CR) + VGPR + partial response (PR)]) was 88.4%, with 19 in 101 evaluable patients achieving CR.

One hundred and fourteen patients received PET/CT scan at the onset of disease and 84.3% presented positive lesions by PET/CT scan. Median SUVmax was 4.22 (interquartile range 3.17–6.00), while 53.2% patients were found with elevated SUVmax (SUVmax > 4.2) at onset (**Supplementary Table S1**). The rates of p53 deletion were higher in patients with SUVmax > 4.2 ($P = 0.002$), while distribution of other baseline characteristics was summarized (**Table 1**). More p53 deletion was also seen in patients with elevated rPET (defined as rPET > 1.46; **Supplementary Table S2**).

Furthermore, 52 patients' BM samples were collected before receiving treatment to uncover the median ratios of BM-derived Bregs within $CD19^{+}$ B cells as 7.5% (interquartile range 1.1%–27.2%). Cutoff of Bregs' ratios of 10% was defined by sequential log-rank tests for discriminating therapeutic response and survival. Decreased Bregs' ratios (defined as Bregs' ratios < 10%) were conformed in 24 (46.2%) patients (**Supplementary Table S1**). A similar trend to higher ratios of p53 deletion was also observed in patients with Bregs' ratios < 10% ($P = 0.056$) (**Table 1**).

Patients With SUVmax > 4.2 or Bregs' Ratios < 10% Had Lower Quality of Response to Treatment

For patients with Bregs' ratios < 10%, 23.1% could not reach PR, while 100% reached beyond PR in the subgroup without Bregs' ratios ≥ 10% ($P = 0.028$) (**Table 1**). A similar trend towards lower quality of best response occurred on patients with SUVmax > 4.2 (**Table 1**). However, the difference between the response of patients with rPET > 1.46 or rPET ≤ 1.46 was small (< PR 5/41 *vs*. 4/42; ≥ PR 36/41 *vs*. 38/42; $P = 0.738$) (**Supplementary Table S2**).

Additionally, as best response to first-line treatment, 6 cases (15.8%) with CR or stringent CR, 15 cases (39.5%) with VGPR, and 17 cases (44.7%) with or less than PR were observed among 38 evaluable patients who received both PET/CT scan and Bregs detection at diagnosis. The patients with both SUVmax > 4.2 and Bregs' ratios < 10% presented the worst response with significant higher rates (41.7% *versus* 3.8%, $P = 0.003$) not reaching PR (**Figure 1**).

SUVmax > 4.2, Bregs' Ratios < 10%, as Well as R-ISS Ⅲ Were Independently Associated With Poorer Survival

On univariate analysis for the whole cohort, R-ISS III, SUVmax > 4.2, and Bregs' ratios < 10% predicted worse PFS, with hazard ratios (HR) (95% CI) of 2.35 (1.11–4.98), 2.05 (0.91–5.50), and 2.72 (0.86–8.59), respectively ($P = 0.026$, $P = 0.074$, and $P = 0.089$, respectively) (**Table 2**). To be specific, PFS for patients with SUVmax > 4.2 or Bregs' ratios < 10% were significantly shorter than those observed for patients with lower SUVmax or higher Bregs' ratios at the time of diagnosis ($P = 0.152$ and $P = 0.131$) (**Figure 2**). Similarly, patients with R-ISS III had median PFS (mPFS) of 23.83 months, in comparison with corresponding values of not reached (NR) ($P = 0.001$) for those who were with R-ISS I and R-ISS II (**Figure 2**). In particular, we compared the stratification ability of rPET and SUVmax for PFS, and elevated rPET (rPET > 1.46) did not perform better than SUVmax > 4.2 (**Supplementary Figure S2**). Age, sex, positive PET/CT findings, and FLs were found less predictive for PFS (**Supplementary Figure S2**). ISS III, p53 deletion, 1q21 gain, and IgH

TABLE 1 | NDMM patients' demographic and clinical characteristics.

	Overall	Patients with PET/CT scan			Patients with Bregs detection		
		SUVmax > 4.2 n/N (%)	SUVmax ≤ 4.2 n/N (%)	P	Bregs' ratios < 10% n/N (%)	Bregs' ratios ≥ 10% n/N (%)	P
Male	64/120 (53.3)	36/59 (61.0)	22/52 (42.3)	0.058	16/28 (57.1)	14/24 (58.3)	0.931
>65 years	38/120 (31.7)	16/59 (27.1)	18/52 (34.6)	0.416	8/28 (28.6)	10/24 (41.7)	0.388
M-component							
IgG	67/114 (58.8)	37/64 (57.8)	27/64 (42.2)		15/26 (57.7)	11/26 (42.3)	
IgA	25/114 (21.9)	10/22 (45.5)	12/22 (54.5)		3/7 (42.9)	4/7 (57.1)	
Light chain	16/114 (16.0)	6/14 (42.9)	8/14 (57.1)		5/11 (45.5)	6/11 (54.5)	
LDH > 220 IU/L	53/113 (46.9)	31/54 (57.4)	21/51 (41.2)	0.096	13/28 (46.4)	9/20 (45.0)	0.922
β2-microglobulin > 5.5 mg/L	38/111 (34.2)	17/53 (32.1)	18/49 (36.7)	0.679	13/26 (50.0)	7/23 (30.4)	0.245
DS, stage III	84/107 (78.5)	42/53 (79.2)	38/46 (82.6)	0.800	22/28 (78.6)	15/18 (83.3)	0.727
ISS, stage III	36/105 (35.2)	18/52 (34.6)	16/45 (35.6)	0.923	11/27 (40.7)	6/17 (35.3)	0.761
R-ISS, stage III	38/101 (37.6)	20/51 (39.2)	16/43 (37.2)	0.842	10/26 (38.5)	9/18 (50.0)	0.542
FISH at diagnosis in MM							
P53 deletion	10/90 (11.1)	10/46 (21.7)	0/37 (0.0)	0.002#	5/26 (19.2)	0/21 (0.0)	0.056
1q21 gain	40/90 (44.4)	20/46 (43.5)	18/37 (48.6)	0.664	11/26 (42.3)	10/21 (47.6)	0.776
IgH translocation	36/90 (40.0)	19/46 (41.3)	14/37 (37.8)	0.823	10/26 (38.5)	9/21 (42.9)	0.775
Karyotype abnormalities	8/84 (9.5)	4/42 (9.5)	4/37 (10.8)	0.850	2/25 (8.0)	4/19 (21.2)	0.378
First-line transplantation	25/117 (21.4)	16/58 (27.6)	7/50 (14.0)	0.102	7/27 (25.9)	5/23 (21.7)	0.754
Best response							
≥PR	90/101 (89.1)	43/47 (91.5)	40/47 (85.1)	0.523	20/26 (76.9)	18/18 (100.0)	0.028
<PR	11/101 (10.9)	4/47 (8.5)	7/47 (14.9)		6/26 (23.1)	0/18 (0.0)	

NDMM, newly diagnosed multiple myeloma; PET/CT, positron-emission tomography/computed tomography; Bregs, regulatory B cells; SUVmax, maximum standard uptake value; LDH, lactate dehydrogenase; DS, Durie and Salmon staging system; ISS, International Staging System; R-ISS, Revised International Staging System; FISH, fluorescence in situ hybridization; PR, partial response. #*P < 0.05, determined by the chi square test and the Fisher's exact test.*

translocation were also found associated with worse PFS (**Table 2**). Female gender, age > 65 year, β2-microglobulin > 5.5 mg/L, with more than 3 FLs, and EMD did not show much significance on the univariate analysis. Thus, R-ISS III, SUVmax > 4.2, and Bregs' ratios < 10% were included in the multivariate analysis, and it showed that the presence of R-ISS III, SUVmax > 4.2, and Bregs' ratios < 10% were independent predictors of worse PFS (**Table 2**).

Univariate analysis for OS showed that SUVmax > 4.2, R-ISS III predicted worse OS with HR (95% CI) of 10.99 (2.29–52.83)

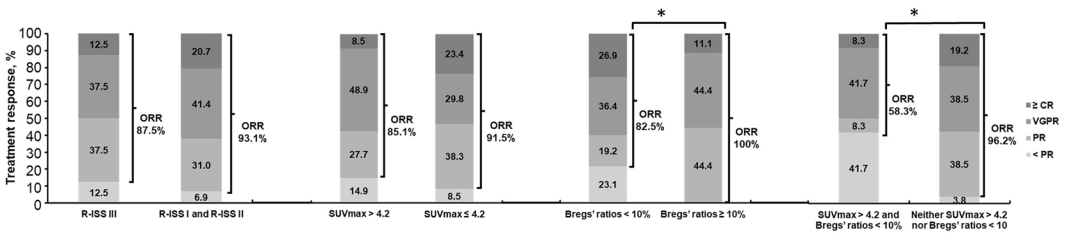

FIGURE 1 | Overall response rates for all patients, by R-ISS III, SUVmax > 4.2, and Bregs' ratios < 10% in NDMM. *P < 0.05, determined by the chi square test and the Fisher's exact test. *NDMM*, newly diagnosed multiple myeloma; *ORR*, overall response rate (ORR = [complete response (CR) + very good partial response (VGPR) + partial response (PR)); *R-ISS*, Revised International Staging System; *SUVmax*, maximum standard uptake value; *Bregs*, regulatory B cells.

TABLE 2 | Univariate and multivariate analysis of PFS and OS according to baseline risk variables for myeloma.

Univariate	**95% CI**				**95% CI**			
	HR	**Lower**	**Upper**	**P**	**HR**	**Lower**	**Upper**	**P**
	PFS				OS			
Sex (female)	1.16	0.57	2.33	0.684	1.29	0.41	4.06	0.665
Age > 65 years	1.03	0.49	2.17	0.943	0.42	0.09	1.90	0.258
LDH > 220 IU/L	1.60	0.78	4.27	0.201	2.30	0.69	7.64	0.176
β2-microglobulin > 5.5 mg/L	1.39	0.66	2.93	0.385	3.34	1.05	10.55	0.040
DS, stage III	1.67	0.64	4.36	0.298	1.58	0.35	7.21	0.557
ISS, stage III	1.88	0.91	3.91	0.090	8.37	2.17	32.28	0.002
R-ISS, stage III[§]	2.35	1.11	4.98	0.026	10.99	2.29	52.83	0.003
SUVmax > 4.2	2.05	0.91	5.50	0.074	4.32	0.93	20.06	0.062
>3 FLs[†]	0.99	0.62	1.59	0.974	0.90	0.43	1.87	0.772
EMD	1.22	0.37	4.05	0.740	4.94	0.86	19.26	0.121
Bregs' ratios < 10%	2.72	0.86	8.59	0.089	2.88	0.85	8.29	0.192
FISH at diagnosis in MM								
P53 deletion	2.91	1.07	7.92	0.036	7.00	2.12	23.09	0.001
1q21 gain	2.37	0.99	5.65	0.052	2.24	0.65	7.66	0.200
IgH translocation	3.88	1.57	9.56	0.003	4.83	1.26	18.52	0.022
Multivariate	**95% CI**				**95% CI**			
	HR	**Lower**	**Upper**	**P**		**HR**	**Lower**	**Upper**	**P**
	PFS				**OS**			
R-ISS, stage III[§]	5.45	1.03	28.81	0.046	R-ISS III§	8.67	1.82	41.20	0.007
SUVmax > 4.2	5.13	2.49	12.31	0.039	SUVmax >4.2	4.41	0.94	20.55	0.059
Bregs ratios < 10%	4.88	1.27	15.21	0.045					

PFS, progression-free survival; OS, overall survival; LDH, lactate dehydrogenase; DS, Durie and Salmon staging system; ISS, International Staging System; R-ISS, Revised International Staging System; SUVmax, maximum standard uptake value; FLs, focal lesions; EMD, extramedullary disease; Bregs, regulatory B cells; FISH fluorescence in situ hybridization.
[§]Referred to R-ISS I and R-ISS II.
[†]Referred to 1-3 FLs.

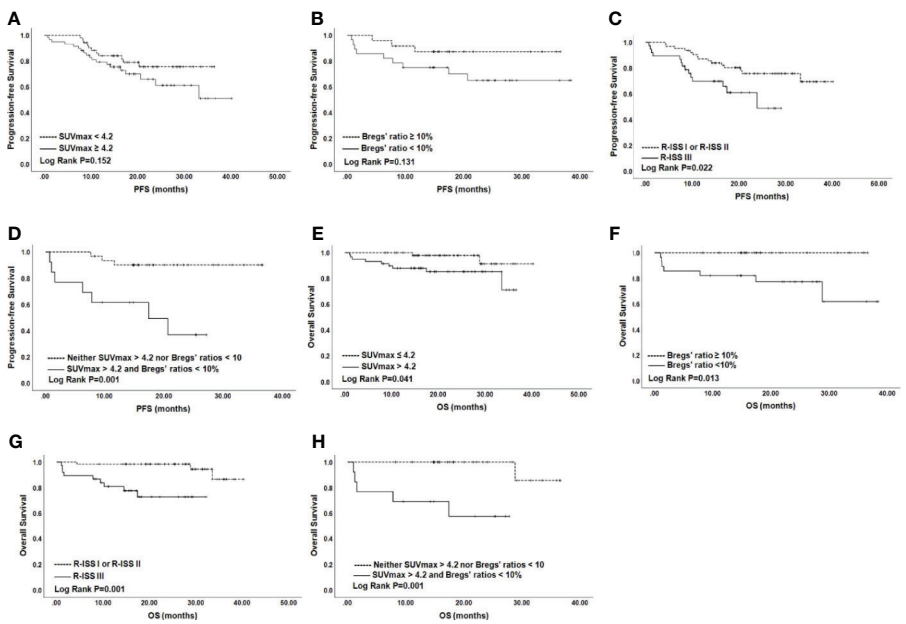

FIGURE 2 | PFS and OS for NDMM patients by SUVmax **(A)**, Bregs **(B)**, and R-ISS **(C)**. OS according to SUVmax **(E)**, Bregs **(F)**, and R-ISS **(G)**. PFS **(D)** and OS **(H)** according to SUVmax >4.2 and Bregs' ratios <10%. *NDMM*, newly diagnosed multiple myeloma; *PFS*, progression-free survival; *OS*, overall survival; *SUVmax*, maximum standard uptake value; *Bregs*, regulatory B cells; *R-ISS*, Revised International Staging System.

and 4.32 (0.93–20.06) (P = 0.003 and P = 0.062). β2-microglobulin > 5.5 mg/L, lactate dehydrogenase (LDH) > 220 IU/L, ISS III, p53 deletion, and IgH translocation were also found associated with worse OS (**Table 2**). Similar to PFS, female gender, age >65 year, with more than 3 FLs, EMD, and rPET > 1.46 showed little impact on OS (**Supplementary Figure S2**). Thus, R-ISS III and SUVmax > 4.2 were included in the multivariate analysis and OS was negatively influenced by R-ISS III and SUVmax > 4.2 with HR (95% CI) of 9.97 (2.02–49.16) and 4.53 (0.97–21.11) (**Table 2**).

Baseline PET/CT in Combination With Bregs as Prognosticators

The independent impact of elevated SUVmax and decreased Bregs' ratios on PFS enabled us to stratify the NDMM patients into three groups, based on the number of risk factors (none of the two adverse factors, 26.8% of the patients; only one of two, 41.5%; two of the two adverse factors, 31.7%). As revealed by the results of Kaplan–Meier analysis and log-rank test, patients with both SUVmax > 4.2 and Bregs' ratios < 10% experienced poorer PFS and OS than those with none factors (P = 0.001 and P = 0.001, respectively), although mPFS was not reached (**Figure 2**). Moreover, quality of response was worse for patients with both SUVmax > 4.2 and Bregs' ratios < 10% (**Supplementary Figure S1**).

DISCUSSION

In this retrospective study of 120 NDMM patients who were evaluated at baseline using PET/CT scan and Bregs detection, we confirmed that imaging technique and TME-derived parameter have a good predictive value on the response of treatment, and both PET/CT > 4.2 and Bregs' ratios < 10% can screen out a group of NDMM patients with poor survival. Furthermore, by combining SUVmax > 4.2 and Bregs' ratios < 10%, a group of high-risk NDMM patients was stratified. To the best of our knowledge, this is the first report providing demonstration that PET/CT and Bregs predict response and survival in NDMM.

The predictive ability of SUVmax > 4.2 for poorer survival was confirmed again in this study, where the adverse influence of high SUVmax on the therapeutic response was explored at the same time. With the advent of PET/CT, bone destruction, tumor metabolism, and systemic tumor burden can be comprehensively evaluated; thus, PET/CT has likewise been combined with other parameters as prognosis prediction factors at baseline (5–7). Transplant-eligible NDMM patients with SUVmax > 4.2 were reported to have shorter PFS and OS (6). EMD and FLs > 3 were also adverse PFS and OS prognosticators for transplant-eligible NDMM patients (5, 6). For transplant-ineligible NDMM patients, PFS and OS were worse with the presence of SUVmax > 4.2, FLs > 3, and EMD (10). FLs > 3 and EMD on PET/CT were also correlated with significantly higher M protein and β2-microglobulin, more cytogenetic abnormalities in NDMM patients (28). In our study, more p53 deletion and elevated LDH were found in patients with SUVmax > 4.2, and we further confirmed the adverse impact of SUVmax > 4.2 on survival of NDMM patients. In the comparison between SUVmax and rPET, we found that the predictive ability of rPET was not stronger than that of SUVmax; it may be because the data of rPET had greater variability and the cutoff brought by the ratios did not have a better stratification effect. The adverse effect of SUVmax > 4.2 on the therapeutic response was explored in this study. As reported, achievement of deeper response within the first four cycles of treatment is an indicator for better survival (29, 30). In a more recent study, patients with a rapid PR or VGPR and gradually achieved CR were found with superior survival than those with early VGPR ≤ 3 months (31). In this study, 26.9% NDMM patients with Bregs ratios < 10% achieved best response of CR or stringent CR; we hypothesized this was because the prognosis of MM was affected by the response kinetics and duration of response in addition to the depth of response.

Positive correlation was found between the ratios of Bregs within CD 19^+ B cells and NDMM patients' outcomes in this study, and Bregs' ratios < 10% was an appropriate cutoff for therapeutic response and survival in NDMM patients. The positive relationship between Bregs' ratios and preserved B cells in NDMM was discovered in our previous study (17, 18). At time of relapse, $CD19^+$ B cells, including Bregs, are too low to be detected (17). This is, to some extent, due to severe acquired immunodeficiency accompanying with a progressive depletion of lymphocytes, including CD19+ B cells, during relapse (32). The clinical behavior of MM is very heterogeneous; ^{18}F-FDG PET/CT scan and Bregs detection can provide a more direct measure of tumor burden and TME and be exploited in an effort to identify newer prognostic factors, therefore improving prognosis.

R-ISS retained its prognostic significance in this study, and it was verified that R-ISS allowed the identification of three different groups of patients with clearly different outcomes. The comparison of the predictive value of R-ISS and other potential predictors in this study showed that R-ISS was an independent prognostic marker. SUVmax > 4.2 and Bregs' ratios < 10% have shown the potential to screen a group of patients with poor PFS from NDMM patients. Moreover, patients with R-ISS III or patients with both SUVmax > 4.2 and Bregs' ratios < 10% account for about 30% of the total NDMM population. PET/CT scan in combination with Bregs detection can complement R-ISS to achieve a good stratification of NDMM patients with poor prognosis. Since Bregs' ratios < 10% was identified as an unfavorable prognostic variable for OS on the univariate analysis but not on the multivariate analysis, more patients are needed to be included and follow-up needs to continue to further consolidate the existing findings.

We acknowledge that there are several limitations to the current study. First, the study is retrospective and only a small number of patients are enrolled. Additionally, due to the short median follow-up time in this study, the predictive value of PET/CT scan and Bregs detection for the survival of NDMM patients cannot be fully revealed. Lastly, because PET/CT is not yet a standardized imaging tool in MM, the prognosis prediction value of SUVmax to response and survival of NDMM patients needs to

be confirmed by more studies. Despite these limitations, the fact that PET/CT findings and frequency of Bregs within $CD19^+$ B cells are well correlated with therapeutic response and survival is quite reliable.

In conclusion, adverse baseline PET/CT findings and low Bregs frequency were positively associated with poor therapeutic response and survival in NDMM patients. More attention is needed for a group of high-risk patients based on the definition of SUVmax > 4.2 and Bregs' ratios < 10%, and risk-adapted treatment is required. On the basis of our results, integrating PET/CT scan and Bregs detection into the algorithm of NDMM staging may improve disease management and supplement risk stratification systems such as R-ISS. More studies are warranted to confirm our findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board/Research Ethics Board of West China Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JC: data analysis and interpretation and writing of manuscript. ZZ: detection of Bregs. JD: collection and assembly of data. WT: data analysis and interpretation. YL: collection and assembly of data. LZ: conceptualization and design, collection and assembly of data, supervision, and approval of final draft. LP: supervision and approval of final draft. TN: supervision and approval of final draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.671904/full#supplementary-material

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Characterization of Organ-Specific Regulatory B Cells Using Single-Cell RNA Sequencing

Si-Yu Yang[1†], Jie Long[2†], Meng-Xing Huang[1], Pan-Yue Luo[3], Zhen-Hua Bian[1], Ya-Fei Xu[1], Cheng-Bo Wang[1], Shu-Han Yang[1], Liang Li[4], Carlo Selmi[5,6], M. Eric Gershwin[7], Zhi-Bin Zhao[4] and Zhe-Xiong Lian[1*]*

[1] Chronic Disease Laboratory, School of Medicine, South China University of Technology, Guangzhou, China, [2] Department of Thoracic Surgery, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [3] School of Biomedical Sciences and Engineering, South China University of Technology, Guangzhou, China, [4] Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [5] Division of Rheumatology and Clinical Immunology, Humanitas Research Hospital IRCCS, Milan, Italy, [6] Department of Biomedical Sciences, Humanitas University, Milan, Italy, [7] Division of Rheumatology, Allergy, and Clinical Immunology, University of California Davis, Davis, CA, United States

Regulatory B cells (Breg) are considered as immunosuppressive cells. Different subsets of Breg cells have been identified both in human beings and in mice. However, there is a lack of unique markers to identify Breg cells, and the heterogeneity of Breg cells in different organs needs to be further illuminated. In this study, we performed high-throughput single-cell RNA sequencing (scRNA-seq) and single-cell B-cell receptor sequencing (scBCR-seq) of B cells from the murine spleen, liver, mesenteric lymph nodes, bone marrow, and peritoneal cavity to better define the phenotype of these cells. Breg cells were identified based on the expression of immunosuppressive genes and IL-10-producing B (B10) cell-related genes, to define B10 and non-B10 subsets in Breg cells based on the score of the B10 gene signatures. Moreover, we characterized 19 common genes significantly expressed in Breg cells, including *Fcrl5*, *Zbtb20*, *Ccdc28b*, *Cd9*, and *Ptpn22*, and further analyzed the transcription factor activity in defined Breg cells. Last, a BCR analysis was used to determine the clonally expanded clusters and the relationship of Breg cells across different organs. We demonstrated that *Atf3* may potentially modulate the function of Breg cells as a transcription factor and that seven organ-specific subsets of Breg cells are found. Depending on gene expression and functional modules, non-B10 Breg cells exhibited activated the TGF-β pathway, thus suggesting that non-B10 Breg cells have specific immunosuppressive properties different from conventional B10 cells. In conclusion, our work provides new insights into Breg cells and illustrates their transcriptional profiles and BCR repertoire in different organs under physiological conditions.

Keywords: Breg cells, scRNA-Seq, BCR, transcription factor, B10 cells

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Reviewed by:
To-Ha Thai,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States
Yaofeng Zhao,
China Agricultural University, China
Yumei He,
Southern Medical University, China

***Correspondence:**
Zhe-Xiong Lian
zxlian@scut.edu.cn
Zhi-Bin Zhao
zzbin@mail.ustc.edu.cn

†These authors have contributed equally to this work

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INTRODUCTION

Regulatory B (Breg) cells have been reported as a special subset of human and murine $CD19^+$ B cells (1), capable of negatively regulating the immune response in mouse models of autoimmune diseases (2), allergy (3), and infections (4), depending on IL-10. In fact, $CD5^{hi}CD38^{low}PD\text{-}1^{hi}$, $CD19^+CD24^{hi}CD27^+$, and $CD19^+CD24^{hi}CD38^{hi}$ Breg cells are involved in cancer, allergic asthma, systemic lupus erythematosus, and rheumatoid arthritis in human beings (5–8). In addition to production of IL-10, Breg cells express other immune-regulatory cytokines to exert immunosuppressive function, including transforming growth factor-β (TGF-β) (9) and IL-35 (10). Breg cells have been identified in various organs of mice like the spleen, draining lymph nodes, and peritoneal cavity (11–14). However, their phenotypes and functions are still obscure and the heterogeneity of Breg cells in different organs warrants further characterization. The definition of Breg cell subsets under different experimental settings and tissues is challenging, based on the frequent changes in cell markers, and is indeed necessary for further studies in disease models. The single-cell RNA sequencing (scRNA-seq) technology provides a unique strategy to understand Breg cell complexity and heterogeneity by identifying cell subsets and functional pathways (15).

We herein report for the first time the scRNA-seq transcriptional profiles of Breg cells in different mouse organs under physiological conditions and propose a novel method for the identification of Breg cells based on the expression of immunosuppressive genes and B10-related genes. Ultimately, we arrayed Breg cells into seven subsets with variable immunosuppressive functions.

MATERIALS AND METHODS

Animals

Four 10-week-old female C57BL/6 mice were maintained on a 12-h-light/dark cycle (light on at 7 a.m. and off at 7 p.m.) and at the temperature of 20°C–26°C with 40%–70% humidity in specific pathogen-free facilities. All animal experiments were performed with the approval of the Guide for the Care and Use of Laboratory Animals, South China University of Technology.

Cell Preparation

Liver tissues were homogenized in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and passed through a steel mesh. Mononuclear cells were separated from hepatocytes through centrifugation (Beckman Coulter Microfuge 20R and Beckman Coulter Allegra X-15R Centrifuge) with 40% Percoll (GE Healthcare, Little Chalfont, UK) at room temperature. Red blood cells were depleted by Red Blood Cell Lysis Buffer (Beyotime). Mouse spleens and mesenteric lymph nodes (mLN) were separately ground using slides in PBS with 0.2% BSA. Cells were filtered and centrifuged, and splenic cells were subjected to red blood cell lysis. Bone marrow (BM) was extracted from tibia and femur bones following removal of the surrounding muscle. BM cells were flushed out using a syringe filled with PBS. Cell clumps were gently disaggregated, then filtered and centrifuged. Red blood cells were depleted by Red Blood Cell Lysis Buffer. To obtain peritoneal cavity (PC) cells, 3–4 ml of cold PBS containing 0.2% BSA was injected into the PC, followed by repeated washing of the abdomen before aspiration of the fluid back into the syringe, and this process was repeated in a second time. PC cells were collected and centrifuged. Cells from all the preparations were counted in the presence of Trypan Blue.

Flow Cytometry and Cell Sorting

Cells are mixed from the isolated liver, spleen, mLN, BM, and PC of four mice separately. Freshly prepared mouse cells were stained for 20 min at 4°C with anti-CD19 PE-Cy7 (6D5, BioLegend) at a ratio of 1:200 and anti-B220 FITC (RA3-6B2, BioLegend) at a ratio of 1:200 antibodies. The stained cells were washed with PBS (containing 0.2% BSA) and then suspended in PBS + 1% FBS + 1 mM EDTA. $CD19^+B220^{+/low}$ cells were sorted by the FACS Aria II cell sorting system (BD Immunocytometry Systems, San Jose, CA, USA).

Cell Hashing and Single-Cell RNA Sequencing

B cells from different organs were incubated in TruStain FcX™ PLUS (anti-mouse CD16/32) (BioLegend) blocking reagent for 10 min at 4°C, and then each sample was stained with 1 μg different hashtag antibodies (BioLegend) and incubated for 30 min at 4°C. Cells were washed three times with Cell Stain Buffer (BioLegend) and resuspended with PBS + 0.04% BSA (Sigma). According to the cell number count, B cells from different organs were mixed at the ratio of 1:1 and cell concentrations were adjusted to about 1,000 cell/μl. For 10× Genomics scRNA-seq, cells were loaded onto a 10× chromium controller to generate Gel beads in emulsion using the 10× genomics Single Cell 5′ Library & Gel Bead Kit. Gene expression libraries were constructed according to instructions from 10× genomics. Three libraries were generated that measure (1) mRNA transcript expression (RNA), (2) mouse-specific hashtag oligos (HTO), and (3) BCR library. Libraries were sequenced by the NovaSeq 6000 sequencing system (Illumina, San Diego, CA, USA).

Single-Cell RNA and BCR Sequencing Data Processing

The raw sequencing data of gene expression were aligned with the mm10 mouse reference genome, using the STAR algorithm in CellRanger software (version 5.0.0; 10× Genomics). BCR reads

Abbreviations: Breg cells, regulatory B cells; scRNA-seq, single-cell RNA sequencing; BCR-seq, BCR sequencing; MZB, marginal-zone B cell; TGF-β, transforming growth factor β; DEG, differential gene expression; SCENIC, single-cell regulatory network inference and clustering; BM, bone marrow; mLN, mesenteric lymph nodes; PC, peritoneal cavity; t-SNE, t-distributed stochastic neighbor embedding; TNF-α, tumor necrosis factor α; IL-10, interleukin 10; IL-12, interleukin 12; IL-6, interleukin 6; IL-4, interleukin 4; IL-35, interleukin 35; IFN-γ, interferon γ; GSEA, gene set enrichment analysis; GSVA, gene set variation analysis; BSA, bovine serum albumin; Th1, T helper cell type 1; Th2, T helper cell type 2; Th17, T helper cell type 17; Tfh, T follicular helper cells.

were aligned with the mouse reference VDJ dataset, using CellRanger (version 5.0.0; 10× Genomics). The gene expression reads, BCR reads, and feature barcodes reads were aligned in one CellRanger pipeline using the command "cellranger multi".

On acquiring the dataset, cells which have more than 8,000 or less than 300 unique genes were excluded from the analysis. Only genes which were expressed in 10 or more cells were used for further analysis. Cells in which the percentages of the mitochondrial genes are more than 10% and log10GenesPerUMI (the gene numbers of per UMI) less than 0.8 were removed from the dataset. Furthermore, we also filtered out the cells which include more than two different BCR clones (means two different heavy chains and two different light chains). A data matrix with 13,116 genes and 21,645 cells was obtained. The data matrix was transformed as a Seurat object using CreateSeuratObject function and normalized using normalization method LogNormalize in Seurat package (version 4.0.1) and the scale factor that uses the default value 10,000. Then, the Seurat object was analyzed for ScaleData, FindVariableFeatures, and RunPCA functions. The dimensionality reduction was performed using the RunTSNE function and then clustering.

To analyze the BCR sequencing data, the BCR data matrix was imported into R language (Version 4.0.1) and clonotype information was inserted into the metadata slot of the RNA data object.

Module Score Calculation

To calculate the module score of the selected Breg gene signature and B10 gene signature, we firstly run the ScaleData function for the Seurat object of total B cells in R. Then, we chose the scaled matrix to calculate the mean values of the Breg gene signature in every B cell and the B10 gene signature in every identified Breg cell. Finally, we run the log1p function for the mean values of the Breg gene signature or B10 gene signature as the Breg cell score and B10 cell score, respectively.

GSVA and GSEA Analysis of scRNA-seq Data

Gene set variation analysis (GSVA) was performed using the GSVA package (Version 1.36.3), and gene set enrichment analysis (GSEA) was performed using the clusterProfiler package (version 3.16.1). Gene sets were downloaded from the MSigDB database or collected from document literatures. The differences in pathway enrichment score between different cell clusters were calculated using the LIMMA package (version 3.44.3).

BCR Data Analysis

The BCR expansion, migration, and transition index of different cell clusters were calculated using the STARTRAC package (version 0.1.0). The shared clonotypes and shared cell counts with the same clonotype of Breg cells between pairwise organs were analyzed in R.

Gene Regulation Network Analysis

To analyze the specific regulatory network of Breg and non-Breg cell clusters, we chose 500 random cells of each cluster to reduce computing resource consumption, employed the SCENIC package (version 1.2.2) in R to infer the regulatory network, and compared the difference between Breg cells and non-Breg cells.

RESULTS

Identification of Breg Cells by High-Throughput scRNA-seq in Different Organs

We performed scRNA-seq analysis of B cells isolated from the liver, spleen, BM, PC, and mLN of four wild type mice. Briefly, live B cells were isolated from fresh samples by FACS sorting and single-cell transcriptomes were obtained using the 10× Genomics platform (**Supplementary Figure 1A**). The final dataset comprised 13,116 single-cell genes merged from 21,645 cells of liver, spleen, BM, PC, and mLN after data filtering (**Supplementary Figures 1B–E**).

Firstly, to define Breg cells, we assessed the expression profile of functional genes that negatively regulate the immune system process and promote the production of inhibitory cytokines, including IL-10, TGF-β, and IL-35 (adopted from the GO database and BioGPS). In addition, significantly upregulated genes of mouse splenic $B10^+$ cells in RNA-seq were obtained from the GEO database (16) (**Figure 1A**). A gene list with 134 genes was eventually selected through profiling (**Figure 1B**). Then, we calculated the module score of these selected genes, and the cells with the score higher than 0.16 were considered as Breg cells (**Figure 1C**). We delineated the distribution of Breg cells in the t-SNE plot of total B cells from the liver, spleen, BM, PC, and mLN (**Figure 1D**), and there was a significantly higher proportion of Breg cells in the PC compared with other organs (**Figure 1E**).

Common Genes and Transcription Factors in Breg Cells

We identified 19 common genes that were upregulated in Breg cells as compared to non-Breg cells in each organ, defined as differential expression genes (DEGs) (**Figure 2A**). Our data showed that *Cd9*, *Ccdc28b*, and *Ptpn22* genes were significantly upregulated in Breg cells from the liver, spleen, BM, and PC, but not in Breg cells from mLN, while *Fcrl5* and *Zbtb20* genes were strikingly expressed in Breg cells from all organs, which indicated that *Fcrl5* and *Zbtb20* would serve as potential suitable marker genes for Breg cells (**Figure 2B**). Furthermore, to identify the cell-intrinsic transcription factors in Breg cells, we performed SCENIC analysis to infer the transcription factor regulatory network between defined Breg cells and non-Breg cells of all organs. Several transcription factor regulators with higher activity were found in Breg cells, including *Sox5*, *Myc*, and *Atf3* (**Figure 2C**). The distribution in the t-SNE plot of total B cells from all organs showed that *Atf3* was specifically expressed in identified Breg cells while *Myc* was extensively expressed in non-Breg cells (**Figures 1D**, **2D**). We further compared the expression of *Atf3*-regulated target genes between Breg cells and non-Breg cells in total B cells (**Figure 2E**). It shows that

A

	Gene Set	Reference
Breg cell definition	positive regulation of interleukin-10 production	GO:0032733
	positive regulation of transforming growth factor beta production	GO:0071636
	negative regulation of immune system process	GO:0002683
	negative regulation of interferon-gamma production	GO:0032689
	negative regulation of interleukin-12 production	GO:0032695
	negative regulation of interleukin-17 production	GO:0032700
	negative regulation of interleukin-1 production	GO:0032692
	negative regulation of interleukin-6 production	GO:0032715
	negative regulation of tumor necrosis factor superfamily cytokine production	GO:1903556
	Il10, TGFβ, Il35 correlated expression genes	BioGPS
	mouse splenic B10 B cell RNA-seq	GSE63426

B

Breg cell definition									
Negative regulation of immune system				Overlapped genes	B10 associated genes				
Cd68	Socs1	Spn	Tlr4	Ctla4	Apoe	Cd9	Clstn1		
Clec2g	Socs6	Zc3h12d	Prkcz	Ptprj	Nrp2	Zbtb32	Rgs13		
Dhx58	Syt11	Nr2f6	Pycard	Cd5	Tbc1d9	Atxn1	Pik3cg		
Dusp3	Tjp2	Twsg1	Hspd1	Fcrl5	Nid1	Mical3	Bcl2l15		
Fbxw7	Tnfrsf4	Pawr	Ebi3	Cd300lf	Rbm47	Pld4	Rab11fip4		
Fgr	Tnfrsf14	Slfn1	Vsir	Ccr1	Lipc	Dmxl2	Mlkl		
Fstl3	Traf3ip1	Fgl2	Sirpa	Lgals3	Slc7a7	Zfp945	Ryk		
Gpr137	Ttll12	Ahr	Slamf1	Il10	Nebl	Acp5	Tnfsf9		
Ldlr	Ubash3b	Gfer	Ptpn22	Pdcd1	Capg	Zdhhc2	Tnfsf8		
Lilrb4a	Zc3h8	Pglyrp2	Furin	Cdkn2a	Ahnak	Alpl	Stk39		
Nbl1	Egr1	Cd1d1	Sirt1	Gpr55	Tnfrsf8	Zap70	Perp		
Nlrc3	Adora2a	Cuedc2	Tlr6	Tigit	S100a11	Fhit	Timd2		
Nod2	Dlg1	Rin3	Tlr2	Sh2d1b1	Csf2rb	Nek6	Cd70		
Nrarp	Dtx1	Nlrx1	Nlrc5		Krt222	Cpd	Elk3		
Prdm1	Cd80	Grn	Cd274		Actn1	Bag3	Crim1		
Prnp	Cd86	C1qbp	Pdcd1lg2		Lysmd2	Hdac9	Stat1		
Rhbdd3	Cd44	Prdx2	Git1		Serpinc1	Cebpd	Nt5e		
Serpinb9					C130026I21Rik				

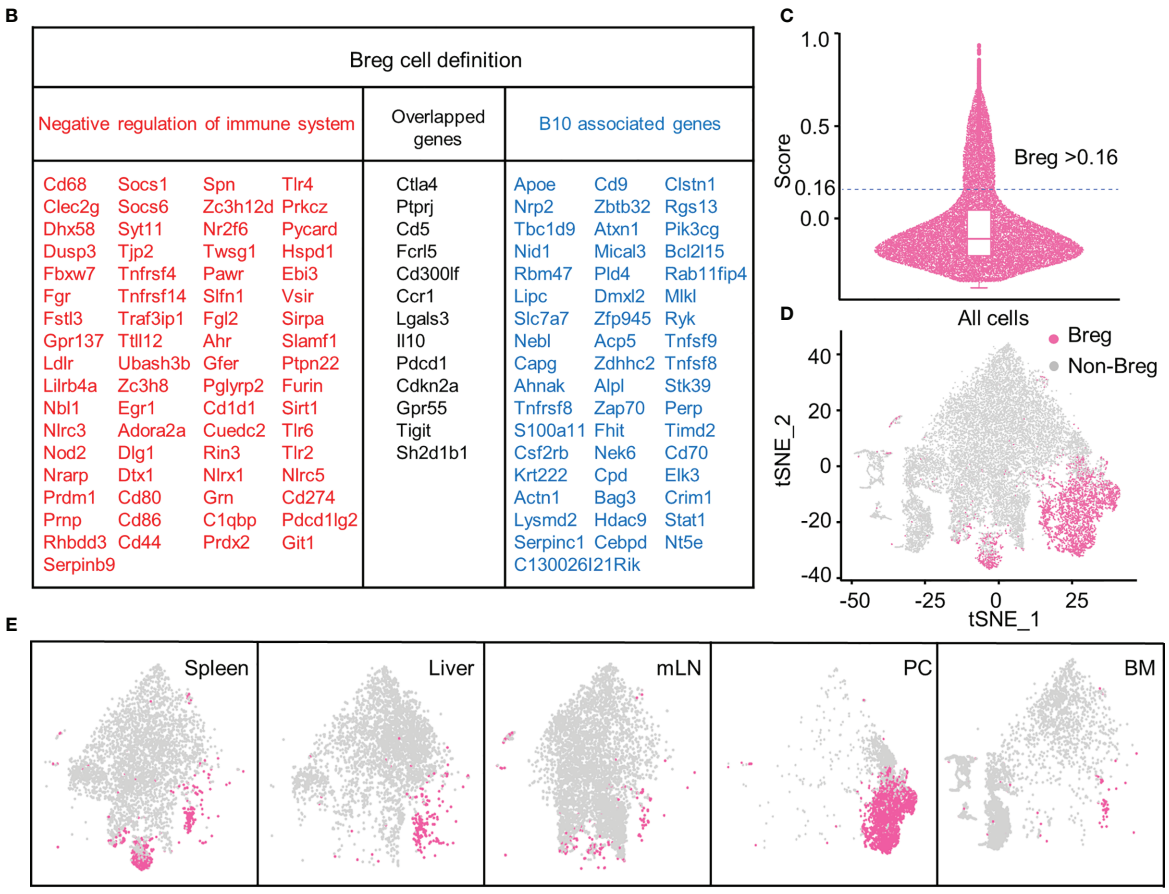

FIGURE 1 | Breg cells analysis by high-throughput scRNA-seq in different organs. **(A)** Gene sets from GO, BioGPS, and GEO databases. **(B)** List of selected genes for identifying Breg cells. **(C)** Evaluation of the Breg cell module score. **(D)** t-SNE plot of Breg cell distribution in total B cells from all organs. **(E)** t-SNE plot of Breg cell distribution in spleen, liver, mLN, PC, and BM.

these genes were dramatically upregulated in Breg cells, including *Ptpn22*, one of the 19 common upregulated genes in DEGs. In mice, reported markers of Breg cells include CD21, CD1d, CD5, CD24, CD23, IgD, IgM, CD138, Tim-1, and PD-L1. We detected the expressions of their corresponding genes (*Cr2*, *Cd1d1*, *Cd5*, *Cd24a*, *Fcer2a*, *Ighd*, *Ighm*, *Sdc1*, *Havcr1*, and *Cd274*) in B cells and observed that the expression only represents part of Breg cells while *Fcrl5*, *Ptpn22*, *Ccdc28b*,

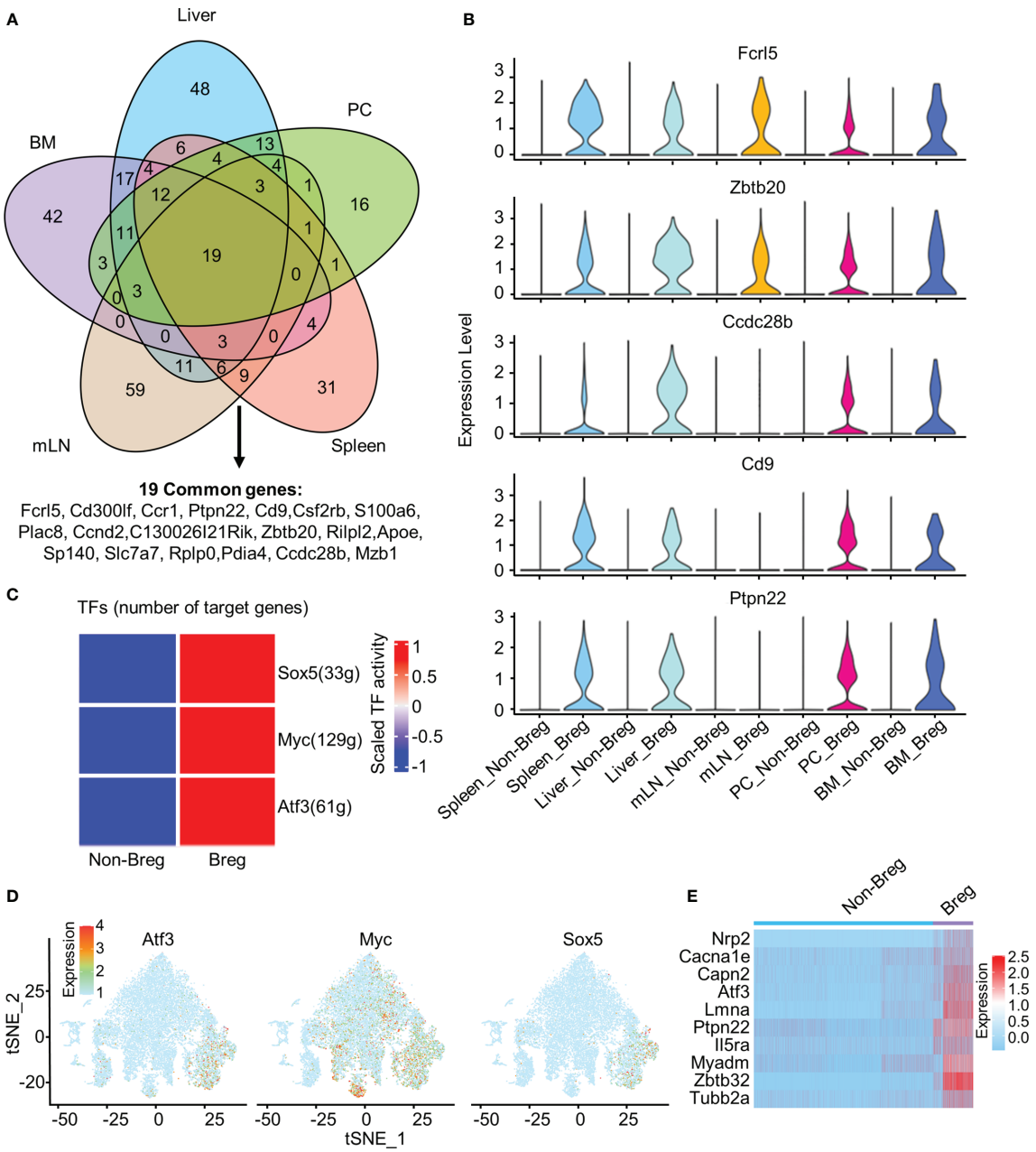

FIGURE 2 | Common marker genes and transcription factors in Breg cells. **(A)** Venn diagram showed the overlap of upregulated DEGs by comparing Breg cells with non-Breg cells in different organs. **(B)** Violin plots represented the normalized expression of *Fcrl5*, *Zbtb20*, *Ccdc28b*, *Cd9*, and *Ptpn22* in Breg cells and non-Breg cells across different organs. **(C)** Heat map of the activation scores of 500 randomly selected Breg cells and 500 non-Breg cells from all organs for expression regulated by transcription factors (TFs). **(D)** Feature plot of *Atf3*, *Myc*, and *Sox5* expression of three TFs on total B cells from all organs. **(E)** Heat map of *Atf3*-related gene expression between Breg cells and non-Breg cells in total B cells from all organs.

Zbtb20, and *Cd9* were expressed specifically in most of the Breg cells (**Supplementary Figures 2A, B**). By comparing the known Breg cell marker genes with the Breg cell genes identified in this study, the newly identified gene expression generally contained the expression of currently known Breg cell marker genes, such as *Cd1d*$^{+}$, *CD5*$^{+}$, and *Cr2*$^{+}$ Breg cells which were included in *Fcrl5*$^{+}$ and *Ptpn22*$^{+}$ Breg cells.

Phenotype and Function of Breg Cell Subsets

Seven Breg subsets were identified and visualized using the t-SNE plot based on the gene expression profiles of identified Breg cells isolated from total B cells of all organs (**Figure 3A**). The top 50 genes for each subset were exhibited by a heat map; this indicated that there were distinct expression profiles among different subsets

(**Figure 3B**), and several typical marker genes showed the distinct expression in seven Breg cell subsets from all organs. The *Apoe* gene was expressed in Breg2, while two immunoglobulin heavy chain variable region genes, *Ighv11-2* and *Ighv1-55*, were specially expressed in Breg3 and Breg5 subsets, respectively. The Breg6 subset was characterized by the *Cr2* gene which encodes protein CD21, a marker of marginal zone B cells, while the Breg7 subset was associated with *Stmn1* (**Figure 3C**). Further, we investigated the potential biological function of different Breg cell clusters of all organs using gene set variation analysis (GSVA) and our analysis indicated that the gene profiles displayed biological process enrichment in negative regulation of IL-12 and IFN-γ production in the Breg1 subset, while the Breg2 subset possessed genes enriched in negative regulation of IL-6 and IL-4 production while promoting the production of TGF-β. The Breg3 subset enriched pathways in negative regulation of Th1-type immune response, and the Breg4 subset enriched in negative regulation of the TNF-mediated signal pathway. The Breg5 subset was demonstrated with enriched pathways in negative regulation of CD8-positive T cell activation while the Breg6 subset was enriched in the IL-10 anti-inflammatory signaling pathway and the Breg7 subset was characterized by the Treg cell differentiation pathway (**Figure 3D**).

