Differential regulation of IgA⁺ B cells in vitro by stromal cells from distinctive anatomical compartments

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Abstract
B cell development is regulated by stromal cells (SCs) that form a supportive microenvironment. These SCs along with other cell types produce cytokines, chemokines, and adhesion molecules that guide B cell commitment and differentiation. BM, spleen (Sp), and the gut lamina propria (LP) constitute distinctive anatomical compartments that support B cell differentiation. In order to characterize and compare the signals necessary to generate IgA⁺ B cells, we developed an in vitro system to co-culture gut LP, BM, or Sp-derived SCs with B lineage cells. Using this co-culture system, we found that gut LP SCs promote IgA⁺ B cell accumulation through the production of soluble stimulatory factors. In contrast to gut LP SCs, BM and splenic SCs were found to impair IgA⁺ B cell accumulation in vitro. Taken together, these observations provide new insights into how SCs derived from different anatomical locations shape IgA⁺ B cell responses.

KEYWORDS
BM, differentiation, gut, spleen

1 | INTRODUCTION

About 80% of the antibody produced in mammals is IgA. The main function of IgA antibodies is to maintain homeostasis. In response to the colonization of commensal microbes, B cells in the gut-associated lymphoid tissue (GALT) produce large amounts of IgA, a dimeric secretory antibody responsible for the blockade of bacterial penetration. IgA class switch recombination (CSR) and plasma cell differentiation in GALT is influenced by metabolic products and cytokines produced by activated epithelial cells, stromal cells (SCs), dendritic cells, B cells, and T cells within the microenvironment.¹⁻⁵ Among all these cell types, the contribution of SCs has until recently been largely neglected. SCs are nonhematopoietic cells that form a supportive matrix, which can also influence the immune response.⁶ Therefore, understanding the interaction between SCs and lymphocytes becomes important. In this study, we focused on SCs derived from gut lamina propria (LP), BM, and spleen (Sp).

Studies comparing humoral immunity at mucosal surfaces to serum immune responses have shown a separation between secretory and systemic immune responses.⁷⁻⁸ Various reports show that IgG is the main Ig isotype found in serum, whereas IgA contributes mostly to mucosal immunity.⁷⁻¹¹ The gut LP, an effector site of GALT for IgA responses, is important for IgA⁺ B cell differentiation. For instance, a proliferation-inducing ligand (APRIL) secreted by gut LP SCs and epithelial cells and B-cell activating factor (BAFF) secreted by gut LP stromal cells induce CD40-independent IgA CSR.¹²,¹³ Unlike the gut LP, BM and Sp constitute different microenvironments for B cell differentiation. On one hand, the BM provides a niche for B cell development with SCs such as osteoblasts, CXCL12hi reticular cells, and IL-7 expressing cells.¹⁴ Besides providing support to early B cell development, the BM is a niche for plasma cell survival. In contrast, multiple human studies indicate that mesenchymal stromal cells (MSCs) from the BM have immunosuppressive effects on B cells.¹⁵⁻¹⁷ The BM has not been considered a site for B cell activation and CSR since it lacks...
germinal centers. However, several studies have found that BM is a site for B cell maturation and T-independent IgM plasma cell differentiation in response to blood-borne microbes.\textsuperscript{18,19} On the other hand, the Sp is a secondary lymphoid organ responsible for the maturation, activation, and differentiation of B cells.\textsuperscript{20} Within the white pulp, follicular dendritic cells, fibroblastic reticular cells, and marginal reticular cells are the most studied SC types.\textsuperscript{20} After infection or immunization, Sp follicular B cells preferentially switch to IgG, and IgG\textsuperscript{+} plasma cells are found in the red pulp.\textsuperscript{21,22} However, to add to the complexity, in mice, both B1 cells and marginal zone B cells in the Sp contribute to IgA production in vitro.\textsuperscript{23} The reason why IgA CSR does not predominantly take place within the BM and Sp is largely unknown. It could be possible that BM and Sp microenvironments actively inhibit IgA plasma cell generation.

In short, SCs from different sites constitute distinctive microenvironments for differential IgA responses. Therefore, we established an in vitro model for analyzing the effects of SCs on IgA\textsuperscript{+} B cells that were generated from Bm-derived B cells. This model enabled us to study and compare the influence of stromal cells derived from the gut LP, BM, and Sp on IgA\textsuperscript{+} B cell development. We found that SCs from distinctive anatomical compartments exert different effects on IgA\textsuperscript{+} B cell generation.

2 | MATERIALS AND METHODS

2.1 | Stromal cell preparation

Female C57BL/6 mice were sacrificed and small intestines were harvested. Detailed protocols for intestine digestion have been described in our previous published article.\textsuperscript{24} Cells from the small intestine were cultured in complete medium, composed of Opti-MEM\textsuperscript{®} Reduced Serum Medium (Life Technologies, Carlsbad, California, United States) with 10% heat-inactivated FBS, β-mercaptoethanol (50 μM), NaHCO\textsubscript{3} (2.4 g/L) and penicillin-streptomycin (100 pg/mL), for 1–2 wk and non-adherent cells were washed off every day with new complete medium until the monolayer of SCs reached confluency. Total cells recovered from femurs and tibias of female C56BL/6 were plated in T25 flask for 1–2 wk with complete medium, and non-adherent cells were washed off every day with new complete medium until a monolayer of SCs reached confluency. Sp SCs were prepared by plating total cells from the Sp in a T25 flask for 1–2 wk with complete medium, and nonadherent cells were washed off every day with new complete medium until a monolayer of SCs reached confluency.

