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# Mast Cells Control the Expansion and Differentiation of IL-10-Competent B Cells

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The discovery of B cell subsets with regulatory properties, dependent on IL-10 production, has expanded our view on the mechanisms that control inflammation. Regulatory B cells acquire the ability to produce IL-10 in a stepwise process: first, they become IL-10 competent, a poised state in which B cells are sensitive to trigger signals but do not actually express the *Il-10* gene; then, when exposed to appropriate stimuli, they start producing IL-10. Even if the existence of IL-10-competent B cells is now well established, it is not yet known how different immune cell types cross talk with B cells and affect IL-10-competent B cell differentiation and expansion. Mast cells (MCs) contribute to the differentiation and influence the effector functions of various immune cells, including B lymphocytes. In this study, we explored whether MCs could play a role in the expansion of IL-10-competent B cells and addressed the *in vivo* relevance of MC deficiency on the generation of these cells. We show that MCs can expand IL-10-competent B cells, but they do not directly induce IL-10 production; moreover, the absence of MCs negatively affects IL-10-competent B cell differentiation. Noteworthy, our findings reveal that the CD40L/CD40 axis plays a significant role in MC-driven expansion of IL-10-competent B cells *in vitro* and highlight the importance of MC CD40L signaling in the colon. *The Journal of Immunology*, 2014, 193: 4568–4579.

**M**ast cells (MCs) are particularly abundant at the surfaces of contact between the body and the external environment where they act as “sentinels” of tissue and immune homeostasis (1). MCs function at the crossroad between innate and adaptive immunity, exerting both effector and immunoregulatory roles, depending on their activation status and on the immunological context (2). MCs express a broad array of

soluble and membrane-bound molecules that enable the cross talk with many immune cells of which they influence phenotype and function (3, 4). MCs prime and regulate adaptive responses by either inducing effector T cells or modulating regulatory T cell suppression (5). In contrast with the well-defined role for MCs in regulating T cell function, little is known about the interaction of MCs with B cells. Although the B–MC interaction has long been considered as likely and worthy of investigation (6, 7), only recently has this line of research been more fully explored.

B lymphocytes were traditionally considered effector cells given their ability to produce Abs. Nonetheless, recent findings from several groups revealed the existence of phenotypically diverse B cell subsets with a regulatory suppressive function in various models of chronic inflammation and autoimmune disease (8). The most widely studied and best understood mechanism by which B lymphocytes accomplish immunosuppression is through secretion of IL-10 (9). In mice, B cells that are competent to express *Il-10* are found in different tissues, although at very low frequencies. The majority of these cells do not produce detectable cytoplasmic IL-10, but they do so after 5 h of *in vitro* stimulation with LPS, PMA, and ionomycin (10). Therefore, the subset of B cells capable of producing IL-10 after this *in vitro* stimulation has been labeled as IL-10 competent. Several studies have shown that upon activation, IL-10-competent B cells exert regulatory suppressive functions (11), and that stimulation through CD40 is essential for their induction (12).

We have previously demonstrated that MCs can enhance the proliferation and survival of B cells, and drive their functional differentiation toward IgA-secreting plasma cells through CD40L stimulation and IL-6 release (13). In light of these findings, we hypothesized that, similar to their interaction with T cells (5), MCs may selectively regulate distinct B cell subsets. In this article, we demonstrate that MCs can expand IL-10-competent B cells and that this expansion is reduced by impairing the CD40L/CD40 axis.

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Abbreviations used in this article: BMDC, bone marrow-derived MC; Breg, regulatory B cell; CM, conditioned media; CPD, Cell Proliferation Dye eFluor 670; DSS, dextran sodium sulfate; LP, lamina propria; LPIM, LPS, PMA, ionomycin, and monensin; MC, mast cell; MZ, marginal zone; o/n, overnight; TBP, TATA-binding protein; T2-MZP, transitional two-MZ precursor; WT, wild-type.

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Moreover, the analysis of CD19<sup>+</sup>IL-10<sup>+</sup> cells in different organs of wild-type (WT) and MC-deficient mice revealed that the absence of MCs negatively affects IL-10-competent B cell differentiation, and this can be seen as a very relevant observation in the context of immunologically mediated inflammatory reactions.

## Materials and Methods

### Mice

Female C57BL/6 (B6) mice were from Harlan Laboratories. *C57BL/6-Kit<sup>W-sh/W-sh</sup> (Kit<sup>W-sh</sup>)* mice were from The Jackson Laboratory and were maintained under specified pathogen-free conditions at the animal facility of Fondazione IRCCS INT, Milan, Italy, in collaboration with the group of M. Colombo. Four- to 6-wk-old female *Kit<sup>W-sh</sup>* mice were reconstituted by i.p. injection of  $5 \times 10^6$  in vitro differentiated bone marrow MCs derived from B6 or *Cd40l<sup>-/-</sup>* mice. Experiments were initiated 8–10 wk later. All animal experiments were performed in accordance with the animal care and use committees of the respective institutes.

### Dextran sodium sulfate-induced colitis

To induce colon inflammation, we administered 1.5% dextran sodium sulfate (DSS; m.w. 40,000–50,000; Affymetrix) to WT and *Kit<sup>W-sh</sup>* mice in place of normal drinking water for 10 d. Inflammation progression was monitored up to 7 d after DSS withdrawal (day 17). To perform histological analyses, we dissected colons from mice at three different time points: steady-state (day 0), acute inflammation (day 10), and recovery from inflammation (day 17).

### Abs

Fluorescence-conjugated Abs to CD1d (1B1), CD19 (6D5), CD40L (MR1), and IL-10 (JESS-16E3) were from BioLegend. PE-Cy7 anti-CD5 (53-7.3) and allophycocyanin anti-CD9 (KMC8) were from eBioscience. Allophycocyanin anti-CD21/CD35 (7G6) was from BD Pharmingen; Alexa Fluor 647 anti-CD23 (B3B4) was from AbD Serotec. Alexa 488 goat anti-rat and Alexa 568 goat anti-mouse Abs were from Molecular Probes. Unconjugated anti-CD45RA (MEM 56) and anti-IL-10 (JESS-16E3) Abs were, respectively, from Merck Millipore and Novus Biologicals. Anti-Hsp60 (LK-2) and anti-Hsc70 (1B5) Abs were purchased from Abcam, whereas the anti-TATA-binding protein (TBP, clone 58C9) Ab was from Sigma-Aldrich and the anti-ATP5B (3D5) Ab from Santa Cruz. Ascites containing the anti-CD40L (MR1) Ab were a gift of M. Colombo.

### Cell preparation

Purified splenic B cells were obtained from 6- to 12-wk-old B6 mice as previously described (14). Bone marrow-derived MCs (BMMCs) were obtained by in vitro differentiation of bone marrow cells taken from femurs of B6 WT or *Cd40l<sup>-/-</sup>* mice as previously described (15). Lymph node cells were purified from either axillary or inguinal lymph nodes by gently disrupting the lymph node in PBS using the sterile plunger of a 1-ml syringe. Peritoneal cells were obtained injecting cold PBS supplemented with 3% FBS in the peritoneal cavity and dislodging any attached cell by massaging the peritoneum. The peritoneal B cell population was enriched by performing an adhesion step of at least 1 h in a six-well flat-bottom culture plate and by collecting only the nonadherent cells. Colonic lamina propria (LP) cells were isolated as described previously (16), through two sequential incubations of 30 min each with, respectively, 1.5 mg/ml Dispase (Life Technologies) and 1.0 mg/ml Dispase plus 1.5 mg/ml collagenase type II (Life Technologies). The obtained cell population was enriched in B and T lymphocytes (B cells were 20% of the enriched lymphocyte population) through Percoll gradient (45–72%) centrifugation.

### B–MC coculture conditions

Purified splenic B cells were cultured for 48 h with an equal number of IgE-sensitized or nonsensitized MCs, in the presence or absence of 100 ng/ml DNP-human serum albumin (Sigma-Aldrich). To study the role of MCs on B lymphocytes from the peritoneum and colonic LP, we cultured  $0.6 \times 10^6$  MCs with an equal number of total peritoneal or colonic cells, enriched in B lymphocytes as described earlier. To evaluate the role of the CD40L/CD40 axis, we added an ascites containing the anti-CD40L blocking Ab to culture medium at 50  $\mu$ g/ml and maintained it for all 48 h of culture. In some experiments, B cells and MCs were separated by a transwell (Corning Costar) with pores of 0.4  $\mu$ m. A total of  $0.6 \times 10^6$  B cells was cultured in the bottom chamber in 0.7 ml medium while  $0.2 \times 10^6$  MCs were placed in the top chamber, in a final volume of 0.2 ml. For proliferation assays, B cells were stained with Cell Proliferation dye eFluor 670 (CPD; eBioscience) before coculture with MCs for 72 h.