We also analyzed the distribution of Breg cell subsets and their proportion within the liver, spleen, BM, PC, and mLN tissues. The results clearly showed that the Breg6 cluster was found only in the spleen, while the Breg1, Breg3, Breg4, and Breg5 clusters almost characterized the PC. Further, the Breg2

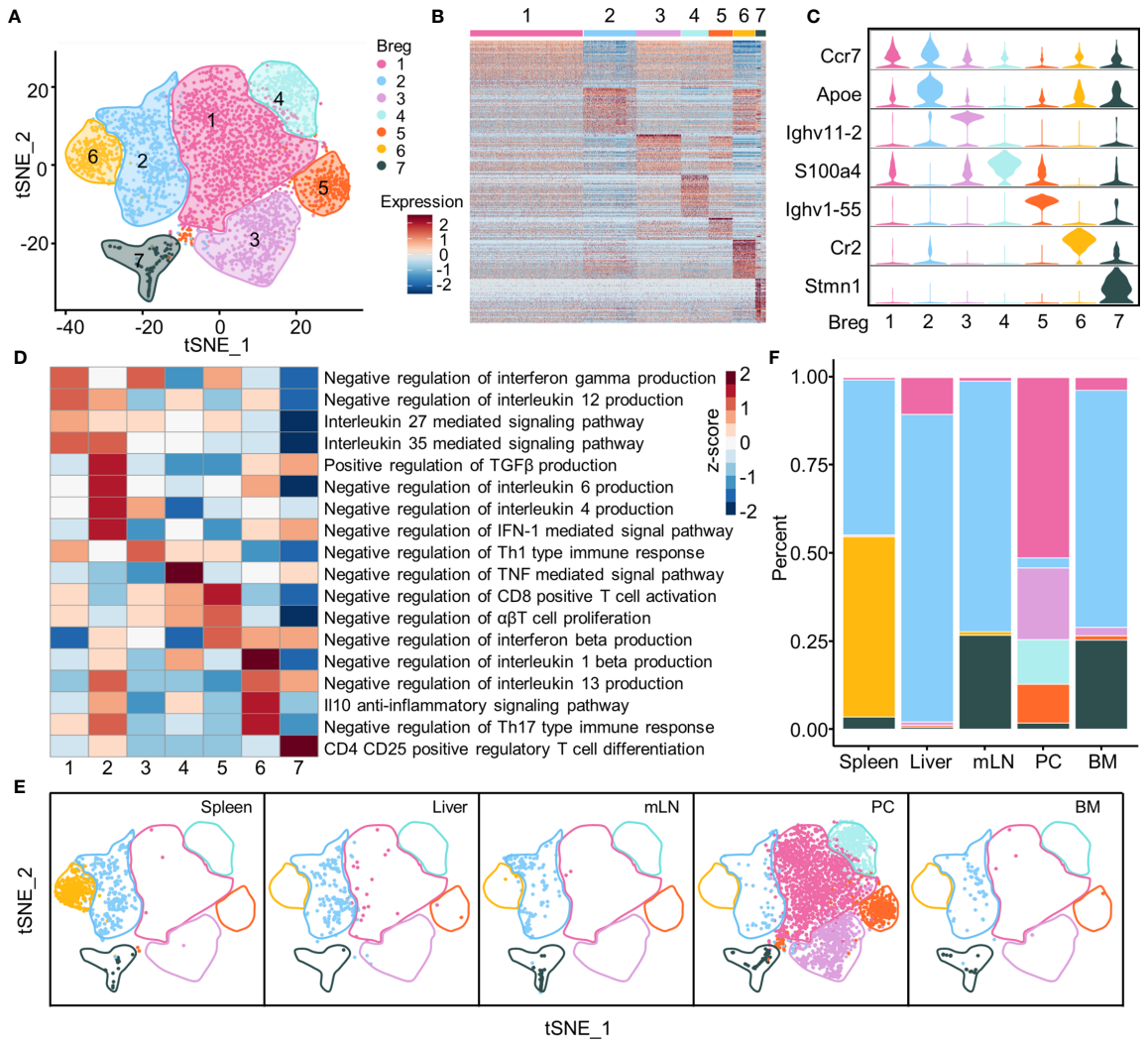

FIGURE 3 | Subsets of Breg cells. **(A)** t-SNE plot of identified Breg cells from all organs showing seven clusters. **(B)** Heat map of top 50 genes in each subset of Breg cells from all organs. **(C)** Violin plots showed the normalized expression of the marker genes in each Breg cell subset from all organs. **(D)** GSVA analysis of immunoregulation pathways in Breg cell clusters from all organs. **(E)** Distribution of Breg cell subsets in spleen, liver, mLN, PC, and BM. **(F)** The proportion of Breg cell subsets in spleen, liver, mLN, PC, and BM.

cluster was universally distributed in all tissues whereas the Breg7 cluster had high proportions in mLN and BM (**Figures 3E, F**).

Clonal Expansion and Potential Migration of Breg Cell Subsets by scBCR-seq

Our single-cell BCR sequencing based on scBCR-seq and scRNA-seq data investigated the distribution of the top 10 BCR clones in the Breg cell t-SNE plot from all organs and demonstrated that PC-specific Breg3 and Breg5 were two most clonally expanded clusters (**Figures 4A, B**). The Breg3 cluster was enriched by clonotype1 with v genes composed of heavy-chain and light-chain *Ighv11-2/Igkv14-126* with CMRYGNYWYFDVW/CLQHGESPYTF as the CDR3 amino acid sequence, while the Breg5 cluster was enriched by clonotype2 with v genes *Ighv1-55/Igkv12-89* in which the CDR3 amino acid sequence was CARRDYGSSYWYFDVW/CQNVLSTPWTF, which consisted with the previous results of *Ighv11-2* and *Ighv1-55* upregulated by Breg3 and Breg5, respectively (**Figure 3C**). Importantly, BCR clonotype1 was also found in the Breg2 cluster and mainly distributed in the liver and spleen, whereas BCR clonotype2 only existed in the Breg5 cluster in the PC (**Figure 4B**). The sharing BCR clonotypes were then compared between organs, and we observed an overlap of BCR clonotypes and cell counts between the liver and PC as well as the spleen and PC (**Figures 4C, D**). Moreover, the Breg2 cluster which distributed in all organs and the Breg3 cluster which only existed in PC displayed the highest transition index score with each other (**Figure 4E**), while Breg3 and Breg5 which were both specifically in the PC exhibited higher expansion and migration indexes as expected (**Figures 4F, G**).

Functionally Specific Non-B10 Breg Cell Cluster in Breg Cells

Approximately 60% of Breg cells with a high B10 signature score were defined as B10 cells (**Figures 5A, B**), and they were most represented among Breg3 and Breg5 clusters while numerous Breg4 and Breg6 cells were regarded as the non-B10 Breg subset (**Figure 5C**). B10 cells accounted for the highest proportion in the PC, followed by the spleen, and minimal in BM (**Figure 5D**).

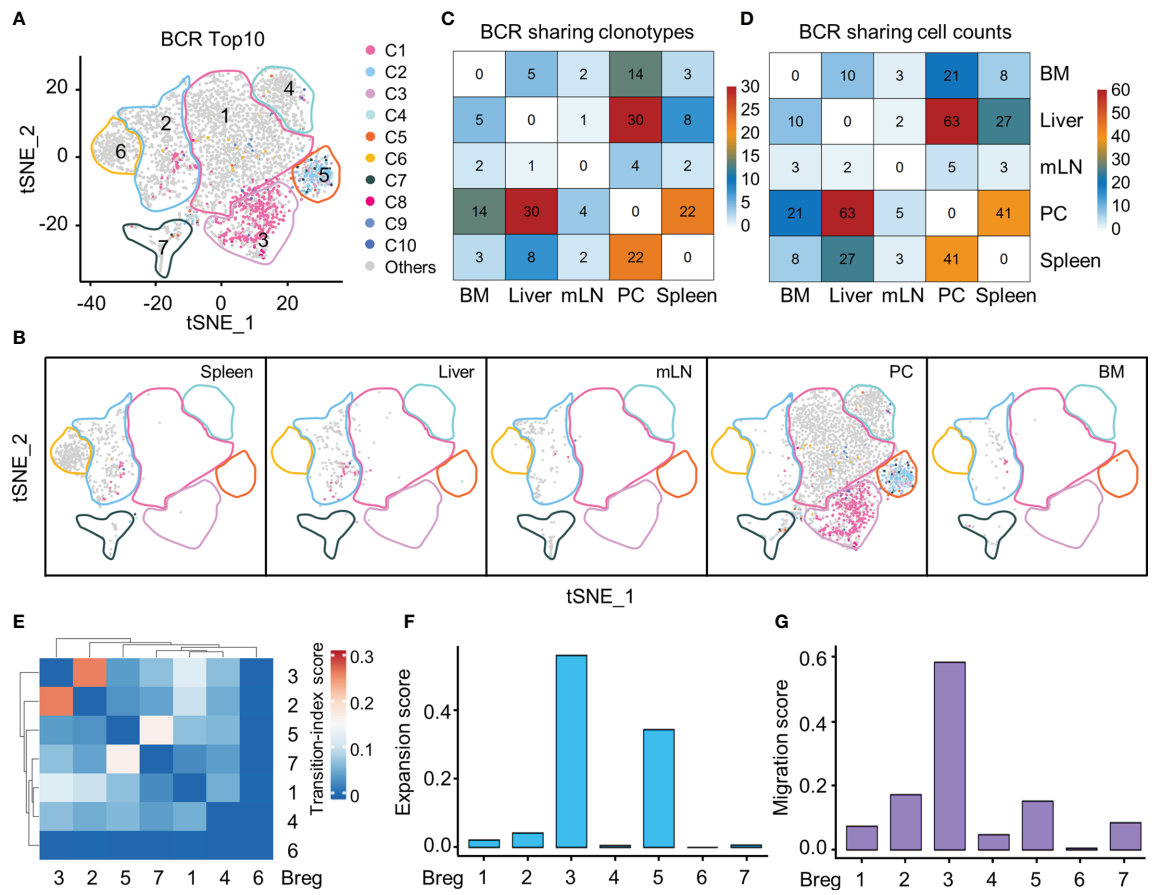

FIGURE 4 | Clonal expansion and potential migration of Breg cells subsets at scBCR-seq. **(A)** The distribution of top 10 BCR clonotypes in Breg cells clusters of all organs. **(B)** t-SNE plot of top 10 BCR clonotypes of Breg cells in each organ. **(C)** Sharing of Breg cell BCR clonotypes across pairwise organs. **(D)** Sharing of Breg cell and BCR cell counts across pairwise organs. **(E)** Heat map of transition-index scores for the pairwise Breg cell clusters from all organs. **(F)** Expansion-index scores of each Breg cluster from all organs calculated using STARTRAC. **(G)** Migration-index scores of each Breg cluster from all organs calculated using STARTRAC.

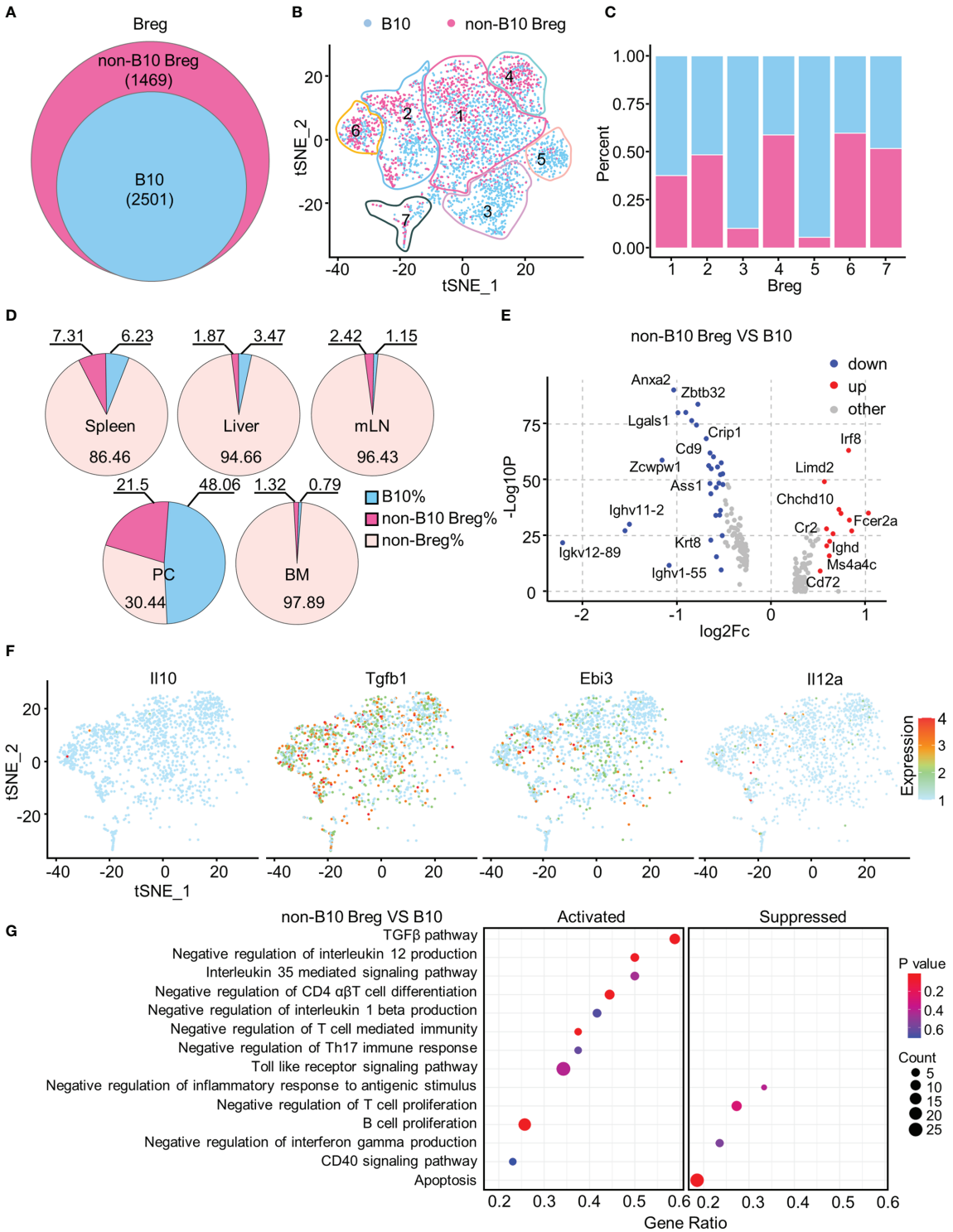

FIGURE 5 | Functionally specific non-B10 *versus* B10 cells among Breg cells. **(A)** Venn diagram showed the B10 and non-B10 cells in Breg cells of all organs. **(B)** t-SNE plots of the B10 and non-B10 cells in Breg cells. **(C)** Proportion of B10 and non-B10 Breg cells in every Breg cell cluster. **(D)** Percent of B10 and non-B10 Breg cells in spleen, liver, mLN, PC, and BM. **(E)** Volcano plot displayed the DEGs between B10 and non-B10 Breg cells from all organs. **(F)** t-SNE plots of the normalized expression of *Il10*, *Tgfb1*, *Ebi3*, and *Il12a* in non-B10 Breg cells of all organs. **(G)** GSEA analysis of B10 and non-B10 Breg cells of all organs.

We depicted in a volcano plot that significant DEGs exist between B10 cells and non-B10 Breg cells from all organs, with *Irf8*, *Fcer2a*, *Chchd10*, and *Cr2* genes dramatically upregulated in non-B10 Breg cells compared to B10 cells (**Figure 5E**). As expected, non-B10 Breg cells isolated from Breg cells of all organs expressed no *Il10* gene, while expressing the Tgfb1 and *Ebi3*, along with *Il12a* in some non-B10 Breg cells (**Figure 5F**). Our gene set enrichment analysis (GSEA) showed the activated TGF-β pathway and IL-35-mediated signaling pathway in non-B10 Breg cells compared to B10 cells (**Figure 5G**).

DISCUSSION

We report herein for the first time a fine characterization of Breg cells in multiple mouse organs using different and comprehensive techniques, particularly scRNA-seq in the mouse spleen, liver, mLN, BM, and PC, and we submit that this has important clinical implications. Prior to our study, the diverse phenotypes of Breg cell subsets and different immune regulatory mechanisms have been determined with conflicting evidence (17). Breg cells are involved in the control of tissue immunopathology (18) by maintaining immune homeostasis, thus leading, when dysregulated, to autoimmunity, allergic diseases, infections, and cancer (19). In the case of rheumatoid arthritis and systemic lupus erythematosus, $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}$ transitional 2 marginal-zone precursor (T2-MZP) B cells significantly ameliorate collagen-induced arthritis (20), while the transfer of T2-like B cells suppresses lupus in MRL/*lpr* mice by restraining Th1 responses and inducing the differentiation of $IL\text{-}10^{+}CD4^{+}$ T cells (21). Further, $CD19^{hi}CD1d^{hi}CD5^{+}$ B cells produce IL10 and inhibit DSS-induced inflammatory bowel disease (22) and experimental Sjögren's syndrome by suppressing Tfh cell responses (23). Plasmablasts in the draining lymph nodes negatively regulate experimental autoimmune encephalomyelitis (12), and IL10-producing B cells depend on dendritic cells to suppress antigen-specific CD8 T which can protect from type 1 diabetes in non-obese diabetic (NOD) mice (24). In the model of allergic asthma, $CD9^{+}$ B cells play a role in airway inflammation suppression by inhibiting Th2- and Th17-driven inflammation in an IL-10-dependent manner (3) while MZB cells reduce the CD8 T cell function and IFN-γ^{+} CD4 T cells during the early stages of *Leishmania donovani* infection (4).

Our data have numerous implications. First, we redefined the Breg cells in different mouse organs based on the score of immunosuppressive genes and B10 cell-related genes. Second, we identified the genes which were widely upregulated in Breg cells as compared to non-Breg cells. Third, we investigated the heterogeneity of Breg cells and identified seven subsets with different prevalence in murine organs. Fourth, we reported that Breg3 and Breg5 in the PC were the most clonally expanded subsets. Fifth, we took advantage of B10-associated genes to calculate the B10 signature score among Breg cells.

Various Breg cells subsets have been described, but there are no general markers for Breg cells (25) and we reported 19 Breg-specific genes, including *Fcrl5*, *Zbtb20*, *Ccdc28b*, *Cd9*, and *Ptpn22*, which are shared in all the five analyzed murine organs. The identification of *Cd9* is consistent with previous reports that CD9 was considered as a marker of Breg cells (16), but we reported that this failed to identify mLN-specific Breg cells. *Fcrl5* can better serve as a general marker to define Breg cells in the liver, spleen, BM, PC, and mLN, similar to previous data that found FCRL5 expressed on MZB and B1 cells (26); it is also related to the expression of several receptors and downstream adaptors with immunosuppressive functions (27). *Zbtb20* is a transcription factor to promote the differentiation and longevity of plasma cells (28) which suppress the pro-inflammatory response during experimental autoimmune encephalomyelitis and *Salmonella* infection (10). *Ptpn22* is rarely reported in B cells but acts as a negative regulator of Src and Syk family kinases downstream of the T cell receptor (TCR) (29), and mutations in *Ptpn22* are associated with an increased susceptibility to autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (30, 31). The importance of transcription factors has been suggested in Breg cells, including HIF-1a (32), STIM1/STIM2 (33), c-Maf (34), IRF4, and Blimp1 (12), but these are not specific for Breg cells, unlike Foxp3 for Treg cells (35, 36). We identified three potential candidate transcription factors, including *Sox5*, *Myc*, and *Atf3* in Breg cells, using SCENIC software, with effects spanning from modulating B cell development (*Myc*) (37) to negatively regulating the inflammatory response (*Atf3*) (38). These findings need to be validated in disease models inducing Breg cells such as experimental autoimmune encephalomyelitis (39), collagen-induced arthritis (40), and systemic lupus erythematosus (41) mouse models.

We also identified seven Breg subsets with markedly different enrichment tendencies in various immune negative regulation pathways including negative regulation of IL-12, IFN-γ, IL-6, and IL-4 production, promoting the production of TGF-β, negative regulation of Th1-type immune response, and negative regulation of the TNF-mediated signal pathway and IL-10 anti-inflammatory signaling pathway, among other effects. We believe that these results may explain the conflicting evidence that was previously reported on the function of Breg cells in different mouse models.

Our single-cell BCR sequencing analysis investigated the clonal expansion of Breg subsets to identify two BCR clonally expanded Breg cell subsets in the PC, with one being the *Ighv11-2/Igkv14-126* Breg3 subset enriching the mouse peritoneal B-1a cells (42), and the other being *Ighv1-55/Igkv12-89* Breg5 reported for the first time. Further, we observed a high degree of BCR sharing between the liver or the spleen and the PC, suggesting that Breg cells may migrate between these organs.

Finally, when we compared B10 and non-B10 Breg cells, we reported that non-B10 Breg cells minimally expressed *Il10* with high levels of Tgfb1 and *Ebi3*, activated TGF-β pathway, and IL-35-mediated signaling pathway.

In conclusion, we characterized Breg cells by scRNA-seq based on the immunosuppressive gene expression profile and B10-related genes and delineated their transcriptional profiles, underlying functions, and BCR clonotypes in different mouse organs under physiological conditions. The scRNA-seq analysis

is of particular value to understand the transcriptional profiles of Breg cells and provides the basis for future Breg cell-based therapeutic avenues for immune-mediated diseases.

DATA AVAILABILITY STATEMENT

The accession number for scRNA-seq datasets reported in this paper is GEO: GSE174739 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE174739). The code used for analysis is available *via* GitHub (https://github.com/jalon9358/Lianlab_Breg).

ETHICS STATEMENT

All animal experiments were performed with the approval of the Guide for the Care and Use of Laboratory Animals, South China University of Technology.

AUTHOR CONTRIBUTIONS

S-YY, JL, Z-BZ, and Z-XL designed the experiments and wrote the manuscript. S-YY, M-XH, Z-BZ, P-YL, and Y-FX carried out the experimental work. S-YY and JL contributed to analyzing the data. M-XH, Z-HB and LL helped with data analysis. C-BW and S-HY edited the manuscript. CS and MG edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.711980/full#supplementary-material

Supplementary Figure 1 | Overview of the study design and data filters. **(A)** The experimental flowchart of this study. **(B)** Data complexity of different organs. **(C)** Gene number (left) and UMI number (right) of different amount of BCR chains. **(D)** Number of genes (top) and proportion of mitochondrial gene counts (bottom) in different organs after data filtering. **(E)** Total number of cells in different organs.

Supplementary Figure 2 | Expression of Breg cells genes and identified common Breg cells genes. **(A)** Gene expression of known Breg cells markers in total B cells from five organs. **(B)** Identified common Breg cells genes *Fcrl5*, *Ptpn22*, *Ccdc28b*, *Zbtb20* and *Cd9* expression in total B cells from five organs.

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Intestinal CD11b$^+$ B Cells Ameliorate Colitis by Secreting Immunoglobulin A

Ying Fu[1†], Zhiming Wang[1†], Baichao Yu[1], Yuli Lin[1], Enyu Huang[1,2], Ronghua Liu[1], Chujun Zhao[3], Mingfang Lu[1], Wei Xu[1], Hongchun Liu[4], Yongzhong Liu[5], Luman Wang[1,6†] and Yiwei Chu[1,7*†]*

[1] Department of Immunology, School of Basic Medical Sciences, and Institutes of Biomedical Sciences, Fudan University, Shanghai, China, [2] Department of Pathology, The University of Hong Kong, Hong Kong, Hong Kong, SAR China, [3] Department of Biomedical Engineering, Columbia University, New York, NY, United States, [4] Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai, China, [5] State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University, School of Medicine, Shanghai, China, [6] Department of Endocrinology and Metabolism, Shanghai Fifth People's Hospital, Fudan University, Shanghai, China, [7] Biotherapy Research Center, Fudan University, Shanghai, China

The intestinal mucosal immune environment requires multiple immune cells to maintain homeostasis. Although intestinal B cells are among the most important immune cells, little is known about the mechanism that they employ to regulate immune homeostasis. In this study, we found that CD11b$^+$ B cells significantly accumulated in the gut lamina propria and Peyer's patches in dextran sulfate sodium-induced colitis mouse models and patients with ulcerative colitis. Adoptive transfer of CD11b$^+$ B cells, but not CD11b$^{-/-}$ B cells, effectively ameliorated colitis and exhibited therapeutic effects. Furthermore, CD11b$^+$ B cells were found to produce higher levels of IgA than CD11b$^-$ B cells. CD11b deficiency in B cells dampened IgA production, resulting in the loss of their ability to ameliorate colitis. Mechanistically, CD11b$^+$ B cells expressed abundant TGF-β and TGF-β receptor II, as well as highly activate phosphorylated Smad2/3 signaling pathway, consequently promoting the class switch to IgA. Collectively, our findings demonstrate that CD11b$^+$ B cells are essential intestinal suppressive immune cells and the primary source of intestinal IgA, which plays an indispensable role in maintaining intestinal homeostasis.

Keywords: CD11b$^+$ B cells, IgA, DSS-induced colitis, TGF-β, Smad

Abbreviations: APC, allophycocyanin; AT, adoptive transfer; BAFF,B-cell-activating factor of the tumor-necrosis-factor family; CCR, CC chemokine receptor; CFSE, carboxyfluorescein succinimidyl ester; CFU, colony-forming units; DAI, disease activity index; DAPI, 4', 6-diamidino-2-phenylindole; DSS, dextran sodium sulfate; FITC, fluorescein isothiocyanate; GALT, gut associated lymphoid tissues; IBD, inflammatory bowel disease; IgA, Immunoglobulin A; LP, lamina propria; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes; p-Smad, phosphorylated Smad; PBS, phosphate-buffered saline; PE, phycoerythrin; PP, peyer's patch; rm, recombinant mouse; TGF-β, cytokine transforming growth factor β.

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Mats Bemark, University of Gothenburg, Sweden
Andrey Kruglov, German Rheumatism Research Center (DRFZ), Germany

***Correspondence:**
Yiwei Chu
yiweichu@fudan.edu.cn
Luman Wang
lumanwang@fudan.edu.cn

[†]These authors have contributed equally to this work

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INTRODUCTION

The intestine is a relatively independent regional immune organ, with bacteria, epithelial cells, and immune cells interacting to maintain homeostasis (1). Gut-associated lymphoid tissue (GALT) is an important site for resident immune cells to function as both a regulator and an effector (2). GALT cells, such as dendritic cells, macrophages, T cells, and B cells are widely known to be involved in maintaining a stable state of the intestinal tract (3, 4). Among the substances produced by immune cells, IgA is considered an essential factor in immune hemostasis (5–7). However, no study has reported whether there are specific subsets of B cells that produce substantial IgA in the inflammatory environment and the underlying mechanisms.

CD11b, a protein subunit that forms the integrin, alpha-M beta-2 molecule (Mac-1), with CD18, is usually expressed on myeloid cells such as macrophages and dendritic cells. CD11b has been functionally implicated in leukocyte suppression, migration, and adhesion (8–10). For instance, CD11b activation promotes pro-inflammatory macrophage polarization and functions as a negative regulator of immune suppression as well as a target for cancer immune therapy (11). In lymphocytes, CD11b is expressed on peritoneal cavity B1 B cells with unrevealed functions (12), and also expressed on some age-associated B cells (13, 14). Kunisawa et al. demonstrated that CD11b is essential for IgA^+ plasma cell production, which is dependent on the Peyer's patch (PP) lymphoid structure, IL-10 and MyD88 pathway (15). Our previous work revealed that CD11b is also expressed on classical B2 B cells, and $CD11b^+$ B cells are essential for maintaining immune tolerance in the liver (16). Thus, whether $CD11b^+$ B cells exist in GALT and whether the expression of CD11b in intestinal B cells determines the function of B cells in the maintenance of intestinal homeostasis are important questions that should be addressed.

In this study, we found that $CD11b^+$ B cells accumulated in GALT under intestinal inflammatory conditions in a dextran sulfate sodium (DSS)-induced colitis model and human ulcerative colitis (UC) patients. Importantly, adoptive transfer (AT) of Peyer's patches (PP)-derived wild-type (WT) B cells, but not $Itgam^{-/-}$ (CD11b KO) B cells, inhibited colitis in $Cd79a^{-/-}$ B cell-deficient mice, suggesting that CD11b is indispensable for the regulatory function of GALT B cells. Furthermore, compared to $CD11b^{-/-}$ B cells, $CD11b^+$ B cells produced higher levels of IgA, and exhibited more intense activation of the TGF-β signaling pathways that is associated with IgA class switching, including higher expression of TGF-β receptor and increased phosphorylation of Smad2/3. In summary, the findings of this study demonstrate that, owing to their high activity and sensitivity to the microenvironment, intestinal $CD11b^+$ B cells are the major source of IgA production, with therapeutic potential for intestinal diseases, such as colitis.

MATERIALS AND METHODS

Mice

Wild type (WT) C57BL/6 mice were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). B6.129S4-Itgam^{tm1Myd}/J (*Itgam*$^{-/-}$) mice were kindly provided by Prof. Xiao Su of the Institute Pasteur of the Chinese Academy of Science (Shanghai, China). C57BL/6-Cd79a^{tm1cyagen} (*Cd79a*$^{-/-}$) and C57BL/6-iga^{em1cyagen} (*Iga*$^{-/-}$) mice were obtained from Cyagen Animal Co., Ltd. (Shanghai, China). B6.129P2-Il10tm1Cgn/J (*Il10*$^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The B6.C(Cg)-Cd79a$^{tm1(cre)Reth}$/EhobJ (*Mb-1*^{cre}) mice were kindly provided by Zhongjun Dong of the School of Medicine, Tsinghua University (Beijing, China). The Tgfbr2$^{flox/flox}$ (*Tgfbr2*$^{f/f}$) mice were obtained from the NCI Mouse Repository (NCI-Frederick, NIH, USA) and kept in B6 background. All mice were 6–12 weeks old and were bred and housed in the animal facility at Fudan University (Shanghai, China) under specific pathogen-free barrier conditions (temperature: 18–29°C; humidity: 40%–70%; air velocity: <0.18 m/s; air pressure: 20–50 Pa). All mice were studied following protocols approved by the Ethics Committee of Animal Care and Use, Department of Laboratory Animal Science, Fudan University (Shanghai, China; approval no. 201901002Z). All animal experiments complied with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised in 1978).

DSS-Induced Colitis

The experimental colitis model was established as described previously (17). Briefly, 2.5% DSS (w/v; MP Biomedicals, Santa Ana, CA, USA) was added to the drinking water administered to mice for 7 days; thereafter, the animals were administered normal drinking water. During the study period, the mice were monitored daily to conduct clinical evaluations, such as their body weight, stool consistency, and gross bleeding. Clinical assessment of disease activity was performed using the following validated scoring system: body weight loss (scored as: 0, none; 1, 1%–5%; 2, 6%–10%; 3, 11%–15%; 4, >15%), stool consistency (scored as: 0, normal pellets; 2, loose stools; 4, diarrhea), and fecal blood (scored as: 0, negative hemoccult test; 1, positive hemoccult test; 2, blood visibly present in the stool; 3, blood visibly and blood clotting on the anus; 4, gross bleeding) (18). The disease activity index (DAI) was calculated using the above parameters (19–21).

Tissue Histopathology

On different days after DSS induction, mice were sacrificed, and their colon tissues were removed and fixed in 10% buffered formalin. Paraffin sections (6 μm) of the distal segments of the colon were cut using a manual rotary microtome (Leica RM2235, Wetzlar, Germany) and stained with hematoxylin and eosin. Histological assessment of colon injury was mainly based on the infiltration of inflammatory cells and epithelial damage and was scored from 0 to 4 as follows: 0, normal tissue; 1, inflammation with scattered infiltrating inflammatory cells and no signs of epithelial degeneration; 2, multiple foci and/or mild epithelial ulcerations; 3, severe inflammation with marked wall thickening and/or ulcerations in >30% of the tissue section; and 4, inflammation with transmural inflammatory cell infiltration and loss of the entire crypt and epithelium. The average fields of view per colon were blindly evaluated for each mouse as

described previously (22, 23). The cumulative DSS histopathological scores of individual *Cd79a*$^{-/-}$ mice treated with DSS were calculated by evaluating several tissue pathology parameters.

Flow Cytometry

Mononuclear cells were first incubated with anti-CD16/32 antibody (BD Bioscience, San Jose, CA, USA) and then reacted with the following anti-mouse antibodies: phycoerythrin (PE)-CD11b (M1/70, eBioscience, San Diego, CA, USA), allophycocyanin (APC)-eFluor780-CD11b (M1/70, eBioscience), APC-CD19 (6D5, BioLegend), APC-Cy7-CD19 (1D3, eBioscience), fluorescein isothiocyanate(FITC)-IgA (C10-3, BD Biosciences), Pacific blue-CD45 (30-F11, BioLegend), APC-IL-10 (JES5-16E3, BioLegend), PE-TGFβ receptorII (TGF-βRII, polyclonal, FAB532P, R&D Systems, Minneapolis, MN, USA), Brilliant Violet 421-TGF-β1 (TW7-16B4, BioLegend), PE-IL-6 (MP5-20F3, BioLegend), PE-Smad1 (pS463/pS465)/Smad8 (pS465/pS467) (N6-1233, BD Biosciences), PE-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (O72-670, BD Biosciences), PE-CD95 (APO-1/Fas) (15A7, eBioscience), FITC-CCR9 (CW-1.2, BioLegend), APC-α4β7 (DATK32, BioLegend), APC-CD62L (MEL-14OX85, BioLegend), FITC-CD103 (2E7, BioLegend), PE-CD69 (H1.2F3, eBioscience), APC-eFluor 780-CD44 (IM7, eBioscience), PE-CCR7 (3D12, eBioscience), PE-PD-L2 (TY25, eBioscience), PE-Cyanine7-CD21/35 (7G67E9, BioLegend), eFluor 450-CD23 (B3B4, eBioscience), APC-CD43 (1G10, eBioscience), eFluor 450-IgD (11-26c, eBioscience), PE-Cyanine7-IgM (II/41, eBioscience), Pacific Blue-CD38 (Clone 90, BioLegend), APC-CD45.1 (A20, eBioscience), FITC-CD45.2 (Clone 104, eBioscience), FITC-CD40 (HM40-3, eBioscience), APC-CD86 (GL-1, eBioscience), eFluor 450-CD80 (16-10A1, eBioscience), APC-CD138 (Clone 281-2, BioLegend), FITC-MHC II (M5/114.15.2, eBioscience), Alexa Fluor 488-PNAd (MECA-79, eBioscience), eFluor 660-GL7 (GL7, Biolegend), PE-S1P1 (Clone 713412, R&D System), APC-CCR10 (248918Polyclonal, R&D System), TGF-β (TW7-16B4A), Smad1 (pS463/pS465)/Smad8 (pS465/pS467) (N6-1233), Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (O72-670), PE-Blimp-1 (5E7, eBioscience), eFluor 450-IL-6 (MP5-20F3, eBioscience), and FITC-Ki67 (SolA15, eBioscience). The human antibodies used were eFluor 450-CD19 (HIB19, eBioscience), PE-Cyanine7-CD11b (ICRF44, eBioscience), FITC-IgA (IS11-8E10, BioLegend), PE-TGF-βRII (25508, R&D Systems), PerCP-Cy5.5-TGF-β (TW4-9E7, BD Biosciences), PE-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (O72-670, BD Biosciences), APC-CCR9 (L053E8, BioLegend), and PE-α4β7 (DATK32, eBioscience).

Mononuclear cells were incubated with anti-CD16/32 antibody (eBioscience) and reacted with the Fixable Viability Dye eFluor 506(Invitrogen) and anti-mouse cell surface antibodies. To stain intranuclear molecules, cells were first fixed and permeabilized with the Staining Buffer set (eBioscience) according to the manufacturer's instructions and then stained with anti-mouse antibodies. To stain intracellular cytokines, cells were stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience) for the last 5 h, followed by fixation and permeabilization with Staining Buffer set (eBioscience) according to the manufacturer's instructions. Finally, the cells were stained with anti-mouse antibodies. For phosphorylation detection, PP-derived CD11b$^+$ B cells and *Itgam*$^{-/-}$ B cells were stimulated with lipopolysaccharide (LPS, 10 μg/mL, Sigma-Aldrich, St. Louis, MO, USA), B-cell-activating factor of the tumor-necrosis-factor family (BAFF, 25 ng/mL, R&D Systems), and TGF-β (2 ng/mL, R&D Systems) for 0, 5, 15, and 30 min. Formaldehyde was added at a final concentration of 2%, and surface staining was performed using anti-CD11b and anti-CD19 antibodies. After permeabilization using phosflow perm buffer (BD Bioscience), the cells were resuspended in incubation buffer containing the antibodies against phosphorylated-Smad1/8(p-Smad1/8) and p-Smad2/3 for 30 min at room temperature and washed with additional antibodies for another 30 min. Finally, phosphorylated detection was performed using flow cytometry.

The concentrations of the matched isotype antibodies were used as the negative controls. Flow cytometric analysis was performed using a CyAn ADP Analyzer (Beckman Coulter, Miami, FL, USA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). The absolute number of cell populations was calculated based on their respective percentages using flow cytometry.

B Cell Isolation and AT

B cells (CD19$^+$) from mouse PPs on day 0 were purified using B cell isolation kits from StemCell Technologies (Vancouver, BC, Canada) according to the manufacturer's recommended protocol. CD11b$^+$ B cells and CD11b$^-$ B cells were sorted using a MoFlo flow cytometer (FCM; Beckman Coulter). The purity of the isolated cell population was at least 90%. To investigate the role of CD11b in B cells in colitis, approximately 1×10^7 purified single-cell suspensions derived from WT or *Itgam*$^{-/-}$ mice were injected into each *Cd79a*$^{-/-}$ mouse through the inferior ophthalmic vein 48 h before DSS consumption. The control group received phosphate-buffered saline (PBS). In the assay of the cell-based therapy for colitis, purified CD11b$^+$ B cells (5×10^6 cells per mouse) and CD11b$^-$ B cells were adoptively transferred in the same manner.

Animal Treatment

2.5% DSS (w/v; MP Biomedicals, Irvine, CA, USA) was added to the drinking water given to the mice for 7 days followed by providing normal drinking water.

Isolation of GALT Cells

To isolate mononuclear cells from MLNs and PPs, the cells were carefully removed with fine scissors and ground with RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 2% fetal bovine serum (FBS, GIBCO BRL, Invitrogen, Carlsbad, CA, USA). Mononuclear cells from colorectal lamina propria (LP) were isolated using the method described by Lefrancois and Lycke (24). Briefly, the colon was lavaged with RPMI-1640 to remove fecal matter and debris, opened longitudinally, cut into 1 cm pieces, and stirred twice in RPMI-1640 medium containing 10 mM HEPES, 25 mM $NaHCO_3$, 1 mM DTT, 1 mM EDTA

(all from Sigma-Aldrich, St. Louis, MO, USA), and 2% fetal calf serum for 30 min at 37°C. To isolate LPs, the remaining pieces were digested with RPMI-1640 medium plus 1 mg/mL collagenase (Roche Applied Science, Upper Bavaria, Germany), 40 μl/mL dispase (Sigma-Aldrich), and 4 μl/mL DNase I (Sigma-Aldrich) for 60 min at 37°C. Mononuclear cells of LPs were then purified with discontinuous Percoll (GE Healthcare, Little Chalfont, Buckinghamshire, UK) gradient centrifugation by collecting interface cells between the 40% and 70% layers.

In Vitro Culture

For $CD11b^+$ B cell stimulation, purified PP-derived B cells were stimulated with LPS(10μg/mL, Sigma-Aldrich), BAFF(25ng/mL, R&D Systems), and/or cytokines(all from R&D Systems). For IgA^+ cell stimulation, $CD11b^+$ B cells and $Itgam^{-/-}$ B cells were stimulated with LPS(10μg/mL), BAFF(25ng/mL),and TGF-β (4ng/mL) for 72 h. All purified cells were plated at 5×10^5/mL in RPMI-1640 medium containing 10% FBS. LY2109761 (5 μM, Selleck, Houston, TX, USA) and anti-TGF-β (1D11.16.8) (4 μg/mL, BioXCell, West Lebanon, NH, USA) were added to some groups. Flow cytometry was performed to measure the expression of IgA isotypes in cultured B cells using PE-IgA (mA-6E1, eBioscience).

For human peripheral blood mononuclear cell (PBMC) stimulation, isolated PBMCs were stimulated with CpG (2μg/mL, ODN 2216, InvivoGen), BAFF (25ng/mL, R&D Systems), and TGF-β (2ng/mL, R&D Systems) for 72 h. All cells were plated at 5×10^5/mL in RPMI-1640 medium containing 10% FBS.

Transfection of Small Interfering RNAs

WT splenic B cells were purified using the EasySep™ MouseB Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). Isolated splenic B cells were washed three times and then transfected with TGF-β small interfering RNA (siRNA) (siTGFβ) or negative control siRNA (siNC, 100nM) by electroporation in Opti-MEM medium. The ECM830 Electro Square Wave Porator (Harvard Apparatus BTX, USA) was set to 400 volt and 700 μs electric shock for the suspension B cells. The transfected cells were cultured in corresponding stimulations (LPS 10ug/mL; BAFF 25ng/mL; LY2109761 5 μM; and anti-TGF-β 4 μg/mL). As siRNAs partially enter suspension cells by electroporation, total mRNA was extracted 24 h after the transfection to detect the transfection efficiency. Based on the significant knockdown efficiency, the RNA level of TGF-β was detected using real-time polymerase chain reaction (PCR) and the percentage of IgA^+ B cells was measured using flow cytometry at 72 h, and the production of IgA in the supernatant was determined using enzyme-linked immunosorbent assay (ELISA).

siRNA:
siTGFβ (forward)- 5′-GAAGCGGACUACUAUGCUAdTdT-3′
siTGFβ (reverse)- 5′-UAGCAUAGUAGUCCGCUUCdTdT-3′
siNC (forward)- 5′-UUCUCCGAACGUGUCACGUdTdT-3′
siNC (reverse)- 5′-ACGUGACACGUUCGGAGAAdTdT-3′

real-time PCR primers:
β-actin (forward)-5′-CTACCTCATGAAGATCCTGACC-3′
β-actin (reverse)-5′-CACAGCTTCTCTTTGATGTCAC-3′
TGFβ (forward)-5′-CCAGATCCTGTCCAAACTAAGG-3′
TGFβ (reverse)-5′-CTCTTTAGCATAGTAGTCCGCT-3′

Colony-Forming Units (CFU) Calculation

WT mice and *Iga*$^{-/-}$ mice were provided drinking water with 2.5% DSS (w/v; MP Biomedicals, Santa Ana, CA) for 7 days, followed by normal drinking water. The SPF faculties, sterile food, and water were strictly controlled during this process. Twenty milligrams of feces were collected every hour on day 10. The quantitative feces were diluted with 1 mL of sterile PBS. After the bacterial solution was mixed evenly, the bacterial solution was diluted 10^4-fold, and 50 uL of the diluted bacterial solution was dropped into the solid LB medium and then evenly smeared with a laboratory coating rod. The coated culture dish was inverted and incubated overnight at 37°C for colony counting and CFU calculation.

IgA-Specific Gut Microbiota Detection

In the DSS-induced colitis model, the mice were bled on day 10. Sera were collected for IgA-specific gut microbiota measurements.

1) ELISA plate with 100 μl/well of capture anti-IgA antibody in Coating Buffer. Seal the plate and incubate overnight at 4°C. Aspirate wells and wash twice with 400 μl/well Wash Buffer. 2) Block wells with 250 μl of Blocking Buffer. Incubate at room temperature for 2 hours. Aspirate wells and wash twice with 400 μl/well Wash Buffer. 3) 10^2-fold-diluted sera was added incubate overnight at 4°C. Aspirate wells and wash five times with 400 μl/well Wash Buffer. 4) For fluorescence conjugation, the gut microbiota was incubated for 5 min with carboxyfluorescein succinimidyl ester (CFSE, ab113853, Abcam) and then washed with 10 ml of 1% FBS-PBS. Then, 50 ul CFSE-labeled gut microbiota was incubated in the sera-coated plate for 1h at room temperature in a biosafety cabinet. Aspirate wells and wash five times with 400 μl/well Wash Buffer. to remove the unruptured microbiota. 5) The plate was read at an excitation wavelength of 492 nm (Tecan). We used OD values to represent the abundance of IgA-specific gut microbiota.

Immunofluorescence Analysis

For mouse colon, after deparaffinization, antigen retrieval, and blocking, the paraffin sections of colon tissues were incubated with the primary antibodies rabbit anti-mouse CD19 (ab227019, Abcam) and rat anti-mouse CD11b (ab8878, Abcam) overnight at 4°C. The tissues were then washed with PBS and incubated with the appropriate fluorophore-conjugated secondary antibodies: Alexa Flour-488 (A-21206, Invitrogen) and 647 (A48265TR, Invitrogen) at a dilution of 1:100 in 5% bovine serum albumin (BSA) for 1 h at room temperature. Additionally, 4′, 6-diamidino-2-phenylindole (DAPI) was used as a counterstain. Images were acquired using a spectral confocal microscope (TCS SP5, TCS SP8; Leica, Wetzlar, Germany).

Colon tissues were obtained from six patients. This study was approved by the Medical Ethics Council of Zhongshan Hospital

(approval number: Y2016-197). Informed consent was obtained from all the patients recruited in this study. After deparaffinization, antigen retrieval, and blocking, paraffin sections of colon tissues were incubated with primary antibodies against hCD19, hCD11b, and hIgA (all from Abcam, Cambridge, MA, USA) overnight at 4°C. The tissues were then washed with PBS and incubated with the appropriate fluorophore-conjugated secondary antibodies, Alexa Flour-488, 594, and 633 (all from Invitrogen) at a dilution of 1:200 in 5% BSA for 1 h at room temperature. DAPI was used as a counterstain. Images were acquired using a spectral confocal microscope (TCS SP5, TCS SP8; Leica).

The density was calculated using Image J software (National Institutes of Health), and the procedures were as follows: For a single-channel (monochromatic) fluorescence image, the gray value of each pixel represented the fluorescence intensity at that point. The fluorescence intensity formula for a specific region was average fluorescence intensity (mean) = total fluorescence intensity of the region (IntDen)/Area of the region (Area) (Mean: Mean gray value; IntDen: Integrated Density). 1) After the image was added, single image-color-split channels were extracted. If the image was stored in RGB format, the channel needed to be divided first; if the image was 16-bit or 8-bit, the threshold operation could be performed directly. 2) The threshold was adjusted, and the appropriate area was selected (image-adjust-threshold). The software automatically selected a default value and selected the default value uniformly (to eliminate the error caused by manually selecting the threshold value for different photos, it is better to use the default threshold value). 3) An appropriate threshold algorithm (image-adjust-auto threshold) and threshold results for all algorithms were selected. According to the results, the default algorithm was chosen here. 4) Parameters to be measured (analyze-set measurements) were set, and the mean gray value was checked and limited to threshold (very important), and OK was clicked. If the threshold limit was not checked, the average fluorescence intensity of the entire image was measured. 5) Measure was clicked, and the test result appeared. The measured results, mean, is the average fluorescence intensity (mean gray value, equal to IntDen/Area), IntDen = Integrated Density (sum of fluorescence intensity).

ELISA

To measure IgA levels in colon tissues, feces and mucus were flushed and homogenized with endotoxin-free PBS. The supernatants were harvested after centrifugation (800G, 15 min) and stored at −80°C for later analysis. The cell supernatants of sort-purified B cells that underwent *in vitro* culture were also collected for cytokine and antibody measurements. The concentrations of IgA, IL-10, IL-6, and IgM were measured using the Mouse Ready-SET-Go! ELISA kits (eBioscience) according to the manufacturer's instructions. The levels of active TGF-β were measured using a human TGF-β1 duo-set (DY240) or mouse TGF-β1 duo-set (DY1679) with a substrate reagent pack (DY999) according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK) (25, 26).

Statistical Analysis

The results of multiple groups were compared using a one-way analysis of variance (ANOVA). Thereafter, Bonferroni correction or the Mann-Whitney U test was conducted to compare two individual groups. Statistical analyses were performed using the GraphPad software (La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

RESULTS

CD11b$^+$ B Cells Are Enriched in the Intestine During DSS-Induced Colitis

Our findings indicate that CD11b is upregulated on B cells during experimental autoimmune hepatitis (EAH) and plays an essential regulatory role in the disease (16). In this study, by using DSS-induced colitis, we detected B cells expressing CD11b at different stages in the PP and LP of GALT, peripheral blood, spleen, and MLN (**Figures 1A–F**). As shown in **Figures 1B, C**, during disease processing, the proportion of CD11b$^+$ B cells increased on day 4 in the PP and LPs compared to that in day 0 control mice (day 0). Thereafter, they remained at high percentage levels and were accompanied by a significant increase in absolute cell numbers. However, there were no changes in CD11b$^+$ B cell frequencies in the spleen or blood cells (**Figures 1D, E**). Moreover, our analysis also confirmed that the proportion of GALT CD11b$^+$ B cells in total lymphocytes also increased (**Supplementary Figures S1A, B**), indicating that CD11b$^+$ B cells were enriched in the GALT, specifically in the colitis stage. Immunofluorescence staining confirmed that CD11b$^+$ B cells were mostly present in the intestinal PP and LP, especially under inflammatory conditions (**Figure 1G**).