To prepare the SCs for staining, SCs were treated with PBS with 1 mM EDTA for 5–10 min at room temperature (25°C) on a shaking platform. Remaining adherent SCs were lifted by cell scrapers. SCs were stained following manufacturer’s instructions.

2.2 | B cell-SC co-culture

B lineage cells were harvested from the femurs and tibias of C56BL/6 mice and were isolated using a CD19 positive selection kit (Mojosort Biolegend, San Diego, California, United States). SCs were trypsinized with 0.25% trypsin and irradiated at 2000 cGy. A total of 20,000 irradiated SCs were plated in a co-culture system with 200,000 CD19-selected BM cells in 500 μL complete medium supplemented with a cocktail composed of the following factors: IL-21 (30 ng/mL, R&D Systems, Minneapolis, Minnesota, United States), TGF-β (2.5 ng/mL, R&D Systems, Minneapolis, Minnesota, United States), IL-7 (0.5 ng/mL), 2 μg of rat IgG2a anti-mouse CD40 (clone 3/23, BD Biosciences, San Jose, California, United States), and fecal matter (extracted according to Fritz et al.\textsuperscript{24} and LPS at 1EU/mL) in a 24-well plate. On day 4, another 500 μL of complete medium with factors was added to each well. Cells were then harvested and ELISPOT or flow cytometry analysis was performed to assess the number of IgA\textsuperscript{+} B cells. Neutralization of BAFF was performed using anti-BAFF blocking antibodies (R&D, Minneapolis, Minnesota, United States). Stimulation with BAFF was performed using mouse recombinant BAFF (Biolegend, San Diego, California, United States). Stimulation with monocyte chemo tactic protein-1 (MCP-1) was performed using mouse recombinant MCP-1 (Biolegend, San Diego, California, United States). Anti-MCP-1 purified NA/LE Hamster anti-mouse antibody (BD Bioscience, San Jose, California, United States) was added at 5 μg/mL.

2.3 | Cell lines

NS451 is a gut LP SC line isolated from the gut LP primary stroma of a B6 CD45.1 mouse. It was established through repeated passaging. Additional gut LP SC lines, LPSC1, LPSC2, LPSC3, LPSC4, LPSC5, LPSC6, and LPSC7, were established by limiting dilution of the adherent cells derived from the gut LP primary stroma from B6 mice.

2.4 | Flow cytometry

Antibodies used for flow cytometry analysis included FITC-anti-IgA (C10-3, BD Biosciences, San Jose, California, United States), PE-anti-IgA (11-44-2, Southern, Birmingham, Alabama, United States), AlexaFluor647-anti-Blimp1 (SE7 BD Biosciences, San Jose, California, United States), FITC-anti-CD45 (30-F11, ebioscience, Waltham, Massachusetts, United States), PE-anti-CD31 (MEC13.3, Biolegend, San Diego, California, United States), and APC-anti-GP38 (8.1.1 Biolegend, San Diego, California, United States). Dead cells were excluded using the Zombie UV Fixable viability dye (Biolegend, San Diego, California, United States). The FOXP3 Staining Buffer Set (ebioscience, Carlsbad, California, United States) was used for intracellular staining. LSRFortessa 5-laser (325; 405; 488; 561; 632) configuration (BD Biosciences, San Jose, California, United States) was used.

2.5 | Enzyme-linked Immunosorbent assay

A total of 100,000 IgA-selected cells (PE-anti-IgA + Stemcell PE selection kit, Vancouver, British Columbia, Canada) were seeded in 24-well plates with 20,000 SCs (gut, BM, Sp) in 500 μL complete OPTI-MEM medium with factor cocktail. A total of 100 μL of supernatant was collected 18hr, 48hr, or 72hr after seeding. A total of 96-well ELISA plates were coated with 10 μg/mL polyclonal anti-IgA coating antibodies (Sigma, St. Louis, Missouri, United States) in PBS overnight, and then subsequently blocked by OPTI-MEM supplemented with 10% FBS. Supernatant was added and incubated for 2hr at room
temperature. IgAs were detected by 1:1000 polyclonal HRP-anti-IgA (Sigma, St. Louis, Missouri, United States) antibodies.

2.6 | Enzyme-linked immunospot

A total of 96-well polyvinylidene difluoride filter plates (Millipore, Burlington, Massachusetts, United States) were coated with 10 μg/mL polyclonal anti-IgA coating antibodies (Sigma, St. Louis, Missouri, United States) or 10 μg/mL polyclonal anti-Ig coating antibodies (Sigma, St. Louis, Missouri, United States) in PBS overnight, and then subsequently blocked by OPTI-MEM supplemented with 10% FBS. A total of 10,000 cells recovered from B cell-SC co-cultures were plated and incubated overnight at 37°C, and IgA secreting cells were detected using 1:1000 polyclonal HRP-anti-IgA (Sigma, St. Louis, Missouri, United States) antibodies. Spots were developed with AEC (Sigma, St. Louis, Missouri, United States), and automatically counted using ImmunoSpot Analysis software (Cellular Technology Ltd., Shaker Heights, Ohio, United States).