### Detection of intracellular and secreted IL-10

For immunofluorescent staining of IL-10, cells were resuspended in medium containing 10  $\mu$ g/ml LPS, 50 ng/ml PMA, 500 ng/ml ionomycin, 2  $\mu$ M monensin (all from Sigma-Aldrich) and cultured for 5 h at 37°C. The protocol for intracellular staining was as described previously (10). In certain experiments, Alexa Fluor 647 anti-CD23, FITC anti-CD5, allophycocyanin anti-CD21/CD35 and Alexa Fluor 647 anti-CD23 or PE-Cy7 anti-CD5 and Pacific Blue anti-CD1d were used to assess the expression of surface markers other than CD19. Stained samples were acquired on FACScan (BD Biosciences), FACSCalibur (BD Biosciences), or Cyan ADP nine-color cytometer (DakoCytomation). Data were analyzed with FlowJo software.

IL-10 levels in cell supernatants were quantified by ELISA. A total of  $0.6 \times 10^6$  B cells and MCs was seeded, either alone or together, in 0.6 ml medium and cultured for 48 h. The mouse IL-10 ELISA Ready-SET-Go kit (eBioscience) was used, following manufacturer's instructions.

### Purification and analysis of exosomes from cell culture supernatants

A total of  $5 \times 10^6$  B cells and MCs was cultured either alone or together in 5 ml medium for 48 h. Culture media were collected and centrifuged at  $300 \times g$  for 10 min. Cleared supernatants were incubated with 1 ml ExoQuick-TC Exosome Precipitation Solution (System Biosciences) overnight (o/n) at 4°C for precipitation. The exosome-containing pellet was resuspended in PBS and stored at  $-80^\circ\text{C}$  until use for analysis. For flow cytometry experiments, isolated exosomes were incubated with 4- $\mu$ m diameter aldehyde/sulfate latex beads (Interfacial Dynamics; 10  $\mu$ l in 0.3 ml PBS), o/n, at room temperature, under gentle movement. Samples were saturated with 1 M glycine, washed, and stained with specific Abs. Samples were acquired on FACSCalibur and analyzed by Summit v4.3 software (Dako Cytomation). For Western blot experiments, exosomes were lysed in radioimmunoprecipitation assay buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) and mixed with Laemmli buffer. Protein extracts were separated on 12% SDS-PAGE, samples were transferred onto nitrocellulose membranes, and Western blot analyses were performed using specific anti-Hsp60, anti-Hsc70, anti-TBP, or anti-ATP5B Abs. HRP-conjugated secondary Abs were revealed using chemiluminescence substrate (Pierce). Blots were captured by using a ChemiDoc XRS imaging system (Bio-Rad), and signals were quantified with the instrument bundled densitometric software.

### Real-time video microscopy and conjugate formation evaluation

The formation of MC–B cell conjugates in real time was analyzed by time-lapse epifluorescent microscopy as previously described (17). At different time points the number of MCs in contact with B cells was counted and the percentage of B-conjugated MCs over total MCs per field was calculated.

### Immunofluorescence microscopy

Tissue samples were fixed in 10% buffered formalin and paraffin embedded. For immunofluorescence analysis, tissue sections were deparaffinized with xylene and rehydrated to water through a graded alcohol series. High-temperature Ag unmasking was performed using citrate buffer pH 6 (Novocastra) in a water bath at 98°C for 30 min. Subsequently, the sections were brought to room temperature and washed in PBS. After Fc blocking using a specific protein block (Novocastra), samples were incubated o/n with rat anti-mouse IL-10 Ab followed by the Alexa 488 goat anti-rat Ab. Subsequently, slides were incubated with anti-mouse CD45RA Ab and marked by the Alexa 568 goat anti-mouse Ab.

### Statistical analysis

Experimental data are shown as means  $\pm$  SEM. Unpaired or paired Student *t* tests (Prism GraphPad Software) were used to analyze the results for statistical significance. The *p* values  $<0.05$  were considered significant.

## Results

### MCs promote the expansion in vitro of IL-10-competent B cells

To assess the ability of MCs to influence the expansion of IL-10-competent B cells, we cocultured murine BMMCs and splenic B cells at a 1:1 ratio for 48 h. To induce cytoplasmic IL-10 production in IL-10-competent B cells and block protein secretion, we added a mixture of LPS, PMA, ionomycin, and monensin (LPIM) to the coculture 5 h before cell harvesting. Compared with

freshly isolated B lymphocytes, the proportion of B cells competent to express cytoplasmic IL-10 after LPIM stimulation was significantly increased after coculture with MCs (Fig. 1A). Coculture of B lymphocytes with non-IgE-sensitized or IgE-Ag-stimulated MCs increased the proportion of IL-10<sup>+</sup> B cells to 8.4% ( $\pm 0.9$ ) and 7.1% ( $\pm 0.8$ ), respectively, an  $\sim 2.5$ -fold increase with respect to the pre-coculture condition ( $2.9 \pm 0.2\%$ ). Both in freshly purified B cells and in B-MC cocultures, no cells positively stained for IL-10 were detected without the 5-h addition of LPS plus PMA and ionomycin (Supplemental Fig. 1).

To test whether the observed increase of IL-10-competent B cells might be driven through enhanced and selective proliferation induced by MCs, we labeled B lymphocytes with the CPD and cocultured them with non-IgE-sensitized MCs for 72 h. As shown in Fig. 1B, MCs induced the proliferation of both IL-10<sup>+</sup> and IL-10<sup>-</sup> B cells. However, the IL-10-competent B cell population showed an enhanced proliferation profile compared with the IL-10<sup>-</sup> counterpart.

Because IL-10 competence and production are differently regulated processes (18), we next evaluated whether MCs could enhance the secretion of IL-10 from B cells in a 48-h coculture without the addition of LPIM during the last 5 h. B cells or MCs alone released low and almost undetectable amounts of IL-10 when compared with B cells stimulated with LPS, which is known to promote IL-10 secretion (10). As shown in Fig. 1C, the secretion of IL-10 was not enhanced when MCs and B cells were cocultured. The result of the ELISA experiment was confirmed by the analysis of the *Il-10* gene expression profile obtained by assessing IL-10 mRNA levels in B cells cultured either alone or with MCs (Supplemental Fig. 2).

#### Phenotypic markers of MC-induced, IL-10-competent B cells

IL-10-competent B cells share phenotypic markers with CD21<sup>hi</sup>CD23<sup>-</sup>IgM<sup>hi</sup>CD1d<sup>hi</sup> marginal zone (MZ) and CD21<sup>hi</sup>CD23<sup>+</sup>IgM<sup>hi</sup>CD1d<sup>hi</sup> transitional two-MZ precursor (T2-MZP) B cells, but also with CD5<sup>+</sup> B-1a and CD1d<sup>hi</sup>CD5<sup>+</sup> B10 cells (11, 19–21).

To address the phenotype of IL-10-competent splenic B cells obtained after coculture with MCs, we analyzed B cells within both the IL-10<sup>+</sup> and the IL-10<sup>-</sup> gates for CD1d and CD5 expression, because their differential expression allows us to distinguish distinct subsets of regulatory B cells (Bregs) described in the literature (11). IL-10-competent B cells induced by non-IgE-sensitized MCs were found within all the four subsets defined by the differential expression of the aforementioned markers. The highest proportion of these cells ( $51.1 \pm 3.6\%$ ) expressed high levels of CD1d and low levels of CD5, thus resembling the phenotype of MZ or T2-MZP B cells (Fig. 2A). Only a small proportion of total IL-10<sup>+</sup> B cells were CD1d<sup>+</sup>CD5<sup>+</sup> ( $7.7 \pm 1.1\%$ ) or CD1d<sup>-</sup>CD5<sup>+</sup> ( $4.3 \pm 1.1\%$ ). In contrast, the majority of IL-10<sup>-</sup> cells expressed low levels of both CD1d and CD5.

Because CD21 and CD23 are commonly used to distinguish MZ from follicular and transitional B cells (22, 23), we further analyzed the expression levels of these markers on IL-10<sup>+</sup> B cells after coculture with MCs. As shown in Fig. 2B, the majority of IL-10<sup>+</sup> B cells expressed high levels of CD19 and CD21 and were CD23<sup>+</sup>, further supporting the evidence that these cells are phenotypically similar to T2-MZP B cells.

#### The CD40L/CD40 axis contributes to MC-driven expansion of IL-10-competent B cells

Both cell–cell contacts and cytokines secreted by MCs could be responsible for the expansion of IL-10-competent B cells. To test the relative contribution of both components, we performed a coculture transwell assay. As shown in Fig. 3A, when the two

populations were physically separated, a partial but significant decrease was observed in the proportion of IL-10<sup>+</sup> B cells. The coculture with non-IgE-sensitized MCs induced 8.1% ( $\pm 0.9$ ) IL-10-competent B cells, whereas only 6.7% ( $\pm 1.0$ ) of the B cells were IL-10<sup>+</sup> in the presence of the transwell. Similar results were seen when MCs were stimulated with IgE-Ag. These findings suggest that cell contacts contribute to the expansion of IL-10-competent B cells, but also imply that soluble factors are involved.

