TLR4 Promotes B Cell Maturation: Independence and Cooperation with B Lymphocyte-Activating Factor

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_J Immunol_ published online 31 March 2010
http://www.jimmunol.org/content/early/2010/03/31/jimmunol.0903253
TLR4 Promotes B Cell Maturation: Independence and Cooperation with B Lymphocyte-Activating Factor

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We have previously shown that TLR4 triggering promotes the generation of CD23⁺CD93⁺ transitional T2-like cells in vitro from mouse B cell precursors, suggesting a possible role for this receptor in B cell maturation. In this study, we perform an extensive study of cell surface markers and functional properties of B cells matured in vitro with LPS, comparatively with the well-known B cell maturation factor B lymphocyte-activating factor (BAFF). LPS increased generation of CD23⁺ transitional B cells in a TLR4-dependent way, upregulating IgD and CD21 and downregulating CD93, without inducing cell proliferation, in a manner essentially equivalent to BAFF. For both BAFF and LPS, functional maturation of the IgM⁺CD23⁺CD93⁺ cells was confirmed by their higher proliferative response to anti-CD40 plus IL-4 compared with IgM⁺CD23⁻CD93⁻ cells. BAFF-R-Fc–mediated neutralization experiments showed that TLR4-induced B cell maturation was independent of BAFF. Distinct from BAFF, maturation by LPS relied on the activation of canonical NF-κB pathway, and the two factors together had complementary effects, leading to higher numbers of IgM⁺CD23⁺CD93⁺ cells with their simultaneous addition. Importantly, BCR cross-linking abrogated the generation of CD23⁺ B cells by LPS or BAFF, indicating that signals mimicking central tolerance act on both systems. Addition of cyclosporin A reverted BCR-mediated inhibition, both for BAFF and LPS, suggesting similar regulation of signaling pathways by calcineurin. Finally, LPS-injected mice showed a rapid increase of mature B cells in the bone marrow, suggesting that TLR4 signaling may effectively stimulate B cell maturation in vivo, acting as an accessory stimulus in B cell development, complementary to the BAFF physiological pathway.

The Journal of Immunology, 2010, 184: 000–000.
and whether signals from TLRs could influence B cell developmental events other than cell activation. It has recently been suggested that immature antichromatin autoreactive B cells could also be activated by CpG-DNA (21). However, total IgM secretion and proliferation in immature B subset were still much lower than in mature subset, even though TLR9 is highly expressed in early immature stage. Likewise, immature B cells poorly proliferate upon LPS stimulation (4, 5), although they express considerable levels of TLR4 (22). In contrast, it has been shown that TLR4 and TLR2 agonists rapidly reach BM in a context of infection, inhibiting lymphoid precursor proliferation (23).

In addition, it has been shown that endogenous molecules with broad distribution in the organism, such as hialuronate and heat-shock proteins, are recognized by TLR4 (reviewed in Ref. 24). In such a context, it is important to investigate the role of TLRs in B cell physiology beyond their mitogenic property, particularly addressing their putative role in B cell maturation and survival. Our previous findings that TLR4 engagement is able to promote the generation of CD23+ transitional B cells with a “T2-like” phenotype from B cell precursor in vitro (25) suggest an alternative mode of action of TLRs on immature B cells, distinct from classical B cell activation and more compatible with the progression in maturation of the developing B lymphocyte.

In this study, we have done a thorough characterization of in vitro B cell maturation triggered by TLR4 signaling, comparatively with the well-described BAFF-dependent B cell maturation process. We found that differentiation promoted by LPS is essentially equivalent to that induced by BAFF in every phenotypic aspects studied in this paper, including changes in molecular developmental markers and functional properties. TLR4-driven B cell maturation was found to be independent of BAFF, relying on the activation of classical NF-κB pathway, whereas BAFF activity did not require that pathway. Both factors together had complementary effects stimulating the generation of higher numbers of CD23+ B cells. Importantly, high-avidity cross-linking of BCR totally inhibited CD23+ B cell generation in the presence of LPS or BAFF, and cyclosporin A (CSA) was able to revert that inhibition, suggesting that both pathways are similarly regulated by BCR-mediated signals controlling central tolerance. Finally, we found evidences in vivo that TLR4 signaling can effectively stimulate generation of mature B cells in BM. These results show consistent evidence that TLR4 can provide alternative or complementary signals to BAFF-R along B cell maturation, respecting developmental control and regulation by BCR-derived signals, raising the question of the physiological role played by this receptor in B cell development.

Materials and Methods

Mice and cells

Adult C57BL/6 and C57BL/10ScCr mice were obtained from animal facilities of Federal University of Rio de Janeiro and maintained under standard pathogen-free conditions. BM cells were flushed from femurs with ice-cooled complete medium (OptiMEM supplemented with 10% FBS, 5 × 10−5 M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin) (Life Technologies, Grand Island, NY). Spleen cell suspensions were obtained by gently teasing spleens onto a cell strainer. After centrifuging cell suspensions at 280 g for 7 min and discarding supernatant, cells were resuspended in ammonium chloride potassium lysis buffer (0.155 M NH4Cl, 10 mM KHCO3, and 0.1 mM sodium EDTA) to deplete erythrocytes, centrifuged for 7 min, and resuspended in complete medium. Cells were counted using a hemacytometer with exclusion of dead cells with trypan blue dye, and processed for flow cytometric analysis or cell sorting. Experimental procedures were approved by the Committee on Ethics for Animal Experimentation of the Federal University of Rio de Janeiro.