2.7 | Preparation of conditioned medium

SCs were irradiated at 2000 cGy, and different cell numbers were plated. SCs were plated on day 0 in 500 μL complete medium supplemented with the cocktail of factors mentioned above. A control well was set up to contain 500 μL of complete medium supplemented with the factors. On day 4, 500 μL complete medium was added to cultures to achieve 1 mL total conditioned medium (CM). On day 6, supernatant was collected from each well, centrifuged at 1250 rpm to remove cells, and stored at -20°C. For subsequent CM experiments, 500 μL CM was added to 200,000 CD19+ BM cell cultures on day 0, and another 500 μL on day 4. On day 6, IgA expression was assessed by flow cytometry.

2.8 | Liquid chromatography–tandem mass spectrometry

Conditioned medium for mass spectrometry analysis was prepared by plating 500,000 stromal cells from gut LP, BM, and Sp in 10 mL complete medium supplemented with the factors in T75 flasks. SCs were irradiated at 2000 cGy on day 0 and cultured for 5 days. On day 5, SCs were washed 3 times using PBS and re-suspended using serum free IMDM medium. On day 6, supernatant was collected. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed by SPARC BioCentre (Toronto, Canada). ThermoFisher LTQ Ion Trap Mass Spectrometer was used to collect data. LC-MS/MS data was analyzed by Scaffold 4, Maxquant, and Perseus Software. Z-score with P ≤ 0.05 was plotted on an expression heatmap.

2.9 | Dual chamber assays

A total of 200,000 CD19+ BM cells and 20,000 SCs from BM, Sp, or gut LP were plated in the bottom chamber in complete medium supplemented with factors. A total of 20,000 SCs were plated in the top chamber of a transwell with a 0.4 μm pore size (Costar, St. Louis, Missouri, United States). Cytodex 1 (GE Health, Chicago, Illinois, United States), a microcarrier for cell attachment, was added to the top chamber at 1 g/L suggested by manufacture. On day 6, IgA expression was assessed.

2.10 | B cell assays

Proliferation assays were performed by adding 5 μM EdU, 5-ethyl-2′-deoxyuridine, a nucleoside analog of thymidine (Click-iTR® EdU Alexa Fluor® 488 Imaging Kit, ThermoFisher, Waltham, Massachusetts, United States), into cultures for 4 h. Proliferation of cells, as defined by EdU incorporation into DNA, was later assessed by flow cytometry.

Plasma cell differentiation was measured by co-culturing 200,000 CD19+ BM cells from B6.Cg-Tg (Pdrtm1-EYFP) 1Mzn/J mouse, a Blimp1-YFP reporter mouse kindly provided by Dr. Jennifer Gomerman’s lab, and 20,000 SCs derived from the gut LP, BM, and spleen of wild type B6 mouse. On day 6, IgA+ B cell differentiation was determined by the number of YFP+ IgA+ B cells (a.k.a Blimp-1+ IgA+ B cells).

BM cells were sorted into pro-B cells (CD19+CD2+IgM−IgD−), pre-B cells (CD19+CD2+IgM+IgD−), immature B cells (CD19+CD2+BP.1−IgD−), and mature B cells (CD19+IgM+IgD+). A total of 200,000 sorted cells were co-cultured with 20,000 irradiated stromal cells with 500μL complete medium supplemented with a cocktail composed of the following factors: IL-21 (30 ng/mL, R&D Systems, Minneapolis, Minnesota, United States), TGFβ (2.5 ng/mL, R&D Systems), IL-7 (0.5 ng/mL), 2 μg of rat IgG2a anti-mouse CD40 (clone 3/23, BD Biosciences, San Jose, California, United States), and feeder matter (extracted according to Fritz et al.24 and LPS at 1EU/mL in a 24-well plate). On day 4, another 500μL of complete medium with factors was added to each well. Cells were then harvested and flow cytometry analysis was performed to assess the number of IgA+ B cells.

2.11 | IgA+ B cell assays

BM IgA+ B cell survival was assessed on sorted IgA+ cells from the BM. Cells were stained with cell tracker dye (CyToxiLux, OncolImmunin, Gaithersburg, Maryland, United States). A total of 10,000 sorted IgA+ B cells were co-cultured with 1000 irradiated SCs in a 96-well plate. Viability of IgA+ B cells was assessed on days 0, 2, 4 using the Zombie UV viability dye and cell counting.

In vitro generated IgA+ B cell survival was assessed on selected IgA+ B cells. IgA+ B cells were selected (IgA-PE and Stemcell PE selection kit, Vancouver, British Columbia, Canada) from a 6-day B cell-gut LP SC co-culture. A total of 100,000 selected IgA+ B cells were co-cultured with 20,000 irradiated SCs in a 24-well plate. Viability of IgA+ B cells was assessed on hours 0, 18, 48, and 72 using the Zombie UV viability dye and cell counting.