We next explored which membrane molecules might be involved in promoting IL-10-competent B cell expansion. MCs express several costimulatory molecules, many of which are known to modulate B cell activation and function. CD40L is constitutively expressed on the surface of MCs (Fig. 3B), and we previously showed that its interaction with CD40 on B cells is important for MC-driven proliferation of B cells and for their differentiation into plasma cells (13).

To evaluate whether the CD40L/CD40 axis is involved in the physical interaction between MCs and B cells, we observed cell–cell interactions by real-time imaging. Time-lapse bright-field video microscopy showed that MC–B cell conjugates were formed after only few minutes of coculture (Fig. 3C). Interestingly, when MCs were derived from *Cd40l*<sup>-/-</sup> mice, the number of conjugates was drastically reduced compared with WT MCs. As soon as 1 min after coculture, the proportion of *Cd40l*<sup>-/-</sup> MCs making contacts with B cells was 5-fold less than that of WT MCs, and this defect in interaction continued for at least 20 min of observation. These findings suggest that the presence of CD40L on MCs is crucial for the formation of cell–cell contacts with B cells.

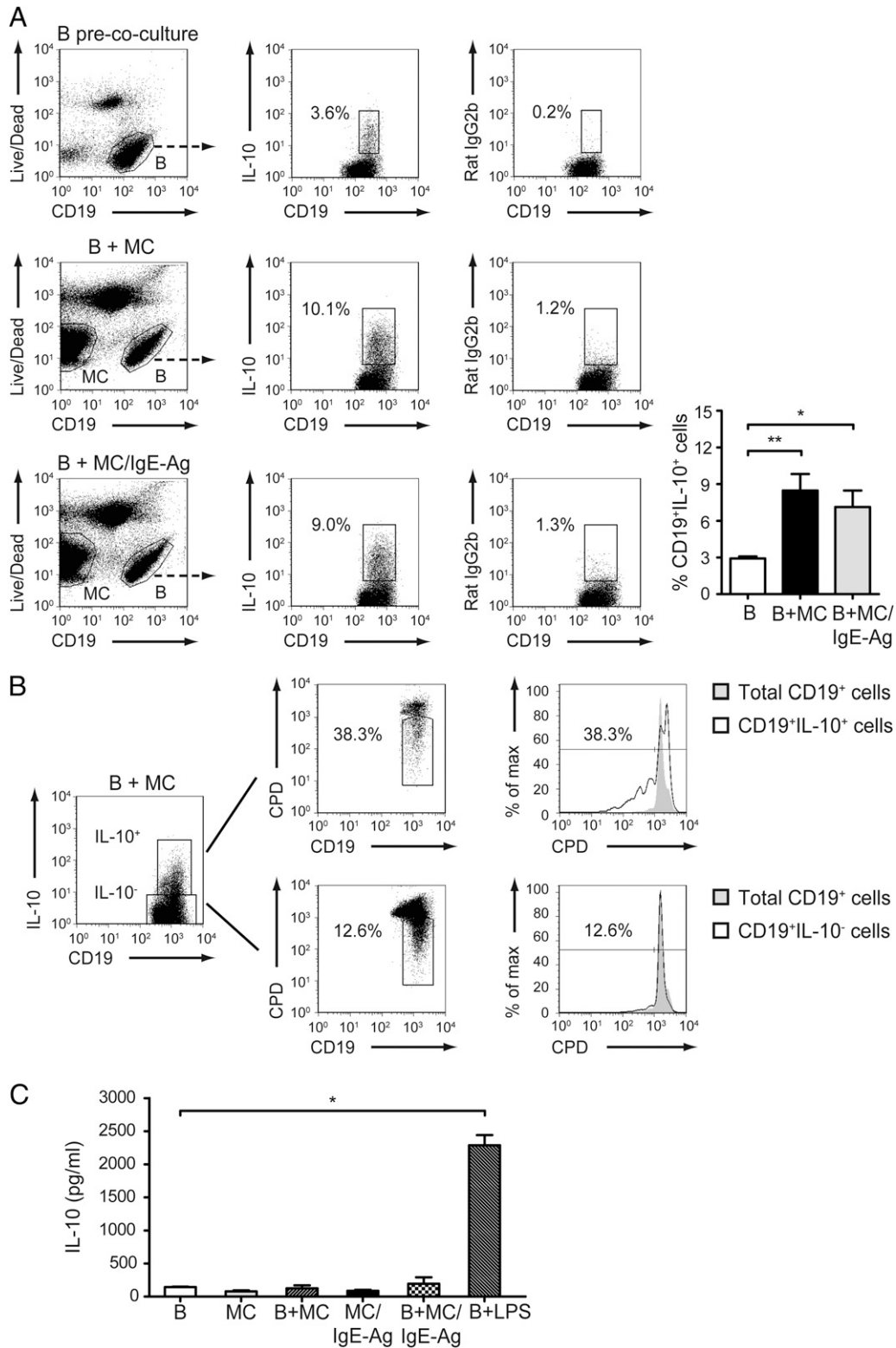
Given the importance of the CD40L/CD40 axis in B–MC interaction, we analyzed its contribution to the expansion of IL-10-competent B cells. In the presence of an anti-CD40L blocking Ab, a significant decrease in the proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells was observed (Fig. 3D). This reduction was  $\sim 30\%$  and occurred with both non-IgE-sensitized and IgE-Ag-stimulated MCs. To further validate these findings, we used *Cd40l*<sup>-/-</sup> BMBCs to perform the B–MC cocultures. As shown in Fig. 3E, in the presence of non-IgE-sensitized or IgE-Ag-stimulated *Cd40l*<sup>-/-</sup> MCs, the proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells was, respectively,  $5.3 (\pm 1.2)$  and  $4.8\% (\pm 1.3)$ ,  $\sim 20\%$  lower than for B lymphocytes incubated with WT MCs.

#### CD40L-exosomes contribute to MC-driven expansion of IL-10-competent B cells

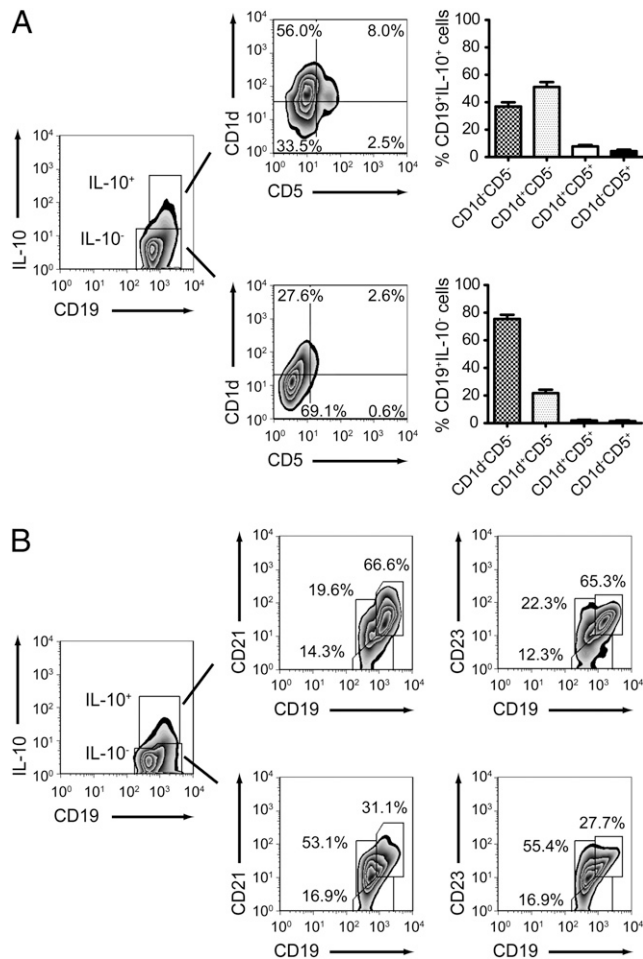
In addition to the expression of cell-surface costimulatory molecules, MCs produce exosomes (Fig. 4A), whose content is quite heterogeneous (24, 25). It has been previously shown that MCs can communicate with B cells through the secretion of these small, diffusible vesicles that induce B cell proliferation and activation (26, 27). Because CD40L is a prominent component of MC-derived exosomes (27), the possible role of exosomes in the expansion of IL-10-competent B cells was investigated.

