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The splenic B cell compartment is comprised of two major, functionally distinct, mature B cell subsets, i.e., follicular mature (FM) and marginal zone (MZ) B cells. Whereas MZ B cells exhibit a robust proliferative response following stimulation with the TLR4 ligand LPS, FM B cells display markedly delayed and reduced levels of proliferation to the identical stimulus. The current study was designed to identify a potential mechanism(s) accounting for this differential responsiveness. In contrast to the delay in cell cycle entry, FM and MZ B cells exhibited nearly identical LPS-driven alterations in the expression level of cell surface activation markers. Furthermore, both the NF-κB and mTOR signaling cascades were similarly activated by LPS stimulation in FM vs MZ B cells, while inducible activation of ERK and AKT were nearly absent in both subsets. MZ B cells, however, exhibited higher basal levels of phospho-AKT and pS6, consistent with a preactivated status. Importantly, both basal and LPS activation-induced c-myc expression was markedly reduced in FM vs MZ B cells and enforced c-myc expression fully restored the defective proliferative response in FM B cells. These data support a model wherein TLR responses in FM B cells are tightly regulated by limiting c-myc levels, thereby providing an important checkpoint to control nonspecific FM B cell activation in the absence of cognate Ag. The Journal of Immunology, 2009, 182: 4065–4075.

Mature splenic B cells can be divided into two subpopulations, follicular mature (FM) and marginal zone (MZ) B cells, based on distinct topographic, phenotypic, gene expression, and functional characteristics (reviewed in Ref. 1). FM B cells reside in the follicles of the splenic white pulp, while MZ B cells are located in the MZ, a region at the border of the splenic red and white pulp. The MZ is delineated by the MZ sinus and a layer of metallophilic macrophages that express MOMA1, thereby surrounding B cell follicles and T cell areas. This architectural structure contributes to the unique function of the splenic MZ to mount a rapid immune response to blood-borne Ags. Phenotypically, MZ B cells are characterized by high expression of IgM, CD21, CD1d, CD9, whereas they are low/negative for IgD and CD23. In contrast, FM B cells are IgMint, IgDhigh, CD21int, CD23low, CD1dint, and CD9low. Multiple gene products are differentially expressed in these two subsets, including, most notably, effectors within the Notch signaling cascade that are essential for MZ B cell development (2, 3).

Mature B cells are relatively unique among immune cells because they express both germline-encoded TLRs as well as a recombination-dependent, clonally rearranged, Ag-specific B cell Ag receptor (BCR). Functionally, FM B cells fit largely within the adaptive arm of the immune system, which is characterized by memory formation and receptor specificity mediated via Ag-specific receptors such as the BCR (4). For full activation, FM B cells require T cell help and, accordingly, they are the main players during T-dependent immune responses. In contrast, MZ B cells have been classified as innate immune cells. Their immune response is rapid, independent of direct T cell help and directed against a great diversity of blood-borne organisms utilizing pathogen-specific pattern recognition receptors like TLR in association with stimulation via the BCR.

Consistent with the classification into the innate and adaptive arms of the immune system, MZ B cells exhibit a strong response to the TLR4 ligand LPS, which promotes cell activation, proliferation, and Ig production (5, 6). FM B cells, in contrast, are readily activated through BCR stimulation in vitro, yet exhibit markedly delayed and reduced cell cycling following LPS stimulation. Notably, although a range of studies have demonstrated differential responsiveness of FM vs MZ B cells to TLR ligand engagement (5–7), the molecular events that limit FM B cell proliferation in response to this key signal remain to be defined. Because TLR engagement can lead to a break in B cell tolerance (8, 9), understanding the mechanism(s) behind this differential response may provide insight into the pathogenesis of autoimmune disease.

In the current study, we have addressed this question in detail. We demonstrate that FM B cells exhibit a specific deficit in cell cycle entry, despite exhibiting normal LPS-dependent proximal signaling events and similar TLR4-induced up-regulation of activation markers. Furthermore, we show that this cell cycle deficit is due to: reduced basal activity within the mammalian target of rapamycin (mTOR) signaling cascade and, most notably, insufficient...
basal and inducible up-regulation of the cell cycle and growth regulator c-myc. Consistent with this conclusion, enforced expression of c-myc rescued this cell cycle defect, leading to efficient FM B cell cycling in response to LPS. Taken together, our findings suggest that limiting c-myc levels may help to restrict FM B cell activation when TLR ligands are encountered in the absence of Ag receptor signaling.

