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Bone Marrow Dendritic Cell-Mediated Regulation of TLR and B Cell Receptor Signaling in B Cells

Vishal J. Sindhava,*1 Halide Tuna,* Beth W. Gachuki,* David J. DiLillo,† Margarita G. Avdiushko,* Thandi M. Onami,‡ Thomas F. Tedder,‡ Donald A. Cohen,*§ and Subbarao Bondada*§

Dendritic cells (DCs) play an essential role in regulation of immune responses. In the periphery, Ag presentation by DCs is critical for adaptive responses; for this reason, DCs are often targets of adjuvants that enhance vaccine responses. Activated mature DCs enhance B cell activation and differentiation by providing cytokines like BAFF and a proliferation-inducing ligand. However, the role of immature DCs in B cell tolerance is not well studied. Recently, mouse immature bone marrow-derived DCs (iBMDCs) have been shown to suppress anti-IgM–induced B cell activation. In this study, we tested the ability of mouse DCs to modulate B cell functions during TLR activation. We found that iBMDCs potently suppressed proliferation and differentiation of various B cell subsets on TLR stimulation. However, iBMDCs did not affect CD40-mediated B cell activation. Optimal suppression of B cell activation by iBMDCs required cell contact via the CD22 receptor on B cells. The B cell suppression was a property of iBMDCs or DCs resident in the bone marrow (BM), but not mature BM-derived DCs or DCs resident in the spleen. Presence of iBMDCs also enhanced the Ag-induced apoptotic response of BM B cells, suggesting that the suppressive effects of iBMDCs may have a role in B cell tolerance. The Journal of Immunology, 2012, 189: 3355–3367.

Abbreviations used in this article: BM, bone marrow; BMDC, bone marrow–derived dendritic cell; BM-RDC, bone marrow resident dendritic cell; DC, dendritic cell; iBMDC, immature BMDC; KO, knockout; mBMDC, mature BMDC; MHC II, MHC class II; PI, propidium iodide; poly(I:C), polyinosinic-polycytidylic acid; Siglec, sialic acid binding Ig-like lectin; Spl-RDC, splenic resident DC; ST6Gal-I, sialyltransferase KO.

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Also induce surface IgA expression in CD40-activated B cells through TGF-β secretion. However, direct interaction of DCs with B cells in the presence of TGF-β and IL-10 is critical for Ig class-switching to IgA1 and IgA2 (8). DCs also secrete B cell–activating factors belonging to the TNF family such as BAFF/BLyS and a proliferation-inducing ligand, which have been shown to enhance B cell survival, proliferation, differentiation, and class-switching (9). Follicular DCs, a very specialized type of DCs, take part in the organization of primary B cell follicles and the germinal center reaction (1). Injection of DCs pulsed with various bacterial, viral, or protein Ags induces Ag-specific Ab production in different in vivo studies, suggesting a role for DC-mediated Ag presentation in B cell responses (10–12). In fact, a study by Qi et al. (13) showed that newly arriving naive B cells examine lymph node DCs for Ags before they enter into lymph node follicles. This study also demonstrated that interaction between Ag-specific B cells and Ag-carrying DCs leads to BCR signaling and extrafollicular activation of B cells. In other studies, DCs have been shown to interact with B cells in lymph nodes and spleen (14) via the integrin LFA-1 (CD11a/18) (15). Blood-derived DCs have been shown to capture particulate Ags and present them to marginal zone B cells in the spleen. This Ag presentation by DCs to marginal zone B cells leads to their differentiation into IgM-secreting plasmablasts (16). These observations altogether suggest a possible role for DCs in the direct activation of Ag-specific B cells during the in vivo immune responses.

TLRs are pathogen-recognition receptors that recognize pathogens via specific components conserved among microorganisms known as pathogen-specific molecular patterns (17). TLRs are differentially expressed among leukocytes and also within the different subsets of DCs. DCs are mainly divided into two major subsets, myeloid DC and plasmacytoid DC, both of which have unique phenotypes and functions (1, 18). Myeloid DCs express CD11b and are involved in Th1-type immune responses through IL-12 production, whereas plasmacytoid DCs express B220 and play an impor-
tant role during viral infection by producing type I IFN or IFN-α (19, 20). Many different TLR ligands such as peptidoglycan, LPS, polyinosinic-polycytidylic acid [poly(I: C)], and CpG induce DC maturation (21, 22). Stimulation of DCs with TLR ligands induces major morphological and functional changes, such as elevated surface expression of MHC II and costimulatory molecules (CD80, CD86) that are essential for DC-mediated activation of the adaptive immune responses (4). In addition to the positive effects of DCs in enhancing immune responses, DCs have been shown to have a role in negative selection of T cells in thymus, peripheral T cell tolerance, and induction of regulatory T cells (23–25). However, their role in inducing B cell tolerance has not been investigated extensively. Recently, it was shown that bone marrow-derived DCs (BMDCs) inhibit BCR-mediated proliferation of B cells (26). However, the role of DCs during the TLR-induced B cell immune responses has not been examined carefully. This is important considering the trend of increasing use of TLR ligands as vaccine adjuvants to activate DCs and B cells to enhance the vaccine-induced Ab response in numerous studies and clinical trials and in the context of several endogenous ligands that appear to act via TLRs (27, 28).

