ROLE OF EXOSOMAL MIRNAS IN IMMUNE CELL COMMUNICATION

by

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ABSTRACT

Immune cells need fine-tuned intercellular communication to properly respond to pathogens. Recently, researchers characterized a novel form of intercellular communication where microRNAs (miRNAs) can be transferred between cells in exosomes. Immune miRNAs, such as miR-155 and miR-146a, are important for post-transcriptional gene regulation and are essential for proper immune cell function. miR-155 is proinflammatory while miR-146a is an anti-inflammatory miRNA. We found that miR-155 and miR-146a were released from bone marrow derived dendritic cells (BMDCs) within exosomes and are transferred to recipient BMDCs. Upon transfer, miRNAs decreased levels of their mRNA targets in recipient cells in a seed dependent manner and reprogrammed the cellular response to endotoxin. Exosomal miR-155 enhanced, whilemiR-146a reduced, inflammatory gene expression. Additionally, injection of miR-146a containing exosomes into mice delivered miR-146a to various tissues and decreased inflammation in response to endotoxin, while miR-155 containing exosomes had the converse effect. Using the Rab27ab-/- (Rab27DKO) mice, which are deficient in producing exosomes, we assayed the importance of exosomes during endotoxin response *in vivo* and observed that these mice have defective response to endotoxin, which could be rescued with injection of WT exosomes. These results suggest that exosome uptake is important for proper response to endotoxin. We are continuing to utilize the Rab27DKO model to investigate the role of exosomes during normal hematopoietic development and during

various disease states. We have found that Rab27DKO mice have a slight resting myeloproliferative disorder as well as over-activated T cells. Their extrameduallary hematopoiesis defects can be rescued by the presence of WT bone marrow (BM) and by injection of WT exosomes in resting mice suggesting that exosome uptake is important for this phenotype. Additionally, in a multiple sclerosis (MS) model, Experimental Autoimmune Encephalomyelitis (EAE), Rab27DKO mice have worsened disease, which we hypothesize is due to their increase in activated T cells at rest. Overall, our results suggest that exosomal communication is important for normal hematopoietic development and is involved in immune responses to challenge.

To my family,
Donald Griffith, Constance Griffith, Pamela Griffith, James Alexander, Nancy Alexander, Rebecca Alexander, Katherine Alexander, and Kevin Hallman

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LIST OF ABBREVIATIONS

AD.....Alzheimer's disease

BM.....Bone marrow

BMDC.....Bone marrow derived dendritic cell

CVD.....Cardiovascular disease

DAMPs.....Damage associated molecular patterns

DC.....Dendritic cell

EAE.....Experimental autoimmune encephalomyelitis

EM.....Electron microscopy

FACS.....Florescence-activated cell sorting

GCB.....Germinal center B cells

GM-BM.....GMCF cultured bone marrow

IBD.....Inflammatory bowel disease

i.p.....Immunoprecipitation

lncRNA.....Long noncoding RNA

LPS.....Lipopolysaccharide

MFI.....Mean fluorescence intensity

miRNA.....microRNA

MS.....Multiple sclerosis

MVB...Multivesicular body

ncRNA...noncoding RNA

NLD...Nod-like receptor

nSMase2...neutral sphingomyelinase 2

NTA...non-templated nucleotide addition

qRT-PCR...Quantitative reversetranscriptase-PCR

RA...Rheumatoid arthritis

Rab27DKO..Rab27a and b double knockout

RISC..RNA-induced silencing complex

SLE. ....Systemic lupus erythematosus

T1D...Type 1 diabetes

T2D...Type 2 diabetes

TCR...T cell receptor

TFH...T follicular helper cell

TLR...Toll-like receptor

TNF...Tumor necrosis factor

Treg...Regulatory T cell

UTR...Untranslated region

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CHAPTER 1

INTRODUCTION

Discovery and characteristics of exosomes

In 1983, the Pan and Johnstone group and the Harding, Heuser, and Stahl team observed that reticulocytes release vesicles containing the transferrin receptor into the extracellular space (1, 2). These were the first descriptions of exosomes, which Johnstone further characterized in 1987 as having activity associated with the plasma membrane. It was initially thought that these shed vesicles were merely a way cells could rid themselves of unwanted content. Indeed, there did not seem to be much interest in these vesicles for many years after their discovery and many believed that the release of exosomes seen by the Johnstone and Stahl labs was an artifact (3). Despite this, the Johnstone and Stahl labs worked to characterize how to isolate these vesicles and began to identify components associated with them. By utilizing electron microscopy (EM) they observed that transferrin localized to both the intraluminal vesicles inside the multivesicular body (MVB) within the cell and was also present on exosomes released from the cell (2, 4). In the EM images, the MVB appeared to fuse with the plasma membrane to release the exosomes into the extracellular space. This suggested a role for the MVB in the biogenesis of exosomes. From the EM images they were also able to estimate the size of exosomes to be approximately 50nm and described the exosomes as being composed of a single lipid bilayer. Since then, the size of exosomes has been characterized to be in the range of 40-100nm (5).

After these initial observations of exosomes, it has been appreciated that exosomes are not the only form of secreted vesicle. Several categories of larger membrane structures have been isolated from cells, including microvesicles that range in size from 150nm-1000nm and apoptotic bodies that range from 1μm-5μm (5). Due to the presence of other secreted vesicles, the isolation of exosomes has been a topic of great discussion.

Classically, exosomes have been isolated via differential centrifugation, where the cell-culture supernatant is subjected to multiple centrifugations at increasing speeds to remove cellular debris and larger vesicles (6). However, there is a push in the field to further isolate exosomes based on various markers properties (6).

As the isolation techniques for exosomes improved, the field was able to investigate the molecular make-up of these vesicles. Initial investigation of exosomal content consisted of western blots of know cellular proteins on exosomes isolated from various cell types (7). Certain cell types have had more extensive characterization performed. For example, dendritic cell (DC) exosomal protein composition has been analyzed with mass spectrometry. This unbiased approach identified unexpected proteins associated with exosomes such as the MHC class II protein. With these approaches both ubiquitous and cell-specific proteins have been identified and these proteins can be contained within the exosomes or spanning the membrane. Many different classes of proteins were identified including cytoskeletal, heat shock, MHC class I and II, and tetraspanins. Tetraspanins such as CD63 and CD81 on exosomes have since been used as marker of exosomes.

Exosome biogenesis, secretion, and uptake

After the initial observation that exosomes were being secreted via the MVB, the biogenesis of exosomes began to be investigated further. Classically, the MVB was thought to fuse with the lysosome to degrade surface proteins. However, the MVB can also traffic to and fuse with the plasma membrane resulting in the release of exosomes into the extracellular space (8). SNARE proteins are involved in the fusion event between the MVB and plasma membrane that results in exosome release (7). Rab27a and Rab27b are two

GTPases that are important for the docking and retention of the MVB at the plasma membrane (9). The knockout of Rab27a and b dramatically reduces exosomes secretion, and thus makes a strong tool for studying these vesicles *in vivo* (9).