MACS

VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) system was used for magnetic cell sorting. For depletion of IgM+ B lymphocytes, BM cells were incubated in MACS buffer (PBS containing 2 mM EDTA, and 5% FBS) with anti-mouse IgM MicroBeads (Miltenyi Biotec) for 20 min on ice, washed, and resuspended in MACS buffer, according to the manufacturer’s specifications. Cells were applied into an LD depletion column (Miltenyi Biotec, Bergisch Gladbach, Germany) and the effluent cells were collected as the BM IgM+ fraction and washed in complete OptiMEM medium. IgM+ fraction was incubated for 20 min on ice with anti-B220 or anti-CD19 MicroBeads (Miltenyi Biotec) and processed as described above. Cells were applied into MS-positive sort column, and after washing, retained cells were collected. Viable cells were scored by trypan blue dye using a hemacytometer, and the purity of cell preparations was verified by flow cytometry. Usually, IgM+ cells corresponded to <1% of B lineage cells after depletion, and B220+ cells corresponded to >95% of recovered cells after positive selection.

Cell staining, flow cytometry, and cell sorting

Fresh BM cells, MACS-purified cells, and cultures were analyzed by flow cytometry. The Abs used for staining were as follows: PE anti-CD23 (clone BB4), PE anti-B220 (clone RA3-6B2), FITC rat anti-mouse IgM (clone R6-60.2), and FITC anti-CD23 (clone 7G6) (BD Pharmingen, San Diego, CA); Alexa 647 anti-mouse IgM (clone b-7-6) and FITC anti-B220 (provided by Dr. J. Cambier, University of Colorado Health Science Center and National Jewish Health Center, Denver, CO); allophycocyanin anti-CD23 (clone BB4B) (Caltag Laboratories, San Francisco, CA); PE-Cy7 anti-CD93 (clone AA4.1), and Alexa 647 anti–BAFF-R; PE rat IgG2a isotype control (clone 22C10); and PE anti-IgM (clone J555-58.1) and PE-Cy7 anti-CD95 (clone R6-60.2), and FITC anti-CD21 (clone 7G6) (Southern Biotechnology Associates, Birmingham, AL); FITC and DyLight 649 goat F(ab’)2 fragments anti-mouse IgM (The Jackson Laboratory, Bar Harbor, ME) and biotin anti-CD93 (493 hybridoma provided by Dr. A. Rolink, Basel University, Basel, Switzerland; the mAb was purified and biotin-conjugated according to standard protocols); and Alexa 488 Annexin V (Molecular Probes, Eugene, OR). Bio- tinylated Abs were revealed with Alexa-Fluor 680-R-PE streptavidin (Molecular Probes) or with APC streptavidin (Caltag Laboratories). Cells were incubated with Abs in FACS buffer (PBS, 5% FBS, and 0.05% sodium azide) for 20 min at 4°C and washed with FACS buffer. When biotinylated mAbs were used, another step of incubation with Alexa 680-PE streptavidin or APC streptavidin was performed under the same conditions as described above except for the use of color-stained cell samples, propidium iodide (PI) was added at 0.5 μg/ml to the samples immediately before data acquisition for dead cell exclusion. Annexin V staining was performed using a calcium-containing buffer as indicated by the manufacturer. Data were acquired by a FACS caliber (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences) or Summit (DakoCytomation, Glostrup, Denmark). For sorting of immature and transitional B cells from BM and cell cultures, MoFlo flow cytometer (DakoCytomation) and EPICS ALTRA (Beckman Coulter, Hialeah, FL) were used.

CFSE staining

For coculture experiments and cell proliferation analysis, cells were stained with CFSE prior to the culture. Briefly, BM-purified B cell precursors and sorted immature and transitional B cells were incubated at 5 × 105 cells/ml with 0.5 μM CFSE (Molecular Probes) in prewarmed PBS for 5 min at 37°C and washed twice with complete OptiMEM medium.

Cultures for B cell differentiation

Purified B220+ IgM+ B cell precursor cells were cultured for 72 h in 96-well flat-bottom plates (Corning Glass, Corning, NY) at 2 × 105 cells/well in 200 μl/well in OptiMEM supplemented with 10% FCS, 5 × 10−5 M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies, Grand Island, NY). Spleen cell suspensions were obtained by gently teasing spleens onto a cell strainer. After centrifuging cell suspensions at 280 g for 7 min and discarding supernatant, cells were resuspended in ammomium chloride potassium lysis buffer (0.155 M NH4Cl, 10 mM KHCO3, and 0.1 mM sodium EDTA) to deplete erythrocytes, centrifuged for 7 min, and resuspended in complete medium. Cells were counted using a hemacytometer with exclusion of dead cells with trypan blue dye, and processed for flow cytometric analysis or cell sorting. Experimental procedures were approved by the Committee on Ethics for Animal Experimentation of the Federal University of Rio de Janeiro.
10% FCS, 5 × 10⁻³ M 2-ME, 100 μg/ml streptomycin, and 100 U/ml penicillin (Life Technologies). Anti-CD40 (clone 1C10) (eBioscience) at 5 μg/ml and IL-4 (0.1% supernatant from cultures of XR63-4 cells) were added to the indicated cultures. After 48 h, cultures were pulsed with 1 μCi (0.037 MBq) [³H]thymidine for 20 h before cells were harvested onto glass microfiber filters (Whatman, Maidstone, U.K.) and analyzed on a scintillation counter (Beckman Coulter).

**In vitro LPS injection**

Adult C57Bl/6 mice were inoculated i.p. with 0.1 μg/μg weight LPS or with PBS only and were killed 20 h later for spleen and BM viable cell counting and flow cytometric analysis of B cell compartments. Cells were processed for counting and flow cytometric analysis as described above. In some experiments, BrdU was injected simultaneously to LPS and control PBS for analysis of in vivo proliferation.