In view of the importance of DC–B cell cross talk during TLR-induced responses, we examined the role of DCs in B cell activation in response to various TLR ligands. Our results show that immature bone marrow-derived DCs (iBMDCs) and bone marrow resident DCs (BM-RDCs) profoundly inhibited TLR-induced, but not CD40-induced, B cell proliferation and differentiation. The inhibition of B cells by iBMDCs was dependent on CD22 expression on B cells. Maturation of iBMDCs with TLR ligands before their interaction with B cells abrogated their inhibitory effect on TLR-induced B cell proliferation. Unlike BM-RDCs, splenic resident DCs (Spl-RDCs) had no effect on TLR-mediated proliferation of B cells. Finally, we present the novel observation that iBMDCs and BM-RDCs induced apoptosis of BM B cells in an Ag- and BCR-dependent manner, suggesting that bone marrow-derived DCs (BMDCs) may influence B cell tolerance mechanisms by providing stronger negative signals when self-Ags are presented by DCs.

Materials and Methods

**Mice and reagents**

C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Hen egg white lysozyme (HEL)-specific BCR-expressing transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CD22 knockout (KO) mice were a generous gift from Dr. Thomas Tedder. ST6Gal-I sialyltransferase KO (ST6-Gal I KO) mice generated by Hennet et al. (29) were a generous gift from Dr. Thomas Tedder. ST6Gal-I sia-

**DC culture**

Bone marrow (BM) cells were collected from femur and tibia bones of C57BL/6 or ST6Gal-I KO mice. A single-cell suspension of BM cells was obtained by flushing the bones with RPMI 1640 medium (HyClone; Thermo Scientific) supplemented with 10% FBS (Atlanta Biologicals), β-glutamine, HEPES, and penicillin/streptomycin (Invitrogen). Cells were plated in 100 × 15-mm petri dishes (BD Falcon) at a concentration of 4 × 10^6 cells in 10 ml DC media (cell culture media supplemented with 20% cell supernatant of F10.9 cells that were transduced with murine GM-CSF gene or with rGM-CSF [20 ng/ml]). Cells were replenished with fresh DC media on days 3 and 6. On day 9, both adherent and nonadherent fractions of cells were harvested and washed free of DC media. For immature and mature DC preparations, day 9 cells were stimulated with media, 1 μg/ml LPS, or 50 μg/ml poly(I:C) for 24 h in IF-12 + 10% FBS media, respectively.

**Purification of B cells, T cells, and DCs**

Splenic B cells were purified as previously described (30) and by positive selection with CD19-magnetic beads and the autoMACS Pro Separator (Miltenyi Biotec, Auburn, CA). BM B cells were purified by positive selection with B220-magnetic beads and the autoMACS Pro Separator. T cells were purified by negative selection using magnetic beads from the CD4 + T cell isolation kit (Miltenyi Biotec), and DCs from BM and spleen were purified by positive selection with CD11c-magnetic beads and the autoMACS Pro Separator (Miltenyi Biotec).

**In vitro cell proliferation assay**

Splenic or BM B cells (1 × 10^6) were stimulated in the presence or absence of different numbers/subsets of DCs with 5 μg/ml LPS, 5 μg/ml Pam3CSK4, 50 μg/ml poly(I:C), PMA (30 ng/ml), ionomycin (100 ng/ml), or anti-CD40 (1C10; 20 μg/ml) ascites in 200 μl IF-12 media (1:1 mixture of Iscove’s DMEM and Ham’s F12 with 10% FCS). CD4 + T cells (1 × 10^5) were stimulated with 10 μg/ml anti-CD3 in RPMI media. Costar 24-well plates were used for the cell cultures. Cultures were pulsed with [3H]Thymidine on day 2 or day 3 and were harvested after 48 h (Packard, Meriden, CT); the incorporated radioactivity was then measured using a Matrix 96 β-counter (Packard, Downers Grove, IL). Results are represented as mean ± SD of cpm from triplicate cultures.

**CFSE labeling and cell cycle analysis**

Splenic B cells were resuspended at 10^6/ml in PBS/0.1% BSA, 10 μM CFSE was incubated at 37°C for 20 min and then washed with IF-12 medium + 10% FBS as described earlier (31). CFSE-labeled B cells (2.5 × 10^6) were cultured with LPS (5 μg/ml) in the presence or absence of DCs for 2 d at 37°C with 5% CO2 and analyzed by flow cytometry. Results are represented as mean ± SD of triplicate cultures. Splenic B cells (2.5 × 10^6) were cultured with 5 μg/ml LPS for 2 d in the presence or absence of iBMDCs. For cell cycle analysis, B cells were fixed in 70% (v/v) ethanol for at least 1 h at 4°C, after which the cells were incubated in a mixture of 1 μg/ml propidium iodide (PI, Sigma-Aldrich) and 25 μg/ml RNase A (Sigma-Aldrich) at 37°C for at least 30 min. The level of PI fluorescence was measured by flow cytometry in the B220- B cell population. Cell populations at subG1, G1, S, G2/M phase were calculated using the ModFit analytical program. Results are represented as mean ± SD of triplicate cultures.

**IgM ELISA assay**

Splenic B cells were cultured with 5 μg/ml LPS for 5 d in the presence or absence of iBMDCs. At the end of culture periods, plates were centrifuged, culture supernatants were collected, and total IgM levels in the supernatants were estimated using ELISA. OD was measured on an ELISA plate reader (Multiskan MCC/340, Thermo Scientific) at dual wavelengths of 405 and 630 nm. Results are represented as mean ± SD of triplicate cultures.

**Cell apoptosis analysis**

BM recirculating mature B cells and pre + immature B cells were sorted purified as live singlet CD19 + B220 + AA4.1 + and B220 + AA4.1 + CD43 cells, respectively. BM-RDCs were sort purified as live singlet CD11c + cells. All the sorted B cells were labeled with CFSE. Sorted CFSE-labeled Fc B cells, recirculating mature B cells, or pre + immature B cells (1 × 10^5) were stimulated with 5 μg/ml LPS in the presence or absence of BM-RDCs (5 × 10^4) for 48 h. After 48 h of culture, recirculating mature B cells were separated from cultured BM-RDCs as AA4.1 + CD19 + CFSE + cells. Immature B cells were separated as IgM + CD19 + CFSE + cells from cultured BM-RDCs and pre-B cells in FACs analysis. B cell death was analyzed by inclusion of live/dead marker-DAPI among earlier mentioned gated B cells.

