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Mast cells crosstalk with B cells in the gut and sustain IgA response in the inflamed intestine

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B lymphocytes are among the cell types whose effector functions are modulated by mast cells (MCs). The B/MC crosstalk emerged in several pathological settings, notably the colon of inflammatory bowel disease (IBD) patients is a privileged site in which MCs and IgA$^+$ cells physically interact. Herein, by inducing conditional depletion of MCs in red MC and basophil (RMB) mice, we show that MCs control B cell distribution in the gut and IgA serum levels. Moreover, in dextran sulfate sodium (DSS)-treated RMB mice, the presence of MCs is fundamental for the enlargement of the IgA$^+$ population in the bowel and the increase of systemic IgA production. Since both conventional B-2 and peritoneal-derived B cells populate the intestine and communicate with MCs in physiological conditions and during inflammation, we further explored this interplay through the use of co-cultures. We show that MCs finely regulate different aspects of splenic B cell biology while peritoneal B cells are unresponsive to the supporting effects provided by MCs. Interestingly, peritoneal B cells induce a pro-inflammatory skewing in MCs, characterized by increased ST2 and TNF-α expression. Altogether, this study uncovers the versatility of the B/MC liaison and highlights key aspects for the resolution of intestinal inflammation.

Keywords: cell-to-cell interplay · colitis · IgA · innate-like B cells · mast cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

The ability of the gastrointestinal immune system to protect the host from harmful foreign microorganisms and antigens but, at the same time, to tolerate the luminal microbiota depends on the effective communication between the diverse immune cell types that populate the gut. As in other autoimmune and allergic disorders, a break of this delicate balance forms the basis for the onset of pathological conditions characterized by chronic or recurring inflammation of the gastrointestinal tract, such as ulcerative colitis (UC) and Crohn's disease. Among the diverse immune cell types known to promote or dampen intestinal diseases, the potential role of mast cells (MCs) is gaining attention [1]. MCs are widely interspersed in the gastrointestinal tract where they act as "sentinels" posed to promptly release a large set of pro-inflammatory and neuro-muscular stimulating factors upon activation. MC mediators can directly regulate physiological and pathological processes such as epithelial cell proliferation, tissue healing, and angiogenesis, and their functions are strongly modulated by the multitude of microenvironmental signals [2]. MCs also act as a rheostat, regulating the functions of other innate and adaptive immune cells, in particular B cells [3]. We and others have previously reported that MCs can regulate discrete stages of B cells' activation and functions: activated MCs induce an increase in IgA production in vitro and the intestine resulted a privileged site of close spatial interactions between MCs and IgA^+ B cells in patients suffering from inflammatory bowel disease (IBD) [4–6]. Moreover, the positive correlation between the accumulation of activated MCs and plasma cells (PCs) in UC patients emerged from the bioinformatical analysis of the present study suggests the establishment of reciprocal interactions between these two cell types in the lesioned intestine. This laid the foundations for the analysis of the mutual support between MCs and B cells under homeostatic and inflammatory conditions. Using the red MC and basophil (RMB) mouse model of MC deficiency, which permits conditional ablation of MCs via diphtheria toxin (DT) injection [7], we demonstrate in vivo that MCs regulate B-cell homeostasis in the intestine and the production of IgA both in physiological conditions and during intestinal inflammation. Our study was not restricted to conventional B-2 cells but, for the first time, analyzed the crosstalk of MCs with the innate arm of the B-cell compartment, represented by B-1 cells. Although in many intestinal pathologies the presence of MCs correlates with the accumulation and activation of B cells, the impact of B cells on MCs' functions remains poorly investigated. To address this missing aspect of the B/MC crosstalk, we established in vitro co-cultures and found that peritoneal B cells were unresponsive to the supporting effects provided by MCs but, unlike splenic B cells, turned out to be good effectors for MC activation. Moreover, we provide preliminary in vivo evidence suggesting that B-1 cells are needed to maintain physiological numbers of MCs in the gut. Collectively, our results provide novel insights in the B/MC crosstalk, emphasizing the intestinal compartment as the core site in which these two partner cell types, and their different functional subsets, reciprocally influence their behavior, contributing to homeostatic and pathological responses.

Results

The accumulation of activated MCs positively correlates with PCs in the inflamed gut mucosa

As the first attempt in our analysis of the B/MC interplay in the intestinal context, we compared the relative proportions of 22 immune cell types (LM22 signature) in non-inflamed and inflamed colon samples of UC patients. In parallel, the normal and cancerous mucosa of colorectal cancer (CRC) patients were also analyzed (Fig. 1A). The LM22 signature was determined on two different transcriptional profile datasets (GSE87211 and GSE107499) using the CIBERSORT algorithm [8]. Figure 1A and B shows that most of the MCs infiltrating the healthy mucosa of UC patients were associated with a resting profile while a significant accumulation of activated MCs was reported in the inflamed counterpart and in the transformed intestinal portion of CRC patients. Regarding the B cell population, naïve and memory B cells and PCs also accumulated in the inflamed UC tissue, while they were less frequent in cancer (Fig. 1A and B). Since both MCs and B cells accumulate in the inflammatory condition, the correlation between activated MCs and diverse B cell subtypes was further analyzed. A negative correlation between activated MCs and naïve B cells was obtained in the inflamed mucosa of UC patients, while the relative proportion of memory B cells was close to zero in all conditions (Fig. 1C). Diverse the situation of PCs that positively correlate with activated MCs in the inflamed UC tissue, in line with our previous observation of the co-localization of MCs and IgA^+ PCs in the inflamed mucosa of IBD patients [4]. Moreover, the opposite tendency between activated MCs and PCs observed in the healthy tissue of UC patients and CRC (Fig. 1C) suggests a specificity of the reciprocal interaction occurring between these two cell types for the colitis setting.

The intestinal B cell distribution is altered in control and inflamed MC-deficient mice

Although the role of MCs in promoting B cell-related functions is widely accepted [4–6,9,10], the impact of the absence of MCs on B cell distribution, phenotype, and effector properties has not yet been investigated in detail. To this aim, we analyzed B cells in control (PBS-) mice and in DT-treated MC-depleted (DT-) RMB mice for which the efficient depletion of MCs was checked by assessing the presence of $Fc\varepsilon RI^+$c-Kit^+ cells in the peritoneal cavity by flow cytometry (Supporting Information Fig. S1). This analysis was performed under physiological conditions and in the context of colitis induced by administering 2% dextran sodium sulfate (DSS) in drinking water for 8 days to both PBS-RMB and DT-RMB mice. Colitis progression was analyzed by following weight loss and comparing colon length at the sacrifice and, in line with the results