Classically, CD11b is a hallmark of peritoneal cavity (PerC) B1 cells, which migrate to the intestine and maintain homeostasis (27, 28). Therefore, we compared intestinal (PP and LP) CD11b$^+$ B cells with classic PerC B1 cells. We first compared the surface markers of PerC B1 cells and intestinal CD11b$^+$ B cells before and after the induction of colitis. As shown in **Supplementary Figure S2A**, intestinal CD11b$^+$ B cells exhibited higher levels of CD21, CD23, IgD, and IgA, as well as lower levels of IgM and CD5 on both day 0 and day 4, expressing an opposite phenotype from that of PerC B1 cells. To investigate whether the intestinal CD11b$^+$ B cells are the PerC B1 B cells migrating to PP, PerC B1 B cells were isolated from CD45.1 mice and intraperitoneally injected into CD45.2 WT mice. An additional group of recipient mice was administered FTY720 to block the migration of injected PerC B1 B cells from the peritoneal cavity as a negative control (**Supplementary Figure S2B**). As shown in **Supplementary Figure S2C**, 4 days after cell transfer, peritoneal cells showed increased expression of S1P1. However, there was no migration of CD45.1 CD11b$^+$ B cells to the PP or LP (**Supplementary Figure S2D**), strongly supporting that intestinal CD11b$^+$B cells were not PerC B1 B cells. We then examined whether CD11b$^+$B cells in LP and PP from day 0 to day 10 were the characteristic of memory B cells, plasmablasts,

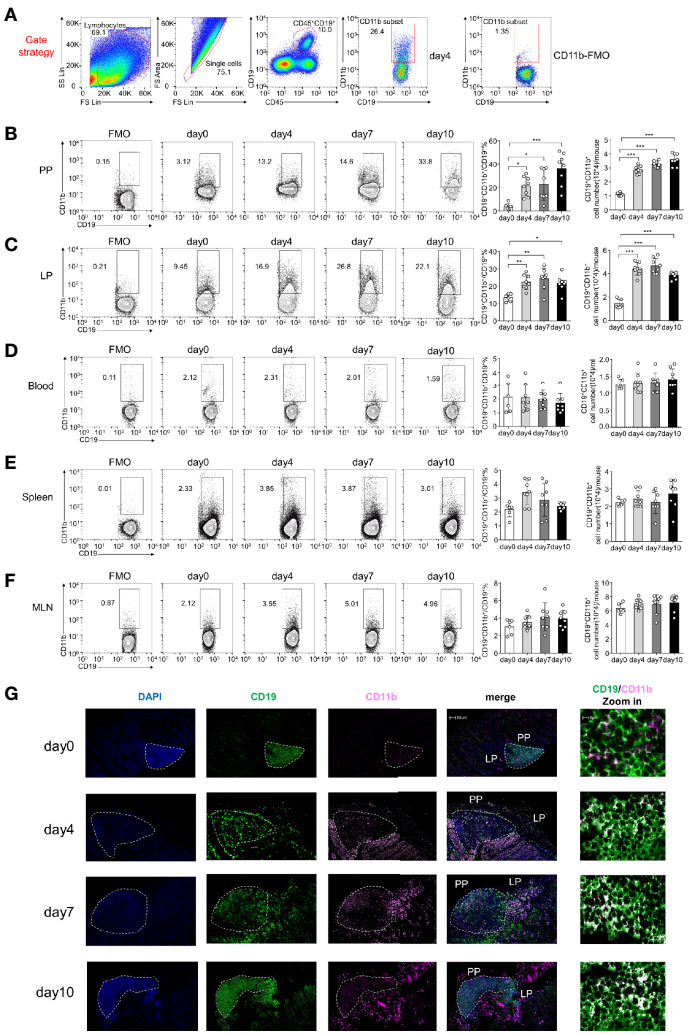

FIGURE 1 | CD11b is induced in Peyer's patches (PPs) and colorectal lamina propria (LP) B cells during dextran sulfate sodium (DSS)-induced colitis. Flow cytometry was used to analyze the frequency and absolute number of $CD11b^+$ B cells. **(A)** Gating strategy for the identification of $CD19^+CD11b^+$ cells in gut-associated lymphoid tissue (GALT). Representative data from the LP of colitis mice on day 4 after DSS induction. Different fluorochrome-labeled isotype control antibodies or anti-CD45, -CD19, and -CD11b antibodies were used for staining. Numbers indicate the percentage of positive cells. The right panel represents CD11b fluorescence minus one (FMO) staining. Cells were isolated from the PPs **(B)**, colorectal LP **(C)**, total blood cell **(D)**, spleen **(E)**, and MLN **(F)** of DSS-induced WT mice on days 0, 4, 7, and 10. The absolute numbers were counting as follow: for PPs, colorectal LP, spleen, and MLN, the number of cells in that organ per mouse times the ratio of $CD11b^+$B cells to obtain absolute count/mouse; for blood cell, 200ul blood cells were counted after red blood cell lysis, the number of 200ul blood cells times five and times the ratio of $CD11b^+$B cells to obtain absolute count/ml. **(G)** Immunofluorescence of $CD11b^+CD19^+$ B cells in PPs and LPs. *P < 0.05, **P < 0.01, ***P < 0.001. Data are expressed as the mean ± SEM of one experiment with six to eight mice, performed in triplicate with similar results.

and germinal center (GC) B cells. We found that the frequency of both PP and LP $CD11b^+$ B cells increased $CD38^{lo}PNA^{hi}$ (GC marker) expression on days 4, 7, and 10, while no changes were observed in the frequency of LP $CD11b^-$ B cells (**Supplementary Figure S2E**). In addition, $CD11b^+$ B cells expressed CD138 and Blimp from day 0 to day 10, and in inflammatory conditions, the frequency of $CD138^+Blimp^+$ plasmablasts increased in colitis (**Supplementary Figure S2F**). We also tested memory B cell markers, CD80 and PD-L2, and found that the levels of both markers were very low on $CD11b^+$ B cells (**Supplementary Figure S2G**), indicating that $CD11b^+$ B cells are not memory B cells. Taken together, these data suggest that $CD11b^+$ B cells exist in all stages of B cell activation and could be expanded in PPs and colorectal LP during colitis and are highly unlikely to be conventional B-1 cells.

Intestinal $CD11b^+$ B Cells Protect Mouse From DSS-Induced Colitis

To determine whether $CD11b^+$ B cells have a protective function in colitis, 1×10^7 $CD19^+$ B cells from the PP of day 0 WT ($Itgam^{+/+}$) mice and CD11b gene-deficient ($Itgam^{-/-}$) mice were purified, and AT was performed in B cell-deficient mice ($Cd79a^{-/-}$) 2 days prior

to the administration of DSS (**Figure 2A**). Compared to control mice (*Cd79a*$^{-/-}$ mice that received PBS), *Cd79a*$^{-/-}$ mice that received B cells from *Itgam*$^{+/+}$ mice exhibited less severe colitis. *Cd79a*$^{-/-}$ mice that received *Itgam*$^{-/-}$ B cells developed severe colitis similar to that in control mice, as measured by body weight loss, DAI score, and histological score (**Figures 2B–D**). *Cd79a*$^{-/-}$ mice that received *Itgam*$^{+/+}$ B cells lost <10% of their body weight on day 9 (the peak of disease progression) and did not experience diarrhea or gross bleeding after day 8. In contrast, *Cd79a*$^{-/-}$ mice that received *Itgam*$^{-/-}$ B cells exhibited persistent disease progression with nearly 20% body weight loss (**Figures 2B, C**). Histologically, control mice exhibited the most severe inflammation throughout the entire colon, with massive loss of crypts and leukocyte infiltration observed on days 7 and 10 (**Figure 2D**). *Cd79a*$^{-/-}$ mice that received *Itgam*$^{-/-}$ B cells displayed a slight improvement. Comparatively, *Cd79a*$^{-/-}$ mice that received WT B cells had an intact colon epithelium and markedly less leukocyte infiltration on days 7 and 10 (**Figure 2D**). These results indicate that the expression of CD11b in activated intestinal B cells is essential for their protective role in this colitis model. Further, we investigated whether intestinal CD11b^{+} B cells could be used to treat colitis. CD11b^{+} and CD11b^{-} B cells were sorted from PP, and an equal number of cells were added to WT mice 3 days after induction of DSS (**Figure 2E**). On day 7, mice that received CD11b^{+} B cells exhibited milder symptoms in the colon than mice that received CD11b^{-} B cells. Similar to mice that received PBS, mice that received CD11b^{-} B cells experienced body weight loss and diarrhea from day 3 (**Figure 2F**) and day 4 (**Figure 2G**), respectively. However, mice that received CD11b^{+} B cells exhibited delayed body weight loss and pathological changes related to colitis (**Figures 2F–H**). These data strongly suggest that intestinal CD11b^{+} B cells alleviate DSS-induced colitis and exhibit therapeutic effects.

Intestinal CD11b^{+} B Cells Are the Main Source of IgA^{+} Cells

IL-10 produced by B cells has been demonstrated to play an important role in the regulation of diseases (29). Thus, the role of IL-10 in CD11b^{+} B cells was investigated. As shown in **Supplementary Figures S3A-C**, the transfer of *Il-10*$^{-/-}$ CD11b^{+} B cells inhibited colitis, suggesting that IL-10 is not essential for CD11b^{+} B cell regulatory function. CD11b has been reported to affect the secretion of IgA in the lymphoid structure of PP (15). Herein, we used IgA-deficient (*Iga*$^{-/-}$) mice to induce colitis and found that *Iga*$^{-/-}$ mice exhibited significantly more severe colitis symptoms than WT mice (**Figures 3A–C**). IgA-deficient (*Iga*$^{-/-}$) mice developed more severe colitis than WT mice, as measured by body weight loss, DAI score, and histological score (**Figures 3A–C**). Consistently, *Iga*$^{-/-}$ mice had significantly higher numbers of bacteria in the PP and LP (**Figure 3D**), with lower bacterial-

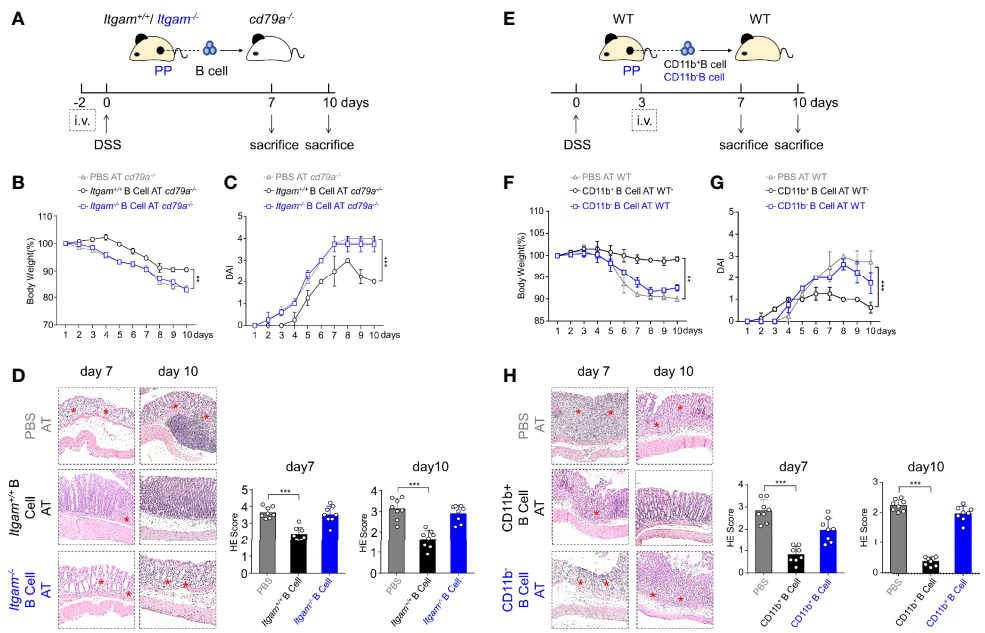

FIGURE 2 | CD11b-deficient B cells exhibit impaired ability to resolve colitis. **(A)** Each *Cd79a*$^{-/-}$ mouse intravenously received 1 × 10^{7} PP-derived B cells from WT mice (*Itgam*$^{+/+}$) or *Itgam*$^{-/-}$ mice 2 days before the induction of colitis. Mice were evaluated daily, and weight loss **(B)** and disease activity index (DAI) scores **(C)** were recorded. **(D)** Representative distal colon histological sections of *Cd79a*$^{-/-}$ mice were stained with hematoxylin and eosin (H&E), and the histological score was calculated. Images are displayed at the original magnification of ×100. Red stars indicate the position of the inflammatory cell infiltration and/or epithelial damage. **(E)** PP-derived B cells of WT mice were sorted and stimulated with lipopolysaccharide (LPS) for 48 h *in vitro*. CD11b^{+} and CD11b^{-} B cells were subsequently purified and intravenously injected (5 × 10^{6} cells per mice) into WT mice treated with DSS for 3 days. Weight loss **(F)** and the DAI scores **(G)** of recipient mice were measured and evaluated from day 0 to day 7. **(H)** H&E-stained histological sections of distal colon tissue are presented. Images are displayed at the original magnification of ×100. Red stars indicate the position of the histological injury. **P < 0.01, ***P < 0.001. Data are expressed as mean ± SEM of eight mice.

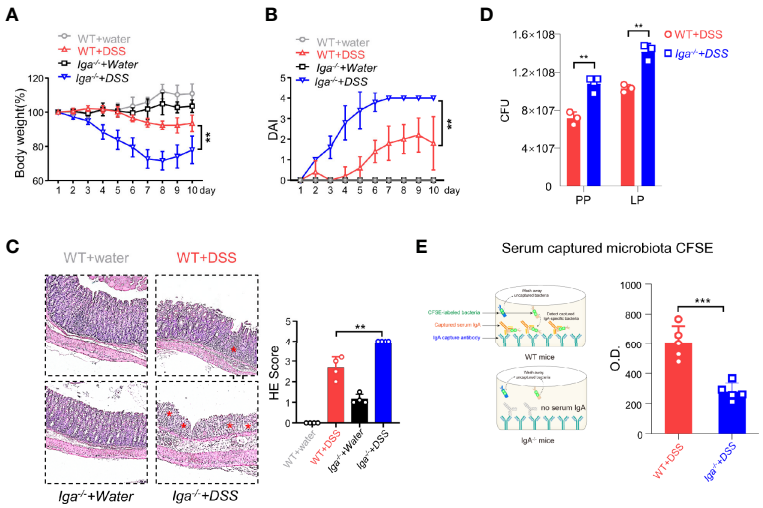

FIGURE 3 | IgA in DSS-induced colitis. **(A)** *Iga*$^{-/-}$ mice and WT mice were administered DSS or regular water (water). Mice were evaluated daily, and weight loss and disease activity index (DAI) scores **(B)** were recorded. **(C)** Representative distal colon histological sections of *Iga*$^{-/-}$ mice were stained with hematoxylin and eosin (H&E), and the histological score was calculated. Images are presented at the original magnification of ×100. Red stars indicate the position of the inflammatory cell infiltration and/or epithelial damage. **(D)** The colony-forming units (CFU) counts of the bacteria in the feces of WT mice or *Iga*$^{-/-}$ mice on day 7 of colitis. **(E)** The O.D. values which indicated bacteria captured by the serum antibodies of WT mice or the *Iga*$^{-/-}$ mice on day 7 after DSS intervention were measured. **P < 0.01, ***P < 0.001. Data are expressed as mean ± SEM of three mice.

binding ability of antibodies in the blood (**Figure 3E**). Thus, IgA, a critical regulatory factor in maintaining gut homeostasis (30), was hypothesized to play a crucial role in CD11b$^+$ B cell-mediated inhibition of colitis. As shown in **Figure 4**, CD11b$^+$ B cells were gated to detect the expression of IgA in this subset, and this expression was compared to that measured in CD11b$^-$ B cells in PPs and the LP. In contrast to CD11b$^-$ B cells, CD11b$^+$ B cells expressed more IgA during colitis, and the number of IgA$^+$ cells was significantly increased in the colitis stage. However, increased numbers of IgA$^+$ cells were not observed in CD11b$^-$ B cells (**Figure 4A**). To confirm these results, we used *Iga*$^{-/-}$ mice as a negative control (**Supplementary Figure S4A**, PP day 10). Similar results were obtained from the transfer experiments with *Itgam*$^{+/+}$ B cells (**Figures 4B, C**) and isolated CD11b$^+$ B cells (**Figures 4D, E**). Higher numbers of IgA$^+$ B cells and levels of sIgA were observed in *Cd79a*$^{-/-}$ mice that received *Itgam*$^{+/+}$ B cells as well as WT mice that received CD11b$^+$ B cells on days 7 and 10 after treatment with DSS, whereas *Itgam*$^{-/-}$ or CD11b$^-$ B cell transfer did not increase the number of IgA$^+$ B cells and sIgA expression (**Figures 4B–E**). In addition, the t-distributed stochastic neighbor embedding (tSNE) clustering visualization diagram also showed that IgA cells were present in CD11b$^+$ B cells, indicating that the production of IgA required CD11b (**Supplementary Figure S4B**). Thus, CD11b$^+$ B cells are the main source of IgA$^+$ cells and represent the vast majority of IgA$^+$ B cells.

Activated TGF-β/p-Smad Signaling Is Mainly Activated in Intestinal CD11b$^+$ B Cells

The mechanisms underlying the ability of CD11b$^+$ B cells to differentiate into IgA$^+$ cells and produce IgA were investigated. PP B cells derived from either *Itgam*$^{+/+}$or *Itgam*$^{-/-}$ mice were cultured in the presence of LPS, BAFF, and TGF-β, all of which are required for class switching to IgA$^+$ B cells. *Itgam*$^{+/+}$ B cells expressed IgA after stimulation compared to *Itgam*$^{-/-}$ B cells (**Figures 5A–C**), indicating that CD11b deficiency impaired the ability to produce IgA. However, the differentiation of IgM$^+$ B cells and the production of sIgM were not affected (**Supplementary Figures S5A, B**). This indicates that CD11b deficiency selectively affected the class switching of IgA$^+$ B cells but not the secretion of antibodies. Furthermore, the percentage of IgA$^+$ cells and the level of sIgA among *Itgam*$^{+/+}$ B cells were significantly increased, even in the absence of TGF-β stimulation, compared with those of the unstimulated cells (**Figures 5A–C**). To investigate whether CD11b$^+$ B cells could produce TGF-β themselves and thus trigger IgA production through an autocrine TGF-β-dependent loop, TGF-β production was measured in *Itgam*$^{-/-}$ PP B cells and WT CD11b$^+$ PP B cells after stimulation with LPS. Almost none of the B cells derived from CD11b-deficient mice were TGF-β-positive, whereas >40% of TGF-β$^+$ cells were detected among CD11b$^+$ B cells derived from day-7 mice after the induction of colitis (**Figures 5D–F**). Furthermore, a significantly higher level of TGF-β was detected in intestinal CD11b$^+$ B cells in day-7 mice with colitis (**Figure 5G**). In addition, **Figure 5G** shows increased TGF-β production in CD11b$^+$ B cells. At the same time, there were no elevated frequencies of CD19$^+$TGF-β$^+$ B cells during colitis (compared to that in day 0). These findings demonstrate that CD11b$^+$ B cells secrete TGF-β to promote the IgA class switch in response to intestinal inflammation in physiological and pathological states. To confirm these results, TGF-β siRNA(siTGF) and TGF-β/TGF-β receptor

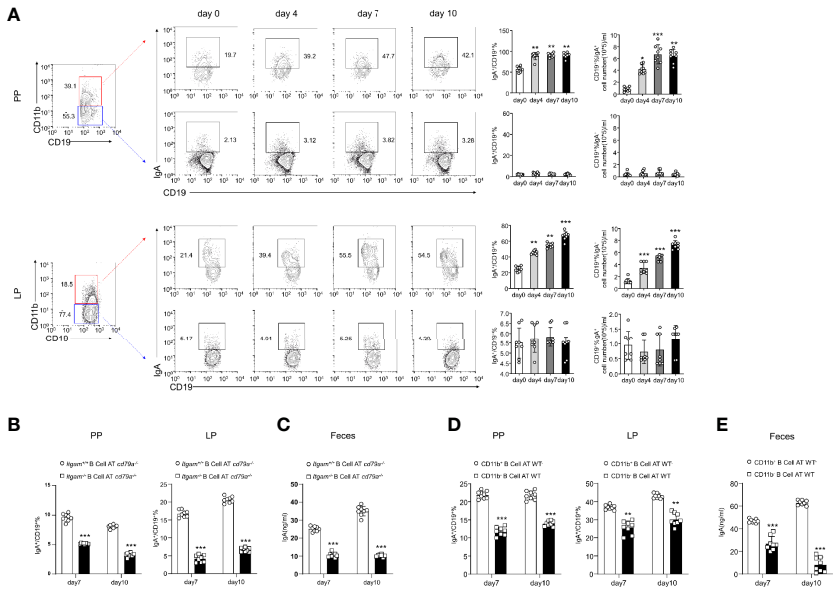

FIGURE 4 | B cells expressing CD11b contribute to the differentiation of IgA in mice with colitis. **(A)** Expression levels of IgA in $CD11b^+$ B cells and $CD11b^-$ B cells in the PPs and colorectal LP at days 0, 4, 7, and 10 after treatment with DSS were detected using flow cytometry. The frequency and absolute number of IgA-expressing cells are presented. **(B)** The adoptively transferred *Itgam*$^{+/+}$ or *Itgam*$^{-/-}$ mice into *Cd79a*$^{-/-}$ mice were as described in **Figure 1**. The percentage and absolute number of IgA^+ B cells in the LP and PPs from DSS-treated *Cd79a*$^{-/-}$ mice were analyzed using flow cytometry. **(C)** The supernatant of the mucus-containing fluid was harvested and transferred into 10 mL phosphate-buffered saline (PBS), and the production of sIgA in the colon was determined using enzyme-linked immunosorbent assay (ELISA). **(D)** The adoptively transferred $CD11b^+$ and $CD11b^-$ B cells into WT mice were as described in **Figure 1**. **(E)** The supernatant of the mucus-containing fluid was harvested and transferred into 10 mL PBS, and the production of sIgA in the colon was determined using ELISA. $^{**}P < 0.01$; $^{***}P < 0.001$. Data are expressed as mean ± SEM of six mice.

agonists were used to inhibit TGF-β/TGF-β receptor expression. Inhibition of the TGF-β/TGF-β receptor expression significantly decreased the expression of IgA in B cells (**Figure 5H** and **Supplementary Figure S5C**). Collectively, these results demonstrate that autocrine TGF-β is a possible mechanism involved in the enhanced ability of $CD11b^+$ B cells to produce IgA.

The TGF-β-Smad signaling pathway has been implicated as a vital regulator of IgA class switching in activated B cells (31). Therefore, we sought to determine whether a deficiency in CD11b impaired the activation and function of the TGF-β-Smad pathway. PP-derived $CD11b^+$ B cells were treated with LPS, BAFF, TGF-β, or anti-TGF-β (αTGF), and the expression levels of the TGF-β receptor and p-Smad were detected at 0, 30, and 120 min after stimulation. In WT $CD11b^+$ B cells, the level of TGF-βRII expression was significantly increased at 30–120 min, p-Smad2/3 was phosphorylated at 120 min, and the expression of the co-factor Smad4 was increased at 120 min. However, the expression of Smad1/5/8 remained largely unchanged (**Figures 5I, J**). The expression of TGF-βRII, Smad4, and p-Smad2/3 was blocked when the anti-TGF-β antibody was used or B cells were obtained from *Itgam*$^{-/-}$ mice (**Figures 5I, J**). These results suggest that the expression of CD11b in B cells plays a positive role in regulating TGF-β signaling pathways, which are important for IgA class switching.

$CD11b^+IgA^+$ B Cells Accumulate in the Human Intestine in UC and Are Associated With the Disease

We obtained intestinal sections from six UC patients and stained them with DAPI and CD19, CD11b, and IgA antibodies for immunofluorescence analysis. The inflammatory site was found to be heavily infiltrated with $CD19^+CD11b^+IgA^+$ cells (**Figure 6A**, upper panel), and the area densities of CD19, CD11b, and IgA were significantly higher at the inflammation site (**Figure 6A**, right panel). In addition, the area density of CD11b was positively correlated with that of IgA (**Figure 6B**). These results indicate that $CD11b^+IgA^+$ B cells exist in the intestinal tract of UC patients to maintain homeostasis. Next, we used CCR9 and α4β7 to screen for intestinal circulation-derived cells, sorted cells with high expression levels of CCR9 and α4β7 from the peripheral blood of healthy donors and induced CD11b and IgA expression *in vitro* (**Supplementary Figure S6**). CD11b expression was upregulated in human B cells at 72 h after CpG stimulation (**Figure 6C**). In addition, IgA and TGF-β expression was upregulated in $CD11b^+$ B cells after 72 h of stimulation compared to that in $CD11b^-$ B cells (**Figures 6D, E**). Furthermore, the number of IgA^+ B cells and sIgA levels increased after 72 h of stimulation (**Figure 6F**). Notably, $CD11b^+$ B cells expressed more IgA than $CD11b^-$ B cells, and the percentage of $CD19^+CD11b^+$ B cells was positively correlated with sIgA levels (**Figure 6F**). These data suggest that,

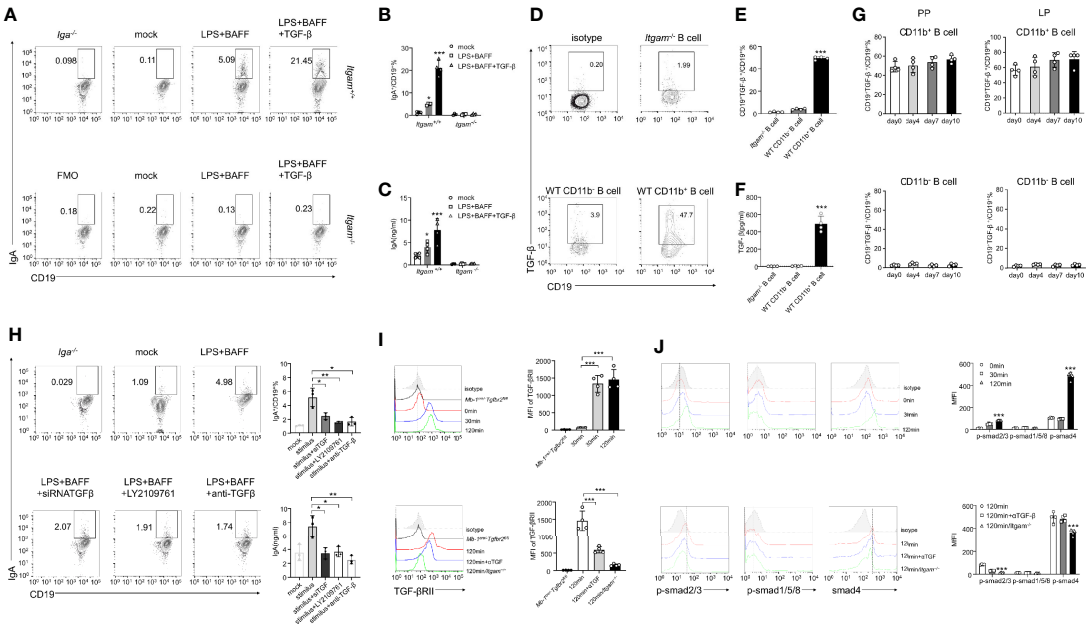

FIGURE 5 | CD11b-deficient altering of Smad signaling in B cells resulted in reduced TGF-β production and IgA isotype switching. **(A)** Purified PP-derived *Itgam*$^{+/+}$ and *Itgam*$^{-/-}$ B cells of WT mice or Iga$^{-/-}$ mice were stimulated with LPS, BAFF, and TGF-β for 72 h Subsequently, IgA^{+} B cells were analyzed using flow cytometry (The *Iga*$^{-/-}$ group was shown as a negative control). **(B)** The frequency of IgA-expressing cells is displayed. **(C)** The culture supernatant was harvested to determine the production of IgA *via* ELISA. **(D)** The population of TGF-β^{+} B cells in the PPs from DSS-treated mice was analyzed using flow cytometry. **(E)** The frequency of TGF-β-expressing cells is displayed. **(F)** Expression of TGF-β in mouse serum was measured using ELISA. **(G)** Expression of TGF-β in CD11b^{+} B cells and CD11b^{-} B cells in the PPs and colorectal LP at days 0, 4, 7, and 10 after treatment with DSS was detected using flow cytometry. The frequency and absolute number of IgA^{+} cells are presented. **(H)** The splenic B cells of mice were purified and transfected with small interfering RNA targeted for TGF-β (siTGF, other groups were transfected with the negative control siRNA for contrast). All groups were subsequently stimulated by LPS, BAFF, and TGF-β for 72 h *in vitro*. LY2109761 (5 μM) and anti-TGF-β (4 μg/mL) were used to inhibit TGF-β or TGF-β receptor, and the percentage of IgA^{+} B cells was measured using flow cytometry, and the IgA level in culture supernatant was measured using ELISA. The splenic B cells from the PP of WT mice, *Mb-1*$^{cre/-}$*Tgfbr2*$^{fl/fl}$, and *Itgam*$^{-/-}$ mice were purified and subsequently stimulated by LPS, TGF-β, or anti-TGF-β (αTGF, 4 μg/mL) for 0, 30, and 120 min. Expression of TGF-βRII **(I)** and the phosphorylation levels of Smad1/5/8 and Smad2/3 **(J)** were analyzed using flow cytometry. *P < 0.05; **P < 0.01; ***P < 0.001. Data are expressed as the mean of three independent experiments.

similar to murine cells, CD11b and IgA expression was upregulated in B cells after stimulation. Furthermore, the TGF-β/TGF-βR/p-Smad signaling pathway was also investigated. As shown in **Figures 6E, G**, a significantly higher level of TGF-β in cultured CD11b^{+} B cells was detected *in vitro* than that in CD11b^{-} B cells. Moreover, the percentage of CD19^{+}CD11b^{+} B cells was positively correlated with the level of secreted TGF-β. The expression levels of TGF-βRII and p-Smad2/3 were significantly increased in CD11b^{+} B cells at 30 min and 120 min (**Figures 6H, I**), indicating that the expression of CD11b in human B cells plays a positive role in regulating TGF-β signaling pathways. Altogether, we demonstrated that GALT CD11b^{+} B cells proliferated under inflammatory conditions, inhibiting intestinal inflammation by secreting sIgA through the TGF-β/p-Smad2/3 signaling axis (**Supplementary Figure S7**).

DISCUSSION

CD11b and other members of the β2 integrin family are usually expressed on myeloid cells and play important roles in provoking or suppressing the inflammatory effects by regulating immune cell migration, interaction, and signal transduction (32). In this study, the DSS-induced colitis model was used to further explore the multiple functions of CD11b^{+} B cells in diseases. To further investigate these functions, the increase in the number of CD11b^{+} B cells in the blood, spleen, and GALT during colitis was first documented. CD11b^{+} on B cells in the LP and PP was highly expressed, consistent with the results of previous studies (33, 34). Furthermore, there was no significant increase in the number of CD11b^{+} B cells in peripheral immune organs, indicating that CD11b^{+} B cells were likely cells originating from the intestine in colitis. Many CD11b^{+} B cells were also recognized in the PP and LP on day 0, possibly because the GALT was constantly in contact with microbiota and foods and was always in a state of physiological inflammation; thus, CD11b^{+} B cells were required to maintain homeostasis.

Traditionally, CD11b is expressed on mouse B-1 cells, which are a small population of B cells preferentially located in the peritoneal cavity and pleural cavity and are rare in lymph nodes (35, 36). The majority of B1 cells have relatively constant phenotypes (CD5 or CD11b) and are normally produced during fetal/neonatal development (37). It has been reported that B1-b B cells can migrate into the colorectal LP and switch

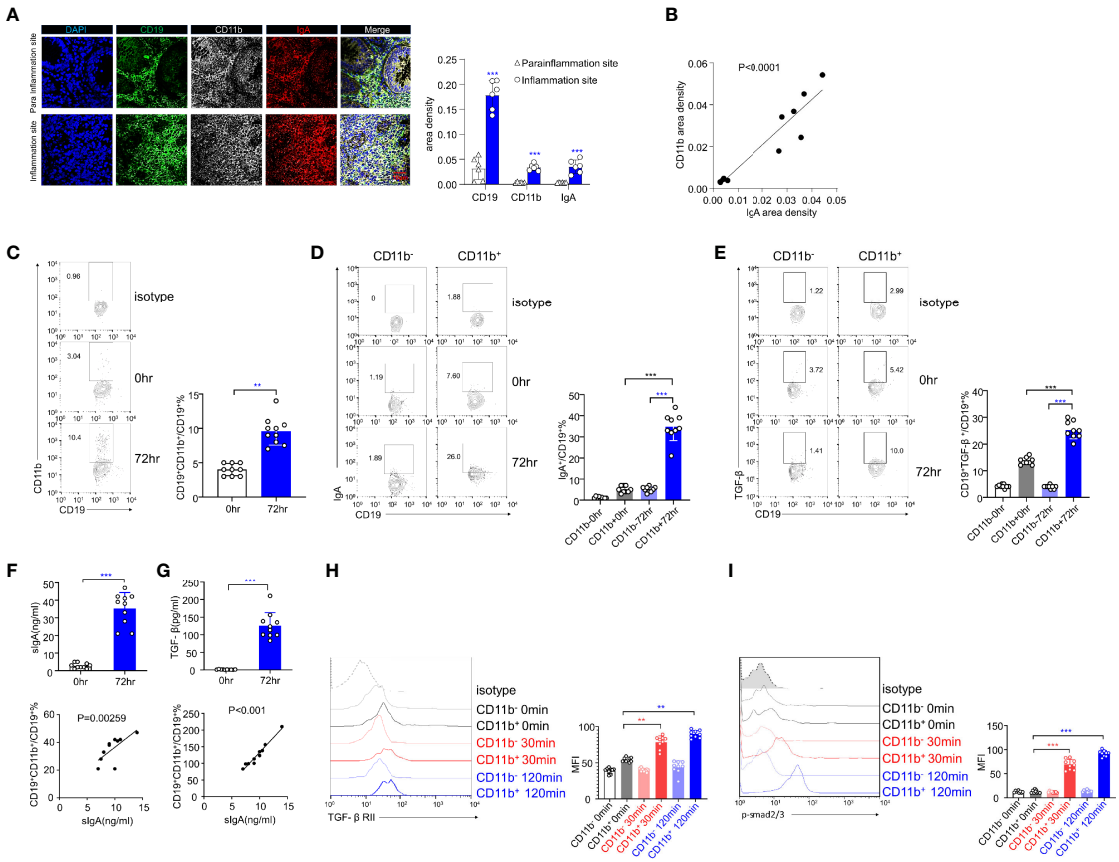

FIGURE 6 | The expression levels of IgA and TGF-β/Smad pathway are upregulated in human CD11b$^+$ B cells *in vitro*. **(A)** Representative staining of CD19 (green), CD11b (white), IgA (red), and 4′, 6-diamidino-2-phenylindole (DAPI) (blue) in the colon tissue of ulcerative colitis (UC) patients. The area density of each marker was calculated. Average data were collected from 20 random fields per patient, n = 6. **(B)** The relative area density of CD11b and IgA was identified. **(C)** Total PBMCs were purified and stimulated with CpG-ODN (2 μg/mL) and recombinant human IL-10 for 72 h *in vitro*. Cells were stained for CD11b detection and analyzed using flow cytometry. **(D)** The percentage of IgA$^+$ B cells in CD11b$^+$ and CD11b$^-$ B cells was analyzed using flow cytometry. **(E)** The percentage of TGF-β$^+$ B cells in CD11b$^+$ and CD11b$^-$ B cells was analyzed using flow cytometry. **(F)** The culture supernatant was harvested to determine the production of IgA (upper panel) *via* ELISA. The relative frequencies of CD19$^+$CD11b$^+$ cells and sIgA level were measured (lower panel). **(G)** The culture supernatant was harvested to determine the production of TGF-β (upper panel) *via* ELISA. The relative frequencies of CD19$^+$CD11b$^+$ cells and TGF-β level were measured (lower panel). The PBMCs from healthy donors were purified and subsequently stimulated by CpG and TGF-β for 0, 30, and 120 min. Expression of TGF-βRII **(H)** and the phosphorylation level of Smad2/3 **(I)** were analyzed using flow cytometry. **P < 0.01; ***P < 0.001. Data represent the mean of more than three independent experiments.

into IgA$^+$ B cells (38, 39). To compare CD11b$^+$ B cells and B-1 B cells, CD19$^+$ cells from the peritoneal cavity, colorectal LP, and PPs of WT mice were purified by FACS and sorted into CD5$^+$ CD11b$^+$ (B-1a), CD5$^-$ CD11b$^+$/CD11b^{hi} (B-1b), and CD5$^+$ CD11b$^-$ (B-1c) cells. After colitis induction, we were able to detect significantly increased numbers of CD5$^-$ CD11b$^+$ B cells, which showed marked proliferation (Ki67$^+$ cells) in colorectal LP and PPs but not in the peritoneal cavity (data not shown). This distinction is important because B1 B cells do not actively proliferate, whereas B2 B cells have the ability to vigorously proliferate in GCs (40). In addition, the surface markers of induced CD11b$^+$ B cells and B1 cells differed significantly. In particular, the PP CD11b$^+$ B cells had exactly the same phenotype as B-2 cells with high expression of CD23 and low expression of CD5/IgM, while colorectal LP CD11b$^+$ B cells are still under debate. It is not clear whether the induced GALT CD11b$^+$ B cells are devoid of B1 cells, but it is highly probable that GALT CD11b$^+$ B cells are B-2 cells that can be induced in response to the inflammatory microenvironment and constitute a new fraction of the intestinal B cell subset. At the same time, we also did BCR diversity analysis to confirm our conclusion (data not shown). We aimed to identify different markers to distinguish CD11b$^+$ B cells from B1 B cells. As we believe that GALT CD11b$^+$ B cells tend to belong to B-2 cells, the stage of B-2 cells needs to be clarified. We detected PP and LP B cells from day 0 to day 10 and divided them into the GC phase to reveal the process and memory B cells or plasmablasts to reveal the outcome. It was found that CD11b$^+$ cells belonged to GC B cells and plasmablasts, which was consistent with their high proliferation characteristics demonstrated previously and also consistent with the results of our previous study (16). Considering that the mucosal immune system was in a state of

chronic inflammation, PPs, as the main site of B cell differentiation, were in a state of activation for the delivery of IgA. Thus, our data showed that PP $CD11b^+$ cells were $CD38^+PNA^+$ and $CD138^+Blimp^+$ on day 0. Microorganisms occupy nearly 50% of the body, and the presence and imbalance of intestinal flora are the key factors inducing many diseases, especially colitis. They have a direct or indirect impact on the initial differentiation of B cells. The components of bacteria, such as LPS and flagellin, are ubiquitous in the intestinal microenvironment. We found that CD11b was highly expressed to some extent after LPS stimulation, but there was no such change after anti-BCR stimulation (data not shown). TLRs on the surface of B cells are involved in the differentiation process of B cells in GC and induce the expression of AID, and it has been reported that LPS mediates long-term primary class-switched antibody responses and memory-like antibody responses *in vivo* (41, 42).

The AT system was subsequently used to verify the role of $CD11b^+$ B cells, demonstrating that *Itgam*$^{-/-}$ B cells could not resolve colitis. To exclude the influence of microbiota, mice from different groups were housed for over one month. As the results were consistent with those of previous studies, CD11b is further indicated to be indispensable for the suppressive role of B cells in immune responses (43, 44). Currently, there are numerous therapeutic strategies for inflammatory bowel disease (IBD) patients. Novel agents that target proinflammatory cytokines (i.e., tumor necrosis factor α, IL-12, and IL-23) are currently undergoing clinical investigation (45). However, these anti-cytokine treatments appear to be effective only in certain subgroups of patients (46, 47). Thus, LPS-induced $CD11b^+$ B cells have been used as therapeutic agents against colitis. AT of these $CD11b^+$ B cells in mice was performed during the early stage of DSS treatment, following the manifestation of symptoms (e.g., diarrhea). Previously, we demonstrated that $CD11b^-$ B cells can be induced to form $CD11b^+$ B cells; thus, they also possess a certain ability to alleviate the disease. However, in the present study, $CD11b^-$ cells were isolated after 48 h of *in vitro* culture. As a result, their ability to transform into $CD11b^+$ B cells was limited, thereby negatively affecting their ability to alleviate the disease. In conclusion, this transferred cell-based therapy in a colitis mouse model may be a potential clinical therapeutic approach for Crohn's disease and UC patients.

Growing evidence indicates that IgA plays an important role in preventing potentially invasive commensal bacteria and neutralizing microbial toxins in the gut (48, 49). IgA deficiency in humans has been associated with chronic intestinal inflammatory disorders (50). However, the exact subset of B cells that possess this IgA-related suppressive function remains debatable. Macpherson et al. reported that a large amount of IgA in the intestinal LP is derived from peritoneal B cells in mice (51), while Thurnheer et al. demonstrated that the upregulation of intestinal IgA level induced by commensal bacteria is mainly mediated by conventional B2 cells and not B1 cells (52). Kunisawa et al. demonstrated that the function of $CD11b^+$ B cells is related to the classic mucosal antibody IgA. They reported that $CD11b^+IgA^+$ plasma cells, unlike $CD11b^-IgA^+$ cells, are the major source of IgA, and the authors did not elaborate on the mechanism (15). In our study, the expression of IgA correlated with the expression of CD11b in B cells in the LP and PPs of mice with colitis. Naïve B cells were also found to be initially induced to form $CD11b^+$ B cells. Thereafter, $CD11b^+$ B cells were differentiated into IgA^+ plasmablasts, referred to as $CD11b^+IgA^+$ plasmablasts, in both the PPs and LP. However, $CD11b^-$ and *Itgam*$^{-/-}$ B cells did not possess this property of IgA class switching. In the *in vitro* assay performed in this study, phosphorylation of Smad2/3 was not observed in *Itgam*$^{-/-}$ B cells derived from PPs, whereas WT $CD11b^+$ B cells demonstrated activated p-Smad2/3 after stimulation. These findings suggest that CD11b may promote the activation of Smad proteins and consequently facilitate the production of IgA. We also found that B cells secrete TGF-β and complete IgA production autocrine. However, TGF-β activation is a multiple-step process, including the release of TGF-β from TGF-β/LAP complexes (25). We did not provide evidence of the activation of TGF-β in B cells, which we may explore in the next project.

In summary, the findings of the present study reveal that intestinal $CD11b^+$ B cells ameliorate DSS-induced colitis and maintain gut homeostasis. Based on *in vitro* and *in vivo* experiments, $CD11b^+$ B cells exhibiting high activation of the TGF-β-Smad signaling pathway were the primary source of IgA, which maintained gut homeostasis and mitigated colitis. These phenomena were also confirmed in UC patients and *in vitro* experiments using human cell cultures, indicating the clinical application value of this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics council of Zhongshan Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by animal facility of Fudan University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YF, ZW and LW designed and performed the experiments, analyzed data, and wrote the manuscript. CY designed the experiments and wrote the manuscript. ML, WX, YLin, and EH improved the manuscript. BY, YLiu, RL, and HL performed the experiments and analyzed the data. The first two and the last two

authors contributed equally to this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.697725/full#supplementary-material

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REVIEW
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Pro- and Anti- Effects of Immunoglobulin A- Producing B Cell in Tumors and Its Triggers

Ziwen Zhong[1,2†], *Ke Nan*[1†], *Meilin Weng*[1†], *Ying Yue*[1], *Wenchang Zhou*[1], *Zhiqiang Wang*[3], *Yiwei Chu*[3,4], *Ronghua Liu*[4*] and *Changhong Miao*[1*]

[1] Department of Anesthesiology, Zhongshan Hospital, Fudan University, Shanghai, China, [2] Department of Anesthesiology, Affiliated Hospital of North Sichuan Medical College, Nanchong, China, [3] Department of Immunology, School of Basic Medical Sciences, Fudan University, Shanghai, China, [4] Shanghai Fifth People's Hospital and Key Laboratory of Medical Epigenetics and Metabolism, Institutes of Biomedical Sciences, Fudan University, Shanghai, China

B cells are well known as key mediators of humoral immune responses *via* the production of antibodies. Immunoglobulin A (IgA) is the most abundantly produced antibody isotype and provides the first line of immune protection at mucosal surfaces. However, IgA has long been a divisive molecule with respect to tumor progression. IgA exerts anti- or pro-tumor effect in different tumor types. In this review, we summarize emerging evidence regarding the production and effects of IgA and IgA$^+$ cells in the tumor microenvironment (TME). Moreover, we discuss that the TME cytokines, host diet, microbiome, and metabolites play a pivotal role in controlling the class-switch recombination (CSR) of IgA. The analysis of intratumoral Ig repertoires and determination of metabolites that influence CSR may help establish novel therapeutic targets for the treatment of cancers.

Keywords: B cells, plasma cells, immunoglobulin A, tumor immunity, class switching

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Wei Luo,
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Mats Bemark,
University of Gothenburg, Sweden

****Correspondence:***
Ronghua Liu
ronghualiu@fudan.edu.cn
Changhong Miao
miao_chh@126.com

†These authors have contributed equally to this work

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INTRODUCTION

Immunoglobulin A (IgA) is the most abundant antibody class present on mucosal surfaces. It plays a key role at these surfaces, which are continuously exposed to antigens, food, and commensal microorganisms (1). IgA-producing plasma cells (IgA$^+$ PCs) are derived from B cells and undergo antibody class-switch recombination (CSR). Emerging evidence shows significant tumor infiltration by B cells. However, the role of B cells and their antibodies in tumors has remained elusive, perhaps due to their phenotype, antibody isotype and production, localization, and the tumor type and tumor microenvironment (TME) (2–5). Multiple lines of evidence now leave no doubt that B cells undergo clonal proliferation, selection for high-affinity antibodies, and isotype switching within tumor-associated tertiary lymphoid structures (TLS) and in less organized structures, ultimately converting into effector/memory B cells and antibody-producing PCs (6–9). However, under certain conditions, tumor-infiltrating B cells, PCs, and their antibodies may exert prominent immunosuppressive effects. Studies have provided well-founded evidence of discrete subsets of immunosuppressive or regulatory B cells (Bregs) in both mice and humans. Their function depends on immunosuppressive factors, such as IL-10, PD-L1, and/or TGF-β (10–13). Moreover, IgA has recently been recognized as a key biomarker of immunosuppressive B cells. However, the effect of IgA$^+$ cells and their antibodies on cancer development remains controversial (1, 11, 14–16). In this

review, we summarize recent findings related to the production and effect of IgA and IgA^+ cells in the TME and discuss their potential roles in novel cancer treatment strategies.

SOURCE AND LOCATION OF IgA

Approximately 80% of all human PCs are located in organized gut-associated lymphoid tissue (GALT). In GALT, IgA is produced at a constant synthesis rate of 50 mg/kg/day, which is more than the total synthesis rate of other Ig isotypes, indicating that continuous secretion of IgA requires an enormous amount of energy in mammalian species (17). In addition, IgA is present in milk and bronchial secretions (18). Serum IgA is predominantly monomeric in humans and is produced by local PCs of the bone marrow, spleen, and lymph nodes (1). IgA at mucosal surfaces is generally produced by local PCs as dimeric molecules, although small amounts of monomers, trimers, tetramers, or polymers can also be present. Dimeric IgA interacts with the polymeric Ig receptor (pIgR), an antibody transporter expressed on the basolateral surface of intestinal epithelial cells (IECs). A secretory IgA (sIgA) complex is formed after a secretory component (SC) of pIgR binds to IgA through a joining (J) chain. SC is a hydrophilic and highly (N- and O-linked) glycosylated negatively charged molecule that protects sIgA from degradation in luminal secretions (19).