MCs and B cells were cultured either alone or together for 48 h. Exosomes were isolated from conditioned media (CM) and analyzed by flow cytometry. As shown in Fig. 4B, both MCs and B cells released exosomes. As expected, CD9 (a characteristic exosome marker) was detected on both MC- and B cell-exosomes, whereas CD40L was found only on MC-secreted exosomes. Interestingly, supernatants from B–MC cocultures showed less detectable exosomes, and those present did not express CD40L at the levels seen in exosomes derived from cultures of MCs alone (Fig. 4B). In light of these results, we quantified the proportion of IL-10-competent B cells when B lymphocytes were cultured for 48 h with MC-CM in the presence or absence of an anti-CD40L





**FIGURE 1.** MCs induce expansion of IL-10-competent B cells, but not IL-10 production. **(A)** IL-10-competent B cells were analyzed in freshly isolated splenic B cells (B pre-co-culture) and in B cells cultured for 48 h with either nonsensitized MCs (B+MC) or IgE-sensitized MCs stimulated with DNP (B+MC/IgE-Ag). In all conditions, LPIM was added for the last 5 h of culture. Dot plots for one representative experiment are shown. CD19 versus Live/Dead plots allow to identify viable B cells (B) and exclude both MCs, which are negative for CD19 staining, and dead cells that are positive for Live/Dead staining. Only viable B cells were analyzed for IL-10 staining, and a perfectly matched isotype control (Rat IgG2b) was used to discriminate IL-10<sup>+</sup> from IL-10<sup>-</sup> B cells. Bar graphs indicate mean ( $\pm$  SEM) percentages of IL-10<sup>+</sup> cells among total B cells ( $n = 5$ ). **(B)** B cells were labeled with CPD and cultured with MCs for 72 h, with LPIM added during the last 5 h of coculture. Dot plots for one representative experiment are shown; the percentages of proliferating cells among both the IL-10<sup>+</sup> and the IL-10<sup>-</sup> populations are indicated. Histograms represent CPD expression in IL-10<sup>+</sup> and IL-10<sup>-</sup> B cells. Filled histograms represent CPD staining of total unstimulated B cells. Data are representative of  $n = 3$  experiments. **(C)** B cells, MCs, or B+MCs were cultured for 48 h with no addition of LPIM. IL-10 concentration in culture supernatants was assessed by ELISA. The same experiment was performed using IgE-Ag-stimulated MCs. B cells activated with 10  $\mu$ g/ml LPS were used as control. Bar graphs indicate mean ( $\pm$ SEM) concentrations of  $n = 3$  experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .



**FIGURE 2.** MC-induced, IL-10-competent B cells express markers typical of T2-MZP cells. **(A)** B cells were cultured with MCs and, after LPIM stimulation, were stained with anti-CD19, CD1d, CD5, and IL-10 Abs. Zebra plots for one representative experiment are shown together with the percentages of IL-10<sup>+</sup> and IL-10<sup>-</sup> B cells that display one of the four phenotypes defined by the differential expression of CD1d and CD5. MCs and dead cells were excluded from the analysis using the CD19 versus Live/Dead dot plot, as in Fig. 1A. Histograms show average percentages ( $\pm$ SEM) of IL-10<sup>+</sup> or IL-10<sup>-</sup> cells, with a CD1d<sup>-</sup>CD5<sup>-</sup>, CD1d<sup>+</sup>CD5<sup>-</sup>, CD1d<sup>+</sup>CD5<sup>+</sup>, or CD1d<sup>-</sup>CD5<sup>+</sup> phenotype from  $n = 4$  experiments. **(B)** Expression of CD21 and CD23 was assessed on both IL-10<sup>+</sup> and IL-10<sup>-</sup> B cells under the conditions described earlier. Zebra plots are representative of  $n = 3$  experiments and show the distribution of total IL-10<sup>+</sup> and IL-10<sup>-</sup> B cells among the subsets defined by CD19 and CD21 or CD19 and CD23 expression.

blocking Ab. Indeed, when B cells were cultured with non-IgE-sensitized MCs supernatants, the proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells was 2.6-fold higher than that seen in the nontreated B cell population (Fig. 4C). When the anti-CD40L blocking Ab was added to MC-CM and cultured with B cells, a significant reduction (25%) in the proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells was observed relative to B cells cultured in MC-CM.

#### MCs influence IL-10-competent B cell differentiation in vivo

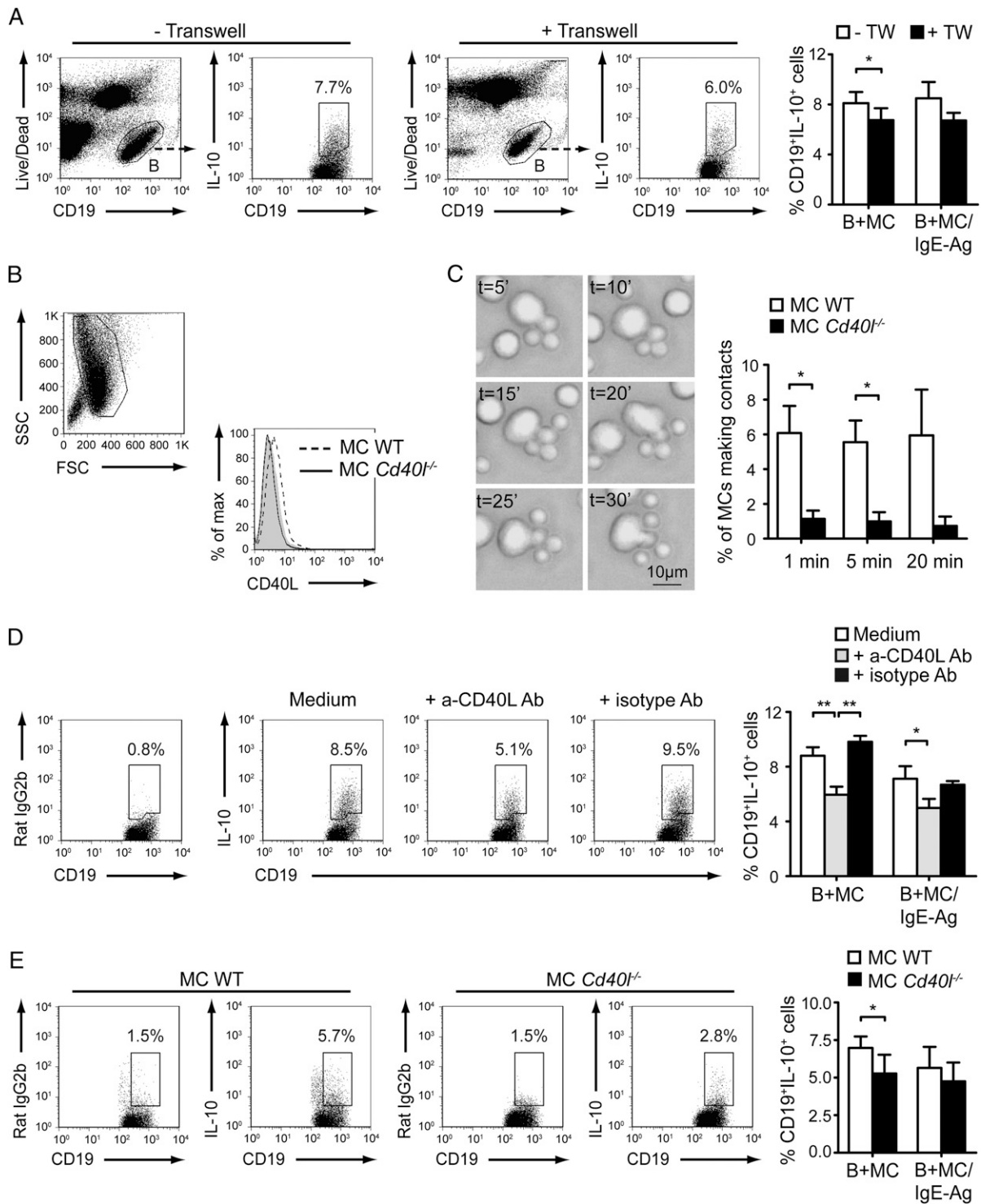
Next, we explored in vivo whether MCs play a role in the differentiation of IL-10-competent B cells. To do so, we used the C57BL/6-*Kit*<sup>W-sh/W-sh</sup> (*Kit*<sup>W-sh</sup>) murine model of MC deficiency, which enabled us to investigate whether the congenital deficiency of MCs could affect the proportions of IL-10-competent B cells in various lymphoid organs. We analyzed the percentage of IL-10-competent B cells in the bone marrow, lymph nodes, peritoneum,

and spleen, which are all known to be important sites of B cell differentiation, maturation, and activation, and we compared them with control C57BL/6 (B6) mice. After stimulation with LPIM for 5 h, CD19<sup>+</sup>IL-10<sup>+</sup> cells were found in all the organs studied of both MC-deficient and -sufficient mice (Fig. 5). In the spleen, the IL-10-competent B cell population seemed not to be affected by the absence of MCs because no significant differences were observed between B6 and *Kit*<sup>W-sh</sup> mice (Fig. 5A). In the bone marrow (Fig. 5B), the percentage of CD19<sup>+</sup>IL-10<sup>+</sup> cells was 30% lower in *Kit*<sup>W-sh</sup> than in WT mice, but this difference was not statistically significant. In the peritoneum (Fig. 5C) and lymph nodes (Fig. 5D), MC-deficient *Kit*<sup>W-sh</sup> mice had significantly lower proportions (a 36 and 33% reduction, respectively) of IL-10-competent B cells than WT controls.