Once secreted, exosomes can be taken up by other cells and deliver their content to the cytoplasm of the recipient cell (10). The mechanism of this uptake is still not completely worked out; however, there are three proposed ways to deliver exosomes (11). One theory contends that the membrane of exosomes can fuse to the plasma membrane to deliver its contents to the cytoplasm. There has been some evidence for this theory where researchers have observed hemi-fusion of the exosome and plasma membranses via EM (12). The second theory suggests that the exosome is taken up via endocytosis that could be mediated by clathrin or caveolin (13). Once the exosome is taken up in the endosome the exosome membrane can fuse to the endosomal membrane. There is also evidence that endocytosis of exosomes is receptor mediated, where proteins on the surface of the cell and exosome are required for uptake (11). The final theory is specific to phagocytic cells, where it is thought that exosomes can be taken up by phagocytosis and then fuse to the phagocytic membrane to release their content into the cytoplasm (11).

Discovery of miRNAs in exosomes

Within the past decade or so miRNAs have been observed in the extracellular space (10). These extracellular miRNAs can be contained within vesicles like exosomes or are free-floating, perhaps bound by protein (14). While there is some debate within the field about the exact source of extracellular miRNA and which source contributes the most to extracellular miRNA content, exosomes have been isolated by various methods and have

been found to contain miRNAs that are resistant to RNase treatment (10, 12). Researchers were initially interested in extracellular miRNAs for their potential as biomarkers for various diseases (15); however, in 2007, Valadi et. al. investigated the ability of exosomal miRNAs and mRNA to be functionally transferred between immune cells as a novel way cells could exchange information (10).

Selective loading of miRNAs into exosomes

The composition of miRNAs secreted from a cell within exosomes is different from the profile of the parent cell (16–20). This observation led to the hypothesis that miRNAs are not merely passively packaged into exosomes, but are selectively loaded into these vesicles. There have been a few studies done investigating the mechanism of how miRNAs are loaded into exosomes. An approach taken by several groups was to immunoprecipitate a biotin tagged miRNA that is normally found within exosomes and a miRNA that is normally not found within exosomes. The proteins that associated with the exosomal miRNA but not with the nonexosomal miRNA were used as candidates for potential factors that could be involved in miRNA loading. In primary human T cells and the Jurkat T cell line, hnRNPA2B1 was implicated in sorting (19) while YBX1 was implicated in 293T cells (20). Another idea is that the level of the mRNA target could affect the loading of miRNAs into exosomes (18) where if more of the miRNA targets are present in a cell, then the miRNA will not be loaded into the cell.

Intercellular communication via exosomal miRNAs

Since the initial observation that miRNAs were contained within exosomes and could potentially be transferred between cells as a form of intercellular communication, many other groups have begun to investigate this idea (21, 22). The bulk of the work investigating the functional transfer of miRNAs is focused on cell culture, cancer cells, and immune cells (21, 22). In cell culture, for example, cancer cells secrete miR-105, which is able to promote metastasis by destroying endothelial barriers (23). In immune cells, Monteclavo et. al. demonstrated that miRNAs could be shuttled between dendritic cells and mediate target knockdown in recipient cells (12). Additionally, miRNAs can be transferred between T cells and DCs at the immune synapse (24). Finally, one of the few studies investigating the function of exosomal miRNA communication *in vivo* focuses on the role exosomal miRNA, Let7d,which contributes to the functional repression of Th1 cell by Tregs (25). These studies highlight the functional transfer of miRNAs via exosomes as a form of intercellular communication.

While previous studies provide important evidence that miRNAs are transferred between immune cells via exosomes, it is still unclear in some cases whether the transfer is merely upregulating the endogenous miRNA. Additionally, the physiological relevance of the transfer of miRNAs still remains uncertain. 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. Our studies focus on miR-146a and miR-155 which are two important immune miRNAs. These two miRNAs play opposing role during inflammation where miR-155 promotes and miR-146a dampens inflammatory responses and our work focuses on the role of the transfer of these miRNAs

during inflammatory responses as discussed in Chapter 2.

Dissertation summary

This dissertation aims to investigate the functional transfer of two immune miRNAs, miR-155 and miR-146a, via exosomes and the importance of exosomal communication *in vivo.* Using specific miRNA knockout cells and mice we investigated the exogenous delivery of miR-155 and miR-146a and determined that their delivery was able to knockdown their respective targets. Additionally, we address whether the delivery of these miRNAs alters the cellular response to endotoxin. The importance of exosome communication *in vivo* remains largely unexplored. Therefore, this dissertation focuses in the second half on the relevance of exosomes during hematopoietic homeostasis and during immune responses using the Rab27DKO mouse model.

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CHAPTER 2

NONCODING RNAS AND CHRONIC INFLAMMATION: MICRO-MANAGING THE FIRE WITHIN

Alexander, M., and R. M. O'Connell. 2015. Noncoding RNAs and chronic inflammation: Micro-managing the fire within. *BioEssays* 37: 1005–1015. © Owned by the authors and published by Bioessays, 2015. With the permission of WILEY periodicals.

Prospects & Overviews

Review essays

Noncoding RNAs and chronic inflammation: Micro-managing the fire within

*Margaret Alexander and Ryan M. O'Connell**

Inflammatory responses are essential for the clearance of pathogens and the repair of injured tissues; however, if these responses are not properly controlled chronic inflammation can occur. Chronic inflammation is now recognized as a contributing factor to many age-associated diseases including metabolic disorders, arthritis, neurodegeneration, and cardiovascular disease. Due to the connection between chronic inflammation and these diseases, it is essential to understand underlying mechanisms behind this process. In this review, factors that contribute to chronic inflammation are discussed. Further, we emphasize the emerging roles of microRNAs (miRNAs) and other noncoding RNAs (ncRNA) in regulating chronic inflammatory states, making them important future diagnostic markers and therapeutic targets. Copyright Line: © 2015 The Authors *BioEssays* Published by Wiley-VCH Verlag GmbH & Co. KGaA.

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Abbreviations:
AD, Alzheimer's disease; **CVD,** cardiovascular diseases; **IBD,** inflammatory bowel disease; **lncRNA,** long noncoding RNA; **ncRNA,** noncoding RNA; **RA,** rheumatoid arthritis; **SLE,** systemic lupus erythematosus; **T1D,** type 1 diabetes; **TLR,** Toll-like receptor.