BM B cells (1 × 10^5) were stimulated with 25 μg/ml anti-IgM F(ab)2; in the presence or absence of iBMDCs. Alternatively, BM B cells (1 × 10^5) were harvested and incubated with FACs-optimized 1 μg/ml LPS and CD19/220-allophycocyanin and Annexin V-FITC at room temperature for 15 min in the dark. Then 2 μl PI solution (0.5 μg/ml) was added and samples were analyzed by flow cytometry within 1 h.
**Results**

**iBMDCs inhibit TLR4-induced proliferation of splenic B cells**

iBMDCs have been recently shown to inhibit BCR-induced proliferation of B cells (26). We questioned whether this was unique to BCR-mediated growth responses and, therefore, tested the effect of iBMDCs on TLR-dependent B cell activation. We found that GM-CSF–cultured iBMDCs (85–95% CD11c+) strongly inhibited the LPS (TLR4)-induced proliferative response of splenic B cells in a dose-dependent manner (Fig. 1A). The inhibition of the TLR response was much stronger than that of the BCR response (data not shown), as 1 iBMDC per 64 B cells inhibited the LPS-induced proliferation significantly compared with 1 iBMDC per 16 B cells required for appreciable inhibition of BCR-induced proliferation, as reported by Santos et al. (26) (Fig. 1A). DCs and B cells have been shown to form tight clusters (6), and DCs can directly enhance growth, differentiation, and Ab production by CD40-mediated activation of B cells (6, 32). This predicted that iBMDCs should not inhibit the B cell responses to CD40 ligation. Accordingly, when splenic B cells were stimulated with a low dose of anti-CD40 that does not induce DC maturation (as shown by lack of CD86 and class II upregulation, data not shown), there was no inhibition of CD40-stimulated B cell proliferation (Fig. 1B).

PMA and ionomycin are known to induce T and B cell activation and proliferation, bypassing the early signaling steps of receptor activation and directly activating Ca\(^{2+}\) fluxes and protein kinase C activity. We stimulated splenic B cells with PMA and ionomycin in the presence of iBMDCs to determine whether the inhibition of B cell proliferation by iBMDCs is a pre- or post-protein kinase C activation event. There was no inhibition of B cell proliferation by iBMDCs over a wide range of DC/B cell ratios when B cells were stimulated with PMA and ionomycin, even as high as one iBMDC per four B cells (Fig. 1C). Immature DCs are deficient at processing and presenting Ags to T cells and at activation of naive T cells (2). We used CD4\(^+\) T cells as control cells to determine whether iBMDCs also inhibit T cell proliferation. CD4\(^+\) T cells were stimulated by anti-CD3 in the presence or absence of a graded number of iBMDCs. There was no inhibition of the anti-CD3–induced T cell proliferation (Fig. 1D), suggesting that inhibitory effects of iBMDCs are specific to TLR4 and BCR-induced B cell responses. Moreover, the lack of inhibition of PMA- and ionomycin-induced B cell proliferation suggests that iBMDCs affect receptor-induced (TLR, BCR) signaling pathways at the proximal receptor level. B cells undergo blast transformation before they enter into S phase of cell cycle. We tested whether this earlier step in B cell response to TLR4 stimulation was affected by iBMDCs. Indeed, TLR4–induced blast transformation response, as measured by light scatter, was suppressed by iBMDCs in a dose-dependent manner (Fig. 1E). In addition, we also measured changes in expression of MHC II, CD80, CD69, and CD86. Interestingly, expression of MHC II, but not CD80, CD69, and CD86, on B cells was suppressed by iBMDCs (data not shown).

Putative differences in the sensitivities of different splenic B cell subsets was tested by phenotyping CFSE-labeled B cells cultured with different ratios of iBMDCs in the presence of TLR4 ligand. Fig. 1F (top panel) shows CFSE dilution of each B cell subset when cultured with or without iBMDCs at a DC/B cell ratio of 1:4. The complete dose response of DC/B cell ratios for each B cell subset is shown in Fig. 1F (lower panel). Clearly, TLR4-induced proliferation responses of all B cell subsets (follicular, marginal zone, and transitional) examined were suppressed by the iBMDCs, although there are some subtle differences. In a separate experiment, these B cell subsets were first separated by FACS sorting and then were cultured with iBMDCs, but the results were similar in that TLR4 responses of all three B cells subsets (Fo, MZ, and Tr) were suppressed by iBMDCs (data not shown).

**Maturation of BMDCs overcomes the inhibition of TLR4-induced proliferation of splenic B cells**

Because mature DCs have been shown to help T and B cell responses (1, 2, 6, 10, 33), we questioned whether this inhibition of the B cell growth response by iBMDCs is abrogated upon maturation. iBMDCs were stimulated with LPS for 24 h in vitro for maturation. There was an upregulation of CD86 and MHC II expression on BMDCs after LPS stimulation compared with unstimulated cells (Fig. 2A), confirming that TLR signaling induced iBMDC maturation. Magnetic bead-purified, CFSE-labeled splenic B cells were stimulated with LPS along with either iBMDCs or mature BMDCs (mBMDCs; LPS matured, as shown in Fig. 2A) at different ratios, and B cell division was determined by CFSE dilution. There was a strong inhibition of cell division when B cells were cultured with iBMDCs (60% inhibition at DC/B cell ratio of 1:8; Fig. 2B, left panel). Interestingly, LPS-induced cell division of B cells was not significantly reduced by mBMDCs except at a high DC/B cell ratio of 1:8 (Fig. 2B, right panel). Similar results were found in a thymidine incorporation assay when we used TLR3 ligand [poly(I:C)]-induced mBMDCs. We confirmed that poly(I:C) significantly increased expression of CD86 and MHC II on iBMDCs compared with media treatment (data not shown). LPS-induced proliferation of splenic B cells was inhibited with iBMDCs, but not with poly(I:C) mBMDCs, over a wide range of DC/B cell ratios (Fig. 2C).