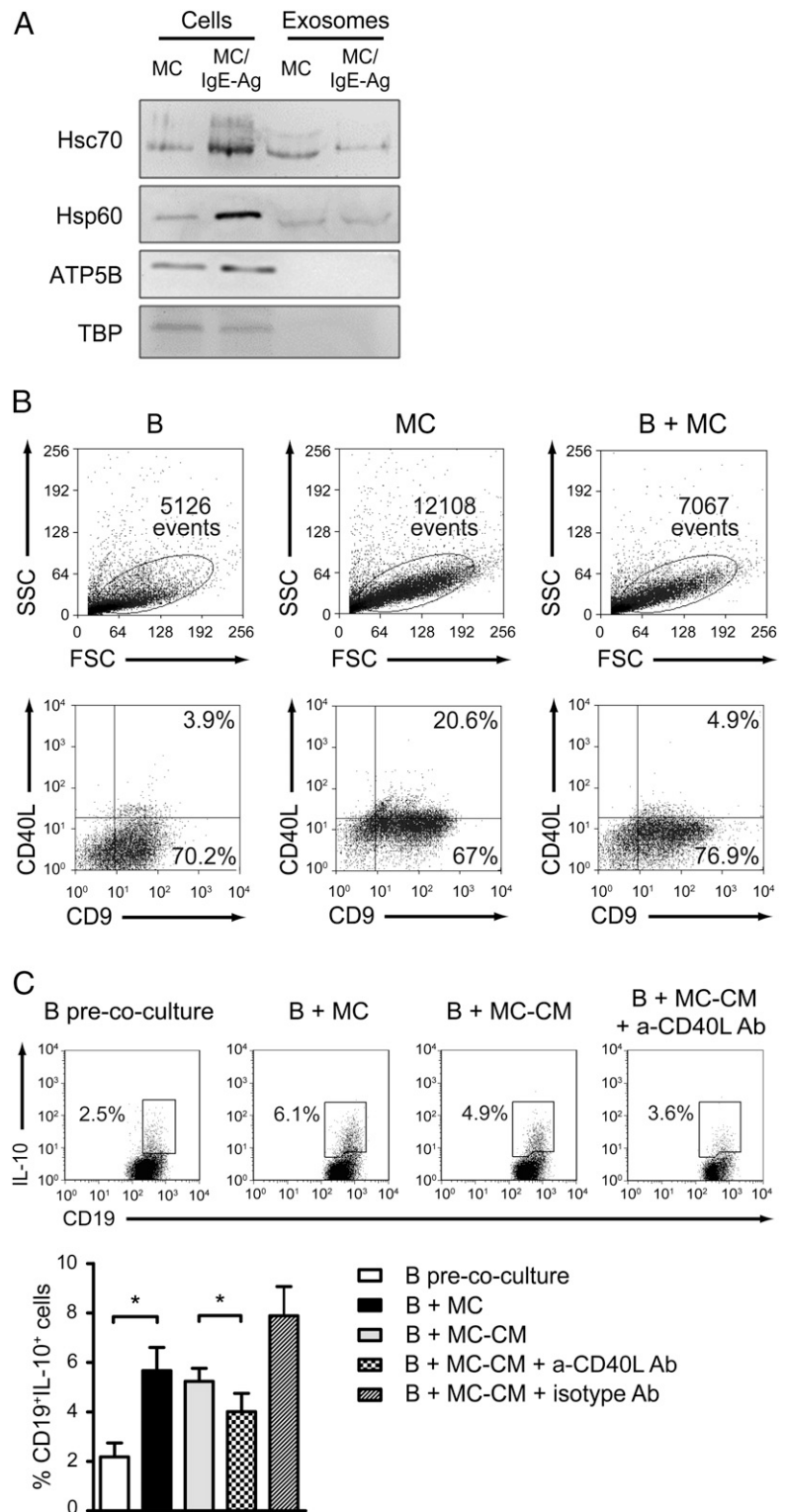
To test whether the observed differences in the percentages of IL-10-competent B cells between B6 and *Kit*<sup>W-sh</sup> mice were truly due to the absence of MCs, we reconstituted *Kit*<sup>W-sh</sup> mice by i.p. injection of in vitro differentiated MCs obtained from B6 mice (BMMC i.p. *Kit*<sup>W-sh</sup>). As shown in Fig. 5, the decrease in the proportion of IL-10<sup>+</sup> B cells was at least partly corrected in all tissues analyzed of BMMC-reconstituted *Kit*<sup>W-sh</sup> mice with the exception of the peritoneum, where reconstituted MCs failed to restore the IL-10-competent B cell population as the percentage of CD19<sup>+</sup>IL-10<sup>+</sup> cells remained significantly lower than in WT mice.

To further investigate the lack of response of peritoneal B cells to MCs, we reproduced the reconstitution experiment in vitro by performing a coculture of peritoneal B cells and MCs, and subsequently analyzing the effect on the IL-10-competent B cell population. Peritoneal cells were isolated from both B6 and *Kit*<sup>W-sh</sup> mice, and B cells were partially purified and cultured for 48 h with MCs. Thus, the percentages of IL-10-competent B cells in the cocultures were compared with those within freshly isolated peritoneal cells of B6 and *Kit*<sup>W-sh</sup> mice. As shown in Fig. 6A, in vitro MCs also failed to induce the expansion of CD19<sup>+</sup>IL-10<sup>+</sup> cells from the total population of peritoneal B lymphocytes, derived from either MC-deficient or -sufficient mice. The cocultures actually yielded decreased percentages of CD19<sup>+</sup>IL-10<sup>+</sup> cells, although the differences were not statistically significant (Fig. 6A, right histograms): for B cells isolated from B6 mice, the proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells decreased from 37.1% ( $\pm$ 6.2) to 20.7% ( $\pm$ 7.0); similarly, B lymphocytes obtained from *Kit*<sup>W-sh</sup> mice showed a 36% reduction in IL-10<sup>+</sup> B cells after coculture with MCs.

The different response of peritoneal B cells to MCs could be because of the specific and peculiar properties of the different B cell subsets present in this site, and because of their different and selective capacity to respond to MC-driven stimuli. The peritoneal cavity is characterized by the presence of two distinct B cell populations, B-1 and B-2, which differ for their developmental origin, anatomical localization, and functional characteristics (28). Therefore, we next investigated whether the different B cell subsets present in the peritoneal cavity responded differently to MCs, in terms of IL-10 competence. The partially purified peritoneal B cell population was cultured for 48 h with MCs. Then IL-10-competent B cells were quantified after the 5-h in vitro stimulation with LPIM, and the anti-CD5 or anti-CD23 Abs were added to the cell-surface staining mixture to distinguish B-1a (CD5<sup>+</sup>) from B-1b and B-2 (CD5<sup>-</sup>) cells or B-2 (CD23<sup>+</sup>) from B-1a and B-1b (CD23<sup>-</sup>) cells. Consistent with what was previously reported by Maseda and coworkers (29), we observed that the percentage of B lymphocytes induced to produce IL-10 was higher among B-1a compared with B-1b and B-2 cells (Fig. 6B, 6C, left panels). Interestingly, the coculture with MCs led to a reduction of the percentage of B cells that responded



**FIGURE 3.** The CD40L-CD40 axis is involved in MC-induced expansion of IL-10-competent B cells. **(A)** B cells were cultured with IgE-sensitized or nonsensitized MCs in the presence or absence of a transwell (TW). **(B)** Flow cytometry analysis of CD40L expression on WT and *Cd40l*<sup>-/-</sup> MCs. **(C)** Time-lapse bright-field video microscopy images showing MC-B cell conjugates. Equal numbers of MCs and B cells were seeded onto glass-bottom Petri dishes, and conjugate formation between the two cell types was monitored for 30 min. The Leica AF6000LX system (microscope: DMI6000 B; camera: DFC350FX; software: LAS AF) was used. Original magnification  $\times 40$ . Scale bar, 10  $\mu$ m. Histograms represent mean ( $\pm$ SEM) percentages of either WT or *Cd40l*<sup>-/-</sup> MCs interacting with B cells from, respectively,  $n = 5$  and  $n = 4$  experiments. Cell-contact formation was analyzed at 1, 5, or 20 min from the beginning of coculture. **(D)** B cells were cultured with either IgE-sensitized or nonsensitized MCs in the presence or absence of an ascites containing the anti-CD40L blocking Ab (a-CD40L Ab) or of the CD40L isotype control Ab, mouse IgG (isotype Ab). **(E)** B cells were cultured with either IgE-sensitized or nonsensitized MCs obtained by in vitro differentiation of bone marrow precursor cells from WT (MC WT) or CD40L-deficient (MC *Cd40l*<sup>-/-</sup>) mice. Representative dot plots in (A), (D), and (E) refer to 48-h B-MC cocultures, with LPIM addition during the last 5 h, and show the percentage of IL-10<sup>+</sup> cells among total viable CD19<sup>+</sup> cells. MCs and dead cells were excluded from the analysis using the CD19 versus Live/Dead dot plot, as in Fig. 1A. Bar graphs indicate mean ( $\pm$ SEM) percentages from at least three experiments for each condition. \* $p < 0.05$ , \*\* $p < 0.01$ .