IgA+ B cell proliferation assay was performed using a CFSE cell proliferation kit (ThermoFisher, Waltham, Massachusetts, United States). A total of 100,000 PE-selected IgA+ B cells were stained with CFSE following manufacturer’s protocol. CFSE-stained IgA+ B cell were co-cultured with 20,000 SCs. Proliferation of IgA+ B cells was assessed on hours 0, 18, 48, and 72 after CFSE staining using FACS.
FIGURE 1  An in vitro system for the comparative analysis on B cell–SC interaction. (A) Accumulation of IgA⁺ B cells in vitro with different SCs was measured by co-culturing 200,000 CD19⁺ BM cells with 0 (No stroma control) or 20,000 SCs from either gut LP, BM, and Sp. On day 6, the expression of IgA was assayed by flow cytometry. Statistical significance was based on one-way ANOVA of 8 independent experiments. Error bars represented SD. (B) ELISPOT detecting IgA-secreting cells was performed on 10,000 suspended cells recovered from cultures of B lineage cells with LP stroma, BM stroma, or Sp stroma. Data was representative of 3 independent experiments. (C) The ratio of Blimp-1⁺ IgA⁺ B cells over IgA⁺ B cells in vitro after 6 days of co-culturing CD19⁺ BM cells with SCs was plotted. Statistical significance was based on one-way ANOVA on 3 independent experiments. (D) Proliferation of IgA⁺ B cells was assessed. On day 6, Edu was added to B cell-SC co-cultures for 4 h, and cell proliferation was assessed by measuring the number of Edu incorporated IgA⁺ B cells. Statistical significance was based on one-way ANOVA on 3 independent experiments. (E and F) Proliferation of CFSE-stained IgA⁺ B cells was assessed on 18, 48, and 72 h. (G) Survival of IgA⁺ B cells co-cultured with gut LP, BM, and Sp SCs was assessed. Statistical significance was assessed by two-way ANOVA. (H) A total of 100,000 selected IgA⁺ B cells were co-cultured with gut LP, BM, and Sp stroma in 500 μL complete medium with factors. ELISA measuring secreted IgA was performed on the supernatant collected on 18, 48, and 72 h. Antibody per 1000 cells was calculated by concentration of IgA antibody (ng/ml) × number of total IgA⁺ B cells in culture × 1000 cells. Statistical significance was assessed by two-way ANOVA.
3 | RESULTS

3.1 | Differentiation of IgA+ B cells is optimized in the presence of gut LP-derived SCs

To test whether SCs from distinctive anatomical compartments exert different effects on IgA+ B cell generation, we followed our published protocol that promotes the differentiation of IgA+ B cells in an in vitro culture system. This entailed seeding BM-derived CD19-selected B lineage cells into cultures containing IL-7, anti-CD40, TGFβ, IL-21, fecal matter, and either gut LP (from small intestines), BM- or Sp-derived SCs for 6 days. Gut LP SCs strongly enhanced the accumulation of IgA+ B cells (Fig. 1A). However, SCs established from the BM or Sp failed to provide similar support (Figs. 1A). This finding was based both on total IgA+ B cells detected on day 6 of cultures using flow cytometry (Fig. 1A) as well as in an ELISPOT assay measuring IgA secreting cells (Fig. 1B).

We next examined the differentiation and proliferation of the IgA+ B cells generated in vitro. The differentiation status of IgA+ B cells was assessed by Blimp-1 expression. Blimp-1 is a transcription factor that drives terminal differentiation of plasma cells. In order to more accurately assess Blimp-1 expression, we cultured CD19+B cells from Blimp1-YFP mice. When CD19+B cells from Blimp1-YFP mice were co-cultured with SCs supplemented with factors, IgA+ B cells were generated. Blimp-1+ IgA+ B cells were detected when B cells were co-cultured with LP stroma, whereas they were almost absent when BM stroma or Sp stroma was present (Fig. 1C). A part of the in vitro generated IgA+ B cells underwent terminal differentiation as evidenced by Blimp-1 expression, while others underwent proliferation. In the presence of gut LP SCs but not with BM or Sp SCs, a large number of IgA+ B cells was proliferating (Fig. 1D).

To accurately establish the basis of the stimulatory effect on IgA+ B cells, we selected IgA+ B cells derived from a 6-day gut LP-CD19+B cell co-culture and plated them with different stromal conditions (Fig. 1E–H). We evaluated the stimulatory activity of gut SCs on IgA+ B cell co-culture and plated them with different stromal conditions (Fig. 1A). However, SCs established from the BM or Sp failed to provide similar support (Figs. 1A). This finding was based both on total IgA+ B cells detected on day 6 of cultures using flow cytometry (Fig. 1A) as well as in an ELISPOT assay measuring IgA secreting cells (Fig. 1B).