**BrdU incorporation**

Adult C57Bl/6 mice were inoculated i.p. with 38 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO) in PBS with or without LPS. Mice were killed 20 h later, and BM and spleen cells were cell surface stained in standard FACS buffer, washed once with protein-free PBS, resuspended in 0.5 ml plus 0.5 ml 2% paraformaldehyde 0.02% Tween 20, used for 20 h in PBS, and incubated for 18 h at 4°C. Subsequently, cells were washed, incubated with DNase I (150 mM NaCl, 5 mM MgCl₂, 10 μM HCl, and 100 KU/ml DNase I) for 20 min at room temperature, washed, then stained with FITC anti-BrdU (eBioscience) before analysis by flow cytometry.

**Results**

**Generation of CD23⁺ B cells in vitro in the presence of LPS does not involve cell proliferation and respects developmental program**

Our group has previously shown that TLR4 agonists promote the generation of CD23⁺ B cells with a transitional T2-like phenotype in a system of B cell differentiation in vitro from purified B cell precursors (25), suggesting a role for TLR4 signal in B cell maturation. We have obtained convincing evidence that such effect is due to differentiation of CD93⁺CD23⁻ immature B cells into CD93⁺CD23⁺ T2-like B cells and does not involve cell proliferation. We considered it important to confirm these results with a more straightforward approach, investigating whether LPS was able to induce short-term differentiation of immature B cells directly obtained ex vivo from fresh BM. To better characterize the effect of LPS on distinct stages of B cell maturation, we tested purified immature (IgM⁻lowCD23⁻neg) and transitional (IgM⁻highCD23⁻neg) B cell subsets separately. Immature and CD23⁻neg transitional T1-type B cells were sorted from adult BM with sort purities of ≥95% (Fig. 1A), stained with CFSE, and cultured for 16 h with or without LPS. LPS stimulation was able to promote a significantly higher generation of CD23⁺ cells from transitional B cells than in immature B cell, both in percentage (Fig. 1B) and cell numbers (Fig. 1C), clearly showing that the responsiveness to LPS is under developmental control. When the CFSE staining was analyzed in CD23⁺ B cells generated in vitro for the last 16 h, we observed a uniform CFSE⁺ population both in LPS-treated and untreated control cultures (Fig. 1B), providing direct proof that LPS was not inducing proliferation of CD23⁺ cells, as suggested by our previous studies (25). Total viable cell recovery after culture was always less than input (70–90%) of transitional B cells, both in control and LPS-stimulated cultures (Fig. 1C and data not shown), in contrast to a large increase in CD23⁺ cell numbers in LPS-stimulated cultures only, showing that TLR4 agonists stimulate the differentiation of immature B cells to CD23⁺ B cells. These data obtained with B cells ex vivo confirmed and extended the results obtained previously with a culture system of B cell differentiation in vitro from purified CD19⁻IgM⁻neg BM B cell precursors (25). Hereafter, we used the later system as our basal approach for the experiments performed in vitro, because of its simplicity and much higher yield of cells. Complementary experiments were also done with sorted BM B cells.

**LPS and BAFF induce similar alterations in surface markers and functional pattern along B lymphocyte maturation in vitro**

BAFF is the most well characterized maturation and survival factor for B lymphocytes and has been shown to induce B cells maturation in vitro as well (11, 26). We thus decided to perform a comparative analysis of the effect of LPS and BAFF on B cell maturation. Purified B cell precursors were cultured for 72 h, in the presence or absence of LPS or BAFF for the last 20 h. The expression of CD23 and other maturation markers were followed by flow cytometry. We could clearly distinguish three main stages of B cell maturation in our system: IgM⁻lowCD23⁻neg immature B cells, IgM⁻highCD23⁻neg transitional B cells, and IgM⁻highCD23⁺ transitional B cells (Fig. 2A). Both BAFF and LPS promoted, very similarly, the increase of the more advanced transitional CD23⁺ B cell population, both in percentage (Fig. 2A) and cell numbers (Fig. 2C), and a decrease in the percentage of immature B cell subset (IgM⁻lowCD23⁻neg). Immature CD23⁻neg transitional and CD23⁺ transitional B cell subset generated by the different treatments were further analyzed for the expression of the maturation markers CD21, IgD, and CD93 (Fig. 2B). We verified that CD23⁺ transitional B cells generated in the presence of either BAFF or LPS corresponded phenotypically to a more advanced maturation state, with higher expression of CD21 and IgD and lower CD93, resembling the CD23⁺ fraction E subset in the BM (27) or its splenic counterpart, the transitional T2 subset (3–5). These results indicate that the expression of CD23⁺ molecule in maturing B cells promoted by LPS, similarly to BAFF, is not due to a simple isolated induction of CD23 expression but to the stimulation of a coherent set of phenotypic maturation changes on B lymphocytes. LPS and BAFF had a similar effect on cultures of sorted immature and transitional B cells as well (Fig. 2D), with a massive increase in CD23⁺ B cell numbers in LPS- and BAFF-treated transitional B cells and a less pronounced increase induced in immature B cells.

Given the close similarities between BAFF and LPS effects in B cell maturation in vitro and the presence of low amounts of B220⁻neg, contaminating cells that could be producing BAFF in our cultures, we aimed to formally exclude the possibility that the LPS-induced maturation would need the presence of BAFF in some extent. We then treated B cell maturation cultures with Fc fusion decoy BAFF-R (BAFF-R-Fc) (Fig. 2E). Addition of BAFF-R-Fc, which blocks the interaction of BAFF with BAFF-R (28), completely abrogated the maturation promoted by BAFF but did not inhibit the maturation promoted by LPS (Fig. 2E), discarding a role of BAFF-R on LPS-induced maturation.