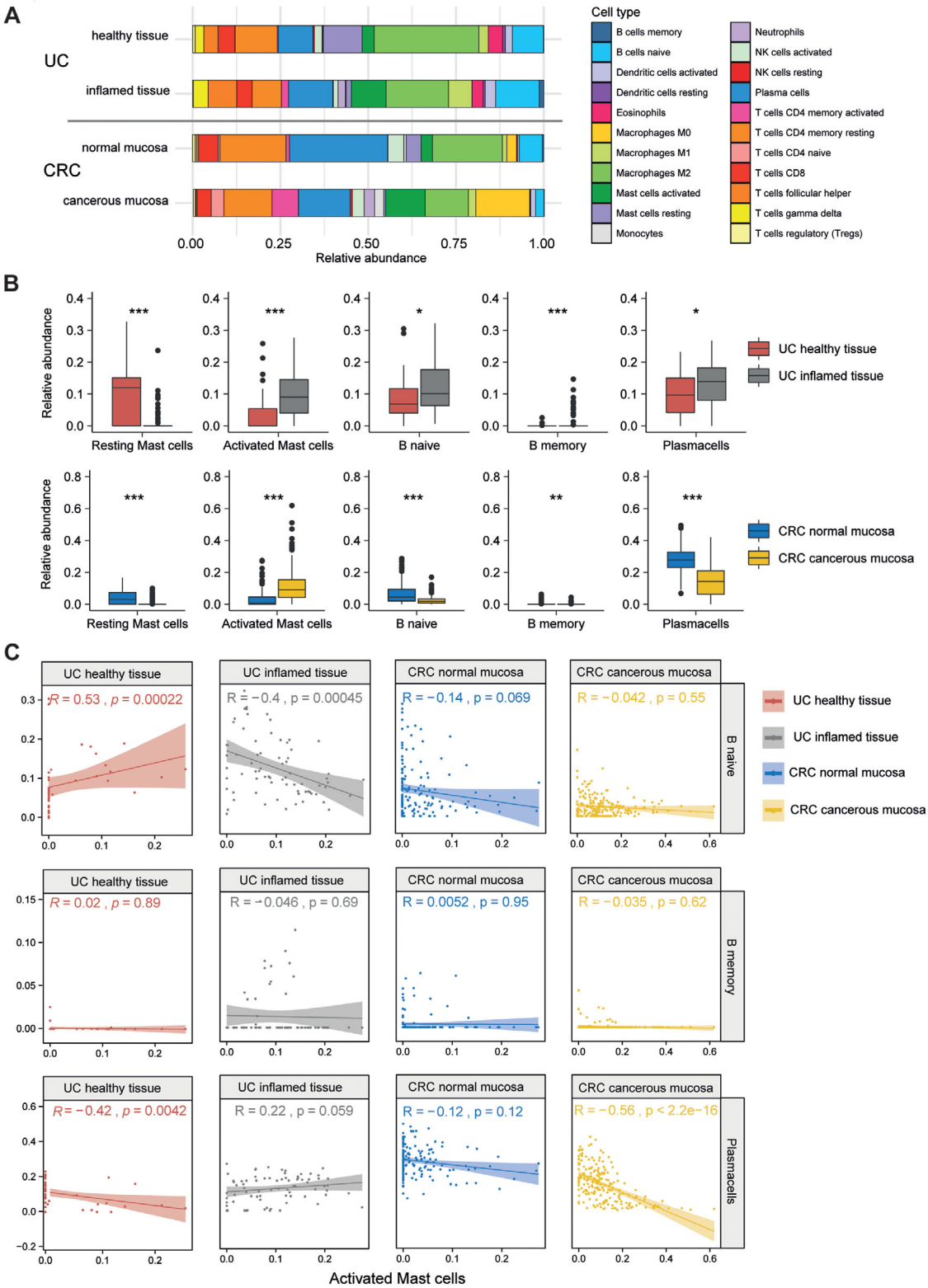

Figure 1. Activated MCs and B cells accumulate in the inflamed tissue of UC patients. (A) Relative proportions of 22 immune cell types were determined on the basis of their gene expression profile in intestinal biopsies from ulcerative colitis (UC) and colorectal cancer (CRC) patients by using the CIBERSORT algorithm. (B) Relative quantifications of resting and activated MCs and of the naïve, memory and plasma cells (PCs) B cell subtypes are compared in healthy *versus* inflamed UC tissues and in the normal versus cancerous mucosa of CRC patients. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired two-tailed Student's *t*-test. Data are represented as median, lower, and upper hinges correspond to the first and third quartiles. (C) The correlation between the proportion of activated MCs and B cell subsets was determined by Spearman correlation in the two indicated pathological sets. *p*-values are indicated in each corresponding graph.

obtained in the *Kit*$^{W\text{-}sh/W\text{-}sh}$ model of MC deficiency [11], DT-RMB mice had a slightly more severe disease progression (Supporting Information Fig. S2A and B). We first compared the distribution of B cells in the spleen, peritoneum, and LNs and observed that total B cell percentages, expressed as CD19$^+$ cells, were not altered in the absence of MCs neither in the control condition nor upon DSS treatment (Fig. 2A). Since Palm and co-workers showed that MCs differently affect diverse B cell subtypes [6], a precise phenotypical analysis of the splenic and peritoneal B cell compartment in MC-deficient and -sufficient mice was conducted. Concerning the spleen, the newly formed (NF), marginal zone (MZ), follicular (FO), CD21^{low}, and transitional two-marginal zone precursors (T2-MZP) B cell subsets were unchanged in the absence of MCs, in both the conditions analyzed (Fig. 2B; gating strategies in Supporting Information Fig. S3A). The analysis of the splenic CD1d^{hi}, CD1d$^+$CD5$^-$, CD1d$^+$CD5^{low}, and CD1d$^+$CD5$^+$ subsets reinforced this result (Supporting Information Fig. S3B). For the peritoneal compartment, the percentages of B-1a (CD19$^+$CD23$^-$CD5$^+$), B-1b (CD19$^+$CD23$^-$CD5$^-$), and B-2 (CD19$^+$CD23$^+$CD5$^-$) cells were assessed but, again, no differences were observed following MC depletion (Fig. 2C; gating strategies in Supporting Information Fig. S3C). Given previous clues of MC/B cell crosstalk in the gut [4,5], we next analyzed the effect of MC depletion on B cell abundance in this site. Interestingly, both in the small intestine and colon, statistically significant higher numbers of CD45R$^+$ elements were detected by immunohistology in DT-RMB compared to PBS-RMB mice under physiological conditions. Additionally, CD45R$^+$ cells accumulated upon colitis and, in the absence of MCs, this increase was much more prominent in the colon, the intestinal site most affected by DSS treatment (Fig. 2D). These data corroborate the existence of active cross-talks between MCs and B cells in the intestine and suggest that these cells may cover an important function in intestinal inflammation.

MCs control IgA increase and PCs expansion in mice carrying intestinal inflammation

MCs were reported to modulate MHC-II, CD86, and CD138 expression on B cells and to increase their antibody production ex vivo [4,6]. We compared the expression of the MHC-II, CD86, and CD138 markers in splenic B cells isolated from MC-deficient and -sufficient mice but no variations were observed (Supporting Information Fig. S4A and B). We then analyzed antibody production in splenic B cell cultures established from control and DSS-treated PBS-RMB and DT-RMB mice, in the presence and absence of anti-CD40 mAb, LPS, or CpG. No changes in IgM and IgG levels were detected both in PBS-RMB and DT-RMB mice under physiological and colitis conditions (Fig. 3A). Yet, in line with previous data showing an increase in IgA-producing cells upon induction of colitis in mice [12], splenic B cells from PBS-RMB mice produced higher amounts of IgA in the colitis condition, both in the presence and absence of stimulation. By contrast, this DSS-induced increase of IgA production was not observed in B cells isolated from the spleen of MC-deficient DT-RMB mice (Fig. 3A). This result was not due to a different response to stimulation in terms of survival or proliferation of splenic B cells derived from PBS-RMB and DT-RMB mice (Supporting Information Fig. S5). In light of the IgA data, we determined serum antibody titers and consistent results were obtained. Indeed, statistically significant higher IgA levels were detected under homeostatic conditions in the absence of MCs, but this was not observed when intestinal inflammation was induced. Again, this effect was restricted to the IgA isotype (Fig. 3B). Of note, IgA alterations were a consequence of the absence of MCs, and not of DT treatment, as revealed by serum antibody titers of PBS- and DT-injected WT mice (Supporting Information Fig. S6). In light of the importance of IgA in the context of intestinal inflammation, we analyzed IgA$^+$ cells in the small intestine and colon. In control conditions, a higher density of IgA$^+$ cells was observed in the small intestine, but not in the colon, of DT-mice. As expected, following DSS treatment, PBS-RMB mice presented an increased number of IgA$^+$ elements both in the small intestine and colon, while this was not observed in MC-deficient mice (Fig. 3C, respectively upper and lower panels). These results were further supported by the immunohistochemical analysis of CD138-expressing cells, whose number in the four different conditions reflects the situation described for IgA$^+$ cells (Fig. 3D; Supporting Information Fig. S4C). Altogether, these results indicate that, in vivo, the absence of MCs leads to a significant alteration of the mucosal B cell compartment and this is particularly relevant in the context of intestinal inflammation.