A total of 200,000 CD19+B cells contained a heterogeneous population including 25% pro-B cells, 24% pre-B cells, 15% immature B cells, 11% mature B cells, 0.50% IgA+ B cells, 10% other cells, and 15% dead cells determined by FACS. In order to clarify which subsets of B cells in this co-culture effectively yield IgA+ B cells, we seeded highly selected B cell subsets with different SCs (Fig. 2C). BM cells were sorted into pro-B cells (CD19+CD21+IgM+IgD−), pre-B cells (CD19+CD21−IgM+IgD−), immature B cells (CD19+CD21+IgM−IgD−), and mature B cells (CD19+IgM+IgD+) (Fig. 2C). All B cell subsets except pro-B cells contributed to the accumulation of IgA+ B cells in vitro (Fig. 2C). To rule out the possibility that the accumulation of IgA+ B cells was due to the expansion of the few IgA+ B cells from the starting population, we assessed the survival of IgA+ B cells derived from the BM over time. Most IgA+ B cells did not survive past day 4 (Fig. 2D), indicating that the proliferation of pre-existing IgA+ B cells was not responsible for the accumulation of IgA+ B cells on day 6. As a result, using a heterogeneous CD19-selected population from the BM was a reasonable approach for future experiments.

Primary stroma contains a heterogeneous population of cells, which includes cell types such as endothelial cells and fibroblasts. To identify the different cell types of the primary stroma, we stained the adherent layers derived from gut LP, BM, and Sp using the common stromal cell marker GP38 and CD31 with lymphatic endothelial cells being CD45−GP38+CD31−, fibroblastic reticular cells being CD45−GP38−CD31−, blood endothelial cells being CD45+GP38−CD31−, and double negative cells being CD45−GP38−CD31+. Gut SCs had a relatively homogenous fibroblastic reticular cell phenotype, while BM SCs and splenic stromal cells contained a heterogeneous population (Fig. 2E). To reduce the level of complexity, we established and tested cell lines to determine if they could recapitulate the effect of primary stroma. We cloned multiple...
FIGURE 2  Development of the in vitro system measuring stromal effects on IgA+ B cells. (A) Factors were titrated. Unless being titrated, factors were provided at the following concentrations: IL-7 at 0.5 ng/mL, αCD40 at 2 μg/mL, TGFβ at 2.5 ng/mL, IL-21 at 30 ng/mL, and FM at 1EU/mL. Statistical significance was based on two-way ANOVA on 3 independent experiments. Error bar was shown in SD. (B) Time course analysis was performed on the accumulation of IgA+ B cells in culture with 200,000 CD19+ BM cells as starting population. Statistical significance was based on two-way ANOVA on 3 independent experiments. (C) B lineage cells gave rise to IgA+ B cells in culture. Different B cell subsets were sorted based on CD19, CD2, IgM, IgD, and BP1 expression (Material and Methods). Sorted B cell subsets were co-cultured with SCs following our usual protocol. IgA+ B cell number was assessed on day 6. (D) Survival of IgA+ B cells was assessed. IgA+ B cells sorted from the BM stained with CyToxiLux® cell tracker dye were co-cultured with 190,000 CD19+ B cells and 20,000 SCs. Viability of IgA+ B cells was assayed on day 0, 2, 4, and 6 by tracing the number of cell-tracker-dye+ IgA+ B cells in vitro. (E) SCs from gut LP, BM, and Sp were stained using CD45, GP38, and CD31. CD45+ population in gut LP SCs is <1%, BM 4%, and Sp 5%. (F) Capacity of different gut LP SC lines to induce IgA+ B cell accumulation was measured in vitro. NS451, LPSC1-7 lines were gut LP cell lines while PA6 was a BM cell line. Statistical significance was based on one-way ANOVA on 3 independent experiments.
cell lines from the gut LP stroma by limiting dilution. Characterizing cell lines using CD45, GP38, and CD31 markers indicated that NS451, PA6, LPSC1, LPSC4, LPSC5, and LPSC6 were CD45+GP38+CD31−, while LPSC2 and LPSC3 were CD45+GP38−CD31+. LPSC7 contained both CD45−GP38+CD31− and CD45−GP38−CD31+ subsets (data not shown). Regarding IgA-promoting effects, there was considerable heterogeneity with some gut LP lines having a positive effect while others did not (Fig 2F). This finding indicated that the gut LP stroma indeed contained a heterogeneous population of cells with different IgA-promoting capacities, and some of which were similar to primary LP stroma.

3.2 Stromal-derived soluble factors play a role in the regulation of IgA+ B cells in vitro

We next wanted to determine whether soluble factors secreted by SCs or contact-dependent mechanisms were responsible for the accumulation of IgA+ B cells in vitro. We determined the number of gut LP SCs needed to elicit IgA-promoting effects by titrating the gut LP SCs. Conditioned medium (CM) from the same number of SCs was provided to the starting B cell population and we found similar levels of IgA+ B cell production, indicating soluble factors played a major role in the regulation of IgA+ B cells (Fig. 3A). CM from 10,000 gut LP SCs was enough to induce a large number of IgA+ B cells accumulated in vitro with no significant difference detected with higher number of SCs plated (Fig. 3A). A total of 20,000 SCs were plated for all co-culture experiments to achieve a 1:10 ratio of 1 stromal cell to 10 input B cells.