Introduction

The mammalian inflammatory response is a double-edged sword. Although immune responses are necessary for efficient pathogen clearance, symbiosis with commensal microbes, wound repair and overall tissue homeostasis, these responses can become dysregulated and initiate a chronic reaction that lacks resolution [1]. This condition, referred to as chronic low-grade inflammation, can fester for long periods of time and adversely contribute to, or possibly even cause, many diseases associated with the aging including obesity [2], type 1 diabetes (T1D) [2], rheumatoid arthritis (RA) [3], systemic lupus erythematosus (SLE) [4], neurodegeneration [5], and cardiovascular diseases (CVD) [6]. In many of these cases chronic inflammatory symptoms, such as elevations in inflammatory cytokines and autoantibodies in the serum, can serve as a prognostic indicator of later disease manifestation and overall morbidity and mortality [7].

As the world's elderly population continues to grow at an alarming rate, there is tremendous need to predict which individuals are at the highest risk for developing many of the disorders mentioned above. However, because chronic inflammation does not typically cause obvious clinical symptoms, and screening for inflammatory markers is not a test performed during routine medical exams, the potentially predictive power of one's chronic inflammatory state is not currently being harnessed. This is in part due to the fact that elevated inflammatory factors in the serum are not markers specific to chronic inflammation, and thus their diagnostic value is currently limited. Further, until a better understanding of the mechanisms underlying this deleterious condition is obtained, therapeutic inhibition of chronic inflammation will remain challenging.

In this article, we review our current understanding of the known causes of chronic low-grade inflammation with a focus on factors distinct from chronic infection. We will also focus on cells of the immune system, although we recognize that non-immune cells also contribute to this state. We also discuss recent evidence that mammalian microRNAs and long noncoding RNAs (lncRNAs) have evolved to regulate chronic

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inflammatory states including those that occur during the aging process, and describe how they provide both diagnostic and therapeutic opportunities moving forward.

Causes of chronic inflammation

During chronic inflammation the resolution phase of the inflammatory response does not occur. This can be a result of either a persisting stimulus and/or the perturbation of molecular mechanisms involved in the resolution of inflammation. Thus, normal "healthy" immune responses can progress to chronic inflammatory states in instances where either of these events takes place.

Immune responses involve a resolution phase where the inflammatory response is shut down once the stimulus, such as a pathogen, is cleared. Mechanisms of resolution have been an intense area of study in recent years, and many important steps in this process have been discovered. Specialized cell types, such as T regulatory cells (Tregs), carry out critical immune repressive functions that are essential in preventing autoimmunity [8]. At the molecular level, the cytokine IL-10 [9, 10], the signaling molecule A20 [11], the signaling receptor PD1 [12], the signaling molecule CTLA4 [13], and the secreted factor IL1RA [14] are all examples of molecules that have evolved to balance and ultimately shut down immune cell activation. In some cases, these pathways are already being exploited therapeutically for such applications as cancer therapy. For instance, antibody disruption of the PD1 pathway, which enhances the immune response against tumors, is proving to be an effective therapy for melanoma [15]. Further, as we will describe below, specific miRNAs such as miR-146a have also evolved to keep immune responses in check.

Initial causes of some forms of chronic inflammatory states are also incompletely understood, yet their identification and prevention is key to avoiding the disease process. Recently, several distinct contributing factors have been described (Fig. 1) and include the following: (i) chronic inflammation can be primarily initiated by immune responses to self-tissues. The recognition of self-antigens by the immune system can result in diseases such as RA [16], SLE [17], multiple sclerosis (MS) [18], and T1D [19], and this self-recognition may or may not have a microbial component; (ii) in other instances, the immune response appears to be a secondary event that emerges in response to damage associated molecular patterns (DAMPs) that are produced following a breakdown in tissue homeostasis where the ensuing immune response driven by DAMPs alters tissue function [20]. Examples of diseases associated with DAMPs include obesity – where nutrient excess and hypertrophic adipocytes are the primary drivers [21], CVD – where lipoprotein buildup initially seeds the pathology [22], and certain neurodegenerative disorders such as Alzheimer's disease (AD) – mediated by protein aggregation [5]; (iii) contributions by the microbiota are also documented in some types of chronic inflammation, as their metabolites can influence both gut and peripheral tissues [23]. Obesity has been associated with alterations to the gut microbiota composition [24]; (iv) finally, the aging process itself leads to changes in immune system phenotypes and correlates with increasing inflammatory status as we grow older [25]. In this section, we will expand on each of these aspects of chronic inflammation.

Self-antigens produce autoimmune responses

A common cause of inflammatory conditions involves the inappropriate immune response to self-tissues, as is the case for RA [16], SLE [17], MS [18], and T1D [19]. For instance, in RA auto-reactive leukocytes attack joint tissues through a variety of mechanisms including production of autoantibodies, reactive oxygen, and nitrogen species as well as secretion of pro-inflammatory cytokines that recruit additional immune cells to the site of tissue damage [16]. Although this phenotype is fairly well characterized, and thought to arise from an inappropriate initial response by the immune system, the underlying triggers of diseases such as RA are still being deciphered. Studies have found a link between a person's genetics, such as MHC type, and disease risk and this is consistent with antigen presentation playing a critical role in the triggering of disease [26, 27]. Additionally, self-antigen responses might also ensue as a result of cleaning up dead or dying cells that may trigger responses against antigens from the tissue where these cells were derived [28].

Additional contributions may also be made by microbial pathogens that produce antigens that are similar to host proteins [29]. This type of molecular mimicry is an attractive hypothesis; however, the identity of pathogens that trigger these responses in different autoimmune settings remains largely elusive. One example of an infectious agent that triggers chronic arthritis is *Borrelia burgdorferi* [30]. Although most people return to health after clearing the infection, about 10% of infected individuals develop chronic arthritis in their joints even after the infection is cleared [31]. Whether this is working through molecular mimicry or a persistent, hard to detect microbial reservoir is unclear and future work is needed to better define this process.

Another interesting theory is that human endogenous retroviruses may also play a role in driving disease onset. Human endogenous retrovirus-K has been implicated in the development of RA [32]. It is thought that this retrovirus impacts the development of RA through molecular mimicry of self antigens. Additionally, human endogenous retrovirus type W envelope expression has been associated with MS [33]. Along with these examples, there have been several implications for human endogenous retroviruses in the development of inflammatory diseases [34].