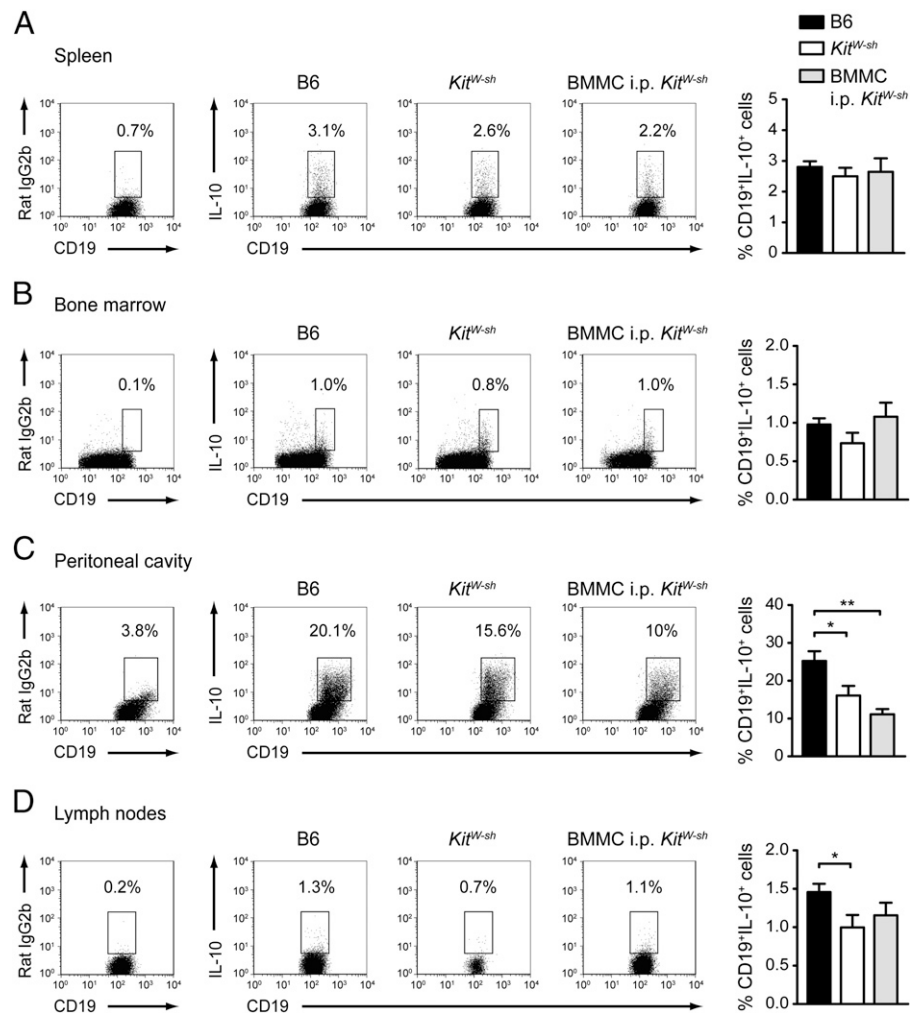
**FIGURE 4.** MC-derived exosomes display CD40L and induce expansion of IL-10-competent B cells. **(A)** MCs were cultured in exosome-depleted medium for 48 h, before collection of supernatants and purification of exosomes. Purified exosomes secreted by equal amounts ( $1 \times 10^6$ ) of nonsensitized (MC) or IgE-Ag-stimulated (MC/IgE-Ag) MCs were analyzed by immunoblotting for the presence of Hsc70, Hsp60, ATP5B, and TBP proteins. Isolated exosomes were confirmed by enrichment of the exosome markers Hsp60 and Hsc70, and by the absence of the nuclear marker TBP and of the mitochondrial marker ATP5B. A total of  $1 \times 10^6$  MCs was lysed and used for comparison. **(B)** Exosome-bead complexes deriving from supernatants of B cells (B), nonsensitized MCs (MC), and B-MC cocultures (B+MC) are identified by forward light scatter (FSC) and side scatter (SSC) properties. CD9 and CD40L expression was analyzed on exosomes identified on the basis of their morphological properties. Dot plots are representative of  $n = 2$  experiments. **(C)** Percentages of IL-10<sup>+</sup> cells before and after treatment with MC-CM, in the presence or absence of the anti-CD40L blocking Ab (a-CD40L Ab) or of its isotype control Ab (isotype Ab). Bar graphs indicate mean ( $\pm$  SEM) percentages from  $n = 4$  experiments. \* $p < 0.05$ .

to LPIM stimulation, regardless of the B cell subset analyzed. Indeed, as shown in the representative experiment in Fig. 6B, the percentage of CD19<sup>+</sup>IL-10<sup>+</sup> cells decreased from 70.6 to 12.3% among CD19<sup>+</sup>CD5<sup>+</sup> cells and from 30.6 to 7.1% among CD19<sup>+</sup>CD5<sup>-</sup> cells. A similar result was obtained comparing the CD19<sup>+</sup>CD23<sup>+</sup> and CD19<sup>+</sup>CD23<sup>-</sup> populations (Fig. 6C). Thus, it appears that MCs not only are unable to restore the levels of IL-10-competent B cells in adult *Kit*<sup>W-sh</sup> mice, but may actually negatively interfere with the induction of IL-10-competent B cells of all subsets in this compartment in both WT and *Kit*<sup>W-sh</sup> mice.

#### MCs finely regulate the IL-10-competent B cell population in the gut microenvironment

The observation that certain anatomical sites of *Kit*<sup>W-sh</sup> mice present a deficiency of IL-10-competent B cells reveals a novel and intriguing role of MCs in the regulation of IL-10-competent B cell differentiation in vivo. Because B cells were shown to play a critical role in different models of autoimmune disease and chronic inflammation (11), we decided to further investigate the effect of MC deficiency on IL-10-competent B cell biology in a mouse model of intestinal inflammation. The choice of the gut





**FIGURE 5.** MC-deficient mice present lower frequencies of IL-10-competent B cells in selected organs. Cells from spleen (**A**), bone marrow (**B**), peritoneum (**C**), and lymph nodes (**D**) of WT (B6,  $n = 7$ ) and MC-deficient ( $Kit^{W-sh}$ ,  $n = 7$ ) mice were cultured with LPIM for 5 h before staining for IL-10. The same experiment was performed in  $Kit^{W-sh}$  mice in which the MC population was reconstituted by i.p. injection of in vitro differentiated BMMCs derived from B6 mice (BMMC i.p.  $Kit^{W-sh}$ ,  $n = 4$ ). Dot plots from one representative experiment show frequencies of IL-10<sup>+</sup> cells among total viable B cells within the indicated gates. Dead cells were excluded from the analysis using the CD19 versus Live/Dead dot plot (data not shown). Bar graphs indicate mean ( $\pm$  SEM) percentages of IL-10<sup>+</sup> B cells from all mice analyzed. \* $p < 0.05$ , \*\* $p < 0.01$ .

microenvironment was due to the fact that the colon represents an anatomical site in which the B–MC interaction is likely to occur (Supplemental Fig. 3), especially in an inflammatory setting (13). Moreover, the differentiation of Bregs actively producing IL-10 has been described in this organ (30).