We next asked whether maturation observed in this study for both BAFF and LPS with the analysis of cell surface markers also corresponded to a functional maturation. It has been reported that B lymphocytes acquire proliferative responsiveness to anti-CD40 plus IL-4 stimulation at CD23⁺ Fraction E stage and that CD23⁻neg stage is still poorly responsive to this stimulus (27). Thus, we tested the functional state of IgM⁻lowCD23⁻neg, IgM⁻highCD23⁻neg, and IgM⁻highCD23⁺ B cells differentiated in the presence of LPS and BAFF in vitro, measuring in vitro proliferation upon stimulation with anti-CD40 plus IL-4 (Fig. 3A). We verified that sorted transitional CD23⁺ B cells obtained from both BAFF- and LPS-differentiated cultures had a significantly higher proliferation upon stimulation, compared with the CD23⁻neg subsets (Fig. 3A), showing that the CD23⁺ subsets generated upon LPS and BAFF stimulation in vitro behave similar to their counterparts generated in vivo. We further investigated whether the acquisition of proliferative responsiveness corresponds to a higher expression of CD40 (Fig. 3B). We observed an increase in CD40 expression along the passage through maturation stages in vitro. That increase is evident comparing IgM⁻lowCD23⁻neg to IgM⁻highCD23⁻neg but only marginal from the IgM⁻highCD23⁻neg to IgM⁻highCD23⁺ subsets (Fig. 3B).
LPS does not depend on induction of secondary factors to generate CD23⁺ B cells

Our previous results with cultures of purified B cell precursors from LPS-unresponsive mice have shown that generation of CD23⁺ B cells is strictly dependent on TLR4 signaling on B lymphocytes. As showed above (Fig. 2), BAFF is not involved in this process, and we sought to investigate whether TLR4 signaling would be inducing the secretion of other soluble factors or upregulation of membrane-bound factors, which would then drive B cell maturation. We addressed this issue with a mixed coculture of CD19⁺IgM⁻ B cell precursors from C57BL/6 mice and precursors from LPS-unresponsive C57BL10/ScCr mice (Fig. 4). To distinguish those two populations, we stained the LPS-unresponsive population with CFSE prior to culture. Only C57BL/6 LPS-responsive cells exhibited a significant augmented percentage of CD23⁺ cells upon addition of LPS, and the presence of C57BL/6 cells could not promote responsiveness of C57BL10/ScCr cells to LPS. This result indicates that no other soluble factor or cell surface ligand potentially produced by LPS-stimulated C57BL/6 cells can act on C57BL10/ScCr immature B cells, strongly suggesting that TLR4 signaling is directly stimulating B lymphocyte maturation.

Blocking of maturation upon BCR cross-linking is maintained in B lymphocytes differentiating in the presence of LPS or BAFF

Immature and transitional B lymphocytes are highly susceptible to negative selection upon BCR cross-linking, which is considered an important mechanism to eliminate autoreactive B lymphocytes (2, 8, 9). To address whether the presence of LPS or BAFF could lead to a change in such a crucial checkpoint of B lymphocyte development, we used F(ab')₂ fragments anti-IgM to mimic Ag-specific interaction and induce BCR cross-linking on B lymphocytes generated in vitro from purified precursors (Fig. 5A–C). As expected, in control cultures, increasing dose of anti-IgM led to a decrease in CD23⁺ B cell percentages (Fig. 5A). Importantly, cells differentiated in the presence of LPS or BAFF were also dose-responsive to anti-IgM inhibition, and at a saturating concentration of 5 μg/ml anti-IgM, the percentages of CD23⁺ B cells dramatically dropped, similarly to unstimulated culture with the same dose of anti-IgM, indicating that negative selection mechanisms mediated by BCR are active in the presence of both factors.

In this study, we could not distinguish whether the decrease of CD23⁺ B cells upon BCR ligation was mainly due to induction of
receptor editing, with a consequent delay in B cell maturation, or to apoptosis. Nonetheless, we found a moderate (10–15%), but significant, decrease in cell survival in cultures with saturating concentration of anti-IgM compared with the cultures without anti-IgM (Fig. 5B), indicating that high-avidity BCR ligation induces B cell apoptosis. It is noteworthy that high-avidity BCR ligation induces the same level of cell death independent of treatment (control, LPS, or BAFF) and that, despite the mild effect in general cell survival, it strongly affects the most advanced B cell maturation states. Altogether, these results indicate that both LPS and BAFF signaling are under strong regulation of BCR-delivered signals.

