Cutting Edge: Regulation of TLR4-Driven B Cell Proliferation by RP105 Is Not B Cell Autonomous

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Cutting Edge: Regulation of TLR4-Driven B Cell Proliferation by RP105 Is Not B Cell Autonomous

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Mechanistic understanding of RP105 has been confounded by the fact that this TLR homolog has appeared to have opposing, cell type-specific effects on TLR4 signaling. Although RP105 inhibits TLR4-driven signaling in cell lines and myeloid cells, impaired LPS-driven proliferation by B cells from RP105−/− mice has suggested that RP105 facilitates TLR4 signaling in B cells. In this article, we show that modulation of B cell proliferation by RP105 is not a function of B cell-intrinsic expression of RP105, and identify a mechanistic role for dysregulated BAFF expression in the proliferative abnormalities of B cells from RP105−/− mice: serum BAFF levels are elevated in RP105−/− mice, and partial BAFF neutralization rescues aberrant B cell proliferative responses in such mice. These data indicate that RP105 does not have dichotomous effects on TLR4 signaling and emphasize the need for caution in interpreting the results of global genetic deletion. The Journal of Immunology, 2012, 188: 2065–2069.

Immunobiological understanding of RP105 was shaped by its discovery and initial analysis in B cells. Ab-mediated cross-linking of RP105 leads to B cell activation and proliferation, providing protection against radiation- and steroid-induced apoptosis (1), but sensitization to apoptosis in response to BCR ligation (2). Anti-RP105–driven proliferation of RP105, and identify a mechanistic role for dysregulated BAFF expression in the proliferative abnormalities of B cells from RP105−/− mice: serum BAFF levels are elevated in RP105−/− mice, and partial BAFF neutralization rescues aberrant B cell proliferative responses in such mice. These data indicate that RP105 does not have dichotomous effects on TLR4 signaling and emphasize the need for caution in interpreting the results of global genetic deletion. The Journal of Immunology, 2012, 188: 2065–2069.

In light of these paradoxical findings—with RP105 appearing to facilitate or inhibit TLR4 signaling, depending on the cell type involved—we reinvestigated the B cell proliferative responses of RP105−/− mice.

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Materials and Methods

Mice

RP105<sup>−/−</sup> mice (6) backcrossed ≥12 generations to a C57BL/6 background, C57BL/6 mice, μMT mice (C57BL/6; Jax), BAFF-Tg mice (C57BL/6; Biogen Idec) (10), and TACI<sup>−/−</sup> mice (C57BL/6) (11) were maintained in specific pathogen-free facilities. Age- and sex-matched mice were used in all experiments. Care was provided in accordance with National Institutes of Health guidelines in studies approved by Institutional Animal Care and Use Committees.

In vitro assays and reagents

Splenic B cells were purified by negative selection with immunomagnetic beads (Miltenyi Biotech B cell Isolation Kit: CD43, CD4, Ter119 microbeads) to ≥98% purity. Purified B cells (1 × 10<sup>6</sup> cells/ml) or sorted marginal zone (MZ) and follicular (FO) B cells (0.45 × 10<sup>6</sup> cells/ml) were plated in triplicate, stimulated for 36 h with repurified E. coli K235 LPS (S. Vogel), CpG DNA (Coley Pharmaceutical), or PBS. Proliferation was quantified by thymidine incorporation over an additional 8 h. Single-cell leukocyte suspensions were incubated with Fluorochrome-labeled Ab for 30 min at 4°C. Events (100–150K per data point) were acquired on an LSR II Flow cytometer and analyzed using FlowJo software. For B cell subset sorting, purified B cells were labeled with Ab to CD19, B220, CD21, and CD23, and sorted to ≥97% purity, using a FACS Aria II. Except for CD24 Ab (BioLegend), all Abs were labeled with Ab to CD19, B220, CD21, and CD23, and sorted to ≥97% purity, using a FACS Aria II. Except for CD24 Ab (BioLegend), all Abs were labeled with Ab to CD19, B220, CD21, and CD23, and sorted to ≥97% purity, using a FACS Aria II. Except for CD24 Ab (BioLegend), all Abs were labeled with Ab to CD19, B220, CD21, and CD23, and sorted to ≥97% purity, using a FACS Aria II.

