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Cutting Edge: miR-223 and EBV miR-BART15 Regulate the NLRP3 Inflammasome and IL-1 β Production

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Although microRNA (miRNA) regulation of TLR signaling is well established, this has not yet been observed for NLR proteins or the inflammasomes they form. We have now validated a highly conserved miR-223 target site in the *NLRP3* 3'-untranslated region. miR-223 expression decreases as monocytes differentiate into macrophages, whereas NLRP3 protein increases during this time. However, overexpression of miR-223 prevents accumulation of NLRP3 protein and inhibits IL-1 β production from the inflammasome. Virus inhibition of the inflammasome is an emerging theme, and we have also identified an EBV miRNA that can target the miR-223 binding site in the *NLRP3* 3'-untranslated region. Furthermore, this virus miRNA can be secreted from infected B cells via exosomes to inhibit the NLRP3 inflammasome in noninfected cells. Therefore, we have identified both the first endogenous miRNA that limits NLRP3 inflammatory capacity during myeloid cell development and also a viral miRNA that takes advantage of this, limiting inflammation for its own purposes. *The Journal of Immunology*, 2012, 189: 3795–3799.

Many different microRNA (miRNA) are regulated by TLR activation, and feedback to amplify or negatively regulate that signal (1). Although TLRs respond to specific pathogen-associated molecular patterns, NLRs are thought to monitor critical intracellular homeostatic parameters. Therefore, they can respond to a variety of pathogens and cell stresses invoked by infection or disease. NLRP3 is the best studied NLR to date, activated in response to toxins, uric acid crystals, amyloid, and a host of other factors (2). A conserved mechanism of activation is yet to be formalized; however, it is clear that many cell types require NLRP3 inflammasome “priming,” such as stimulation with a TLR ligand. This functions both to provide pro-IL-1 β as a sub-

strate for the inflammasome and to induce NLRP3 expression above a critical threshold required for activation (3). Although NLRP3 is TLR inducible, very little is known about what regulates the expression of NLRP3 in different tissues and cell types, or the stability of the mRNA and protein once it is generated. In this work, we investigated NLRP3 expression and found that it can be regulated by miR-223, which is likely to be important early in the myeloid lineage. Furthermore, we identified a virus miRNA that targets the miR-223 binding site in the *NLRP3* 3'-untranslated region (UTR) and also inhibits inflammasome production of IL-1 β .

Materials and Methods

Cell culture

Primary monocytes were isolated using anti-CD14 magnetic beads (Miltenyi Biotec) and then differentiated into macrophages by cultivation with 100 ng/ml M-CSF or 100 ng/ml GM-CSF for 7 d. Macrophages were classically activated (M1) by stimulation with 100 ng/ml LPS (Alexis) and 20 ng/ml IFN- γ , and alternatively activated (M2) by 20 ng/ml IL-4 for 18 h each. Macrophages were transfected with 25 nM control scramble or miR-223 mimic using Dharmafect reagent 3 (Dharmacon-Thermo Scientific) and activated with 1 μ g/ml LPS and 5 mM ATP (Sigma). Thp-1 cells were differentiated with 20 nM PMA (Sigma) overnight. Cells were then transfected with 100 nM small interfering RNA, 50 nM synthetic miRNA, or negative control precursors (Applied Biosystems) using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, transfected Thp-1 cells were primed with 100 ng/ml LPS for 3 h; then the inflammasome was activated with 1 mM ATP, 50 μ M nigericin for 30 min or 250 ng/ml monosodium urate (MSU; Invivogen), 100 μ g/ml Alum (Brenntag Biosector), 10 μ M human islet amyloid polypeptide (Sigma), 10 μ g/ml poly-dAdT, *Salmonella* (multiplicity of infection = 10) overnight. For the transwell assay of EBV miRNA, B cells were added to the top of a 3- μ m transwell dish (Corning) seeded below with PMA-treated Thp-1 cells. Cells were cultured in the same well for 24 h before the addition of 20 μ M monensin (Sigma) for 6 h with or without 100 ng/ml LPS for the final 3 h. To collect exosomes, we treated B cells with monensin for 3 h, the supernatant was clarified by centrifugation at 500 \times g for 10 min, then 10,000 \times g for 20 min, and finally exosomes were collected by centrifugation at 50,000 \times g for 150 min. Exosomes from 15 ml supernatant were resuspended in 200 μ l RPMI and added to one well of a six-well plate of PMA-differentiated Thp-1 cells for 48 h.