MCs support activation and IgA production of splenic but not peritoneal B cells

The in vivo data demonstrate that MCs control B cell homeostasis in the gut and that they play a relevant role in the regulation of IgA production. However, the results shown so far do not provide any indication as to whether it is a direct or indirect effect and whether a specific B cell subset is involved. PC differentiation and IgA secretion were shown to be the result of the in vitro cross-talk between MCs and B cells, but only the B-2 population was examined [4]. Conversely, the mouse gut is populated not only by conventional B-2 lymphocytes but also by innate-like B-1 cells that are known to generate IgA$^+$ PCs [13,14]. For this reason, purified splenic and peritoneal B cells, enriched in B-2 and B-1 lymphocytes respectively, were cultured with resting or IgE/Ag activated BM-derived MCs (BMMCs). In first instance, antibody production by the two B cell subtypes in the presence of BMMCs was analyzed. Activated BMMCs were able to enhance antibody production by splenic B cells but did not influence antibody release by peritoneal B cells (Fig. 4A). Therefore, we investigated other known effects of MCs on B-2 cells such as survival, proliferation, and surface markers modulation. Figure 4B and C shows that the survival and proliferation rates of splenic B cells in culture with both resting and IgE/Ag triggered MCs were enhanced while viability and proliferation of peritoneal B cells were not affected. Concerning the modulation of receptors important for B cell activation, we examined CD80, CD86, and

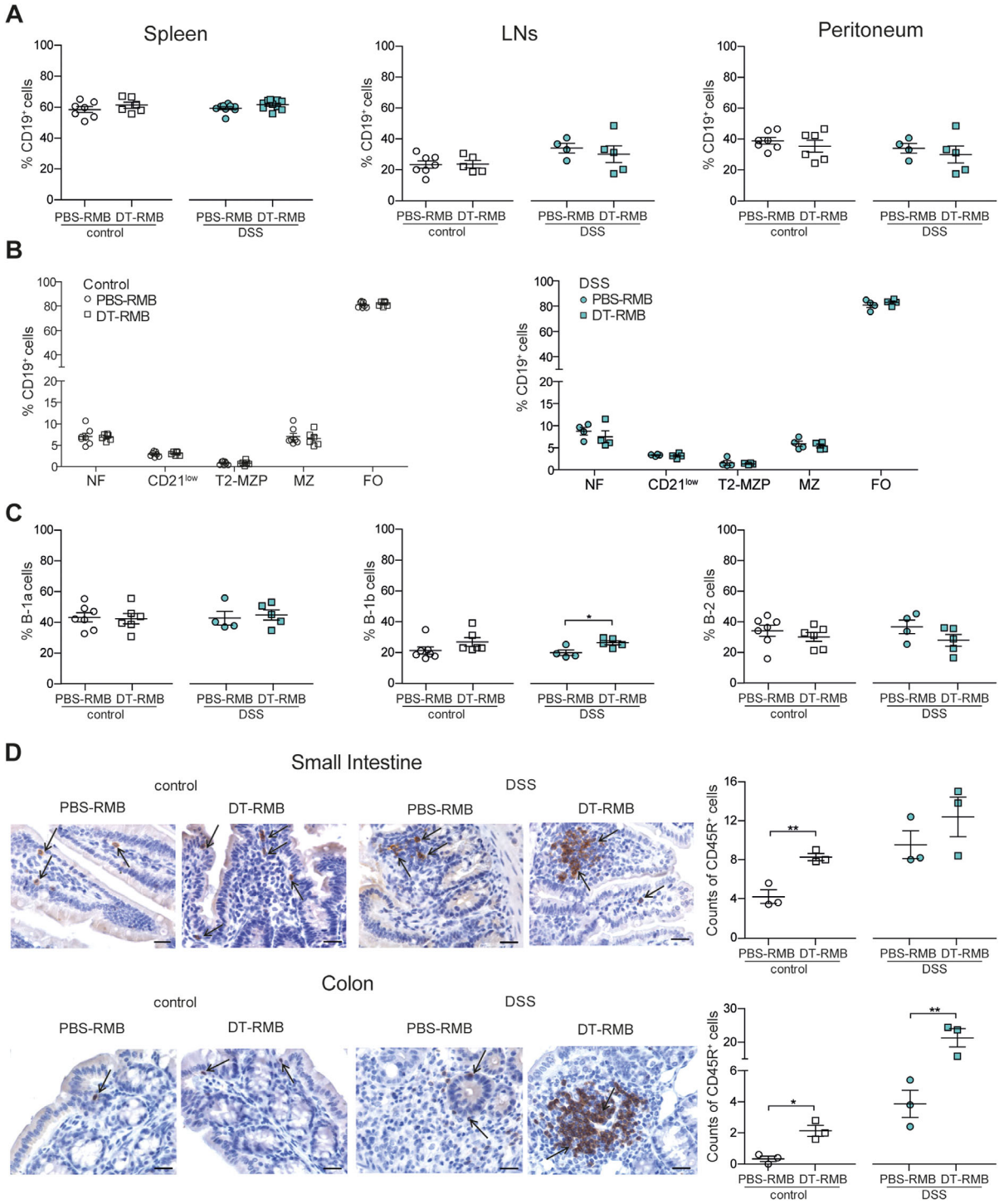

Figure 2. Enlargement of the B cell population in the intestine of DT-RMB mice under physiological conditions and during DSS-induced colitis. Comparisons of PBS-RMB and DT-RMB control and DSS-treated mice are reported for each analysis. Symbols depicts individual mice among the different groups and at least two independent experiments were performed for each analysis with at least three mice per group in total. (A) Frequencies of B cells in the spleen, lymph nodes (LNs, pooled in the healthy condition, mesenteric in DSS-treated mice), and peritoneum were detected by flow cytometry and are shown as percentages of $CD19^+$ cells. (B) Splenocytes were stained with anti-CD21, -CD23, and -CD19 mAbs and analyzed by flow cytometry. $CD19^+$ cells with the phenotype of newly formed (NF), $CD21^{low}$, transitional 2-marginal zone precursors (T2-MZP), marginal zone (MZ), and follicular (FO) cells are shown as percentages among the total splenic B cell population. (C) Cells recovered from peritoneal lavages were stained with anti-CD19, -CD23, and -CD5 mAbs and $CD19^+$ B cells were identified as B-2, B-1a, and B-1b subsets respectively as $CD23^+CD5^-$, $CD23^-CD5^+$, and $CD23^-CD5^-$. (D) Counts of $CD45R^+$ cells were determined by immunostaining of formalin-fixed paraffin-embedded small intestine and colon sections. Each symbol of the scatter plot is the mean count of five fields and represents a single animal. Representative immunohistochemical analyses (scale bar = 50 μm; 40×) are shown on the left. Data represent means (±SEM). $^*p < 0.05$, $^{**}p < 0.01$ by unpaired two-tailed Student's *t*-test.

