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Cutting Edge: Ezrin Regulates Inflammation by Limiting B Cell IL-10 Production

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IL-10 produced by B cells is important for controlling inflammation, thus underscoring the need to identify mechanisms regulating its production. In this study, we demonstrate that conditional deletion of ezrin in B cells increases IL-10 production induced by TLR4 ligation. The MyD88-independent Toll/IL-1R domain–containing adapter inducing IFN-β–IFN regulatory factor 3 pathway is required for Ezrin-deficient B cells to produce higher IL-10 upon LPS stimulation. Treatment of B cells with a novel small-molecule inhibitor of ezrin induces its dephosphorylation and increases LPS-induced NF-κB and IFN regulatory factor 3 activation and IL-10 secretion, indicating a role for threonine 567 phosphorylation of ezrin in limiting IL-10. Loss of ezrin in B cells results in dampened proinflammatory response to a sublethal dose of LPS in vivo, which is dependent on increased IL-10 production. Taken together, our data yield new insights into molecular and membrane-cytoskeletal regulation of B cell IL-10 production and reveal ezrin as a potential therapeutic target in inflammatory diseases. The Journal of Immunology, 2016, 196: 000–000.

In addition to their essential role in Ab production, B cells regulate immune responses by secreting effector cytokines. Of these, IL-10 has gained attention due to its ability to suppress the functions of macrophages, dendritic cells, and T cells (1). As B cell–secreted IL-10 has potential for therapeutic suppression of autoimmune inflammation (2–4), identification of proteins that regulate its production in B cells is clinically significant. B cells secrete IL-10 in response to TLR ligands LPS, CpG, and Pam3CSK4 (5). TLR4 employs both MyD88-dependent and -independent pathways to signal downstream, whereas TLR2 and TLR9 operate exclusively, indicating a role for threonine 567 phosphorylation of ezrin in limiting IL-10. Loss of ezrin in B cells results in dampened proinflammatory response to a sublethal dose of LPS in vivo, which is dependent on increased IL-10 production. Taken together, our data yield new insights into molecular and membrane-cytoskeletal regulation of B cell IL-10 production and reveal ezrin as a potential therapeutic target in inflammatory diseases. The Journal of Immunology, 2016, 196: 000–000.

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Cytokine assays

Mouse IL-10, IL-1β, IL-6, and TNF-α ELISA Ready-SET-Go! ELISA kits (eBioscience) were used according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using primers for IL-10 (forward, 5'-CCAAGCTTATCAGGAAATG-3' and reverse, 5'-TCTGAAAGGGTCCTCACGCTTC-3'), and β2-microglobulin (forward, 5'-TCAGTCGCCGTCGCCCTTC-3' and reverse, 5'-CAAGCACCAGAAACATGAGTGC-3'). Fold induction was calculated using MB1<sup>cre<sup> cells cultured in media as a reference.

Flow cytometry

TLR4 was detected by staining B cells with purified Ab to TLR4 (Abcam), followed by Alexa Fluor 488–conjugated anti-mouse IgG (Molecular Probes). Surface BCR was detected as described before (11) using PE-conjugated F(ab) fragment of anti-mouse IgM (BD Pharmingen). For detection of intracellular IL-10, purified B cells were stimulated with 10 μg/ml LPS for 48 h at 37°C, with PMA, ionomycin, and monensin added for the last 5 h (15). Cells were fixed with 4% paraformaldehyde, stained with fluorochrome-conjugated Abs with PMA, ionomycin, and monensin added for the last 5 h (15). Cells were stained with fluorochrome-conjugated anti–IL-10 Ab (BD Pharmingen) (15).

Flow cytometry

CD19<sup>+</sup>CD21<sup>lo</sup>CD23<sup>+</sup>, permeabilized, and stained with allophycocyanin-conjugated anti–IL-10 Ab (BD Pharmingen) to identify B cell subsets (regulatory B10 cells, CD19<sup>+</sup>CD5<sup>+</sup>CD1d<sup>+</sup>; plasmablasts, CD19<sup>+</sup>B220<sup>+</sup>CD138<sup>+</sup>; B1, CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> marginal zone [MZ] B, CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> and follicular, CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>); permeabilized, and stained with allophycocyanin-conjugated anti–IL-10 Ab (BD Pharmingen) (15).