DAMPs are initiators of chronic inflammatory states

In other instances, sustained inflammatory responses can be driven by DAMPs produced as a result of tissue damage or stress, or other events that disrupt tissue homeostasis. Examples include lipoproteins in the vasculature that drive atherosclerosis [35], and protein aggregates in the CNS that are associated with AD [36]. In such cases, DAMPs are produced and are recognized by Toll-like receptors (TLRs) or Nod-like

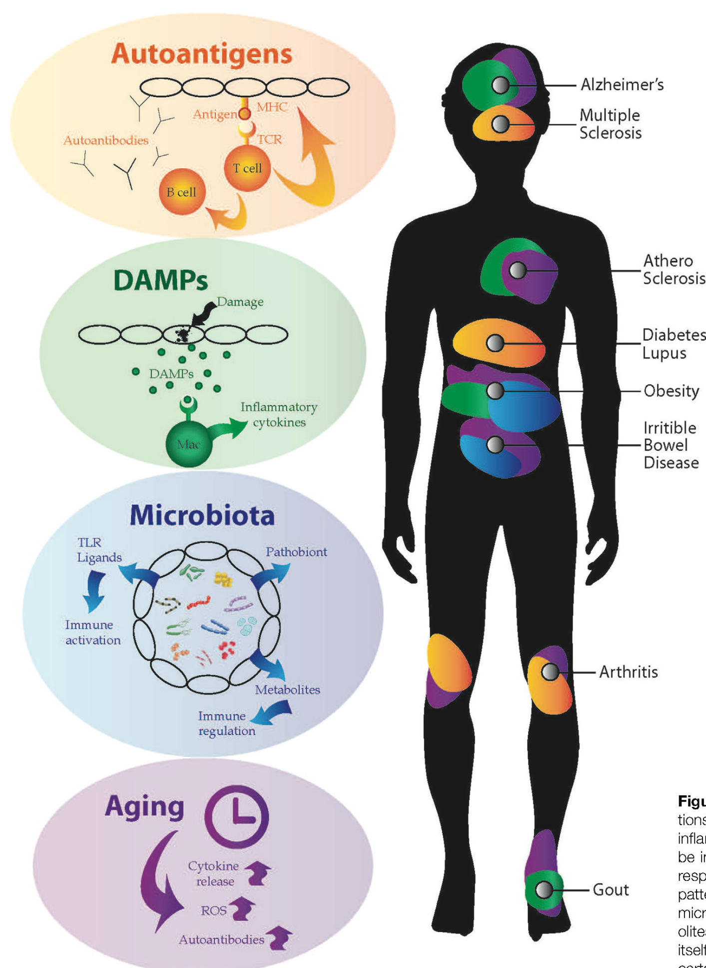


Figure 1. Mechanisms, anatomical locations, and disease types involving chronic inflammation. Chronic inflammation can be initiated by autoantigens or in response to damage associated molecular patterns (DAMPs). Furthermore, the microbiota – via the action of their metabolites, etc. – as well as the aging process itself have been shown to be involved in certain types of chronic inflammation.

receptors (NLRs) on innate immune cells [37]. In these instances, the innate immune system plays a secondary role as it responds to the inappropriate buildup or localization of certain molecules that signal disruptions to tissue homeostasis.

In the case of metabolic syndromes, such as obesity and diabetes, nutrient excess drives adipocyte hypertrophy, production of adipokines as well as inflammatory cytokines leading to eventual necrosis [38]. As adipocytes begin to die, their contents are taken up by tissues macrophages that are activated by products such as secreted cytokines and fatty acids that are detected by macrophage TLRs [39]. This response can change the nature of the tissue macrophage from an M2 to an M1 subtype, which subsequently initiates low-grade inflammation within adipose tissues. This includes the recruitment of a variety of immune cell mediators that reinforce the inflammatory state and promote insulin resistance, an early step in the development of type 2 diabetes (T2D). This is a good example of how a stressed tissue can induce an inflammatory response that is self-sustaining.

The microbiota and their products impact chronic inflammatory states

It is now widely recognized that the human microbiome, most of which resides in the intestinal tract, has an enormous

impact on our health. Thus, it is not surprising that commensal bacteria have been linked to a variety of chronic inflammatory conditions including inflammatory bowel disease (IBD), T1D, obesity, and neuroinflammatory diseases such as MS [40]. In each case, the microbiome of diseased individuals differs from that of healthy controls. Further, animal models have shown that the microbiota can cause or inhibit disease, based on its composition. This influence is mediated, at least in part, through the production and delivery of TLR ligands and other metabolites to either intestinal or extra-intestinal tissues that alter tissue homeostasis [41]. Many factors can influence the microbiota including diet, exposure to animals and agriculture, stress, and geographical location, indicating that lifestyle choices play an important role in microbiota composition [42]. However, while the importance of the microbiota in human health, including chronic inflammation, has become clear in recent years, the contribution of different microbial members to disease phenotypes is still under intense study as it is a complex mixture of distinct species.

The immune system changes with aging

Like other systems in our body, our immune system undergoes a variety of alterations as we grow older. Our thymus produces fewer naïve T lymphocytes [43], restricting one's ability to adequately respond to novel antigens and form memory against new pathogens or in response to vaccination. There is also an elevated amount of serum autoantibodies against self-tissues [44] and memory phenotype T cells can produce higher levels of inflammatory cytokines as they respond to persistent/chronic viral infections [45]. Hematopoietic output of innate immune myeloid cells becomes more prevalent [46], and senescent macrophages secrete higher amounts of inflammatory cytokines and produce ROS spontaneously [47]. Thus, the aging process creates an immune system that is less specific and more deregulated leading to a higher prevalence of autoimmunity in older versus younger individuals. It is also not surprising that a majority of diseases that emerge in the elderly are correlated with high levels of chronic inflammatory markers during middle age. As the world's aging population continues to grow larger, the need to prevent or treat disease in the elderly has become vital.

The regulatory potential of noncoding RNAs in the immune system

With the long-term goal of being able to prevent or reverse the pathological outcomes of chronic inflammatory states, much work has gone into understanding how these debilitating conditions are initially triggered, as we have just discussed. Further, we have developed a good understanding of various mechanisms that provide resolution to immune responses following necessary and protective responses to infection. This knowledge has led to the emergence of a wide range of possible therapeutic targets that are either currently being exploited clinically or still being tested and developed to reduce inflammation. Examples of therapeutic targets include anti-TNFα treatment for inflammatory arthritis [48], anti-IL-1 as a therapy for gout [49], anti-IL1R as treatment for a wide range of inflammatory diseases [50], anti-PD1 and anti CTLA4 for cancer treatment [15], and steroids for a variety of inflammatory conditions [51].

However, while these approaches hold much promise, they are based almost exclusively on targeting, activating, or inhibiting cellular protein factors that we know are involved in chronic inflammatory responses. Yet, approximately 3/4 of the human genome is transcribed into RNA, with only about 1% of these transcripts encoding proteins. Thus, most of the RNA diversity in our cells is made up of ncRNA. In recent years, it has become clear that different types of ncRNAs play important regulatory roles, not only in the immune system, but in all mammalian organ systems. In particular, miRNAs and lncRNAs have emerged as critical regulators of immune system development and function [52–54], including several new studies that have linked specific miRNA and lncRNA species to the control of chronic inflammatory conditions (Table 1). The role of lncRNAs in the immune system is reviewed further by Heward and Lindsay [54].