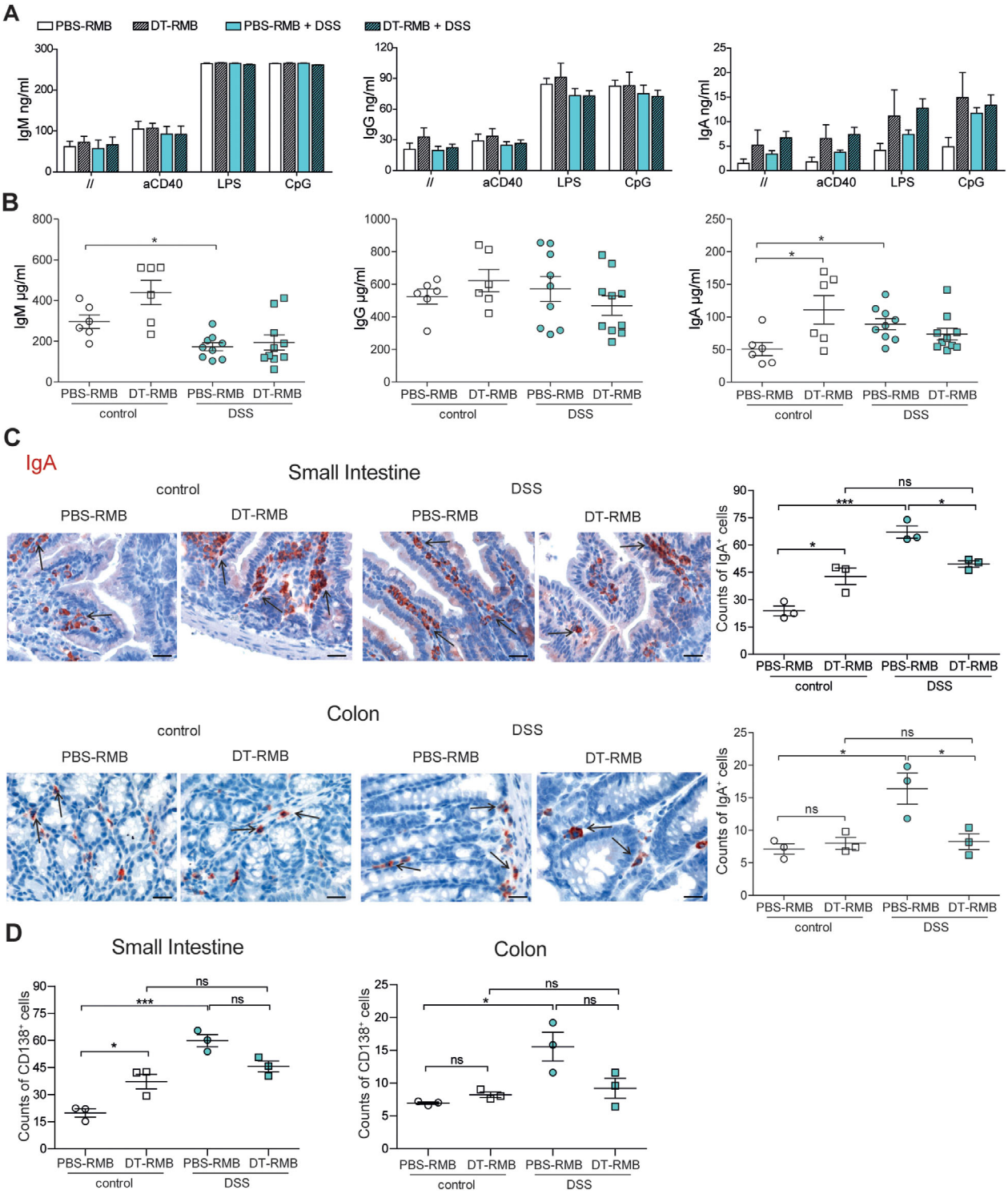

Figure 3. IgA production in DSS-treated mice is sustained by the presence of MCs. (A) IgM, IgG, and IgA concentrations were measured by ELISA in supernatants collected from the 48 h culture of purified splenic B cells with or without a-CD40 mAb, LPS, or CpG. B cells were isolated from both control and DSS-treated PBS-RMB and DT-RMB mice and put in comparison. Means (+SEM) from at least three independent experiments are shown with at least three mouse per group in total. (B) IgM, IgG, and IgA were detected by ELISA in serum samples of PBS-RMB and DT-RMB mice that were administered normal or DSS-supplemented drinking water. Each symbol indicates individual mice among the groups analyzed. Counts of IgA^+ (C) and $CD138^+$ (D) elements were determined by immunostaining of formalin-fixed paraffin-embedded small intestine and colon sections from both control and DSS-treated PBS-RMB and DT-RMB mice. Each symbol of the scatter plot is the mean count of five fields and represents a single animal. Representative immunohistochemical analyses of IgA (scale bar = 50 µm; 40×) are shown in panel (C). (B–D) Shown are means (±SEM) from at least two independent experiments with at least three mouse per group in total. $^*p < 0.05$, $^{***}p < 0.001$ by one-way ANOVA. ns, not significant.