In myeloid cells, signals delivered by ITAM-coupled receptors were shown to inhibit TLR signals by the recruitment of calcineurin (29, 30). As BCR is associated with ITAM-bearing molecules, we next asked whether a similar mechanism could play a role in BCR-mediated regulation of LPS responsiveness in developing B lymphocytes. To test this hypothesis, we used CsA as a well-described calcineurin inhibitor in an attempt to revert BCR-mediated block on the generation of CD23+ B cells by LPS and

FIGURE 2. Comparative analysis of the effect of LPS and BAFF on B lymphocyte differentiation in vitro. A–C, CD19+ IgMneg B cell precursors purified from mouse BM were cultured for 72 h. LPS (12.5 μg/ml) or BAFF (100 ng/ml) was added 18 h before the end of culture, and the cells were recovered for viable cell counting and analysis by flow cytometry. A, CD23 versus IgM profile of viable cells recovered from cultures with the indicated treatments. Numbers indicate the percentage of IgMhighCD23neg, IgMhighCD23+, and IgMlowCD23+ subsets within the total IgM+ population. B, Histograms show expression levels of CD21 (left panels), IgD (middle panels), and CD93 (right panels) of the IgMhighCD23+, IgMhighCD23neg, and IgMlowCD23+ subsets, defined as shown in A. C, Numbers of viable pre-B cells (left panel), IgM+ cells (middle panel), and CD23+ B cells (right panel) recovered per well in untreated control or LPS- or BAFF-treated cultures. Bars show mean ± SEM of four independent experiments. *p < 0.05. D, Immature and CD23neg transitional B lymphocytes were sorted by FACS from mouse BM as described in Fig. 1 and cultured for 16 h without or with LPS at 12.5 μg/ml or BAFF at 100 ng/ml. Panels show numbers of viable total (left panel) and CD23+ B cells (right panel) per well in 0 h and after 16 h of culture of sorted immature and transitional B cells. E, Purified BM B cell precursors were cultured for 72 h with or without BAFF, LPS, and the decoy BAFF-R (BAFF-R-Fc). Cells were stained for IgM, CD23, and B220 and analyzed by FACS. Plots show CD23 versus B220 profile of IgM+ cells for each culture condition, and the numbers indicate percentages of CD23+ cells in IgM+ B cell population. Results are representative of four (A–C) or two (D, E) independent experiments. Dead cells were excluded from analyses with PI staining.
BAFF (Fig. 5C). We found that CsA partially recovered the percentage of CD23+B cells in nonstimulated cultures incubated with anti-IgM and promoted a remarkable reversion of the inhibition of anti-IgM on both LPS- and BAFF-stimulated cultures. CsA had no effect on cultures without anti-IgM (Fig. 5C), suggesting that its effect is a specific consequence of the interference on BCR pathway.

We also tested the effect of BCR cross-linking on differentiation stimulated by LPS and BAFF of IgMlowCD23neg immature and IgMhighCD23neg transitional B lymphocytes sorted from BM. As expected, even in the presence of LPS or BAFF, anti-IgM inhibited the appearance of CD23+B lymphocytes in the 16-h cultures of both sorted immature and transitional B cells (Fig. 5D), confirming the experiments done with purified B cell precursors, shown in Fig 5B. To better define the involvement of apoptosis in anti-IgM-mediated inhibition of B cell maturation, we also stained those cells with Annexin V and PI. We found that cell viability was barely affected by anti-IgM in immature B cell cultures, despite the strong inhibition of CD23+B cells (Fig. 5D). In contrast, there were significant and similar cell death rates (35–50%) in transitional B cell cultures upon anti-IgM treatment among control, LPS-, and BAFF-stimulated cultures relative to the respective untreated cultures (Fig. 5D). These results indicate that IgMhigh B cells are more susceptible to anti-IgM-induced cell death than IgMlow B cells, in agreement with previous results by others (8), and that induction of apoptosis by anti-IgM probably occurs prior to appearance of CD23+ cells by LPS and BAFF, because the cell death rates among control, LPS-, and BAFF-stimulated cultures are equivalent. Considering that the sorted transitional B cells are already competent for response to BAFF and LPS with differentiation to CD23+ cells (Figs. 2D, 5D), we interpret these data as evidence that BCR signaling prevails over the triggering of BAFF-R and TLR4.

**Classical NF-κB activation pathway is involved in the LPS-induced increase of CD23+ transitional B cells in vitro**

NF-κB plays central roles in BAFF-mediated maturation of B cells and lymphocyte survival. Importantly, NF-κB is also a critical messenger of LPS activation in myeloid cells and mature B lymphocytes. Therefore, we considered this transcription factor as a major candidate for mediating B cell maturation by LPS. TLR4 activation leads to recruitment of classical pathway of NF-κB (24), whereas the main roles of BAFF-R on B cell maturation has been attributed to the activation of alternative pathway of NF-κB (31). We thus tested the role of NF-κB activation on LPS activity in our culture system by using SN50, a peptide that specifically inhibits classical NF-κB activation pathway. We observed that increase of CD23+ B cells promoted by LPS was abrogated in the presence of SN50 (Fig. 6), whereas the activity of BAFF, as well as the basal levels of CD23+B cells in control cultures, was not affected by the presence of that inhibitor, confirming that distinctly to BAFF, NF-κB activation through canonical pathway is crucial for the effect of LPS.

**LPS and BAFF have complementary activities on B lymphocyte differentiation in vitro**

Our data show that LPS-stimulated B cell maturation has significant similarities with BAFF activity but is independent of that factor and uses a distinct signaling pathway. These results indicate that TLR4 signaling could play an alternative or complementary role to BAFF in B cell development. We thus verified whether those factors could cooperate to stimulate B lymphocyte differentiation. In fact, when we added simultaneously LPS and BAFF into the culture, we observed that an increase in the percentage of CD23+B cells was roughly twice that promoted by the factors separately (Fig. 7A). The use of four times higher concentrations of LPS or BAFF than the usual established for our cultures did not result in further increase in the effect observed for none of the factors.
alone, discarding the possibility that we were working below saturating levels (data not shown). These data indicate that signals from LPS and BAFF stimulations do cooperate with each other to increase B cell maturation rates.

