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Rab27-dependent exosome production inhibits chronic inflammation and enables acute responses to inflammatory stimuli

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Abstract

Extracellular vesicles, including exosomes, have recently been implicated as novel mediators of immune cell communication in mammals. However, roles for endogenously produced exosomes in regulating immune cell functions *in vivo* are just beginning to be identified. Here, we demonstrate that Rab27a and Rab27b double knockout (Rab27DKO) mice that are deficient in exosome secretion have a chronic, low-grade inflammatory phenotype characterized by elevated inflammatory cytokines and myeloproliferation. Upon further investigation, we found that some of these phenotypes could be complemented by WT hematopoietic cells or administration of exosomes produced by GM-CSF expanded bone marrow cells. Additionally, chronically inflamed Rab27DKO mice had a blunted response to bacterial LPS, resembling endotoxin tolerance. This defect was rescued by bone marrow exosomes from WT but not miR-155−/− cells suggesting that uptake of miR-155-containing exosomes is important for a proper LPS response. Further, we found that SHIP1 and IRAK-M, direct targets of miR-155 that are known negative regulators of the LPS response, were elevated in Rab27DKO mice and decreased following treatment with WT but not miR-155−/− exosomes. Together, our study finds that Rab27-dependent exosome production contributes to homeostasis within the hematopoietic system and appropriate responsiveness to inflammatory stimuli.

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Author contributions

M.A. and R.M.O. designed the study and interpreted the data. M.A. carried out all experimental work with assistance from A.R., K.B, S.L., M.C.R., T.H., J.W., D.L., and guidance from R.M.O., D.M.W. and J.L.R. T.T. and M.C.S. contributed the *Rab27 DKO* mice. M.A. and R.M.O. wrote the manuscript with contributions from all authors.

Introduction

The mammalian inflammatory response must maintain an intricate balance between pro- and anti-inflammatory signals. Proper control of inflammation is needed to clear infections as well as maintain commensal microbe populations and overall homeostasis of tissues. When this balance is disrupted, a chronic, low-grade inflammation develops that over time can contribute to a variety of diseases associated with aging, including obesity and type 2 diabetes (1). Chronic inflammation and associated diseases are expanding at an alarming rate putting enormous stress on the US medical system and overall economy (2). There is a pressing need to improve our understanding of the underlying mechanisms that contribute to chronic inflammation in order to develop effective therapies moving forward. Non-coding RNAs, including microRNAs (miRNAs), are one such mechanism for regulating chronic inflammation as demonstrated by an age-dependent, chronic inflammatory disease seen in mice lacking miR-146a that is driven by another miRNA, miR-155 (2, 3). However, several aspects of how these miRNAs are regulated and function is still being explored.

Over the past decades, researchers have begun to investigate a novel form of intercellular communication between immune cells mediated by small lipid vesicles called exosomes (4–7). The transfer of cellular contents including proteins, RNAs, and other molecules by exosomes has been reported (8). Exosome-mediated transfer of miRNAs has become of particular interest to the field (7, 9, 10) because miRNAs can be transferred between different cell types, including immune cells, to regulate cellular responses (6, 11, 12). For instance, miRNAs can be shuttled between dendritic cells (DCs) and mediate target knockdown in recipient cells (13) as well as be transferred between T cells and DCs at the immunological synapse (14). Further, transfer of miRNAs via exosomes conveyed resistance to hepatitis B and contributed to interferon-α antiviral responses in mice (15). There are also examples of specific miRNAs being transferred between hematopoietic cells and altering responses. For example, the exogenous delivery of exosomal miR-155 leads to an enhanced response to inflammatory challenges, while administration of exosomal miR-146a reduces this response (11). Such effects have been observed both systemically and in specific tissues, including those that make up the central nervous system (16). Additionally, miR-155 can also be transferred from acute myeloid leukemia (AML) cells to healthy blood cells, resulting in the suppression of c-MYB and compromised hematopoiesis in the context of cancer (17). These examples clearly demonstrate that miRNAs contained within exosomes are involved in the communication between immune and other hematopoietic cells, during both physiological and pathological situations.

Despite the expanding amount of research investigating the functional transfer of miRNAs between immune cells, the key question of whether endogenously produced exosomes and their miRNA cargos are important for cellular communication *in vivo* is just beginning to be addressed as new tools emerge. Rab27a and Rab27b double knockout (Rab27DKO) mice provide one such reagent with which to study the roles of exosomes *in vivo* as these mice exhibit significantly reduced exosome release, among other phenotypes, due to the role of these GTPases in the docking and retention of the multivesiclar body to the plasma membrane (18–21). Rab27DKO regulatory T cells (Tregs) have previously been used to study the function of an exosomal miRNA, Let7d, in suppression of Th1 cells by Tregs

using an adoptive transfer model (9). However, it remains unclear whether endogenously produced exosomes play important roles in other immune cell functions, including innate immunity.

In our current study, we found that Rab27DKO mice have a chronic, low-grade inflammatory condition characterized by increased baseline inflammatory cytokines and myeloproliferation. Some of these phenotypes were cell extrinsic and could be rescued, at least in part, by WT BM cells or injections of WT exosomes isolated from BM cells. This indicates that exosome uptake is important for maintenance of hematopoietic homeostasis and prevention of aberrant chronic inflammation. Chronically inflamed Rab27DKO mice were also hypo-responsive to challenge with LPS, and thus resembled a state of endotoxin tolerance (22). Further, we found that the response of Rab27DKO mice to LPS can be rescued by delivery of WT but not miR-155−/− exosomes, suggesting that miR-155 contained within exosomes is essential for the proper responses to inflammatory cues. SHIP1 and IRAK-M, miR-155 targets that negatively regulate inflammatory responses (23, 24) and are involved in establishing tolerance to LPS (25), are elevated in Rab27DKO mice compared to WT mice. SHIP1 and IRAK-M levels were reduced following administration of WT but not miR-155−/− exosomes in mice challenged with LPS implicating miR-155 targeting of SHIP1 and IRAK-M in the exosomal rescue of Rab27DKO mouse response to LPS. Together, these results provide evidence that exosomal communication is important for proper maintenance of homeostasis in the immune system, and subsequent responses to challenge with LPS.

Materials and methods

Mice

CD45.2 WT (Jackson Labs), *CD45.1 WT* (Jackson Labs), and *Rab27DKO* (Rab27a ash/ash Rab27b−/−) mice (Tanya Tolmachova and Miguel C. Seabra, Imperial College London) are on a C57BL6 genetic background and housed in the animal facility at the University of Utah. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Utah. Mice were age matched and sex matched, and were in the age range of 6–16 weeks old for all experiments. Both males and females displayed observed phenotypes.

Exosome isolation and procedures

Differential centrifugation was performed to isolate exosomes from BM conditioned medium. Initial spins consisted of a 10 min spin at 1,000*g*, a 2,000*g* spin for 10 min and a 10,000*g* spin for 30 min. The supernatant was retained each time. The supernatant was then spun at 100,000*g* for 70 min and the pellet was re-suspended in 25ml 1 × PBS, to dilute remaining soluble factors, followed by another centrifugation at 100,000*g* for 70 min. The final pellet contained the exosomes, which were re-suspended in PBS. This protocol is based on a previous exosome isolation methods(26). We used a Thermo Scientific Sorvall Lynx 6000 with a T26-8 × 50 rotor. For the *in vitro* and *in vivo* experiments, exosomes were isolated from BM cultured in GM-CSF (GM-BM cells) from WT, Rab27DKO, or miR-155−/− mice. BM cells were incubated for 3 days with 20ng ml^{-1} GM-CSF and then

given an additional 5ml of medium with 20ng ml^{-1} GM-CSF for a total of 7 days of culture. For the *in vitro* experiments, exosomes isolated from 3 million GM-BM cells were transferred to the same amount of recipient cells. For the *in vivo* experiments, each mouse was i.p injected with exosome pellets resuspended in 100 ul of 1x PBS. These exosomes were derived from 3×10cm plates of 3 million GM-BMs per plate and re-suspended in 100ul PBS, which yields approximately 10^9 exosomes as previously quantified (11). Protein concentrations in the exosome preparations were also quantified and similar protein levels were injected except for Rab27DKO exosome pellets and exosomes were previously characterized to be CD63+ and of normal size and morphology (11).