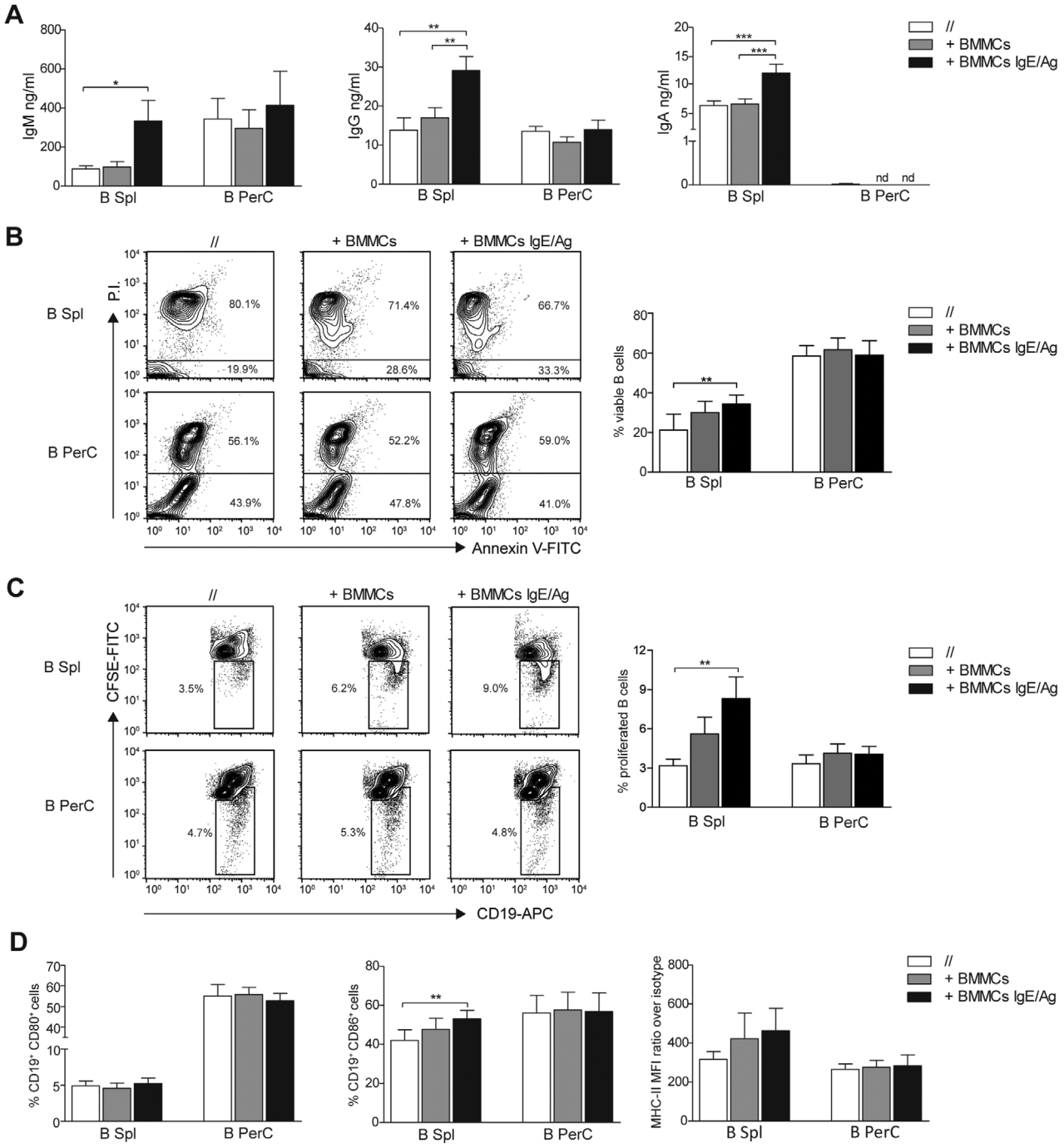

Figure 4. The functional support of MCs is important for the splenic B cell population but dispensable for peritoneal cavity B cells. Naïve splenic (Spl) or peritoneal cavity (PerC) B cells were cultured either alone (//) or in the presence of resting (+ BMMCs) or activated (+ BMMCs IgE/Ag) MCs for 48 (A, B, and D) or 72 (C) h. (A) Soluble Igs were quantified by ELISA in the co-culture medium. (B) Percentages of viable B cells were determined by flow cytometry following Annexin V/Propidium Iodide staining. (C) Percentages of B cells with diminished CFSE intensity were detected among total $CD19^+$ cells by flow cytometry following co-culture with MCs. (D) B cells were stained with anti-CD80, -CD86, and -MHC-II mAbs and analyzed by flow cytometry. Percentages of $CD19^+$ cells expressing the surface molecules are reported for the analysis of CD80 and CD86, while the MFI ratio over isotype control among the $CD19^+$ population is indicated for the MHC-II analysis. Means (±SEM) from at least three independent experiments are shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA. nd, not detectable.

MHC-II expression. While CD80 was unaffected on both B cell populations, a slight increase in MHC-II and a more pronounced upregulation of CD86 was detected in the presence of activated BMMCs on splenic B cells only (Fig. 4D; gating strategies in Supporting Information Fig. S4B). Altogether, these observations support the hypothesis that, in vivo, activated MCs can directly sustain the activation and antibody production of conventional B-2 cells but not of innate like-B-1 cells.

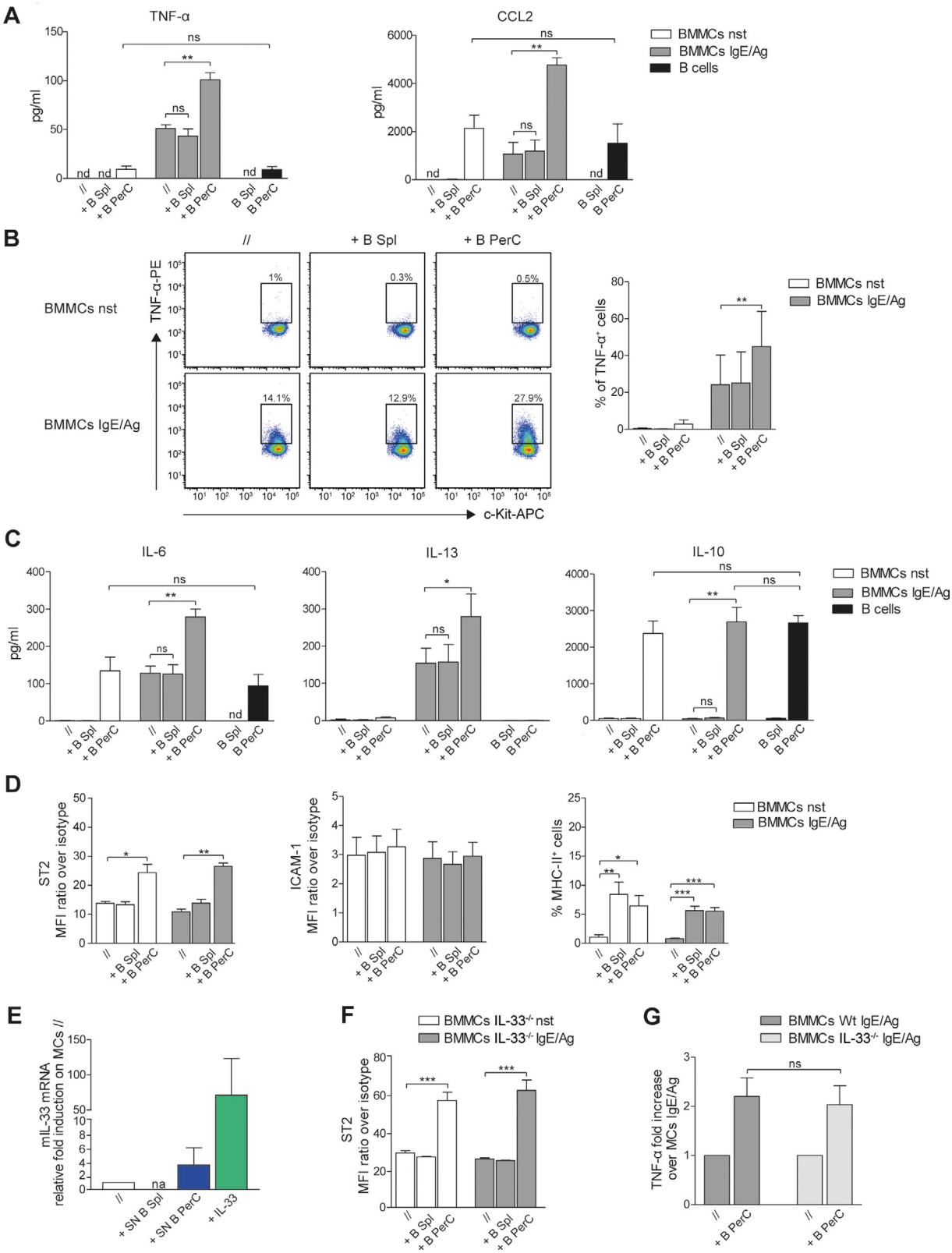

Figure 5. Peritoneal B cells promote the production of pro-inflammatory cytokines by activated MCs and upregulate ST2 expression. (A) Secreted TNF-α and CCL2 were detected by ELISA in the supernatants of resting (BMMCs nst) and activated (BMMCs IgE/Ag) MCs cultured alone (//) or with splenic (Spl) or peritoneal cavity (PerC) B cells for 24 (TNF-α) or 6 h (CCL2). The B cell alone condition was also evaluated. (B) TNF-α intracellular staining performed after 1 h of co-culture between MCs and B cells in the presence of brefeldin A and analyzed by flow cytometry. A representative experiment showing the gating strategy is reported on the left while the bar plot summarizing all the performed experiments is shown on the right.

Peritoneal B cells promote the acquisition of a proinflammatory cytokine signature by MCs

In intestinal inflammation, the enlargement of the B cell population is related to the accumulation of activated MCs (Fig. 1). Moreover, MCs increase in number and acquire an activated phenotype in many B cell-related inflammatory pathologies and tumors [15–17]. Therefore, we surmised that, during colitis, B cells could not only be supported by MCs in their antibody production but that they could also reciprocally enhance MC activation. To assess this possibility, we tested the effect of B cells on MC's degranulation but no relevant result was observed neither with splenic nor peritoneal B cells (Supporting Information Fig. S7A). The analysis of LAMP-1, a surface marker of degranulation, further confirmed this result (Supporting Information Fig. S7B). Next, we analyzed soluble mediators relevant in the pathogenesis of IBD, focusing on TNF-α and CCL2 that are known to be released by MCs in early stages of inflammation and that acquire importance in the sustaining of colitis [18,19]. The amount of both these mediators was strongly enhanced when IgE/Ag activated MCs were co-cultured with peritoneal, but not splenic B cells (Fig. 5A). Deepening the TNF-α result, TNF-$\alpha^{-/-}$ BMMCs were used to verify that MCs were the source of the increased mediator amounts, and the kinetic analysis showed that the effect reached a statistical significance at 1 h of co-culture and was maintained at 24 h (Supporting Information Fig. S7C). The interaction with the peritoneal B cell population promoted the neo-synthesis of TNF-α as demonstrated by measuring intracellular cytokine accumulation. Indeed, following brefeldin A treatment, the percentage of TNF-α^{+} BMMCs increased from 25% to 45% after 1 h of co-culture with peritoneal B lymphocytes (Fig. 5B). Likewise, the production of the pro-inflammatory cytokines IL-6 and IL-13 were sustained by the co-culture with peritoneal but not splenic B cells (Fig. 5C). By contrast, secretion of the anti-inflammatory cytokine IL-10, which is produced in high amounts by peritoneal B cells, was not enhanced. Altogether, these results strongly suggest that peritoneal B cells promote the acquisition of a proinflammatory cytokine signature by activated MCs.