MicroRNAs, immune responses, and the regulation of cellular physiology

MicroRNAs modulate immune cell differentiation and responses

MicroRNAs are small, single-stranded ncRNAs that were first discovered in *C. elegans* [55] approximately one decade before they were appreciated in mammalian cells [56, 57]. Since then, research involving miRNAs has exploded over the past 10–15 years and much has been learned regarding their biogenesis, expression patterns and functions at the molecular, cellular and organismal levels. miRNAs clearly function to repress gene expression and influence virtually all organ systems in vertebrates [58]. Much of this has been extensively reviewed elsewhere [59]. However, there are certain fundamental attributes of miRNAs that make them ideally suited to regulate chronic inflammatory conditions.

Through their ability to modulate gene expression networks by adjusting the levels of dosage sensitive target genes, miRNAs are able to shift thresholds that dictate whether a cellular response will occur or not, how strong it will be, and if it will be resolved (Fig. 2) [60]. For instance, miR-146a is induced in response to TLR signaling and forms a negative feedback loop that inhibits Traf6 and Irak1, two critical upstream TLR-signaling mediators that promote macrophage activation (Fig. 2A). While miR-146a is a repressor of immune cell signaling [61], miR-155 and miR-181a are activators of inflammation. miR-155 is induced in activated myeloid cells and represses both Socs1 and Ship1 to enhance cytokine production by dendritic cells and macrophages (Fig. 2B) [62]. miR-155 has also been shown to enable CD8+ T cells to respond to limiting doses of γ-chain cytokines, which enables robust immune responses in lymphoreplete hosts [63]. T cell receptor (TCR) signaling strength is regulated by miR-181a, which modulates expression of several phosphatases

Table 1. Selected examples of ncRNAs with roles in regulating inflammation

Species	Type	Disease	Cell types	Targets	Reference
miR-155	miRNA	CVD, viral infection, MS, RA, SLE, tumor immunity, chronic low-grade inflammation	Tfh, Th17, Th1, Th2, Macs, B cells, Treg, DCs	SHIP1, SOCS1, BACH1, PU.1, JARID2, PELI1, FOSl2, ETS1	[62, 63, 66–70, 77–79, 88, 106–108]
miR-146a	miRNA	Autoimmunity, dermatitis, chronic low-grade inflammation	Th1, Tfh, Treg, B cell, Macs, DCs, HSC	TRAF6, IRAK1, STAT1	[61, 68, 86, 89, 108, 109]
miR-17∼92	miRNA	Tumor immunity asthma, MS, viral Infection	Tfh, Th17, Th1, Th2, Treg, B cell	PTEN, PHLPP2, SOCS1, RORA, A20, IKZF4	[65, 80–82, 110, 111]
miR-181a	miRNA	Autoimmunity, aging-related inflammation	T cells	DUSP6, SHP2, DUSP5, PTPN22	[64, 112]
miR-182	miRNA	Tissue inflammation	T cells	FOXO1	[113]
miR-29a	miRNA	Crohn's disease	Th1, DC	TBET, EOMES, IL-12p40	[114, 115]
miR-125	miRNA	IBD, SLE	Macs	KLF13, IRF4	[71, 72]
miR-223	miRNA	Inflammatory lung pathology	Macs, granulocytes	Mef2c, Pknox1	[73, 74]
miR-124	miRNA	Neuro-inflammatory	Microglia	C/EBP-α, PU.1	[116]
LincRNA-Cox2	LncRNA	–	Macs	CCL5, IL-6	[117]
NeST	LncRNA	Microbial infection	T cells, NK cells	IFNG	[118]
LncDC	LncRNA	–	Macs	STAT3 target genes	[119]
CCR2	LncRNA	–	TH2	TH2 genes	[120]
E330013P06	LncRNA	Diabetes	Macs	–	[103]
Thril	LncRNA	Kawasaki disease	Macs	TNFα, IL-8, CXCL10, CCL1, CSF1	[121]

DC, dendritic cells; HSC, hematopoietic stem cell; Macs, macrophages; NK cell, natural killer cell; Tfh, T follicular helper cells; Tregs, regulatory T cells.

that inhibit TCR-induced signaling pathways resulting in activation of T cell genes (Fig. 2C) [64]. Thus, miRNAs can both enhance or hinder signaling pathways that control innate and adaptive immune responses that underlie inflammation.

Further, miRNAs have also been shown to confer robustness to cellular states. In these scenarios, specific miRNAs play important roles in determining the extent to which differentiation occurs. For example, the miR-17∼92 cluster of miRNAs repress Pten and Phlpp2, inhibitors of Icos signaling in activated T cells, resulting in the skewing of cells into T follicular helper cells (Tfh cells) (Fig. 2D) [65]. In the absence of the miR-17∼92 cluster, these proteins are at higher levels and reduce the amount of Tfh cells produced during inflammatory responses. Another example is miR-155, a miRNA that is necessary for both T cell homeostasis and optimal differentiation of multiple T cell types including Th17 [66, 67], Tfh [68], Th2 [69], and Th1 cells [70]. In these cases, miR-155 appears to be working through repression of multiple targets including Jarid2, Socs1, Ship1, Ets1, Peli1, Fosl2 (and possibly others). However, additional work is needed to determine if unique target/s are used by miR-155 depending on the Th cell type produced. Because T cells are central regulators of inflammatory responses, their modulation by miRNAs is of significant relevance to chronic inflammatory states, as described below.

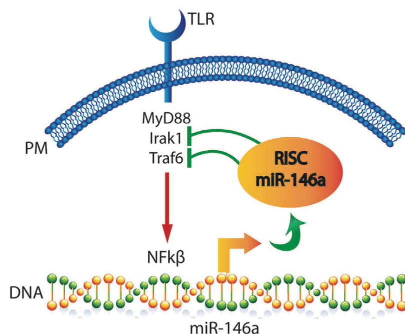
Additionally, several miRNAs have been implicated in regulating macrophage lineage skewing during inflammatory responses. Macrophages can be skewed toward either pro-inflammatory subtypes (M1), or toward more reparative and less inflammatory subtypes (M2). miR- 125 has been shown to repress M1 skewing while promoting the M2 fate [71, 72]. miR-223 has also been implicated in macrophage skewing where miR-223 promotes macrophage polarization toward the M2 subtype [73]. Additionally, miR-223 has been implicated in control of granulocyte activation, and miR-223-/- mice display overactive immune responses and develop inflammatory lung pathology [74].