TLR4 is unique among TLRs, as it can activate both MyD88- and TRIF-dependent signaling, and is the only known TLR that uses all four TIR domain-containing adaptors (34). These unique characteristics of TLR4 led us to examine whether iBMDCs can inhibit proliferation of B cells mediated by other TLRs and whether maturation of iBMDCs can overcome the inhibitory effects. We found that iBMDCs inhibited the proliferation response of splenic B cells when stimulated with the TLR2 ligand, Pam3CSK4 (Fig. 2D), or the TLR3 ligand, poly(I:C) (Fig. 2E). Similar to LPS, maturation of iBMDCs with poly(I:C) overcame the inhibitory effects of iBMDCs on B cells stimulated with Pam3CSK4 (Fig. 2D) or poly(I:C) (Fig. 2E).

**iBMDCs inhibit B cell proliferation and differentiation by G1/S growth arrest**

Proliferation is usually arrested when B cells begin to differentiate into plasma cells or memory cells. DCs are known to enhance the differentiation of naive B cells into plasma cells upon CD40-mediated activation (6). Therefore, it is possible that iBMDCs help B cells become plasma cells during TLR-mediated activation of B cells, which results in decreased proliferation of B cells. To examine this possibility, we evaluated plasma cell formation upon CD40 stimulation of B cells when stimulated with the TLR2 ligand, Pam3CSK4 (Fig. 2D), or the TLR3 ligand, poly(I:C) (Fig. 2E). Similar to LPS, maturation of iBMDCs with poly(I:C) overcame the inhibitory effects of iBMDCs on B cells stimulated with Pam3CSK4 (Fig. 2D) or poly(I:C) (Fig. 2E).

**Statistical analysis**

Paired Student t test was used to determine statistical significance of differences between various groups.
FIGURE 1. iBMDCs specifically inhibit TLR4-induced B cell proliferation. (A) B cells were cultured with LPS, (B) anti-CD40, or (C) PMA and ionomycin for 48 h in the presence or absence of different ratios of iBMDCs, and proliferation was measured by [3H] thymidine incorporation. (D) CD4+ T cells were cultured with anti-CD3 for 48 h in the presence or absence of different ratios of iBMDCs, and proliferation was measured by [3H] thymidine incorporation. Data points represent mean ± SD values from triplicate cultures and are representative of three independent experiments. (A) *p < 0.005 when comparing responses of B cells in the presence and absence of iBMDCs. (E) Magnetic bead-purified CD19+ splenic B cells were labeled with CFSE and then cultured with LPS in the presence or absence of different ratios of iBMDCs for 72 h, and blast formation was measured by forward and side scatter measurements. (F) CFSE dilution was measured in different splenic B cell subsets, follicular B cells (Fo B cells, CD19+B220+IgM+AA4.1+CD23+CD21lo/hi), marginal zone B cells (MZ B cells, CD19+B220+IgM+AA4.1+CD23−CD21hi), and transitional B cells (Tr B cells, CD19+B220+IgM+AA4.1+) after 72 h of culture with LPS in the presence or absence of different ratios of iBMDCs. FACS histograms in the top row show CFSE fluorescence.
FIGURE 2. TLR-induced maturation of iBMDCs overcomes their inhibitory effect on B cell proliferation. (A) GM-CSF cultured BMDCs were stained for CD11c (all panels), CD86 (middle panel), and MHC II (right panel) after 10 d of culture, with or without LPS treatment for last 24 h. Numbers represent mean fluorescence intensity (MFI) of staining. (B) CFSE-labeled B cells were cultured with LPS for 48 h in the presence or absence of different ratios of iBMDCs or mBMDCs and were analyzed for CFSE dilution after gating for B220⁺ cells. The percentage of cells in left area gate represent CFSE dilution from the second and subsequent divisions of cells based on CFSE fluorescence. Data (mean ± SD) are representative of triplicate cultures. (B) *p < 0.05 shows statistical significance of the differences in CFSE dilution in LPS-treated B cells ± iBMDCs or mBMDCs. B cells were cultured with (C) LPS, (D) poly(I:C), or (E) Pam3CSK4 in the presence or absence of iBMDCs or poly(I:C) mBMDCs, and proliferation was measured by [³H]thymidine incorporation after 48 h. Data are representative of three independent experiments. *p < 0.05 signifies the differences between B cell proliferation responses with iBMDCs and mBMDCs.
cultures inhibited plasma cell formation up to 76% (Fig. 3A). There was also a significant decrease in total IgM production after day 5 by LPS-stimulated B cells on addition of iBMDCs (Fig. 3B). These results suggest that iBMDCs do not help in B cell differentiation but inhibit B cell activation.