Peritoneal B cells increase MC responsiveness to IL-33

MCs express a variety of activating and inhibitory surface receptors that can fine-tune their level of activation [20]. Particularly relevant in the context of colitis is the IL-33 receptor (ST2) that is abundantly expressed on MCs and can strongly support the release of pro-inflammatory mediators in inflammatory settings driven by IL-33 [21]. Interestingly, co-culture experiments showed that ST2 was upregulated on BMMCs in the presence of peritoneal, but not splenic B cells (Fig. 5D; Supporting Information Fig. S7D). We also tested the modulation of MHC-II and ICAM-I whose expression is known to be enhanced in the context of IL-33 stimulation of MCs [22,23]. While ICAM-1 was not modulated, MHC-II expression was increased by both the splenic and peritoneal B cell populations, suggesting that B cells can enhance the ability of MCs to process and present antigens to $CD4^{+}$ T cells. We further investigated the hypothesis of an autocrine loop in which the mediators released from peritoneal B cells induce IL-33 production in MCs that in turn may be responsible for the increase of both ST2 expression and inflammatory cytokines production. According to our hypothesis, BMMCs cultured for 5 h in peritoneal B cell-derived conditioned medium showed a slight increase of *il33* gene transcription (Fig. 5E). However, MC-derived IL-33 neither impacted ST2 upregulation nor TNF-α release, since similar results were obtained when co-culturing peritoneal B cells with WT and IL-$33^{-/-}$ BMMCs (Fig. 5F and G).

Altered MC distribution in IκBNS-deficient bumble mice displaying reduced B-1 cells

Our data show a mutual relationship between MCs and the B-1/B-2 populations relevant in the inflammatory context, where B-2 cells are fostered *versus* IgA production while reciprocally B-1-enriched cells may sustain proinflammatory functions of MCs. In this light, the impact of the physiological absence of B-1 cells on MC distribution in different mouse organs was investigated. The IκBNS-deficient *bumble* mouse model was chosen due to its drastically reduced frequencies of B-1 cells [24]. First, percentages and absolute numbers of peritoneal MCs and, as a control, of peritoneal B cell subsets were assessed. In parallel, the expression of the MC specific markers FcεRI and c-Kit was also investigated. As shown in Fig. 6A and B, the absolute numbers and phenotype of peritoneal MCs were not affected in this B-1-lacking mouse model. However, interesting results emerged from the analysis of mucosa-lined luminal organs: a significant decrease of MCs was detected by immunohistology in the colon and lungs of *bumble*

(C) Secreted IL-6, IL-13, and IL-10 were detected by ELISA in the culture media of resting (BMMCs nst) and activated (BMMCs IgE/Ag) MCs cultured alone (//) or with splenic (Spl) or peritoneal cavity (PerC) B cells for 48 h. The B cell alone condition was also evaluated. (D) Following co-culture with either splenic (Spl) or peritoneal cavity (PerC) B cells, c-Kit^{+} BMMCs were analyzed by flow cytometry for the expression of ST2, ICAM-1, and MHC-II. ST2 and ICAM-I are indicated as MFI ratio over the isotype control. For the MHC-II molecule that is not uniformly expressed by the MC population, the percentage of positive cells among the total population analyzed is reported. (E) BMMCs were cultured for 5 h in normal medium or in the 1 h supernatant (SN) derived from either splenic (Spl) or peritoneal cavity (PerC) B cells. The graph reports *il33* mRNA gene expression shown as fold induction over resting (//) MCs. MC stimulation with recombinant IL-33 was used as positive control. na, not amplified. (F) The modulation of ST2 expression on IL-$33^{-/-}$ BMMCs was performed by flow cytometry after co-culture with either splenic (Spl) or peritoneal cavity (PerC) B cells. (G) Released TNF-α was measured by ELISA in co-cultures between peritoneal B cells and IgE/Ag activated WT or IL-$33^{-/-}$ BMMCs and expressed as fold increase over the BMMCs IgE/Ag alone condition. (A–G) Means (±SEM) from at least three independent experiments are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA (A–D, F) performed on nst and IgE/Ag conditions. No statistically significant differences are detected by one-way ANOVA (E) or by unpaired two-tailed Student's *t*-test (G). ns, not significant, nd, not detectable.