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S.L.M., M.H., M.G., A.-A.R., D.P., and M.K.-S. designed and performed experiments, analyzed data, and wrote the manuscript; I.B.M., W.H., and L.A.J.O. provided advice and reagents.

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The online version of this article contains supplemental material.

Abbreviations used in this article: miRNA, microRNA; MSU, monosodium urate; UTR, untranslated region.

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Quantitative PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen), using 100% rather than 70% ethanol, and omitting buffer RW1 to retain miRNA. *NLRP3* (Hs00918082_m1) mRNA levels were determined using TaqMan Gene Expression Assays (Applied Biosystems) with *GAPDH* as an internal control. miR-223 and RNU6B levels were determined using TaqMan miRNA assays (Applied Biosystems).

Western blot

Cells were cultured in six-well plates, lysed directly in 70–80 μ l SDS loading buffer, then separated by 8% Tris-glycine PAGE and blotted according to standard protocol. Membranes were probed for NLRP3 (rabbit anti-human NLRP3, HPA012878; Adas Antibodies), IL-1 β (goat anti-human IL-1 β), and β -actin as a loading control.

Luciferase experiments

NLRP3 3'-UTR luciferase construct was purchased from Switchgear Genomics. 293T cells were transfected in 96-well plates in triplicates with 100 ng 3'-UTR luciferase constructs and 50 ng TK-*Renilla* luciferase control vector using Lipofectamine 2000 (Invitrogen). In addition, 50 nM pre-miRNA precursors were cotransfected. Cells were lysed in Passive Lysis Buffer (Promega) after 24 h; then luciferase activity was measured after the addition of luciferin or coelenterazine.

Cytokine measurement

Thp-1 cells (4×10^5 /ml) were cultured in 96-well plates, then transfected and stimulated as indicated in triplicate. Human macrophages (2.5×10^5 /ml) were cultured in 24-well plates, then transfected and stimulated as indicated for seven individual donors. Supernatants were collected and cytokine secretion was determined by ELISA for human IL-1 β and TNF- α (R&D Systems).

Statistical analysis

Data are presented as mean \pm SD. Significance was determined by two-tailed unpaired *t* test.

Results

NLRP3 and miR-223 expression during macrophage differentiation

Primary human monocytes (CD14⁺ cells) were differentiated into macrophages and then polarized toward either an M1 or M2 phenotype. The NLRP3 protein level in monocytes was only very low but increased as the cells were differentiated into macrophages (Fig. 1A). Surprisingly, we observed that *NLRP3* mRNA expression was very high in monocytes and actually much lower in macrophages, both M1 and M2 (Fig. 1B). A number of posttranscriptional mechanisms could account for this difference in NLRP3 protein compared with mRNA levels, and we have not yet investigated all of them. In particular, we were interested to determine whether miRNA

inhibition of mRNA translation (4) could contribute to this effect. We analyzed the human *NLRP3* 3'-UTR and found a predicted miRNA binding site for miR-223. The seed sequence of the predicted site has perfect conservation across all mammalian species (Supplemental Fig. 1A). Furthermore, we confirmed that miR-223 expression is inversely correlated with NLRP3 protein levels during macrophage differentiation (Fig. 1C), and the Ab was validated by small interfering RNA directed against *NLRP3* (Supplemental Fig. 1B). These data would also agree with the expression pattern of NLRP3-GFP mice where GFP under the *Nlrp3* promoter is a surrogate marker or NLRP3 expression, but not regulated by the *Nlrp3* 3'-UTR (5). In that case, NLRP3-GFP protein expression is very high in monocytes, which corresponds to high *NLRP3* mRNA levels in our study.