Cells culture

BMDCs were derived from mouse BM by culturing red blood cell-depleted BM in complete RPMI (10% fetal bovine serum, 100 units per ml penicillin and 100 units per ml streptomycin, glutamate, sodium pyruvate, HEPES and non-essential amino acids) with 20 ng ml^{-1} GM-CSF for 3–4 days at 37 °C with 5% CO_2. The cells were then cultured in 5 ml complete RPMI with 20 ng ml^{-1} GM-CSF for an additional 3–4 days for a total of 7 days in culture. LPS stimulation was performed at a concentration of 500 ng ml^{-1}.

miR-155 Mimic

miRNA mimics were purchased from Qiagen. Scrambled, seed mutant and miR-mimic sequences are as follows:

miR-155 seed mutant (5′-UUUGCUAAAAUUGUGAUAGGGGU-3′) and

miR-155 mimic (5′-UUAAUGCUAAUUGUGAUAGGGGU-3′). Rab27DKO BMDCs were transfected with 30μl of the Tran-IT siQUEST reagent (Mirus) in 2 ml of OPTI-MEM media with 60ng of each mimic. After 24 hours, the Rab27DKO BMDCs were treated with or without LPS at a concentration of 500 ng ml^{-1} and media was isolated at 2 and 6 hours for ELISAs.

Flow cytometry

Fluorophore-conjugated antibodies against the indicated surface markers (eBioscience) were used to stain RBC-depleted splenocytes and BM cells. Stained cells were analyzed with a BD LSR Fortessa flow cytometer, and further data analysis was carried out with FlowJo software.

ELISA

The enzyme-linked immunosorbent assay (ELISA) used to quantify mouse IL-6 and TNFα concentrations were obtained from eBioscience and were performed using the manufacturer's suggested protocol.

Immunoblotting

Cellular extract was size fractionated via SDS-PAGE and immunoblotting was performed in accordance with standard protocols. Specific antibodies were used to detect SHIP1 (Santa Cruz sc-1964) and GAPDH (Abcam ab9485).

RNA isolation and qRT–PCR

RNA isolation was performed using the miRNeasy kit (Qiagen), according to manufacturer's instructions. cDNA from total RNA was made with qScript using 90ng of RNA from each sample (Quanta). qPCR was performed with Promega GoTaq qPCR master mix. Primer sequences are as follows:

SHIP1-F (5′-GAGCGGGATGAATCCAGTGG-3),

SHIP1-R (5′-GGACCTCGGTTGGCAATGGTA-3),

IRAK-M-F (5′-CCTGAACATAATGAAAAAGGAACAC -3)

IRAK-M-R (5′-ATGCTTGGTTTCGAATGTCC -3)

L32-F (5′-AGCTCCCAAAAATAGACGCAC-3) and

L32-R (5′-TTCATAGCAGTAGGCACAAAGG-3). L32 levels were used to normalize mRNA expression levels.

BM reconstitution

CD45.1 WT mice were lethally irradiated (1,000 rads) using an X-ray source (Rad Source RS200 biological system). Following irradiation, mice were injected with three million RBC depleted BM cells via retro-orbital injection and aged for 2 months before analysis.

In vivo LPS administration to mice

Escherichia coli LPS (Sigma) was administered through i.p. injections at a sub-lethal concentration of 50μg in 1x PBS per mouse. In exosome injection experiments, exosomes were i.p. injected 24 hours before LPS injection and again at 48 hours post LPS injection. Mice were harvested at 72 hours post LPS injection for analysis of immune cell populations.

Statistics

Data were analyzed using either Welsh's corrected *t*-tests or one-way ANOVA with Tukey multiple comparisons and p adjusted values are reported. These statistics were done using Graphpad Prism software. *P*-values were either listed or represented by the following number of asterisks: *p<0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

Results

Rab27DKO mice have a chronic, low-grade inflammatory phenotype

Upon phenotyping Rab27DKO mice, we observed that granulocyte-monocyte (GM) myeloid populations, marked by surface expression of CD11b+ and GR1+, were expanded in the Rab27DKO mice in both the spleen and bone marrow (BM) compartments with a corresponding decrease in the B220+ B cell population compared to WT controls (Figure 1a–c, e–g). There were signs of extramedullary hematopoiesis indicated by the expansion of Ter119+ erythroid precursor cells in the spleen, and a reduction of these cells in the BM (Figure 1a, d, e, h). Increased spleen weights were also observed in the Rab27DKO vs. WT control mice (Figure 1i). These data suggest that the Rab27DKO mice have a mild myeloproliferative disorder. In contrast, no apparent differences in splenic and thymic T cell

populations, hematopoietic stem and progenitor cells (Linage negative Kit+ Sca+ LKS), and overall hematopoietic cell numbers in the BM and spleen of Rab27DKO mice were observed when compared to WT mice (Supplemental Figure 1). Beyond hematopoietic population differences, the pro-inflammatory cytokines TNFα and IL-6 were elevated above baseline in the serum of Rab27DKO mice (Figure 1j–k). These results indicate that mice deficient in Rab27a/b develop a chronic, low-grade inflammatory condition.

Some Rab27DKO mouse chronic inflammatory phenotypes are cell extrinsic

In order to determine whether these phenotypes are cell intrinsic or extrinsic we utilized BM radiation chimeras. CD45.1 WT mice were lethally irradiated and reconstituted with either a 1:1 mixture of CD45.1 WT/CD45.2 WT, Rab27DKO (CD45.2)/WT (CD45.1), or only Rab27DKO BM cells. After two months, we found that CD45.1 and CD45.2 populations were fairly equivalent in both groups (Figure 2a–b), indicating that Rab27DKO hematopoietic stem cells (HSCs) are able to engraft with similar efficiency as WT HSCs. However, Rab27DKO/WT reconstituted mice no longer had an overall accumulation of CD11b+ GR1+ GM myeloid cells in the spleen as seen in Rab27DKO reconstituted mice (Figures 2c–d). To investigate whether Rab27DKO or WT cells composed this population, we gated on CD45.1 and CD45.2 and found that within the CD11b+ GR1+ population there were not significantly more WT or Rab27DKO cells making up this population, suggesting that the presence of WT cells was able to rescue the accumulation of these cells (Figure 2e). Additionally, there was some rescue of B220+ B cell populations in the spleens of mice reconstituted with the WT/Rab27DKO BM mixtures which was also composed of WT and Rab27DKO derived cells at equal proportions (Figures 2f–h). The Ter119+ erythroid precursor population was restored back to normal low levels in the spleen in the WT/Rab27DKO BM chimeras (Figure 2i). This was also accompanied by a decrease in spleen weights (Figure 2j). Similar trends were seen in the BM with the exception of the Ter119+ population, which was not rescued with the presence of WT BM (Supplemental Figure 2). These results provide evidence that certain Rab27DKO phenotypes are cell extrinsic and potentially regulated by exosomes, while others may be intrinsic and regulated by exosome independent mechanisms.

Delivery of WT exosomes can complement specific Rab27DKO mouse phenotypes

Due to our observations that some of the Rab27DKO mouse chronic, low-grade inflammatory phenotypes are a result of cell extrinsic mechanisms, we investigated whether exosome uptake was an important contributing factor to homeostasis within the immune system. To test this, we intraperitoneally (i.p.) injected Rab27DKO mice with exosomes produced by WT GM-CSF cultured bone marrow (GM-BM) cells or a control “exosome” pellet from Rab27DKO GM-BM cells which have been previously shown to produce significantly fewer exosomes (11, 18). We have previously characterized these WT exosomes and found they are CD63+ and display a typical morphology and size as observed by EM imaging (11). Injections were performed two times per week over the course of four weeks.