Materials and Methods

Mice

C57BL/6, c-myc transgenic (tg) (10), MyD88−/− (11), and TRIF−/− (12) (all on a C57BL/6 background) were bred and maintained in the specific-pathogen-free animal facility of the Seattle Children’s Hospital Research Institute (Seattle, WA) and handled according to the Institutional Animal Care and Use Committee-approved protocols. Mice used in all experiments were between 6 and 16 wk of age.

Reagents and Abs

Abs used in this study included reagents specific for: CD24 (M1/69), CD21 (7G6), trinitrophenyl (TNP; 49, 2), and B220 (RA3-6B2) from BD Pharmingen; CD23 (B3B4) from Caltag Laboratories; CD62L (MEL-14) from eBioscience; IgD (11-26) and IgM (B93/8) from Southern Biotechnology Associates; and CD19 (ID3) from BioLegend. Polyclonal F(ab)2 anti-IgM for BCR stimulation was purchased from Jackson ImmunoResearch Laboratories. Additional reagents included pyronin Y and 4′,6-diamidino-2-phenylindole (DAPI) from Molecular Probes; LPS from Sigma, BAFF from Alexis, and TNP-Ficol from Biosearch Technologies.

Cell culture

Splenocytes were cultured in RPMI 1640 with 10% FCS, 55 μM 2-ME, 10 mM HEPES, penicillin, and streptomycin (complete medium) at 37°C. Cells were stimulated with polyclonal goat anti-mouse IgM Fab’2, or LPS in concentrations as indicated.

Flow cytometry and cell sorting

Single-cell suspensions from BM and spleen were incubated with fluorescence-labeled Abs for 20 min at 4°C in staining buffer (PBS with 0.5% BSA or 2.5% FCS). Data were collected on a FACSCalibur or LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). For LSR II experiments, the data were analyzed using bi-exponential transformation function for complete data visualization.

For cell sorting, CD43 depletion was performed using magnetic bead-conjugated anti-CD43 Abs according to the manufacturer’s instructions (Miltenyi Biotec) and enriched cells were labeled with specific Abs in staining buffer. Sorting was performed using a FACSAria sorter with Diva software (Miltenyi Biotec) and enriched cells were labeled with specific Abs for 20 min at 4°C in staining buffer (PBS with 0.5% BSA). For cell sorting, CD43 depletion was performed using magnetic bead-conjugated anti-CD43 Abs according to the manufacturer’s instructions (Miltenyi Biotec) and enriched cells were labeled with specific Abs in staining buffer. Sorting was performed using a FACSAria sorter with Diva software (Miltenyi Biotec) and enriched cells were labeled with specific Abs for 20 min at 4°C in staining buffer (PBS with 0.5% BSA). Cells were washed and stimulated with a stimulatory anti-IgM Ab or LPS, and Ca2+ flux was measured by flow cytometry within each B cell subset.

Western blot analysis

For whole cell lysates, stimulated B cells were lysed at indicated time points in a buffer containing 0.5% Nonidet P-40, 0.5% Triton X-100, 50 mM NaCl, 0.5% SDS gel, transferred to a polyvinylidene difluoride-FL# membrane (Millipore) and blots were probed with anti-IκBα (SC-203), c-Rel (SC-71), p65 (SC-372-G), perK (SC-7383), ERK1 (SC-93), HDAC1 (SC-6298), c-myc (SC-764) (all from Santa Cruz Biotechnology), and/or pS6 (2211S; Cell Signaling) Abs.

Flow cytometry analysis of phosopho-AKT (pAKT)

Spleenic B cells were incubated in RPMI 1640 with 10% FCS for 30 min, stimulated with 15 μg/ml polyclonal goat F(ab)2, anti-mouse IgM, or 20 μg/ml LPS, and fixed with 2% paraformaldehyde for 30 min at room temperature. Cells were then surface stained with Abs to B220, CD21, CD24, and CD23, permeabilized with Perm Wash (BD Pharmingen) for 20 min at 4°C, and incubated with un conjugated anti-phospho-AKT-Ser473 (Ab Cell Signaling) for 45 min at room temperature, followed by anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch Laboratories).