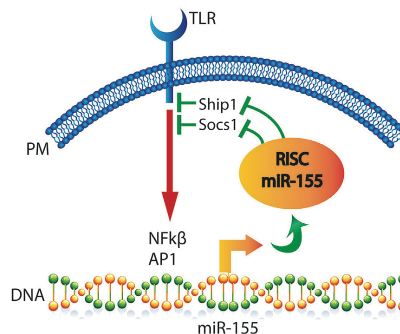
Further highlighting the importance of miRNAs in human systems, greater numbers of miRNAs have emerged throughout evolution, in addition to increased target diversity [75]. This suggests that miRNAs are among the regulatory mechanisms that enable increased human complexity despite a genome size that is similar to less complex organisms. This appears to include critical roles in establishing proper inflammatory set points and facilitating optimal responses and resolution by our immune system. In the next section, we will assess our current understanding of how miRNAs influence distinct types of chronic inflammatory conditions.

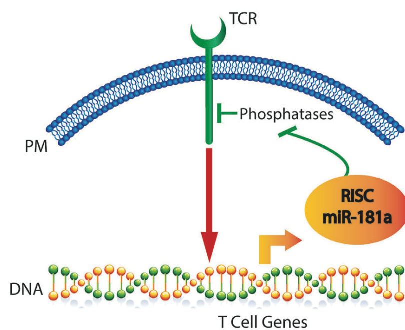
Functional roles for miRNAs during chronic inflammation

MicroRNAs regulate antigen specific responses

There has been a substantial amount of work to date assessing how miRNAs control different types of acute inflammatory responses following infection, immunization, tumor challenge, and induction of several antigen-dependent autoimmune conditions that are all rooted in inflammation [76]. In each case, specific miRNAs have been shown to play pivotal roles during disease onset, peak magnitude, and rate of resolution by influencing the immune cell populations that mediate these steps (Table 1). Examples include a pro-







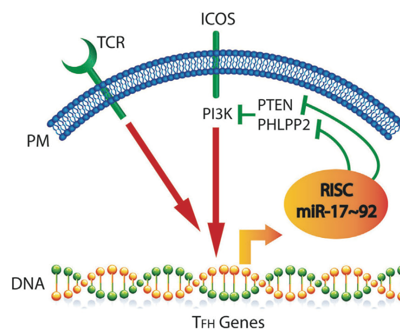


Figure 2. Mechanisms of miRNA function in the immune system. miRNAs can have various functions within the immune system and can act in different manners. **A:** For example, miR-146a acts as an anti-inflammatory miRNA via feedback inhibition of an inflammatory pathway. **B:** miR-155, on the other hand, is a pro-inflammatory miRNA that acts in a feed-forward manner bolstering the immune response. **C:** miR-181a is also a pro-inflammatory miRNA that acts by inhibiting phosphatases that block the activation of T cell genes thus activating T cells. **D:** Finally, the miR-17~92 cluster is involved in promoting the differentiation of a subset of inflammatory T cells called T follicular helpers cells (T_FH).

inflammatory role for miR-155 in T cells during antigen-induced experimental autoimmune encephalomyelitis (EAE) in mice [70], in B and T cells during collagen-induced arthritis in mice [77], in licensing CD8+ T cell responses against viruses and tumors [78], and in B cells during murine lupus [79]. miR-17~92 has been shown to enhance antibody responses against viral infections through its promotion of Tfh cell differentiation [65, 80], to promote Th1 cell responses against solid tumors [81], and provoke asthma through its augmentation of Th2 cell development [82]. miR- 146a has been shown to play a critical role in preventing the onset of arthritis following infection by *Borrelia burgdorferi* by influencing macrophage responses [83]. Importantly, this body of work strongly implicates miRNAs in the human iterations of these disorders where their altered expression is often observed. It is also relevant to note that while many of these studies are based upon induced disease states in mice, several of these disorders have been linked to pre-conditions of low-grade inflammation characterized by elevated titers of self-reactive antibodies and/or pro-inflammatory cytokines.

Although studies continue to unravel roles for different miRNAs in autoimmune disease states mediated by auto-antigens, far fewer studies have determined the role of miRNAs during chronic low-grade inflammation triggered by DAMPs, aging and the microbiota, and the diseases that emerge as a result of these triggers. We next turn to these emerging areas.

MicroRNAs are involved in inflammaging

As described above, the aging process itself is associated with inflammatory phenotypes. Early studies in *C. elegans* have functionally linked miRNAs to lifespan, perhaps offering a first clue that miRNAs are involved in different aspects of the aging process. For instance, lin-4 loss-of-function mutants

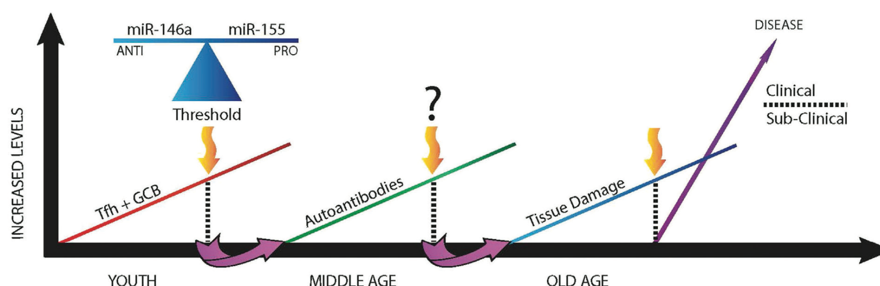


Figure 3. Sequential threshold model of age-dependent diseases linked to miRNA regulated chronic inflammation. During youth, the immune system has little chronic inflammation; however, as the aging process occurs, levels of inflammatory cells, such as T follicular helper cells (T_FH) and germinal center B cells (GCB), start to rise. Once the levels of these cells reach a certain threshold, they trigger increased inflammation and the production of autoantibodies. As levels of autoantibodies rise this might trigger tissue damage, which can accumulate and result in the clinical manifestation of chronic inflammatory diseases.

have shortened lifespans while lin-14 loss-of-function mutants have increased lifespans [84]. Additionally, mouse studies have shown that certain miRNAs alter expression patterns with aging. For example, in the mouse brain miR-22 and miR-101a are up-regulated in aged mice [85]. These studies reveal that miRNAs can both affect the aging process as well as be affected by it.

Recently, the roles of miRNAs in age-dependent inflammatory phenotypes have started to be explored. In particular, it has been discovered that mice lacking miR-146a develop an age-dependent, chronic inflammatory disease that is spontaneous, life-shortening, associated with inflammatory cytokines and autoantibodies, and that involves a variety of hematopoietic abnormalities and/or malignancies typically associated with the aging process [68]. Further, the condition has been shown to involve activated lymphocytes and be largely dependent upon NF-kB [86].

Our group and others have recently explored this miR-146a deficiency phenotype further and found that it involves the spontaneous development of T follicular helper cells that play a pivotal role in facilitating germinal center (GC) B cell development, production of high affinity, class-switched antibodies, and formation of B cell memory [68]. Consistent with this, both Tfh and GC B cells begin to spontaneously arise in younger miR-146a-/- animals, and this precedes most other phenotypes in this model. Upon reaching middle age, these animals begin to produce anti-dsDNA autoantibodies as a consequence of their deregulated GC response, and begin to display inflammation in a variety of different peripheral tissues. Of relevance, this phenotype was largely dependent on T cell expression of miR-155 indicating that these two miRNAs counter-regulate chronic inflammation. Although more work remains, it is plausible that these autoantibodies contribute to tissue stress and ultimately the onset of disease upon reaching old age (Fig. 3).