Many groups have found that in mature B lymphocytes, TLR signaling stimulates the responsiveness to BAFF by inducing increased expression of its receptors (32, 33). We verified in this study whether LPS could be inducing increased expression of BAFF-R in developing B lymphocytes as well, comparing the expression levels of BAFF-R on the IgM lowCD23neg, IgM highCD23neg, and IgM highCD23
+ subsets grown in control and LPS-stimulated cultures (Fig. 7B). As expected, we could observe a gradual increase of BAFF-R expression along differentiation, with the highest expression of BAFF-R on IgM highCD23
+ subset, both in LPS and control cultures, coherent with the sequence of normal B cell maturation (6). However, LPS did not induce any higher levels of BAFF-R compared with control cultures, discarding the possibility that the cooperation effect of LPS with BAFF, observed above, was due to the augmented expression of BAFF-R.

In vivo injection of LPS increases mature B lymphocyte compartment in the BM

In our B cell differentiation culture system, as in other previously described in vitro systems (8, 34), we could not observe significant numbers of B lymphocytes with fully mature phenotype in culture, probably because of the lack of still unknown stimuli or micro-environment factors necessary for complete B cell maturation. To verify whether the partial advance in development found in vitro could actually result in complete maturation in vivo, we injected adult mice with sublethal doses of LPS and analyzed B cell...
compartments in the BM 20 h after injection. We found that percents of B cell precursors within B lineage cells in the LPS-injected group were significantly reduced compared with the control group injected with PBS only (Fig. 8A). This result is in agreement with previous findings that TLR signaling inhibits growth of lymphoid precursors (23). Interestingly, we observed no change or slight augmentation in immature and transitional B cell compartments and a significant increase both in percentage (Fig. 8A) and in cell numbers (Fig. 8B) of a BM mature B cell compartment in a LPS-injected group. We could not detect increase neither in percentage nor in numbers of B lymphocyte compartment in spleen at low doses (0.1 μg/g) of LPS (Fig. 8C). Finally, results from in vivo BrdU treatment indicate that mature B cell compartment increase after that short period of time was not consequence of cell proliferation (Fig. 8D).

Discussion

We have previously found evidence that TLR4 agonists favor late developmental progression of B lymphocytes, increasing generation of CD23+ B cells with a transitional B cell phenotype in vitro (25). In the present work, we confirmed and extended these results, showing that engagement of TLR4 in immature B cells directly obtained from fresh BM resulted in the generation of CD23+

![FIGURE 7. Collaborative effect of LPS and BAFF on B lymphocyte differentiation in vitro. CD19+IgM- BM B cell precursors were cultured for 72 h. LPS, BAFF, or both were added 18 h before the end of culture. A. Plots show CD23 versus B220 profile of IgM+ cells for the indicated treatments, and the numbers are the percentages of CD23+ cells in IgM+ B cell population. Representative of four experiments. B. Histograms show expression levels of BAFF-R of the IgMhighCD23+, IgMhighCD23neg, and IgMlowCD23neg subsets, defined as shown in Fig. 2A, in control (hatched) and LPS-treated cultures (thick line). Representative of two independent experiments.](http://www.jimmunol.org/)

![FIGURE 8. Effect of TLR4 signaling on B lymphocyte differentiation in vivo. Adult C57BL/6 mice were injected i.p. with LPS (0.1 μg/g weight) or PBS and sacrificed 20 h later for analysis by flow cytometry of B cell compartments. Percentages relative to total BM B220+ population (A) or absolute cell numbers per femur (B) are shown for pre-B (B220lowIgMneg), immature (B220lowIgMlow), transitional (B220dimIgMhigh), and mature (B220highIgMlow) B cells in PBS- (○) and LPS-injected (●) mice. In C, percentage (left panel) and absolute cell numbers (right panel) of B220+ cells in the spleen of PBS- and LPS-injected mice are shown. Bars show mean values ± SEM of five mice per group. *p < 0.05; **p < 0.01. D. Adult C57BL/6 mice were injected i.p. with LPS (0.1 μg/g weight) or PBS plus BrdU (38 mg/kg) and sacrificed 20 h later for assessing BrdU incorporation of the B lineage subsets in the BM as defined in A or mature B lymphocytes in the spleen (last two columns) of PBS- and LPS-injected mice (five per group). The data showed are representative of two different experiments with similar results.](http://www.jimmunol.org/)
B cell maturation in vivo: immature CD23\textsuperscript{neg} fraction E (or T1, its we considered to be equivalent to the main consecutive steps of increase of CD23\textsuperscript{+} B cells induced by LPS reflects an isolated staining relative to control cultures, indicating that proliferation is not occurring.

An important question we addressed in this paper is whether the increase of CD23\textsuperscript{+} B cells induced by LPS reflects an isolated phenomenon of upregulation of CD23 expression or is part of a coherent set of developmental changes characterizing progression in maturation. This point was investigated comparing the effects of LPS with BAFF, the main factor involved in B cell maturation in vivo and in vitro (11, 26). We performed a detailed analysis of the B cells generated in vitro using CD21, IgD, and CD93 as maturation markers for characterization of the IgM\textsuperscript{low} CD23\textsuperscript{neg}, IgM\textsuperscript{high}CD23\textsuperscript{neg}, and IgM\textsuperscript{high}\textsuperscript{CD23}\textsuperscript{+} B cell subsets that we considered to be equivalent to the main consecutive steps of B cell maturation in vivo: immature CD23\textsuperscript{neg}\textsuperscript{fraction E (or T1, its counterpart in spleen) and CD23\textsuperscript{+} fraction E (or T2, its counterpart in spleen) (4, 27), respectively. The three subsets generated in the presence of LPS had the same characteristics as their equivalent subsets in untreated control cultures and BAFF-treated cultures, following the pattern observed in vivo, with a gradual increase in CD21 and IgD and lowering of CD93 in the passage from IgM\textsuperscript{low} CD23\textsuperscript{neg} to IgM\textsuperscript{high}\textsuperscript{CD23}\textsuperscript{+} stages (Fig. 2).