WT exosome injections fully rescued the splenomegaly of the Rab27DKO mice while the splenic GR1+ CD11b+ GM myeloid population was partially reduced towards WT levels

(Figure 3a–c). Ter119+ erythroid precursor cell expansion in the Rab27DKO mouse spleens was also reduced following injections of WT exosomes, but the B220+ B cell population was not rescued by WT exosome treatment (Figure 3d–f). The GR1+ CD11b+ GM population in the BM also recovered after WT exosomes were injected (Figure 3g–h); however, the Rab27DKO Ter119+ and B220+ BM cellular populations were not altered following delivery of WT exosomes (Figure 3i–k). Levels of IL-6 and TNFα in the Rab27DKO serum were also reduced to normal levels following injection of WT exosomes (Figure 3l and Supplemental Figure 3). This experiment was repeated with PBS and miR-155−/− exosome injections as additional controls and we observed that the injections with PBS displayed similar results as the injections of Rab27DKO exosomal pellets, while the injection of miR-155−/− exosomes showed results similar to WT exosome injections (Supplemental Figure 3). These results suggest that Rab27DKO mouse steady state phenotypes that are regulated by exosomes are independent of exosomal miR-155. Of note, miR-155−/− exosomes have been previously shown to be made at similar levels as WT exosomes (11), which is corroborated by similar protein concentrations in our WT and miR-155−/− exosomal preparations (Supplemental Figure 3). Rab27DKO exosomal pellets have significantly decreased protein concentration as well as significantly decreased levels of miR-155 and miR-146a in their exosomal pellet (Supplemental Figure 3). A summary of which cellular phenotypes are rescued by both WT BM and WT exosomes can be found in Supplemental Table 1. These results suggest that some Rab27DKO mouse phenotypes are dependent on GM-BM exosome uptake, such as Ter119+ cell accumulation in the spleen and splenomegaly, increased GM myeloid cells and elevated cytokine levels, while others appear to be independent of GM-BM exosome uptake.

Rab27DKO mice are refractory to treatment with LPS

Due to the chronic inflammation observed in the Rab27DKO mice, we next wanted to investigate how these mice respond to a LPS challenge. To do this, we i.p. injected WT or Rab27DKO mice with a non-lethal dose of LPS and analyzed the inflammatory response by isolating serum at 2 and 6 hours post-LPS injection and performing IL-6 and TNFα ELISAs. Despite starting out with increased TNFα and IL-6 basal levels, Rab27DKO mice could not elevate these levels in response to LPS to the same extent as WT mice (Figure 4a–f). Normally, during a LPS challenge mice will undergo a switch in hematopoietic development in the BM compartment, termed emergency granulopoiesis, where the GR1+ CD11b+ GM myeloid population expands and the B220+ B and Ter119+ erythroid precursor cell populations contracts by 72 hours after LPS stimulation. Rab27DKO mice were unable to shift their myeloid, B, and erythroid precursor populations in response to LPS, which was observed in their WT counterparts (Figure 4g–k). These results indicate that chronically inflamed Rab27DKO mice are refractory to stimulation with LPS.

Exosome injections restores the Rab27DKO response to LPS

To determine if the failure of Rab27DKO mice to respond to LPS could be rescued by exosome uptake, we i.p. injected WT exosomes into Rab27DKO mice, or PBS as a control, 24 hours before LPS injection (Figure 5a). Serum levels of TNFα and IL-6 were significantly increased when Rab27DKO mice were pre-treated with WT exosomes compared to PBS (Figure 5b–e). To analyze changes in emergency granulopoiesis, mice

were injected a second time with exosomes 48 hours after LPS administration and then harvested 24 hours later (Figure 5a). Rab27DKO mice given WT exosomes were able to expand their CD11b+ GR1+ myeloid populations and reduce their B220+ B cell and Ter119+ erythroid precursor populations in response to LPS (Figure 5f–j) suggesting that the uptake of WT exosomes is able to restore the ability of Rab27DKO mice to perform emergency granulopoiesis in response to LPS. It is important to note that the two-injection regiment did not rescue the baseline levels of myeloid, B, and erythroid precursor cells, but did rescue the responsiveness of these populations to LPS (Figure 5f–j). These results demonstrate that uptake of GM-BM derived exosomes can complement the refractory response to LPS in Rab27DKO mice, and suggests that exosomes are involved in proper responsiveness to endotoxin *in vivo*.

Exosomal miR-155-dependent rescue of LPS responsiveness by Rab27DKO bone marrow derived dendritic cells

Based on our findings that Rab27DKO mice have a refractory response to endotoxin that involves exosome uptake, we next wanted to investigate which factor in the exosome could be responsible for this outcome. Based on our previous findings that miR-155-containing exosomes were able to bolster the response to endotoxin (11), and recent observations that exosomal miR-155 levels increase in response to LPS *in vivo* (27), we performed the following experiment. Rab27DKO, WT, or miR-155−/− GM-BM derived exosome pellets were administered to recipient Rab27DKO BMDCs, followed by LPS administration 24 hours later. 2 hours post-LPS treatment, TNFα levels were increased in the Rab27DKO bone marrow derived dendritic cells (BMDCs) given WT exosomes but not the Rab27DKO BMDCs that received their own exosome pellet or miR-155−/− exosomes (Figure 6a–b) indicating that WT exosomes can rescue this defect through a miR-155-dependent mechanism. We next investigated whether SHIP1, a target of miR-155 that negatively regulates inflammation and responses to LPS, were affected by the administration of WT exosomes. We found that WT but not miR-155−/− exosomes were able to reduce SHIP1 and IRAK-M levels in Rab27DKO BMDCs, corresponding to increased TNFα levels (Figure 6c–d), consistent with previous reports of SHIP1 negatively regulating TNFα production (28). Further, another negative regulator of inflammation and target of miR-155 (29), IRAK-M, displays a similar pattern as SHIP1 (Figure 6e), suggesting that miR-155 targets multiple negative regulators of inflammation to promote the LPS response in Rab27DKO BMDCs.

To determine whether miR-155 is sufficient to rescue Rab27DKO BMDC response to LPS, we utilized miR-155 mimics. Rab27DKO BMDCs were transfected with either a mature miR-155 mimic or a miR-155 mimic where the seed site had been mutated (seed) using a lipid based reagent. 24 hours post mimic transfection, the cells where treated with or without LPS. Rab27DKO BMDCs that received the miR-155 mimic had increased IL-6 and TNFα levels compared to those that received the seed mutant mimic (Figure 6f–i). These results demonstrate that miR-155 is sufficient to rescue Rab27DKO BMDC LPS response, which further supports the idea that the delivery of miR-155 via WT exosomes is responsible for rescuing this response.

Rescue of Rab27DKO mouse responses to LPS is dependent on miR-155-containing exosomes

Based on our *in vitro* observations, we investigated the requirement of miR-155 in exosomes for the rescue of Rab27DKO mouse response to LPS. To study this, we injected WT, miR-155−/− or Rab27DKO GM-BM derived exosome pellets into Rab27DKO mice 24 hours before LPS injection and again 48 hours after LPS injection with the same timeline as Figure 5a. Rab27DKO mice treated with WT exosomes had enhanced TNFα production by 2-hours post LPS challenge, that was restored back to WT levels, while TNFα levels in LPS treated Rab27DKO mice given miR-155−/− or Rab27DKO exosome pellets were not rescued (Figure 7a–b). We also found that resting Rab27DKO mice had increased levels of SHIP1 and IRAK-M (Figure 7c–f), suggesting that the Rab27DKO mice are trying to compensate for their chronic inflammatory status by upregulating negative regulators of inflammation. The increase in SHIP1 and IRAK-M in resting conditions could explain why the Rab27DKO mice are hypo-responsive to LPS, and why WT exosome administration aids in the rescue of Rab27DKO response to LPS through a miR-155 dependent mechanism. This hypothesis is supported by the observed reduction in SHIP1 and IRAK-M levels when LPS treated Rab27DKO mice received WT but not miR-155−/− exosomes (Figure 7g–h). Additionally, the GM myeloid population was increased in response to LPS administration following pretreatment with WT but not miR-155−/− exosomes (Figure 7i–j). These results provide evidence that miR-155 is required for exosomes to rescue LPS responsiveness by Rab27DKO mice, and that reductions in miR-155 targets SHIP1 and IRAK-M are involved in mediating this rescue.