MicroRNAs are involved in other chronic inflammatory contexts

A role for miRNAs in controlling commensal bacteria populations and their production of metabolites that influence inflammatory conditions is beginning to emerge [87]. This has important implications both within the gut and in peripheral tissues. miRNA specific knockout mice have been shown to have altered gut microbiota. For example, miR-155-/- mice have increases in pathobionts within the gastrointestinal tract due to defective humoral immunity [88]. This suggests that the function of miRNA within host immune cells can help shape the composition and control of commensal microbes. However, it remains unclear if miRNAs shape populations that influence chronic inflammatory disease states.

Although specific miRNAs have been shown to regulate metabolic syndromes, there is little functional evidence thus far linking miRNAs, inflammation, and obesity/diabetes. However, based upon our understanding of miRNA functions in other contexts, it is highly likely that miRNAs regulate low-grade inflammatory conditions that influence weight gain and insulin resistance. Further, clinical evidence is beginning to emerge. For instance, there have been reports linking altered miR-146a expression and T2D [89].

MicroRNAs are emerging biomarkers and therapeutic targets in chronic inflammation

As miRNAs have been functionally connected to the development of chronic inflammation, it follows that alterations to miRNA levels could be a reasonable way to detect the presence of chronic inflammatory states in patients. miRNAs are currently being used as diagnostics for at least some types of diseases, including some forms of chronic inflammation such as colitis and IBD [90, 91]. With some diseases, miRNAs can even be used for both diagnosis and prognosis [92]. The appeal of using miRNAs as diagnostic markers comes from the high sensitivity that miRNA biomarkers possess as well as the ability to use miRNA profiles to stratify distinct downstream disease outcomes. The hope is that miRNAs associated with chronic inflammation can be used to diagnose chronic inflammation before clinical manifestations appear. This

would allow for preventative treatment of diseases that stem from chronic, low-grade inflammatory states.

Recently, the identification of miRNAs in blood serum as well as other biological fluids has opened the door for diagnosis of various diseases using these samples obtained through non-invasive methods. Serum miRNAs can either exist cell-free in association with the RISC complex or within small lipid vesicles such as exosomes [93]. Recently, there has been significant interest in utilizing these extracellular miRNAs as biomarkers because of their specificity and sensitivity of detection. Specific secreted miRNAs in the serum can be used to diagnose chronic inflammatory diseases such as IBD [94] where each disease has a unique profile of secreted miRNAs. This could also be an approach to diagnosing other forms of chronic inflammatory states, such as those described above.

The presence of extracellular miRNAs raises questions regarding the biological role of these secreted miRNAs. One current theory of the role of extracellular miRNAs, especially those within exosomes, is that they constitute a novel form of intercellular communication [95, 96]. This idea is supported by several reports providing evidence that secreted miRNAs are functionally passed between various cell types including immune cells [97–99]. It is possible that these extracellular miRNAs could play a role in the development, advancement, or inhibition of chronic inflammatory states. However, further investigation is needed to determine the role of extracellular miRNAs during chronic inflammation and diseases derived from this condition.

miRNAs have also begun to emerge as therapeutic targets. Currently, several anti-miRNA therapeutics are in clinical trials: most notably antisense inhibitors of miR-122 are being used to combat HCV infections [100, 101]. The therapeutic targeting of miRNAs is discussed in further detail in Li and Rana's 2014 review [100]. Based on the success of these approaches, it is our view that miRNAs that regulate chronic inflammation, such as miR-155 or miR-146a, could also be targeted therapeutically with optimal doses of anti-miRs or miRNA mimics. Ideally, chronic inflammatory disease could be diagnosed early using miRNA detection in blood serum samples, and then possibly treated with specific cocktails targeting the particular miRNAs that are dysregulated. However, there are some barriers to utilizing miRNAs as therapies. One of the biggest hurdles is targeting the miRNA therapeutics to the cells of interest. Exosomes and other lipid carriers have received a lot of attention lately as possible ways by which miRNAs and other therapies may be delivered to specific cell types [102]. However, there is still additional understanding that is required before these approaches can be effectively used in the clinic to provide specificity and sufficient dosing.

Long non-coding RNAs are involved in chronic inflammation

MicroRNAs are currently the best characterized ncRNAs involved in chronic inflammation; however, there are also other emerging classes of ncRNAs, such as lncRNAs, that are also involved (Table 1) [54]. LncRNAs appear to function through a variety of different molecular mechanisms, and most commonly play a scaffolding role to promote proper recruitment and positioning of protein regulators both in the nucleus and in the cytoplasm. Loss-of-function approaches have found that lncRNAs regulate the biology of both innate and adaptive immune cells during inflammatory responses. For example, there have recently been reports linking macrophage lncRNAs and obesity [103], as well as other studies that have implicated certain lncRNAs in macrophage and DC inflammatory functions [104]. LncRNA have also been connected to the regulation of T cell homing and differentiation into effector subtypes [105]. These and other classes of ncRNAs must be further characterized to better understand their roles in chronic inflammation. The role of lncRNAs in the immune system is reviewed further by Heward and Lindsay [54].

Conclusions and outlook

The association of chronic inflammation with a variety of diseases emphasizes the importance of gaining a deeper understanding of the underlying mechanisms behind this phenomenon. In this review, we have highlighted factors that contribute to chronic inflammation emphasizing the newly identified roles of miRNAs and other ncRNAs. As we move forward, it will be essential to consider contributions by both coding and noncoding factors in order to formulate an optimal approach for diagnosing, treating, and/or preventing diseases associated with chronic inflammation.

Despite the potential that ncRNAs pose as therapeutic reagents or targets, there are significant barriers that must be overcome in order to achieve therapeutic efficacy. Cell-specific delivery of reagents to manipulate ncRNAs remains a significant challenge. Although progress has been made on cell targeting therapies utilizing lipid vesicles and nonlipid carriers containing antisense miRNAs or miRNA mimics, this remains a substantial challenge facing the field of ncRNA therapy. Additionally, delivery of effective doses of ncRNAs or their inhibitors is also a major hurdle for therapy. For diseases brought on by chronic inflammation, it may also be difficult to determine when to begin treatment, although the use of ncRNA biomarkers in patients' biofluids should held to indicate when treatment should begin. Despite these barriers, the manipulation of ncRNAs represents a potential way to treat chronic inflammatory diseases, and hopefully surmounting these barriers will lead to more efficacious uses of ncRNAs as therapeutics.

The authors have declared no conflicts of interest.