Inhibitor studies

The PI3K inhibitor wortmannin (Sigma-Aldrich) and the mTOR inhibitor rapamycin (Sigma-Aldrich) were used at 100 nM. Cells were preincubated with inhibitors for 60 min before stimulation.

In vitro analysis of Ab production

Sort-purified FM or MZp/MZ cells were plated at ~150,000 cells/well in 96-well plates and stimulated with 20 μg/ml LPS. Culture supernatants were collected at day 3, diluted 1/100 in PBS, incubated in triplicate on anti-IgM (10 μg/ml), coated, washed, and followed by incubation with HRP-conjugated secondary Ab. ELISA was performed using a tetramethylbenzidine ELISA kit (BD Biosciences) and absorbance was measured at 450 nm on a Victor 3 plate reader (PerkinElmer). IgM levels were quantified using titrated standards.

TNF-Ficol-binding assay

Mice were injected i.v. with 500 μl of 1 mg/ml TNF-Ficol. Thirty minutes later, splenocytes were isolated from injected mice, surface stained with B220, CD21, and CD24, and TNF-Ficol binding was determined in each B cell subset using an anti-TNF Ab.
Statistical evaluation

Values of \( p \) were calculated using the Student \( t \) test.

Results

**Limited cell cycle entry of FM B cells in response to LPS**

It has previously been shown that FM B cells proliferate only weakly, if at all, to LPS stimulation (5, 6, 14). Using a tritiated thymidine proliferation assay, BCR stimulation of sort-purified, CFSE-labeled FM and MZ B cells with varying doses of LPS (0.001–10 \( \mu \)g/ml) results in rapid thymidine incorporation within 48 h of stimulation. The identical population, however, exhibits a negligible response to stimulation across a broad range of LPS doses (Fig. 1A and data not shown). In contrast, MZ B cells exhibit a rapid and robust proliferative response to LPS, but undergo apoptosis in response to BCR engagement in vitro. 

Sort purities in these and all subsequent experiments were >95% and >90% for FM and MZ B cells, respectively. The near absence of FM proliferation in response to LPS differs from some previous reports and likely reflects higher FM B cell purity in our studies. Similar results were also obtained by analyzing dilutions of CFSE: 48 h after LPS stimulation, MZ B cells have undergone one to two divisions, while very few FM B cells exhibit CFSE dilution in response to this stimulus even at very high doses (up to 50 \( \mu \)g/ml; Fig. 1B). Analysis at 72 h demonstrates that a subset of FM B cells have divided to a similar extent as MZ B cells. However, whereas nearly all MZ B cells had divided at least once in response to 1 or 10 \( \mu \)g/ml LPS, even at 50 \( \mu \)g/ml LPS a significant proportion of FM B cells (10–15%) consistently fail to enter the cell cycle.

We also performed FACS-based cell cycle analysis in anti-IgM- vs LPS-stimulated mature B cell subsets. To obtain sufficient numbers of MZ-lineage cells, we conducted this analysis using sorted cells comprised of both MZp, a subset that exhibits a nearly identical response to LPS as well as MZ B cells (7, 15). Although FM B cells rapidly entered the cell cycle in response to BCR ligation, very few cells entered G1 phase even 48 h after LPS stimulation (Fig. 1 C and D). In contrast, LPS-stimulated MZp/MZ B cells rapidly entered the cell cycle, resembling the response of FM B cells to BCR engagement. As an additional control, incubation with BAFF promoted survival of FM B cells but did not promote cycling with or without LPS stimulation (Fig. 1D and data not shown). Together, these data demonstrate that FM B cells have a marked delay or block in cell cycle entry in response to LPS stimulation.