miR-223 targets the *NLRP3* 3'-UTR and inflammasome activation

We next used a luciferase-based reporter system to test the effect of miR-223 on the human *NLRP3* 3'-UTR. Overexpression of miR-223 reduced luciferase expression from the vector containing the wild-type *NLRP3* 3'-UTR compared with the empty vector (Fig. 2A). Mutating the seed sequence of the predicted miR-223 binding site, which is known to block miRNA binding (6), releases the reporter from repression by miR-223 (Fig. 2A). This indicates that the predicted interaction is functional and mediated by a single binding site. A common polymorphism, rs10802501, is present in the *NLRP3* 3'-UTR and could theoretically influence binding of miR-223 (Supplemental Fig. 1A); however, this had little effect on luciferase expression when miR-223 was overexpressed (Fig. 2A). Next, we wanted to investigate whether miR-223 can regulate endogenous NLRP3 levels in the monotypic Thp-1 cell line. PMA differentiates Thp-1 cells into macrophages, and again NLRP3 protein expression is greatly increased (Fig. 2B, left). Transfection of differentiated Thp-1 with pre-miR-223 decreased NLRP3 protein levels compared with a control precursor miRNA (Fig. 2B, right). Because the cellular function of NLRP3 is to induce IL-1 β protein processing by caspase-1, we also tested whether miR-223 can influence IL-1 β secretion on inflammasome activation. Indeed, IL-1 β production was reduced because of miR-223 overexpression during NLRP3 inflammasome activation (nigericin, MSU crystals, alum or amyloid), but not AIM2 (poly dAdT) or NLRC4 (*Salmonella*) activation (Fig. 2C). Pro-IL-1 β levels in cell lysates

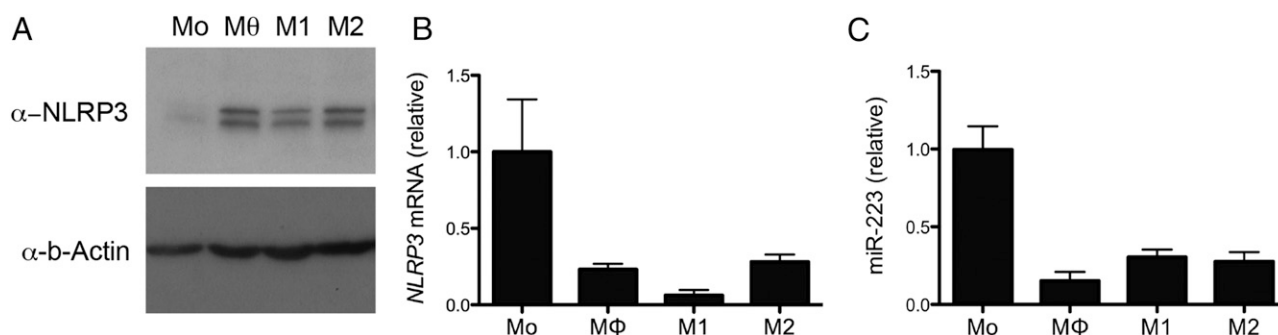


FIGURE 1. NLRP3 and miR-223 expression during macrophage differentiation and polarization. Human peripheral blood CD14⁺ cells (Mo) were differentiated into macrophages using M-CSF (M0) and polarized toward M1 (IFN- γ +LPS) or M2 (IL-4) phenotypes. (A) Western blotting for NLRP3 and β -actin loading control. (B) Quantitative PCR for *NLRP3* mRNA relative to *GAPDH*. (C) Quantitative PCR for miR-223 relative to RNU6B. Means \pm SD. All data are representative of at least three independent experiments.