To examine the roles of soluble factors from different stromal environment, we utilized a dual chamber culture system. While optimizing the dual chamber experiment, we found that the addition of Cytodex I microcarriers to the upper chambers was necessary as SCs could not effectively adhere to the membranes of the upper wells, and SCs required attachment to surfaces to survive and secrete factors (data not shown). The addition of gut LP stroma in the top chamber had equal IgA-promoting effects as when gut LP stroma was in the bottom chamber with B cells (Fig. 3B), indicating again that soluble factors played a major role in the induction of IgA+ B cells in vitro. In contrast, both BM stroma and Sp stroma exhibited inhibitory effects on IgA+ B cell accumulation in a dual chamber system with gut LP stroma (Fig. 3B). When the BM or Sp SCs were in the top chamber and gut LP stroma with B cells were placed in the bottom chamber, the number of IgA+ B cells recovered from a 6-day co-culture was significantly lower than when B cells were with gut LP SCs in the bottom but without BM or Sp SCs in the top chamber (Fig. 3B). When gut LP stroma was in the top chamber and BM or Sp stroma with B cells in the bottom chamber, there was a trend, albeit not significant, toward a reduction on IgA+ B cell numbers (Fig. 3B). This suggests that B cells in contact with gut LP stroma might have an additional influence on the IgA-promoting capacities of gut LP stroma. Fig. 3B demonstrates that there may be direct inhibitory effect from BM or Sp stroma on IgA+ B cells, or there may be indirect inhibitory effect on gut LP SCs.

To avoid potential cross-talk between SCs, we isolated CM from various stromal cultures. Using CM, we could examine whether direct or indirect inhibition took place. CM was prepared by adding 1 mL of complete medium supplemented with IL-7, TGFβ, αCD40, IL-21, and FM to 20,000 irradiated SCs for 6 days. A total of 500 μL CM was added to the B cell (200,000 cells)-SCs (20,000 cells) co-culture on day 0 and day 4. CM from BM stroma and Sp stroma suppressed the positive effects of the gut LP stroma (Fig. 3C). This indicates that the inhibitory effect of BM stroma or Sp stroma in Fig. 3B was due to the soluble factors secreted by BM stroma or Sp stroma that might inhibit the secretion of pro-IgA factors from gut LP. Consistent with this, the positive effect from the addition of gut LP CM to B cell-BM SC or B cell-Sp SC co-cultures was not diminished (Fig. 3C), further
FIGURE 4  Effects of BAFF on IgA⁺ B cell accumulation. (A) The effect of the neutralization of BAFF on IgA⁺ B cell accumulation was determined. Anti-BAFF antibodies were added every 2 days to co-cultures. Statistical significance was based on two-way ANOVA on 3 independent experiments. Left panel: statistical analysis was performed comparing IgA⁺ B cell number derived from gut LP with and without anti-BAFF treatment. Right panel: statistical analysis was performed comparing IgA⁺ B cell number between gut LP versus BM, Sp, and No stroma when cultures were treated with 5 μg/mL anti-BAFF. Error bar represented SD. (B) The effect of the addition of BAFF on IgA⁺ B cell accumulation in vitro with no, BM, and Sp stroma was assessed. A total of 5 ng/mL of BAFF was added every 2 days to B cell cultures. Statistical significance was based on two-way ANOVA on 3 independent experiments. (C and D). The effect of BAFF on the proliferation of IgA⁺ B cells with different SCs was assessed. Cell proliferation was assessed by measuring the number of Edu incorporated cells (C). Flow plots showed the staining of EdU and IgA (D). Data was representative of 3 independent experiments (C and D) demonstrating that the inhibition was an indirect inhibitory effect on gut LP stromal cells.

3.3 | BAFF stimulates the accumulation of IgA⁺ B cells in vitro

We were interested in elucidating the role of BAFF in the co-culture system, as it is an essential positive regulator of IgA⁺ B cell development. To examine the effect of BAFF in culture, we performed a BAFF neutralization assay (Fig. 4A). The addition of anti-BAFF blocking antibodies decreased the number of IgA⁺ B cells recovered from B cell-gut LP stroma co-cultures, indicating that BAFF was one of the major contributors of IgA⁺ B cell development (Fig. 4A).

Since BAFF played a role in the accumulation of IgA⁺ B cells in vitro, we wondered if the addition of BAFF to cultures with BM or Sp stroma increased the number of IgA⁺ B cells. Interestingly, the addition of BAFF resulted in an increase in IgA⁺ B cells in cultures without stroma but not in cultures containing either BM or Sp stroma (Fig. 4B). To elucidate the mechanism behind this antagonistic effect, we examined B cell proliferation. We found that the cocktail of factors (containing
IL-7, IL-21, TGFβ, αCD40, and FM) with BAFF alone (and no stroma) was sufficient to induce proliferating IgA⁺ B cells, but in the presence of BM or splenic stroma, proliferating IgA⁺ B cells were not increased (Fig. 4C). Flow plots of Fig. 4C demonstrated that the addition of BAFF increased the percentage of both total proliferating cells and proliferating IgA⁺ B cells in control wells without SCs (Fig. 4D). However, in the presence of BM or Sp SCs, such increase was not observed, suggesting that BM and Sp stroma exerted a negative influence on the proliferation of BAFF-treated IgA⁺ B cells (Fig. 4D). Such inhibitory effect could be either directly impacting on IgA⁺ B cells responding to BAFF, or indirectly via BAFF neutralization. It led us to investigate the presence of inhibitory factors using mass spectrometry.