**FM B cells are highly sensitive to LPS stimulation**

Based on these observations, we next asked whether FM B cells exhibit a global defect in response to LPS signaling. Consistent with this idea, previous studies have shown that the TLR4 co-receptor RP105 is expressed at significantly higher levels on MZ compared with FM B cells; suggesting that this difference might impact the overall LPS responsiveness of FM B cells. As an initial test of the sensitivity of FM B cells to LPS, purified
FM vs MZp/MZ B cells were stimulated with varying doses of LPS and the relative expression level of a series of activation markers was determined by FACS (Fig. 2, A and B). Most activation markers including CD86, CD25, MHC class II (MHC II), and peanut agglutinin were expressed at higher levels basally in unstimulated MZp/MZ compared with FM B cells. LPS stimulation, however, led to a similar fold change in each of these activation markers in both cell types at 48 h after stimulation. FM B cells also consistently up-regulated CD23 expression even at very low doses of LPS. This marker is not expressed on MZ B cells and therefore is not evaluated in that population. Alteration in surface markers was dependent upon the expression of the TLR signaling adaptors MyD88 and TRIF, consistent with a direct role for LPS signals in modulating their expression (data not shown). Notably, like MZp/MZ cells, FM B cells readily responded to LPS doses as low as 0.1 μg/ml. However, at these lower LPS doses, MZp/MZ B cells showed relatively greater responses, indicating that they are slightly more sensitive to LPS.

We also compared the relative expression of candidate activation markers in nonproliferating vs total FM B cells (identified based on relative size and forward scatter as shown in Fig. 2).
2B) at 48 h after LPS stimulation. Whereas CD25 and peanut agglutinin were nearly exclusively up-regulated in enlarged FM B cells, MHC II and CD23 were equivalently up-regulated in both proliferating and nonproliferating FM B cells (Fig. 2B). CD86 was also up-regulated in nonproliferating FM B cells although to a slightly lesser extent than in the proliferating population.

In addition, we directly compared the kinetics of candidate LPS target genes by assessing mRNA expression levels for AID and BAFF-R in response to LPS stimulation in FM vs MZp/MZ B cells. Although LPS-induced AID expression was higher in MZ B cells, significant expression was achieved only after 48 h and this timing was similar to that observed for FM B cells (Fig. 2C). Basal BAFF-R transcript expression levels were slightly higher in MZ B cells. However, BAFF-R mRNA induction was nearly identical with regard to kinetics and maximal expression levels up to 24 h after stimulation in FM vs MZ B cells.

Taken together, these data demonstrate that although FM B cells are highly sensitive toward LPS stimulation as assessed by activation marker and target gene expression; this subset exhibits a clear deficit in cell cycle entry and proliferation in response to LPS.

**FM and MZ B cells exhibit similar proximal biochemical responses to LPS stimulation**

Based upon our findings, we next sought to determine why FM B cells failed to enter the cell cycle at early time points following LPS stimulation. To begin to address this question, we analyzed proximal signaling cascades triggered in response to TLR4 ligation in FM vs MZp/MZ B cells.

First, we measured activation of the NF-κB pathway as assessed by relative degradation of IκBα, a repressor of NF-κB nuclear translocation and activity (Fig. 3A). Basal IκBα levels were higher in FM compared with MZp/MZ B cells (Fig. 3A), consistent with the suggestion that basal, canonical NF-κB signaling may be greater in the MZp/MZ population (16, 17). However, the relative rate of IκBα degradation following stimulation with either 20 μg/ml LPS or 10 μg/ml anti-IgM was similar in both subsets. Moreover, nuclear translocation of p65 and c-Rel were comparable in these subsets (Fig. 3B). Although activation marker changes were clearly evident, we were unable to identify IκBα degradation following stimulation with lower doses of LPS (e.g., 1 μg/ml) in either FM or MZp/MZ B cells (data not shown).

We next investigated Ca2+ flux and signaling via the PI3K pathway. Although we observed a clear anti-IgM-induced Ca2+ flux, LPS stimulation did not result in Ca2+ flux in either MZp/MZ or FM B cells (Fig. 4A). Phosphorylation of AKT was evaluated using a FACS-based assay. Consistent with a previous report (18), basal pAKT levels were ~2-fold higher in MZp/MZ compared with FM B cells (Fig. 4C). As anticipated, BCR engagement resulted in rapid phosphorylation of AKT in both populations. In contrast, stimulation with LPS failed to induce any increase in pAKT in either FM or MZp/MZ B cells for up to 20 min after stimulation (Fig. 4B and data not shown).
Recent work has suggested that the mTOR pathway is activated via PI3K-independent signals in response to LPS (18). Therefore, we also analyzed mTOR activation by determining relative phosphorylation of the ribosomal protein S6. Basal levels of pS6 were significantly higher in MZp/MZ B cells and this phosphorylation was further enhanced in response to TLR4 engagement. FM B cells, however, exhibited an equivalent fold increase in S6 phosphorylation following LPS stimulation (H110112-fold), demonstrating that this pathway was also similarly activated in this population (Fig. 4, D and E). We were also unable to demonstrate a significant change in S6 phosphorylation following low-dose LPS stimulation in either population (data not shown), consistent with the idea that these subsets are not markedly different with regard to these biochemical responses.