Statistical analysis

All analyses were performed using Prism 4 (GraphPad Software) using an α level of 0.05. The p values were *p < 0.05, **p < 0.01, and ***p < 0.0001.

Results and Discussion

Ezrin-deficient B cells produce higher levels of IL-10

To investigate the role of ezrin in IL-10 production by B cells, we employed mice in which the deletion of ezrin is restricted to the B cell lineage (hereafter referred to as Ez-def) (11). We compared the ability of MB1<sup>cre<sup> cells and Ez-def B cells to produce IL-10 by first stimulating them with anti-IgM and different TLR ligands that are known to induce IL-10 secretion. Anti-IgM did not induce detectable levels of IL-10 in naive MB1<sup>cre<sup> or Ez-def B cells (Fig. 1A), consistent with previous reports (16, 17). Ez-def B cells secreted 2-fold more IL-10 than MB1<sup>cre<sup> B cells in response to LPS (TLR4), but not to CpG (TLR9) or Pam3CSK4 (TLR2) (Fig. 1A). TLR4 was similarly expressed on MB1<sup>cre<sup> and Ez-def B cells (Supplemental Fig. 1A) and hence excluded as a reason for differential IL-10 secretion. siRNA-mediated knockdown of ezrin expression in purified B cells from C57BL/6 mice also caused significantly higher induction of IL-10 production upon LPS treatment (Supplemental Fig. 1B), further supporting the potentiating effect of ezrin deficiency on IL-10 production. We performed qPCR to determine if higher IL-10 production by Ez-def cells was due to altered IL-10 gene expression. Ez-def B cells showed 3- to 4-fold higher IL-10 mRNA induction than MB1<sup>cre<sup> B cells in response to LPS (Fig. 1B), indicating that the absence of ezrin in B cells enhances IL-10 gene expression in response to TLR4 stimulation. LPS-primed Ez-def B cells that were subsequently stimulated with anti-IgM produced even greater levels of IL-10 than similarly stimulated MB1<sup>cre<sup> B cells (Supplemental Fig. 1C) and showed higher IL-10 mRNA expression (Supplemental Fig. 1D). The levels of surface IgM on LPS-primed MB1<sup>cre<sup> or Ez-def B cells were similar (Supplemental Fig. 1E). As several subsets of B cells, including regulatory B10, plasmablasts, B1, MZ, and follicular B cells can produce IL-10 under different circumstances, we examined their relative contribution to higher IL-10 production in the absence of ezrin. Purified MB1<sup>cre<sup> and Ez-def B cells were treated with LPS for 48 h and intracellular IL-10 detected by flow as described (15). The MB1<sup>cre<sup> B cells showed a significant increase in only the IL-10<sup>+</sup> B10 subset upon LPS stimulation, whereas in the Ez-def group, the percentages of IL-10<sup>+</sup> B10, MZ B cells, and plasmablast subsets were all significantly upregulated in response to LPS (Fig. 1C). All B cell subsets showed higher IL-10-producing cells in the absence of ezrin (Fig. 1C). The biggest fold induction in IL-10<sup>+</sup> cells in MB1<sup>cre<sup> was observed in the B10 subset (8-fold), whereas among the Ez-def B cell subsets, the B10 (21.1-fold), MZ (3.8-fold), and plasmablasts (9.2-fold) showed the strongest fold induction. These data suggest that the B10 B cells, MZ B cells, and plasmablasts are the most sensitive to ezrin deletion.

Higher IL-10 production by Ezrin-deficient B cells is due to stronger NF-κB signaling

IL-10 production is regulated by multiple pathways including, p38, PI3K, ERK, and NF-κB (1). Therefore, we employed immunoblotting with phospho-specific Abs to report proteins to examine if any of these pathways was altered in Ez-def B cells. Phosphorylation of p38, ERK, and Akt was similarly induced upon stimulation of MB1<sup>cre<sup> and Ez-def B cells with LPS (Fig. 2A). However, phosphorylation of the p65 subunit of NF-κB was at least 2-fold higher, and degradation of IkB was accelerated in Ez-def B cells as compared with MB1<sup>cre<sup> B cells (Fig. 2B). Although TLR2 and TLR9 only signal through MyD88, TLR4 can additionally recruit a MyD88-independent pathway that relies on the adaptor TRIF that further activates TANK-binding kinase 1 (TBK1), IRF3, and NF-κB (6). Because ezrin deletion did not affect the activation of p38, ERK and PI3K downstream of TLR4 and also did not alter TLR2- and TLR9-dependent IL-10 secretion, we hypothesized that the MyD88-independent pathway may be