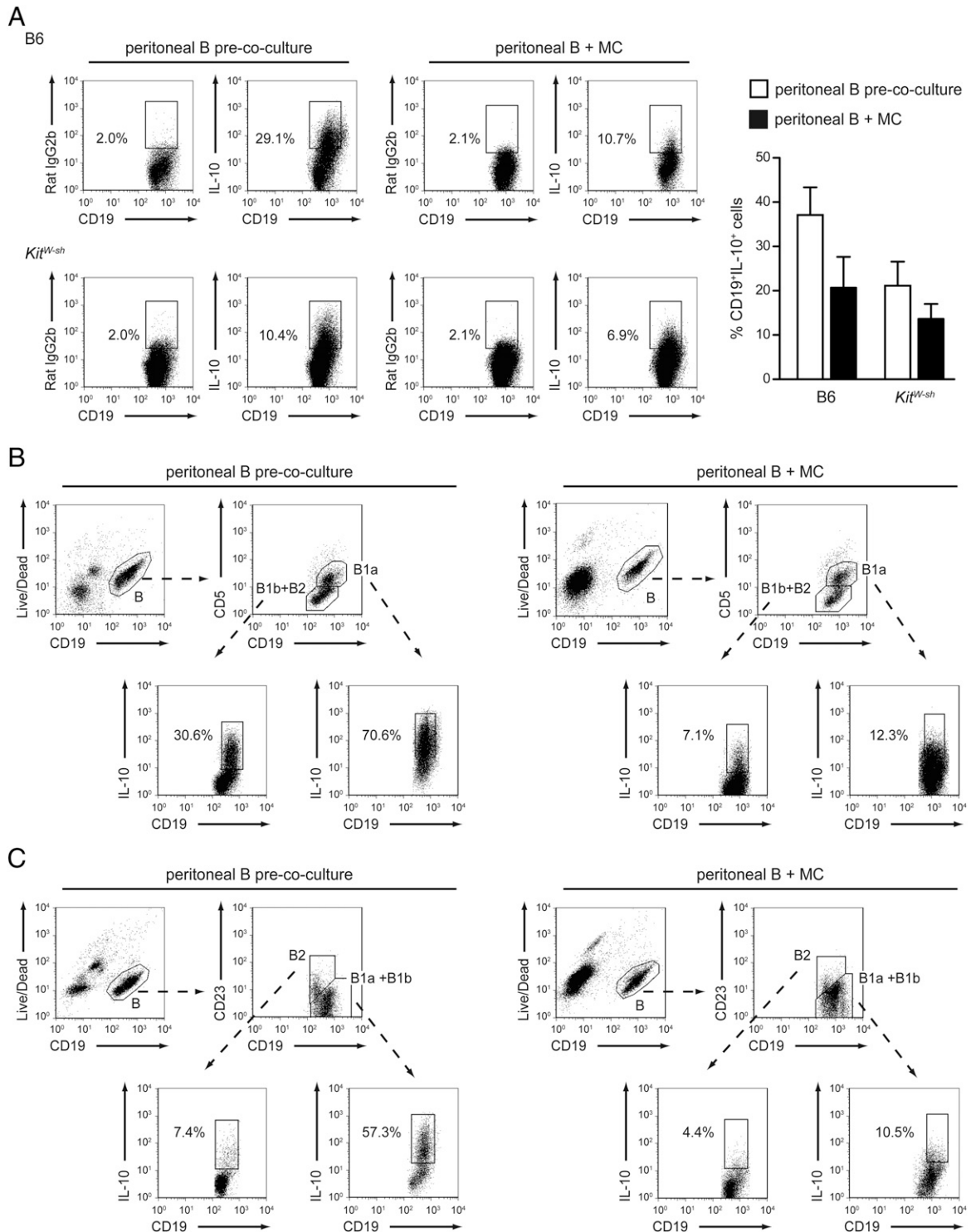
First, we confirmed the ability of MCs to affect IL-10-competent B cells in a normal intestinal microenvironment. Although in this condition the proportion of CD19<sup>+</sup>IL10<sup>+</sup> cells present among the enriched lymphocyte population of the colonic LP is lower compared with other mouse organs, MCs were still able to augment the percentage of IL-10-competent B cells in a 48-h coculture assay. After the 5-h in vitro stimulation with LPIM, only 0.8% of the B cells present among the enriched lymphocyte population of the LP responded expressing IL-10, but this percentage is doubled after coculture with MCs (Fig. 7A). This in vitro finding was further validated by looking at the presence of IL-10-producing B cells in colon sections of WT and  $Kit^{W-sh}$  mice by in situ double immunofluorescence. As a control, the same experiment was performed in specimens of  $Kit^{W-sh}$  mice that were i.p. reconstituted with WT MCs and in which MC reconstitution was checked by analyzing the distribution of MCs in the colon 8–10 wk after adoptive transfer of BMMCs (Supplemental Fig. 4). As shown in Fig. 7B, WT mice had more B cells positively stained for IL-10 compared with  $Kit^{W-sh}$  mice. Reconstitution of  $Kit^{W-sh}$  mice with WT BMMCs significantly increased the number of IL-10-producing B cells relative to  $Kit^{W-sh}$  mice, but it did not completely restore the number of such cells to the levels of WT mice.

Interestingly, the same effect was not obtained by reconstituting  $Kit^{W-sh}$  mice with  $Cd40l^{-/-}$  BMMCs, further supporting the evidence that MC-provided CD40L is an important posing signal for IL-10 competence in B cells.

We then examined the effect of the absence of MCs on IL-10-producing B cells in the context of DSS-induced intestinal inflammation. Yanaba and coworkers (31) have previously shown that during DSS-induced intestinal injury, IL-10 expression was enhanced in splenic B cells, but not in B cells from the intestinal LP. Accordingly, we found a higher number of B cells positively stained for IL-10 in colon samples of untreated WT mice than of WT mice sacrificed in the acute phase of inflammation that is 10 d after initiation of DSS administration (day 10). This result was not dependent on MCs because the same outcome was observed also in  $Kit^{W-sh}$  mice (Fig. 7C, upper and middle panels). Interestingly, the number of IL-10-producing B cells began to increase in WT mice 7 d after DSS withdrawal (day 17), suggesting that this Breg population might be expanded in the colon or recruited from other anatomical sites during the recovery phase to resolve inflammation. Notably, this process was dependent on MCs because no increase was observed at day 17 in colon samples of  $Kit^{W-sh}$  mice (Fig. 7C, lower panels).

## Discussion

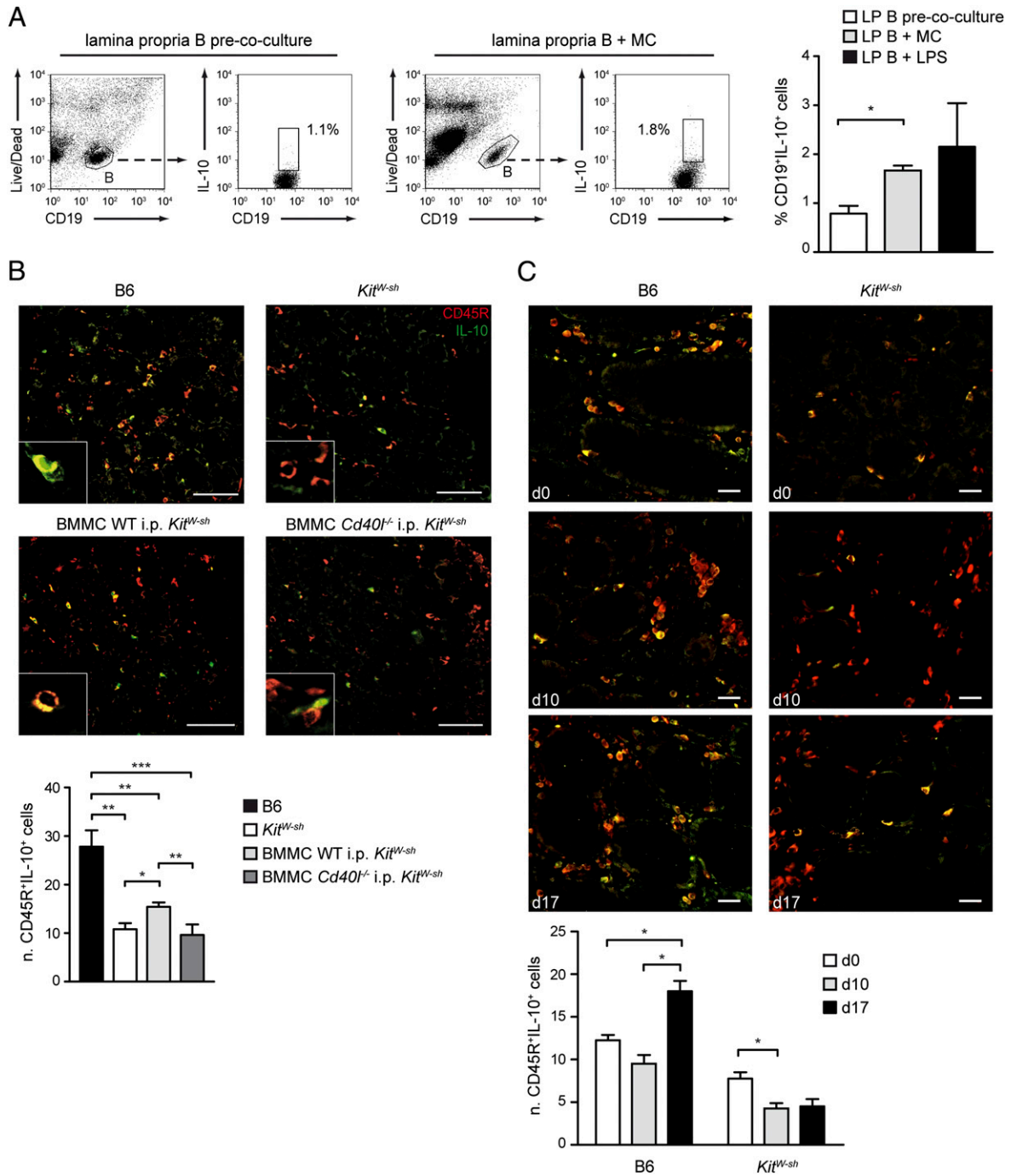
It is now widely accepted that B cells can exert a regulatory role in autoimmunity, inflammation, and infection, and that IL-10 production is pivotal to Breg-mediated tolerance (11). B cells that