Discussion

While it is clear from the literature that exosomes are important for intercellular communication between immune cells (6, 30), the roles of endogenously produced exosomes *in vivo* are just beginning to be investigated. Previous studies have shown that endogenous exosome production and content are affected by disease states in humans (31, 32) and can be used as biomarkers for diseases such as obesity and type 2 diabetes (33). However, it is unclear from these studies whether exosomes are playing a role in disease or if they are mere byproducts. Additionally, manipulated tumor exosomes have been shown to activate CD8+ T cells *in vivo* (34), while endogenous exosomes can mediate Treg suppression of Th1 cells via Let7d in an adoptive transfer system (35). While these studies provide evidence for the importance of endogenously produced exosomes during immunity, we designed our approach utilizing the Rab27DKO mouse model where we were able to study the relevance of endogenous exosome production with minimal manipulation because these mice have defective exosome production (18, 35).

Although useful for exosome studies, it is important to note that the Rab27DKO mouse model has defects beyond exosome release. Rab27a and/or b deficient mice have been shown to have defective granule release by platelets, cytotoxic T cells and neutrophils as well as improper neutrophil chemotaxis in certain contexts (19–21, 36). There may also be a role for the Rab27 proteins in recycling of membrane proteins and phagosome function (37, 38). Based on these additional functions of the Rab27 proteins in immune and cellular

functions it is critical to distinguish these from their role in exosome secretion. To address this, we attempted to rescue observed phenotypes with either WT hematopoietic cells or the administration of WT exosomes. If the defect was rescued by both approaches, then it suggests that a lack of exosome uptake contributes to the phenotype.

Our data demonstrate that exosome uptake is essential for hematopoietic homeostasis and the prevention of chronic inflammation in Rab27DKO mice. Corresponding to increased basal inflammation, the Rab27DKO mice were hypo-responsive to LPS, consistent with previous findings that Rab27a knockout mice (ashen) are protected from LPS sepsis (39). We hypothesized that the Rab27DKO mice are less responsive to LPS because they have increased negative regulators of inflammation, such as SHIP1 (25, 28), as a way to combat their chronic inflammatory condition. This would explain why WT exosomes could rescue Rab27DKO response to LPS while miR-155−/− exosomes could not as SHIP1 is a known miR-155 target (24) and TNFα and IL-6 have been previously shown to be inhibited by SHIP1 (28). Further, previous studies have demonstrated that miR-155 upregulation increases TNFα production through repression of its targets SHIP1 and IRAK-M (27, 29), which agrees with our findings that exosomally delivered miR-155 is able to restore Rab27DKO cellular response to LPS via a mechanism that involves these targets. Our model for how exosome uptake is affecting the LPS response is summarized in Figure 8. Together our findings implicate endogenously produced, miRNA-containing exosomes in the regulation of the innate immune response *in vivo*.

Although our findings indicate that exosomal communication is needed to maintain hematopoietic homeostasis during the steady state, it remains to be clarified which component of the exosome contributes to these important physiological effects. Previous studies have highlighted the importance of certain contents of exosomes for immune cell responses (6, 30). Exosomes can directly present antigen via MHC II molecules on their surface to T cells (40, 41). Activation of NK cells can be mediated by NKG2D ligands and IL-15Rα on DC exosomes (42). Further, mRNAs and miRNAs can be transferred between cells via exosomes and modulate immune responses (7, 13, 35). These examples and more summarized in Théry et al. and Robbins et al. (6, 30) show that there are many potential molecules contained on or within exosomes that could be contributing to baseline phenotypes observed in the Rab27DKO mice.

Our experiments provide strong evidence that the transfer of exosomes containing miR-155, which is increased within exosomes in response to LPS (43), is important for proper responsiveness to LPS. However, while our experiments indicate that miR-155 itself is involved in this, it is important to note that other exosomal factors could be altered by the deletion of miR-155 in exosome donor cells. This possibility will be explored in future studies. Despite these possibilities, our data strongly support the hypothesis that exosomes are critical for limiting chronic inflammation and the range of phenotypes involved in this condition as well as for enabling proper responses to inflammatory cues through a miR-155 dependent mechanism.

In order to further understand the role of endogenously produced exosomes in immune cell communication novel reagents are required moving forward. The development of additional

ways to specifically decrease exosome production *in vivo* would be helpful to see if the phenotypes observed in a different model of defective exosome production mimics the phenotypes seen in Rab27DKO mice. To specifically delve into the role of miRNAs within exosomes a reagent where the production of miRNA-containing exosomes can be specifically blocked *in vivo* is needed. As we begin to identify factors involved in miRNA loading, such as Ybx1, hnRNPA2B1, and SYNCRIP (44–46), novel mouse strains can be created to address these questions. Furthermore, the production of exosomes containing only the miRNA of interest would make a strong tool both for further understanding of that miRNA within exosomes and for therapeutic applications down the road.

Our study implicates exosomes as key regulators of chronic inflammation, and presents a valuable model that can be used to better understand the role of exosomes in this condition. Chronic inflammation and associated diseases pose an enormous economic and medical burden making it essential to understand the underlying mechanisms of these disorders. Due to our observations that exosome delivery could rescue some aspects of chronic inflammation and responsiveness to LPS, we propose that exosomes could potentially be used in a therapeutic manner to promote these outcomes clinically. Additionally, the differential response of WT versus miR-155−/− exosome treatment suggests that miRNAs can impact the function of the exosomes and their ability to alter response to LPS. Therefore, it stands to reason that the miRNA contents of exosomes could be manipulated to alter the therapeutic outcome desired during exosome treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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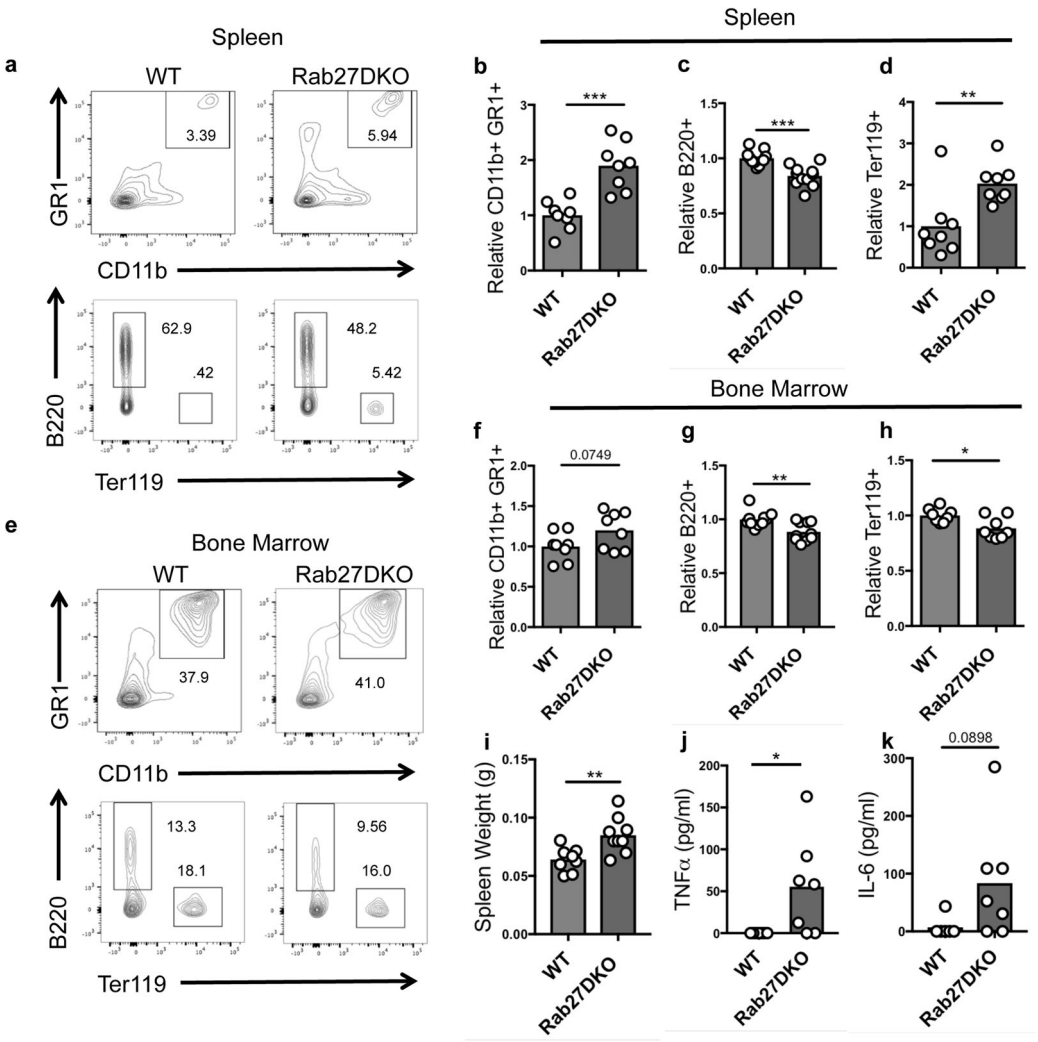