![Graph showing the results](http://www.jimmunol.org/)
dominant in Ez-def B cells. Indeed, Ez-def B cells stimulated with LPS showed stronger phosphorylation of TBK1 and IRF3 (Fig. 2B). Together, these data suggest that deletion of ezrin promotes MyD88-independent NF-κB signaling. To further delineate the relative requirement of each of the above pathways in IL-10 production in the absence of ezrin, we employed pharmacological inhibitors of PI3K, p38, MEK, IκB kinase (IKK), and siRNA-mediated knockdown of Myd88, TRIF, and IRF3. We pretreated MB1cre/+ and Ez-def B cells with inhibitors of the kinases p38 (SB203580), MEK1/2 (U0126), PI3K (LY294002), and IKK (PS1145) for 1 h before stimulating them with LPS for 48 h. p38 and PI3K inhibition completely abolished IL-10 production in both MB1cre/+ and Ez-def B cells, and inhibition of ERK signaling lowered IL-10 production in both groups by 50% (Fig. 3A).

In contrast, IKK inhibition had a more pronounced effect on IL-10 secretion by Ez-def B cells (Fig. 3A). Next, MB1cre/+ and Ez-def B cells were incubated with MyD88 or TRIF siRNAs for 48 h, followed by stimulation with LPS for 48 h. Interestingly, knockdown of MyD88 expression (Supplemental Fig. 1F) severely affected IL-10 production by MB1cre/+ B cells but not as much in Ez-def B cells (Fig. 3B).

Inhibition of ERM phosphorylation increases B cell IL-10 production

As phosphorylation of ERM proteins regulates their membrane-cytoskeleton crosslinking ability, and hence their activity, we next tested if ezrin phosphorylation was required for IL-10 suppression. For this, we employed a novel small-molecule inhibitor of ezrin (NSC668394), which directly binds to ezrin (and also to moesin and radixin, albeit less well) and causes its dephosphorylation (14, 18). B cells purified from C57BL/6 mice were pretreated with DMSO or 0.1 or 0.5 μM NSC668394 for 1 h before stimulation with LPS for 48 h. Pretreatment with NSC668394 inhibited IL-10 production in MB1cre/+ and Ez-def B cells by 50% and 75%, respectively (Fig. 3C). These data indicate that under normal conditions the MyD88-dependent pathway, which activates p38, PI3K, ERK, and NF-κB signaling, is the dominant pathway for IL-10 production and that ezrin suppresses MyD88-independent TRIF-dependent IL-10 secretion. Deletion of ezrin releases the brakes on the TRIF pathway, which takes over IL-10 production via activation of MyD88-independent NF-κB and IRF3. Thus, a stronger TRIF–TBK1–IKK–IRF3 axis appears to be the dominant mode of increased IL-10 in LPS-stimulated Ez-def B cells.
0.5 μmol NSC668394 for 1 h, followed by incubation with 10 μg/ml LPS. Treatment of B cells with LPS led to ERM dephosphorylation, consistent with our previous data (12). This dephosphorylation was further enhanced in the presence of NSC668394 (Fig. 4A). LPS stimulation also induced phosphorylation of TBK1, p65, and IRF3 and degradation of IκB, and each of these was further increased by 2- to 3-fold in the presence of 0.5 μmol NSC668394 (Fig. 4A). Accordingly, treatment of purified C57BL/6 B cells with 0.5 μmol NSC668394 significantly increased the production of LPS-induced IL-10 by 2-fold (Fig. 4B). These data show that the ability of ezrin to limit MyD8-independent signaling and IL-10 production by B cells is mediated by its phosphorylated, active conformation.