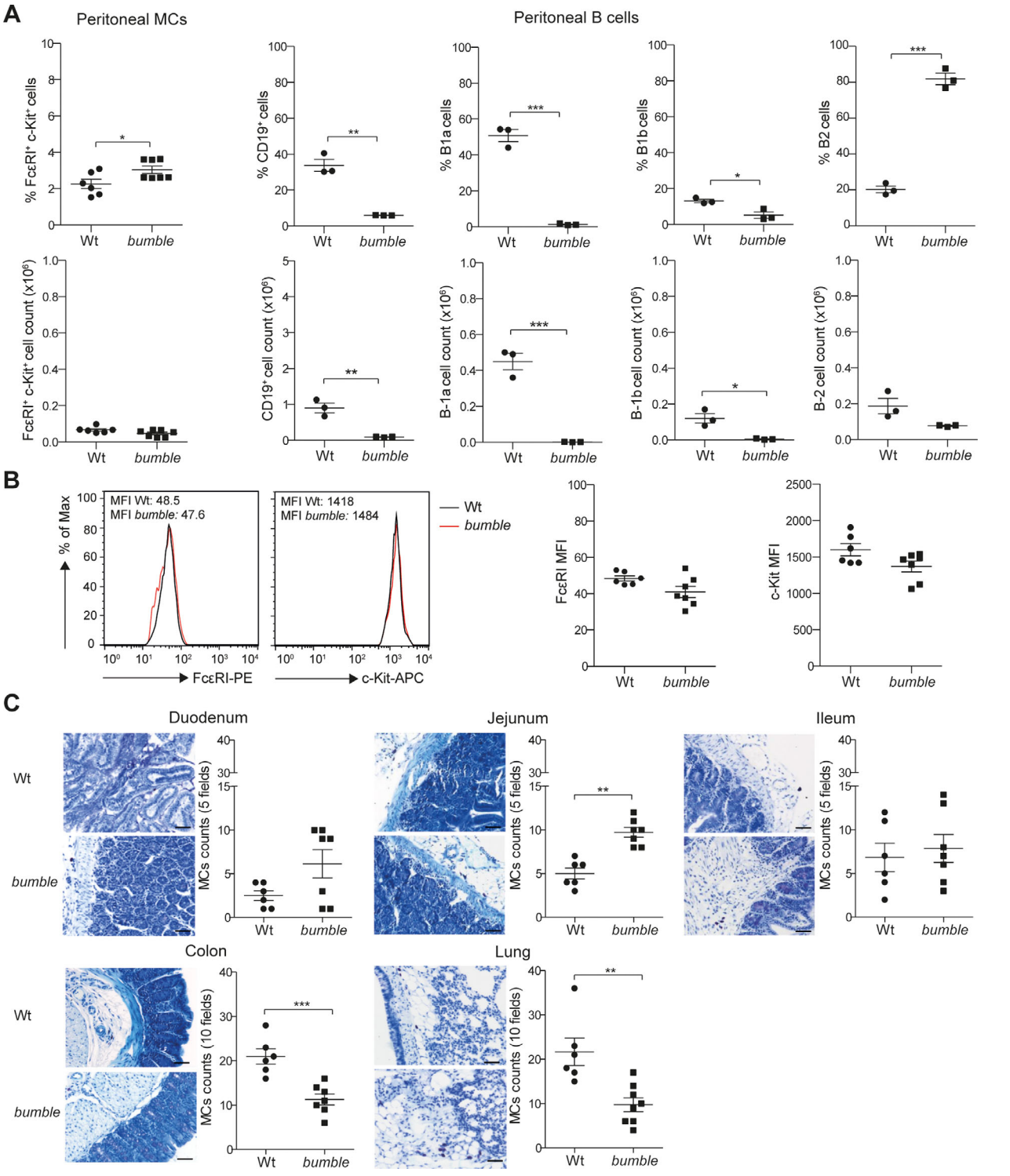

Figure 6. MC numbers are decreased in the colon of B-1 cells-lacking *bumble* mice under homeostatic conditions. (A) Flow cytometry analysis of FcεRI$^+$c-Kit$^+$ MCs and of CD19$^+$, CD19$^+$CD5$^+$CD23$^-$ (B-1a), CD19$^+$CD5$^-$CD23$^-$ (B-1b), and CD19$^+$CD5$^-$CD23$^+$ (B-2) cells among the peritoneal population of WT and *bumble* mice. Percentages among total peritoneal cells are indicated in the upper panels, absolute numbers are shown in the lower panels. (B) The MFI for FcεRI and c-Kit receptors on MCs are reported. A representative experiment is shown on the left while the summary of at least six mice per condition is reported on the right. (C) Tissue samples were collected from WT and *bumble* mice and Toluidin Blue staining for MCs was performed. Representative histological images are shown at a 20× magnificence (scale bar = 100 μm). Graphs report the results from at least six animals per group. In the case of jejunum, ileum, and duodenum, each symbol indicates the total number of MCs counted in five fields (40×), while in the lung and colon each symbol is the total number of the MCs counted in ten fields (40×). (A–C) Each symbol represents individual mice among the different groups and results of two independent experiments are shown with at least three mice per group in total. Means (±SEM). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired two-tailed Student's *t*-test.

mice respect to the WT counterpart (Fig. 6C, lower panels), while an opposite tendency toward increased numbers of MCs was observed in the small intestine (Fig. 6C, upper panels). This in vivo preliminary study of the *bumble* mouse strengthens our in vitro data showing that B-1 cells are relevant modulators of MCs functions and corroborates the idea that the gut represents a privileged site of the B/MC interplay.

Discussion

The majority of the MCs in the body are located in the gastrointestinal tract and their numbers are further increased in the mucosa of patients with irritable bowel syndrome, mastocytic enterocolitis, and systemic mastocytosis [25]. Hence, this represents an intriguing field of investigation for the research of new therapeutic options for these pathologies. MCs regulate the function of different immunological cell types that play key roles in gastrointestinal diseases. Concerning the crosstalk with the adaptive immune system, the interplay between B cells and MCs has been shown by multiple pieces of evidence [3]. We have previously reported in vivo data showing that MCs are in close contact with IgA-expressing PCs within inflamed tissues of IBD patients [4]. This observation is of particular interest given the result of our bioinformatics analysis revealing that the accumulation of activated MCs in the inflamed gut mucosa of UC patients positively correlates with the enlargement of the PC population. This result was specific to the inflammatory condition since the simultaneous increase of activated MCs and PCs was not observed in the context of CRC. Indeed, all B cell populations considered in our analysis were less represented in the cancerous compared to the normal mucosa of CRC patients, which is in accordance with our recently published data showing the accumulation of IgA^+ elements in areas of low-grade dysplasia and their extrusion from the adenoma tissue [26]. Altogether these data call for the need for a better understanding of this crosstalk that bridges the innate and adaptive immune system, with particular attention on the implications that this bidirectional interaction has on MC function. Accordingly, we analyzed the effect of the conditional ablation of MCs on B cell biology both in physiological conditions and in DSS-induced colitis, which represents the most widely used experimental model of UC [27]. In this context, we report that MCs contribute to the maintenance of B cell homeostasis in the intestine while they do not impact these cells in other secondary lymphoid organs. The higher numbers of B cells detected in the small intestine and colon following MC depletion is not in contrast with the well-known sustaining effect of MCs on B cell survival and proliferation, but rather it might be the consequence of MC interaction with other B cell-helping immunological partners such as Tregs [28]. Moreover, the mouse gut is populated to a great extent not only by conventional B-2 lymphocytes but also by innate-like B-1 cells [13,14] that, as demonstrated in the present study, are not affected by the co-culture with MCs. Although the effect of MC depletion on intestinal B cell numbers was observed independently of the inflammatory context, it was even stronger in DSS-treated mice. Under normal conditions, commensal microbiota and intestinal immune cells cooperate to maintain intestinal homeostasis and retain the ability to elicit an efficient immune response to invading pathogenic agents [29]. Following epithelial erosion, this equilibrium is disturbed and dysbiosis and immunological abnormalities develop. In line with several studies highlighting the importance of the IgA isotype in colitis [30,31], an enhanced IgA production was detected in DSS-treated compared to healthy mice. MCs contribute to this outcome since DT-RMB mice failed to increase both the number of IgA^+ and $CD138^+$ cells in the small intestine and colon, and IgA levels in the serum. This is in accordance with published in vitro and in vivo reports [4–6,32,33] suggesting a major role of MCs in fostering IgA^+ cells in the context of intestinal inflammation. In this regard, attention must be paid on the different outcomes that MC depletion has on IgA production in the normal and inflammatory setting. Indeed, the result that under physiological conditions MC-deficient mice present higher titers of serum IgA compared to the normal counterpart may define a Janus-faced MC that acts as a cellular handbrake of IgA synthesis under homeostatic conditions while unleashing IgA production following the onset of colitis. This is a direct consequence of the most intriguing function of the MC that, endowed by receptors involved in cell-cell interaction and bacterial pathogen recognition, acts as an antenna of the microenvironment and responds to multiple stimuli [34,35]. Moreover, whether this outcome on IgA production is the result of the direct B/MC interaction or rather an indirect effect of MCs involving a third cellular partner (e.g., Treg, Th17) remains to be investigated. Corollary to the opposite role of MCs about a homeostatic or activating microenvironment was the second part of the present study in which we assessed whether the interaction with the B cell could impact MC responses. Indeed, this is an understudied aspect in the field of B/MC research, which needs to be addressed. In the current study, we demonstrate the role of peritoneal, but not of splenic, B cells in shifting the MC phenotype toward a proinflammatory signature. Yet, the MC is not the only cell type affected by this change in phenotype after interaction with peritoneal B lymphocytes, as demonstrated by B-1 cell-mediated modulation of macrophage function during fungal infection [36]. The peritoneal B cell-driven upregulation of ST2 expression on MCs is particularly relevant in the context of colitis due to recent findings demonstrating that IL-33-dependent activation of MCs is fundamental for beneficial mucosal tissue healing and recovery in the MC-deficient Kit^{Wsh} DSS-mouse model [11]. Our analysis of *bumble* mice, which lack B-1 cells [24], corroborate our in vitro results since they presented altered MC numbers in different anatomical sites. The significantly lower number of MCs in the colon of *bumble* compared to WT mice is a further observation suggesting the relevance of the B/MC interaction in the context of colitis. Although the present study represents the first step toward an improved understanding of the B/MC interaction and its possible implications in IBD, our results highlight the crucial importance of unraveling the tangled immune cells network to learn more about the situation we are

dealing with and to design successful therapeutic options for these pathologies.