Figure 1. Rab27DKO mice display chronic, low-grade inflammation

6–8 week old WT or Rab27DKO mouse hematopoietic populations were analyzed in the bone marrow and the spleen. (A) Granulocyte-monocyte myeloid (GR1+ CD11b+), erythroid precursor (Ter119+), and B cell (B220+) populations were analyzed in the spleen via flow cytometry. Representative flow plots are displayed. (B–D) Relative levels of GR1+ CD11b+, B220+, and Ter119+ populations in the spleen were quantified and set relative to WT controls. (E) Representative flow plots of myeloid (GR1+ CD11b+), erythroid precursor (Ter119+), and B cell (B220+) populations in the bone marrow. (F–H) Relative levels of GR1+ CD11b+, B220+, and Ter119+ populations were quantified in the bone marrow. (I) Spleen weights of WT and Rab27DKO mice. (J–K) TNFα and IL-6 protein levels were quantified via ELISA from the serum of WT and Rab27DKO mice. Dots represent individual mice and the bar represents the mean. Relative levels are relative to the WT condition where the average of the WT condition is set to 1. Data are representative of at least 3 individual experiments. *P* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; Welsh's corrected *t*-Test.

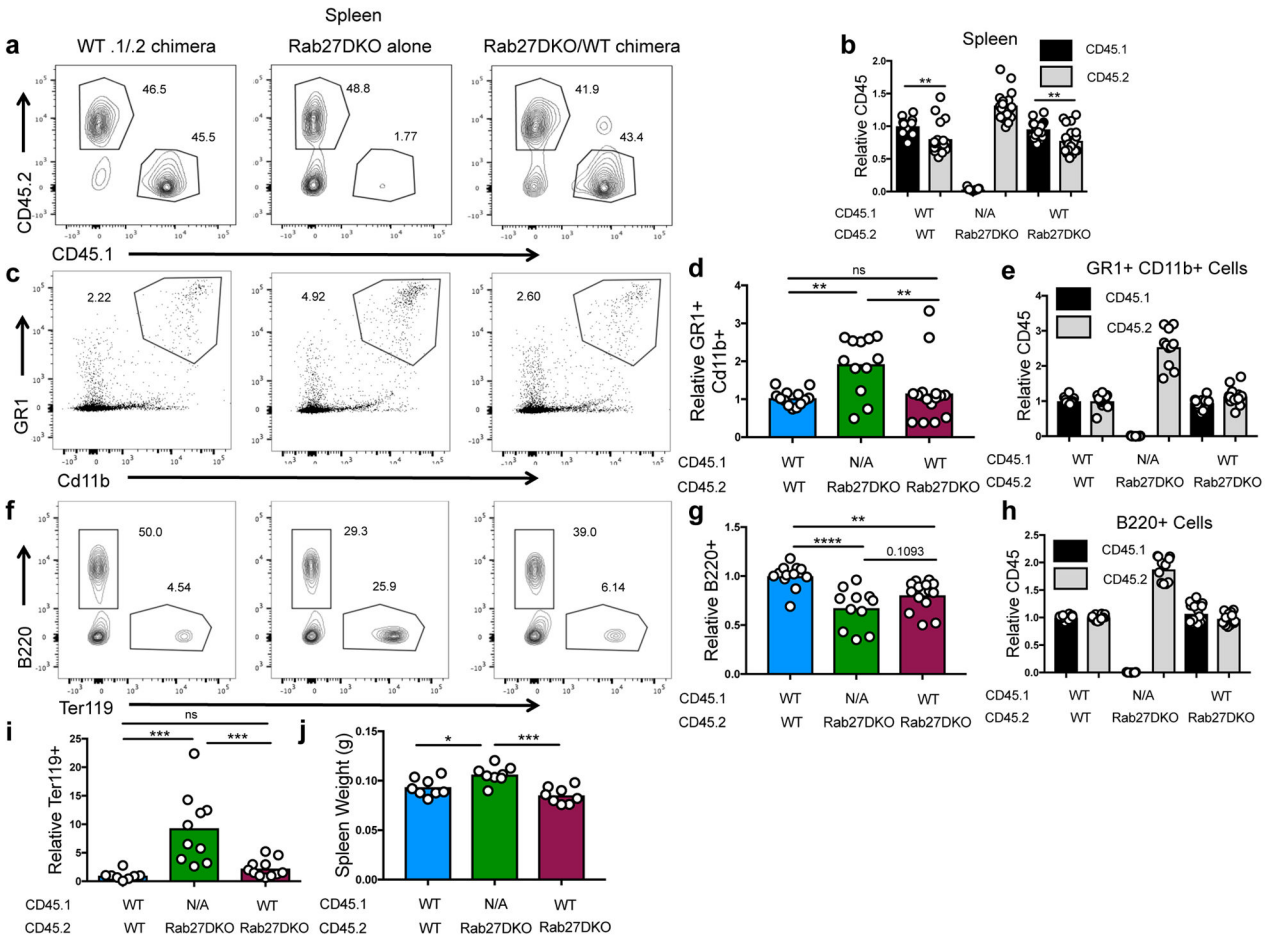

Figure 2. A subset of Rab27DKO phenotypes are cell extrinsic

WT CD45.1 mice were lethally irradiated and reconstituted with either a 1:1 mix of WT (CD45.1+) and Rab27DKO (CD45.2+), Rab27DKO (CD45.2+) alone, or 1:1 mix of CD45.1+ and CD45.2+ WT bone marrow for 2 months. (A) Representative flow plots of reconstitution efficiency in the spleen using CD45.1/.2 as a marker. (B) Reconstitution efficacy was quantified by CD45 markers in the spleen, genotype of CD45 marker is indicated below the graph. (C–D) GR1 CD11b+ representative flow plots and percentages with quantification of relative levels to the right. (E) CD45 markers within the GR1+ CD11b + population. (F–G) Ter119+ and B220+ representative flow plots are shown for the spleen and relative levels are quantified to the right. (H) CD45 markers within the B220+ population in the spleen. (I) Relative Ter119+ cells. (J) Spleen weight in grams. Dots represent individual mice and the bar represents the mean. Relative levels are relative to the WT condition where the average of the WT condition is set to 1. Data are representative of 4 individual experiments. Adjusted p values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison.