To evaluate the inhibitory effect of iBMDCs on B cell activation, we examined the effect of iBMDCs on cell-cycle progression. Splenic B cells were cultured with LPS in the presence or absence of different doses of iBMDCs for 48 h, stained with PI, and analyzed by flow cytometry. Representative results are shown in Fig. 3C, and a summary of the analysis is shown in Fig. 3D. The presence of iBMDCs induced predominantly G1 arrest in B cells (cells in G1 phase increased from 41 to 75%; Fig. 3C, 3D). Accordingly, there was a significant decrease in the percentage of B cells in S phase of the cell cycle (from 41 to 13%; Fig. 3C, 3D). In the absence of iBMDCs, the G1/S ratio of LPS-stimulated B cells was 1, and it increased to 5.8 in the presence of iBMDCs when cultured at a DC/B cell ratio of 1:4. These results suggest that iBMDCs inhibit progression of LPS-activated B cells from G1 to S phase. To determine whether G1/S arrest leads to apoptosis, we isolated B220+ cells by magnetic beads and quantified apoptotic cells (Annexin and PI staining) upon TLR stimulation in the presence of iBMDCs. Fig. 3E shows that iBMDCs induce apoptosis in these highly purified splenic B cells activated via TLR4.

DCs have been shown to enhance growth and differentiation of CD40-activated B cells (6). Furthermore, it has been shown that DCs can activate T cells that subsequently induce activation and differentiation of B cells through CD40 signaling (1). In this study, iBMDCs did not inhibit CD40-stimulated B cell proliferation (Fig. 1B), but rather increased CD40-stimulated B cell proliferation when cultured with mBMDCs (data not shown), which parallels results reported by Dubois et al. (6). Therefore, we evaluated whether CD40 costimulation, a surrogate for T-dependent B cell activation, can overcome the iBMDC-mediated inhibition of TLR-induced B cell activation. B cells were cultured with LPS or LPS+anti-CD40 in the presence of iBMDCs. There was an 80% inhibition of B cell proliferation when stimulated with LPS alone in the presence of iBMDCs (1:8 ratio), which was abrogated when anti-CD40 was included in these cultures (Fig. 3F). This result suggests that cognate signaling from T cells can overcome the inhibitory effect of iBMDCs on B cell activation.

**BM-RDCs inhibit the TLR4 responses of BM and splenic B cells, but help CD40 responses**

BM is a primary site for B cell development and also provides a niche for mature B cells and plasma cells (36, 37). Sapoznikov and colleagues (38) have shown that BM-RDCs play an important role in the survival of mature B cells in BM by producing macrophage migration inhibitory factor. Because the effects of BM-RDCs on BCR- or TLR-induced B cell responses have not been well studied, we examined whether BM-RDCs also have an inhibitory effect on BM B cells. We used CD11c and B220 magnetic beads to purify BM-RDCs and BM B cells, respectively. BM B cells were cultured with LPS in the presence or absence of BM-RDCs. Interestingly, BM-RDCs behaved like the iBMDCs in inhibiting the LPS-mediated BM B cell proliferation response in a dose-dependent manner (Fig. 4A, left panel). Similar to cultured iBMDCs, BM-RDCs also inhibited splenic B cell response to TLR4 stimulation (Fig. 4A, right panel). However, BM-RDCs helped the proliferative response of B cells from either BM or spleen when stimulated via CD40 (Fig. 4B), suggesting that the inhibitory effects of BM-RDCs are TLR4 specific.

DCs in humans and mice are mainly divided into two subsets: plasmacytoid DCs and myeloid DCs. Myeloid DCs are potent APCs and help in initiation of the adaptive immune response by activating T cells, whereas plasmacytoid DCs have a modest capacity to activate T cells (39), but plasmacytoid DCs produce very high amounts of type I IFN on viral stimulation (40, 41). Both plasmacytoid and myeloid DCs are present in mouse BM. To identify the inhibitory subset of iBMDCs, we first characterized the DCs in GM-CSF–treated BM cell cultures and found that >70% of the DCs in the culture were myeloid DCs (CD11c+CD11b+, data not shown). We purified the CD11c+CD11b+ myeloid DCs from these cultures by flow cytometric sorting and tested their ability to inhibit B cell proliferation. Highly purified myeloid DCs inhibited the LPS-mediated, but not anti-CD40–mediated, splenic B cell proliferation (Fig. 4C, 4D), suggesting that myeloid DCs play a major role in regulation of TLR4-induced B cell responses.

**Spl-RDCs do not inhibit TLR4-induced proliferation of splenic B cells**

Spl-RDC function has been shown to be regulated by microbial products such as LPS, which induce maturation of Spl-RDCs and migration to T cell areas where they regulate T cell function (42). Spl-RDCs also interact with B cells in the red pulp (14) and T cell–B cell borders (16) in the spleen. This interaction supports plasmablast formation, survival, and their differentiation into plasma cells (14, 16). One report suggests that Spl-RDCs can also tolerize B cells and inhibit activation of B cells with low doses of Ag (43). To understand whether Spl-RDC–B cell interaction can also inhibit B cell responses, as did iBMDCs, we evaluated sort-purified Spl-RDCs (>95% CD11c+). Upon maturation with LPS, Spl-RDCs showed upregulation of CD86 and MHC II expression when compared with just media-treated cells (Fig. 5A). Unlike iBMDCs or BM-RDCs, Spl-RDCs did not inhibit the splenic B cell response to LPS (Fig. 5B). LPS matured Spl-RDCs behaved like media-treated Spl-RDCs and had neither a stimulatory nor an inhibitory effect on splenic B cell proliferation (Fig. 5B). Similarly, the splenic B cell response to anti-CD40 and receptor-independent (PMA + ionomycin) stimulation remained unchanged when cultured with Spl-RDCs (Fig. 5C). Moreover, Spl-RDCs neither inhibited nor enhanced CD4+ T cell proliferation when cultured at different DC/T cell ratios (Fig. 5D).