In vivo transfers and proliferation assays

Splenic B cells were purified by negative immunomagnetic separation (CD43, CD4, Ter119 microbeads; Miltenyi Biotech), and 30–40 × 10<sup>6</sup> were injected i.v. into C57BL/6 mice (C57BL/6) (11) were maintained in specific pathogen-free facilities. Age- and sex-matched mice were used in all experiments. Care was provided in accordance with National Institutes of Health guidelines in studies approved by Institutional Animal Care and Use Committees.

Results and Discussion

Impaired TLR4-driven proliferation is recapitulated in MZ B cells from RP105<sup>−/−</sup> mice

The B cell proliferative phenotype of RP105<sup>−/−</sup> mice has remained robust despite extensive crossing onto a C57BL/6 background: impaired LPS-driven proliferation, but normal proliferation to other stimuli (Supplemental Fig. 1A). This proliferative inhibition is not due to decreased B cell expression of TLR4/MD-2 (7); data not shown). Note, blunting of LPS-driven proliferation is not associated with increased B cell death (Supplemental Fig. 1B). The lack of genotype-specific effects on apoptosis, in the face of an apparent dose-response relationship in the ability of TLR signaling to prevent apoptosis (no stimulation—LPS—CpG; Supplemental Fig. 1A, 1B), suggested that impaired LPS-driven proliferation might not be due to suppressed TLR4 signaling in RP105<sup>−/−</sup> B cells. Impaired LPS-driven proliferation was also seen in vivo: FACS analysis revealed that significantly fewer splenic B cells in RP105<sup>−/−</sup> mice, compared with B cells in wild-type controls, underwent proliferation in response to LPS challenge (Fig. 1A).

Marked variability is observed in LPS proliferative responsiveness across splenic B cell populations. Despite the fact that they represent a minor subset numerically, MZ B cells constitute the dominant such subpopulation, proliferating robustly after LPS stimulation. By contrast, LPS-stimulated proliferation by FO B cells is much weaker, indeed nearly absent when sort purities are high (12). Transitional type 2 B cells exhibit intermediate sensitivity (13). Although B cell development proceeds normally in RP105<sup>−/−</sup> mice (6), it remained possible that these proliferative abnormalities were a function of alterations in the development or survival of B cell subsets variably responsive to LPS-driven proliferation. The proportions of these subpopulations were, however, indistinguishable between RP105<sup>−/−</sup> and wild-type mice (Supplemental Fig. 1C).

We subsequently directly tested the ex vivo proliferative responses of highly purified B cell subpopulations. Notably, MZ B cells from RP105<sup>−/−</sup> mice recapitated the blunted response to LPS observed in unfraccionated splenic B cells, despite normal proliferative responsiveness to CpG DNA (Fig. 1B), data that underscore the fact that this phenotype is not due to altered B cell subset representation. As expected, FO B cells from RP105<sup>−/−</sup> and wild-type mice exhibited little to no proliferation (and no differential proliferation) in response to LPS (Supplemental Fig. 1D). It should be noted that it is not possible to analyze LPS-driven proliferation of these subsets in vivo, as LPS stimulation alters expression of surface markers that can be used in their definition, including CD23, CD1d, and IgD (14; data not shown).

Despite proliferative differences, MZ and FO B cells exhibit similar changes in LPS-driven surface activation marker expression and signaling pathway activation (12). Similarly, we found that LPS-driven CD69 and MHC class II expression was comparable in MZ B cells (and FO B cells) purified from RP105<sup>−/−</sup> and wild-type mice (Supplemental Fig. 1E, 1F; and data not shown). Further, no significant genotype-specific differences in LPS-driven B cell secretion of IL-10 or IL-6 were observed (Supplemental Fig. 1G, 1H). These findings of altered LPS-driven proliferation, in the absence of differences in other LPS-driven responses (including prevention of apoptosis; see above), are difficult to reconcile with a fundamental difference in responsiveness at the level of the LPS receptor.