We next assessed ERK signaling because this pathway plays a key role in cell growth in response to a range of cell surface receptors. Consistent with previous data (19), stimulation with LPS resulted in weak phosphorylation of ERK at late time points (10–30 min) relative to BCR stimulation. This analysis again failed to reveal any major difference in S6 phosphorylation following low-dose LPS stimulation in either population (data not shown), consistent with the idea that these subsets are not markedly different with regard to these biochemical responses.

Although proximal LPS-driven signals have been extensively studied in other cell types, analyses using primary B cells have been more limited. Our combined findings indicate that LPS strongly promotes activation of the NF-κB and mTOR pathways, fails to activate calcium or PI3K signaling, and promotes delayed ERK activation. Surprisingly, despite the failure to the enter cell cycle, FM B cells exhibit no appreciable deficit in these proximal signals following TLR4 ligation.

**Basal mTOR signaling in MZ B cells facilitates the proliferative response to LPS**

Because we failed to observe major differences in proximal LPS-driven signals, we next asked whether the differential capacity for cycling might reflect key differences in the basal activation status of FM vs MZ B cells. Although the PI3K/AKT pathway plays a crucial role in cell proliferation, it is not directly activated upon LPS stimulation as shown above. Thus, if the increased basal PI3K/AKT signals permitted LPS-driven cycling in MZp/MZ B cells, blocking these signals should limit LPS-triggered proliferation, but not alter the capacity to upregulate activation markers. To test this idea, we treated total splenic B cells using either the PI3K-specific inhibitor wortmannin or the mTOR-specific inhibitor rapamycin. Cells were pretreated with inhibitor (or DMSO as control) for 1 h and subsequently stimulated with LPS or anti-IgM. Cell survival was minimally effected with either inhibitor at the doses used (data not shown). In anti-IgM-stimulated cells, wortmannin treatment lead to near complete ablation of up-regulation of all activation markers, whereas rapamycin mainly altered MHC II levels (Fig. 5A). BCR-induced proliferation was abrogated in response to either inhibitor (Fig. 5B). In contrast, after LPS stimulation, up-regulation of CD86, CD25, and MHC II were minimally impacted by either inhibitor (Fig. 5A). However, LPS-induced proliferation was completely abrogated
by rapamycin and only minimally impacted by wortmannin treatment (Fig. 5B).

These data imply that proliferation in response to LPS, but not up-regulation of activation markers, is mTOR dependent. Moreover, in contrast to BCR ligation, activation of the mTOR pathway in response to LPS is mainly PI3K independent. These data also show that high basal and/or triggered mTOR signals are crucial for the LPS-driven proliferative response in MZ B cells.

c-myc expression levels limit LPS proliferative responses in FM B cells

NF-κB target genes, including c-myc, A1, and Bcl-xL, play a crucial role in BCR- and TLR-triggered cell survival and proliferation (20–22). Therefore, we next investigated the expression of NF-κB target genes in these cell subsets. In unstimulated, freshly isolated FM B cells, c-myc transcript levels were consistently 3- to 4-fold lower compared with those in MZp/MZ B cells (Fig. 6, A, D, and E). In contrast, basal A1 and Bcl-xL levels were relatively similar, and LPS stimulation promoted nearly identical kinetics and absolute fold changes for both transcripts in both cell populations. Importantly, although the relative fold change in c-myc levels was similar or even greater at each time point in FM B cells, absolute c-myc transcript levels remained significantly lower at all time points in this population (Fig. 6A). Anti-IgM stimulation of FM B cells resulted in higher levels of all three transcripts, and the rate of increase and the overall level of c-myc transcripts was markedly higher compared with LPS-stimulated FM B cells.