3.4 | Mass spectrometric analysis of stromal cell secreted factors

We demonstrated that soluble factors play a role both in the stimulation and inhibition of IgA⁺ B cell accumulation in vitro. Specifically, BAFF is a major contributor to the stimulatory effect. However, the addition of anti-BAFF antibodies even at high concentrations could not fully ablate the induction of IgA⁺ B cells, suggesting that other factors besides BAFF might play a role (Fig. 4A). We also determined that BM and Sp stroma secreted soluble inhibitory factors. As a result, a proteomic analysis to identify these factors is necessary.

In order to screen for other possible soluble factors, we performed a LC–MS/MS analysis on the supernatant of SCs (Figs. 5A and B). Approximately 1593 proteins were quantified using the LC–MS/MS method, and a large proportion (420/1593) of the detected proteins was found across all the 3 SC subsets (Fig. 5A). BM and Sp stroma shared a more similar secretion pattern than gut LP stroma (Fig. 5B). Among all the differentially secreted proteins, a group of proteins from the complement system (C1s, C1r, C4b) was highly expressed by gut SCs. Other proteins either upregulated or exclusively expressed by gut SCs included metalloproteinases (MMP3/10/14), growth factors (insulin growth factor-1, vascular endothelial growth factor [VEGF]-D), myeloid-derived growth factor), chemokines (CCL2 and CXCL5), and a group of proteins specializing in cell adhesion and extracellular matrix formation (Fig. 5B). BM and Sp stroma but not gut LP shared several highly secreted proteins: proteins of lysosomal activities (cathepsin H and cathepsin B), chemokines (CCL3 and CCL6), soluble receptors (IFNαR2), cytokine antagonist (IL-1Ra), and a group of protein involved in inflammatory processes (SAA3, CD14, S100-A8, apoptosis-associated speck-like protein containing A card) (Fig. 5B). The factor cocktail (IL-21, IL-7, TGFβ, and CD40L) was not produced by any of the SCs, indicating gut LP stroma secreted additional factors that enhanced IgA production. BAFF was produced by gut LP stroma, but not by BM or Sp stroma.

MCP-1 (or CCL2), monocyte chemotactic protein-1, is one of the key chemokines that regulate the migration of monocytes/macrophages. MCP-1 secreted by gut LP stroma may have an inductive role on IgA⁺ B cell accumulation in vitro as the addition of anti-MCP-1 neutralization antibodies to the culture decreased the number of IgA⁺ B cells recovered (Fig. 5C). MCP-1 itself was not sufficient to induce IgA⁺ B cell accumulation since the addition of recombinant MCP-1 protein to B cell cultures showed no significant inductive effect (Fig. 5C).

4 | DISCUSSION

Our previous published work has detailed an important role for gut LP SCs in the generation of IgA⁺INOS⁺ plasma cells. This current work extends these findings by analyzing how SCs from different anatomical sites regulate IgA⁺ B cells. The role of SCs on shaping immune responses is being increasingly appreciated. As a result, it is of interest to examine stromal-B cell and stromal-IgA⁺ B cell interactions. In this report, we characterize the optimal conditions for in vitro generation of IgA⁺ B cells with gut LP SCs as well as the gut LP cell lines. We find that SCs from distinctive anatomical compartments exert different effects on IgA⁺ B cell generation. Gut LP SCs secrete various soluble stimulatory factors for IgA⁺ B cell development. We also demonstrate that BM and Sp SCs exert inhibitory effects on IgA⁺ B cell accumulation in vitro.

In the presence of gut LP SCs, the ability of B lineage cells to become IgA⁺ B cells is greatly enhanced. This suggests that besides the known pro-IgA factors (IL-7, IL-21, TGFβ, αCD40, and fecal matter) that we added to cultures, additional positive regulators are produced by gut LP SCs that support IgA⁺ B cell generation. Through experiments using CM, we confirmed that positive soluble regulators are secreted. BAFF, consistent with many recent studies, is found to be an important contributor in the in vitro system. BAFF acts differently depending on its receptors: BCMA is involved in post-switch events given its ability to enhance PC survival; BAFF-R is involved in providing a survival signal to peripheral B cells; TACI (Transmembrane activator and calcium modulator and cyclophilin ligand interactor) is involved in CSR (84, 85, 86). The addition of anti-BAFF neutralization antibodies greatly reduces IgA⁺ B cell accumulation, but does not completely ablate it, indicating that other factors besides BAFF are involved. MCP-1 is also one of the contributors to IgA⁺ B cell accumulation in vitro. MCP-1, a chemokine that recruits monocytes and dendritic cells to sites of inflammation, is secreted by macrophages, dendritic cells, and mesenchymal SCs.

In this study, we have found that MCP-1 is necessary but not sufficient to induce IgA⁺ B cell accumulation in vitro. MCP-1 may act through priming B cells to be more sensitive to other pro-IgA stimulating factors or localizing B cells to a microenvironment favorable to plasma cell differentiation, but such mechanism demands further experiments to verify.