Acquisition of proliferative responsiveness to stimulation with anti-CD40 plus IL-4 was described as a functional hallmark that distinguishes CD23\textsuperscript{+} transitional B cell subset from the CD23\textsuperscript{neg} counterparts both in BM and spleen (5, 27). We confirmed that CD23\textsuperscript{+} cells generated in the presence of LPS or BAFF in vitro were much more responsive than the CD23\textsuperscript{neg} cells to anti-CD40 plus IL-4 stimulation, showing that functional maturation of B cells is also very likely occurring in our culture system (Fig. 3A). We investigated whether CD40 expression levels could be responsible for the distinct abilities among those subsets and detected a slight increase in CD40 levels along the passage from CD23\textsuperscript{neg} to CD23\textsuperscript{+} stage. We believe, however, that such a gradual change in CD40 expression alone could not explain the clear-cut difference in responsiveness of CD23\textsuperscript{+} subset, and an additional set of maturation events is probably required. Thus, we conclude that CD23\textsuperscript{+} cells generated in the presence of LPS constitute a population that has passed through complex changes in phenotype and functional capacities that correspond to effective advance in maturation. More important, the results observed with LPS were essentially equivalent to those obtained in BAFF-treated cultures.

Many groups have pointed out that survival signals from BAFF are highly susceptible to negative selection upon BCR cross-linking (2, 8, 9), in apparent opposition to the maturation effect of BAFF and LPS. In this paper, we studied the effect of BCR signaling on in vitro B cell maturation and found that the high-avidity BCR cross-linking completely blocked LPS- and BAFF-induced increase of CD23\textsuperscript{+} B cells and neither BAFF nor LPS could inhibit cell death induced by anti-IgM (Fig. 5). The physiological implication of those findings is that the checkpoint of the negative selection of highly autoreactive B cells is respected even in the presence of nonspecific positive stimuli from TLR4 agonists or BAFF. Our results with BAFF are in agreement with a previous report showing that BAFF overexpression does not rescue B cells from deletion by high-affinity self-reactive interaction in BM (36); one explanation raised by the authors was that those cells are still unable to fully respond to BAFF stimulation. However, we observed in this study that the highly purified transitional B cell subset, containing a large fraction of cells responsive to both LPS and BAFF, remains extremely susceptible to blocking of maturation by BCR engagement even in the presence of those factors (Fig. 5D). This result suggests that concurrent signaling from BCR on developing B lymphocytes is inhibiting BAFF-R– and TLR4-delivered signals.

Immature B lymphocytes, different from the mature ones, have deficient or inadequate recruitment of NF-\kappaB by BCR engagement (31) but strongly respond with calcineurin activation and Ca\textsuperscript{2+} mobilization (37). We found that the calcineurin inhibitor CsA was able to revert the blocking effect of BCR triggering on LPS activity (Fig. 5C). In myeloid cells, calcineurin activation without NF-\kappaB recruitment by ITAM-coupled receptors was shown to inhibit TLR activation (29, 30). Thus, a similar mechanism could play a role in BCR-mediated control of the LPS responsiveness in developing B cells, where the acute signal from the BCR, an ITAM-associated receptor, seems to inhibit TLR4 signaling through a calcineurin-dependent way. In addition, BCR signal in mature B cells has been shown to be able to rescue B cells from the hyporesponsiveness to TLR restimulation through PI3K activation (38), which is an upstream event of the calcineurin recruitment, reinforcing the notion that BCR directly modulates TLR-mediated signals through this pathway. Interestingly, our results indicate that CsA also reverses the inhibition mediated by BCR cross-linking on maturation induced by BAFF (Fig. 5C). These results bring new clues for the comprehension of the mechanisms of central tolerance. Of note, we also observed that low concentrations of anti-IgM that can still significantly inhibit the generation of CD23\textsuperscript{+} B cell in nonstimulated cultures seem to have much milder or no effect on the generation of CD23\textsuperscript{+} cells in LPS- or BAFF-treated cultures (Fig. 5A), suggesting a dual role for BCR triggering on B cell selection, with distinct presence of LPS. The inability of decay BAFF-R-Fc, which totally blocks the action of BAFF, to inhibit LPS-mediated B cell maturation showed that BAFF is not participating in TLR4-dependent B cell maturation (Fig. 2E). Moreover, coculture of LPS-responsive with LPS-nonresponsive B cell precursors showed that the presence of LPS-responsive cells was not able to stimulate the maturation of LPS-nonresponsive B cells, indicating that no other factor would be mediating LPS effects (Fig. 4). The possibility that LPS induces both an unknown factor and its own receptor on the responding cells could not be excluded, although we believe it is unlikely. Our results strongly support the notion that the maturation changes induced in immature B cells in vitro by LPS are independent of BAFF or other unknown mediators and are direct consequence of TLR4-delivered intracellular signals.

TLR signaling has been shown to synergize with BAFF-delivered signals for mature B cell activation (17–20). In contrast to mature B cells, immature and transitional B lymphocytes are highly susceptible to negative selection upon BCR cross-linking (2, 8, 9), but BAFF seems also to induce some maturation changes independent of survival stimulation (26, 35). We observed that low concentrations of anti-IgM that can still significantly inhibit the generation of CD23\textsuperscript{+} B cell in nonstimulated cultures seem to have much milder or no effect on the generation of CD23\textsuperscript{+} cells in LPS- or BAFF-treated cultures (Fig. 5A), suggesting a dual role for BCR triggering on B cell selection, with distinct...
interactions with survival or maturation signals depending on its signal strength. This question must be further explored with more detailed analysis of signaling events involved in this phenomenon.