Materials and methods

Animals and treatments

C57BL/6J mice were purchased from Envigo and maintained at the animal facility of the Department of Medical Area, University of Udine, Italy. The study on RMB mice (official name, B6.Ms4a2^{tm1Mal}) was performed in collaboration with Prof. Blank (INSERM UMRS1149, Paris, France). Eight-weeks old RMB mice were injected twice intraperitoneally (2 days apart) with 1 μg DT (Merch Millipore) in PBS and sacrificed 3 weeks after MC depletion. Control mice were injected with PBS only. Where indicated, 14 days after DT injection mice were administered 2% DSS (MW 36 000–50 000; MP BIOMEDICALS) in drinking water for 8 days to induce acute intestinal inflammation (further details in the Supporting Information). TNF-α$^{-/-}$ and IL-33$^{-/-}$ femurs and tibiae were kindly provided by Prof. Kollias (BSRC "Alexander Fleming", Vari, Greece) and Prof. Bulfone-Paus (University of Manchester, United Kingdom), respectively. Organs from Wt and IκBNS-deficient bumble mice were kindly provided by Prof. Karlsson Hedestam Gunilla B. Karlsson Hedestam(Karolinska Institutet, Stockholm, Sweden). Mice were maintained under pathogen-free conditions and experiments were performed following institutional guidelines and national laws of respective institutes.

Cell preparation and B/MC co-cultures

Single-cell suspensions of spleens, peritoneum, and LNs were obtained as previously described [26]. Peritoneal and splenic CD19$^+$ B cells for B/MC co-cultures were obtained using mouse CD19 MicroBeads (Miltenyi) while the B cell isolation kit (Miltenyi) was used to isolate B lymphocytes from total RMB splenocytes. Where indicated, purified splenic B cells were cultured at 1×10^6 cell/mL in the presence or absence of 1 μg/mL anti-mouse CD40 mAb (BD Pharmigen), 10 μg/mL LPS (Sigma–Aldrich) or 5 μg/mL CpG (Sigma–Aldrich). BMMCs were obtained by in vitro differentiation of BM cells isolated from femurs and tibiae of 6- to 8-weeks old mice and cultured in the presence of 20 ng/mL IL-3 (PeproTech) as described previously [4,5]. For B/MC co-cultures, MCs were starved for 3 h in complete medium in resting conditions or sensitized with DNP-specific IgE. Cells were then washed and re-suspended in fresh medium alone (resting MCs) or with 100 ng/mL DNP to challenge the cells. Finally, MCs were put in culture in a 1:1 ratio with freshly purified B cells, each cell type at a final concentration of 1×10^6 cell/mL. B cells and MCs cultured alone were included among the experimental conditions. Cells and supernatants were collected and analyzed at different time points.

Histology and immunohistochemistry

Four micrometers thick tissue sections of paraffin-embedded samples from either WT and *bumble* or PBS-RMB and DT-RMB mice were deparaffinized and rehydrated. Hematoxylin-Eosin (Bioptica) or Toluidine Blue (Histo-Line) stains were performed on the WT and *bumble* samples. MCs frequency was determined by counting purple cells in the Toluidine Blue stains in five to ten non-overlapping high-power microscopic fields (×400). Results were expressed as the sum of counted fields. Antigen unmasking was performed using Target Retrieval Solutions pH 9 EDTA-based buffer in a thermostatic bath at 98°C for 30 min. Subsequently, sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H_2O_2 and Fc-blocking by a specific protein block (Novocastra UK), small intestine and colon samples from untreated and DSS-treated PBS-RMB and DT-RMB mice were incubated 2 h at room temperature with the HRP-conjugated goat anti-mouse IgA alpha chain antibody (1:400, Abcam, ab97235). Alternatively, rat anti-mouse CD45R (1:10, BD Pharmigen, RA3-6B2) and anti-CD138 (1:100, Santa Cruz, DL-101) primary antibodies were incubated overnight at 4°C and immunostaining was revealed by the HRP-conjugated secondary antibodies goat anti-rat IgG (H+L) and rabbit anti-mouse IgG, respectively. The binding of the primary antibody was revealed by 3-amino-9-ethylcarbazole (Dako) and 3,3'-diaminobenzidine (Leica) as substrate chromogens. Slides were counterstained with Harris hematoxylin (Novocastra).

All sections were analyzed under a Zeiss AXIOScope.A1 optical microscope (Zeiss, Germany) and microphotographs were collected using a Zeiss Axiocam 503 Color digital camera using the Zen 2.0 imaging software. CD45R, IgA, and CD138 were analyzed on small intestine and colon samples belonging to the same animal.

Cytometry

Whenever possible, flow cytometry data acquisition and analysis were performed following Cossarizza et al. [37]. Total splenic, peritoneal, or LNs cell suspensions or purified MCs and B cells were surface stained using the anti-mouse mAbs listed in Supporting Information Table S1. BMMCs were stained for intracellular TNF-α after 1 h of co-culture with B cells in the presence of BFA (eBioscience, 1000× solution). The Live/Dead Fixable Dead Cell Stain Probe (Invitrogen) was used to discriminate viable cells while c-Kit staining allowed to select MCs in the gating strategy. Cytofix/Cytoperm (BD Biosciences) was used before TNF-α staining following manufacturer's instructions. The Annexin V-FITC Apoptosis Detection kit (eBiosciences) was used to determine the viability of cultured B cells. To analyze proliferation, freshly purified B cells were stained with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), re-suspended in complete medium, and cultured alone or with MCs for 72 h. The CellTrace Violet Cell Proliferation Kit (ThermoFisher) was used to assess the proliferation of purified splenic B cells from RMB mice. FACSCalibur

or FACSCanto flow cytometers (Becton Dickinson) were used for sample acquisitions. Data were analyzed with the FlowJo software (Tree Star).

RNA extraction and RT-qPCR

RNA was extracted with EUROGOLD TriFast (Euroclone) according to manufacturer's instructions and quantified using NanoDropTM (ThermoFischer). One microgram of RNA was reverse transcribed with the SensiFAST^{TM} cDNA Synthesis Kit (Bioline). RT-qPCR analyses were performed with SYBR Green chemistry (iQTM SYBR Green Super Mix, BioRad) using the BioRad CFX96 real-time PCR detection systems. Target gene expression was quantified using G3PDH as normalizer gene. Primers (Sigma–Aldrich) used for RT-qPCR are listed in Supporting Information Table S2.

Quantification of secreted mediators

Serum samples or cell supernatants were analyzed by ELISA kits for the detection of mouse IL-6, IL-10, IL-13, TNF-α, IgM (eBiosciences), and CCL2 (PeproTech), according to manufacturer's instructions. For mouse IgG and IgA measurements, home-made sandwich ELISAs were developed as described previously [26].

Bioinformatics

The profiling of immune cells in the GEO datasets GSE87211 and GSE107499 was performed taking advantage of the CIBERSORT algorithm by using the LM22 matrix and applying 1000 permutations. Spearman correlation analysis was performed with Rstudio Version 1.2.5019 by using the ggpubr 0.2.4 package.

Statistical analyses

Results are expressed as mean (±SEM) of the indicated replicates of each independent experiment. Two-tailed paired or unpaired Student's *t*-test, or one-way ANOVA with the Bonferroni correction as post hoc analysis, were performed to assess statistical significance with the GraphPad Prism 5 Software. A confidence level of 95% was used. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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Abbreviations: **BMMCs**: BM-derived mast cells · **CRC**: colorectal cancer · **DSS**: dextran sulfate sodium · **DT**: diphtheria toxin · **FO**: follicular · **IBD**: inflammatory bowel disease · **MZ**: marginal zone · **MC**: mast cell · **NF**: newly formed · **PC**: plasma cell · **RMB**: red MC and basophil · **T2-MZP**: transitional 2-marginal zone precursors · **UC**: ulcerative colitis

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