BM MSCs display an immunoregulatory function on B cell proliferation and differentiation. Our study indicates BM SCs are able to inhibit BAFF-induced IgA⁺ B cell response through inhibiting the proliferation of IgA⁺ B cells. Sp SCs also show inhibitory effects on IgA⁺ B cell accumulation. BM stroma or Sp stroma may inhibit B cell response to stimulating factors such as BAFF. A recent study shows that some factors (e.g. SLPI) can actively attenuate NF-κB activation in BAFF-induced B cells. Whether or not NF-κB signaling in B cells is affected in cultures requires further investigation.

Besides antagonizing the effects of BAFF, BM and Sp SCs affect the IgA⁺-promoting capacity of gut LP Scs. The addition of CM from BM stroma or Sp stroma reduces the number of IgA⁺ B cells detected in gut.
FIGURE 5  Mass spectrometry analysis on secreted factors. (A) A Venn diagram showed the number of protein detected by LC–MS/MS in each SCs sets (BM, Sp, and gut LP) and the overlap among them. (B) Heatmap of the differentially secreted proteins of BM, gut LP, and Sp was plotted based on z-score calculated by Maxquant. Each row represented one protein identified by LC–MS/MS, blue indicating lower and yellow higher expression. Selected proteins were displayed based on one-way ANOVA ($P \leq 0.05$) on 3 independent experiments. (C) Roles of MCP-1 on IgA+ B cell accumulation were assessed. A total of 200,000 CD19+ BM cells were co-cultured with 20,000 SCs +/- MCP-1 (5 ng/mL every two days) or +/- anti-MCP-1 antibodies (5 µg/mL every 2 days). Statistical significance was based on two-way ANOVA on 4 independent experiments. Error bar represented SD.

In contrast, the addition of CM from gut LP stroma to BM stroma – B cell co-cultures or Sp stroma – B cell co-cultures can significantly enhance IgA+ B cell accumulation as shown in Fig. 3C. This suggests that factors produced by the BM stroma or splenic stroma inhibit gut LP’s IgA-promoting capacity. This indicates that there is a potential cross-talk between different SCs and between SCs and the microenvironment. In addition, we have found that soluble receptor (IFNαR2) and cytokine antagonist (IL-1Ra) are secreted by BM and Sp SCs. Whether these factors are playing a role in this system requires further examination. In addition, it would be of further interest to examine the presence of soluble decoy receptors such as sBCMA or sTACI as they were found in physiological conditions to neutralize BAFF and APRIL.27–29 It has been shown in both in vitro and in vivo studies that both BM and Sp sites promote plasma cell

| Protein | BM | Sp | LP | P-value |
|---------|----|----|----|---------|
| Factor 1 | 100 | 90 | 80 | 0.05    |
| Factor 2 | 90  | 85 | 80 | 0.06    |
| Factor 3 | 85  | 80 | 75 | 0.07    |
survival and differentiation. In particular, BM niche promotes long-term survival of IgA+ plasma cells. As a result, there are positive regulators in both BM and Sp microenvironments. However, in this in vitro setting, we propose that the inhibitory effect by BM or Sp stroma on IgA+ B cell generation dominates.

Inevitably, this in vitro system has some caveats. First, gut LP, BM, and splenic primary stroma contains a heterogeneous population of SCs, and our in vitro system might not fully recapitulate the contribution and interaction of all SCs in vivo. Our data confirmed previous publications that freshly isolated gut stroma contain four populations: CD45+GP38+CD31+ lymphatic endothelial cells, CD45+GP38+CD31+ fibroblastic reticular cells, CD45+GP38+CD31+ blood endothelial cells, and CD45+GP38+CD31+ double negative cells. However, gut LP SCs after culture become mostly fibroblastic reticular cells. In regards to BM and Sp SCs, as suggested before, we might miss the IgA-promoting component of BM and Sp SCs in vitro. Nonetheless, an in vitro system permits a more detailed analysis of SC and IgA+ B cell interaction, enabling screening methods for identifying any stimulatory or inhibitory molecules. Second, analysis of secreted factors is based on irradiated SCs. There is evidence that irradiation can change the secretion pattern of cells, but most of the changes are on stromal cell growth, such as VEGF levels and platelet-derived growth factor (PDGF)-AA and PDGF-AB/BB levels. Third, there might be macrophages present in the CD19-selection and stromal preparation. However, the percentage of non-B cells and CD45− SCs in culture was minimal and thus negligible.

Altogether our findings indicate that the interaction between IgA+ B cells and SCs is dynamic in vitro. We propose that IgA+ B cell development is tightly controlled by the anatomical microenvironment in which B cells are activated. We have provided evidence that gut LP SCs secrete positive factors that stimulate IgA+ B cell generation, while some factors supplied by the BM and Sp SCs are inhibitory. These findings rationalize future in vivo studies on interactions between SCs and B cells in the generation of IgA-secreting cells.

AUTHORSHIP
SYC and CJP conceived and designed the study. SYC performed all experiments and JMM, AB, and CF contributed to experiments. SYC and CJP analyzed data and wrote the manuscript.

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DISCLOSURE
The authors have no conflicts of interest to declare.

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