Because c-myc is a key regulator for cell proliferation (reviewed in Ref. 23), these data suggested that failure to reach a threshold level of c-myc might represent a rate-limiting bottleneck in LPS-dependent FM B cell cycling. Furthermore, these data suggested that increasing c-myc dosage might rescue this proliferative defect. To test this idea, we sort-purified FM vs MZp/MZ B cells from c-myc tg mice and stimulated these cell populations with anti-IgM or LPS. As shown in Fig. 6, B and C, c-myc tg FM B cells, unlike wild-type (wt) cells, exhibited a robust, dose-dependent LPS-driven proliferative response as assessed by either [3H]thymidine incorporation or CFSE dilution. In contrast, the proliferative response to BCR engagement was only slightly enhanced in c-myc tg FM B cells, and we observed no significant difference in LPS-driven cycling in c-myc tg vs wt MZp/MZ B cells (except at very low doses of LPS stimulation). Of note, increased c-myc expression alone, however, was not sufficient to trigger cycling as shown by the dependence for cell cycling on LPS dosage.

We also assessed relative c-myc levels in FM and MZp/MZ B cells from wt and c-myc tg mice using Western blotting and semi-quantitative real-time PCR. In wt mice, c-myc protein expression was ~3- to 4-fold higher in MZp/MZ B cells compared with FM B cells and this correlated with ~3-fold higher levels of c-myc mRNA in MZp/MZ cells (Fig. 6, D and E). FM B cells from c-myc tg mice expressed ~4- to 6-fold higher levels of c-myc compared with wt FM B cells. These data correlated with a similar increase in c-myc mRNA and rescue of the LPS-driven proliferative response. In contrast, c-myc protein and mRNA levels were minimally increased (<2-fold) in c-myc-tg MZp/MZ B cells and correlated with the minimal change in proliferative responses to LPS.

Finally, we asked whether increased c-myc expression in MZ B cells might correlate with increased basal mTOR signaling. To address this question, we determined whether PI3K or mTOR signals were required for c-myc up-regulation in response to mitogen stimulation. Total splenic B cells were incubated with wortmannin or rapamycin before stimulation with anti-IgM or LPS, and c-myc expression was determined by quantitative PCR (Q-PCR) analysis. Neither drug inhibited c-myc expression in response to LPS (Fig. 6F). In contrast, c-myc levels after stimulation with anti-IgM were significantly decreased by wortmannin but not rapamycin treatment. These
results suggest that c-myc up-regulation is not linked to the mTOR pathway.

c-myc tg FM B cells exhibit partial T cell-independent immune responses

Because overexpression of c-myc in FM B cells leads to functional responses similar to those present in MZ B cells, we next asked whether this change was sufficient to promote participation in T cell-independent immune responses. To begin to address this question, we sort-purified FM and MZp/MZ B cells from wt and c-myc tg mice and determined the level of IgM production in response to LPS stimulation. As previously described (5), our results show that LPS-stimulated MZ B cells produce much higher levels of IgM compared with wt FM B cells (Fig. 7A). In addition, c-myc tg MZp/MZ B cells produced nearly identical levels of IgM as their wt counterparts (Fig. 7A). In contrast, c-myc tg FM B cells produced significantly more IgM than wt FM cells, although these levels remained less (approximately one-third) than those produced by MZp/MZ B cells.

We also determined whether FM B cells derived from wt vs c-myc tg mice gained the capacity to bind the T-independent type II Ag, TNP-Ficoll, following i.v. in vivo immunization. Consistent with previously published data (7), binding of TNP was significantly higher in MZp/MZ B cells compared with FM B cells and was equivalent for MZ cells derived from either wt and c-myc tg mice. Notably, c-myc tg FM B cells bound more TNP than wt FM B cells, although still less than MZp/MZ B cells (Fig. 7B). Together, these data indicate that overexpression of c-myc within FM B cells results in an increased capacity to participate in T cell-independent immune responses similar to the functional responses of MZ B cells.

Discussion

The current study was designed to address the potential events that limit the capacity of FM B cells to enter cell cycle in
response to LPS. Surprisingly, this delay in cell cycle entry is not predominantly due to differential sensitivity of FM vs MZ B cells to LPS. Indeed, our data indicate that both proximal LPS signaling pathways and downstream LPS-driven transcriptional activity are similarly activated in FM and MZ B cells. In contrast, the basal activation status of MZ B cells was significantly enhanced, thereby enabling this population to rapidly enter the cell cycle after LPS stimulation. Most notably, basal c-myc transcript and protein levels were significantly lower in FM compared with MZ B cells. Together, our findings suggest that in the majority of FM B cells, LPS stimulation fails to promote a sufficient level of c-myc to permit cell cycle entry. In contrast, MZp/MZ B cells attain this threshold via LPS-driven c-myc transcription in combination with higher basal levels of c-myc. Consistent with this interpretation, provision of higher basal levels of c-myc was sufficient to rescue the proliferative defect in FM B cells.