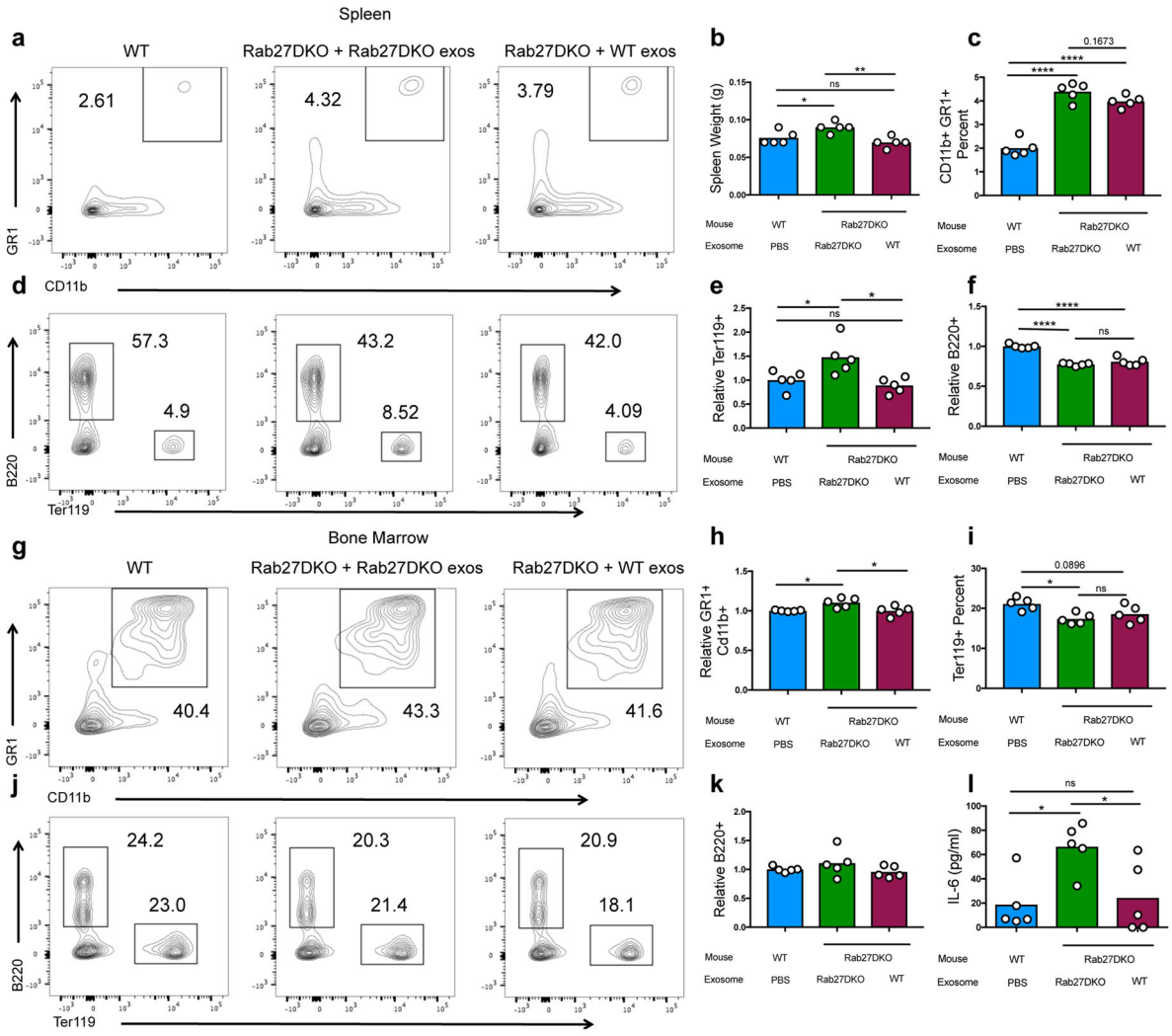

Figure 3. WT exosome treatment can complement certain Rab27DKO phenotypes

Rab27DKO mice were injected 2 times per week for 4 weeks with the exosomal pellet from WT or Rab27DKO GM-BMs. (A) GR1+ CD11b+ representative flow plots in the spleen. (B) Spleen weights in grams of the treatment groups. Mouse genotype and exosome treatment are indicated below the graphs. (C) Quantification of GR1+ CD11b+ relative levels. (D–F) Representative flow plot of Ter119+ and B220+ cells in the spleen with quantification of relative levels to the right. (G–H) GR1+ CD11b+ representative flow plots in the bone marrow and quantification of these percentages. (I–K) Representative flow plot of Ter119+ and B220+ cells in the bone marrow and relative levels are quantified to the right. (L) IL-6 levels were quantified via ELISA from the serum. Relative levels are relative to the WT condition where the average of the WT condition is set to 1. Data are representative of 3 individual experiments. Dots represent individual mice and the bar represents the mean. Adjusted *p* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison.

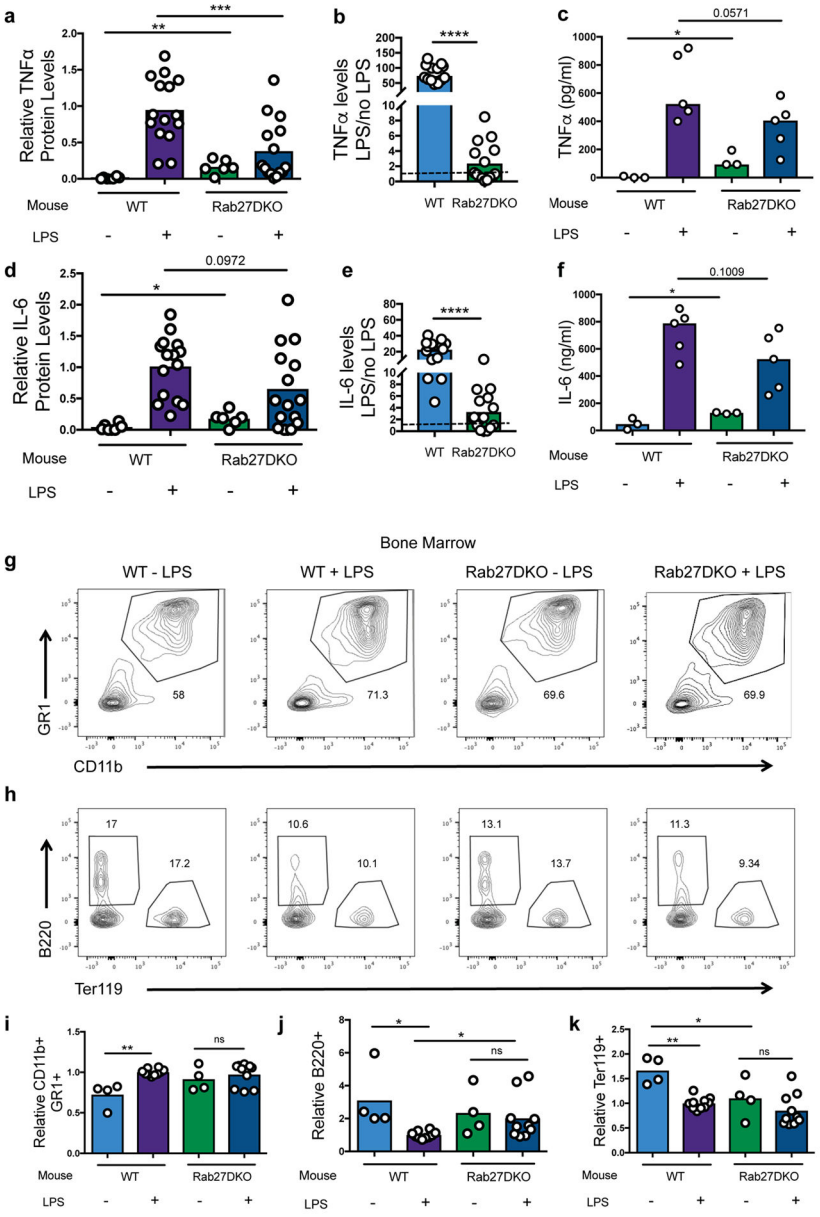

Figure 4. Rab27DKO mice have a refractory response to LPS

WT or Rab27DKO mice were challenged with or without LPS. Serum was taken 2 and 6 hours post LPS challenge while immune populations were examined 72 hours post LPS. (A) Relative levels of TNFα in the serum at 2 hours post LPS administration with WT LPS treatment group set to 1. (B) Fold change in TNFα levels in response to LPS where LPS levels of the cytokines are set relative to the no LPS treatment from same experiments shown in A. Dotted line represents no response to LPS. *P* value from Welsh's corrected *t*-test ****, $p < .0001$. (C) Representative TNFα concentrations from WT and Rab27DKO mice treated with or without LPS (D–F) Same analysis as A–C but for IL-6. (G–H) Representative flow plots of myeloid (GR1+ CD11b+), B cell (B220+) and erythroid precursor (Ter119+) populations for each experimental condition in the bone marrow compartment. (I–K)

Relative levels of CD11b+ GR1+, B220+, and Ter119+ population are shown with the WT +LPS average set to 1. Data are representative of 3 separate experiments. Dots represent individual mice and the bar represents the mean. Adjusted p values are either stated or *, $p < 0.05$; **, $p < 0.01$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison unless otherwise noted.