It has been confirmed that various structural compartments differ in the mode of IgA induction. Mucosal antigens initiate IgA production through both T cell-dependent (TD) and T cell-independent (TI) reactions (20–22). Most antigens produce IgA by activating follicular B cells in the germinal center of Peyer's patches (PPs) and mesenteric lymph nodes (MLNs). This TD pathway involves a cognate interaction between follicular B cells and antigen-activated $CD4^+$ T cells, including TH2 cells, regulatory T (Treg) cells, and T follicular helper (TFH) cells (22, 23). Together with follicular dendritic cells (FDCs), this interaction fosters proliferation, differentiation, somatic hypermutation (SHM), and IgA CSR of B cells in the germinal center. Ultimately, this TD pathway leads to the emergence of long-lived memory B cells and PCs that release high-affinity IgA antibodies in the lamina propria (LP) (20). However, TD antibody responses require 5-7 days, which can be too much of a delay to control pathogens. To compensate for this limitation, a TI reaction occurs in PPs, MLNs, isolated lymphoid follicles (ILFs), and LP. Commensals trigger multiple Toll-like receptors (TLRs), which stimulate FDCs, DCs, and stromal cells to release BAFF, APRIL, TGF-β, and other IgA-inducing cytokines, thereby triggering the induction of TI IgA CSR and production in PPs, MLNs, ILFs, and LP (24, 25). Class-switched B cells emerging from these pathways ultimately release both low- and high-affinity IgA antibodies in LP. Moreover, it is likely that the generation of IgA^+ PCs depends on the nature of IgA and the initial location of B cells. A precise combination of different leukocyte and mesenchymal cell types may also influence the generation of IgA^+ PCs (23) (**Figure 1**).

IMMUNOSUPPRESSIVE B CELLS IN TUMORS MARKED WITH IgA

The tumor-promoting effects of B cells and PCs are generally associated with the presence of heterogeneous cell populations called Bregs and immunosuppressive plasma cells (ISPCs), which may accelerate tumor progression through various mechanisms. Multiple studies have shown that Bregs suppress tumor immunity through immunosuppressive factors, such as IL-10, TGF-β, PD-L1, FASL, IL-35, and Tim1 (11, 14, 26–28). This type of tumor immunity suppression is activated by signals from CD40, TLRs, and B cell receptors (BCRs) (10, 29). Furthermore, Bregs reportedly cause cytotoxic T lymphocyte (CTL) suppression by inducing M2 macrophage polarization (30, 31) and Tregs in squamous carcinomas (SCCs) and pancreatic ductal adenocarcinoma (PDAC) (32).

However, no specific transcriptional marker has been identified that exclusively defines the Bregs phenotype in mouse or humans. Numerous studies have now demonstrated that Bregs mainly suppress inflammatory responses *via* IL-10-depengdent and -independent mechanisms (33). Subsets that are enriched for IL-10-producing B cells include transitional-2 marginal zone precursor (T2-MZP) B cells, peritoneal B-1 B cells, and antibody-producing B cell subsets in mice, as well as antibody-producing and immature B cell subsets in humans. In a recent series of studies, recirculating IgA^+ regulatory B cells have been shown to suppress inflammation *via* expression of IL-10 (34, 35). Shalapour et al. discovered in 2015 that IgA^+ PCs suppress anti-tumor immunity. These immunosuppressive B cells are IgA-producing PCs that express PD-L1, IL-10, and Fas-L (11). Most tumoral $CD19^+$ B cells in oxaliplatin-treated pancreatic cancer are IgA-positive, and successful eradication of tumors requires removal of IgA^+ cells (11). In a murine study of hepatocellular carcinoma, IgA^+ cells expressing high levels of PD-L1, IL-10, and TGF-β were shown to directly repress $CD8^+$ T cell proliferation and activation (14). IgA^+ PCs within prostate tumors induce $CD8^+$ T cell exhaustion and suppress anti-tumor CTL responses through PD-L1 and IL-10, either of which can induce anergy or exhaustion (36, 37). In general, IgA^+ cells promote the expansion of Treg cell populations, whereas Treg cells produce TGF-β, which mediates the isotype class switch to IgA (9, 38). This regulatory loop induces a state of relative immune suppression and may further promote tumor progression in at least some cancer types or subtypes (**Figure 2**).

DUAL EFFECT OF IgA ON TUMOR IMMUNITY

Although there is more than sufficient evidence indicating that IgA^+ B cells and PCs may exert prominent immunosuppressive effects, the antitumor effect of IgA^+ cells has also been reported; this may have resulted from the dual effect of IgA. The expression of IgA by tumor-infiltrating PCs has been linked to poor outcomes in colorectal cancer (CRC) (44) and melanoma (45, 46). Elevated levels of intratumoral IgA have been shown to be

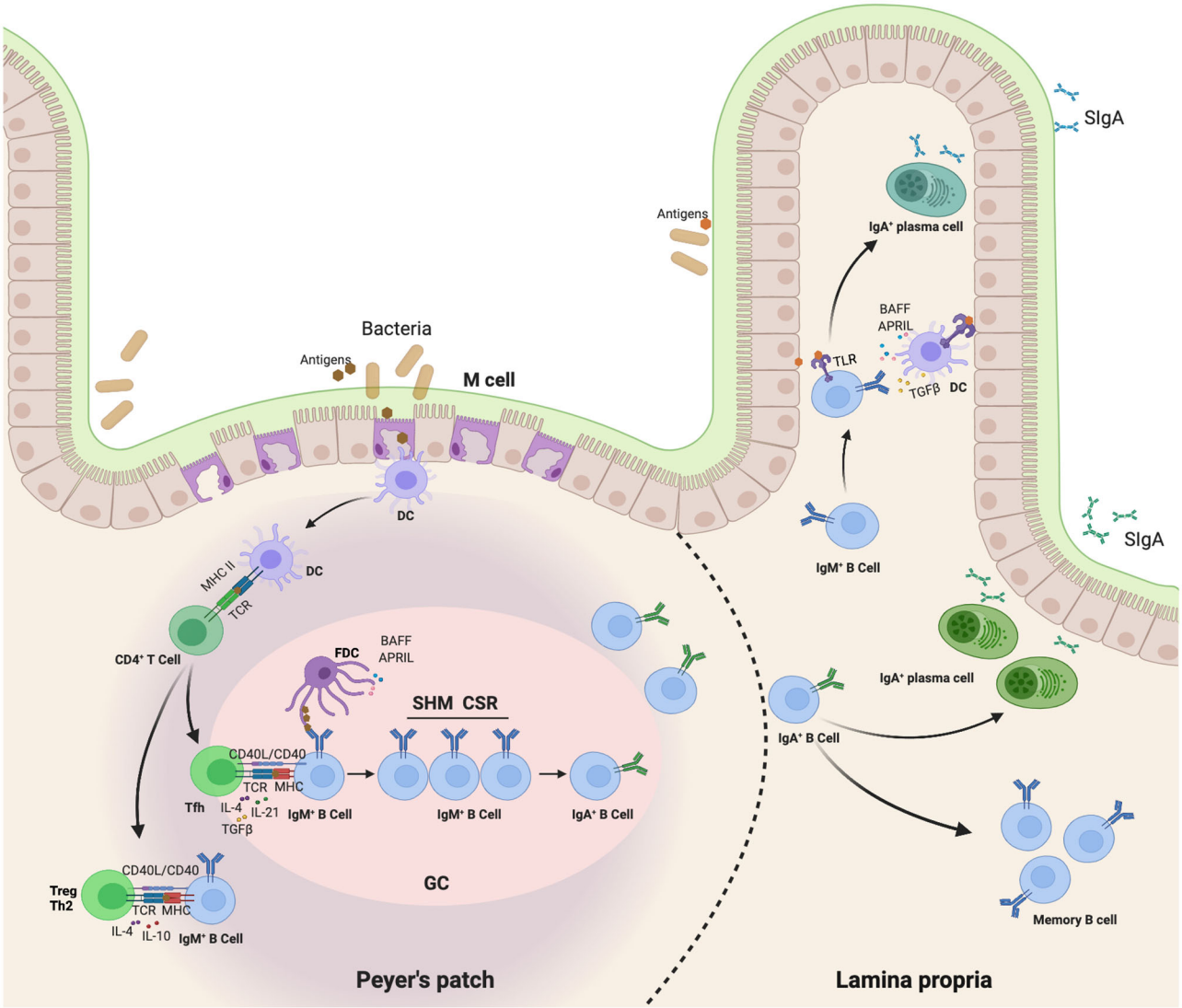

FIGURE 1 | Map of IgA class switching through both TD and TI reaction in the gut. This TD pathway involves a cognate interaction of follicular B cells with antigen-activated $CD4^+$ T cells, including TH2 cells, regulatory T (Treg) cells, and T follicular helper (TFH) cells (22, 23). First, Dendritic cells (DCs) in the subepithelial dome (SED) of the PP capture and present antigen to follicular $CD4^+$ T cells, thereby inducing them to differentiate into effector T cells releasing IgA-inducing cytokines. These T cells also interact with antigen-specific IgM^+IgD^+ naive B cells. Together with follicular dendritic cells (FDCs), this interaction fosters a proliferation, differentiation, somatic hypermutation (SHM) and IgA class-switch recombination (CSR) of B cells in germinal center. Ultimately, TD pathway leads to the emergence of long-lived memory B cells and plasma cells that release high-affinity IgA antibodies in the lamina propria (20). In addition, commensals trigger multiple Toll-like receptors (TLRs) to stimulate FDC, DC and stromal cells release of BAFF, APRIL, TGFβ and other IgA-inducing cytokines, thereby triggering direct IgA CSR in lamina propria IgM^+ B cells in a T-cell-independent manner (24, 25).

associated with poor prognosis and shorter survival in patients with bladder cancer (47). A recent meta-analysis indicated a strong association between IgA CSR and solid cancer diagnosis, and confirmed its immunosuppressive and pro-tumorigenic roles (48). The aforementioned study showed that, compared with healthy individuals, patients with solid malignancies had significantly higher serum IgA levels, which further increased in patients with advanced cancer. However, Biswas et al. demonstrated that tumor-antigen-specific and tumor-antigen-independent IgA responses antagonize the growth of ovarian cancer by governing coordinated tumor cell, T cell, and B cell responses (15). These results suggest that IgA may play various roles in different tumor types or in the tumor immune microenvironment (**Table 1**).

Pro-Tumor Effect of IgA

A systematic review and meta-analysis revealed that a 2-fold increase in the standardized mean difference (SMD) of the IgA level occurred in advanced cancer compared with early carcinoma, further supporting the possibility that serum IgA

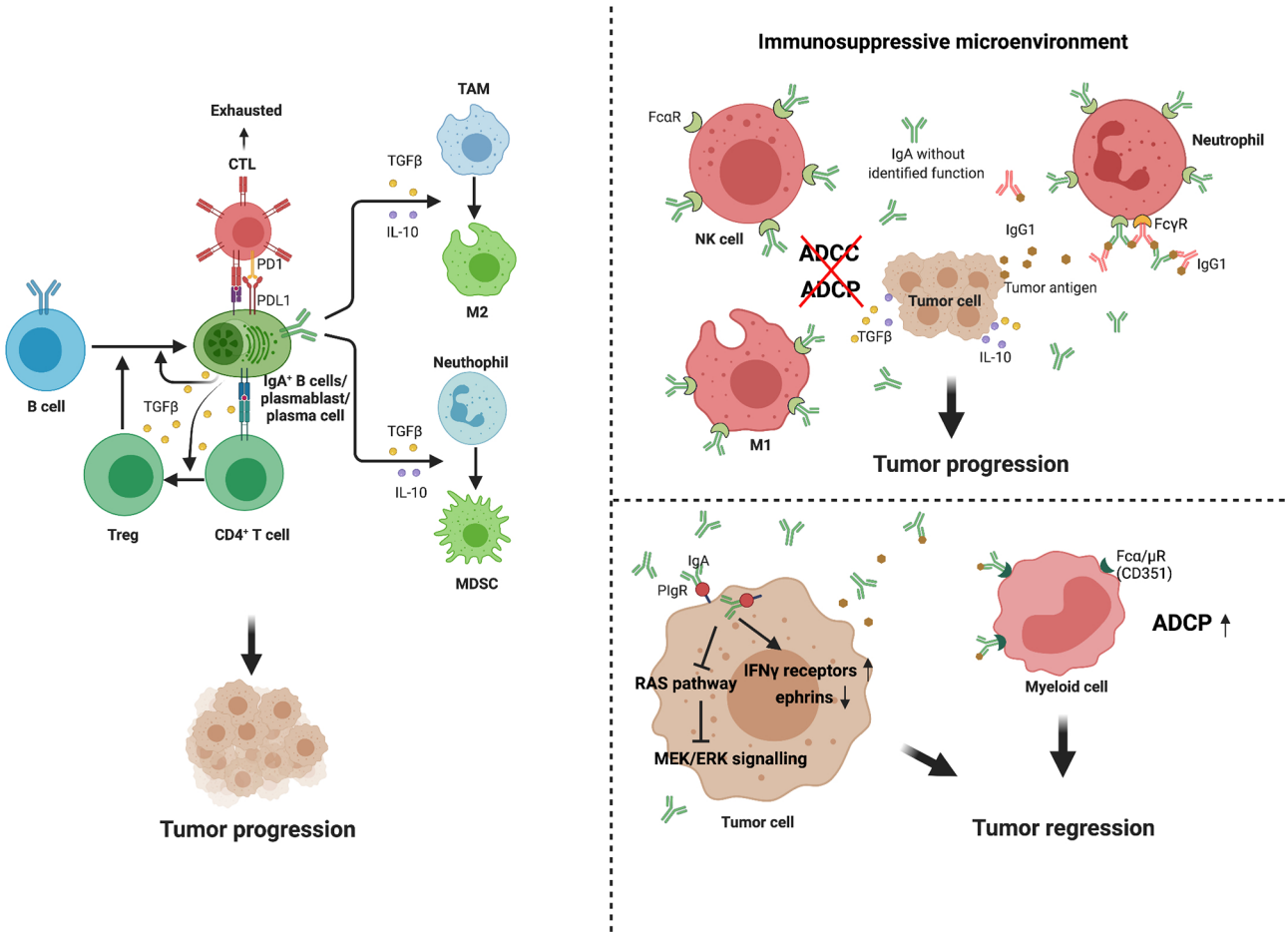

FIGURE 2 | Multiple functions of IgA$^+$ cells and IgA in tumor. IgA$^+$ B cells and plasma cells may promote tumor progression through several mechanisms. First, they can release high level of PD-L1, IL-10 and TGFβ, which directly induce CD8$^+$T cell exhaustion and suppress anti-tumor CTL responses (14, 36, 37). Second, the IgA$^+$ cells promote expansion of Treg cell populations, while Treg cells produce TGFβ, which mediates isotype class switch to IgA (9, 38). In addition, these immunosuppressive cytokines secreted by IgA$^+$ cells, such as IL-10 and TGFβ, can promote immunosuppressive phenotypes in macrophage and myeloid cells. IgA performs dual effects on tumor immunity. In immunosuppressive environment, due to the binding of IgA without identified function and Fcα receptors (FcαR) expressed on macrophages, natural killer (NK) cells and neutrophils, high levels of production of IgA block ADCC and ADCP (39, 40). Meanwhile, such antibodies can form immune complexes with tumor or non-tumor antigens, promoting chronic inflammation, tissue remodeling (41–43). However, in some specific tumor types, IgA transcytosis induced broad transcriptional changes in inflammatory pathways in tumor cells, contributing to hindering malignant progression. In further support of antibody-dependent cellular phagocytosis, tumor-derived IgA redirection of Fcα/μ$^+$(CD351) myeloid cells against extracellular oncogenic drivers, which causes tumor cell death (15).

levels may correlate with immune escape and tumor burden (48). A high intratumoral proportion of the IgA isotype has a close relationship with a negative prognosis in the KRAS-mutant subtype of lung adenocarcinoma (4). TCGA RNA-seq data of human melanoma indicate that high proportions of IgA, IgD, and IgE are related to a poor prognosis (2).

The tumor-promoting mechanism of IgA can be summarized as follows. Firstly, it is a tumor immunosuppressive environment and not a tumor antigen that promotes IgA production. IgA with unfocused specificity is ineffective in mediating antitumor responses, for which it cannot facilitate antigen presentation or mediate antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) of tumor cells (39, 40). On the other hand, such antibodies can form immune complexes with tumor or non-tumor antigens, promoting chronic inflammation and tissue remodeling, and thus directly or indirectly favoring the myeloid-derived suppressor cell phenotype (41–43) (**Figure 2**). Another possible role of mucosal IgA in tumor immunity depends on its dynamic relationship with environmental factors, such as microbiota or diet (56). Tissue-specific immunoregulation may determine the ability of different tumors to exploit the IgA–microbiota axis to facilitate immune escape (56, 57). Furthermore, IgA fails to activate the complement system but mediates the regulatory effects of its main inducer, TGF-β (58). In particular, monomeric IgA exerts inhibitory effects on many immune cell subsets by activating the myeloid-cell-specific type I

TABLE 1 | Mechanism and prognostic impact of IgA in cancers.

Cancer type	Number of patients	Animal models	Methods	Prognostic	Response to therapy	Mechanism		Ref.
						IgA-mediated effects	Beyond IgA production	
Prostate	87	Transplanted tumor models TRAMP transgenic tumor models	IHC, Serum	IgA↓	oxaliplatin↓	N/A	TGFβ receptor signaling↑	(11)
Hepatocellular	598	MUP-μPA, STAM, CCl4, MCD models	IHC, FC, Serum	IgA↓	N/A	N/A	PD-L1↑, IL-10↑	(14)
Ovarian	534	Transplanted tumor models	IHC, TCGA, Single-cell V(D)J (BCR) sequencing	IgA↑	N/A	IgA transcytosis	N/A	(15)
Colorectal	90	AD model	IHC	IgA↓	N/A	N/A	PD-L1↑, IL-10↑, TGFβ↑	(44)
Melanoma	710	N/A	IHC	IgA↓	N/A	Inability to activate the complement cascade, low affinities for activating FcRs、 low potency in triggering ADCC and ADCP	N/A	(45, 46, 49)
Bladder	110	N/A	IHC	IgA↓	N/A	N/A	N/A	(47)
LUAD	442	N/A	TCGA	IgA↓	N/A	N/A	PD-L1↑, IL-10↑	(4)
AML	13	N/A	TCGA, scRNA-seq	IgA↓	N/A	N/A	PD-L1↑, TGFβ↑	(50)
Breast	66	N/A	Serum, IHC	IgA→ IgA-Fc-folate conjugate↑	N/A	ADCC	N/A	(51, 52)
Head and neck	97 226	N/A	Serum	IgA→ IgA↓	N/A	N/A	N/A	(53, 54)
Pancreatic	812073	N/A	Serum, Urine	IgA→	N/A	N/A	N/A	(55)

Prognostic: favorable/mostly favorable ↑ no impact → unfavorable ↓.

Fc receptor for IgA (FcαRI; also known as CD89) (59, 60) and inducing IL-10 production (61), as well as by regulating proinflammatory cytokines (62).

Anti-Tumor Effect of IgA

IgA also plays an anti-tumor role in some specific cancers. Recent studies have demonstrated that tumor-derived IgA abrogates tumor growth through antigen-dependent redirection of Fcα/μ$^+$ myeloid cells and antigen-independent, pIgR-mediated transcytosis (15). IgA can bind to polymeric IgA receptors that are universally expressed on ovarian cancer cells. IgA transcytosis induces broad transcriptional changes in inflammatory pathways in tumor cells, including the upregulation of IFN-γ receptors and downregulation of tumor-promoting ephrins. In addition, IgA transcytosis through malignant epithelial cells can antagonize the RAS pathway and sensitize tumor cells to cytolytic killing by T cells, which also helps prevent malignant progression (**Figure 2**). Thus, tumor-antigen-specific and tumor-antigen-independent IgA responses inhibit the growth of ovarian cancer cells by regulating coordinated B cell, T cell, and tumor cell responses.

These results indicate that IgA plays a completely different role in governing ovarian cancer than that in some other types of tumors. The causes may be related to the tumor type and TME. It can be hypothesized that most solid tumor cells do not express polymeric IgA receptors that are universally expressed on ovarian cancer cells, and hence IgA fails to exert anti-tumor effects through transcytosis. Furthermore, some tumor antigens can be specifically recognized by IgA in ovarian cancer. However, in some other tumor types, tumor antigens may not be exposed and thus cannot be recognized on the surface of tumor cells, or do not contain immunogenic peptides suitable for presentation to T cells on major histocompatibility complex class (MHC) molecules, which may lead to failure of recognition by IgA and thus mislead the immune response and favor pro-tumor processes (40, 63). Therefore, the analysis of intratumoral IgA expression and clonality is crucial for evaluating the role of IgA in tumors.

TUMOR-RELATED IgA CLASS SWITCHING

Emerging evidence points that a combination of host, environmental, and tumor factors mediates IgA class switching and determines the efficiency of cancer surveillance or promotion (48, 64). At the host level, this implies that increased serum IgA levels are related to older age, male gender, metabolic syndrome, etc. (48, 64, 65). Recent study has shown that environmental factors, including commensal bacteria and diet, and tumor immunosuppressive cytokines, including IL-10 and TGF-β, affect the production of IgA (66–68).

CSR Triggers in Immunosuppressive TME

In the TME, most tumor-infiltrating TH/TFH cells present a regulatory phenotype, which secretes immunosuppressive cytokines and supports the development of immunosuppressive and anti-inflammatory isotypes (69). Furthermore, activated cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) in the TME can produce TGF-β, which represses NK cell and CTL activation, promotes Tregs, and supports IgA CSR (38, 70, 71). More precisely, naïve B cells are recruited into the TME by CAFs *via* CXCL13 and CXCL12 and further exposed to TGF-β, IL-21, IL-33, lymphotoxin β (LTβ), and IL-10, which favors IgM-naïve BCR class switching to IgA (11, 70). Emerging evidence shows that tumors can also induce IgA CSR through a regulatory loop formed by myeloid-derived suppressor cells (MDSCs) and IgA. A recent study showed that $CD11b^+$ MDSCs induced the differentiation of B cells into IgA^+ PCs by secreting IL-10 and TGF-β (72). IgA with unfocused specificity can form immune complexes simultaneously with tumor or non-tumor antigens, promoting immunosuppressive phenotypes in myeloid cells (40).

CSR Triggers From Host Diet, Microbiome, and Metabolites

Diet is universally considered a mechanism that can affect energy metabolism and immune responses, including intestinal IgA production (73). Among the various dietary components, vitamins are the most important factors for immune responses. For example, vitamin B1 has been confirmed to play a pivotal role in intestinal IgA responses as a cofactor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which are essential enzymes in the TCA cycle (74). Similar to vitamins, dietary fatty acids influence B cell metabolism and the production of IgA (73, 75). A study showed that intestinal IgA production increased in mice maintained on a palmitic acid-enriched oil (76). Palmitoyl-CoA and serine act as substrates of serine palmitoyltransferase (SPT) and are subsequently converted into sphingolipids, such as ceramide, sphingosine, sphingomyelin, and S1P (77). Sphingolipids are a class of membrane lipids that also have biologic functions (78). For instance, an increase in ceramide concentrations may induce the proliferation of IgA PCs. Moreover, S1P can recruit IgA PCs into the intestine. These activation pathways may coincide with the changes in energy metabolism that occur in intestinal IgA PCs (76). Furthermore, metabolic intermediates of glycolysis, such as glucose monophosphate and fructose bis-phosphate, were detected preferentially in IgA PCs compared with naïve B cells, which implied that the shift to glycolysis-mediated energy metabolism likely is useful for the generation and production of IgA (73). For example, the glucose monophosphate and 3-phosphoglycerate generated through glycolysis are used in the pentose phosphate and serine biosynthetic pathways for nucleotide and amino acid synthesis, respectively (79). In addition, pyruvate, the metabolic product of glycolysis, can subsequently be converted to acetyl coenzyme A (acetyl-CoA) and used in fatty acid synthesis, which is required for B cell differentiation (80) (**Figure 3**).

In addition, dysbiosis and changes in microbiome diversity were recently shown to regulate the IgA–microbiota axis and influence multiple aspects of antitumor immunity by altering the risk of developing cancer and regulating responses to immunotherapy (81–83). It was shown that gut and lung microbiota themselves are capable of promoting IgA CSR through antigen presentation by $CD103^+$ DCs and induction of IL-10 and TGF-β (84). Conversely, a decrease in intestinal IgA responses and structural immaturity of PPs occurred in germ-free mice (85). Gut commensal microbes can produce biologically active metabolites, represented by the short-chain fatty acids (SCFAs) acetate, propionate and butyrate, which are all reported to promote IgA CSR (86, 87). Recently, Isobe et al. discovered that commensal-bacteria-derived butyrate up-regulated the production of TGF-β and all-trans retinoic acid by $CD103^+CD11b^+$DCs, both of which promote the T-cell-independent IgA response in the colon (88) (**Figure 3**).

A possible role of IgA in tumor immunity arises from its dynamic relationship with diet and the microbiota. Hyper-nutrition and alcohol consumption not only regulate the magnitude of B cell activity and effector function but also change the levels and diversity of IgA, which may further enhance tumor development (14, 89, 90). In addition, Piper et al. identified that the aryl-hydrocarbon receptor (AhR), a ligand-activated transcription factor that senses dietary, microbial, and metabolic cues, binds to the IL-10 locus in murine cells (91). The supplementation with microbially derived SCFA butyrate supports Breg to produce IL-10 by amplifying AhR activation (91). IL-10 upregulates the expression of AID and triggers the induction of SHM and CSR from IgM to IgA, which may mediate tumor progression (20, 33).

INTRATUMORAL IgA REPERTOIRES

RNA- and DNA-based analyses of Ig repertoires and intratumoral T-cell receptors have received considerable attention as powerful tools for identifying prognostic and predictive cancer biomarkers, such as clonality metrics (2, 92). In particular, assessment of the degree of clonality and the amount of somatic hypermutations in Ig gene segments can offer prognostic value for some types of cancer (93). Clonality reflects mainly the presence of plasma cell clonal expansion. TCGA RNA-seq data on melanoma samples have demonstrated that a high IgG1 proportion is strongly associated with the presence of large clonal expansions, contributing to a better prognosis. In contrast, a high IgA proportion correlates with low clonality, resulting in a negative outcome (2). These results can be explained by a focused immune response leading to the production of high-affinity, tumor-specific IgG1 antibodies; conversely, switching of B cells to the IgA isotype is not driven by particular antigens but is a passive consequence of the intratumoral suppressive cytokine environment (40). Therefore, it can be hypothesized that dual effect of IgA on tumor immunity is highly associated with clonal expansion of PCs. High IgA expression and high clonality, implying that IgA

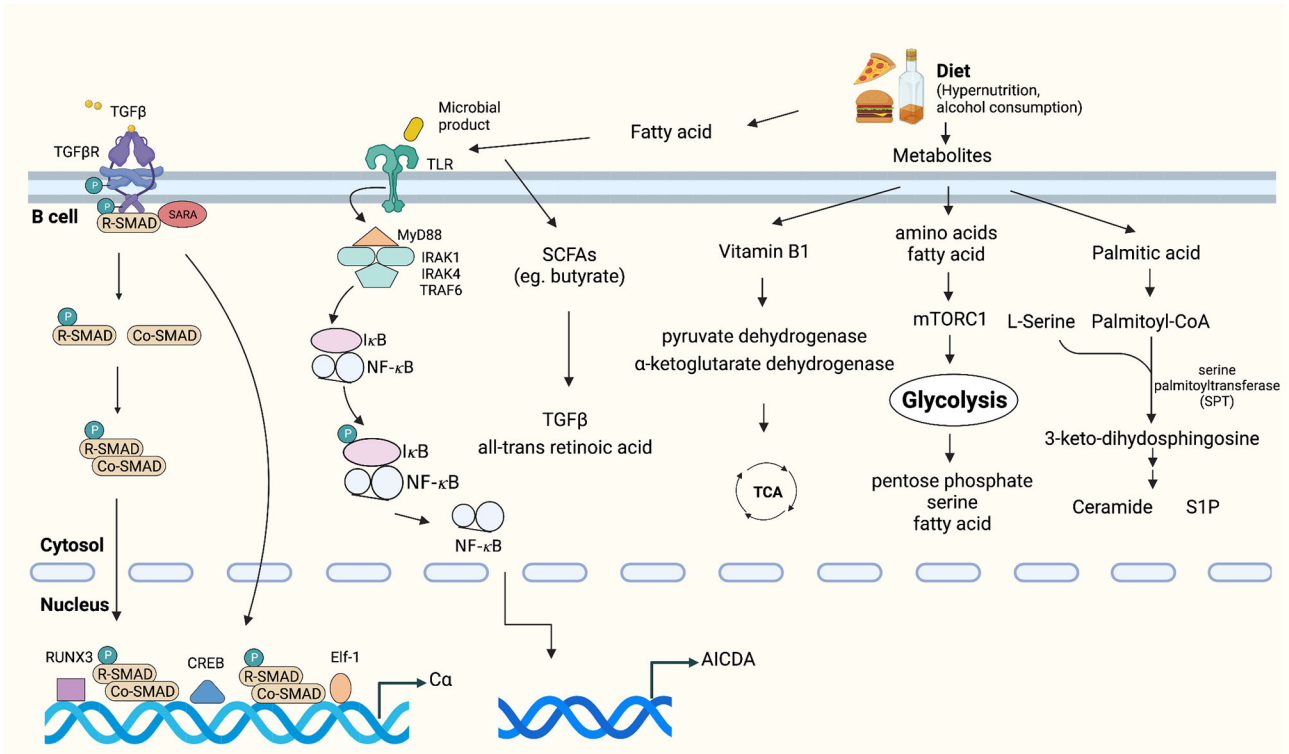

FIGURE 3 | Tumor microenvironmental stimuli that induce IgA class switching. TGFβ is highly expressed in the tumor microenvironment, which activates the constant heavy chain α (Cα) gene promoters (38, 70, 71). Exposure to dietary extracellular metabolites within the immediate microenvironment may play an important role in controlling the production of IgA (73–75, 77). Fatty acid or microbial products can activate TLRs, which promote activation of IκB kinase (IKK) complex, phosphorylation and degradation of IκB [inhibitor of nuclear factor-κB (NF-κB)]. Subsequently, IκB-free NF-κB translocates to the nucleus and initiates CSR by binding to κB motifs on the AID gene promoter (81–83).

may be driven by a focused immune response and may result in a better prognosis, whereas high IgA expression and low clonality mean that an immunosuppressive microenvironment drives IgA production and is likely to contribute to poor outcomes in some specific malignancies.

CONCLUSION

IgA and IgA$^+$ cells are active participants that can fundamentally orchestrate the immune response in the TME. IgA can exert pro-tumorigenic roles in cancer by inducing the release of immunosuppressive cytokines, forming immune complexes with tumor or non-tumor antigens, and interacting with immunosuppressive cells, such as MDSCs or Tregs. However, IgA can also promote anticancer immunity and suppress tumor growth. These opposing effects can be explained by the influence of different tumor types, environmental factors, and host factors, which elicit a different antigen specificity of IgA, as well as the inconsistent interaction between IgA and tumor cells. Decisions on the application of IgA-targeted therapies and their combination with other therapies should be based on an advanced understanding of the prevailing nature of IgA involvement in tumor–immune interactions in each cancer subtype or even in each patient. Therefore, analysis of intratumoral IgA repertoires and determination of their metabolites are becoming powerful tools for identifying prognostic and predictive cancer biomarkers.

AUTHOR CONTRIBUTIONS

Conceptualization: ZZ, RL, and CM. Writing original draft: ZZ, KN, and MW. Supervision: YC, RL, and CM. All authors contributed to the article and approved the submitted version.

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REVIEW
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Characterization of Definitive Regulatory B Cell Subsets by Cell Surface Phenotype, Function and Context

Savannah D. Neu[1,2] *and Bonnie N. Dittel*[1,2]*

[1] Versiti Blood Research Institute, Milwaukee, WI, United States, [2] Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI, United States

Regulatory B cell or "Breg" is a broad term that represents the anti-inflammatory activity of B cells, but does not describe their individual phenotypes, specific mechanisms of regulation or relevant disease contexts. Thus, given the variety of B cell regulatory mechanisms reported in human disease and their animal models, a more thorough and comprehensive identification strategy is needed for tracking and comparing B cell subsets between research groups and in clinical settings. This review summarizes the discovery process and mechanism of action for well-defined regulatory B cell subsets with an emphasis on the mouse model of multiple sclerosis experimental autoimmune encephalomyelitis. We discuss the importance of conducting thorough B cell phenotyping along with mechanistic studies prior to defining a particular subset of B cells as Breg. Since virtually all B cell subsets can exert regulatory activity, it is timely for their definitive identification across studies.

Keywords: regulatory B cell (Breg cell), IL-10, Treg, autoimmunity, EAE (experimental autoimmune encephalomyelitis), multiple scleorsis (MS), BD_L

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****Correspondence:***
Bonnie N. Dittel
bdittel@versiti.org

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INTRODUCTION

Alongside T cells, B cells or B lymphocytes make up the adaptive immune system that recognizes and retains memory of foreign antigen encounters. The B cell receptor (BCR) or immunoglobulin (Ig) is a cell surface protein that recognizes intact antigens. BCR binding to cognate shapes activates B cells and leads to downstream protective functions including antigen presentation, upregulation of effector cell surface proteins and soluble factors and B cell differentiation into specialized effector subsets (1, 2). B cells are continuously produced in the bone marrow (BM) to replenish and replace expended populations in peripheral tissues (3, 4). High B cell turnover sustains a large and diverse BCR repertoire, that can protect against a vast array of foreign antigens, but can also be self-reactive (3, 5, 6). To minimize pathological autoimmunity regulatory cell subsets, which include T cells, macrophages and B cells, control and resolve inflammation and reestablish homeostasis through many mechanisms (7–10). Targeting regulatory cells for depletion to boost anti-cancer responses or expansion to dampen autoimmunity is a current therapeutic strategy (9, 11–13).

Although there are differences between the human immune system and animal models, B cells have similar functions and residence across species. B cells are divided into two broad subsets based

on origin. Conventional B2 B cells in mouse and human are BM-derived and account for most B cell populations in circulation and in the secondary lymphoid organs including lymph nodes, spleen and mucosal sites (14). In mice, B1 B cells were shown to be derived from the yolk sac/fetal liver and largely self-renew in the peritoneal cavity and surrounding abdominal tissues and secrete large amounts of IgM for innate-like immune protection (15, 16). B1 B cells are subdivided into B1a and B1b in mice (17). Both B2 and B1 B cells can engage in antigen-specific immune responses, secrete protective antibodies and become further specialized based on 1) localization to tissue types/structures and proximity to other cells; 2) BCR affinity for self or foreign ligands and 3) availability of growth signals, stimulation, and differentiation factors. In both mice and humans, BM-derived B cells follow a maturation trajectory from immature states (pre-B, pro-B, transitional) to mature marginal zone and follicular subsets (18). Naïve B1 and B2 B cells become activated through their BCR and related signaling (co-stimulation, cytokines) and differentiate into long-lived memory or antibody secreting plasma cells (19, 20). During differentiation into plasma cells, activated B cells in both mice and humans, downregulate canonical B cell markers (CD19, surface Ig, Pax5) and upregulate transcriptional programs needed for efficient protein folding and secretion (IRF4, Blimp-1, XBP1) (19, 21–24). In this process, B cells mature through an intermediary plasmablast stage characterized by high proliferation and migration to effector sites (19, 21).

While the term "Breg" denotes a unique B cell subset, regulatory activity can be found in virtually all B cell subsets (25–28). Thus, Breg describes the general capacity for B cells to dampen immune responses but does not detail their exact nature, i.e., phenotype, mechanism of regulation or functional context. Many recent reviews summarize a multitude of mechanisms by which B cells are anti-inflammatory. For simplicity, these mechanisms are often divided into IL-10-dependent and -independent activities (10, 26, 28, 29). We support the concept that while all B cell subsets have contextual regulatory capacity, some subsets are inherently better suited than others. Because of this complexity, characterization of specific B cell subsets that exert regulatory activities has been incomplete and inconsistent across model systems and between research groups. Thus, there is a need for a definitive characterization strategy to assess novel regulatory B cell subsets. Here, we will review the history of B cell immune regulation research with focus on the discovery and testing of B cell subsets whose phenotype and mechanism of action have been clearly delineated. These include IL-10 producing plasmablasts/plasma cells (30–32) (**Figure 1**) and B cell IgD low (BD_L) that interact with and induce proliferation of $CD4^+Foxp3^+$ T regulatory cells (Treg) (33) (**Figure 2**). In each of the four articles highlighted, negative immune regulation was assessed using the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), and measured as the level of recovery from the signs of disease. Although B cell negative immune regulation has been reported in a variety of human disease models, for brevity and continuity, we are limiting this review to MS and its animal model EAE.

THE DISCOVERY OF B CELL NEGATIVE IMMUNE REGULATION

The discovery of a regulatory role for B cells in autoimmunity, ironically, was a serendipitous finding that was the outcome of a study in the laboratory of Dr. Charles A. Janeway, Jr. to determine whether B cell antigen presentation of peptides could induce CD4 T cell priming. This question was asked using EAE, which was well suited to address the question because of its dependence upon CD4 T cell priming to a self-antigen (34). EAE was induced in B10.PL ($H\text{-}2^u$) mice by immunization with the myelin basic protein (MBP) immunodominant peptide $Ac_{1\text{-}11}$ emulsified in complete Freund's adjuvant accompanied by two doses of pertussis toxin (day 1 & 2) (35). This model was chosen because it was the first peptide active immunization model of EAE (36). To address the original question, B10.PL mice rendered deficient of mature B cells by disruption of the IgM heavy chain (B10.PLμMT) (37) were immunized with $Ac_{1\text{-}11}$ (35). Now as part of scientific history, we showed that μMT mice not only succumbed to EAE, but failed to recover from the signs of EAE (35). This later finding is an advantage of using B10.PL mice because WT mice spontaneously recover from EAE. Although this result did not answer the original question of whether B cells can take up and present peptide antigens to T cells, it did indicate that B cell antigen presentation was not required for EAE induction. The observation that the mice did not recover from EAE provided the first evidence that B cells play a regulatory role in autoimmune responses. Remarkably, this finding was obtained prior to the identification of $CD4^+Foxp3^+$ Treg. The question then became, through what mechanisms do B cells facilitate resolution of EAE or other inflammatory conditions?

IL-10/IL-35-SECRETING PLASMABLASTS/ PLASMA CELLS

IL-10 is a strong anti-inflammatory cytokine, which can potently suppress antigen presentation, among other functions, leading to attenuated immune responses (38, 39). IL-10-deficient mice develop a severe, nonrecovery EAE phenotype much like B cell-deficient animals (40, 41). Fillatreau and colleagues, in 2002, reproduced our findings that B cells are required for EAE recovery in C567BL/6 mice using the myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide immunization model (42). Mechanistically, they were the first to demonstrate a role for B cell production of IL-10 in EAE recovery (42). This was accomplished utilizing IL-10-deficient mice and a combination of mixed BM chimeras and B cell transfers. Since this landmark finding, a multitude of subsequent studies have assessed B cell production of IL-10 through methods such as ELISA and ELISPOT, flow cytometry, fluorescent reporters and quantitative PCR (43). IL-10 production was commonly assessed following potent antigen non-specific *in vitro* B cell stimulation (44). While many, if not all, B cell

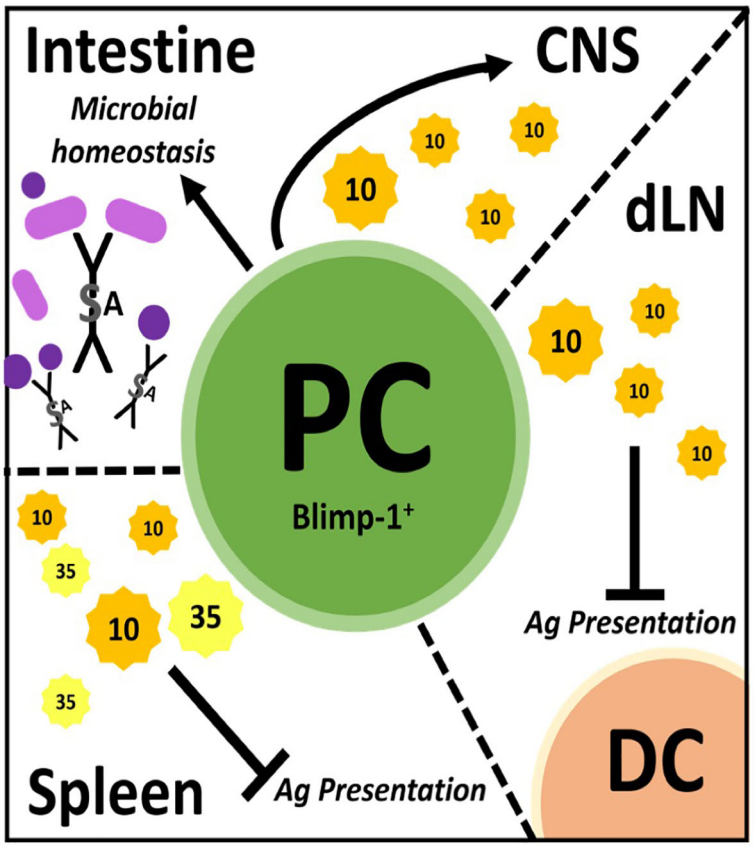

FIGURE 1 | Plasmablast/plasma cells (PC) regulate through many mechanisms in different tissues. PC facilitate microbial homeostasis at mucosal surfaces like the small and large intestine through secretion of IgA, a dimerized Ig with added features. These PC can migrate to the central nervous system (CNS) and secrete IL-10 to dampen inflammation therein. In draining lymph nodes (dLN), PC secrete IL-10 to inhibit antigen (Ag) presentation by dendritic cells (DC), thereby dampening immune responses. PC secrete IL-10 and IL-35 in the spleen which is believed to inhibit Ag presentation therein.

subsets can produce IL-10 after *in vitro* stimulation, concurrent secretion of pro-inflammatory cytokines (IL-6, TNF-α) has been reported, suggesting that *in vitro* activation is not sufficient to assess true regulatory activity *via* IL-10 (25, 45). Indeed, B10 B cells named for their production of IL-10, are not a specific subset of B cells, but defined by IL-10 production, regardless of whether the IL-10 is regulatory in a disease context (28). Thus, measuring IL-10 production alone is not sufficient to define/phenotype B cell subsets with regulatory activity. The later requires mechanistic studies utilizing well-defined and/or purified B cell subsets, and as shown by Fillatreau and colleagues (42), the utilization of IL-10-deficient B cells.

Once it was recognized that B cell production of IL-10 was anti-inflammatory in EAE, the search commenced to identify the phenotype of the regulatory B cell. This was a daunting task given that all B cell subsets have the capacity to produce IL-10. In particular, B cells produce copious amounts of IL-10 following TLR stimulation (44, 46–49). Interestingly, CFA used to induce EAE has numerous TLR ligands and B cells stimulated *via* TLR ligands were shown to suppress EAE (44, 46). It took until 2014 for the identification of plasmablasts/plasma cells as the major source of B cell-derived IL-10 during EAE (30). In the first of two articles, Fillatreau and colleagues, used both EAE and *Salmonella* infection along with BM chimeras utilizing knockout (KO) mice, to demonstrate that IL-35 production was also a critical B cell-derived anti-inflammatory cytokine (30). IL-35 as a potential B cell regulator was identified *via* a microarray approach utilizing B cells expressing IL-10 as detected using IL-10 reporter mice. They went on to show that splenic plasma cells were the major contributors of IL-10 and IL-35. Here, we want to emphasize that thorough cell surface phenotyping ($CD138^{hi}TACI^{+}CXCR4^{+}CD1d^{int}Tim1^{int}$) combined with intranuclear detection of the plasma cell transcription factor Blimp were utilized (30). This was the first time a "definitive" B cell phenotype was identified exhibiting regulatory activity *via* secretion of IL-10/IL-35 (**Figure 1**). IL-10/IL-35 production was used as the starting point to identify the B cells, not as the endpoint.

In the same year, Matsumoto and colleagues also reported that plasmablasts were the primary producers of IL-10 in EAE (31). IL-10 reporter mice (Venus) were used as the strategy to identify IL-10 producing B cells, which were highly prevalent in the draining lymph nodes, but not in the spleen or spinal cord on

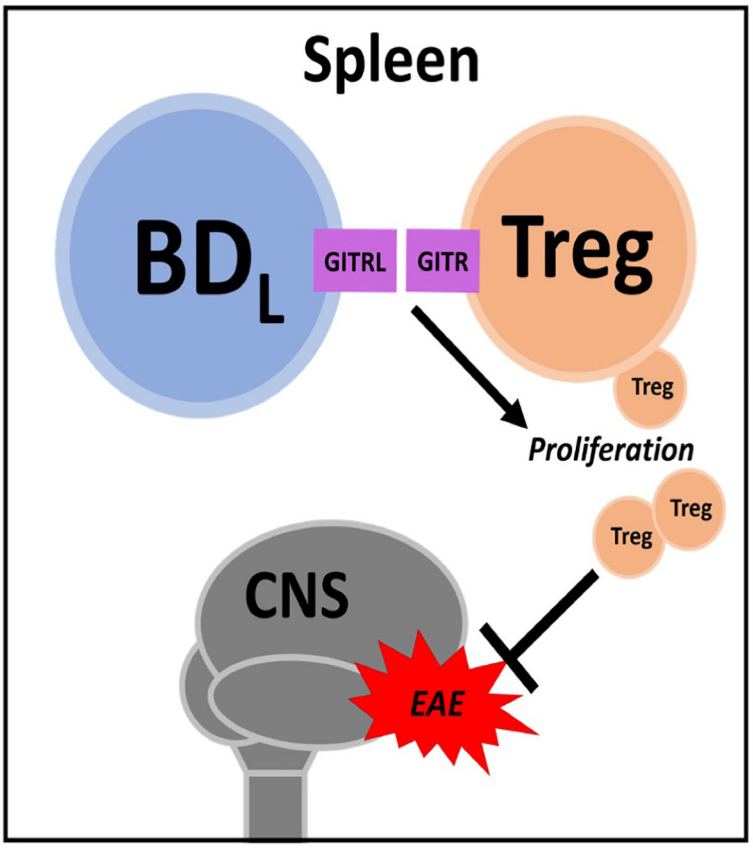

FIGURE 2 | BD_L. In the spleen BD_L promote regulatory T cell (Treg) proliferation through GITR-GITRL interactions. Treg attenuate experimental autoimmune encephalomyelitis (EAE) severity.

day 14 of EAE (~peak of disease). Using cell surface phenotyping and intranuclear Blimp-1 staining, $CD138^+$ plasmasblasts/plasma cells were identified as the major IL-10 secreting cells during EAE. Confirmation that the plasmablasts/plasma cells were negative regulatory in EAE came from studies utilizing Blimp-1 B cell conditional KO mice. Additional studies indicated that lymph node, but not splenic plasmablasts/plasma cells were responsible for the negative regulation. To resolve the splenic (30) versus lymph node origin of the regulatory plasmablasts (31), adoptive transfer experiments were conducted, which ultimately demonstrated that the negative immune regulation occured in the lymph node independent of the germinal center (i.e., plasmablasts). In retrospect, the lymph node as the location of B cell-derived IL-10 immune regulation makes intuitive sense, due to IL-10s' potent ability to suppress antigen presentation. MOG_{35-55} EAE is induced by immunization driving T cell priming due to delivery of the peptide to the draining lymph node *via* dendritic cells. IL-10 has been reported to negatively regulate the upregulation of MHC class II, co-stimulatory molecules and cytokines important for CD4 T cell priming (50, 51). Indeed, *in vitro* studies suggested that dendritic cells were the target of the plasmablast-derived IL-10 (31). TLR2, 4 and/or 9 agonists in the CFA likely induced the IL-10 production by the plasmablasts/plasma cells (30, 31). Here, again, IL-10 production was the starting point and the implementation of multiple strategies were utilized to definitively identify the B cell subset that produced the regulatory IL-10 (**Figure 1**).

In addition to autoimmunity, B cell activation and differentiation into antibody-secreting plasma cells is also associated with IL-10 competency and immunosuppressive activity in cancer (31, 52–54). While plasmablast/plasma cell characterization has narrowed the phenotype for IL-10-secreting B cells, several plasma cell populations exist between the BM, secondary lymphoid organs (spleen/lymph nodes) and mucosal sites (i.e., gut) (55–60). Of importance, not all PC can produce IL-10, such as IgG-secreting plasma cells (53). Interestingly, microbial sensing through TLR (54) and the presence of gut commensals (61) seems required for effective plasmablast/plasma cell immunosuppression (30, 31). The gut microbiome and its role in MS has been investigated using models like germ-free, single microbe monocolonized and specific pathogen-free animals in tandem with EAE and by sequencing microbiomes from human patients (62–64). While a definitive MS microbiome is yet unclear, these cumulative studies have

identified microbes associated with disease activity as well as quiescence and elucidated further mechanisms through which the gut microbiome impacts immune regulation (65–67). While a multitude of mechanisms exist, IgA production in the gut has emerged as an important regulator of the composition of the gut microbiome. IgA is the most abundant Ig in the body. In human adults, around 5 mg is secreted at mucosal surfaces per day (68, 69). IgA is produced by gut plasma cells residing in the lamina propria of the small and large intestine and is secreted as a dimer joined covalently by the J-chain protein which acquires an additional secretory component during translocation into the gut lumen *via* the epithelium (70–72) (**Figure 1**). In the gut, IgA canonically targets microbes through antigen-specific binding but can also participate in non-specific binding *via* glycan-, J-chain-, and secretory component-driven adherence (72–74). Through these combined mechanisms, IgA facilitates clearance of potentially pathogenic microbes while allowing beneficial commensals to thrive (75, 76).