**iBMDC-mediated inhibition of TLR-induced splenic B cell proliferation requires CD22 expression on B cells, but not α2-6–linked sialic acid on iBMDCs**

Immature DCs have been shown to induce tolerance in T cells (25, 43) and to inhibit BCR-mediated proliferation of B cells (26) through contact-dependent events. When B cells were separated from iBMDCs by a membrane barrier in a transwell culture, there was a significant decrease in the inhibition of LPS-induced B cell proliferation (Fig. 6A). Thus, when iBMDC and B cells were cocultured at a DC/B cell ratio of 1:32, the LPS response of B cells was reduced by 60%, and this inhibition was almost completely abrogated when B cells were separated from iBMDCs by a membrane. However, at the high DC/B cell ratio (1:10), there was still appreciable inhibition of B cell proliferation even when iBMDCs and B cells were not in contact, suggesting a role for a soluble mediator (Fig. 6A). Because of our recent data about the potent inhibitory effects of IL-10 on TLR-induced B cell responses (31), we tested whether Abs to the IL-10R could overcome iBMDC-mediated inhibitory effects. However, inhibition of IL-10 signaling did not overcome the iBMDC-induced growth inhibition (data not shown). Thus, at low DC/B cell ratios, iBMDC-mediated inhibition of B cells required cell-to-cell contact between iBMDCs and B cells, possibly through inhibitory surface receptor(s).

A number of inhibitory receptors have been described on B cells (44), and among these, CD22 has been shown to inhibit the BCR-
FIGURE 3. iBMDCs inhibit B cell differentiation by blocking their cell-cycle progression at G1 to S phase, which can be overcome by CD40 co-stimulation. (A) B cells were cultured with media alone or LPS for 3 d in the presence or absence of iBMDCs. Cells were stained for B220 and CD138 at the end of 3 d of culture. Numbers in selected regional gate represent plasma cells (B220<sub>low</sub>CD138<sup>+</sup>). Data are shown as mean ± SD of triplicate cultures and are representative of two independent experiments. (B) B cells were cultured with LPS for 5 d in the presence or absence of different ratios of iBMDCs. At the end of 5 d, culture supernatants were collected and assayed by ELISA for total IgM. Results (mean ± SD) are representative of two experiments. *p < 0.005 when comparing total IgM production by B cells in the presence and absence of iBMDCs. (C) B cells were cultured with LPS in the presence or absence of different ratios of iBMDCs for 48 h, and cell-cycle analysis was performed by PI staining. The fraction of cells in G1, S, or G2/M was determined from triplicate cultures. In these panels, solid histograms on the left represent cells in G1 phase, whereas those on the right represent cells in G2/M phase of cell cycle. Striped histograms in the middle represent cells in S phase of the cell cycle. (D) The mean values of B cells in G1, S, and G2/M phases of cell cycle are presented. *p < 0.005 when comparing percentage of B cells in G1 (*) or S phase (#) in the presence of iBMDCs with percentage of B cells in G1 or S phase in the absence of iBMDCs, respectively. (E) Magnetic bead-purified total B220<sup>+</sup> B cells were cultured with LPS in the presence or absence of different ratios of iBMDCs for 48 h, and cell death was measured by PI and Annexin V staining. Graph represents percentage of PI and Annexin V<sup>+</sup> cells. (F) B cells were cultured with LPS or LPS + anti-CD40 for 48 h in the presence or absence of different ratios of iBMDCs. Cell proliferation was measured by [3H]thymidine incorporation. Results are shown as percentage of mean ± SD responses of triplicate cultures (LPS 100% = 101,500 cpm and LPS + anti-CD40 100% = 209,667 cpm). Data are representative of two independent experiments. *p < 0.005 when comparing responses of B cells in the presence and absence of iBMDCs.
mediated B cell response when engaged by iBMDCs (26). CD22, a member of the Siglec (sialic acid binding Ig-like lectin) family, is known to recruit the Src homology region 2 domain-containing phosphatase-1 upon activation and to negatively regulate BCR-mediated signaling in B cells (45, 46). CD22 can participate in cell–cell interactions by binding to its ligand, α2-6–linked sialic acid (47). Several cell types, including DCs, express ST6Gal I, which is required for the production of α2-6–linked sialic acid. We examined whether CD22 signaling plays an inhibitory role in iBMDC-mediated regulation of B cells upon TLR signaling.

B cells from wild type and CD22 KO mice were stimulated with different TLR ligands in the presence or absence of iBMDCs. Unlike wild type mice, CD22 KO B cell proliferation was not inhibited by iBMDCs when stimulated with Pam3CSK4 (Fig. 6B) or LPS (Fig. 6D) at different DC/B cell ratios. These results demonstrate that iBMDCs inhibit various TLR-induced B cell proliferation responses via CD22. We also examined whether, in the absence of CD22-mediated negative regulatory signaling, mBMDCs aid in the B cell proliferation response. We did not see any significant increase in proliferation of CD22 KO B cells or

FIGURE 4. BM-RDCs and purified myeloid DCs inhibit TLR- but not CD40-mediated B cell proliferation response. (A) B220 bead-purified BM or splenic B cells were cultured with (A) LPS or (B) anti-CD40 for 48 h in the presence or absence of CD11c bead-purified BM-RDCs. Splenic B cells were cultured with (C) LPS or (D) anti-CD40 for 48 h in the presence or absence of CD11c/CD11b− sort-purified myeloid DCs from cultured BMDCs. (A–D) Cell proliferation was measured by [3H]thymidine incorporation. Results (shown as mean ± SD responses of triplicate cultures) are representative of two experiments. *p < 0.005 when comparing responses of B cells with DCs to B cells in the absence of BM-RDCs (A, B) or myeloid DCs (C).
wild type B cells when cocultured with mBMDCs and stimulated with Pam3CSK4 (Fig. 6C) or LPS (Fig. 6E). Because the major CD22 ligands are generated by the sialyltransferase ST6Gal I (29), we tested whether iBMDCs from ST6-Gal I KO mice are deficient in their ability to regulate B cell responses to TLR4. Surprisingly, but in agreement with the previous studies on BCR responses (26), iBMDCs from ST6-Gal I KO mice were as efficient as wild type iBMDC in suppressing TLR4 responses (Fig. 6F).