**FIGURE 1.** The LPS-driven proliferative defect of B cells from RP105<sup>−/−</sup> mice is recapitulated in MZ B cells. (A) Mice were injected i.p. with LPS (40 μg), and splenic B cell proliferation was quantified by BrdU incorporation 48 h later. Means ± SE are depicted; n = 10 mice per genotype, pooled from two independent experiments. *p < 0.005, unpaired two-tailed t test. (B) FACS-sorted MZ B cells were stimulated as indicated, and proliferation was quantified by thymidine incorporation. Means ± SE of triplicate cultures are depicted; n = 4 pooled mice per genotype; representative of two separate experiments. *p < 0.0001, unpaired two-tailed t test.
RP105-associated defects in LPS-driven B cell proliferation are not B cell autonomous

Of note, splenic size and leukocyte numbers were significantly increased in RP105+/− mice (Fig. 2A, 2B). That said, RP105+/− mice exhibited no disproportionate changes in cell composition in either B cell or non-B cell compartments (Supplemental Fig. 1C, 1D): all leukocyte populations were proportionately increased. Further, despite blunted LPS-driven in vivo B cell proliferation, basal in vivo B cell proliferation was significantly increased in RP105+/− mice (Fig. 2C). Increased BrdU incorporation was specifically observed in RP105+/−/MZ B cells (Fig. 2D), indicating increased basal B cell proliferation in the RP105+/−/MZ B compartment—the same compartment exhibiting blunted LPS-driven proliferation.

Taken together, these data suggested that proliferative differences between wild-type and RP105+/− B cells might not be due to B cell expression (or not) of RP105. To test this, we purified splenic B cells from wild-type and RP105+/− mice and transferred them into B cell-deficient, RP105-sufficient μMT mice. Eight wk later, splenic B cells were purified and stimulated in vitro. Given the contrasting proliferative responsiveness of MZ and FO B cells, we first quantified the engraftment of these subsets. Skewing toward MZ B cell engraftment, reported previously, was observed; however, no genotype-specific differences in the proportions of MZ and FO B cells were seen (Fig. 3A). Notably, as shown in Fig. 3B, engraftment of RP105-deficient and -sufficient B cells in an RP105-sufficient environment obviated differences in LPS-driven B cell proliferative responses. These data strongly suggest that RP105-associated defects in LPS-driven B cell proliferation are not a function of B cell autonomous lack of expression of RP105.

Integral role for BAFF in altered proliferative responses of B cells from RP105+/− mice

BAFF is essential for B cell maturation and survival. Overexpression drives B cell hyperplasia; neutralization leads to B cell death (15). As TLR signaling induces BAFF expression (15), we tested whether compromised control of TLR signaling in RP105+/− mice led to increased BAFF activity. Indeed, RP105+/− mice, compared with wild-type controls, exhibited significantly increased basal serum BAFF levels, a difference exacerbated by LPS stimulation (Fig. 4A). No genotype-specific differences in splenic APRIL expression were observed (data not shown). B cell expression of two of the three BAFF receptors, TACI and BAFF-R, can also be upregulated by TLR stimulation (15). As might be expected, B cells from RP105+/− mice exhibited significantly increased basal expression of TACI (Fig. 4B), although genotype-specific differences in BAFF-R expression were not observed (data not shown). TACI expression is thought to limit the amount of soluble BAFF available for signaling (15). That said, no in vivo data bearing directly on the subject appear to have been published. TACI+/− mice did indeed have significantly increased serum BAFF concentrations at baseline—despite their expanded B cell compartment (11)—and after LPS stimulation (Fig. 4C). Increased basal TACI expression may thus lead to underestimation of basal BAFF production in RP105+/− mice. Consonant with this, quantitative RT-PCR–mediated quantification of splenic BAFF mRNA revealed a mean 30% increase in basal expression levels in RP105+/− mice (data not shown).