Rojas and colleagues, investigated whether there was a link between gut IgA, IL-10 and EAE penetrance (32). They identified gut-derived IgA-secreting plasma cells as a potent source of IL-10 and that these cells ameliorated EAE by trafficking from the gut to the brain. In their work, IgA PC were characterized by multiple techniques including fluorescent reporters with parabiosis tracking, flow cytometry and microscopy and were functionally assessed *via* ELISPOT and adoptive cell transfer analysis. These plasma cells were shown to be IgA- and IL-10-secreting, expressed Blimp-1, had low levels of canonical B cell markers (B220) and reacted with gut-derived antigens *in vitro* (32) (**Figure 1**).

While each of the three highlighted studies discussed above were comprehensively and elegantly conducted using state-of-the-art techniques, one can ask how does induction of EAE with immunization utilizing a potent TLR stimulating adjuvant (CFA) have relevance to human MS? The nature of this question is relevant to most animal models of human disease. Nevertheless, interestingly, all FDA approved therapies for MS have shown efficacy in the EAE model of MS. Although little is known regarding the role of IL-35 in MS, it has been shown to be elevated in the serum of treated patients as compared to the control group (77). A second study found that IL-35 was reduced in relapsing-remitting MS patients as compared to healthy controls (78). A third study reported that IL-35 was higher in MS patients as compared to healthy controls (79). In a fourth study, MS patients infected with helminths had higher levels of IL-35-producing B cells in the CSF, which correlated with reduced MRI lesions, as compared to uninfected patients (80). *In vitro* studies, showed that IL-35 could drive IL-35 and IL-10 production by $CD19^+$ B cells and induce a Treg-like phenotype (80). While these studies are interesting, the lack of definitive B cell phenotyping limits impact. Taken together, the collective IL-35 data indicate that much remains unknown regarding the source of IL-35 in MS and its mechanism of action. Human relevance investigated by Matsumoto and colleagues utilized an *in vitro* culture system whereby healthy human peripheral blood B cells were stimulated through TLR9 with CpG along with IFN-α/IFN-β to induce IL-10 production (31). It was found that of the various plasmablast populations generated, those derived from naïve B cells, with a $CD27^{int}CD38^+$ phenotype, expressed the highest level of IL-10 and remained IgM^+. Whether these cells are regulatory in MS is not known. In the Rojas study, MS patients were utilized to demonstrate that IgA bound bacteria was reduced during active disease (32). The presence of IgA-producing plasma cells in the MS brain has since been confirmed (81). While the gut microbiota is closely associated with IgA-secreting plasma cells, the signals required for IL-10 secretion and migration to sites of autoimmune inflammation require further exploration.

In summary, although plasmablasts/plasma cells identified by Blimp-1 expression were shown to be the regulatory B cell in EAE, each study identified a different mechanism. In the spleen, they regulated *via* IL-35, *via* an unknown mechanism (30) (**Figure 1**). In the lymph node, IL-10 was the primary immunosuppressive cytokine that likely targeted dendritic cell antigen presentation (31) (**Figure 1**). While in the gut, IgA plasma cells trafficked to the CNS and suppressed *via* IL-10 (32) (**Figure 1**). While the IL-10 target in the CNS was not investigated, it was shown that overexpression of IL-10 in the CNS *via* transfer of immortalized fibroblasts producing IL-10 or an adenovirus expressing IL-10 into the CNS potently attenuated EAE severity (82, 83). These collective studies illustrate the complexity of B cell regulation within just the plasmablast/plasma cell subset, which itself is a complex group of cells with differential production of Ig isotypes, anatomical location, cytokine profiles and longevity.

MAINTENANCE OF TREG HOMEOSTASIS BY BD_L

As discussed above, we were the first to demonstrate a negative regulatory role for B cells in promoting recovery from EAE (35). This seminal study was conducted using the active immunization model utilizing CFA. To avoid bystander effects of CFA (i.e., TLR agonism), we first confirmed that B10.PLμMT mice did not recover from EAE induced by the passive transfer of MBP-specific encephalitogenic $CD4^+$ T cells generated from MBP-TCR transgenic mice (84, 85). Interestingly, in this study utilizing BM chimeras, we did investigate IL-10 expression, but in the context of the recently described $CD4^+Foxp3^+$ Treg, not B cells (85). In a follow up study, we utilized anti-CD20 B cell depletion as an alternative to genetic disruption (μMT) to render mice B cell deficient, to further support our findings that B cells are required for EAE recovery (86). In this study, we moved from a BM chimera approach to B cell adoptive transfer and showed that neither IL-10 nor MHC class II were required for B cells to drive EAE recovery. Keeping with our previous findings, we investigated a role for Treg and found that B cells induced their proliferation in a glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL)-dependent manner (**Figure 2**). This mechanism was supported by findings that mice deficient in B cells (either μMT or anti-CD20 depleted) had a significant reduction in splenic Treg and that B cell adoptive transfer into

μMT mice restored Treg numbers and recovery from EAE. Antibody blocking of GITRL prior to B cell adoptive transfer, attenuated Treg expansion and EAE recovery.

By utilizing two different EAE models (active & passive) and two means to render mice deficient in B cells (genetic & antibody depletion), we were confident that B cells were required for recovery from EAE (85, 86). We then required a strategy by which to identify and subsequently purify the regulatory B cell subset. Unlike IL-10, we could not utilize GITRL due to its very low expression on B cells (86). We did perform some targeted adoptive transfers using FACS purified B cell subsets, but found that strategy too laborious, costly and unfocused. Thus, we chose anti-CD20 partial B cell depletion. By swabbing the IgG_{2a} heavy chain of the totally B cell depleting anti-CD20 to IgG_1, B cell depletion efficiency was reduced (33, 87). We would like to thank Biogen Idec for generously providing these antibodies. Anti-CD20 IgG_1 depleted the majority of follicular B cells while sparing marginal zone B cells (33). Thus, we were surprised that the B cell regulatory activity resided within the few follicular B cells that were not depleted (33). We then employed extensive flow cytometry cell surface phenotyping to determine that the non-depleted follicular B cells were enriched for IgD^{low} expressing B cells. Using FACS purified follicular IgD^{low} B cells, we showed that upon transfer into μMT mice, they drove Treg proliferation and EAE recovery. Importantly, neither follicular IgD^{hi} nor marginal zone B cells exhibited regulatory activity. Given that only follicular IgD^{low} B cells were regulatory in the context of our EAE model in conjunction with RNAseq studies, this evidence supported that we had identified a new B cell subset and named them B cell IgD low (BD_L). Several strategies were utilized to determine that BD_L are not part of the marginal zone B cell lineage. Developmental studies showed that BD_L emerge in the spleen after the transitional 2 subset, similar to follicular B cells. However, how BD_L fit into follicular B cell development is not known. A more comprehensive review of how BD_L were discovered and the assays used to identify then were recently published (88, 89).

We too, were interested in whether our findings were relevant to humans. In that regard, we found that BD_L activity existed in IgD^{low} B cells in both human spleen and peripheral blood, as determined using an *in vitro* Treg expansion assay (33). However, a definitive human BD_L phenotype was not identified nor were studies in MS performed. Thus, much remains to be discovered regarding B cell biology, particularly in the context of disease. In summary, by utilizing a variety of experimental approaches combined with comprehensive cell surface phenotyping and mechanistic studies, like IL-10 secreting plasmablasts/plasma cells, we have successfully identified a definitive B cell subset with regulatory activity (33, 88).

B CELL PHENOTYPING TO IDENTIFY REGULATORY B CELL SUBSETS

Previous research has uncovered many B cell subsets with regulatory potential that utilize a large cadre of regulatory mechanisms (10, 26). This highlights the complexity and challenges in targeting these populations for in-depth testing and therapeutic development. Here, we describe a strategy for identifying regulatory B cell subsets using gold-standard and state-of-the-art methodology that has three steps: 1) in-depth B cell subset phenotyping, 2) determination of B cell subset origin and 3) functional/mechanistic analysis. These steps do not need to be performed in a specific order, and the process of definitively characterizing a novel subset is often iterative and cyclic in nature (**Figure 3**).

In-Depth B Cell Subset Phenotyping

For in-depth B cell subset phenotyping, flow cytometry is a powerful tool that allows for comparative analysis of many cell surface and intracellular/intranuclear markers combined. However, conventional flow cytometry is limited in breath due to an upper limit of 12-14 colors (markers) with a conventional flow cytometer equipped with four lasers. If the flow cytometer also has a UV laser, 20 colors are possible. However, realistically, most laboratories have a upper limit of 6-8 colors. In our studies, identification of BD_L required six colors that includes B220 or CD19, IgM, IgD, CD21, CD23 and CD93 (33) (**Figure 4**). In addition, this combination of cell surface markers can be used to identify splenic transitional subsets, the marginal zone lineage and follicular B cells (**Figure 4**). In unpublished studies, we have now identified an additional BD_L marker making our panels seven colors. We also routinely phenotype Treg ($CD4^+Foxp4^+$) and if done in combination with B cell phenotyping would be nine colors. If B cells are enzymatically obtained from tissues the hematopoietic marker CD45 along with a live/dead stain need to be included. Determining IL-10 expression requires either a reporter or intracellular staining. Similarly

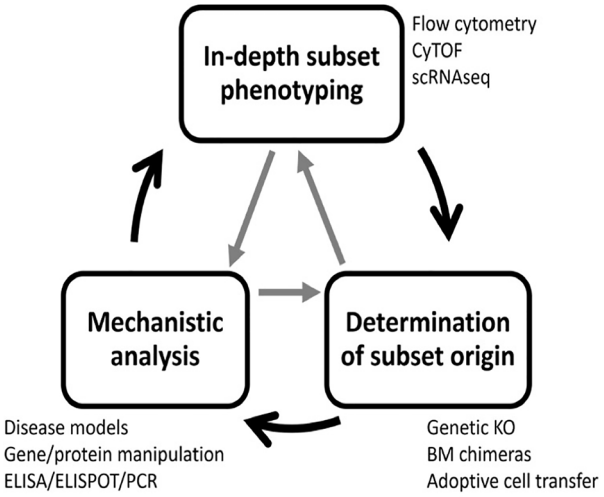

FIGURE 3 | A three-step process to identify regulatory B cell subsets. Complete characterization of novel regulatory B cell subsets requires three steps: subset phenotyping, lineage determination, and functional analysis. To accomplish this goal, many experimental techniques are required. The process is often iterative, and steps can be completed in any order and revisited as new information is gained.

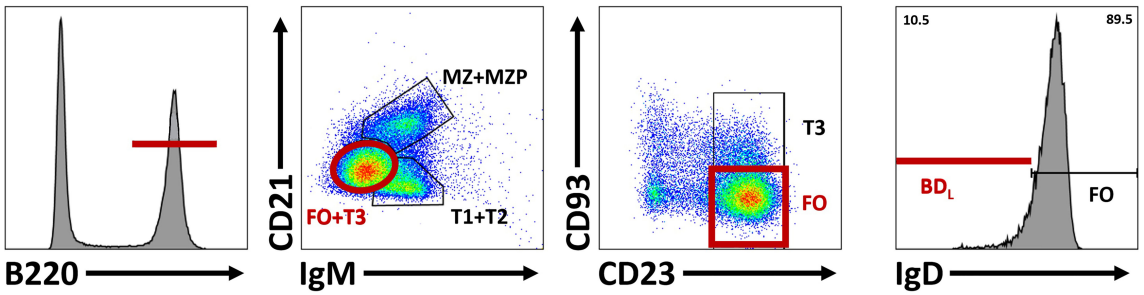

FIGURE 4 | Differentiation of BD_L from other splenic B cell subsets. To identify BD_L, $B220^+$ cells are selected and subsequent comparison of IgM and CD21 subdivides B cells into three groups based on maturity: marginal zone (MZ) and marginal zone precursors (MZP), transitional 1 (TI) and T2 cell and T3 and follicular (FO) B cells. CD23 and CD93 are subsequently used to identify FO B cells. Within the FO B cell subset, BD_L are selected for their low/negative expression of IgD.

complex, delineation between mouse plasmablasts and plasma cells from other B cell subsets requires both cell surface phenotyping, intracellular (IL-10/IL-35) and intranuclear transcription factor identification (i.e., Blimp-1, IRF4), because no cell surface markers alone identify these subsets (**Figure 5**). Surface markers including $CD19^{low/neg}$, $B220^{low}$, $CD20^-$, $CD38^+$, $CD44^{hi}$, $CD27^+$, $CD138^+$, $TACI^+$, $CXCR4^+$, $Tim1^+$, cell surface $Ig^{low/-}$ are used in addition to transcription factor identification to further characterize subsets of plasmablast/plasma cells (**Figure 5**). One major hurdle in identifying BD_L and plasmablasts/plasma cells is that they are rare, making their purification for downstream analysis challenging. Because transcription factor staining for flow cytometry kills target cells, Blimp-1 reporter animals are necessary to FACS purify plasmablasts/plasma cells (90). Additionally, IgM-targeting can activate B cells so care must be taken when purifying cell subsets using this marker. Other markers associated with B cell regulation to consider are CD5, CD1d, PD-L1 and FasL (10, 26). In mouse studies, it is important to analyze several lymphoid tissues to determine if the novel B cell regulatory subsets are sequestered to a portion of the body or more generally found in multiple tissue types.

B subset markers in mouse and human are relatively similar, but differences do exist. CD10 is a human B cell marker not used in mice and CD38 is used more often in humans than mice (60). Human tissues beyond peripheral blood can be difficult to obtain and B cells in circulation do not allow the analysis of specialized B cells subsets such as marginal zone B cells, germinal center B cells, long-lived plasma cells or mucosal B cell subsets. Human B cell subsets are also infinitely more complex than mice due to exposure to environmental and microorganism-derived antigens leading to isotype class switching and memory and plasma cell generation. Here, BD_L will be used to illustrate human B cell complexity. Although, we examined a number of B cell markers

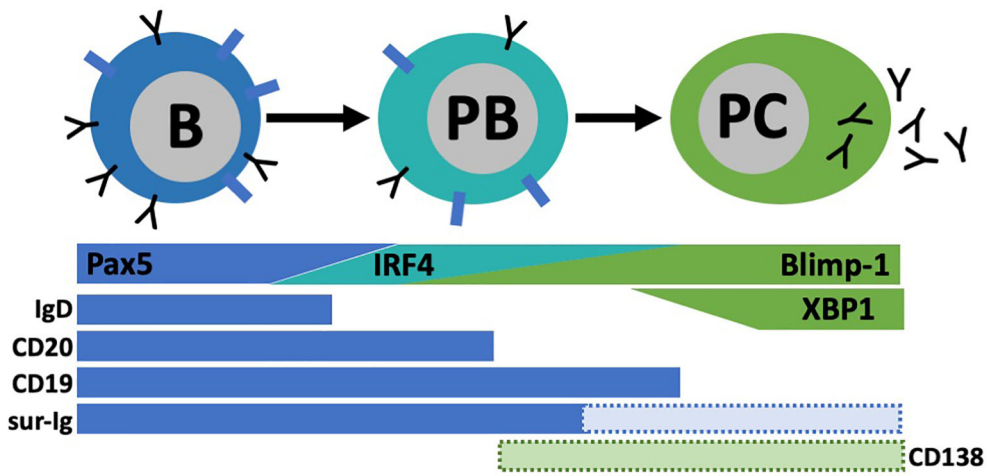

FIGURE 5 | Plasmablast (PB)/plasma cell (PC) differentiation. B cells are characterized by their high expression of transcription factor Pax5 and surface markers IgD, CD20, CD19, and surface Ig (sur-Ig). When B cells differentiate into PC, they transverse through a PB stage known for its high proliferative capacity. As PC maturation continues, Pax5 expression is lost and other transcriptional programs (IRF4, Blimp-I, XBPI) are upregulated. Concurrently, most B cell surface proteins are lost. Some subsets of PB and PC have expression of CD138 among other markers, but not all express these.

to narrow the BD_L phenotype in humans (unpublished observations), in the end, the only reliable phenotype was $CD19^+$ (or equivalent B cell marker) and IgD^{low}. In addition to BD_L, the $CD19^+IgD^{low/-}$ phenotype can include: 1) isotype class switched effector B cells, 2) isotype class switched memory B cells, 3) IgM memory B cells, 4) circulating transitional B cell subsets and 5) circulating marginal zone B cells. It is unlikely that flow cytometry alone could differentiate between the various $IgD^{low/-}$ human B cell subsets. Thus, newer technologies may need to be applied. These include imaging mass cytometry (CyTOF), which is a mass spectrometry approach that utilizes metal conjugated antibodies to identify ~50 parameters/cells with minimal overlap in the metal signals (91). Primary disadvantages include its lack of mainstream use, slow flow rate and destruction of the analyzed cells. Spectral flow cytometry is a newer technology that also has the advantage of minimal overlap of signals. While similar to conventional flow cytometry, its fluorescent emission spectrum is captured by detectors across several defined wavelength ranges. In essence, every molecule's fluorescent spectrum is recorded as a spectral signature that can be discriminated from other fluorescent signatures (92). Spectral flow cytometers are now commercially available and are slowly being incorporated into basic research studies. Finally, single cell RNA sequencing (scRNAseq) has become a strategy of choice to identify rare subsets of immune cells (93). Detection of cell surface proteins *via* antibodies (i.e., TotalSeq™) can be used alongside scRNAseq strategies (i.e., 10X Genomices) to analyze the transcriptional profiles of pre-marked immune cell subsets (94). In addition, full-length BCR sequences can also be obtained (95). The best platform to choose is dependent upon the experimental question. 10X Genomics allows the analysis of high numbers of individual cells (thousands) and can be combined with cell surface identification. However, its primary weakness is that sequence read depth is limited to high copy number transcripts, so unique markers expressed at low levels may be washed out during analysis. In comparison, both the Fluidym and Takara systems can analyze sequences with low copy number transcripts, but fewer cells can be analyzed per run (hundreds) (93, 96, 97).

Determination of B Cell Subset Origin

B cell phenotyping can provide strong evidence of the lineage from which a particular B cell subset derives. However, additional developmental studies should be performed. In comparison with conventional B2 B cells from the BM, B1 B cells are derived from the yolk sac/fetal liver, thus they can be eliminated with BM transplantation (98). Marginal zone B cells require Notch-2 signaling for development and any disruption in that pathway prevents their differentiation (99). Plasmablast/plasma cell differentiation can be prevented by loss of Blimp-1 expression (100). The loss of XBP-1 in plasmablasts/plasma cells can be used interrogate relevance of Ig secretion with other mechanisms (101). Germinal center B cells require BCL6 and its deficiency leads to truncation of the germinal center response (102). The transcription factor ABF-1 prevents plasma cells differentiation, without disrupting memory (103). Bach2 was recently shown to be essential for the transition from germinal center B cells to memory B cells (104). Undoubtably, other transcription factors can also be targeted to disrupt the development of specific B cell subsets. The developmental timing/kinetics of any B2 subset can be tracked after BM transplantation or sublethal irradiation (33). In addition, adoptive transfer is also an effective strategy for monitoring B cell subsets, especially when used in tandem with congenic markers (CD45.1/2) (33). For instance, the adoptive transfer of transitional 2 cells leads to the emergence of both the follicular and marginal zone lineages, as well as BD_L (33). When used together, these KO and cell reconstitution strategies can experimentally interrogate the lineage of regulatory B cell activity.

Functional/Mechanistic Analysis

Beyond identifying definitive markers of regulatory B cells subsets and where they come from, characterization of their functional capacity is equally important. Functional capacity includes 1) the mechanism through which the regulatory B cell yields an altered immune response and 2) the context in which this is applicable (type of disease, target cells). As evidenced through the story of B cells in EAE, not every regulatory B cell activity is relevant in all model systems and potentially multiple B cell subsets can be regulatory through different mechanisms at the same time. Thus, choosing and accurately describing the disease context is important for characterizing the regulatory B cell subset. Mechanisms of regulatory B cells can be investigated using many of the approaches discussed above where cellular activities (i.e.; IL-10/IL-35 secretion) are altered by gene/transcript-targeted deletion/overexpression or protein inhibition/stimulation with drug compounds. Genetic animal strains (including B cell-conditional varieties), BM chimeras, adoptive cell transfers and *in vitro* co-culture systems are used in tandem with these approaches to minimize confounding effects of gene manipulation on other, non-B cells. Further methods for investigating regulatory B cell mechanisms include measuring cytokines and effector molecule production *via* reporters (i.e.; IL-10, Blimp-1), ELISA, ELISPOT, quantitative PCR and RNAseq. While conducting these analyses, it is important to keep in mind the purity of tested cell subsets, this is where in-depth B cell phenotyping can aid in and strengthen the interpretation of results.

CONCLUDING REMARKS

To ensure the accurate identification of novel B cell subsets with regulatory activity and their mechanism, consistent and comprehensive phenotyping along with mechanistic studies are necessary. The four laboratories, including our own, whose work was discussed in this review were chosen because they all utilized multiple strategies to identify a definitive B cell subset whose mechanism of action was confirmed using multiple mechanistic approaches. These included KO mice, BM chimeras, genetic approaches and adoptive cell transfer, among others. Although each story was years in the making, cumulatively, they provide a path by which others can follow to discover novel regulatory B cell subsets and mechanisms in the context of other diseases. Similar investigations have been made in models beyond MS/EAE that researchers interested in B cell regulation should explore. The

identification of regulatory B cell subsets and their mechanism leads to the question of how they can be exploited for the treatment of anti-inflammatory diseases. The main hurdle with utilizing B cells as an adoptive cell therapy is that they do not self-renew, like their T cell counterparts. Thus, longevity must be addressed. In the context of IL-10, adoptive transfer of long-lived plasma cells that produce IL-10 is being actively explored. We are actively exploring how BD_L can be utilized as an adoptive cell therapy to increase Treg numbers in autoimmunity. These are exciting times for research in B cell regulation and given the complexity of the entire B cell lineage new B cell subsets and regulatory mechanisms are surely to be discovered.

AUTHOR CONTRIBUTIONS

BD and SN both wrote and edited the article. All authors contributed to the article and approved the submitted version.

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Altered Tim-1 and IL-10 Expression in Regulatory B Cell Subsets in Type 1 Diabetes

Yikai Liu, Zhiying Chen, Junlin Qiu, Hongzhi Chen and Zhiguang Zhou**

National Clinical Research Center for Metabolic Diseases, Key Laboratory of Diabetes Immunology, Ministry of Education, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, Changsha, China

Background: Type 1 diabetes (T1D) is an autoimmune disease with a complex aetiology. B cells play an important role in the pathogenesis of T1D. Regulatory B cells (Bregs) are a subset of B cells that produce and secrete the inhibitory factor interleukin-10 (IL-10), thereby exerting an anti-inflammatory effect. It was recently discovered that T-cell immunoglobulin mucin domain 1 (Tim-1) is essential for maintaining Bregs function related to immune tolerance. However, the detailed understanding of Tim-1$^+$ Bregs and IL-10$^+$ Bregs in T1D patients is lacking. This study aimed to characterize the profile of B cell subsets in T1D patients compared with that in controls and determine whether Tim-1$^+$ Bregs and IL-10$^+$ Bregs play roles in T1D.

Materials and Methods: A total of 47 patients with T1D, 30 patients with type 2 diabetes (T2D) and 24 healthy controls were recruited in this study. Flow cytometry was used to measure the levels of different B cell subsets (including B cells, plasmablasts, and Bregs) in the peripheral blood. Radiobinding assays were performed to detect the antibody titres of T1D patients. In addition, the correlations between different B cell subsets and patient parameters were investigated.

Results: Compared with healthy controls, differences in frequency of Tim-1$^+$ Bregs were significantly decreased in patients with T1D (36.53 ± 6.51 *vs*. 42.25 ± 6.83, P=0.02*), and frequency of IL-10$^+$ Bregs were lower than healthy controls (17.64 ± 7.21*vs*. 24.52 ± 11.69, P=0.009**), the frequency of total Bregs in PBMC was also decreased in patients with T1D (1.42 ± 0.53*vs*. 1.99 ± 0.93, P=0.002.**). We analyzed whether these alterations in B cells subsets were associated with clinical features. The frequencies of Tim-1$^+$ Bregs and IL-10$^+$ Bregs were negatively related to fasting blood glucose (FBG) (r=-0.25 and -0.22; P=0.01* and 0.03*, respectively). The frequencies of Tim-1$^+$ Bregs and IL-10$^+$ Bregs are positively correlated with fast C-peptide (FCP) (r=0.23 and 0.37; P=0.02* and 0.0001***, respectively). In addition, the frequency of IL-10$^+$ Breg was also negatively related to glycosylated haemoglobin (HbA1c) (r=-0.20, P=0.04*). The frequencies of Tim-1$^+$ Bregs, IL-10$^+$ Bregs and Bregs in T2D patients were reduced, but no statistically significant difference was found between other groups. Interestingly, there was positive correlation between the frequencies of Tim-1$^+$ Bregs and IL-10$^+$ Bregs

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Edited by:
Luman Wang,
Fudan University, China

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Aaron Michels,
University of Colorado, United States
Guangbo Zhang,
Soochow University, China

***Correspondence:**
Zhiguang Zhou
zhouzhiguang@csu.edu.cn
Hongzhi Chen
chenhongzhi2013@csu.edu.cn

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in T1D (r=0.37, P=0.01*). Of note, it is worth noting that our study did not observe any correlations between B cell subsets and autoantibody titres.

Conclusions: Our study showed altered Tim-1 and IL-10 expression in regulatory B cell in T1D patients. Tim-1, as suggested by the present study, is associated with islet function and blood glucose levels. These findings indicate that Tim-1$^+$ Bregs and IL-10$^+$ Bregs were involved in the pathogenesis of T1D.

Keywords: Tim-1, IL-10, T1D (type 1 diabetes), regulatory B (Breg) cells, autoantibody

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease mediated by T cells that selectively destroys insulin-producing β cells (1). It is generally believed that B cells, an important type of antigen-presenting cell that expresses costimulatory signalling molecules, participate in the activation and expansion of autoreactive CD4$^+$ T cells and CD8$^+$ cytotoxic T cells, thereby contributing to the occurrence of T1D (2).

In animal model experiments, B cells have been shown to infiltrate the islets of young nonobese diabetic (NOD) mice and mediate the autoimmune response that causes β cell destruction (3). In clinical trials, the use of rituximab (an anti-CD20 monoclonal antibody) was shown to effectively postpone the decline in pancreatic β cell function in patients with T1D (4). These results demonstrated that B lymphocytes play an indispensable and important role in the pathogenesis of T1D. This finding opened up a new way to explore T1D. Multiple subsets of B cells may be involved in immune homeostasis and prevent autoimmunity. Regulatory B cells (Bregs) are characterized by a cell-surface CD19$^+$ CD24^{hi} CD38^{hi} phenotype (5). It has recently been established that Bregs have regulatory functions in infections, allergies, transplantation and autoimmune diseases (6). Bregs down-regulate the immune response by producing the inhibitory cytokine interleukin-10 (IL-10). Therefore, Bregs play important roles in immune tolerance. IL-10 is an anti-inflammatory cytokine mainly produced by Bregs. It regulates cell growth and differentiation and participates in inflammatory and immune responses. This cytokine is currently recognized as an immunosuppressive factor (7). T cell Ig and mucin domain (Tim-1), a transmembrane glycoprotein, has been identified as one of the three human Tim family members (Tim-1, Tim-3, and Tim-4) (8). Tim-1 was previously reported to be expressed and function in T cells; in addition to its role in T cells, Tim-1 signalling on B cells was recently shown to play roles in maintaining the stability of the immune system and inhibiting autoimmune diseases (9). Researchers have generated Tim-1 mutant mice [Tim-1 (Δmucin)], in which the mucin domain of Tim-1 is genetically deleted. The function of Bregs in these mutant mice to produce IL-10 is defective (10).

There is a growing list of autoimmune diseases that are associated with Breg dysregulation, including multiple sclerosis (MS), systemic lupus erythaematosus (SLE) and rheumatoid arthritis (RA) (11–13). In addition, many studies have demonstrated that there are negative correlations between the frequency of Bregs and the activity of some diseases. However, little is known about the frequency of Tim-1 or IL-10 in Bregs in patients with T1D. Therefore, the aim of this study was to compare the frequencies of Tim-1$^+$ Bregs and IL-10$^+$ Bregs in patients with T1D to those in healthy controls.

MATERIALS AND METHODS

Patients and Controls

Forty-seven patients with T1D (28 males and 19 females; mean age=30.79 ± 9.05), 30 T2D (17 males and 13 females; mean age=46.63 ± 9.43) and 24 healthy controls (15 males and 9 females; mean age=33.33 ± 6.20) were enrolled in this study at the Second Xiangya Hospital. The diagnosis of T1D was made based on the World Health Organization (WHO) criteria (14). The diagnosis of T1D was based on acute onset ketosis or ketoacidosis, and all patients diagnosed with T1D required immediate insulin replacement therapy. At least one classic pancreatic islet autoantibody (anti-GAD antibodies [GADA], anti-zinc transporter 8 antibodies [ZnT8A] or anti-insulinoma-associated protein-2 antibodies [IA-2A]) was positive, or patients exhibited impaired C-peptide secretion. The diagnostic criteria for T2D is a typical history of hyperglycaemia according to WHO criteria, islet autoantibodies are negative and insulin therapy is not needed immediately. 24 healthy controls had no history of autoimmune diseases, pregnancy, or malignant diseases. All patients were confirmed to be negative for chronic infection disease, allergic or cancer and did not receive any immunosuppressive medication. Our study was approved by the ethics committee of Second Xiangya Hospital, Central South University. All subjects voluntarily joined this study and provided informed consent. The characteristics of all subjects are summarized in **Table 1**.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Venous blood samples (5 mL) were collected from each subject into heparinized sodium tubes, and PBMCs were isolated by Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. After centrifugation at 800 rcf for 23 min, PBMC was separated from the whole blood.

TABLE 1 | General characteristics of study subjects.

	Type 1 diabetes	Type 2 diabetes	Healthy control
Sex(male/female)	28/19	17/13	15/9
Age (years)	30.79 ± 9.05****	46.63 ± 9.43####	33.33 ± 6.20
TG (mmol/L)	0.81(0.54-1.17)****	1.66(1.18-2.43)	1.12 (0.75-1.77)Δ
TC (mmol/L)	4.20 ± 0.98	4.72 ± 0.95	4.36 ± 0.80
LDL-C (mmol/L)	2.35 ± 0.71**	2.95 ± 0.83	2.56 ± 0.82
HDL-C (mmol/L)	1.56 ± 0.48***	1.17 ± 0.21#	1.45 ± 0.49
FBG (mmol/L)	7.06(5.43-10.37)	8.33(6.52-11.12)####	4.80(4.45-5.18)ΔΔΔΔ
HbA1c(%)	7.45 ± 1.45*	8.16 ± 1.74####	5.31 ± 0.34ΔΔΔΔ
FCP (pmol/L)	39.30 (16.5-154.1)****	436.9(252.4-567.0)	556.8(332.9-734.9)ΔΔΔΔ
Duration (months)	40.66 ± 24.69*	68.47 ± 50.46	NA
GADA	27/47 (57.4%)	NA	NA
IA-2A	11/47 (23.4%)	NA	NA
ZnT8A	11/47 (23.4%)	NA	NA

Data are expressed as (n) for qualitative data, the mean ± standard deviation for parametric data and the median (interquartile ranges) for nonparametric data. NA, not applicable.
**P < 0.05 compared to T2D, **P < 0.01 compared to T2D, ***P < 0.001 compared to T2D, ****P < 0.0001 compared to T2D.*
#P < 0.05 compared to healthy control, ####P < 0.0001 compared to healthy control.
ΔP < 0.05 compared to T1D, ΔΔΔΔP < 0.0001 compared to T1D.

Islet Autoantibody Assays

Radiobinding assays for GADA, ZnT8A and IA-2A were carried out as previously described (15, 16). Positive test results for GADA, IA-2A or ZNT8A were defined as 18.5 units/mL, 3.3 units/mL (WHO unit), and 0.011 (ZnT8A index), respectively.

Glycosylated Haemoglobin (HbA1c) and β Cell Function

HbA1c levels were measured by liquid chromatography (Tosoh Corporation, Tokyo, Japan). Fasting C-protein (FCP) was measured by a chemiluminescence method (Siemens, Munich, Germany).

Flow Cytometry

For intracellular staining of cytokines, PBMCs were stimulated with 1 μl/ml cell activation cocktail (BioLegend, San Diego, CA, USA) and 1 μl/ml monensin (BioLegend, San Diego, CA, USA) for 6 hours. PBMCs were harvested and stained with fixable viability stain (BD, Franklin Lakes, NJ, USA) and various monoclonal antibodies (anti-CD19-FITC, anti-CD24-PE, anti-CD38-APC, and anti-IL-10-PerCP-Cy5.5) (BD, Franklin Lakes, NJ, USA). Immunofluorescence staining for flow cytometric analysis was performed following the manufacturer's instructions.

To determine Tim-1^+ Breg frequencies, PBMCs were stained for CD19, CD24, CD38, and Tim-1 (anti-CD19- FITC, anti-CD24-FE, anti-CD38-APC, and anti-Tim-1-PerCP-Cy5.5) as surface markers. Data were collected on a Canto flow cytometer (BD, Franklin Lakes, NJ, USA) and analysed with FlowJo X software (Tree Star, Ashland, OR, USA). Dead cells were excluded from flow cytometric analysis on the basis of their forward- and side-light scatter properties and staining with Fixable Viability Stain 780 (BD, Franklin Lakes, NJ, USA). The gating strategies for all B cell subsets are described in **Figure 1**. PBMCs were isolated, and $CD19^+$ B cell subsets were defined as follows: $CD19^+$ (total B cells), $CD19^+$ $CD24^{hi}$ $CD38^{hi}$ (Bregs), and $CD19^+$ $CD24^-$ $CD38^{hi}$ (plasmablasts). The results are expressed as the percentages of Tim-1^+ Bregs and IL-10^+ Bregs (17).

Statistical Analysis

All statistical analyses were performed with IBM SPSS version 24 (IBM Corporation, Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). In the correlation analysis, the frequency of various B cell subsets was logarithmically transformed, the clinical features conforming to the normal distribution were analyzed by Pearson correlation, and the non-parametric data was analyzed by Spearman. Data are presented as the mean ± standard deviation of the mean. Continuous variables were compared by one-way ANOVA. $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Demographic and Clinical Characteristics

One hundred and one participants were enrolled in our study, and **Table 1** summarizes the features of the subjects in each group. The T1D patients included 28 males and 19 females. The mean duration of T1D after diagnosis was 40.66 ± 24.69 months. T2D patients were older than T1D and healthy control groups, and the duration of diabetes was longer than T1D (68.47 ± 50.46 months). Compared with other groups, T2D have higher levels of triglycerides(TG), total cholesterol(TC), LDL cholesterol(LDL-C) and HDL cholesterol (HDL-C). The fasting blood glucose levels of the two groups of diabetic patients were similar, but the HbA1c of T2D was higher than that of T1D. Of note, the FCP of T1D was significantly lower than that of T2D and healthy controls. There was no statistical difference in gender or age among the three groups.

Frequencies of B Cell Subsets Among T1D, T2D and Healthy Controls

The gating strategy for B cell subset quantification was based on CD19, CD24 and CD38 frequency, as shown in **Figure 1**. Flow cytometric analysis indicated that Tim-1^+ Bregs were significantly decreased in T1D patients (36.53 ± 6.51 *vs.* 42.25 ± 6.83, P=0.02*) (**Figure 2A**). In contrast, the frequency of IL-10^+ Bregs was lower

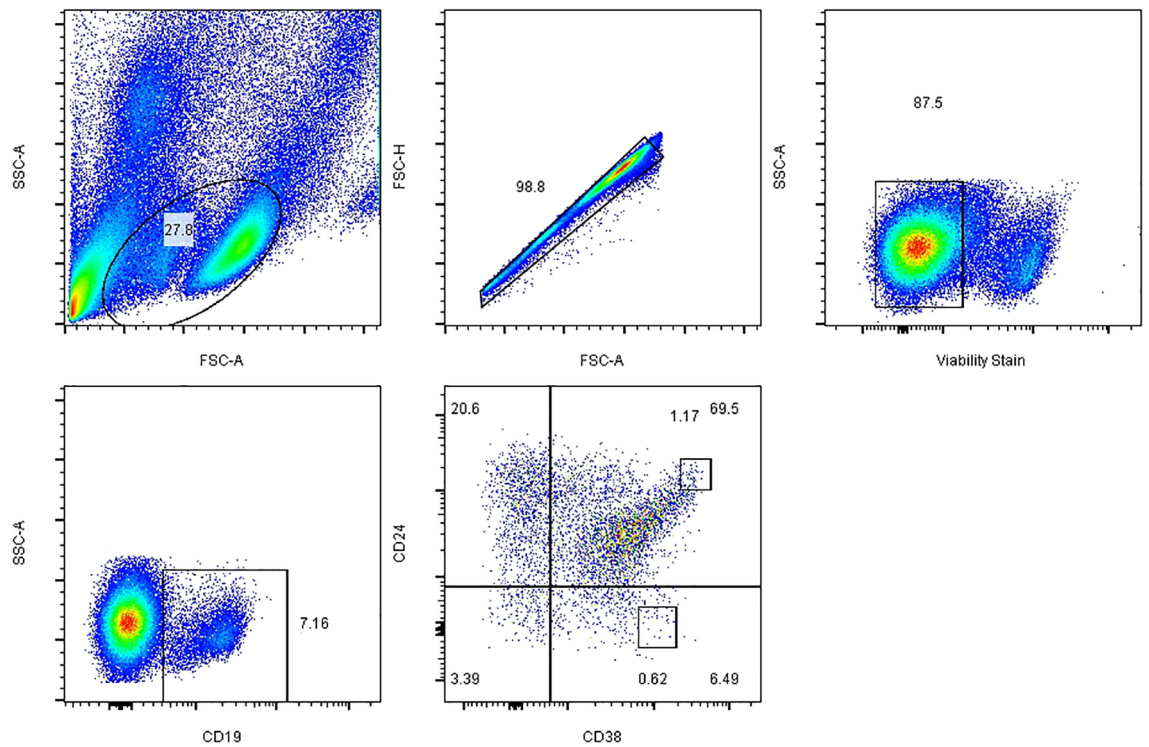

FIGURE 1 | Representative plots and the gating strategy for fluorescence-activated cell sorting analysis of B cell subsets. Initial $CD19^+$ B cells were sequentially gated on total lymphocytes, single cells, live cells and $CD19^+$ B cells, and the expression of Breg and plasmablast markers on $CD19^+$ cells was further analysed. FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area.

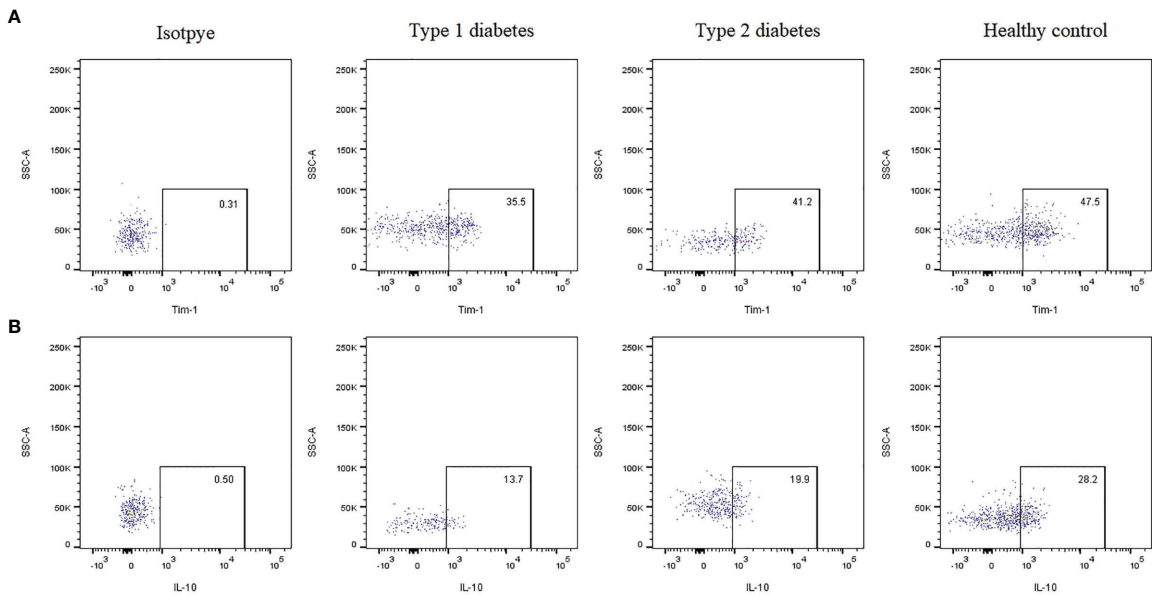

FIGURE 2 | Representative dot plots showing the expression of Tim-1 or IL-10 by Bregs in T1D, T2D patients and healthy controls evaluated using flow cytometric analysis. **(A)** Frequency of Tim-1^+ Bregs in various groups. **(B)** Frequency of IL-10^+ Bregs in various groups.

in T1D patients than that of healthy controls (17.64 ± 7.21 *vs.* 24.52 ± 11.69, *P*=0.009**) (**Figure 2B**). The frequency of Tim-1$^+$ Bregs and IL-10$^+$ Bregs in T2D patients was amid that of T1D and healthy controls (38.11 ± 11.30, 20.67 ± 9.19, respectively). But there was no statistical difference between T2D and other two groups(*P*>0.05 for both).

The differences in the frequencies of different B cell subsets are summarized in **Figure 3**. There were no significant differences in the frequencies of total B cells (CD19$^+$) or plasmablasts (CD19$^+$ CD24$^-$ CD38^{hi}) between T1D, T2D and controls (10.87 ± 4.56 and 1.07 ± 0.42 in T1D patients, 9.17 ± 4.3 and 1.00 ± 0.44 in T2D patents, 9.20 ± 4.4 and 1.03 ± 0.39 in healthy controls for total B cells and plasmablasts, respectively). Interestingly, Bregs (CD19$^+$ CD24^{hi} CD38^{hi}) were significantly lower in T1D patients than that of healthy controls (*P*=0.002**). The mean frequency of Bregs in the T1D patients was 1.42 ± 0.53, while the value in the controls was 1.99 ± 0.93. Compared with healthy controls, the frequency of Bregs in T2D patients showed a decreasing trend (1.78 ± 0.61), but no significant difference was found between the T2D and other two groups.(*P*=0.06 compared to T1D, *P* =0.48 compared to controls, respectively).

Correlations Between the Frequencies of B Cell Subsets and Clinical Features of All the Study Subjects

Given that B cells play an important role in the development of diabetes, we next determined whether the changes in various B cell subsets in all participants had any correlation with clinical

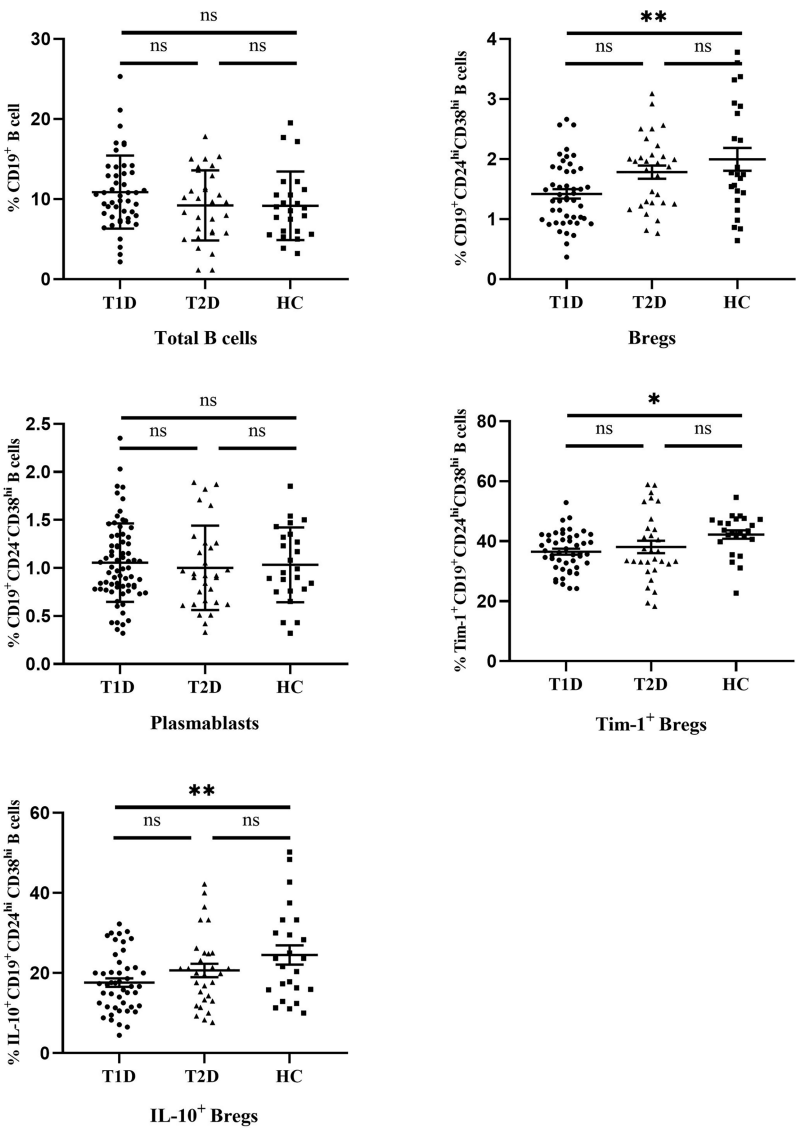

FIGURE 3 | The frequencies of different B cell subsets in T1D, T2D patients and healthy controls. Horizontal lines show the medians. T1D, type 1 diabetes; T2D, type 2 diabetes; HC, healthy control. ns, no significant; *$P < 0.05$; **$P < 0.01$.

features (**Table 2**). The frequencies of Tim-1$^+$ Bregs and IL-10$^+$ Bregs were negatively correlated to fasting blood glucose (FBG) (r=-0.25 and -0.22; P=0.01* and 0.03*, respectively). and positively correlated with FCP (r=0.23 and 0.37; P=0.02* and 0.0001***, respectively). Moreover, the frequency of IL-10$^+$ Bregs was also negatively related to glycosylated haemoglobin (HbA1c) (r=-0.20, P=0.04*). In addition, we also found a positive correlation between Tim-1$^+$ Bregs and IL-10$^+$ Bregs in T1D patients (r=0.37, P=0.01*) (**Figure 4**).

Correlation Between Tim-1$^+$ or IL-10$^+$ Bregs With Autoantibodies in T1D Patients

We then examined the relationships between Tim-1$^+$ Bregs or IL-10$^+$ Bregs and the studied autoantibodies. There were no significant differences in the frequencies of Tim-1$^+$ Bregs or IL-10$^+$ Bregs among T1D patients who were positive for one, two, or three of the antibodies (**Figure 5A**). Additionally, no difference in Tim-1$^+$ Bregs or IL-10$^+$ Bregs was observed in patients regardless of autoantibody testing results(**Figure 5B**).

DISCUSSION

In the present study, we investigated the frequencies of various B cell subsets in patients with T1D. Our study showed that the frequency of Bregs, Tim-1$^+$ Bregs and IL-10$^+$ Bregs in diabetic patients showed a declining trend, which was statistically significant in the T1D group. FBG and HbA1c respectively reflect the short- and long-term blood glucose level of diabetic patients, although the T2D had higher FBG and HbA1c levels compared with the T1D patients, the frequencies of Bregs, Tim-1$^+$ Bregs and IL-10$^+$ Bregs in the T2D appears not to be lower than that in the T1D. This discrepancy may be due to heterogeneous pathogenesis of the two diabetes groups. T1D is a chronic autoimmune disease that is mainly caused by the destruction of pancreatic islet β cells mediated by T lymphocyte. Impaired pancreatic islet β cells exhibits a decreased FCP levels (18). B cells are important antibody-producing cells, antigen-presenting cells, and expressing costimulatory signals involved in the activation of CD4$^+$ and CD8$^+$ T cells. The dysfunction of B cells has an important impact on the pathogenesis of type 1 diabetes. However, metabolic dysfunction and chronic inflammation are the main clinical symptoms in T2D patients (19). TG, TC, HDL-C, and LDL-C are known for common metabolic indicator (20). We did not identify any correlation between Breg subsets and these indices in all subjects. This result ties well with previously published research (21). All these results indicate that changes in B cell subsets may contribute to immune disorders. In addition, correlations between the frequencies of B cell subsets and clinical features were confirmed in all subjects. The frequencies of Tim-1$^+$ and IL-10$^+$ Bregs were negatively correlated with FBG and positively correlated with the FCP levels. The frequency of IL-10$^+$ Bregs was also negatively correlated with HbA1c levels, However, neither Tim-1$^+$ Bregs nor IL-10$^+$ Bregs were related to the number and types of islet-related autoantibodies.