**FIGURE 6.** MCs do not induce the expansion of mature peritoneal IL-10-competent B cells. **(A)** The frequency of IL-10<sup>+</sup> B cells was analyzed among the partially purified B cell peritoneal population of B6 and *Kit<sup>W-sh</sup>* mice (peritoneal B pre-coculture) and in the same B cells cultured with nonsensitized MCs (peritoneal B+MC). In all conditions, LPIM was added during the last 5 h of culture. Dot plots from one representative experiment show the percentages of IL-10<sup>+</sup> B cells within the indicated gates. Non-B and dead cells were excluded from the analysis using the CD19 versus Live/Dead dot plot (data not shown). Samples colored with the rat IgG2b isotype control Ab served as negative controls. Bar graphs indicate mean ( $\pm$  SEM) percentages of IL-10<sup>+</sup> cells among total B cells from  $n = 3$  experiments. **(B and C)** Frequency of IL-10-competent B cells among B-1a, B-1b, and B-2 peritoneal cells, before and after coculture with MCs. The cell-surface CD5 was used to distinguish B-1a from B-1b+B-2 cells (B), whereas CD23 allowed to discriminate between B-2 and B-1a+B-1b cells (C). Reported plots are representative of  $n = 3$  (B) and  $n = 2$  (C) experiments.

produce IL-10 are found at different anatomical sites where they exert their suppressive function after encountering an appropriate and context-specific signal. This response can be mimicked in vitro by the short-term stimulation with LPS, PMA, and ion-

omycin, which promotes *Il-10* expression only in poised B cells already competent for cytokine production, allowing their detection ex vivo (10). Over the past decade, a considerable effort has been made toward identification of signals that induce IL-10-



**FIGURE 7.** MCs regulate the IL-10-competent B cell population in the gut microenvironment. **(A)** IL-10-competent B cells were analyzed among the freshly isolated LP cell population (LP B pre-coculture) and among the same cell population cultured for 48 h with nonsensitized MCs (LP B+MC). In all conditions, LPIM was added for the last 5 h of culture. Dot plots for one representative experiment are shown. Only viable B cells were analyzed for IL-10 staining, and a perfectly matched isotype control was used to discriminate IL-10<sup>+</sup> from IL-10<sup>-</sup> B cells. Bar graphs indicate mean ( $\pm$ SEM) percentages of IL-10<sup>+</sup> cells among total B cells ( $n = 3$ ). \* $p < 0.05$ . **(B)** Immunofluorescence analysis of B cells positively stained for IL-10 on formalin-fixed, paraffin-embedded colon sections of WT (B6) and MC-deficient (*Kit<sup>W-sh</sup>*) mice and MC-deficient mice reconstituted with BMMCs from either B6 (BMMC WT i.p. *Kit<sup>W-sh</sup>*) or CD40L-deficient (BMMC *Cd40l<sup>-/-</sup>* i.p. *Kit<sup>W-sh</sup>*) mice. Scale bars, 100  $\mu$ m. Bar graphs indicate mean ( $\pm$  SEM) numbers of CD45R<sup>+</sup>IL-10<sup>+</sup> cells for one of two independent experiments. B cells positively stained for IL-10 were detected and counted out of five  $\times 400$  high-power microscopic fields in each case. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . **(C)** Immunofluorescence analysis of IL-10<sup>+</sup> B cells on formalin-fixed, paraffin-embedded colon sections of WT (B6) and MC-deficient (*Kit<sup>W-sh</sup>*) mice at steady-state (day 0), during acute DSS-induced intestinal inflammation (day 10), or 7 d after DSS withdrawal (day 17; recovery from inflammation). Scale bars, 50  $\mu$ m. Bar graphs indicate mean ( $\pm$  SEM) numbers of CD45R<sup>+</sup>IL-10<sup>+</sup> cells for one of two independent experiments. B cells stained for IL-10 were detected and counted out of four  $\times 400$  high-power microscopic fields in each case. \* $p < 0.05$ . (B and C) Slides were evaluated using a Leica DMI6000 microscope, and microphotographs were collected using a Leica DFC350FX digital camera. Original magnification  $\times 200$ . Red represents CD45R; green represents IL-10.

competent B cells in vivo (12). Nonetheless, little is known about how the cell types promote IL-10-competent B cell expansion.

In this article, we show that MCs contribute to the expansion of IL-10-competent splenic B cells in vitro. The proportion of CD19<sup>+</sup>IL-10<sup>+</sup> cells was significantly increased after culturing B cells with MCs, and proliferation assays demonstrated that MC-derived signals promote the preferential proliferation of IL-10-competent B cells with respect to other B cell subsets. However, a process of de novo differentiation of IL-10-competent B cells cannot be excluded. Although MCs induced the expansion of IL-10-competent B cells, they did not enhance the overall production of the cytokine. IL-10 mRNA and protein levels in B lymphocytes were not increased in the presence of MCs. This is not unexpected because the expansion of IL-10-competent B cells and the production of IL-10 are two distinct processes, differentially regulated. A superb example comes from direct CD40 activation of B cells: although CD40 signaling promotes an increase in the proportion of IL-10-competent B cells, it does not itself induce IL-10 secretion (32). Thus, consistent with our findings, the signals provided by MCs seem to be preparatory in nature, instructing the B cell to become competent for a suppressive function when encountering an appropriate and context-specific second signal.

We show that CD40L on MCs is important for IL-10-competent B cell expansion because the presence of anti-CD40L blocking Ab or of *Cd40l*<sup>-/-</sup> MCs led to a significant reduction of CD19<sup>+</sup>IL-10<sup>+</sup> cells in the cocultures. However, it is also clear that other membrane-bound or soluble factors are likely to contribute to this expansion. MCs release cytokines known to affect important physiological functions such as B cell growth, differentiation, and Ig class switching (33, 34). Moreover, MCs can release leukotriene B<sub>4</sub>, to which it has recently been attributed a role in the generation of tumor-evoked Bregs (35). These multiple factors constitute fertile ground for further investigation. The role of exosomes in cell-cell communication is noteworthy (36), and the finding that MCs produce exosomes suggests that this may be an important mechanism by which MCs communicate to other cells. Importantly, we show that MC-derived exosomes express CD40L and contribute to the expansion of IL-10-competent B cells.

Taking advantage of the *Kit*<sup>W-sh</sup> murine model of MC deficiency, we investigated the role of MCs on IL-10-competent B cell differentiation and maturation in vivo. If on one hand our results argue that MCs are involved in these processes (*Kit*<sup>W-sh</sup> mice had significant lower percentages of CD19<sup>+</sup>IL-10<sup>+</sup> cells in the colon, peritoneum, and lymph nodes compared with WT mice), then on the other hand they revealed the complexity of the in vivo regulation of IL-10-competent B cell differentiation, which appeared to be tissue specific. No differences were observed in the spleen, a site in which B-MC interactions are less likely and where other immune cell types may play specific inductive functions, compensating the role of MCs. The IL-10-competent B cell deficiency in the peritoneum of *Kit*<sup>W-sh</sup> mice is peculiar in that MC reconstitution in adult mice does not restore normal percentages of these cells. This difference is not due to the selective sensitivity of the B-1 versus the B-2 cell subsets to MCs because B cells that belong to both subsets appear insensitive to the inductive signals from MCs. Given the developmental features of this immunological site (the peritoneum is populated early during ontogenesis and the resident B cell population is mainly self-replenishing) (37), it is still possible that MCs influence the development of IL-10-competent B cells earlier in life and that the deficiency observed in adult *Kit*<sup>W-sh</sup> mice is the result of a defect during ontogenesis. Nonetheless, once B cells have developed and established themselves in the peritoneum, their interaction with MCs does not lead to the expansion of IL-10-competent B cells.

Thus, the cross talk between peritoneal B cells and MCs appears to produce different effects compared with the other organs analyzed. Again, this probably reflects the peculiar immunological features of this compartment and the specific functions of peritoneal MCs and B cells within.

Finally, we observed that the IL-10-producing B cell population is significantly reduced in the colon of *Kit*<sup>W-sh</sup> mice and that the population of IL-10-competent B cells resident in the LP responds to the inductive signals of MCs as CD19<sup>+</sup>IL-10<sup>+</sup> cells are expanded when cocultured in vitro with MCs. Moreover, MCs could play a role in expansion of IL-10-competent B cells resident in the LP and/or in the recruitment of circulating IL-10-competent B cells to this site because in *Kit*<sup>W-sh</sup> mice we did not observe an increase of these cells during the resolution phase of DSS-induced intestinal inflammation. The function of IL-10-producing B cells in this organ represents a key mechanism for the maintenance of gut homeostasis and for the control of the immune response against Ags (38). MC regulation of IL-10-competent B cells in the colon contributes to this homeostatic process, and the alteration of this mechanism could play a role in pathological conditions such as chronic inflammation and cancer.

In conclusion, for the first time, to our knowledge, we provide the evidence of a complex cross talk between MCs and B cells that is important for the expansion and differentiation of IL-10-competent B cells. We show that MCs are able to modulate the IL-10-competent B cell population according to the specific tissue microenvironment, and we provide evidence that in the absence of MCs the proportions of IL-10-competent B cells are significantly reduced in several immunological sites, both under homeostatic conditions and at resolution of inflammation.

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## Disclosures

The authors have no financial conflicts of interest.

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