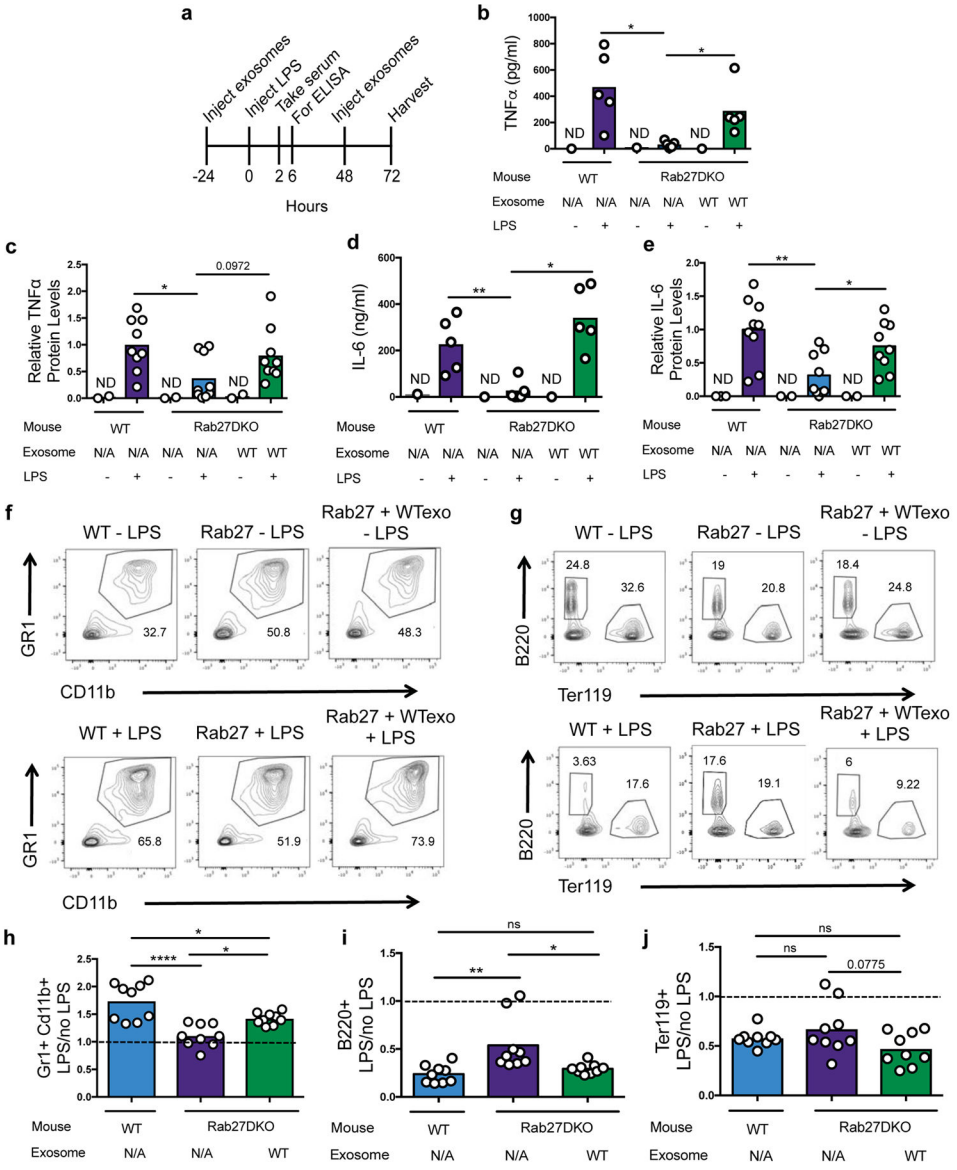

Figure 5. Injection of WT exosomes restores responsiveness to LPS by Rab27DKO mice

(A) Rab27DKO mice were either i.p injected with a PBS mock control or WT exosomal pellets 24 hours before an LPS challenge. Serum was taken 2 or 6 hours post LPS injection for ELISAs. 48 hours after LPS administration exosomes were injected again and 72 hours post LPS immune populations were analyzed. (B) Representative TNFα concentration at 2 hours post LPS treatment. Mouse genotype and exosome treatment are indicated below the graphs. (C) Relative levels of TNFα at 2 hours post LPS with WT treated with LPS was set to 1. (D) Representative IL-6 concentration at 6 hours post LPS treatment. (E) Relative levels of IL-6 at 6 hours post LPS with WT treated with LPS was set to 1. (F–G) Representative flow plots of myeloid (GR1+ CD11b+), B cell (B220+), and erythroid precursor (Ter119+) populations in the bone marrow compartment in each experimental condition. (H–J) Quantification of changes to the CD11b+ GR1+, B220+, and Ter119+ populations where the LPS treated group was set relative to the no LPS group to show the

responsiveness of the population. Dotted line marks no change between LPS and no LPS groups. Data are representative of 2 independent experiments. Dots represent individual mice and the bar represents the mean. Adjusted p values are either stated or *, $p < 0.05$; **, $p < 0.01$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison.

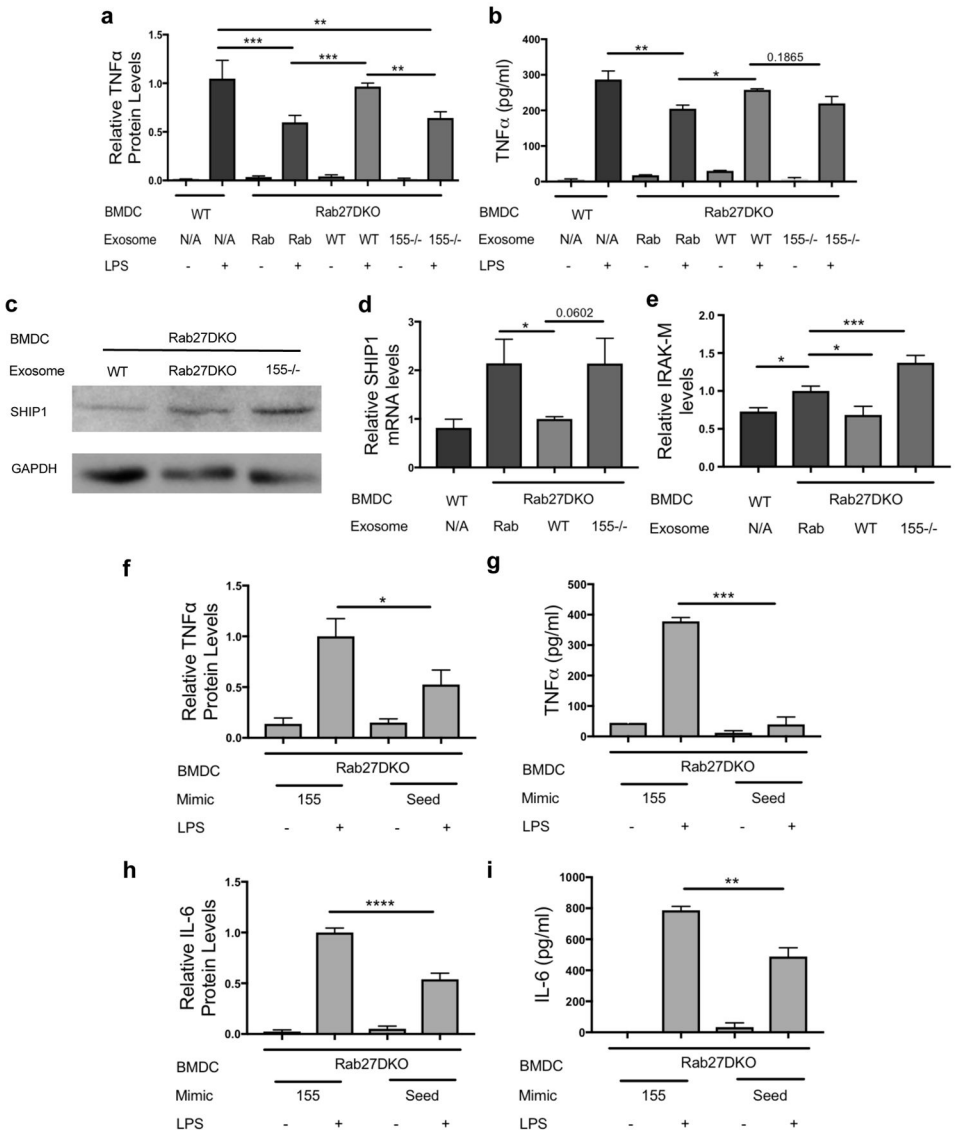

Figure 6. Restoration of Rab27DKO BMDC LPS responsiveness by miR-155 containing exosomes or miR-155 mimic