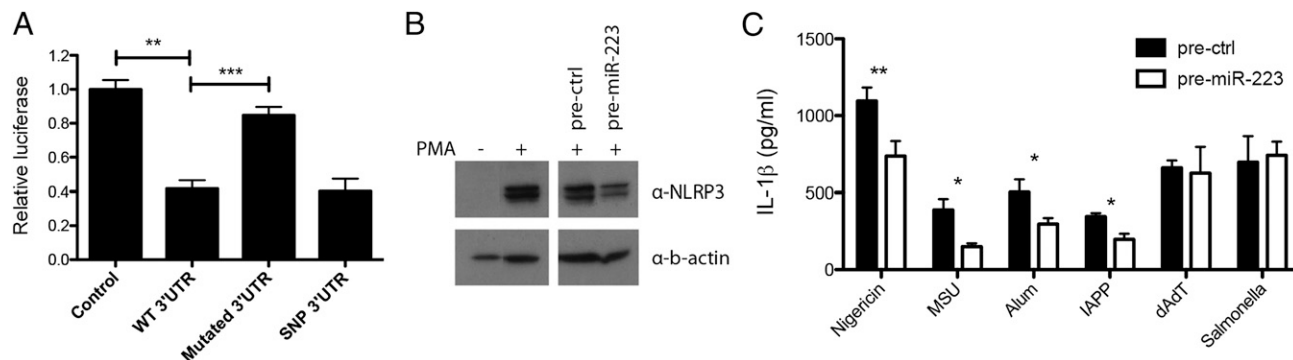


FIGURE 2. miR-223 targets the *NLRP3* 3'-UTR and inflammasome activation. **(A)** Luciferase constructs fused to the *NLRP3* 3'-UTR (WT), mutated in the miR-223 seed region (mutated), or with the polymorphism rs10802501 introduced (SNP) were transiently transfected into 293T cells along with pre-miR-223. Firefly luciferase activity was measured relative to TK *Renilla* as an internal control. **(B)** Western blotting for NLRP3 and β-actin from Thp-1 cells differentiated into macrophages with PMA and transfected with control pre-miRNA (pre-ctrl) or pre-miR-223. **(C)** PMA-differentiated Thp-1 cells were primed with LPS; then the NLRP3 inflammasome was activated with nigericin, MSU crystals, Alum, or islet amyloid polypeptide, the AIM2 inflammasome was activated by transfected poly dAdT, and the NLRC4 inflammasome was activated by *Salmonella*. Means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data are representative of at least three independent experiments.

were not decreased by miR-223 overexpression (Supplemental Fig. 1C). Finally, we showed that miR-223 also inhibits the NLRP3 inflammasome in primary human macrophages, and that neutralizing miR-223 activity had the reverse effect, increasing NLRP3 levels and IL-1β production (Supplemental Fig. 2).

EBV miR-BART15 targets the NLRP3 3'-UTR and inflammasome activation

Many viral inhibitors of the IL-1 pathway have been described, and recently, viral proteins targeting NLRP3 specifically have been identified. Gamma herpes viruses typically encode 30–40 miRNA species, and by sequence homology, we found two miRNA encoded by EBV, miR-BART11-5p and miR-BART15, that could potentially target the same site as miR-223 (Supplemental Fig. 1A). Targeting host miRNA binding sites is likely to be a preferred method of action for virus miRNA because variation of this sequence to avoid virus miRNA binding would also decrease the host miRNA affinity and could lead to inflammatory pathology, as for mice lacking miR-223

(7). We formally tested the ability of the viral miRNA to decrease the expression of the *NLRP3* 3'-UTR luciferase construct and observed that only EBV miR-BART15 targeted this construct, and it was specific for the miR-223 binding site (Fig. 3A). Transfection of EBV miR-BART15 into PMA-treated Thp-1 cells reduced endogenous NLRP3 protein levels (Fig. 3B), and this was functionally relevant because IL-1β production after NLRP3 inflammasome activation was also decreased (Fig. 3C).

Exosomal EBV miR-BART15 from B cells targets the NLRP3 inflammasome in noninfected cells

The B cell is the primary cell type infected by EBV during the natural course of infection. Currently, there is no known role for NLRP3 in B cells; however, it has been observed that EBV miRNA can be released via exosomes from infected B cells to have an influence on other cell types (8). We used the EBV transformed Namalwa B cell line, compared with the EBV naive Ramos B cell line, to test whether EBV miR-BART15 was secreted via exosomes and taken up by PMA-treated Thp-1