The Tim gene family contains three Tim proteins (Tim-1, Tim-3, and Tim-4). Tim-1 is a glycoprotein involved in the immune response. Tim-3 expression has been detected on innate and adaptive immune cells. Tim-4 is a natural ligand of Tim-1. Emerging evidence suggests that the binding of Tim-1 to Tim-4 is involved in T cell proliferation (22). However, whether Tim-3 or Tim-4 is expressed in Breg cells is not yet clear (23). Early studies found that Tim-1 is expressed in T cells participates in the activation of T cells and regulates the immune response of T helper cells (8). Recent studies revealed that Tim-1 also expressed in other immune cells, such as mast cells (24), Tregs and B cells (25, 26). Interestingly, Tim-1 signal transduction is necessary for the maintenance and induction of IL-10 in Bregs (9). Tim-1-mutant B cells exhibit a reduced apoptotic cells binding capacity and incapable to produce IL-10 effectively (25). Aravena et al. evaluated the frequency of Tim-1 in different B cell subpopulations. Their study presented that human transitional B cells, as a phenotype associated with Bregs, have the highest content of Tim-1$^+$ cells, and most Tim-1$^+$ Bregs also express IL-10 (27). It is worth noting that in their study, the frequency of

TABLE 2 | Correlations between the frequencies of B cell subsets and clinical features of all the study subjects.

	CD19$^+$ B cells		Bregs		Plasmablasts		Tim-1$^+$ Bregs		IL-10$^+$ Bregs	
	r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Male[a]	0.04	0.68	0.02	0.81	-0.06	0.56	-0.13	0.19	-0.01	0.89
Age	-0.18	0.07	0.15	0.11	-0.17	0.08	0.10	0.33	0.76	0.45
TG[a]	-0.14	0.16	0.08	0.43	-0.10	0.34	0.11	0.29	0.15	0.13
TC	-0.07	0.49	0.11	0.28	-0.15	0.14	0.05	0.63	0.01	0.95
LDL-C	-0.10	0.35	0.18	0.07	-0.07	0.50	0.02	0.86	-0.02	0.83
HDL-C	0.14	0.15	-0.12	0.25	-0.11	0.28	0.01	0.91	-0.00	0.99
FBG[a]	-0.08	0.44	-0.19	0.06	-0.12	0.23	-0.25	0.01*	-0.22	0.03*
HbA1c	-0.01	0.93	-0.14	0.16	-0.08	0.44	-0.15	0.12	-0.20	0.04*
FCP[a]	-0.18	0.07	0.17	0.08	-0.02	0.86	0.23	0.02*	0.37	0.00***
GADA[a]	-0.09	0.55	0.19	0.20	0.26	0.07	-0.01	0.92	-0.10	0.48
IA-2A[a]	-0.01	0.93	-0.07	0.62	0.25	0.09	0.14	0.34	0.10	0.48
ZnT8A[a]	0.19	0.20	-0.07	0.63	0.02	0.91	-0.05	0.73	-0.07	0.64

Correlation analyses between various B cell subset frequencies (after log transformation) and clinical features were performed by Pearson test.
*[a]compared by spearman correlations. * indicates significance (P < 0.05), *** indicates significance (P < 0.001).*

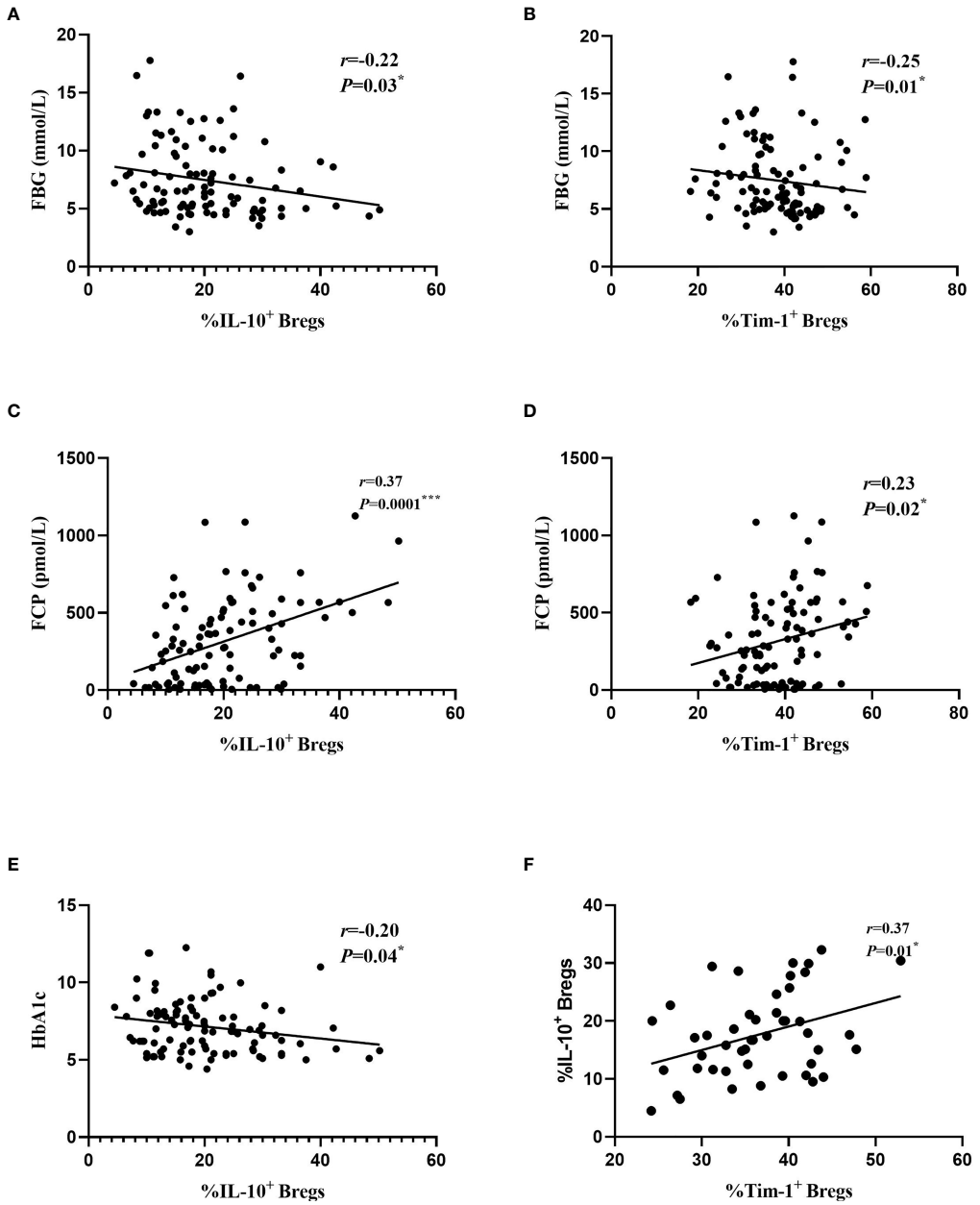

FIGURE 4 | Relationships between different B cell subsets and clinical data from all the study subjects. **(A)** Correlations between IL-10$^+$ Bregs and FBG. **(B)** Correlations between Tim-1$^+$ Bregs and FBG. **(C)** Correlations between IL-10$^+$ Bregs and FCP. **(D)** Correlations between Tim-1$^+$ Bregs and FCP. **(E)** Correlations between IL-10$^+$ Bregs and HbA1c. **(F)** Correlations between IL-10$^+$ Bregs and Tim-1$^+$ Bregs in T1D. Each point represents an individual patient with T1D, T2D and healthy controls. *$P < 0.05$. ***$P < 0.001$.

Tim-1$^+$ Bregs in systemic sclerosis patients was lower than that in healthy people. At the same time, the competence of the Tim-1$^+$ Bregs in patients was compromised. All these results indicate that Tim-1 is essential for regulating immune tolerance in Bregs.

As a major immunomodulatory cytokine, IL-10 affects many cells of the immune system (28). It has a powerful anti-inflammatory function, mainly targeting antigen-presenting cells (APCs) such as monocytes and macrophages. It also inhibits the release of pro-inflammatory cytokines. IL-10 can reduce the expression of major histocompatibility antigen II (MHC II) on the surface of monocytes. Thus it reduces antigen presentation by these cells, downregulates the activity of T lymphocytes, and inhibits the activation, migration and adhesion of inflammatory cells. At the same time, IL-10 can also inhibit the synthesis and release of inflammatory factors in Th cells (29). Moreover, IL-10 can directly act on T cells to inhibit their proliferation and cytokine production. Thereby reducing inflammation. Meanwhile, IL-10 plays an important role in promoting the activation and

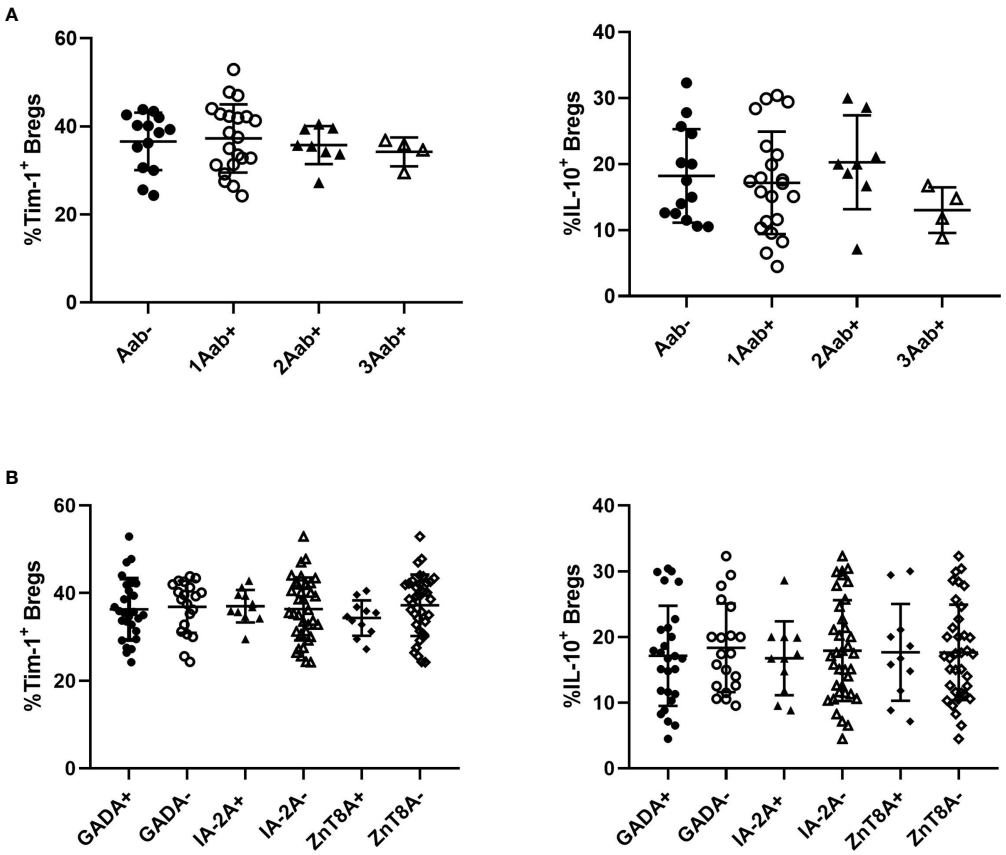

FIGURE 5 | Relationships between Tim-1 or IL-10 expressed by Bregs and autoantibodies in T1D patients. **(A)** Correlations between the number of positive antibodies and the frequency of Tim-1$^+$ or IL-10$^+$ Bregs. **(B)** Correlations between the types of positive antibodies and the frequency of Tim-1$^+$ or IL-10$^+$ Bregs.

proliferation of human B cells (7). The immunomodulatory properties of Bregs depend on the production of IL-10. The importance of IL-10 has been confirmed both *in vivo* and *in vitro*. However, due to the low frequency of Bregs and the lack of specific transcription markers, research on how Bregs are induced is still limited.

Deng et al. illustrated that changes in the phenotypes of B cell subsets are related to the onset of autoimmune diabetes. The frequencies of B cell subsets that produce IL-10 (defined as B10) in T1D patients were found to be reduced, and the frequency of B10 cells was positively correlated with FCP and negatively correlated with HbA1c (21). Wang et al. showed that the frequencies of CD24^{hi} CD38^{hi} B cells in T1D patients were reduced. In addition, the frequency of CD24^{hi} CD38^{hi} B cells was negatively correlated with HbA1c (30) which was similar to the current study. Our data showed that compared with healthy controls, T1D patients exhibit decreased levels of Tim-1$^+$ Bregs, IL-10$^+$ Bregs and total Bregs. Moreover, the frequency of IL-10$^+$ Bregs was positively correlated with Tim-1$^+$ Bregs in T1D patients.

Diabetes-related autoantibodies can be used to assess the progression of T1D (31), and the titres of autoantibodies also seem to affect the complications of T1D (32, 33). High-titre antibodies are more likely to be accompanied by other autoimmune diseases or autoimmune-related antibody positivity. Islet autoantibodies (including GADA, IA2A and ZnT8A) can predict the risk of T1D and improve the sensitivity of diagnosing T1D patients (34, 35). Nevertheless, we did not find a correlation between B cell subsets and islet-related autoantibody in the current study. Considering that there are differences in measured GADA titres among different laboratories, it is difficult to set a threshold for distinguishing high and low levels. Furthermore, GADA titres may also be related to the age, disease course, and medication level of the patient.

Overall, our work suggests that the frequencies of Bregs, Tim-1$^+$ Bregs and IL-10$^+$ Bregs in T1D patients are reduced, and the latter two were correlated with islet function and blood glucose levels. The current data indicates that alterations in B cell subsets are related to the pathogenesis of autoimmune diabetes. Our results highlight the potential of selective B cell-targeted therapy aimed at specifically eliminating pathogenic B cells but promoting Bregs in T1D patients, which is a method being reconsidered. Our work has certain limitations. First, it is a small sample size study. Attention may need be paid when extending the conclusion to large population. Second, patients in this study possess relatively long courses of disease. In view of the low islet-related antibody positivity rate, it is very likely that

autoantibodies turned negative in some patients prior to this study, which may affect the correlation study of Tim-1 and autoantibodies. Third, our study was cross-sectional. We did not track our patients longitudinally, nor did we perform a cohort study. Long-term and large-scale experiments, such as experiments on B cell subset functional levels and metabolic pathway level changes, need to be performed in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Second Xiangya Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL searched the references, wrote the first draft of the paper, and revised the text. ZC and JQ critically revised the text and provided substantial scientific contributions. ZZ and HC proposed the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Chloroquine Suppresses Effector B-Cell Functions and Has Differential Impact on Regulatory B-Cell Subsets

*Xin Ma[1,2], Yang Dai[1], Oliver Witzke[3], Shilei Xu[1], Monika Lindemann[4], Andreas Kribben[1], Sebastian Dolff[3] and Benjamin Wilde[1]**

[1] Department of Nephrology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [2] Department of Nephrology, First Affiliated Hospital of Chengdu Medical College, Chengdu, China, [3] Department of Infectious Diseases, West German Centre of Infectious Diseases, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [4] Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Objectives: Chloroquine (CQ) is approved for treatment of B-cell mediated diseases such as rheumatoid arthritis and systemic lupus erythematosus. However, the exact mode of action in these diseases has not been studied and it remains unclear which effect CQ has on B-cells. Thus, it was the aim of this study to investigate to which extent CQ affects functionality of effector and regulatory B-cell.

Methods: For this purpose, B-cells were isolated from peripheral blood of healthy controls and renal transplant patients. B-cells were stimulated in presence or absence of CQ and Interleukin-10 (IL-10) and Granzyme B (GrB) secretion were assessed. In addition, effector functions such as plasma cell formation, and Immunoglobulin G (IgG) secretion were studied.

Results: CQ suppressed Toll-Like-Receptor (TLR)-9 induced B-cell proliferation in a dose-dependent manner. IL-10^{pos} regulatory B-cells were suppressed by CQ already at low concentrations whereas anti-IgG/IgM-induced GrB secreting regulatory B-cells were less susceptible. Plasma blast formation and IgG secretion was potently suppressed by CQ. Moreover, purified B-cells from renal transplant patients were also susceptible to CQ-induced suppression of effector B-cell functions as observed by diminished IgG secretion.

Conclusion: In conclusion, CQ had a suppressive effect on IL-10 regulatory B-cells whereas GrB secreting regulatory B-cells were less affected. Effector functions of B-cells such as plasma blast formation and IgG secretion were also inhibited by CQ. Effector B-cells derived from renal transplant patients already under immunosuppression could be suppressed by CQ. These findings may partly explain the clinical efficacy of CQ in B-cell mediated autoimmune diseases. The application of CQ in other disease contexts where suppression of effector B-cells could offer a benefit, such as renal transplantation, may hypothetically be advantageous.

Keywords: chloroquine, regulatory B (Breg) cells, renal transplantation, B-cells, effector B-cells

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Yiwei Chu,
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Reviewed by:
Yoshiko Matsuda,
National Center for Child Health and Development (NCCHD), Japan
Takashi Matsushita,
Kanazawa University, Japan

***Correspondence:**
Benjamin Wilde
benjamin.wilde@uk-essen.de

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INTRODUCTION

Chloroquine and its derivate hydroxychloroquine (CQ) have originally been developed as antimalarial drugs. In addition, CQ was investigated in multiple clinical trials for efficacy in inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (1, 2). In these trials, CQ was shown to reduce disease activity and thus it is now an established therapeutic drug which is used regularly. The mechanisms by which CQ reduces disease activity have not been unraveled completely; experimental evidence indicates that it interferes with lysosomal acidification, intracytoplasmatic calcium mobilization, antagonizes nucleic acid ligands for toll-like-receptors, inhibits autophagy and suppresses the cyclic GMP-AMP-synthetase/stimulator of interferon genes (cGAS/STING) pathway (3). Whereas the effects of CQ on T-cells have been studied by several groups, less is known on the impact of CQ on B-cell effector and regulatory function (3, 4). It was the aim of this study to assess the effect of CQ on effector and regulatory B-cell populations.

MATERIAL AND METHODS

Patients and Samples

Buffy coats from healthy blood donors provided by the Institute for Transfusion Medicine were used for the experiments. In addition, 22 patients after renal transplantation were enrolled. Details on the patient demographics are given in **Table 1**. This study was approved by the local ethics committee and all patients provided informed consent.

TABLE 1 | Patient's characteristics.

	Renal Transplant Patients (n = 22)
Age, mean ± SD, years	58 ± 14
Sex (female/male)	5/17
Time since transplantation (months)	142 ± 106
# of patients ≥1 previous RTX	0
# of HLA-mismatches (HLA-A,-B,-DR)	3 ± 2
Immunosuppressants at the time of sampling (# of patients treated)	
Tacrolimus	15
Cyclosporine A	4
Mycophenolate	18
mTOR inhibitor	5
Steroids	11
CMV status at the time of RTX (D/R)	
+/+	4
+/-	3
-/+	6
-/-	5
unknown	4

Peripheral Blood Mononuclear Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Stemcell, Cologne, Germany). In case purified B-cells were used for the experimental procedures, B-cells were isolated from PBMCs using a bead-/column-based magnetic separation method (B-cell isolation kit II, Miltenyi Biotec, Bergisch Gladbach, Germany). B-cells were purified by negative selection and purity was typically above 90%. In case B-cells were isolated from renal transplant patients, B-cells were directly isolated from whole blood using a magnetic bead-based negative selection system (MACSXpress Whole Blood B cell isolation kit, Miltenyi Biotec) and purity was typically above 90%. To track proliferation, cells were labeled with 2 µM carboxyfluorescein-succinimidyl-ester (CFSE, Sigma Aldrich, Taufkirchen, Germany).

Cell Culture

PBMCs or isolated B-cells were cultured in RPMI1640 Glutamax (Thermo Fisher Scientific, Darmstadt, Germany) supplemented with 100 U/ml Penicillin (Sigma Aldrich), 100 µg/ml Streptomycin (Sigma Aldrich), 10% fetal calf serum (Greiner-Bio one, Frickenhausen, Germany), NEAA (Sigma Aldrich) and sodium pyruvate (Sigma Aldrich) at a concentration of $0.5x10^6$ cells/ml in 96-well U-bottom plates (Sigma Aldrich). Depending on the assay, cells were cultured for three to six days in a 5% CO2 atmosphere at 37°C. To induce Interleukin (IL)-10 producing Breg, cells were stimulated with CpG (ODN2006, 0.1 µM, Invivogen, Toulouse, France), Poly-S (resiquimod plus IL-2, 1:1000 final dilution, CTL Europe GmbH, Bonn, Germany) or anti-human Immunoglobulin G/M (IgG/IgM, 6 µg/ml and 6.5 µg/ml, Jackson Immunoresearch Europe Ltd, Cambridge, United Kingdom) for 72 hours, followed by restimulation with phorbol 12-myristate-13-acetate (PMA) (10 ng/ml) and ionomycin (1 µg/ml, both Sigma-Aldrich) in presence of brefeldin A (5 ug/ml, BFA, BD Biosciences). For detection of Granzyme B producing (GrB^{pos}) B-cells, B-cells were stimulated with stimulated with CpG (ODN2006, 0.1 µM) or anti-human IgG/IgM (6 µg/ml and 6.5 µg/ml, Jackson Immunoresearch Europe Ltd) in presence of IL-21 (50 ng/ml, Miltenyi Biotec) for 72 hours, followed by restimulation with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml, Sigma Aldrich) and ionomycin (1 µg/ml, Sigma-Aldrich) in presence of brefeldin A (BFA, BD Biosciences). For the plasma blast formation assay, cells were stimulated for six days with either CpG (ODN2006, 0.1 µM, Invivogen) or anti-human IgG/IgM (6 µg/ml and 6.5 µg/ml, Jackson Immunoresearch Europe Ltd) in presence of IL-2 (50 ng/ml, Miltenyi Biotech) and IL-21 (50 ng/ml, Miltenyi Biotech). To detect IgG secretion, isolated B-cells or PBMCs were stimulated with Poly-S (resiquimod plus IL-2, 1:1000 final dilution, CTL Europe GmbH) for four days and then transferred to a prepared EliSpot plate to be incubated for further 24 hours. CQ (chloroquine, Cyto-ID kit, Enzo Life Sciences, Lörrach, Germany) was used at different concentrations for the assays. Tacrolimus (1.25 ng/ml, Sigma Aldrich) and rapamycin (12.5 ng/ml, Sigma Aldrich) were used in selected experiments as comparators with a known immunosuppressive effect on B-cells. CNIs are also commonly used in other autoimmune diseases such as systemic lupus erythematosus as well as CQ.

EliSpot Assay

For detection of IgG-secreting human cells, a commercially available EliSpot kit was used (Human IgG single-color

EliSpot, CTL Europe GmbH) according to the manufacturer's instructions. For the final incubation step, fixed numbers of cells were used (500 or 1000 cells per well) and spots were counted using an EliSpot Reader (AID, Straßberg, Germany).

Flow Cytometry

For GrB and IL-10 detection, cells were harvested at the end of the culture period and stained with anti-CD19 Pacific Blue (clone J3-119, Beckman Coulter, Krefeld, Germany) and 7AAD (BioLegend, Eching, Deutschland) followed by fixation/permeabilization (Cytofix/Cytoperm kit, BD Biosciences, Heidelberg, Germany). Cells were then stained intracellulary for GrB (anti-GrB, clone GB11, PE, eBioscience) or IL-10 (anti-human IL-10, APC, clone JES3-9D7, Biolegend). For the plasma blast formation assay, cells were harvested after culture and stained with anti-human CD19 (clone J3-119, FITC, Beckman Coulter), anti-human CD38 (clone HIT-2, PE, Biolegend), anti-human CD27 (clone O323, APC-H7, Biolegend). Appropriate isotype controls were used to confirm specificity of staining. Flow cytometric measurement was performed the same day with a fluorescence activated cell sorter (FACS) NAVIOS™ from Beckman Coulter. Kaluza Version 2.1 (Beckman Coulter) was used to analyze FACS data.

Statistics

All values are expressed as mean ± standard deviation (SD). The significance for the differences between groups was determined by ANOVA and Dunnetts test. Differences were considered statistically significant at a p-value <0.05.

RESULTS

Chloroquine Suppresses CpG-Induced B-Cell Proliferation

To study the effect of CQ on B-cells, purified B-cells or PBMCs were used. First, it was determined whether CQ had impact on the proliferation of stimulated B-cells. For this purpose, CFSE-labeled cells were stimulated with CpG in presence or absence of CQ. CQ suppressed B-cell proliferation in a dose-dependent manner (**Figure 1**). This effect was observed in purified B-cell and PBMCs cultures. Enhanced cell death was not observed after stimulation in presence of CQ (**Figures 1C, D**). Next, it was determined whether the type of stimulus has impact on CQ-mediated suppression. The type of stimulus was chosen according to the expected strengths of stimulation and to the biological context in which B-cell activation occurs. It is generally thought that full B-cell activation requires different signals under physiological circumstances *in vivo* (5, 6). Binding of the cognate antigen to the B-cell receptor (BCR) may serve as initial signal and promotes antigen processing (5, 6). Additional signals amplifying B-cell activation can be mediated by T-cells or by pathogen-associated molecular patterns (PAMP) being recognized by TLR (7, 8). TLR7 and TLR9 bind different ligands. While TLR7 senses single-stranded RNA (ssRNA), TLR9 senses hypomethylated CpG DNA motifs (9–11). Both ssRNA and CpG are pathogen-associated molecular patterns (PAMP) and recognition of these PAMP is pivotal to establish a sufficient immune response. ssRNA is a PAMP mostly associated with viral pathogens such as influenza whereas CpG motifs as PAMP can be found in bacterial and viral pathogens (9–11). However, there is some evidence that also host-derived ssRNA and CpG acting as damage-associated molecular patterns (DAMP) may activate the respective TLR under specific circumstances (9). As both TLR-pathways have an important role in host defense and may have a role in the pathogenesis of B-cell mediated diseases, TLR7 and TLR9 agonists were used for *in vitro* experiments. Thus, established BCR- and TLR-based *in vitro* activation protocols were chosen for comprehensive characterization of B-cells. B-cells stimulated with CpG were more susceptible to CQ-induced suppression than B-cells stimulated *via* the B-cell receptor (BCR) using anti-IgG/IgM or *via* TLR7/8 using Poly-S, i.e. resiquimod (**Figure 2**). Rapamycin reduced proliferation of B-cells stimulated with CpG or Poly-S whereas Tacrolimus-mediated suppression was minor (**Figures 2A, C**).

IL-10^{pos} Breg Are More Susceptible to CQ-Mediated Suppression Than Anti-BCR Induced GrB^{pos} Breg

It was then tested whether CQ inhibits regulatory functions of B-cells. Therefore, IL-10 production as wells as GrB synthesis of B-cells was assessed. IL-10^{pos} Breg were induced by CpG, Poly-S or anti-IgG/IgM stimulation. As expected, CpG and Poly-S were potent inducers of IL-10^{pos} Breg whereas the fraction of IL-10^{pos} Breg was low upon anti-IgG/IgM stimulation (**Figure 3** and **Figure S2**). CQ lowered the fraction of CpG-induced Breg significantly at a concentration of 0.5 uM (**Figure 3A**). The same concentration had no significant effect on Poly-S induced Breg (**Figure 3B**). However, at a CQ concentration of 10 uM, the fraction of Poly-S induced Breg was significantly reduced (**Figure 3B**). There was no effect on Breg induced by anti-IgG/IgM stimulation. Next, it was investigated whether Breg producing GrB are affected by CQ. GrB^{pos} Breg were potently induced by stimulation with CpG or anti-IgG/IgM in presence of IL-21 (**Figure 4**). However, BCR-stimulation lead to a higher fraction of GrB^{pos} Breg than CpG-stimulation. CQ suppressed CpG-induced GrB^{pos} Breg whereas inhibition of anti-IgG/IgM-induced GrB^{pos} Breg was much less effective (**Figures 4** and **S1**).

Effector B-Cell Functions Such as Plasma Blast Formation and IgG Secretion Are Suppressed by CQ

Furthermore, it was investigated to which extend CQ has impact on effector B-cell function. Specifically, plasma blast formation and IgG secretion was studied. Plasmablast formation was induced by either stimulation with CpG or Poly-S in presence of IL-21 and IL-2 for six days. The fraction of plasmablasts defined as CD19^{+}CD27^{++}CD38^{++} was then determined by flow cytometry (**Figure S3**). CQ inhibited plasmablast formation efficiently at low concentrations upon TLR9 stimulation with

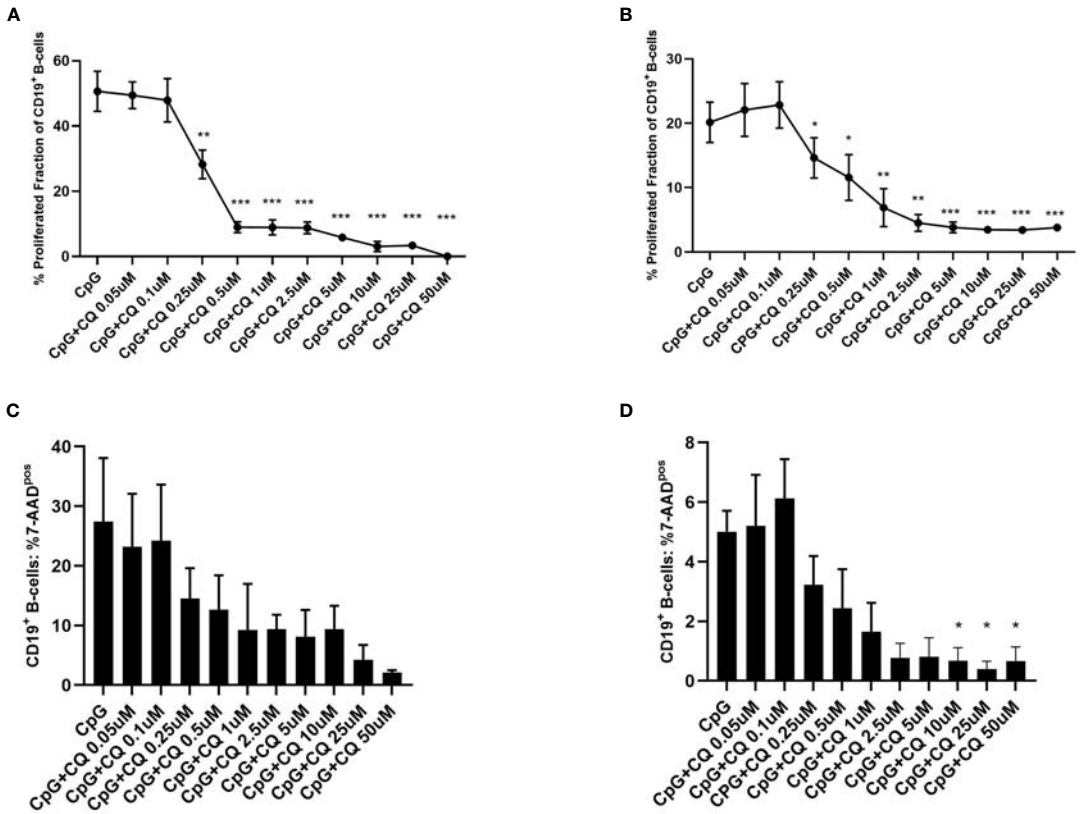

FIGURE 1 | Dose-dependent suppression of TLR9-induced B-cell proliferation by CQ. PBMCs or purified B-cells were labeled with CFSE and stimulated with CpG in presence of different concentrations of CQ. After 72 hours, $CD19^+$ B-cell proliferation was determined by CFSE-dilution. **(A)** Impact of CQ on B cell proliferation with CpG stimulation in PBMCs. **(B)** Impact of CQ on B cell proliferation with CpG stimulation in purified B cells. **(C)** Impact of CQ on vitality of B-cells after stimulation with CpG in PBMCs. **(D)** Impact of CQ on vitality of B-cells after stimulation with CpG in purified B-cells, P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.0001 (against CpG).

CpG being as potent as rapamycin (**Figure 5A**). CQ was slightly less suppressive upon TLR7-stimulation with Poly-S and higher concentrations were necessary (**Figure 5B**). In the same conditions, rapamycin suppressed plasmablast formation efficiently. Subsequently, it was studied if IgG secretion can be inhibited by CQ. Purified B-cells were stimulated for four days with Poly-S in presence of CQ, tacrolimus or rapamycin. Then, B-cells were transferred to EliSpot plates and SFU were determined after 24 hours. CQ significantly lowered the number of SFU already at a concentration of 1 uM and was equally potent to rapamycin at higher concentrations (**Figure 6**). Tacrolimus did not influence IgG secretion. To assess if CQ also shows significant effects under diseased conditions, B-cells derived from patients after renal transplantation were assayed. CQ inhibited IgG secretion to a similar extend as seen in healthy controls (**Figure 7**).

DISCUSSION

CQ is used as treatment in autoimmune diseases such as SLE and RA (1, 2). The exact mechanisms of action have not been deciphered completely and the specific effect on B-cells has been investigated scarcely. Thus, this study aimed to assess the impact of CQ on regulatory and effector B-cells. Furthermore, it was tested whether CQ suppresses B-cells to the same extent under diseased conditions. It was found that CQ potently suppressed B-cell proliferation; moreover CQ had a differential impact on regulatory B-cells with a more substantial inhibition of $IL\text{-}10^{pos}$ Breg whereas GrB^{pos} Breg were less susceptible to suppression by CQ. CQ inhibited effector B-cell functions such as plasmablast formation and antibody production not only in healthy controls but also in renal transplant patients.

It has been demonstrated previously that CQ exerts its effects on mononuclear cells by different mechanisms of action. CQ antagonizes the TLR-mediated stimulation of mononuclear cells; elevation of intra-lysosomal pH preventing acidification necessary for activation, binding of nucleic acid ligands and interference with downstream signaling seem key mechanisms (3). The impact of CQ on TLR9-mediated effector functions of B-cells was studied by Torigoe et al. (12). CQ blocked the maturation of B-cells into plasma cells, IgG secretion and TNFα production (12). Likewise, in a murine lupus model, CQ blocked TLR9-mediated proliferation of autoantigen-specific

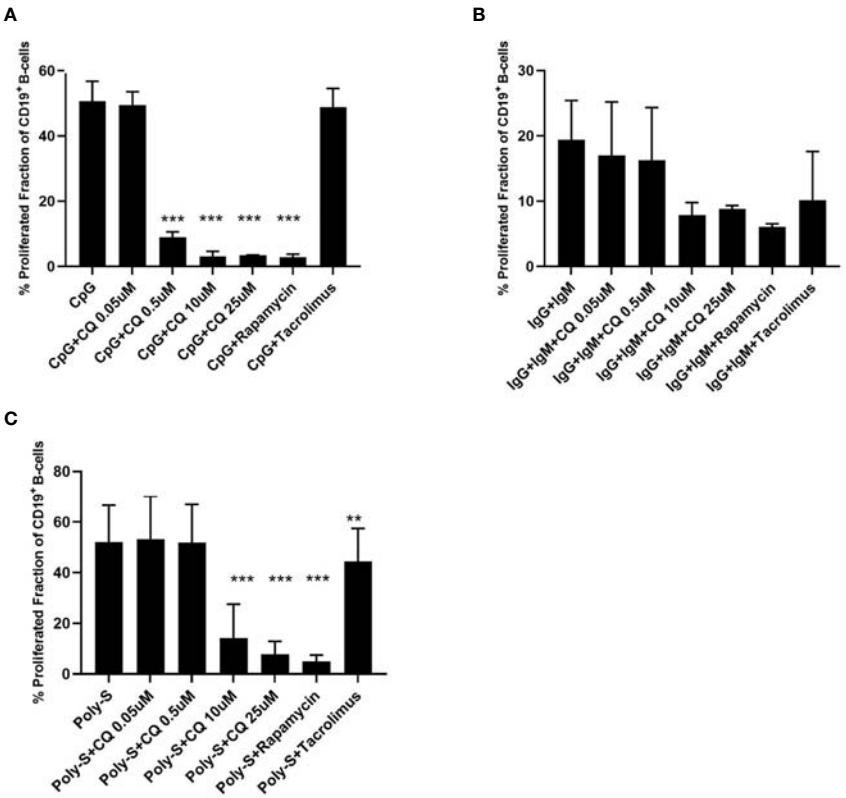

FIGURE 2 | CQ affects B-cell proliferation depending on the stimulus. PBMCs were isolated and labeled with CFSE followed by stimulation with different stimuli in presence of CQ at four concentrations (0.05 μM, 0.5 μM, 10 μM, 25 μM). Rapamycin and tacrolimus were also used as controls. After 72 hours, $CD19^+$ B-cell proliferation was determined by CFSE-dilution. **(A)** Impact of CQ on B cell proliferation upon stimulation with CpG. **(B)** Impact of CQ on B cell proliferation upon IgM/IgG stimulation. **(C)** Impact of CQ on B cell proliferation upon Poly-S stimulation. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. $^{**}p < 0.01$, $^{***}p < 0.0001$ (against CpG, IgG+IgM or Poly-S as control conditions).

B-cells (13). There is fewer data on antagonism of TLR7/8 mediated mononuclear cells. Resiquimod-induced NK cell activation was dampened by CQ in an *in vitro* model (14). Our study confirms the existing data on suppression of TLR9-stimulated effector B-cells by CQ. CQ suppressed TLR9-induced B-cell proliferation and plasmablast formation. Furthermore, our study extends the body of evidence further and provides novel, robust data on CQ-mediated suppression of TLR7/8-stimulated effector B-cells. CQ suppressed resiquimod-induced B-cell maturation into plasmablasts and IgG production suggesting a wider range of inhibitory capacity. TLR7/8 and TLR9 also recognize endogenous ligands known as damage associated molecular patterns (DAMP) which are released upon tissue damage (9–11, 15, 16). In the setting of renal transplantation, TLR7 also recognizes host-derived miRNA; overexpression of specific types within the renal allograft have been associated with reperfusion injury, acute and chronic rejection (17–19). TLR9 signaling is induced by intra-graft release of high mobility group boxed protein-1 (HMGB-1) and mitochondrial DNA (9). Therefore, TLR7/8 and TLR9 induced B-cell activation may contribute to sensitization of B-cells in renal transplant patients. Indeed, despite broad immunosuppressive therapy, allograft rejection occurs in renal transplant patients and especially antibody-mediated rejection is problematic leading to loss of graft function (20). B-cell directed therapy is one of the therapeutic measures that can be considered but comes at the expense of increased risk of infection (21). Thus, the effect of CQ on TLR-stimulated B-cells derived from renal transplant patients was assessed and a profound suppressive effect on effector B-cells was demonstrated. As CQ suppressed maturation and subsequent IgG secretion of B-cells derived from renal transplant patients *in vitro*, CQ could have the potential to further optimize prophylaxis against antibody-mediated rejection (ABMR). This is also interesting as CQ does not increase the risk for infection (4). Still, CQ acts right in the end of a complex cascade leading to formation and maturation of donor-specific B-cells (22). As has been demonstrated earlier, there are several other factors driving maturation of donor-specific B-cells such as the B-cell activating factor (BAFF), IL-6 and interactions with other immune cells (22, 23). It might be more beneficial to interfere very early with this complex cascade and targeting effector B-cells during the already initiated maturation process could be too late.

To which extend Breg are influenced by CQ has not been studied in detail in human B-cells. In our study, we investigated two different subsets of Breg. $IL\text{-}10^{pos}$ Breg are pivotal to

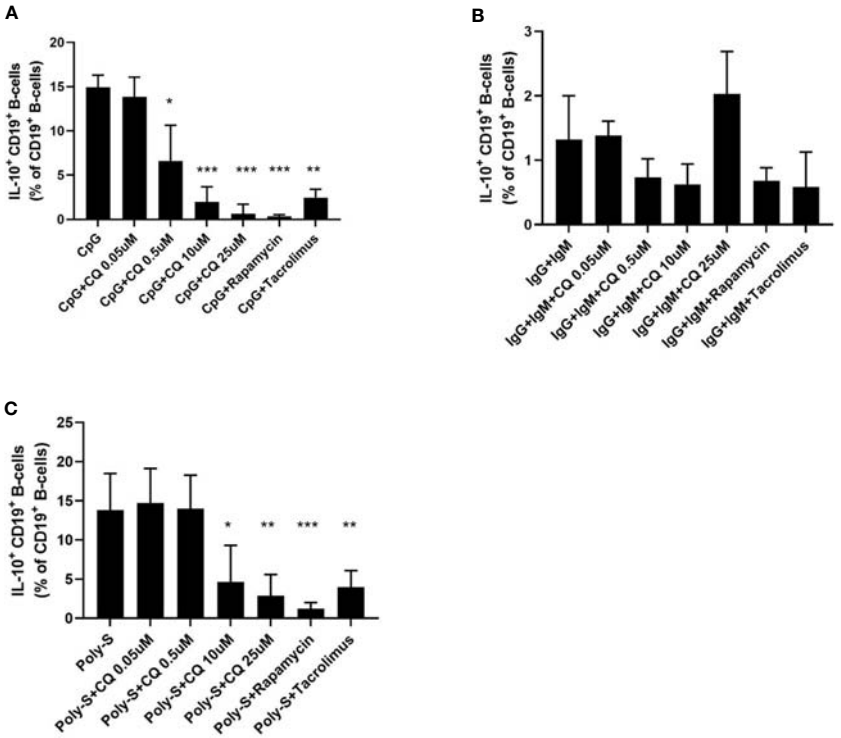

FIGURE 3 | Effect of CQ on IL-10^{pos} regulatory B-cells. PBMCs were cultured in presence of different stimuli and CQ at four concentrations (0.05 µM, 0.5 µM, 10 µM, 25 µM) for 72 hours. Rapamycin and tacrolimus were also used as controls. After 72 hours, the production of IL-10 by CD19$^+$ B-cells was determined by flow cytometry. **(A)** Impact of CQ on IL-10$^+$ CD19$^+$ B-cells upon CpG stimulation in PBMC. **(B)** Impact of CQ on IL-10$^+$ CD19$^+$ B-cells upon IgG+IgM stimulation in PBMCs. **(C)** Impact of CQ on IL-10$^+$ CD19$^+$ B-cells upon Poly-S stimulation in PBMCs. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. *p < 0.05, **p < 0.001, ***p < 0.0001 (against CpG, IgG+IgM or Poly-S as control conditions).

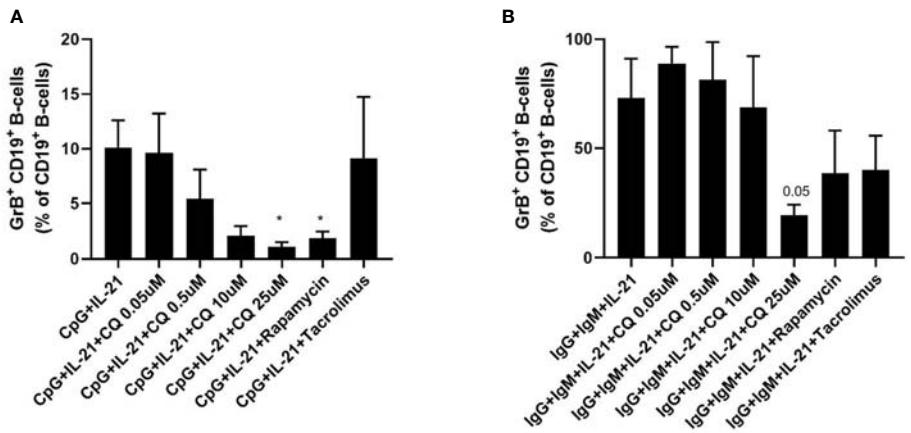

FIGURE 4 | Effect of CQ on GrB^{pos} regulatory B-cells. Purified B cells were isolated and stimulated *via* TLR9 or BCR plus IL-21, in presence or absence of CQ, rapamycin or tacrolimus for 72 hours. After 72 hours, GrB$^+$ CD19$^+$ B-cells were determined by flow cytometry. **(A)** Impact of CQ on GrB$^+$ CD19$^+$ B-cells upon CpG plus IL-21 stimulation. **(B)** Impact of CQ on GrB$^+$ CD19$^+$ B-cells upon IgG/IgM plus IL-21 stimulation. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. *p < 0.05 (against CpG+IL-21 or IgG+IgM+IL-21 as control conditions).

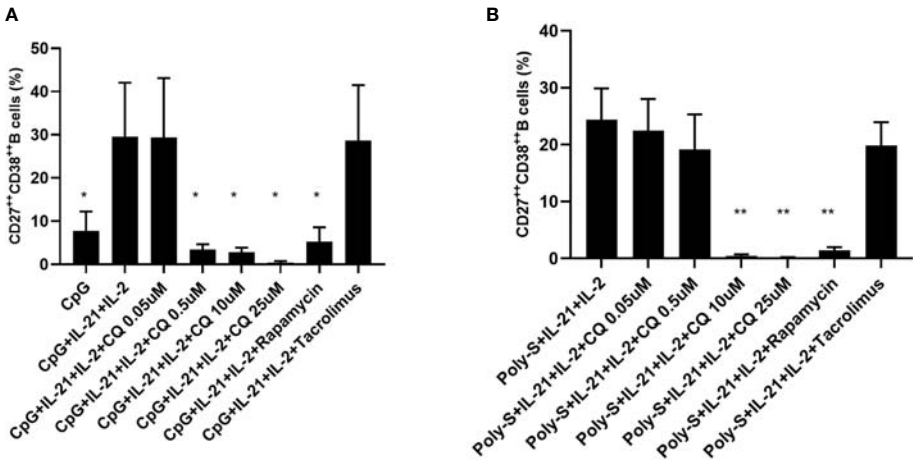

FIGURE 5 | Effect of CQ on plasmablast formation depending on the stimulus. Purified B cells were stimulated for 6 days and were monitored for differentiation into plasma cells by flow cytometry. **(A)** Impact of CQ on plasma cells upon CpG plus IL-21 and IL-2 stimulation. **(B)** Impact of CQ on plasma cells upon IgG+IgM plus IL-21 and IL-2 stimulation. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. *p < 0.05, **p < 0.001 (against CpG+IL-2+IL-21 or Poly-S+IL-2+IL-21 as control conditions).

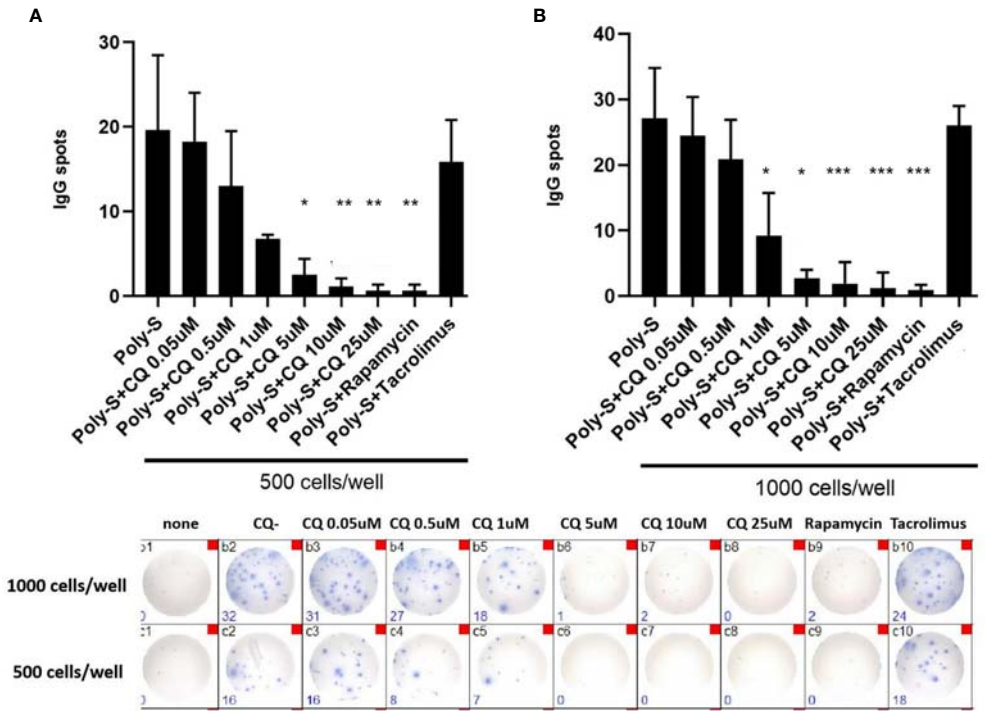

FIGURE 6 | Chloroquine suppresses antibody-synthesis by plasma cells. To assess if CQ has impact on antibody-secretion by B-cells, a human IgG-specific ELISpot analysis was performed. Isolated CD19^{+}B cells were initially seeded at 5×10^{4} cells/well under Poly-S stimulation in presence of CQ at different concentrations for four days. Then, cells were harvested and transferred to ELISpot plates at a density of 500 cells/well or 1000 cells/well to be cultured for another 24 hours. **(A, B)** Impact of CQ on IgG-secretion of plasma cells using purified B cells. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. *p < 0.05, **p < 0.001, ***p < 0.0001 (against Poly-S as control condition).