Ezrin-deficient B cells suppress proinflammatory cytokine production in mice

To understand the in vivo significance of higher IL-10 production by Ez-def B cells, we employed a mouse model of endotoxic shock induced by a sublethal dose of LPS. In this model, LPS quickly induces copious amounts of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, but these are not sufficient to cause lethality and mice recover fully within 24 h (19, 20). We injected MB1cre/+ and Ez-def mice i.p. with 10 μg LPS and harvested peritoneal fluid (PF) at 1.5 h and serum at 1.5, 3, 6, and 24 h. The cytokines TNF-α and IL-1β peaked at 1.5 h, whereas maximal IL-6 levels were induced at 6 h in both groups (Fig. 5A). However, the Ez-def mice showed significantly lower levels of all three cytokines at all times in both serum (Fig. 5A) and PF (Supplemental Fig. 2A) samples. In contrast, IL-10, which was induced rapidly upon LPS administration, was significantly higher in the serum (Fig. 5B) as well as in PF (Supplemental Fig. 2A) of Ez-def mice. These data suggest that increased levels of local and serum IL-10 may mitigate the production of proinflammatory cytokines. B cells purified from PF of LPS-injected Ez-def mice also responded more robustly than those from MB1cre/+ mice upon restimulation with PMA and ionomycin ex vivo (Supplemental Fig. 2B). Neutralization of IL-10 by coadministering an anti–IL-10 blocking Ab with LPS restored all three proinflammatory cytokine levels in PF (Supplemental Fig. 2C) and serum (Supplemental Fig. 2C) of both MB1cre/+ and Ez-def mice, with the latter showing greater sensitivity to IL-10 ablation. Together, these data show that greater production of IL-10 by Ez-def B cells suppresses proinflammatory cytokine production in response to low doses of endotoxin.

Taken together, our study demonstrates that the membrane-cytoskeleton linker protein ezrin is a novel regulator of IL-10 production by B cells. TLRs have been implicated as playing a

**FIGURE 4.** Inhibition of ezrin phosphorylation increases IL-10 production by LPS-treated B cells. Purified B cells from C57BL/6 mice were treated with DMSO or the indicated concentrations of NSC668394 (Ez-Inh) for 1 h, centrifuged, and then incubated with 10 μg/ml LPS for 48 h. Cell lysates were subjected to immunoblotting with Abs to p-ThrERMT, ezrin, p-TBK1, TBK1, p-p65, p65, IκB, actin, p-IRF3, and IRF3 (A), and IL-10 was quantified in the supernatants by ELISA (B). Bar graphs in (A) show ratio of phosphoprotein to total protein and IκB to actin. The data are representative of two independent experiments (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 5.** B cell–specific deletion of ezrin in mice leads to decreased proinflammatory cytokine production in response to LPS. MB1cre/+ and Ez-def mice were injected i.p. with 10 μg LPS (n = 3 experiments, three to five mice per genotype), and levels of TNF-α, IL-6, and IL-1β (A) and IL-10 (B) in the serum were quantified by ELISA. (C) MB1cre/+ and Ez-def mice were injected with 10 μg LPS alone or LPS + 50 μg anti–IL-10 Ab (n = 3–5 mice/genotype). Peritoneal fluid was harvested after 1.5 h and assayed for IL-10, TNF-α, IL-6, and IL-1β by ELISA. The data are representative of two independent experiments (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.
dual role in mediating the balance between tolerance and autoimmunity through provision of different cytokines (21, 22). Our data support the conclusion that ezrin fine-tunes this balance by limiting MyD88-independent IL-10 production. We (9, 11) and others (8) have reported that ezrin undergoes rapid dephosphorylation upon BCR stimulation, followed by local rephosphorylation, which enables the organization and motility of BCR signaling microclusters. Given that ezrin undergoes dephosphorylation upon LPS stimulation (12), it may impose similar spatial constraints on TLR4 molecules/clusters in naive B cells. We show that ezrin dephosphorylation is sufficient to increase LPS-induced NF-κB and IRF3 activation, suggesting that phosphorylated ezrin may restrain TLR4 aggregation, association with the MyD88-independent pathway adaptors TRIF and TRAM, or internalization of TLR4 and consequent engagement of endosomal IRF3 signaling. In the future, it will be important to carefully examine the dynamics of TLR4 during LPS stimulation to fully understand the spatial control exerted by ezrin. Different B cell subsets vary in their signaling and transcriptional regulation, but the mechanisms of TLR-dependent activation in these subsets have not been fully elucidated. Our data demonstrate that MZ B cells, plasmablasts, and regulatory B10 populations are the most affected by deletion of ezrin with regard to IL-10 production, suggesting that the MyD88-independent signaling pathway may be regulated differentially in these subsets. In this manuscript, we focus on the role of ERM proteins. Our data support the conclusion that ezrin fine-tunes the magnitude of humoral immunity.

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References