Several previous reports have demonstrated that FM B cells, compared with MZ B cells, proliferate poorly in response to LPS (5–7). These findings, in association with data showing higher levels of activation markers and reduced LPS-driven IgM production, have led to the interpretation that FM B cells are inherently less responsive to LPS. Consistent with this idea, we and others have also reported that RP105/MD-1, the key TLR4 coreceptor adaptor that confers LPS responsiveness in B cells (24), is expressed at significantly higher levels on MZ compared with FM B cells (14, 25). However, in contrast to this view, our current data indicate that LPS stimulation (even at doses as low as 0.1 μg/ml) leads to a similar fold change in activation markers in FM vs MZ B cells and that FM B cells differ in relative responsiveness only at very low doses of LPS. Activation of early signaling pathways was also comparable in FM and MZ B cells upon LPS stimulation: the kinetics of IκBα degradation and inducible phosphorylation of the ribosomal protein S6 (as a measurement for activation of the mTOR pathway) were similar in both B cell subsets. In addition, the induction of the TLR target genes AID and BAFF-R also occurred with similar kinetics in both mature populations. Together, these findings indicate that the observed delay in cell cycle entry in FM B cells was unlikely due to a failure to respond to LPS. Notably, previous work also suggests that a similar cycling-specific deficit may also exist in naive human peripheral blood B cells. In contrast to memory B cells, naive human B cells fail to proliferate in response to the TLR9 ligand CpG, yet both populations exhibit up-regulation of CD69 and CD86 and an increase in cell size following TLR9 stimulation (26).

Importantly, and as suggested by a recent report (18), our data indicate that the mTOR signaling cascade can be activated independently of proximal PI3K signaling in B cells in response to LPS. Although the fold induction of mTOR activation upon LPS stimulation was similar in FM and MZ cells, MZ B cells exhibited much higher basal PI3K and mTOR activity based upon assessment of relative phosphorylation of AKT and S6, respectively. To test whether this preactivation state was important in promoting LPS-driven cell cycle entry in MZ B cells, we pharmacologically blocked the PI3K vs the mTOR pathways and assessed relative expression of activation markers as well as cell proliferation. Inhibition of the PI3K pathway had little effect on these events. In contrast, mTOR inhibition significantly reduced proliferation yet had little effect on activation marker expression, indicating that mTOR signaling is essential for LPS-driven cell cycle entry and that basal mTOR signaling in MZ B cells contributes substantially to the ability of this subset to rapidly enter cell cycle entry in response to LPS.

The most striking observation in this study is the role for c-myc levels in controlling FM B cell cycle entry upon LPS stimulation. The protooncogene c-myc is a member of the myc transcription factor family (c-, N- and L-myc) and is involved in multiple cellular functions, including cell cycle regulation, proliferation, growth, apoptosis, differentiation, and metabolism (reviewed in Ref. 27). c-myc is crucial for normal B cell growth and differentiation and deregulated c-myc expression contributes to development of a range of human malignancies (28, 29).
Accordingly, c-myc deficiency results in impaired B cell development at the pre-B cell stage leading to reduced numbers of B cells in both the bone marrow and periphery, and c-myc tg mice exhibit an increase in bone marrow B cells and develop pre-B cell leukemias. c-myc is also crucial for proliferation of mature B cells in response to mitogenic stimuli including both BCR and TLR4 stimulation (22, 30), and stimulation of bulk splenic B cells with LPS results in rapid up-regulation of c-myc transcript levels (31, 32).

Grumont et al. (22) have also previously demonstrated that total c-myc levels are important for controlling B cell cycle entry. Furthermore, a threshold level of c-myc is required for cells to enter S phase and previous work has indicated that c-myc controls the decision of cells to divide or not (33, 34). Notably, our analyses show that distinct mature splenic B cell subsets exhibit a major difference in basal c-myc transcript and protein levels. Basal c-myc protein levels were significantly higher in MZ compared with FM B cells and correlated with mRNA expression as determined by quantitative real-time PCR. Although c-myc transcripts were similarly induced in both subsets in response to all doses of LPS, our combined findings suggest that this initial difference in basal c-myc expression is crucial in dictating the cycling response of MZ vs FM B cells. Consistent with this idea, increased expression of c-myc rescues the cycling deficit in FM B cells and permits a robust FM B cell proliferative response similar to that of wt MZ B cells in response to LPS.