To define the relevance of BAFF overexpression to B cell proliferation in RP105+/− mice, we turned first to BAFF-Tg mice (10). Such mice also exhibit significantly increased basal B cell proliferation (data not shown). As the even more

**FIGURE 2.** RP105+/− mice exhibit increased baseline spleen size, splenic leukocyte number, and in vivo B cell proliferation. Spleen size (A) and splenic leukocytes (B) were quantified in wild-type mice (white bars) and RP105+/− mice (black bars). Means ± SE from a single experiment are depicted. For (A), n = 28 mice per genotype. *p < 0.0001, unpaired two-tailed t test. For (B), n = 12 mice per genotype. *p < 0.05, unpaired two-tailed t test. In vivo baseline B cell proliferation was assessed by flow cytometric quantification of BrdU incorporation, 48 h after ip injection of PBS. n = 6 mice per genotype, representative of three independent experiments. *p < 0.005, unpaired two-tailed t test. (C) In vivo baseline B cell proliferation was assessed by flow cytometric quantification of BrdU incorporation, 48 h after ip injection of PBS. n = 6 mice per genotype, representative of three independent experiments. *p < 0.005, unpaired two-tailed t test. (D) In vivo baseline B cell proliferation was assessed by flow cytometric quantification of BrdU incorporation in MZ (CD19+ B220− CD21high CD233−) and FO (CD19+ B220− CD21high CD233+) B cell subsets from wild-type mice (white bars) and RP105-deficient mice (black bars). Means ± SE are depicted; n = 6 mice per genotype. *p < 0.05, unpaired two-tailed t test.

**FIGURE 3.** RP105-deficient and -sufficient B cells have similar TLR4-driven proliferative responses after adoptive transfer into an RP105-sufficient environment. Splenic B cells were purified from wild-type mice (white bars) and RP105+/− mice (black bars), transferred into μMT (B cell deficient, RP105+/−) mice, and allowed to reconstitute for 8 wk. (A) Following engraftment, MZ B and FO B cell subsets were quantified by flow cytometry. (B) B cell proliferative responses were quantified by thymidine incorporation. Means ± SE are depicted. n = 12−15 mice per genotype, pooled from three independent experiments. NS, unpaired, two-tailed t test.
FIGURE 4. Dysregulated BAFF expression is integral to the altered TLR4 proliferative responsiveness of RP105<sup>−/−</sup> B cells. (A) Kinetic analysis of serum BAFF levels from wild-type mice (white bars) and RP105<sup>−/−</sup> mice (black bars) in response to i.p. challenge with LPS (40 µg). Means ± SE are depicted; n = 6 mice per genotype. *p < 0.05, **p < 0.005, unpaired two-tailed t test. (B) Splenic B cell expression of TACI, 48 h after i.p. injection of PBS in wild-type mice (white bars) or RP105<sup>−/−</sup> mice (black bars). Means ± SE are depicted; n = 9–12 mice per genotype, pooled from two independent experiments. *p < 0.05, **p < 0.0001, unpaired two-tailed t test. (C) Kinetic analysis of serum BAFF levels from wild-type mice (white bars) and TACI<sup>−/−</sup> mice (black bars) in response to i.p. challenge with LPS (40 µg). Means ± SE are depicted; n = 5 mice per genotype from a single experiment. *p < 0.05, **p < 0.005, unpaired two-tailed t test. (D–F) RP105<sup>−/−</sup> mice (black bars) and wild type mice (white bars) were treated i.p. with BCMA-Fc (300 ng) or IV-Ig (300 ng). Six days later, splenic MZ B and FO B cell subsets were quantified by flow cytometry (D), and splenic B cell proliferation was quantified by BrdU incorporation (E, F) 48 h after i.p. challenge with LPS (40 µg) (E) or PBS (F). Black bars, RP105<sup>−/−</sup> mice; white bars, wild-type mice. Means ± SE are depicted; n = 8–11 mice per group (E, F), pooled from two independent experiments. *p < 0.05, **p < 0.005, ANOVA/Bonferroni post hoc test.