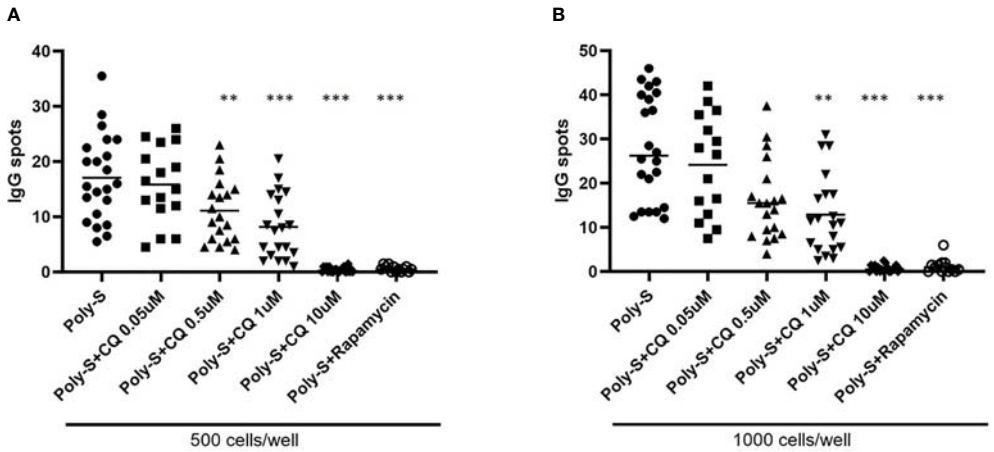

FIGURE 7 | Effect of CQ on IgG-secretion of plasma cells derived from renal transplant patients. To further investigate the effect of CQ on B-cells from renal transplant patients, 22 patients were enrolled. Isolated $CD19^+$ B cells derived from renal transplant patients were seeded at a concentration of 5×10^4 cells/well under Poly-S stimulation in presence of CQ for 4 days. B cells were then harvested and transferred to ELISpot plates at a density of 500 or 1000 cells/well for further 24 hours. **(A)** Impact of CQ on IgG-secretion of plasma cells at a density of 500 cells/well. **(B)** Impact of CQ on IgG-secretion of plasma cells at a density of 1000 cells/well. P-values were calculated by repeated-measures ANOVA and correction for multiple comparisons were done by Dunnett's test. $^{**}p < 0.001$, $^{***}p < 0.0001$ (against Poly-S as control condition).

maintain immunological tolerance and functional or numerical deficiency promotes unwanted inflammation (24–27). IL-10^{pos} Breg promote the development of regulatory T-cells (Treg), directly suppress pro-inflammatory T-cells *via* IL-10 as well as *via* contact-dependent mechanisms and inhibit maturation of antigen-presenting cells (28). Dominance of IL-10^{pos} Breg over effector B-cells has been associated with a more favorable outcome after renal transplantation (29, 30); likewise, patients with antibody-mediated rejection showed a profound deficiency of circulating IL-10^{pos} Breg (31, 32). GrB^{pos} Breg have been studied less extensively but the current evidence suggests a similar role in restraining pro-inflammatory immune responses (33–35). Circulating GrB^{pos} Breg are expanded in renal transplant patients with operational tolerance and during rejection, numbers seem diminished (36). It has been hypothesized that local GrB release by Breg without concomitant expression of perforin may lead to degradation of the T-cell receptor (TCR) of neighboring T-cells (37). We could show that CQ interferes with CpG-induced maturation of Breg, no matter if IL-10 or GrB producing. Likewise, TLR7/8-induced maturation of IL-10^{pos} Breg was hampered. This is in line with data from Miles et al. showing that murine B-cells secret less IL-10 in presence of CQ (38). In another study by Cepika et al. SLE patients were followed before and after beginning of CQ therapy. After initiation of CQ therapy, IL-10 serum levels decreased and IL-10 secretion of total mononuclear cells was diminished upon CpG-stimulation (39).

In contrast, anti-BCR-mediated induction of GrB^{pos} Breg was much less susceptible to CQ treatment consistent with the current concept of CQ-mediated mechanism of action. Rabani et al. studied GrB^{pos} Breg in a cohort of SLE patients (34). In these patients, the fraction of GrB^{pos} Breg was reduced in comparison to healthy controls. However, patients receiving CQ showed a similar fraction of GrB^{pos} Breg as compared to patients who were not treated with CQ.

Thus, Breg show a differential response to CQ with IL-10^{pos} Breg being very susceptible to CQ-mediated suppression whereas GrB^{pos} Breg are nearly unaffected. If CQ would selectively affect Breg sparing effector B-cells, this would have a detrimental effect in B-cell mediated diseases. However, as effector B-cells are also inhibited by CQ, the whole B-cell compartment most probably shifts towards a less pro-inflammatory polarization consistent with the observed clinical efficacy of CQ in B-cell mediated autoimmune diseases.

In summary, we demonstrated that CQ not only suppresses CpG-induced effector B-cell maturation but also resiquimod i.e. TLR7/8-mediated effector B-cell function. Furthermore, this effect was not limited to B-cells from healthy controls but was also reproducible in B-cells from patients after renal transplantation. In addition, we provided novel data that CQ differentially impacts subsets of regulatory B-cells extending the knowledge on the biology and role of these regulatory subsets. These novel insights may allow to further investigate CQ as additional treatment for other patient cohorts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local institutional review board, Ethik-Kommission Medizinische Fakultät Universität Duisburg-

Essen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XM designed the study, performed the experiments, performed the data analyses, and wrote the manuscript. YD, SX, SD, ML, AK, and OW designed the study and critically edited the manuscript. BW designed the study, supervised the study, performed data analyses, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.818704/full#supplementary-material

Supplementary Figure 1 | Effect of CQ on GrB producing regulatory B-cells: Representative flow cytometric data. Plots are gated on vital B-cells.

Supplementary Figure 2 | Effect of CQ on IL-10 producing regulatory B-cells: Representative flow cytometric data. Plots are gated on vital B-cells.

Supplementary Figure 3 | Effect of CQ on plasma blast formation of B-cells: Representative flow cytometric data. Plots are gated on vital B-cells and plasma blasts were defined as $CD27^{++}CD38^{++}$.

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Molecular Mechanisms Driving IL-10-Producing B Cells Functions: STAT3 and c-MAF as Underestimated Central Key Regulators?

Magalie Michée-Cospolite[1†], Marina Boudigou[1†], Alexis Grasseau[1], Quentin Simon[1], Olivier Mignen[1], Jacques-Olivier Pers[2], Divi Cornec[2], Laëtitia Le Pottier[1] and Sophie Hillion[2]*

[1] U1227, LBAI, Univ Brest, Inserm, Brest, France, [2] U1227, LBAI, Univ Brest, Inserm, and CHU Brest, Brest, France

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Edited by:
Luman Wang, Fudan University, China

Reviewed by:
Avijit Ray, GlaxoSmithKline, United States
Hong Zan, The University of Texas Health Science Center at San Antonio, United States

***Correspondence:**
Sophie Hillion
sophie.hillion@univ-brest.fr

[†]These authors have contributed equally to this work and share first authorship

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Regulatory B cells (Bregs) have been highlighted in very different pathology settings including autoimmune diseases, allergy, graft rejection, and cancer. Improving tools for the characterization of Bregs has become the main objective especially in humans. Transitional, mature B cells and plasma cells can differentiate into IL-10 producing Bregs in both mice and humans, suggesting that Bregs are not derived from unique precursors but may arise from different competent progenitors at unrestricted development stages. Moreover, in addition to IL-10 production, regulatory B cells used a broad range of suppressing mechanisms to modulate the immune response. Although Bregs have been consistently described in the literature, only a few reports described the molecular aspects that control the acquisition of the regulatory function. In this manuscript, we detailed the latest reports describing the control of IL-10, TGFβ, and GZMB production in different Breg subsets at the molecular level. We focused on the understanding of the role of the transcription factors STAT3 and c-MAF in controlling IL-10 production in murine and human B cells and how these factors may represent an important crossroad of several key drivers of the Breg response. Finally, we provided original data supporting the evidence that MAF is expressed in human IL-10- producing plasmablast and could be induced *in vitro* following different stimulation cocktails. At steady state, we reported that MAF is expressed in specific human B-cell tonsillar subsets including the IgD^+ $CD27^+$ unswitched population, germinal center cells and plasmablast.

Keywords: regulatory B cells, molecular drivers, c-MAF, STAT3, B-cell differentiation

INTRODUCTION

B cells with immunosuppressive function (Bregs) have been highlighted for the first time in the murine experimental autoimmune encephalomyelitis (EAE) model (1). Although the induction of the disease was not influenced by the absence of B cells, B-cell depleted mice exhibited no substantial recovery suggesting a regulatory role. During the past decade, various studies have reported this regulatory B cell-induced immune response suppression in very diverse pathophysiological settings

like autoimmune diseases, transplantation, virus immunity, and cancer. The heterogeneity of the Breg subsets is a subject of interest for many years (2–5) and consensus opinions suggest that Bregs may arise from multiple progenitors depending on the microenvironment. Beyond their phenotypic status, Bregs used different immunosuppressive mechanisms such as the production of interleukin (IL)-10, granzyme B (GZMB), and transforming growth factor (TGF) β to regulate other immune cells (3, 6, 7). IL-10 has become the most documented suppressive mechanism by B cells and in many cases, IL-10 can be often co-expressed with other regulatory molecules such as TGFβ or IL-35 (8). New studies are now starting to clarify the different molecular mechanisms that may control the acquisition of the Breg function. In this review, we described the last reports establishing new insights into the molecular control of TGF β, GZMB, and IL-10 producing B cells (B10) with a specific focus of an understudied transcription factor (TF) in B cells: *the avian musculoaponeurotic fibrosarcoma oncogene* c-MAF (MAF).

MOLECULAR CONTROL OF THE PRODUCTION OF TGFB AND GZMB IN B CELLS

TGFβ -Producing Regulatory B Cells

TGFβ-producing B cells with regulatory properties were first described in cancer, especially in lung metastasis from breast cancer in mouse models (9). TGF-β^+ Bregs that exhibited a particular phenotype ($CD19^+$, $CD25^{high}$, IgD^{high}, $CD21^{low}$, $CD23^{low}$, $CD43^-$, IgM^{int}, and $CD62L^{low}$) were able to decrease T cell proliferation and induce *de novo* regulatory T cells (Tregs) ($Foxp3^+$) *in vivo* and *in vitro*. In this study, they demonstrated that the tumor-microenvironment was necessary to both mouse and human Breg development. These TGFβ-expressing Bregs expressed high levels of phosphorylated-STAT3 (pSTAT3). The same group further demonstrated that the inhibition of STAT3 phosphorylation by resveratrol (phytoalexin found in some plants as mulberries or peanuts) induced the decreased expression of TGFβ by Bregs leading to the reduced expansion of Tregs *in vivo* and *in vitro* (10). Inhibitory effect of the resveratrol as a potent inhibitor of STAT3 phosphorylation and acetylation is mostly mediated by the Sirtuin 1 protein (SIRT1) an NAD^+-dependent class III histone deacetylase (HDAC). Although SIRT1/STAT3 axis has been extensively studied in cancer cells (11, 12), there are few reports on its role on B-cells. However, it was recently demonstrated that B-cell specific SIRT1 deficient cells displayed an increase of IL10 expression *in vitro* following LPS stimulation (13). Moreover, SIRT1 is highly expressed in resting naïve mouse B cells but dramatically reduced following cell activation and immunization. In this context, SIRT1 suppressed AICDA expression and the class switch DNA recombination through the regulation of the acetylation of STAT3 and the NFkB p65, another important target of SIRT1 (14). Underlying mechanisms of these different interactions with other molecular drivers of TGFβ are still unclear, especially in B cells.

Some studies highlighted the role of the different TGFβ isoforms in the regulatory properties of B cells. A first mouse study revealed the co-expression of TGFβ1 and TGFβ3 at similar levels in resting B cells but in IgM-activated B cells, TFGβ1 was increased and TGFβ3 was highly reduced. In a coculture model with resting B cells and T cells, Treg expansion was only dependent on the TGFβ3 isoform (15). However, in the EAE mouse model, TGFβ1-deficient B cells led to increased EAE score level and the disease severity was linked to the expansion of T cell helper (Th)1 in conserved non-coding sequences (CNS) (16). So, the two isoforms TGFβ1 and TGFβ3 seem to be implicated in Breg properties but with microenvironment-dependent mechanisms. Unlike in mice, the activation of human B cells upon BCR and Toll-like receptor (TLR) 9 induced decreased expression of TGFβ1 (highly expressed in resting B cells) but an increase of IL-10 expression (17). Unfortunately, in most of the studies on TGFβ-expressing Bregs, the nature of the isoform involved is still elusive.

The co-expression of TGFβ and IL-10 by Bregs is frequently reported in the literature but the role of TGFβ is controversial. One study demonstrated that B cells infiltrating tumors expressed TGFβ and IL-10 and inhibited T cell proliferation and the generation of interferon (IFN)γ-$CD8^+$ T cells. This mechanism was TGFβ-dependent (18). However, in the model of low-dose methotrexate-induced tolerance in mice, the generated Bregs co-expressed TGFβ and IL-10, but only IL-10 seemed to be implicated in the tolerance mechanism (19). Two additional studies performed in humans demonstrated the role of TGFβ in Bregs-mediated regulation. In the first study, *in vitro* Bregs were induced following a CD40 ligand (CD40L), CpG, and IL-4 stimulation and were able to decrease T-cell proliferation and induce the Tregs expansion. FoxP3 expression and Tregs generation were reduced in the presence of the anti-TGFβ blocking antibody (Ab) but not after IL-10 blocking (6). We also demonstrated that *in vitro*-induced Bregs used a TGFβ-dependent mechanism in controlling T -cell proliferation whereas the decrease of IFNγ-Th1 cells was IL-10-dependent (20).

Other information puzzled the understanding of the generation of TGFβ-producing-Bregs because TGFβ was first described as able to inhibit B cell proliferation and Ig production (21). Moreover, a recent study highlighted the synergy between IL-10 and TGFβ3 to inhibit B-cell proliferation and Ig production after TLR4 or TLR7 activation by decreasing energy metabolism of B cells such as glycolysis. Interestingly, pSTAT3 was also expressed in these B cells (22). However, the synergistic role of IL-10 and TGFβ in controlling Bregs function remains to be explored.

Expression of GZMB in B Cells

Initially, the GZMB had been described as a compound of the cytoplasmic granules of cytotoxic cells that induced targeted cell death when accompanied by perforin (23). However, over the last decade, the secretion of GZMB by B cells, Tregs, or plasmacytoïd dendritic cells, has been found to exert an immunosuppressive effect through performing-independent mechanisms. This GZMB-expressing Breg subset has been

detected in a variety of pathological contexts: chronic lymphocytic leukemia (CLL) (24), solid tumor infiltration (7), autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (25), viral infections (26) and in the context of the transplantation (27). Although GZMB-expressing B cells were found to be enriched in differentiated B cells like plasmablasts (PB), less than 3% of circulating B cells seemed to express GZMB in healthy individuals (28).

One of the key inducers of the production of GZMB in B cells is IL-21. Jahrsdörfer's group first reported that IL-21 directly induced the secretion of enzymatically active GZMB by human B cells. The effect of IL-21 is synergically enhanced by CpG-ODN or BCR engagement (24, 29). Moreover, they demonstrated that GZMB and IL-21 serum levels are highly correlated in SLE and that $CD5^+$ B cells from SLE and Sjögren syndrome patients, which constitutively expressed GZMB, displayed higher expression of IL-21 receptor (30). IL-21 is mainly secreted by activated $CD4^+$ T cells, especially Th17 and follicular helper T cell (Tfh) subsets and natural killer T cell (NKT) (31). A study has shown that macrophages-derived IL-15 synergized with IL-21 to promote GZMB expression and B cell differentiation into $CD38^+$ $CD20^-$ PB. While IL-21 alone induced a quick and massive transcription of GZMB mRNA but a minimal production of the protein, the addition of IL-15 allowed efficient translation and/or secretion of the GZMB protein (32).

Little is known about the molecular control of GZMB production in B cells and the generation of $GZMB^+$ Bregs remains to be understood. Besides, the inability of mouse B cells to secrete GZMB further complicates addressing this question (33). Nevertheless, it would appear that pSTAT3 signaling is implicated in GZMB expression. IL-21 signaling is known to activate JAK/STAT pathways, especially pSTAT1 and pSTAT3, and to a lesser extent pSTAT5a and pSTAT5b (34). An up-regulation of pJAK1, pJAK3, and pSTAT3, but not pTYK2 or pSTAT1, was observed in B cells stimulated with IL-21 and anti-BCR, and the use of a JAK inhibitor abrogated the GZMB production (29). The use of STAT1, STAT3, or STAT5 inhibitors confirmed the involvement of STAT3 signaling but not STAT1 and STAT5, in GZMB synthesis. Moreover, B cells failed to produce GZMB in patients with the autosomal-dominant hyper-IgE syndrome which exhibit heterozygous STAT3 mutations, confirming the unique role of STAT3 in GZMB production (32).

Recently, Sophie Brouard's group has developed an expansion mixture containing IL-21, anti-BCR, CpG ODN, IL-2, and CD40L, to induce GZMB expression in more than 90% of B cells. While the presence of IL-21 and anti-BCR seemed indispensable to induce a strong expression of GZMB, the presence of CpG ODN and IL-2 seemed to be necessary for the maintaining of B-cell survival in culture (28). To better characterize GZMB-producing Bregs, they performed RNAseq analysis of sorted *in vitro*-expanded GZMB-positive and GZMB-negative B cells (35). Thirty-six genes were found to be differentially expressed between these two populations, including the top differential expressed genes (DEGs) *GZMB*, *LAG3*, and *FASLG*, which were already described for their role in immune regulation. They revealed a higher frequency of $LAG\text{-}3^+$ cells but a similar expression of IL-10 in GZMB-positive Bregs when compared to GZMB-negative B cells. In this transcriptomic analysis, *SOX5* was the only gene encoding a TF found to be differentially expressed. While *SOX5* was found to be downregulated during the proliferation of B cells, its high expression might limit proliferation allowing PB differentiation (36). However, as an autocrine production in $GZMB^+$ Bregs, the GZMB stimulates the proliferation of these cells (28). At this point, it is difficult to say whether the *SOX5* down-regulation may play a role in the generation and function of GZMB-positive Bregs or whether it is only a consequence of the autocrine production of GZMB itself.

STAT3 AND ITS PARTNERS IN THE GENERATION OF IL-10- PRODUCING B CELLS

IL-10 is a pleiotropic cytokine produced by virtually all immune cell types and possesses immunosuppressive properties. IL-10 is an inhibitor of a broad spectrum of monocytes/macrophages and T-cell functions, including cytokine synthesis, effector subset differentiation, and expression of co-stimulatory molecules such as CD80/CD86. However, the role of IL-10 on B cells was described as ambivalent. First reports described IL-10 as a growth factor for B cells. It promotes B-cell proliferation, Ab production, and class II MHC expression depending on their activation state (37). The addition of IL-10 in CD40-activated B cells results in a very high immunoglobulin production indicating differentiation of B cells into plasma cells (38, 39). IL-10 was an essential factor for isotype switching in presence of anti-CD40 stimulation leading to the production of IgM, IgG, and IgA (IgA1 and IgA2) in combination with TGFβ (40). Subsequent important reports described the role of IL-10 in promoting the differentiation of memory B cells into PB and plasma cells (41, 42).

B cells secrete a very low amount of IL-10 at a quiescent state however specific stimulatory signals were described to be involved in the induction of IL-10 producing B cells with suppressive functions: the BCR, CD40, TLR, and cytokine signaling including IL-21, IL-6, or TGFβ (43–45). Although the time-sequence and location of these events have to be clarified *in vivo*, the molecular signals leading to IL-10 production by B cells have been studied less thoroughly than in Th cells and macrophages (reviewed in (46)). Among the key factors involved in the control of IL-10 production, STAT3 appears to be involved in the different activation cascades leading to IL-10 secretion by B cells (**Figure 1**). It was demonstrated in $CD1d^{hi}$ $CD5^+$ B10 cells, that whilst the absence of the B-cell linker protein (BLNK) had no consequence on the distribution of Bregs in tissues, its expression was indispensable to IL-10 production. After TLR4 activation by lipopolysaccharide (LPS), phosphorylated BLNK interacts with the Bruton tyrosine kinase (Btk) leading to the phosphorylation of STAT3 that next translocates to the nucleus to transactivate the *IL10* gene (47) (**Figure 1**).

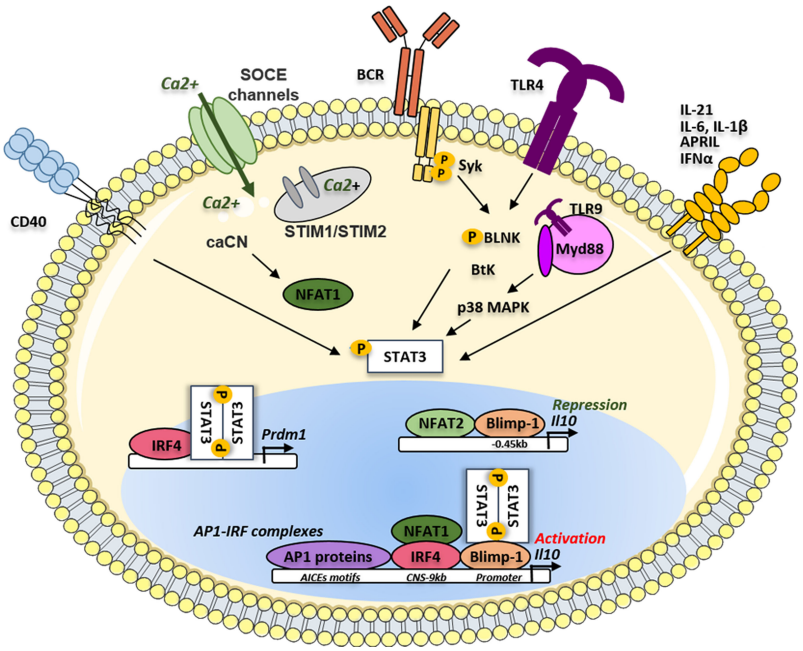

FIGURE 1 | IRF4/Blimp-1/STAT3 activation pathways on *Il10* gene regulation in Bregs. The phosphorylation of STAT3 could be activated following different microenvironment signals such as different cytokines (APRIL, IL-21, IL-6, IL-1β and IFNα), the antigen or TLR ligands. Phosphorylated STAT3, translocates to the nucleus and could bind IRF4 to induce the transcription of the Prdm1 gene. The translation of Prdm1 gene leads to the production of the Blimp-1 protein supporting the binding of STAT3 on the *Il10* gene promoter. The binding of Blimp-1 together with NFAT2 could induce repression of the *Il10* gene transcription. The different AP1 proteins such as BATF, Jun B and Jun D could bind the AICES motifs on the Il10 gene to promote its transcription through the cooperation of IRF4. IL, Interleukin; IFNα, Interferon alpha; APRIL, A Proliferation inducing ligand; SOCE, Store operated calcium entry; TLR, toll-like receptor; CNS, conserved non-coding sequence; TLR, toll-like receptor BCR, B-cell receptor; BtK, Bruton tyrosine kinase; BLNK, B-cell linker protein; MAPK, Mitogen-activated protein kinase; Syk, Spleen associated tyrosine kinase; STAT3, Signal transducer and activator of transcription 3; NFAT, Nuclear factor of activated T-cells; STIM, Stromal interaction molecules; CaCN, active calcineurin A; Myd88, Myeloid differentiation primary response protein; Blimp-1, B lymphocyte-induced maturation protein 1; Prdm1, PR domain zinc finger protein 1; AP1, Activator protein 1; IRF, Interferon regulatory factor; AICEs, AP1-IRF-composite elements.

In SLE patients, $CD24^{high}$ $CD38^{high}$ Bregs exhibited a defect in CD40 signaling with impairment of STAT3 phosphorylation and IL-10 production (48). Similar results were described in patients with systemic sclerosis where TLR9-induced IL-10 production by B cells was impaired because of a defect in p38 MAPK and STAT3 signaling (49, 50). Various cytokines increased *in vitro* the generation of human B10, often in combination with TLR activation in a STAT3-dependent manner including APRIL (51), IFNα (52, 53), IL-35 (54), IL-21 (55), IL-6, or IL-1β (56). Interestingly mice with the specific deletion of STAT3 in B cells developed a severe experimental autoimmune uveitis and displayed exacerbated EAE (57). In both diseases, the deficiency of STAT3 in B cells induced a defect not only in the induction of B10 cells but also in the generation of Tregs. Moreover, the authors demonstrated that expression of the CD80 and CD86 costimulatory molecules was markedly upregulated on B cells in STAT3-deficient mice, suggesting that STAT3 signaling may also contribute to restraining excessive activation of T cells.

Cooperation of BLIMP1 and IRF4 in the Generation of IL10 Producing B Cells

Once activated, STAT proteins can directly bind on a single motif on the *Il10* promoter (58) or may interact with other TF to induce IL-10 expression. The mouse *Il10* locus has been extensively studied in naive and differentiated T cells (59–61) mostly using DNase I hypersensitivity approaches highlighting at least nine genomic regions with potent high transcriptional activity. These several conserved noncoding sequences (CNS) presented a great homology between mice and humans. Together with the promoter, the CNS-9, CNS-4.5, located at 9 kb and 4.5 kb, respectively, upstream the *Il10* gene transcription start site (TSS), and the CNS+3, and CNS+6.5 Kb, located downstream the TSS, have been described as the main activator sites of the *Il10* transcription in T-cell subsets (62, 63). In Th1 cells, the B-lymphocyte-induced maturation protein 1 (Blimp-1) binds the CNS-9 site in a STAT4-dependent manner, and mice with a T-cell-specific Blimp-1 deficiency had a severe inflammatory response during *T. gondii* infection, suggesting that Blimp-1 was involved in the IL-10–dependent control of Th1 immune responses (64)

However, the role of Blimp-1 in the generation of B10 is conflicting. In a recent study, *Prdm1* mRNA expression was shown upregulated in IL10-positive compared to IL-10-negative B cells following LPS stimulation. This was then confirmed *in vivo* using a *Prdm1*-EYFP reporter mouse model. This group also demonstrated that the deletion of *Prdm1* led to the increased

frequency of B10 cells following LPS injection suggesting a negative role of Blimp-1 on the *Il10* transcription. Using luciferase reporter assays and chromatin immunoprecipitation (ChIP), these authors demonstrated that the CNS-0.45 kb site on the *Il10* gene was the major Blimp-1 suppressive site of the *Il10* transcription (**Figure 1**). They further demonstrated that STAT3 needed the Blimp1 DNA-binding domain to efficiently transactivate the *Il10* promoter indicating that Blimp1 could exhibit alternatively a positive role and negative role depending on the presence or absence of its molecular partners (65). However, these binding sites were not reported in primary studies identifying the pattern of regulatory regions within the *Il10* gene in T cells suggesting a B cell-specific regulation of the *Il10* transcription (46, 59, 61).

The Blimp-1 regulated genes have been extensively studied during the B-cell differentiation, revealing that Blimp1 could act as both transcriptional repressor and activator. Using streptavidin-mediated chromatin precipitation coupled with deep sequencing (Bio-ChIP), the *Il10* gene was robustly identified among the 93 genes potentially directly activated by Blimp-1 (66, 67). Regulatory plasma cells producing IL-10 have been reported in mice in EAE and during the *Salmonella Typhimurium* infection (3, 4) In EAE, the PB ($CD138^{+}$ $CD44^{high}$) subset was the main IL-10 producer population, generated through a germinal center (GC) independent pathway as the EAE course remained unchanged in $Bcl6^{yfp/yfp}$ knock-out mice. By investigating the mechanisms by which PB produced IL-10, it was demonstrated that B cells lacking IRF4 but not Blimp-1 have an impairment of IL-10 production. The TF IRF4 binds to the *Il10* CNS-9 enhancer site in mouse PB as previously described in T cells (68). This region highly conserved in the vertebrate genome has been also described as a major site of fixation of the nuclear factor of activated T cells (NFAT1 also named NF-ATc2) (69). The involvement of different CNS in the regulation of *Il10* transcription was significantly studied in different Th cell populations. Whereas the CNS-9 seemed to be potentially active in both Th1 and Th2 lineages, the CNS+6.45 and CNS-26 are specific for Th2 cells (68), where the CNS+6.45 region is a binding site for the activator protein 1 (AP1) proteins JunB, and c-Jun. However, such distinctions are not yet clearly established in human B10 compared to other B-cell subsets. Furthermore, the TF IRF4 itself binds to the DNA in a weak-dependent manner due to its carboxy-terminal auto-inhibitory domain. IRF4 has to form a complex with AP1 family proteins like BATF or JunB to increase its binding affinity and thus regulates genes expressing the AP1–IRF consensus motif elements (AICEs) (70, 71). In B cells, IRF4 through the cooperation with BATF, JunB, and JunD was described to bound to AICEs of *Il10* and *Ebi3* (coding for an IL-35 subunit) *loci* (70, 72). In T cells, Blimp-1 expression was required in a subset of IL-10 expressing Tregs localized at mucosal sites (73). In this subset, IRF4 directly regulated Blimp-1 expression at the transcriptional level. These data were also observed in B cells following IL-21 stimulation showing that STAT3 and IRF4 cooperate to promote *Prdm1* expression (74) (**Figure 1**). More recently an elegant study investigated the IL27-driven transcriptional network highlighting several key IL-10 molecular regulators (63) suggesting that *Prdm1, Irf4*, and *Irf8* may have a suppressive role in the production of IL-10 in type 1 Tregs (Tr1). Interestingly, IRF4 and IRF8 antagonized during B-cell activation and differentiation (75) suggesting opposite roles in the control of B-cell-specific *Il10* transcription.

We thus could hypothesize that IRF4 by utilizing distinct binding partners could mediate cell-type and *stimulus*-specific IL-10 production in different lymphocyte subsets. Furthermore, the time-frame of the molecular control of IL-10 is crucial and may explain contradictory relationships. The molecular mechanisms required for the induction of *Il10* in B cells may change throughout time to ensure its maintenance leading to important modification in the cooperative binding factors. Further studies, especially kinetics studies have to be designed to identify how the core triad including BLIMP-1, IRF4 and STAT3 may cooperate or act independently in the generation of B10 cells (**Figure 1**).

STAT3 and the NFAT Family in the Generation of IL-10 Producing B Cells

STAT3 signaling is involved in the early activation of B cells, especially in the BCR signalosome recruitment and the STAT3 deficiency led to reduction of pAkt and pErk1/2 and both mTORC1 and mTORC2 activity following BCR stimulation (76) Furthermore, STAT3 has also a non-canonical function and its localization to the endoplasmic reticulum (ER) modulates ER-mitochondria calcium (Ca^{2+}) release by interacting with the Ca^{2+} channel IP3R3 (77).

The implication of the BCR signaling in the generation of B10 is still discussed. Although *in vitro* activation of splenic B cells with anti-IgM Ab failed to induce IL-10 production compared to TLR signaling, there is a dramatic decrease of B10 cell numbers in the MD4 transgenic mice with a fixed BCR rearrangement suggesting that BCR diversity may direct B10 development (43). A report using TgVH3B4 mice that express a VH derived from an actin-reactive natural Ab, 3B4 revealed that B10 cells mainly targeted self-antigens (Ag). This study also suggest that B10 could be positively selected by self Ag and that high BCR signaling could promote B10 development and IL-10 production (78). In contrast to the MD4 model where there is no virtually Ag recognition, the interaction with the Ag seems mandatory for the maintenance and generation of B10. This study is in agreement with others showing that B10 induction is negatively or positively modulated in $CD19^{-/-}$ and $CD22^{-/-}$ KO mice, respectively where BCR signaling is directly altered.

B cells from mice that are deficient for the ER calcium sensors stromal interaction molecules 1 and 2 (STIM1 and STIM2) have normal B-cell development but display defective activation of NFAT1 and aberrant calcium (Ca^{2+}) signaling following BCR ligation (79) (**Figure 1**). In these mice, the BCR-induced dephosphorylation of NFAT1 was markedly impaired in B cells leading to a defect in IL-10 production, observations that were recapitulating with NFAT1 inhibitors such as cyclosporine or Tacrolimus (FK506). The authors further demonstrated that the double STIM1/STIM2 KO mice developed an exacerbated EAE

due to a dramatic depletion of B10. These data support that a Store Operated Calcium (SOC) influx induced by BCR stimulation drove IL-10 expression in a STIM-dependent NFAT1 activation manner. Several years later, the same group demonstrated that the transfection of active calcineurin A markedly increased BCR-induced IL-10 production through activation of NFAT1 in B cells using an *Il10-GFP* reporter mouse model. Furthermore, they showed that this induction is repressed in *Irf4* KO mice suggesting that NFAT1-dependent IL-10 production required IRF4 and was dependent on the Ca^{2+} influx (3).

On the other hand, another report showed that mice with an NFAT2 deficiency in the B-cell compartment have two-fold more B10 cells compared with wild-type B cells and lead to the amelioration of EAE (80). More recently, similar results showed that NFAT2 ablation in B cells suppressed the induction of skin inflammation by an increase of B10 ($CD1d^+$ $CD5^+$ and $CD138^+$ PB) in the spleen, the lymph nodes, and the blood (81) They have further shown that NFAT2 binds directly to the *Il10* gene *in vivo* and suppresses its transcription. However, the role of NFAT1 and NFAT2 in IL-10 production by B cells remains complex and differs between normal and malignant B cells. We previously demonstrated that the CD5 molecule, overexpressed in CLL B cells, promoted IL-10 expression and cell survival through a STAT3 and NFAT2-dependent pathway (82) and that IL-10 expression by B cells was directly connected with the aggressivity of the disease (83). These studies were further extended, demonstrating that NFAT2 controls multiple anergy-associated genes, BCR expression, and Ca^{2+} response in CLL B cells (84) and that knockdown of STAT3 significantly impaired the ability of CLL B cells to produce IL-10 and reverse T-cell dysfunction (85). Interestingly similar observations were made in diffuse large B-cell lymphoma (DLBCL) showing that STAT3 and NFAT2 interaction induced an immunoregulatory phenotype in malignant B cells with the upregulation of IL-10 and programmed Death-Ligand 1 (PDL1) (86).

This fine-tune regulation of Ca^{2+} signaling in B cells by its different partners including the balance between NFAT1 and NFAT2 and their interaction with STAT3 may be an important crossroad in the generation of B10 in normal and malignant B cells.

STAT3 and Metabolic Pathways in the Generation of IL-10 Producing B Cells

The STAT3 dimers translocate to the nucleus and regulate promoter genes with STAT3-binding elements exhibiting a broad range of cellular processes (87). Among the different STAT3 responsive genes, STAT3 induces the transcription of the Hypoxia-inducible factor (HIF) 1a in BCR and LPS-activated B cells through the ERK and NFkB-dependent pathways, respectively (88, 89) (**Figure 2**). HIFs are heterodimeric TF, consisting of an oxygen-labile alpha subunit (HIF-α) and a stable beta subunit (HIF-β). Three O2-sensitive HIFα proteins have been described so far, HIF-1α, HIF-2α -also known as EPAS1, and HIF-3α (90). The B-cell-specific deletion of HIF-1α, but not HIF-2α, resulted in a defect in IL-10 production by B cells (88). Except for an important decrease of the B1a population in the peritoneum observed in $Mb1^{cre}Hif1a^{f/f}$ mice, no other defect in the B-cell maturation was observed. This group further demonstrated that in BCR-stimulated B cells, HIF-1α cooperated with pSTAT3 to bind to hypoxia-responsive element (HRE) I and HRE II regions on the *Il10* promoter under hypoxic conditions. These authors showed that HIF-1α controlled the glycolytic metabolism required for the normal expansion of $CD1d^{high}$ $CD5^+$ Bregs *in vivo*. This report was supported by an additional study demonstrating that the suppression of HIF-1α in B cells led to exacerbated colitis through CD11b and IL-10 downregulation (89). The hypoxic microenvironment has been described in the physiological situation as during the GC reaction in secondary lymphoid organs (91), but also in solid tumors, transplantation, or inflammatory diseases. The fine characterization of Bregs in normal or pathophysiological tissues promises new stimulating insights in the better comprehension of these pathophysiological mechanisms.

Beyond the control of the glycolysis pathway, another metabolic driver involved in the control of IL-10 expression in B cells was recently described implying lipid metabolism through the multi-step process of transformation of Acetyl CoA to Cholesterol. The abrogation of the HMG-CoA reductase enzyme activity by atorvastatin led to the suppression of TLR9-induced regulatory capacities of human B cells (92). It was further demonstrated that IL-10 production was dependent on the geranylgeranyl transferase (GGTase) I (GGTi) that catabolizes conversion of Geranyl-Geranyl-PP (GGPP) toward the isoprenylation pathway. By controlling the downstream Ras-dependent PI3K-AKT signaling following TLR9 stimulation, the GGTase *via* inhibitory phosphorylation on the Serine 9 prevented the activity of the glycogen synthase kinase 3 (GSK3β) and the subsequent *Il10* transcription inhibition (**Figure 2**). GSK3 was already described as an important repressor of *Il10* among the Th1, Th2, and Th17 subsets (93, 94) and in dendritic cells (95) at the epigenetic level. The comparative RNA-sequencing analysis of TLR9 stimulated B cells in the presence or absence of GGTi revealed the strong downregulation of several TF including *Prdm1, Batf* (Basic Leucine Zipper ATF-Like Transcription Factor), and the *Aryl-hydrocarbon receptor (AhR)*. Finally, the siRNA-knockdown of Blimp-1 *in vitro* led to the reduction of the TLR9-mediated IL-10 production by B cells. This study is, so far, the only report that established the direct involvement of Blimp-1 in the generation of B10 cells.

In B cells, different studies have shown the AhR implication in IL-10 production in collaboration with numerous transcriptional cofactors (96) (**Figure 3**). The AhR is an environmental sensor that binds on a variety of ligands such as exogenous toxins (dioxin), xenobiotics, and endogenous derivatives metabolites from cells or microbiota. Mauri's group has established the involvement of the AhR in the control of *Il10* transcription in the immature $CD19^+$ $CD21^{high}$ $CD24^{high}$ B cell population in mice. *In vivo*, B cell-specific deletion of AhR caused exacerbated arthritis and promoted excessive inflammation by depleting B10. The AhR binding site seems to be located at -3.5 kb upstream of the TSS on the Il10 gene in a non-CNS region suggesting putative difference with human Bregs (97). This study was further extended by demonstrating that change in the short-chain fatty

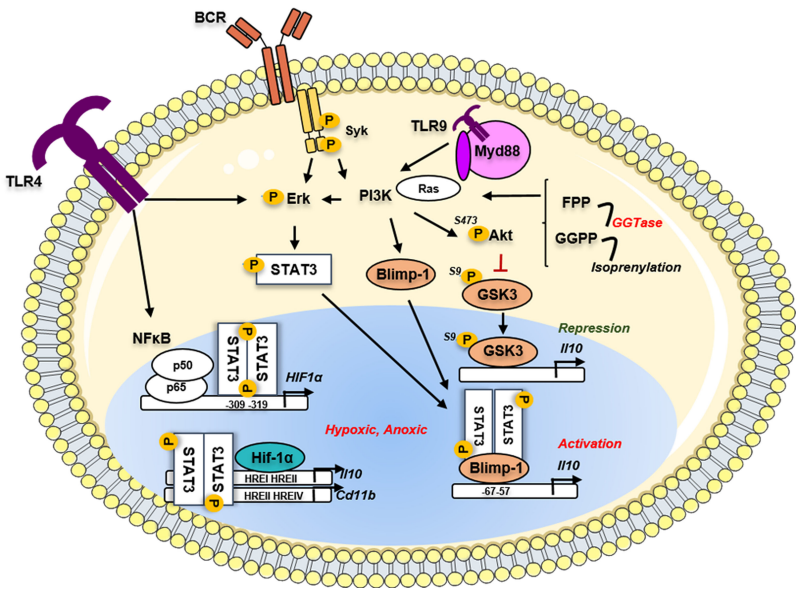

FIGURE 2 | Implication of the metabolic pathways in the regulation of the *Il10* gene in Bregs. Phosphorylated STAT3 could induce metabolic pathways such as the Hypoxia sensing by the transcription of Hif-1α. The Hif1α protein binds on the HRE sites located on the *Il10* gene to induce its transcription and its production by Bregs. In parallel, another metabolic driver implicated in the multi-step transformation of acetyl-CoA to cholesterol, the geranylgeranyl transferase I (GGTi) catalyses the conversion of geranylgeranyl-PP (GGPP) to drive the isoprenylation process. This mechanism controls the Ras-dependent downstream signal of the PI3K-Akt pathway, leading to the restriction of the inhibitory GSK3 activity and thus activation of the *Il10* transcription. The GGP-dependent pathway *via* the regulation of the PI3K downstream signaling regulated the *Prdm1* gene translation and the Blimp-1 translation directly promoting *Il10* production. TLR, toll-like receptor; BCR, B-cell receptor; Myd88, Myeloid differentiation primary response protein; Syk, Spleen associated tyrosine kinase; STAT3, Signal transducer and activator of transcription 3; Blimp-1, B lymphocyte-induced maturation protein 1; PI3K, Phosphoinositide 3 Kinase; Erk, Extracellular signal-regulated kinase; GSK3, glycogen synthase kinase; Akt, Protein kinase B; Ras, Rat sarcoma virus protein; FPP, Farnesyl pyrophosphate; GGPP, Geranylgeranyl pyrophosphate; GGTase, geranylgeranyl transferase; HIF1a, hypoxia inducible factor 1 subunit alpha; NFκB, Nuclear factor kappa B; p50, p50 protein; p65, p65 protein; HRE, hypoxia response element.

acid (SCFA) butyrate levels influenced the generation of $IL10^+$ $CD19^+$ $CD24^{high}$ $CD38^{high}$ B cells in patients with rheumatoid arthritis. By using an antigen-induced mouse model of arthritis, the authors established that the butyrate supplementation was sufficient to suppress the disease by increasing Bregs suppressive ability through an AhR dependent-manner (98) Moreover, AhR seems to also regulate the inhibitory receptor TIGIT and *Il10* expression in $TIM1^+$ B cells (99). Several years before, the fatty acid palmitate was demonstrated in synergy with CXCL12 able to induce IL-10 production in adipose B cells through the PI3K and NFkB signaling (100). News studies have recently demonstrated the role of SCFA in controlling B10 generation with some discrepancies mainly due to the different *in vitro* stimulation cocktails used for the B10 induction. The SCFA acetate, known to influence the lipid and the tricarboxylic acid (TCA) cycle, was demonstrated directly promoting the differentiation of IL-10 producing B cells from B1a progenitors in mice or from $CD24^{high}$ $CD27^+$ B cells in humans with a more potent activity than the butyrate (101). The inhibition of the conversion of acetate to acetyl-CoA by the repression of acetyl-CoA synthetase member 2 (ACSS2) decreases the IL-10 production suggesting that acetate is an essential regulator fueling the energy production necessary to B10 differentiation. In another report, when B cells were pre-activated with CD40, LPS, or CpG, the addition of butyrate, and pentanoate, but not acetate, increased the percentage of B10 cells (102). They demonstrated that the molecular mechanisms of butyrate-dependent B10 expansion are independent of the mammalian G-protein-coupled receptor pair activity but acted through HDAC inhibitory action *via* a p38 MAPK and PI3K-dependent pathway. However, the precise role of histone acetylation in B10 generation remains to be established. To note, the epigenetic regulation of *Il10* expression especially in B cells is still an unresolved field of research.

Overall, these reports about metabolism control of regulator B cells have opened an exciting new area of investigation, recently reviewed by the Mauri's group and ours (103, 104).

C-MAF AS A KEY DRIVER OF THE B-CELL IMMUNOREGULATORY FUNCTION?

The Transcription Factor c-MAF

c-MAF (MAF) is a basic region-leucine zipper (bZIP) TF belonging to the AP-1 superfamily and more specifically to the large MAF family proteins. Large MAF proteins include MafA, MafB, MAF, and Nrl, which possess a transactivation domain and activate transcription by forming a homodimer. MAF contains basic regions allowing the recognition of a palindromic sequence

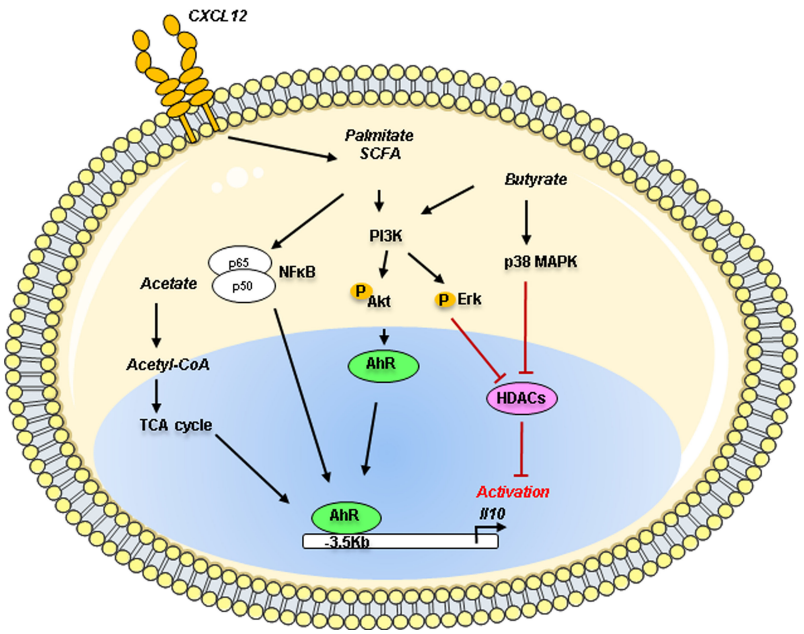

FIGURE 3 | Metabolites action on the Il10 gene regulation in Bregs. Metabolites such as acetate, palmitate and butyrate can control IL-10 production in B cells through the activation of the environmental sensor Aryl-hydrocarbon receptor (AhR) known to bind on a variety of derivative metabolites. Palmitate in synergy with CXCL12 induces IL-10 production through the PI3K and NFκB pathways. Acetate through its conversion into acetyl-coA could act as a regulator of the TCA cycle, fueling the differentiation of murine B1a cells or human $CD24^{high}$ $CD27^{+}$ into IL-10 producing B cells (B10). Butyrate can mediate B10 expansion *via* the p38 MAPK and PI3K pathways using the histone deacetylases (HDACs). PI3K, Phosphoinositide 3 Kinase; ErK, Extracellular signal-regulated kinase; Akt, Protein kinase B; MAPK, Mitogen-activated protein kinase; NFκB, Nuclear factor kappa B; AhR, Aryl hydrocarbon receptor; TCA, tricarboxylic acid; Acetyl-CoA, Acetyl-coenzyme A; SCFA, short-chain fatty acid; HDACs, histone deacetylases; p50, p50 protein; p65, p65 protein.

named MAF Recognition Element (MARE) in the promoter region of target genes of which there are two types: a 13-bp T-MARE (TGCTGAG/CTCAGCA) with a TPA-Responsive Element (TRE) and a 14-bp C-MARE (TGCTGAGC/CGTCAGCA) with an AMP-Responsive Element (CRE). The MAF gene is located on chromosome 16q23.2 in humans and on chromosome 8 in mice, on the reverse strand for both (105).