Recruitment of transcriptional factors of the NF-κB family is central for maintenance of B lymphocytes, and both classical and alternative activation pathways are required for normal B cell development (31, 39). Alternative pathway is essential for the BAFF-R signaling (40), whereas classical NF-κB pathway activation seems to be essential for the BCR-dependent cell survival (41). TLR4 is able to elicit components of classical NF-κB pathway (24). Remarkably, we found that the inhibition of the classical NF-κB pathway mobilization severely impaired the activity of LPS on the developing B cells in our cultures, while BAFF activity, as expected, was unaffected (Fig. 6). These data lead us to the tempting hypothesis that TLR4 can provide, through classical NF-κB activation, maturational and survival signals alternative or complementary to BAFF-R. Indeed, we found that simultaneous addition of LPS and BAFF induced twice as many CD23⁺ B cells as each factor alone, indicating an additive effect between LPS and BAFF (Fig. 7). An alternative explanation for the cooperation of LPS with BAFF in B cell maturation is that TLR4 signaling could lead to the up-regulation of BAFF-R, increasing BAFF responsiveness. Many groups have recently described that TLR4 and TLR9 signaling upregulate receptors for BAFF in mature B cells, increasing survival and activation events mediated by BAFF (32, 33). Nonetheless, in our system, transitional B cells were not able to upregulate BAFF-R expression upon LPS stimulation (Fig. 7B). Thus, it seems that at this maturation stage BAFF and LPS are probably complementing each other directly through the downstream recruitment of NF-κB.

Our results obtained in vitro clearly indicate that direct TLR4 signaling on immature or transitional B cells favor their maturation and raise the question of a possible role for TLR4 in the B cell development in vivo either through the engagement by endogenous or exogenous ligands. Only few studies have addressed this question. B cell subsets in BM and spleen of B10ScCr LPS-nonresponsive and MyD88-knockout mice are apparently normal in numbers and distribution (data not shown and Ref. 42); however, the survival and turnover rates in B cell compartments have not been studied, and a careful analysis of population kinetics of B lymphocytes must be performed in those mice. Freitas et al. (43) have shown in BM competition chimeras bearing both LPS-responsive and -nonresponsive cells that LPS responsiveness confers competitive advantages for the entry into plasma cell compartment, but the analysis of the different subsets of BM and splenic naïve B cells in those chimeras was not done at that time. A more recent study, using a previously described mouse model bearing a positively selected autoreactive BCR transgene, has addressed the role of TLRs in the selection of transgenic B cells (44). The authors compared the transgenic system on a normal or TLR-deficient background and found that TLR4 deficiency leads to a significantly decreased selection of BCR-transgenic B cells into mature B cell compartment in BM but not in spleen, suggesting a role for TLR4 in BM B cell development. Recent evidence showed that BM, in parallel to and independently of spleen, can sustain complete B lymphocyte maturation (27, 45). Survival of splenic B cells depends strongly on BAFF, whereas BM B cell development and survival are relatively spared in the absence BAFF (10), with evidence that other factors could provide the necessary stimulus for maturation and survival (46). The results presented in this paper suggest that TLRs agonists are potential candidates to fulfill this role.

In this study, we tested a possible role for exogenous TLR4 agonist on B cell maturation in vivo by injecting a sublethal dose of LPS. In a short period after injection (20 h), there was a significantly increased mature B subset in BM of LPS-injected mice (Fig. 8A, 8B) without proliferation (Fig. 8D). Although we could not discard the possibility that many other factors are acting to induce such a change in vivo, this result argues in favor of the idea that TLR4 signaling can directly drive B cells to the final maturation in vivo. We observed that, in parallel, TLR4 signaling leads to decrease in B cell precursor compartment (Fig. 8) (25), in agreement with other reports showing that LPS-treated mice have a significant depletion of B lymphocytes from BM after 3 d (23, 47), and direct TLR4 and TLR2 signaling on hematopoietic precursors lead to a myeloid-biased hematopoiesis.

LPS can gain access to BM in bacterial infections and modulate lymphopoiesis and myelopoiesis (23). It is interesting to speculate about the role of TLR4 signaling as a feedback regulation upon B cell maturation. An interesting hypothesis for optimization of the immune response has been proposed (48) suggesting that BCR/BAFF-R dual system evolved to give the best balance between Ag-specific and unspecific pressures, so that available repertoire is diverse enough to cover exogenous Ags, but limited enough not to spend energy and space with excessively random or autoimmune specificities. According to this proposal, BAFF would be acting following the principle of the strength of BCR signaling. Analogously, TLR4 could be modulating the strength of BCR signaling, eventually augmenting the threshold for negative selection. In carriers of infectious diseases, for instance, the TLR4-dependent tuning of BCR signaling could lead to reduced negative selection of low-avidity autoreactive clones and consequent enlargement of the clonal diversity of the newly formed B cell repertoire, augmenting the probability of an effective immune response.

Acknowledgments
We thank Dr. John Cambier for providing FITC anti-B220 and Alexa 647 anti-mouse IgM Abs, Dr. Antonius Rolink for providing anti–CD93-producing 493 hibridoma, Eduardo Aguilar for technical assistance, and Drs. Marcelo Bozza and Fabricio Montalvão for helpful discussion and suggestions for this manuscript.

Disclosures
The authors have no financial conflicts of interest.

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