Interestingly, although the majority of FM B cells failed to cycle at early time points following LPS stimulation, a fraction of sort-purified cells consistently entered the cell cycle within 48 h. Assuming that c-myc up-regulation occurs in a stochastic manner within the overall pool of FM B cells, we would predict that a limited number of cells might attain threshold c-myc levels necessary to enter the cell cycle. Because it is challenging to directly prove this idea, we sorted proliferating vs nonproliferating FM B cells 3 days after LPS stimulation based on CFSE dilution (CFSE<sup>high</sup> vs CFSE<sup>low</sup>) and determined c-myc transcript expression. Although c-myc was higher in proliferating compared with nonproliferating cells, overall transcript levels were relatively low (data not shown). This is not surprising since highest levels of myc expression occur within hours of LPS stimulation. An alternative explanation for the observed proliferation of a limited fraction of FM B cells is that these cells may have encountered local costimulatory signals in vivo, such as CD40 or Notch ligand, thereby leading to higher basal c-myc levels before LPS stimulation in vitro.

Notably, although our data document differential c-myc expression in FM vs MZ B cells, the signal(s) that establishes this basal difference remains to be identified. Although the mTOR pathway plays a crucial role in supporting LPS-induced proliferation, inhibition of the mTOR pathway does not lead to reduced c-myc expression levels. Thus, although the mTOR pathway promotes cycling, it does not appear to directly regulate c-myc levels. A key candidate pathway in this regard is BAFF-R signaling. BAFF is the most important B cell survival factor in the periphery and BAFF signals are crucial for MZ B cell development (35). BAFF-R-dependent noncanonical NF-κB signals strongly promote the mTOR cascade (36, 37), and it is possible that in parallel BAFF-triggered canonical NF-κB signals may directly modulate c-myc expression. MZ B cells express significantly higher levels of BAFF-R compared with FM cells (15); thus, increased canonical NF-κB signals in this subset may be sufficient to explain their higher basal levels of c-myc expression. Finally, it is important to point out that increased c-myc expression alone is not sufficient to drive FM B cells into cycle: c-myc tg B cells (MZ or FM B cells) do not spontaneously proliferate and the proliferative response of c-myc tg FM B cells remains LPS dose dependent. Additional studies will be required to define the signals that synergize to promote cycling including, in particular, the specific components of the mTOR cascade necessary for these events.

In summary, our findings contribute to a better understanding of the regulation of TLR signals in mature B cell subsets. Although MZ B cells have been shown to function primarily as innate-like immune cells, FM B cells largely generate an adaptive immune response that can be amplified by TLR costimulation. In FM B cells, the tight control of TLR4-dependent proliferative response likely functions to limit nonspecific amplification of self-reactive cells and thereby help prevent the development of autoimmunity. Consistent with this idea and the concept that clonal expansion is important before Ab production, our data show that overexpression of c-myc in FM B cells also results in an increased facility to produce IgM after stimulation with LPS in vitro and in a greater capacity for T-independent, type II Ag binding in vivo. The importance of TLR signals (in the absence of exogenously delivered TLR ligands) for the pathogenesis of autoimmune diseases has recently been shown by crossing BAFF tg mice with MyD88-deficient animals. Under these conditions, the development of the lupus-like autoimmunity characteristic of BAFF tg mice is largely eliminated, thereby highlighting the importance for B cell-intrinsic TLR signals in these events (38). Taken in concert with other reports showing the role of B cell autonomous TLR signaling in autoimmunity (reviewed in Ref. 39), our findings suggest that tight regulation of TLR signaling in FM B cells may be critical for B cell tolerance. Notably, FM B cells proliferate robustly to LPS in association with either BCR or CD40 costimulation (14). These combined observations suggest that B cells that receive an Ag-specific signal may be synergistically amplified via TLR signals. Thus, approaches aimed at limiting TLR signals in activated mature FM B cells may provide an important therapeutic avenue for use in patients with a range of autoimmune diseases.

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Disclosures

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