**BM-RDCs and iBMDCs induce apoptosis of B cells**

The role of DCs during T cell negative selection is well studied (24, 25). However, it is not known what role DCs play during B cell negative selection in BM. We asked whether interactions with DCs can lead to apoptosis of BM B cells during the TLR- or BCR-mediated responses. We observed increased death of BM immature B cells in the presence of BM-RDCs upon TLR4 stimulation. Similarly, BM-RDCs also induce death of BM recirculating mature B cells (Fig. 7A). We also examined the survival of highly purified BM B cells upon BCR stimulation in the presence or absence of iBMDCs. Immature B cells from BM have been shown to undergo apoptosis in vitro when cultured with anti-IgM Abs (48, 49). As expected, we found a modest increase in apoptosis of BM B cells in the presence of low-dose anti-IgM compared with untreated BM B cells (Fig. 7B). Notably, addition of iBMDCs induced a dose-dependent increase in BM B cell apoptosis during BCR stimulation. iBMDCs alone even at the highest DC/B cell ratio did not increase BM B cell apoptosis (Fig. 7B). These results suggest that iBMDCs increase apoptosis of BM B cells when they...
are triggered via BCR signaling. To examine the Ag-specific ap-
optosis of BM B cells by iBMDCs, we used transgenic mice that
have high frequencies of B cells expressing a high-affinity BCR
against HEL. Goodnow and colleagues (50) demonstrated elegantly
that soluble Ag induces BM B cell anergy in HEL transgenic mice,
whereas membrane-bound self-Ag has been shown to induce clonal
deletion (51). Therefore, we determined whether the presentation of
HEL to transgenic B cells via iBMDCs can induce apoptosis of
these Ag-specific B cells. Purified BM B cells from HEL transgenic
mice were cultured with HEL pulsed iBMDCs. iBMDCs alone
reduced spontaneous apoptosis of BM B cells but strongly en-
hanced the apoptosis of BM B cells when they were pulsed with
HEL (Fig. 7C). Pulsing of iBMDCs with HEL alone did not alter
expression of MHC II, CD80, and CD86 on DCs (data not shown).
These results suggest that DCs may play a crucial role during the
B cell negative selection in the BM.

FIGURE 6. iBMDCs inhibit TLR-in-
duced B cell response through CD22 en-
gagement on B cells. (A) Splenic B cells
were cultured with LPS in the presence or
absence of different ratios of iBMDCs in
direct contact or transwell culture. Data are
presented as percentage of [3H]thymidine
incorporation in LPS-treated B cell cultures
only (contact 100% = 91,155 cpm and
transwell 100% = 104,000 cpm). (B–E)
Splenic B cells from C57BL/6 (WT) or
CD22 KO mice were cultured with (B, C)
Pam3CSK4 or (D, E) LPS in the presence or
absence of different ratios of iBMDCs (B–
D) or mBMDCs (E), and proliferation was
measured by [3H]thymidine incorporation
after 48 h. Data are representative of two
independent experiments. *p < 0.05 sig-
nifies the differences between prolifera-
tion responses with WT and CD22 KO B
cells. Data are presented as percentage of
[3H]thymidine incorporation in either (B, C)
Pam3CSK4-treated B cell cultures only
(WT 100% = 85,458 cpm and CD22 KO
100% = 190,666 cpm) or (D, E) LPS-treated
B cell cultures only (WT 100% = 130,666
cpm and CD22 KO 100% = 96,490 cpm).
(F) WT splenic B cells were cultured with
LPS in the presence or absence of different
ratios of iBMDCs derived from either WT
or ST6Gal-I KO mice for 48 h, and prolif-
eration was measured by [3H]thymidine
incorporation.
Discussion