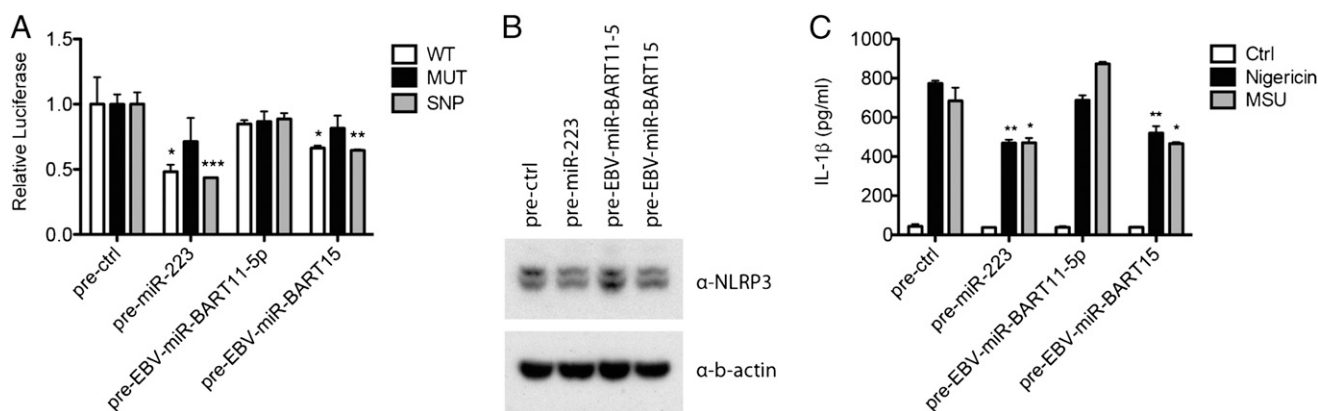


FIGURE 3. EBV miR-BART15 targets the *NLRP3* 3'-UTR and inflammasome activation. **(A)** Luciferase constructs fused to the *NLRP3* 3'-UTR (WT), mutated in the miR-223 seed region (MUT), or with the polymorphism rs10802501 introduced (SNP) were transiently transfected into 293T cells along with pre-miR-223, pre-EBV-miR-BART11-5p, or pre-EBV-miR-BART15. Firefly luciferase activity was measured relative to TK *Renilla* as an internal control. **(B)** Western blotting for NLRP3 and β-actin from Thp-1 cells differentiated into macrophages with PMA and transfected with pre-miRNA as in (A). **(C)** PMA differentiated Thp-1 cells were primed with LPS; then the NLRP3 inflammasome was activated with nigericin or MSU crystals. Means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data are representative of at least three independent experiments.

cells. The B cells were separated from the Thp-1 cells using a 3- μ m transwell filter, and exosome secretion was stimulated using monensin as described previously (9). Using this method, we confirmed that a small but significant amount of EBV miR-BART15 was indeed taken up by the Thp-1 cells (Fig. 4A). Furthermore, exosomes shed by these B cells were purified by ultracentrifugation and added to PMA-treated Thp-1 cells, which led to a decrease in NLRP3 protein expression, as determined by Western blot (Fig. 4B). To measure the effect of exosomes shed across a transwell on IL-1 β production, we first primed Thp-1 cells with LPS; however, there was no need to additionally activate NLRP3 because of the use of monensin, which has previously been shown to trigger the inflammasome (10). In this fashion, there was a specific decrease in IL-1 β production caused by EBV miR-BART15 that was not seen for TNF- α (Fig. 4C).

Discussion

In all of our experiments, the effect of miR-223 or EBV miR-BART15 on the NLRP3 inflammasome is modest. However, this is to be expected for any miRNA that typically only give rise to 2-fold differences in biological responses (1). Notably, the experiments we performed only modulate miRNA levels transiently, and it is likely that consistently high levels, such as for miR-223 in monocytes, will have a more pronounced effect on NLRP3 in vivo. Some evidence for this can be found in the supplementary data to a study by Baek et al. (11), where proteomic analysis of miR-223-deficient mice does indeed find an increased amount of NLRP3 in vivo. The phenotype of mice lacking miR-223 is entirely consistent with what might be expected from this increase in NLRP3 expression, namely, neutrophilia, inflammatory lung disease, and an increased susceptibility to endotoxin lethality (7). Some of these neutrophil-mediated phenotypes are attributed to other miR-

223 targets, like Mef-2c; however, inflammatory pathology remains in *miR-223*^{-/-} *Mef-2c*^{-/-} compound deletion mice. Analysis of *miR-223*^{-/-} *Nlrp3*^{-/-} compound deletion mice will formally determine the contribution of NLRP3 in future studies. The miR-223-deficient granulocytes also display increased fungicidal activity when cocultured with *Candida albicans* (7). Importantly, NLRP3 is a critical regulator of fungal infection, where the IL-1 β produced by this inflammasome drives antifungal Th17 responses (12). Furthermore, miR-223 and NLRP3 are both associated with influenza, HIV-1 infection, and diabetes. It will be interesting to see whether the effect of miR-223 is mediated by NLRP3 in any of these cases.