Robust BAFF overexpression seen in BAFF-Tg mice is associated with a significant increase in the proportion of MZ B cells (10), we analyzed proliferation of purified MZ B cells. Similar to RP105<sup>−/−</sup> mice, MZ B cells from BAFF-Tg mice exhibited blunted LPS-driven, but intact CpG-driven, proliferation (Supplemental Fig. 2). These data suggested a possible role for BAFF overexpression in the B cell proliferative abnormalities of RP105<sup>−/−</sup> mice—mice with considerably less dramatic perturbations in immune homeostasis than BAFF-Tg mice. We thus used a huBCMA-Fc fusion protein (16) to test whether partial BAFF neutralization would rescue B cell proliferative responses in RP105<sup>−/−</sup> mice. Robust neutralization of BAFF by high doses of huBCMA-Fc leads to B cell depletion (16). We thus quantified in vivo B cell proliferation 6 d after treating mice with a low dose of huBCMA-Fc, defined by preliminary dose-titration experiments (300 ng), that altered neither B cell numbers nor B cell subset composition (Fig. 4D), nor the proliferative responsiveness of B cells in wild type mice (data not shown). Notably, such treatment reversed the LPS proliferative hyporesponsiveness of RP105<sup>−/−</sup> B cells (Fig. 4E). It also reversed their basal increase in B cell proliferation (Fig. 4F). Thus, BAFF concentrations are increased in RP105<sup>−/−</sup> mice, and partial neutralization of BAFF rescues aberrant B cell proliferation in such mice.

These data indicate that the B cell proliferative abnormalities of RP105<sup>−/−</sup> mice are not the result of B cell-autonomous (lack of) expression of RP105, and implicate dysregulated BAFF expression in the generation of these abnormalities.

Broader biological relevance for the pathways outlined in this article may well exist. In line with recent data showing BAFF overexpression in diverse human autoimmune diseases (15), it was reported almost 30 y ago that patients with lupus exhibit defective LPS-driven B cell proliferation (17). Whether these two findings are mechanistically related, as suggested by the current data, remains to be tested.

Although TACI facilitates T cell-independent type II Ab responses, TACI negatively regulates B cell compartment size. Current data support the existence of both indirect (restriction of BAFF availability) and direct (B cell signaling) mechanisms for the latter activity (15). Given increased basal TACI expression by RP105<sup>−/−</sup> mice, it is tempting to speculate that the mechanisms underlying restriction of LPS-driven proliferation by BAFF overexpression in such mice involve TACI signaling. Concordant with this, despite the fact that TACI<sup>−/−</sup> mice exhibit increased serum BAFF levels, MZ B cells from TACI<sup>−/−</sup> mice [subset purification performed because of elevated percentages of MZ B cells in such mice (18)] do not exhibit significant blunting of LPS-driven proliferation (data not shown).

We have focused in this article on B2 B cells; indeed, all in vitro studies have employed a negative selection approach that depletes B1 cells. Whether the pathways defined in this article also affect B1 cell function [B1 cell numbers are unchanged in RP105<sup>−/−</sup> mice (6)] remains to be defined.

Our data resolve some of the apparent biological complexities associated with RP105. There is no need to postulate...
dichotomous, cell type-specific effects on TLR4 signaling. Generation of mice with cell type-specific deletions in RP105 expression should allow for clear definition of whether RP105 is a biologically relevant inhibitor of TLR4 signaling in B cells, as it is in myeloid cells. More broadly, the critical function(s) of B cell-intrinsic RP105 expression remain to be defined. In particular, it is not known whether the pathways of B cell activation and proliferation stimulated by Ab-mediated cross-linking of RP105 model the effects of any natural ligands for RP105. If so, identification of such ligands and mechanistic analysis of the roles of the pathways activated by such ligands in homeostasis and disease would be of obvious biological, and potentially therapeutic, interest.

Disclosures
The authors have no financial conflicts of interest.

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