(A) Rab27DKO BMDCs were treated with Rab27DKO, WT, or miR-155−/− exosomal pellets from GM-BMs 24 hour before LPS administration. Relative media TNFα levels 2 hours post LPS administration are shown with the WT group treated with LPS was set as 1. n = 7. (B) Representative TNFα concentrations shown. n = 4. (C) Representative western blot of SHIP1 in Rab27DKO BMDCs that have been given WT, Rab27DKO, or miR-155−/− exosome pellets from GM-BMs and then treated with LPS. (D–E) Rab27DKO BMDCs were treated with the Rab27DKO, WT, or miR-155−/− exosomal pellet from GM-BMs 24 hours before LPS administration. 6 hours after LPS treatment RNA was harvested and SHIP1 and IRAK-M levels were assayed with qRT-PCR with L32 as a loading control. Data is set relative to the Rab27DKO BMDC treated with WT exosomal pellets which is set to 1. n = 5 (F–I) Rab27DKO BMDCs were treated with either miR-155 mimic (155) or a miR-155 mimic where the seed region was mutated (Seed) 24 hour before LPS administration. (F)

Relative TNFα levels. n = 7 (G) TNFα concentration. n = 4. (H) Relative IL-6 levels. n = 7 (G) IL-6 concentration. n = 4. The error bars represent +/− S.E.M. Adjusted *p* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison.

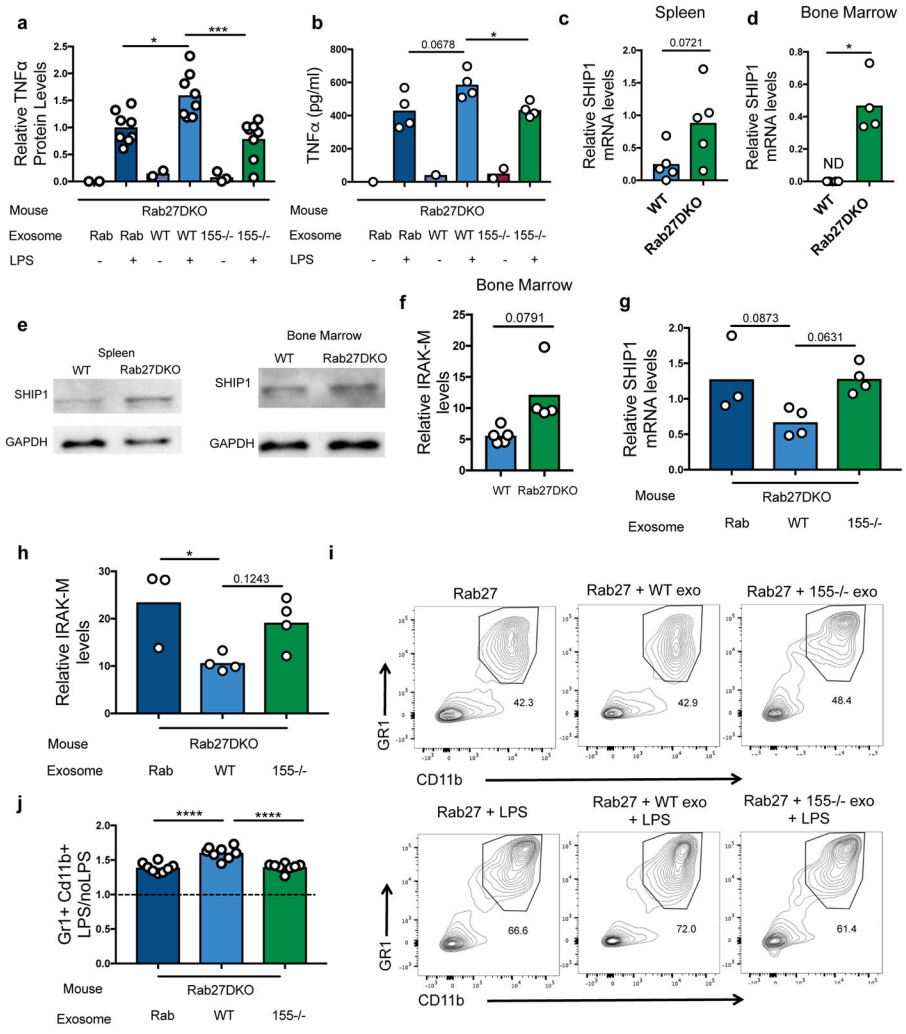

Figure 7. miR-155 containing exosome rescue LPS responsiveness in Rab27DKO mice

(A) Rab27DKO mice were either i.p injected with a Rab27DKO, WT, or miR-155−/− exosome pellets 24 hours before an LPS challenge. Serum was taken 2 hours post LPS injection for ELISAs. Values are set relative to Rab27DKO +Rab27DKO exosomal pellets +LPS set to 1. (B) Representative TNFα concentrations. (C–D) Levels of SHIP1 mRNA in resting WT and Rab27DKO mice in the spleen and bone marrow relative to L32 loading control. *P* values are from Welsh's corrected *t*-tests. (E) Westerns of SHIP1 in resting spleen and bone marrow with GAPDH as a loading control. (F) IRAK–M mRNA levels in resting bone marrow relative to L32. *P* values are from Welsh's corrected *t*-tests. (G–H) Levels of SHIP1 and IRAK-M mRNA in Rab27DKO mice BM that received Rab27DKO, WT, or miR-155−/− exosomal pellets then were treated with LPS for 72 hours. (I) Representative flow plots of the myeloid (GR1+ CD11b+) population for each condition in the bone marrow compartment from the same experiment conditions of H. (J) Ratio of the CD11b+ GR1+ population is shown of the LPS treatment group compared to the no LPS treatment group. Dotted line marks no change between LPS and no LPS treatments. Adjusted *p* values

are either stated or *, $p < 0.05$; ***, $p < .001$; ****, $p < .0001$; one-way ANOVA Tukey multiple comparison unless otherwise noted.

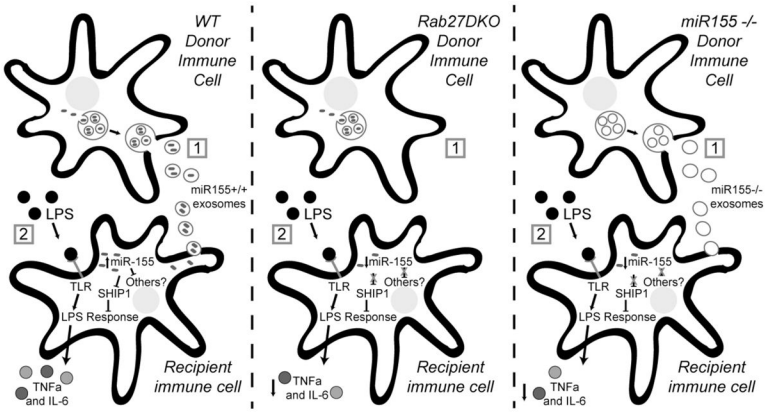

Figure 8. Model of the contribution of exosomal miR-155 to the LPS response

In a WT scenario, miR-155 can be transferred to a recipient cell leading to the knockdown of targets like SHIP1. Then the cell receives an LPS signal and can respond properly. However, in the Rab27DKO model, cells are defective in producing the appropriate amounts of exosomes, therefore the recipient cells do not receive miR-155 and cannot down-regulate targets such as SHIP1 and thus respond improperly to LPS. In the last scenario, the cells are producing exosomes but they do not contain miR-155 resulting in the lack of transferred miR-155, increasing levels of miR-155 targets, and an improper response to LPS.