Western blotting for NLRP3 reveals two predominant isoforms at ~110 kDa, which could correspond to four of five different *NLRP3* transcripts. All four of these full-length transcripts contain the same *NLRP3* 3'-UTR, which can be subject to regulation by miR-223 and EBV miR-BART15. However, there is also a truncated isoform of *NLRP3* that involves another 3'-UTR and may not be regulated by these miRNAs. Unfortunately, Western blotting did not detect a band at the size of this transcript, around 80 kDa. Further analysis of this isoform will be important because it lacks the leucine-rich repeats, which may be autoinhibitory, and could therefore explain auto-activated caspase-1 in cell types where miR-223 is high, such as monocytes (13). Furthermore, expressed sequence tags suggest that the *NLRP3* 3'-UTR uses alternative polyadenylation sites, which can alter the possibility of miRNA regulation. Overall, this means that the posttranscriptional regulation of NLRP3 is complex and may be highly cell-type specific, as our experiments on myeloid differentiation suggest.

Our data showing that EBV miR-BART15 can inhibit the NLRP3 inflammasome suggests that EBV may first trigger inflammasome activity. Although this remains to be formally proven, increased IL-1 β and IL-18 have been associated with EBV infection in certain circumstances (14, 15). This is also true of several other viruses, where an inflammasome inhibitor has been identified, but the molecular mechanism by which the host recognizes virus infection and initiates inflammasome activation is not yet clear (16). This may indicate that targeting IL-1 β and IL-18 has a more general rather than a specific proviral effect.

Our study makes two major conclusions. First, miR-223, a hematopoietic-specific miRNA, can target the *NLRP3* 3'-UTR. This restricts inflammasome activation, which is likely to be important in monocytes, where miR-223 expression is high and NLRP3 protein low compared with mRNA levels. Second, EBV miR-BART15 targets the same site in the *NLRP3* 3'-UTR. This enables EBV-infected B cells to dampen inflammasome activation in noninfected cells by transfer of the miRNA through exosomes.

From a therapeutic perspective, it might be interesting to target *NLRP3* with an miRNA mimic if the other targets (such as Mef2c for miR-223) are also suitable to target for the same disease state. This would be more appropriate if an miR-223/NLRP3 imbalance was identified in a particular disease or, preferably, a particular patient. Alternatively, miR-223 and EBV-miR-BART15 could themselves become targets with antisense therapeutics to recover inflammasome activity or fight EBV and associated malignancy.

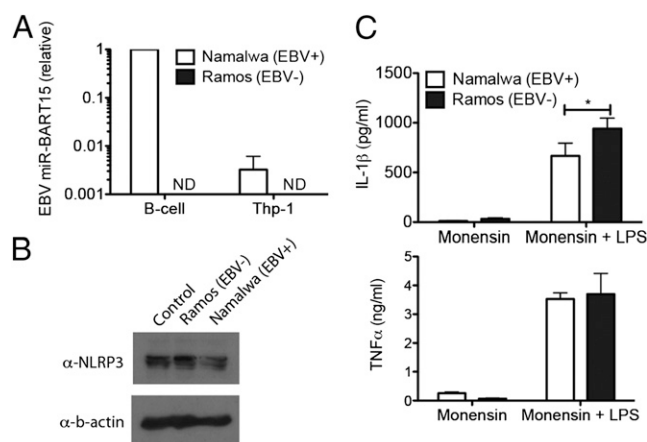


FIGURE 4. EBV miR-BART15 secreted in exosomes from infected B cells inhibits the NLRP3 inflammasome in noninfected cells. (A) B cell lines were cultured with PMA-treated Thp-1 cells, separated by a 3- μ m filter. After 24 h, exosome secretion was promoted by the addition of monensin for 6 h. Quantitative PCR was performed for EBV miR-BART15 relative to RNU6B from either the B cells or Thp-1 cells. (B) Exosomes were purified by centrifugation from the supernatant of monensin-treated B cell lines. These were then added to PMA-treated Thp-1 cells for 48 h, and Western blotting for NLRP3 and β -actin was performed. (C) Cells cocultured as in (A) were further treated with 3-h LPS; then IL-1 β or TNF- α were measured by ELISA. Means \pm SD. * p < 0.05. All data are representative of at least three independent experiments. ND, None detected.

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Disclosures

The authors have no financial conflicts of interest.

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