In recent years, natural or synthetic TLR ligands have been used to boost DC-driven, Ag-specific immune responses in mice and humans (27, 28). A better understanding of the role of DCs during TLR-mediated B cell activation would provide important information about the use of TLR ligands as adjuvants during DC-based vaccine development. In this regard, we performed comprehensive in vitro studies, including proliferation, cell-cycle progression, differentiation, Ab production, and apoptosis of B cells in the presence or absence of BM-RDCs. Among various mitogens used in our study, iBMDCs specifically inhibited TLR2, TLR3, and TLR4, as well as BCR-induced proliferation, but did not inhibit anti-CD40 or PMA-ionomycin-induced B cell proliferation. These results suggest that iBMDC-mediated inhibition of B cells is signaling pathway specific and does not have global growth inhibitory effects. The cell-cycle analysis data revealed that iBMDCs block B cell proliferation by inducing G1-S growth arrest. Furthermore, our study showed that the cell-cycle arrest of B cells in the presence of iBMDCs is not due to their differentiation, because plasma cell formation and Ab production were also decreased in the presence of iBMDCs. Immature DCs have been shown to inhibit and tolerize T cells in vivo because of the absence of a second (costimulatory) signal, whereas maturation of DCs by TLR or CD40 overcomes this inhibitory effect on T cells (25, 52, 53). Similar to the DC-mediated regulation of T cells, maturation of iBMDCs with TLR ligands overcame the inhibitory effect of DCs on B cells. Many different TLR ligands can mature DCs in either a MyD88-dependent or -independent manner (21). Even though different TLR ligands are able to induce DC maturation, noticeable differences were observed in terms of cytokine and chemokine secretion patterns depending on the TLR ligand (54). We found that the maturation of iBMDCs via the MyD88-dependent pathway (LPS, peptidoglycan; data not shown) or the MyD88-independent pathway [poly(I:C)] had similar effects in overcoming the inhibitory effect of iBMDCs on B cells. Costimulating B cells with anti-CD40 also overcame inhibitory effects of iBMDCs on B cells.
The iBMDC-mediated inhibition of B cells was significantly dependent on cell contact. This is in agreement with Santos et al. (26), who showed that iBMDCs inhibited BCR-mediated B cell responses in a contact-dependent manner through CD22-mediated signaling on B cells. We observed that CD22 expression is also critical for the iBMDC-mediated inhibition of TLR-induced B cell proliferation. The absence of CD22 on B cell surfaces completely abrogated iBMDC-mediated inhibitory effects during TLR3- or TLR4-induced B cell proliferation. Interestingly, maturation of BMDCs has been reported to decrease the expression of sialic acid ligands (55), which is consistent with the role of CD22 in DC–B cell interaction. In parallel with the findings of Santos et al. (26), we found that expression of ST6Gal I, one of the enzymes that induces sialylation, was not required for DC–B cell interaction. Currently, we cannot rule out the possibility that CD22 on B cells binds some other ligand on iBMDCs and inhibits their response to BCR- and TLR-mediated signaling. Despite the importance of CD22, current studies also cannot rule out a role for other sialic acid binding proteins such as Siglec G, which have been shown to be important for B cell responses and for Ag-induced tolerance in B cells (56). Moreover, the inability of mature DCs to inhibit B cells could also be caused by generation of rescue signals such as the gene expression pattern induced by CD40 ligation.

Our study with resident DCs from spleen and BM showed that inhibitory effect of DCs on B cells is a characteristic of BM-RDCs, but not Spl-RDCs. To our knowledge, such a difference between BM-RDCs and Spl-RDCs in affecting B cell activation has not been reported previously. Spl-RDCs without stimulation were immature, characterized by low CD86 and class II expression on the surface, compared with LPS matured Spl-RDCs. However, Spl-RDCs did not inhibit TLR-induced B cell responses. In contrast, DCs isolated from the BM showed strong inhibition of TLR-induced proliferation of B cells from both BM and spleen. BM-RDCs resembled iBMDCs in enhancing CD40-mediated proliferation of B cells from both BM and spleen. This difference between BM-RDCs and Spl-RDCs is interesting in the context of the requirement for CD22, because there are no data about differences in the ubiquitously expressed ligands containing sialic acids on these two DC populations. Although iBMDCs normally are a heterogeneous population of DCs, flow cytometric sorting established that myeloid DCs in this population have potent inhibitory effects on B cells. It is of interest that Kilmon et al. (57) found that myeloid DCs and macrophages, but not plasmacytoid DCs, inhibited autoantibody production by self-reactive B cells, but not Ab production by normal B cells.

Finally, the inhibitory effects of iBMDCs in BM B cell growth responses led us to examine the role of iBMDCs in Ag-dependent selection of B cells. Negative selection of self-reactive B cells may occur via receptor editing, anergy, or deletion. B cell tolerance involves anergy when soluble Ag is present but uses clonal deletion mechanisms when self-Ags are membrane bound. This was well established by the studies of Goodnow et al. (58) using soluble and membrane-bound HEL, and by Nemazee and Bürki (51) using membrane-bound MHC I molecules. Therefore, DCs may provide an opportunity to present self-Ags in a membrane-bound form leading to deletion of self-reactive B cells. We found that HEL-specific BM B cells showed increased apoptosis when HEL was presented by iBMDCs. Currently, we do not know the underlying mechanism. In the literature there is evidence for presentation of native Ag by DCs (59). Hence one scenario for increased apoptosis with HEL pulsed BM B cells could be simultaneous binding of Ag to BCR and CD22 ligands on DCs to CD22 on B cells, although other explanations cannot be ruled out at this time. In thymus, DCs present self-Ag in the context of their MHC and play a decisive role in the negative selection of T cells (60). To our knowledge, our study is the first to suggest that DCs in the BM might play a similar role in B cell negative selection by presenting self-Ags to B cells in the context of CD22/Siglec-mediated inhibitory signals. In this context, recently it has been shown that decoration of T-independent Ags with sialic acid epitopes made them tolerogenic through their ability to crosslink Siglec family proteins (56). It was proposed that self-Ags that behave like T-independent Ags may use this pathway for self-tolerance. In support of such an idea, mice doubly deficient for CD22 and Siglec G developed autoantibodies and a moderate form of immune complex-mediated glomerular nephritis (61). Although the significance of inhibition of TLR responses of BM B cells is currently unclear, it must be noted that several endogenous TLR ligands have been identified and have been implicated in the breakdown of self-tolerance in several autoimmune models (62–65). Some of the endogenous TLR ligands such as high mobility group box 1 and heat shock proteins have their origin in cell death (66), which is known to occur extensively during B cell development. Defects in clearance of dead cells have a critical role in development of autoimmune diseases such as lupus (67). In the periphery, DCs in mucosal areas and epidermal Langerhans cells (sites of extensive cell turnover) have anti-inflammatory properties. Further in vivo studies are warranted to address the potential role of DCs in the negative selection of B cells in the BM.

Overall, our study gives valuable insight on the role that immature DCs play in B cell function. Because there is increasing use of BM-derived DC vaccines and TLR ligands as adjuvants, a careful selection of DC subset and maturation state is warranted to generate B cell-specific immune responses.

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