Two forms of MAF mRNA have been described in humans, the variant MAF-201 referred to as the small transcript and the MAF-202 variant referred to as the long transcript. The small transcript MAF-201 (2 646 bases) was made up of the 2 exons of the MAF gene after splicing of the intron (4 231 bases) which was not spliced in the long MAF-202 transcript (**Figure 4A**). The intron retention in MAF-202 has resulted in the introduction of a premature stop codon in the open reading frame leading to the production of a short protein isoform (c-MAF b), whereas the small transcript MAF-201 encoded a longer protein isoform (c-MAF a). The c-MAF a and c-MAF b proteins from these transcripts comprised 403 and 373 amino acids, respectively. A putative third protein and mRNA (referred to as medium variant MAF-203) have been described in the *Ensembl* database, however, neither the mRNA nor the protein have been reported in experimental data. The c-MAF a protein is only found in humans whereas the c-MAF b is present in both murine and human species. The different constitutive domains of the c-MAF proteins are highly conserved between the different isoforms (**Figure 4B**). The basic domain is requisite for DNA binding while the leucine zipper allows dimerization. The EHR domain (extended homology region) is specific for the MAF proteins and is also required for MAF binding to DNA. Finally, the transactivation domain which is rich in acidic residues responsible for protein's transcriptional activator functions is separated from the bZIP domain by a Hinge region (106).

The c-MAF proteins are subject to some post-translational modifications that could modulate their activity (**Figure 4C**). In T cells, the phosphorylation of c-MAF by the tyrosine-protein kinase Tec or by the CARMA1-dependent activation of the IκB kinase (via IKKβ) was required for c-MAF nuclear translocation and DNA binding (107–109). The serine-threonine protein kinase GSK3β-dependent c-MAF phosphorylation has been described with antagonist outcomes. GSK3β mediated c-MAF-phosphorylation and subsequent degradation through the ubiquitin-proteasome pathway in multiple myeloma. However, in presence of a coactivator complex, the phosphorylation and possibly its mono-ubiquitination may also result in the increase of its transcriptional activity (110, 111). At least nine phosphorylation sites located specifically in the activation domain of c-MAF (**Figure 4C**) were described, supporting their essential role in c-MAF activity. The SUMOylating, a post-translational modification resulting in the covalent binding of one or more SUMO proteins to a lysine motif was reported on the c-MAF protein at the lysine 33 leading to a

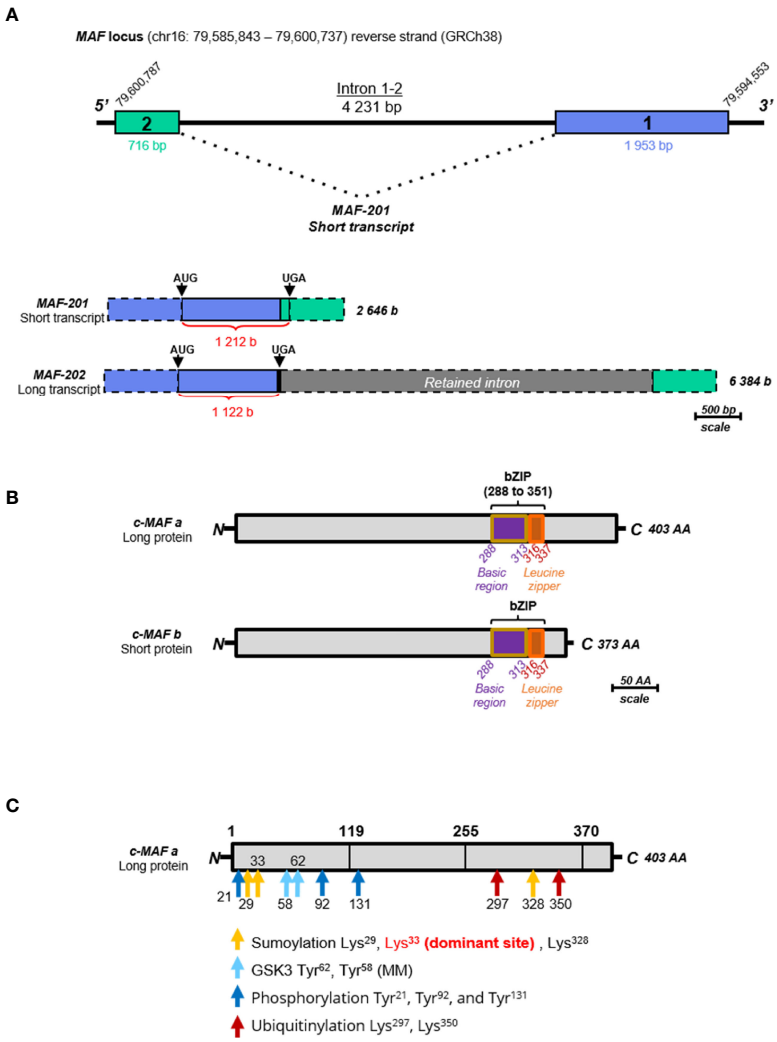

FIGURE 4 | Schematic representation of the MAF gene, transcripts and proteins. **(A)** Schematic representation of the MAF gene and transcripts. The scale used is 1 cm for 500 bases (b) or base pairs (bp). The blue rectangle represents the exon 1 (1 953 bp) and the green rectangle represents the exon 2 (716 bp). The only intron (4 231 bp) is represented by a black line. The two transcripts, MAF-201 (2 646 b) and MAF-202 (6384 b) are represented below, keeping the color code of the exons. The retained intron in the long transcript MAF-202 is represented by a grey rectangle. The untranslated parts of the exons are identified by dotted borders and the length of the open reading frame of each transcript is indicated in red. These open reading frames are delimited by "start" codons (AUG) and "stop" codons (UGA). **(B)** Schematic representation of c-Maf protein isoforms. The scale used is 1 cm for 50 amino acids (AA). The long protein (c-MAF a) is composed of 403 AA and the small protein (c-MAF b) is composed of 373 AA. The entire bZIP (basic leucine zipper) domain is indicated on each isoform within violet, the basic motif of 25 AA, and in orange the leucine zipper of 21 AA. All these data are extracted from databases "Ensembl" (ensemble.org) and "Uniprot" (uniprot.org). **(C)** Schematic representation of post-translational modifications of the c-MAF proteins, using the long protein as template. Post-translational modification sites are indicated by arrows: the sumoylation sites are in yellow, GSK3 phosphorylation sites are in blue, other phosphorylation sites are in dark blue, and ubiquitination sites are in red. There are three sites of sumoylation located on the lysine 29, lysine 328, and lysine 33 which is the dominant site. There are two sites of phosphorylation by GSK3 located on tyrosines 58 and 62. Three other sites of phosphorylation are indicated in dark blue located on the tyrosines 21, 92 and 131. Two sites of ubiquitination are located on the lysines 297 and 350 and indicated in red.

decrease of the c-MAF transcriptional activity on the *IL-4* and *IL-21* genes (112, 113).

MAF Regulation of the IL-10 Locus

MAF binds directly the MARE sequences of the *Il10* promoter in human macrophages (114), mouse B cells (115), Tr1 cells (96), or Th17 (116). However, several reports suggested that c-MAF could not induce *Il10* or even bound the *Il10 locus* in the absence of other co-regulatory factors like BATF or IRF1 as described in Tr1 cells (117). The protein c-MAF binds the CNS -9 together with BLIMP-1 and STAT4 and induces *Il10* in Th1 cells, but the binding of MAF is independent of BLIMP-1 expression (64). Although *Il10* represents a main target of the MAF gene, this target is far from being the only gene regulated by c-MAF. Indeed, c-MAF regulates a broad range of immune molecules as described for *RoRa, Il21, Il2, Il22* in Th17 (118),

colony-stimulating factor 1 receptor (*Csf-1r*), and metabolic pathways in macrophages (119) or *Tbx21* (encoding the TF T-bet) in group 3 innate lymphoid cells (120) underlined its crucial regulatory role in the immune system (121).

MAF Expression in IL-10 Producing T Cells and Macrophages

In T cells, extensive literature exists on the role of c-MAF and its relationship with IL-10 production [recently reviewed in (122)]. One recent elegant study underlined the context-specificity of the role of MAF in the T-cell response across different diseases model demonstrating that beyond the control of IL-10 expression, MAF acted as a negative regulator of *Il2* (123). One study has examined the role of both isoforms in the modulation of IL-10 production in human stimulated Th17 cells (116). Only the overexpression of c-MAF b (short protein) led to upregulation of IL-10 production suggesting a unique specific function of the different isoforms. Interestingly, extensive ChIP-sequencing studies revealed that c-MAF binding domains are mostly localized outside the promoters and may target enhancer sequences. Furthermore, c-MAF overexpression alone was not sufficient to convert Th17 IL-10^{-} cells into Th17 IL-10^{+} highlighting its primary role as a co-activator or co-repressor factor. The availability or competition of other cooperating factors together with the epigenetic accessibility of regulatory regions seem to be crucial for the c-MAF activity supporting its highly versatile broad range of action in many immune cells.

c-MAF is also essential during innate immunity and was first reported as a key element in macrophage polarization leading to IL-10 production and establishing the immunoregulatory M2 program (124). A recent report demonstrated that c-MAF contributes to the tumor-associated macrophages (TAMs) dampening T-cell effector function during lung cancer (119). Through ChIP-sequencing analysis, c-MAF was shown to directly regulate *Csf-1r* transcription and control important M2 markers as *Il12, Il1b*, *Il6, Arg1, Il10, Tgfb, Irf4*, and *Ccr2*. This study further demonstrated that c-MAF may act as a metabolic switch in macrophage inhibiting the glycolysis and promoting the TCA cycle and UDP-GlcNAc activity to favor macrophage conversion into immunosuppressive M2 within the tumor suggesting an important role of MAF on cell metabolic activity (119).

MAF in B Cells

The role of MAF in B cells is very less documented and a large gap in knowledge remains regarding the intrinsic function of c-MAF in B cells. Two recent papers suggested that c-MAF could exert an activity by controlling the IL-10 production in murine and human B cells suggesting an underestimated role in Breg function.

The first study demonstrated that MAF was constitutively expressed in B220^{+} B cells isolated from the spleen and could be upregulated following LPS stimulation together with IL-10 expression (115). ChIP-PCR identified that c-MAF directly bound a MARE sequence located at the CNS-0.5 on the *Il10* gene. The knockdown of MAF using specific shRNA in LPS-stimulated B cells resulted in the decrease but not a complete abrogation of *Il10* production. A second group has more recently suggested the role of c-MAF in regulating B10 during EAE. The B-cell specific deletion of the signaling lymphocytic activating molecules (SLAM)F5 (CD84) led to an important amelioration of the disease with delayed onset and attenuate clinical score (125). This disease improvement is accompanied by an increase in the different IL-10 producing Breg populations (CD5^{+} CD1d^{high}, T2-MZP, and CD138^{+} PB) in the spinal cord but also the spleen and lymph nodes. Using the Vert-x mice which express a GFP reporter on the *Il10* gene, they analyzed the transcriptome of CD19^{+} GFP^{+} cells in mice treated or not with a SLAMF5 blocking Ab revealing that SLAMF5 downmodulation increased c-MAF and AhR expression. Interestingly, these observations were also confirmed in humans showing that c-MAF is upregulated *in vitro* following SLAMF5 inhibition in total B cells. Although indirect, these results suggest that MAF could exert a key role in regulating Breg populations both in humans and mice. One interesting point is that MAF expression seems not restricted to a specific Breg subset but is broadly associated with *Il10* expression whereas AhR was demonstrated as specific to the T2/MZP subset (97). Interestingly, SLAMF5 (CD84) was also found to be downregulated in the human signature of GZMB^{+} B cells (35), suggesting maybe a broad inhibitory role in multiple Breg populations.

We recently developed an *in vitro* model of B-cell polarization to induce pro-inflammatory B (Be1) cells and Bregs suppressing CD4^{+} T cell proliferation (126). *In vitro*-induced Breg cells included mostly activated B cells (IgM^{+} CD38^{+} CD27^{-}) and unswitched PB (CD19^{+} IgM^{+} CD27^{low} CD38^{+} CD20^{+}) able to simultaneously produce IL-10 and IgM. To further characterize the co-cultured B-cells, we performed RNA-sequencing (RNA-seq) transcriptional analyses on three pooled samples of B cells sorted from the two different co-culture conditions. We assessed the differential expressing genes (DEGs) between Bregs and Be1 (**Figure 5A**). We observed 953 DEGs (P-value < 0.01) and 223 genes with a FC > $\left|1.5\right|$ (**Figure 5A**). We underlined a significant up-regulation of genes involved in immune regulation and PB differentiation including *IL-10*, *ELL2*, *IgJ*, *PRDM1*, and the transcription factor *MAF* (**Figure 5B**). We also highlighted several key molecules that could contribute to the immunoregulatory phenotype observed *in vitro* such as BATF, FOSL2, GATA3, CTLA4, TIGIT, or EPAS1 (HIF2-α). Interestingly, FOSL2 (also called Fra-2, an IL-2 regulator), and BATF are AP1 family transcription factors that have already been described as able to interact with MAF proteins (118). We confirmed at the protein level that c-MAF is upregulated together with BLIMP-1 and IRF4 in Bregs induced in our co-culture, suggesting that in these conditions c-MAF is associated with IL-10^{+} regulatory PB (**Figure 5C**).

To examine the conditions required for c-MAF induction, we stimulated peripheral B cells with CpG and IL-2 and or anti-CD40 Ab for two days, both simulations described as potent IL-10 inductors (127, 128). Whereas c-MAF is low expressed in resting peripheral B cells, c-MAF could be induced following stimulation with an optimal response through the combination

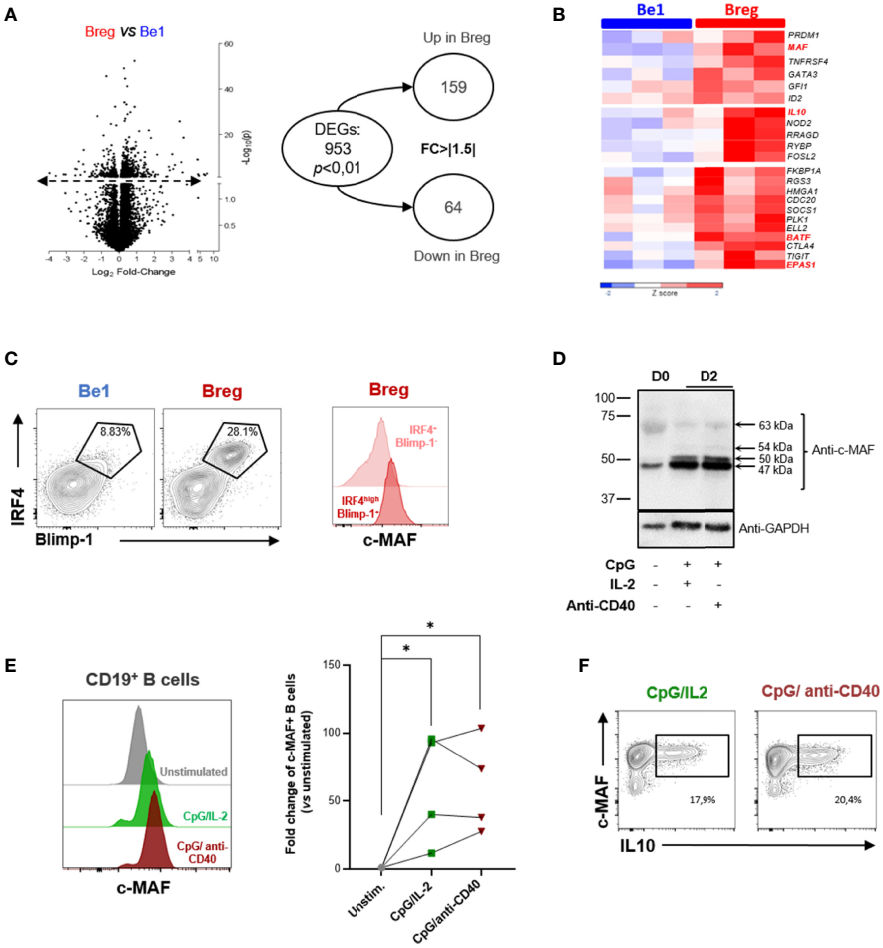

FIGURE 5 | MAF is upregulated in *in vitro*-induced human B10. **(A)** Differential expressing genes between pro-inflammatory B (Be1) cells and Bregs. RNA-sequencing transcriptional analysis was performed on three pooled samples of *in vitro* induced Be1 and Bregs. Left: Volcano plot reporting p value (-log10[P], y axis) as a function of log2 fold change (FC) between samples (x axis). Transcripts that were identified as significantly differentially expressed are represented above the dotted line. On the right, the circle chart shows numbers of differentially expressed genes (DEGs) with a p value < 0.01 and a FC > |1.5|. **(B)** Differential expression of genes implicated in immunoregulation between Breg and Be1. Expression of genes is represented in z score with a color gradient from dark blue for down-regulated expression to red for up-regulated expression. The MAF gene highlighted in red is the transcription factor more differentially expressed between Be1 and Breg. Other genes related to immunoregulation appear upregulated in Breg compared to Be1. **(C)** Left: Assessment of the differentiation state of Be1 and Breg using IRF4 and Blimp-1 expression. Black gates indicate IRF4^{high} BLIMP-1^{+} differentiated B cells. Right: Representative histogram of MAF expression in gated differentiated IRF4^{high} BLIMP-1^{+} (dark red) and IRF4^{+} BLIMP-1^{-} (light red) cells from *in vitro* generated Bregs. The expression of MAF is higher in regulatory plasmablasts than in IRF4^{+} Blimp-1^{-} Bregs. **(D, E)** c-MAF protein expression in unstimulated or two-days stimulated B cells assessed by western blotting **(D)** or flow cytometry **(E)**. **(D)** Western Blot showing the c-MAF expression in resting unstimulated peripheral B cells (D0) and in stimulated B cells simulated (D2) by CpG with IL-2 or anti-CD40 for two days. GAPDH expression was used as an endogenous control. Black arrows indicate the calculated sizes of the protein recognized by the anti-c-MAF antibody. **(E)** Left: Representative histograms of c-MAF protein expression in unstimulated (grey) or stimulated B cells by CpG/IL-2 (green) or CpG/anti-CD40 (brown) for two days. Right: Plot representing differential expression of c-MAF between unstimulated and stimulated B cells (N = 4). The c-MAF expression is represented in fold change of unstimulated B cells. The difference of MAF expression between unstimulated and stimulated B cells have been evaluated using Kruskal Wallis test combined with Dunn's multiple comparison test. *p-value < 0,05. **(F)** Assessment of the simultaneous expression of c-MAF and IL-10 expression production in stimulated B cells. Representative dot plots of c-MAF and IL-10 expression in the two-days stimulated B cells by CpG/IL2 (green) or CpG/anti-CD40 (brown). Black rectangles indicate the c-MAF^{+} IL10^{+} B cells. The percentage of this population is indicated below the rectangles.

of TLR9 with IL-2 or CD40 signaling (**Figure 5D**). As previously described, the western blotting analysis revealed different sizes of proteins recognized by anti-MAF Ab suggested some post-translational modifications (107, 129). The main form (47 kDa) observed in resting peripheral B cells was preferentially increased after stimulation. Two other c-MAF isoforms of 50 and 54 kDa were absent in resting B cells but induced following stimulation. The higher form (63 kDa) could correspond to the SUMOylated c-MAF described in mice (**Figure 5D**) (112, 113). Further investigations will be necessary to elucidate the post-translational modifications and the transcripts implicated in different c-MAF proteins. We further confirmed these results

by flow cytometry, with an expression 10 to 100 times higher (p-value < 0,05) compared with resting B cells (**Figure 5E**). We finally analyzed the production of IL-10 together with the c-MAF expression in stimulated B cells (**Figure 5F**). IL-10 intracellular detection was shown to be associated only with MAF-expressing B cells but was not observed in MAF$^-$ negative B cells. However, such observation only suggested a correlative phenomenon and further experiments are mandatory to understand the precise role of MAF on IL-10 production.

We next addressed whether MAF could be preferentially induced from a specific mature B-cell population (**Figure 6A**) and could be associated with B-cell differentiation. We thus sorted four B-cell populations according to IgD and CD27 expression and stimulated them for three days following BCR, TLR9, and IL-2 stimulation. We observed that c-MAF was upregulated in all mature B-cell subsets, however, we showed that the co-expression of c-MAF and BLIMP-1 expression is mainly observed in CD27$^+$ mature differentiated B cells. Finally, as c-MAF was weakly expressed in circulating resting B-cells but could be induced following activation, we hypothesized that c-MAF could be expressed in the secondary lymphoid tissues as described in mice. We then examined MAF expression in human tonsils, showing that MAF was barely detectable in naive B cells but was found significantly increased in IgD$^+$ CD27$^+$ unswitched memory, GC cells, and PB(**Figure 6B**).

Although preliminary and mostly correlative, our analyses emphasized that the TF MAF is expressed in human mature B cells and could be observed in IL-10$^+$ producing B cells as well as in BLIMP-1$^+$ PB. We also showed that c-MAF could be physiologically observed in secondary lymphoid organs open

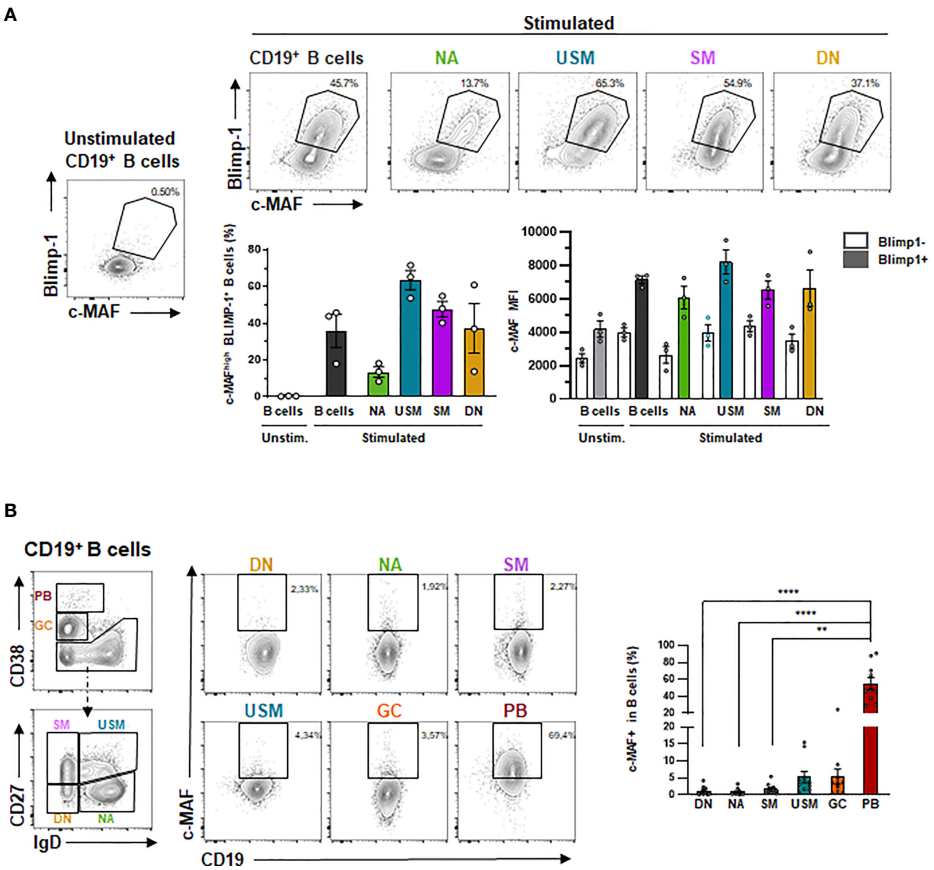

FIGURE 6 | MAF is up-regulated following B-cell differentiation and expressed in human tonsils. **(A)** Expression of BLIMP-1 and c-MAF in differentiated B cells. Up: Representative expression of c-MAF and BLIMP-1 by flow cytometry in unstimulated B cells or stimulated naive (NA), unswitched memory (USM), switched memory (SM), double-negative (DN) B cells and total B cells. Black polygons indicate c-MAF$^+$ BLIMP-1 $^+$ B cells. Down- Left: Percentage of MAF$^+$ BLIMP-1 $^+$ B cells in unstimulated B cells (light grey) or stimulated total B cells (dark grey), naives (green), unswitched memory (blue), switched memory (purple), double-negative (yellow) B cells (N = 3). Down – Right: Mean Fluorescence intensity of c-MAF in differentiated B cells (N = 3). **(B)** Expression of MAF in the different tonsillar B-cell populations. Left: Gating strategy for defining the different of differentiated B cell subpopulations is presented. Differentiated B-cell populations are determined delimited according to the expression of CD38, CD27 and IgD in six subpopulations: double-negative cells IgD$^-$ CD27$^-$ (DN, yellow), naïve IgD$^+$ CD27$^-$ (NA, green) switched memory IgD$^-$ CD27$^+$ (SM, purple), unswitched memory IgD$^+$ CD27$^+$ (USM, blue), germinal center cells IgD$^-$ CD38^{high} (GC, orange) and CD27^{high} CD38^{high} plasmablasts (PB, red). Right: Representative expression of c-MAF in the previously described subpopulations of tonsil B cells. Black rectangles indicate c-MAF$^+$ B cells. The percentage of c-MAF$^+$ B cells in each subpopulation is represented on the associated plot (N = 10). The difference of MAF expression between the populations have been evaluated using Kruskal Wallis test combined with Dunn's multiple comparison test. ****p-value < 0.0001; **p-value < 0,001.

new directions about its putative role in human B-cell homeostasis. However, further experiments are needed to fully understand its role in these different B-cell subsets. Furthermore, as described in T cells, we anticipated that MAF function will may be highly dependent on its different interacting molecular partners.

DISCUSSION: MAF ASSOCIATION WITH OTHER REGULATORS

A study providing a highly comprehensive view of the TF network regulating Th17 has underlined the key role of MAF in adaptive immunity (118). In Th17, c-MAF was shown as a negative regulator attenuating expression of several pro-inflammatory genes such as *Rora, Runx1, Il1r1, Ccr6*, or *Tnf* while upregulating immunoregulatory other ones such as *Il10* or *Ctla4* suggesting that its role is not restricted to cytokine regulation. As a bZIP TF, c-MAF could virtually interact with all AP1 protein members. We reported as follows the MAF relationships that may be relevant in B cells (**Figure 7**).

STAT3

STAT3 represents a key element in the transcriptional regulation of MAF expression in all helper T cells, but the mechanism remains unclear. MAF and SOX5 were demonstrated to be regulated by IL-6-mediated STAT3 activation driving Th17 expansion (130). Moreover, STAT3 binding on class II IL-6 responsive elements was observed on the MAF promoter in Th2 cells after TCR and IL-6 activation (131). This observation was also reproduced in $CD69^{+}$ $FOXP3^{+}$ Tregs where the treatment of cells with STAT3 and STAT5 siRNA reduced MAF expression (132). The TGFβ seems to synergically act with IL-6 to induce MAF expression by sustaining the STAT3 expression (133). IL-21 and IL-6 are upstream inducers of STAT3 activation and highly involved in plasma cell differentiation suggesting that they could also act in inducing MAF in B cells (134). Moreover, dysregulated STAT3 signaling has been reported in B cells in autoimmune diseases associated with abnormal GC reaction or plasma cell expansion (135). Does STAT3 influence B10 generation by regulating MAF expression? It is an ongoing question and several reports have already suggested that STAT3 deficient signaling contributes to impair IL-10 production in SLE B cells (48, 53).

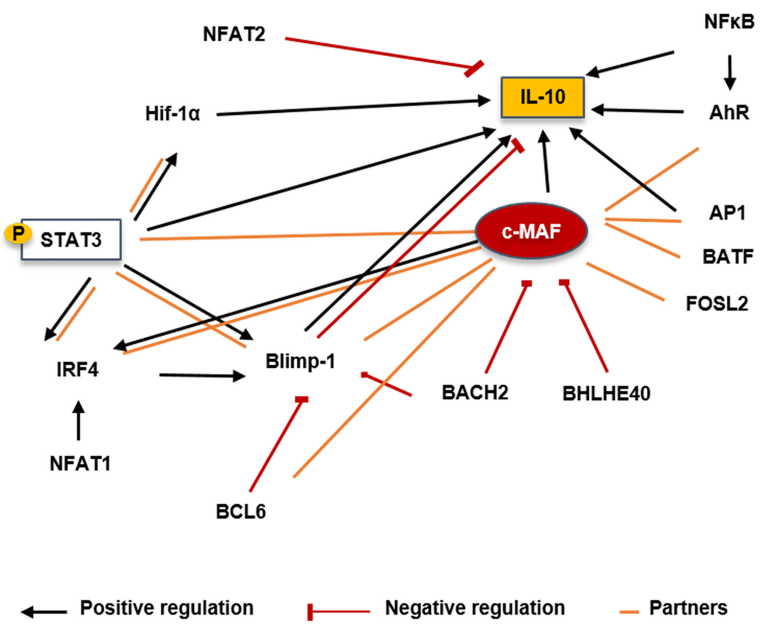

FIGURE 7 | Putative molecular factors involved in controlling *Il10* transcription in Bregs. STAT3 could induce expression upon IL-6 activation and could also bind on the class II IL-6 responsive elements present on the c-MAF promoter. BACH2 has been shown to directly repress c-MAF transcription by binding its promoter in Tregs. BHLHE40 is a major repressor of IL-10 production through the inhibition of c-MAF in T cells and macrophages. BCL6 cooperates with c-MAF to regulate Tfh-associated genes. Tfh-cell differentiation and BCL6 expression is promoted through the control of c-MAF expression. IRF4 could be induced by c-MAF with the cooperation of BATF. c-MAF could directly bind on the IRF4 locus but needs a partner to transactivate its promoter. BLIMP-1 may not directly interact with c-MAF but seems to form a complex with it in T cells and both seem to have compensatory roles. Positive regulations are represented by black arrows, negative regulation by red lines and partners by orange lines. IRF, Interferon regulatory factor; Blimp-1, B lymphocyte-induced maturation protein 1; NFAT, Nuclear factor of activated T-cells; HIF1α, hypoxia inducible factor 1 subunit alpha; pSTAT3, Phosphorylated signal transducer and activator of transcription 3; AhR, Aryl hydrocarbon receptor; NFκB, Nuclear factor kappa B; AP1, Activator protein 1; IL, Interleukin; BHLHE40, basic helix-loop-helix transcription factor 40; BACH2, Broad complex-tramtrack-bric a brac and Cap'n'collar homology 2; BCL6, B-cell lymphoma 6 protein; FOSL2, Fos-related antigen 2; BATF, Basic leucine zipper activating transcription factor-like.

BACH2

BACH2 is a TF involved in B cell differentiation and is controlled in human B cells by the IL-2 signaling (136, 137). BACH2 was mostly described as able to form heterodimers with small c-MAF proteins (136) binding to MARE domains. BACH2 interacts with MAFK to negatively regulate the immunoglobulin heavy chain gene 3' enhancer and the PRDM1 gene (138) controlling B cell differentiation and isotype switching. The BACH2 and the large MAF proteins interaction was not reported in B cells but BACH2 deletion in T cells led to an increase of the generation of Tfh-producing IL-4 and subsequent generated humoral autoimmunity (139). In this study, BACH2 was shown to directly repress *MAF* transcription by binding its promoter. BACH2 inhibits the differentiation of mature Tregs by competition with IRF4-recruiting AP-1 complexes for MARE genomic binding sites resulting in the decrease of Treg-associated genes such as *Il10, Tigit*, and, *Maf*. Interestingly, our previous data have shown that B-cell differentiation is associated with an upregulation of a similar pattern of genes suggesting that BACH2 could also repress *MAF* and *IRF4* expression in B cells.

BHLHE40

The basic helix-loop-helix transcription factor 40 (Bhlhe40) is a member of the basic helix-loop-helix transcription factor family. BHLHE40 is a central mediator of both inflammation and pathogen control in T cells and macrophages (review in (140)). BHLHE40 was found as a major repressor of *Il10* in IL-27 driven Tr1 or Th1 cells through the inhibition of MAF (63, 141). During *Mycobacterium tuberculosis* infection, BHLHE40 was shown to bind directly to the +6kb (CNS3) enhancer of the *Il10 locus* in T cells and myeloid cells (142). BHLHE40 also named DEC1, STRA13, or Clast5, was first described as a negative regulator of B-cell activation and is downregulated following LPS or BCR stimulation (143). BHLHE40 overexpression led to cell cycle arrest in WEHI231 B lymphoma cells. By studying Bhlhe41$^{-/-}$ mice, and DKO Bhlhe41$^{-/-}$ Bhlhe40$^{-/-}$, it was demonstrated that compared to BHLHE41, BHLHE40 contributes slightly to B1 development and it is still not clear whether BHLHE40 possesses intrinsic action on murine B-cell development (144). However, it was shown that BHLHE40 was induced in human B cells following BCR or TLR9 stimulation restraining proliferating capacities (145). Moreover, BHLHE40 is overexpressed in anergic CD21^{low} B cells of patients with hepatitis C virus, a subset recently underlined as pathogenic in autoimmune diseases. The role of BHLHE40 in controlling Breg expansion has to be determined and interestingly, we recently underlined that BHLHE40 is a common factor downregulated during the PB differentiation (67).

BCL6

The BCL6 protein was first described as a master regulator of B cells during the GC reaction (146, 147). There exists a reciprocal regulatory loop between Blimp-1 and BCL6 during the B-cell differentiation, whereby both TF antagonize the expression of each other (148, 149). This antagonism between BCL6 and BLIMP-1 was also described as a critical mechanism regulating the development of Tfh (150). BCL6 and c-MAF cooperate to regulate the expression of Tfh-associated genes like *PD1* or *CXCR5* (151). Furthermore, T cells lacking MAF expression failed to develop into Tfh after immunization (152). More recently the regulatory circuit between c-MAF, BLIMP-1, and BCL6 during the Tfh differentiation was examined in *Thpok* (encoded by the Zbtb7b gene) deficient mice (153). *Thpok* promoted Tfh cell differentiation and *Bcl6* expression through controlling *MAF* expression but independently of the repression of BLIMP-1.

IRF4

IRF4 is a key factor in the induction of regulatory plasma cells. Through a multi-layer -omics approach, c-MAF was suggested to interact with IRF4 in normal and myeloma plasma cells although no experimental data supported this idea (154). In T cells BATF and c-MAF cooperated to induce IRF4, to promote IL-4 production, and it was observed that c-MAF could bind to the IRF4 locus although it could not transactivate the promoter alone (155). To note, the action of c-MAF and IRF4 was also described in the M12 B cell lymphoma line acting in synergy with NFAT1 to enhance the IL-4 transcriptional activity (156)

BLIMP-1

The relationship between *MAF* and *PRDM1* has been studied with attention in Th1 and Th17. Although both factors seem to be required for *Il10* expression in both subsets, there are contradictory reports about their putative interaction as previously discussed (64). Whereas some reports suggested no physical interaction between both TFs, another study using co-immunoprecipitation assay suggested that MAF, Blimp-1, and RORγt form a complex in IL-27 stimulated T cells (157)

Moreover, a study that recently examined the regulatory network involved in the control of T-cell exhaustion in tumors underlined that although *MAF* and *PRDM1* are together involved in controlling several co-inhibitory receptors expression in T cells, both factors do not interact together and that *PRDM1* function in tumor-infiltrating T cells is independent of MAF suggesting that both TFs may have a compensatory role (116) Indeed, regression of tumor was observed more significantly in *MAF*, *PRDM1* double KO mice than in mice with a single gene deletion. Our data reported an association of *MAF* and *PRDM1* during the B cell differentiation, thus a role of MAF in the B-cell central regulatory network involving *BACH2*, *BCL6*, *IRF4*, and *PRDM1* is likely to also operate in the B cell differentiation and need to be further examined.

Role of the MicroRNAs miR155 and miR 21

MicroRNAs (miRNAs) are small non-coding RNA molecules that control gene expression by binding messenger RNA (mRNA). Whereas most of the studies demonstrated that the binding of miRNAs to 5′ UTR and coding regions have silencing effects, some others have also suggested that miRNA interaction with promoter region could have a positive effect and induce transcription (158). Of particular interest in B-cell physiology are the miR-155 and the miR-21.

First observations in mir155-deficient mice suggested that miR-155 control the GC formation, as well as, the extrafollicular response (159). It was further demonstrated that one main target of the miR155 was a member of the Ets domain-transcription factor family the TF PU.1 (160). Interestingly, the deletion of miR155 led to the overexpression of PU.1 resulting in the impairment of class switch recombination and plasma cell formation mostly by controlling the TF Pax5 (161).

The failure to downregulate Pax5 in PU.1-overexpressing cells prevented them from upregulating Blimp1 and thus initiating the plasma cell differentiation program. However, no direct role of miR155 on Blimp1 has been established so far.

The role of miRNA on IL-10 production is not clear. A recent report suggested that miR155 increased the expression of IL-10 by both upregulating the STAT3 phosphorylation and repressing the epigenetic regulator Jarid2 leading to the decrease in histone H3 lysine 27 trimethylation of the IL-10 promoter (162). On the other hand, the IL-10 production following LPS stimulation could inhibit miR155 in B cells in a STAT3-dependent manner (163). These complex interactions between IL-10, mir155, and STAT3 at the crossroad between B-cell differentiation and B-cell regulatory function need to be further elucidated but underlined their close relationship.

In one seminal paper, MAF has been described as an important target of miR-155 in T cells, and the increase of the Th2 profile observed in the miR-155 KO mice ($bic^{m1/m1}$ and $bic^{m2/m2}$) was partially attributed to the upregulation of the MAF protein in absence of its antagonist (164). The miR-155 suppressed MAF expression in microglia leading to the decrease of IL-10 thus promoting neuroinflammation (165). In Group 2 innate lymphoid cells (ILC2), miR-155 targets the 3′-UTR of the Maf mRNA and increased the production of IL-5, IL-9, and IL-13 Th2 cytokines in culture. Moreover, the treatment of mice with induced-allergic rhinitis with miR-155 antagomir significantly increased the expression of c-Maf and reduced allergic symptoms (166). Furthermore, another report described that c-MAF binding to the *Il10* promoter is restricted by *jarid2* in miR-155-deficient Th17 cells, emphasizing the importance of this network in regulating IL-10 expression.

Considering the key role of miR-155 in controlling the GC reaction, PB differentiation, IL-10 production, and MAF expression, we could anticipate that its expression may highly vary throughout the B-cell maturation. An in-depth investigation of these interactions could decipher important regulator mechanisms of the B-cell effector function.

The miR-21 was also described as a critical regulator of the B-cell differentiation and IL-10 production. Analysis of miR-21 levels in B cells revealed higher expression in activated B cells, especially in GC and memory B cells, and decreased expression in plasma cell differentiation (167). BLIMP-1 binds to the promoter of the pre-miR-21 to repress its expression upregulating miR-21 target genes, BTG2, PDCD4, and RHOB.

The miR-21 downregulated IL-10 expression in LPS-stimulated B cells *in vitro* and its expression is reduced in $CD1d^{hi}CD5^{+}$ and $Tim\text{-}1^{+}$ regulatory B cells. Furthermore, the administration of miR-21 antagonist *in vivo* decreased neuroinflammation and ameliorated the clinical symptoms of the EAE mostly through the increase of IL-10 producing B cells (168). However, the fine-tune mechanisms of miR-21 regulating IL-10 expression appear more elaborate, as one of its main targets the PDCD4 protein was also described as a potent IL-10 inhibitor (169). Interestingly, PDCD4 controls MAF expression by sequestering the Basic helix-loop-helix (bHLH) transcription factors Twist2 from the MAF promoter (170).

The list of miRNAs that could potentially control IL-10 producing B cells described here is far from being exhaustive, and it is not clear how the interplay between these different actors could influence the B-cell function. However, this entire field remains an exciting area of investigation still uncovered.

CONCLUDING REMARKS

The idea of a unique TF regulating all Breg populations seems unlikely, the diversity of precursors and mechanisms of action of Bregs are more compatible with microenvironmental plasticity and multiple molecular mechanisms. However, the extreme flexibility of the c-MAFs proteins to directly or indirectly cooperate with diverse partners modulating cell specification makes it a promising candidate in regulating B-cell effector functions such as Ab production or immune regulation.

MATERIAL AND METHODS

Patients and Samples

The study was performed in accordance with the Declaration of Helsinki and was approved by ethical committees. The patients/participants provided their written informed consent to participate in this study.

RNA Sequencing Sample Preparation

The *in-vitro*-induced Be1 and Be2/Breg were obtained in coculture with naive and memory T cells, respectively, as previously described (126). After coculture, B cells were sorted with the "FACS MoFlo XDP cell sorter" (Beckman Coulter) with a purity greater than 98%.

RNA-Sequencing

RNA-seq (50 bp paired-end) was performed on a Nextseq500 instrument (Illumina). The library preparation was done using the Illumina TruSeq® Stranded mRNA Sample preparation kit. The starting material (1 μg) of total RNA was mRNA enriched using the oligodT bead system. The isolated mRNA was subsequently digested using enzymatic fragmentation. Then, the first strand and second strand synthesis were performed and purified (AMPure XP, Beckman Coulter). Next, the double-stranded cDNA was end repaired, 3' adenylated, and Illumina sequencing adaptors were ligated onto the fragment ends. Finally, purified mRNA-stranded libraries were pre-amplified by PCR and the library size distributions were validated and quality inspected on a Bioanalyzer (high sensitivity DNA chip). High quality libraries were quantified using qPCR, the

concentration was normalized, and the 12 samples from six independent experiments were randomly pooled in six final libraries, three for the Be1 conditions and three for the B reg conditions. The library pools were re-quantified with qPCR and the optimal concentration of the library pools was used to generate the clusters on the surface of a flow cell before sequencing on a Nextseq500 instrument using a High Output sequencing kit (2 x 50 cycles) according to the manufacturer's instructions (Illumina). The quality of the Raw.fastq files was analyzed using the bcl2fastq software (Illumina), with a Q-score greater than 30. Reads mapping to the reference genome (GRCh37/hg19) were performed on quality-checked reads using STAR 2.4.1c. The reference annotation used was Ensembl_75. The overlap of reads with annotation features found in the reference *gtf* file was calculated using featureCount. The output computed for each sample (raw read counts) was then used as the input for Edge R analysis. Genes that had an expression level of under 10 counts per million (cpm) were excluded from the analysis. The quantile-adjusted conditional maximum likelihood (qCML) method was used to determine differentially expressed genes (DEGs) with p-value filters <0,01 and |fold-change (FC)|≥1,5.

Peripheral Blood B-Cell Isolation, Sorting, and Culture

The leukoreduction system chambers (LRSC) of Peripheral blood from healthy donors were obtained during routine plateletpheresis at the "Etablissement français du sang". The peripheral Blood Mononuclear Cells (PBMCs) were isolated using density gradient centrifugation on lymphocyte separation medium, Pancoll human (PAN Biotech). $CD19^+$ B cells were purified from human PBMCs using the REAlease® CD19 Microbead Kit (Miltenyi Biotec) with a purity greater than 98%, according to the manufacturer's instructions.

Isolated B cells were cultured in 96-well plates (BD Falcon) at $2x10^5$ cells per 200 µL/wells in complete medium (RPMI 1640 medium (Sigma-Aldrich)) supplemented with 10% of heat-inactivated Fetal Calf Serum (FCS) (BD Biosciences), 2 mM Glutamax (Gibco) and penicillin (200 U/mL) and streptomycin (100 µg/mL). B cells were stimulated for 48 h (2 days) in the presence of CpG oligodeoxynucleotide (ODN-2006) (1 µM; *In vivo*Gen), recombinant human Interleukin-2 (rh IL-2) (20 ng/mL, ImmunoTools) or anti-CD40 (1 mg/mL) (MAB-59; Beckman Coulter).

For the sorting of B cell subsets, isolated B cells were stained for 30 min at 4°C into the dark with IgD (IA6-2, Biolegend), CD10 (ALB1, Beckman coulter), CD27 (1A4CD27, Beckman coulter), CD19 (J3-119, Beckman coulter) Abs. CD10-positive B cells, which correspond to the transitional subset, were excluded from the cell sorting. Naive (NA, IgD^+ $CD27^-$ $CD10^-$), unswitched memory (USM, IgD^+ $CD27^+$ $CD10^-$), switched memory (SM, IgD^- $CD27^+$ $CD10^-$) and double negative B cells (DN, IgD^- $CD27^-$ $CD10^-$) were sorted. Sorted cells were cultured in 96-well plates (Falcon) at 2×10^5 cells per 200 µL/well in complete medium (RPMI 1640 medium supplemented with 10% of heat-inactivated FCS, 4 mM L-Glutamine (Gibco) and penicillin and streptomycin. B cell subsets were stimulated for 84 h (3.5 days), in the presence of the AffiniPure Goat anti-Human IgG+IgM (H+L) (2 µg/mL), the AffiniPure F(ab') 2 Fragment Goat Anti-Human Serum IgA, α Chain Specific (2 µg/mL; Jackson ImmunoReasearch Laboratories), CpG oligodeoxynucleotide (ODN-2006) (0,25 µM), recombinant human Interleukin-2 (rh IL-2) (20 ng/mL), as previously described (https://www.biorxiv.org/content/10.1101/2021.03.31.437810v1).

Isolation of Tonsil B Cells

Tonsils were collected during tonsillectomy. The Tonsillar Mononuclear Cells (TMCs) were isolated by mechanical disruption of tonsil tissue followed by Pancoll gradient centrifugation. Isolated TMCs were diluted in FCS up to a concentration of 10.10^6 TMCs/mL. Sheep red blood cells (Boehringer Ingelheim Therapeutics) were pre-incubated with Alserver solution (114 mM of dextrose, 27 mM of sodium citrate, 71 mM of sodium chloride, 1 M of citric acid diluted in distilled water) for 5 minutes and washed before an incubation at 37°C with 1 U/mL of neuraminidase (Sigma-aldrich) for 1 h. Neuraminidase-treated sheep red blood cells were washed and then incubated with the diluted TMCs during 10 minutes at 37°C. B cells from TMCs were then isolated following Pancoll gradient centrifugation, with a purity greater than 96%.

Flow Cytometry

All staining was performed using the Navios II flow cytometer (Beckman Coulter). The Abs with the following specificities were used: CD19 (J3-119), CD38 (LS198-4-3), CD27 (1A4CD27), IgD (IA6-2) c-MAF (T54-853, BD Biosciences), BLIMP-1 (646702, R&D systems) and IRF4 (REA201, Miltenyi Biotec), IL-10 (JES3-9D7, Biolegend). Intranuclear staining of IRF4, BLIMP-1 and c-MAF (T54-853, BD Biosciences) were performed using the Transcription Factor Buffer Set according to the manufacturer's instructions (BD Biosciences). For the detection of IL10 and c-MAF together, we used the CytoFast Perm Fix Set (BioLegend) according to the manufacturer's instructions. The c-MAF expression was analyzed using an unconjugated rabbit anti-MAF (BLR045F, Bethyl laboratories) revealed by a Fluorescein (FITC) AffiniPure $F(ab')_2$ Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories). Flow cytometry data were analyzed using Kaluza Analysis (v2.1) and FlowJo (v10.7.2) softwares.

Western Blot

Total proteins were extracted with RIPA Lysis Buffer (Thermo Fisher Scientific) supplemented with 1% of protease inhibitor cocktail (Sigma Aldrich). Samples treated with reducing Laemmli buffer (1.8% β-mercaptoethanol; Sigma Aldrich) were separated on SDS-PAGE with 10% acrylamide/bis (37,5:1) solution (Biorad) with the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Biorad) at 120 volts for 20 minutes and then at 160 volts for 45 minutes. Separated proteins were then transferred to a 0,2 µm PVDF membrane (Biorad) using the Trans-Blot® SD Semi-Dry Transfer Cell (Biorad) at 20 volts during 90 min. After washing in Tris Buffered Saline 0,1% Tween (TBST), the membrane was blocked for 1 hour in TBST containing 5% of fat dry milk (for c-MAF) or 5% of BSA (for GAPDH) at room temperature. The polyclonal rabbit c-MAF Ab

(ProteinTech) at 0,7 µg/mL in TBST containing 1% of fat dry milk and the membrane was incubated in this solution for 2 hours at room temperature. The secondary antibody used was a peroxydase conjugated donkey anti-rabbit IgG (H+L) Ab (GE Healthcare) at 0,2 µg/mL in 1% of fat dry milk TBST during one hour at room temperature. The HRP-Rabbit polyclonal to GAPDH (Abcam) in TBST containing 1% of BSA and the membrane was incubated overnight. The PVDF membranes were revealed by chemiluminescence (Immobilon Fort Western HRP substrate, Merck Millipore) by using the ChemiDoc™ XRS system (Biorad, Quantity One® Software).

Statistics

All statistics and graph representation were performed with GraphPad Prism (v.8.8.2). The Kruskal Wallis test combined with Dunn's multiple comparison test was used.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE112448.

ETHICS STATEMENT

The study was performed in accordance with the Declaration of Helsinki and was approved by the Brest Hospital ethical committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LLP and SH designed the study. MB, MM-C, and LLP wrote the first draft of the manuscript and figures. AG, MB, MM-C, and QS performed and analyzed the experiments. SH wrote the final version of the manuscript. DC, J-OP, and OM participated in the text edition. All authors contributed to the article and approved the submitted version.

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