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CHAPTER 3

EXOSOME-DELIVERED MICRORNAS MODULATE THE INFLAMMATORY RESPONSE TO ENDOTOXIN

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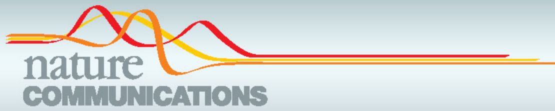

ARTICLE

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Exosome-delivered microRNAs modulate the inflammatory response to endotoxin

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MicroRNAs regulate gene expression posttranscriptionally and function within the cells in which they are transcribed. However, recent evidence suggests that microRNAs can be transferred between cells and mediate target gene repression. We find that endogenous miR-155 and miR-146a, two critical microRNAs that regulate inflammation, are released from dendritic cells within exosomes and are subsequently taken up by recipient dendritic cells. Following uptake, exogenous microRNAs mediate target gene repression and can reprogramme the cellular response to endotoxin, where exosome-delivered miR-155 enhances while miR-146a reduces inflammatory gene expression. We also find that miR-155 and miR-146a are present in exosomes and pass between immune cells *in vivo*, as well as demonstrate that exosomal miR-146a inhibits while miR-155 promotes endotoxin-induced inflammation in mice. Together, our findings provide strong evidence that endogenous microRNAs undergo a functional transfer between immune cells and constitute a mechanism of regulating the inflammatory response.

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Intercellular communication is essential for immune cells to coordinate inflammatory responses. Cytokines, chemokines and cell surface receptors are well-known mediators of this process. In addition to these classical signalling molecules, emerging evidence suggests that immune cells can signal by secreting small lipid packages called exosomes, which carry a variety of different molecules that can be taken up by recipient cells[1–4]. The functional relevance of exosomes in many different biological systems, including the immune system, is beginning to be demonstrated[5–11].

MicroRNAs (miRNAs) are important modulators of gene expression that function by targeting messenger RNAs for degradation or preventing translation. Typically, miRNAs are thought to function within the cells in which they are made; however, recently, miRNAs have been observed in secreted exosomes[12–14]. Immune cells, including antigen-presenting dendritic cells (DCs) and T lymphocytes, can both secrete and take up exosomal miRNAs, suggesting that exosomal transfer of miRNAs could be a novel mechanism for intercellular communication[12,14,15]. Furthermore, recent studies indicate that the loading of miRNAs into exosomes is a selective process where specific motifs in miRNA sequences are recognized by the RNA-binding protein, hnRNPA2B1 (ref. 16). Other reports find that miRNA loading into exosomes is dependent on 3′-end uridylated isoforms[17] and on the levels of miRNA targets in producer cells[18]. Consistent with this, exosomal miRNA signatures do not simply reflect the miRNA composition of the parent cell, but are composed of a distinct set of miRNAs[16,18–21]. This argues that certain miRNAs have evolved to be packaged into exosomes to carry out their biological functions.

Exosomally transferred miRNAs are emerging as novel regulators of cellular function. There is evidence in both immune cells and other cell types that transferred miRNAs repress target mRNAs in recipient cells[12–14,22–24]. The transfer of miRNAs can also cause physiological changes in recipient cells[5–7], as demonstrated by miRNAs moving from cancer cells to endothelial cells, which promotes tumour metastasis[5]. Cancer cells can also receive exosomal miRNAs secreted from immune cells, which were shown to have an anti-proliferative effect on the tumour cells[7]. These data suggest that different cell types can secrete or receive miRNAs as a form of communication and have set the stage for investigating the functional roles of transferred miRNAs in the context of immune responses.

Within the immune system, several specific miRNAs have recently emerged as important regulators of immune cell function. Among these, miR-155 is a promoter of inflammatory responses, while miR-146a is a mediator of immune suppression[25–28]. Despite significant progress in our understanding of how these miRNAs influence immunity *in vivo*, there are many aspects of their regulation and function that remain unclear. In the current study, we investigate whether endogenous miR-155 and miR-146a are functionally transferred between primary bone marrow-derived DCs (BMDCs). We find that both of these miRNAs are released within exosomes and are taken up by recipient BMDCs. On uptake, the miRNAs are associated with Ago proteins, knock down their respective targets and reprogramme the response of BMDCs to endotoxin challenge. We also show that miR-155 can be transferred between immune cells *in vivo*. Finally, we demonstrate that injection of miR-146a-containing exosomes into mice inhibits their inflammatory response to endotoxin, whereas injection of miR-155-containing exosomes promotes inflammation following exposure to the same inflammatory stimulus. Our study supports a model whereby exosomal miRNAs participate in the regulation of inflammatory responses.

Results

miR-155 is found in exosomes and transferred between BMDCs. miR-155 is an immunomodulatory miRNA expressed by many types of immune cells including DCs[27]. We sought to determine whether miR-155 could be passed between cultured BMDCs. Co-cultures of primary mouse BMDCs derived from *CD45.1*$^+$ *Wt* mice and *CD45.2*$^+$ *miR-155−/−* mice were set up at a 1:1 ratio with and without lipopolysaccharide (LPS) treatment (Fig. 1a). As a control, *miR-155−/−* BMDCs were also cultured under the same conditions without *Wt* cells. After 24 h, the co-cultured *Wt* and *miR-155−/−* CD11c$^+$ BMDCs were separated based on their differential CD45 markers using fluorescence-activated cell sorting (FACS) (Fig. 1b). Quantitative reversetranscriptase–PCR (qRT–PCR) was performed on RNA isolated from the CD45.2$^+$ *miR-155−/−* BMDCs. miR-155 was detected in *miR-155−/−* BMDCs that were cultured with *Wt* cells and the signal was clearly above background levels established using *miR-155−/−* BMDCs cultured alone (Fig. 1c). When cells were treated with LPS, the transfer of miR-155 to *miR155−/−* cells was increased, consistent with previous findings that cellular miR-155 concentrations are elevated following LPS stimulation[29] (Fig. 1c).

To determine whether cell–cell contact is necessary for the transfer of miR-155, we used 0.4-μm filters to separate *miR-155−/−* and *Wt* BMDCs that were co-cultured in the presence or the absence of LPS for 24 h. The 0.4-μm pore size allows for small molecules and vesicles such as exosomes to pass through but prevents cell-contact-mediated exchange of material[23]. We detected miR-155 in the *miR-155−/−* BMDCs that were cultured with *Wt* BMDCs, which was above background (Fig. 1d). Our data indicate that miR-155 is passed between cells, and that cell–cell contact is not necessary for transfer to occur between BMDCs.

As miRNAs have recently been shown to be transferred between immune cells within exosomes, we investigated whether miR-155 is contained within these secreted vesicles. To address this question, we isolated the exosomal pellet from *Wt* or *miR-155−/−* BMDC conditioned media using differential centrifugation. Both electron microscopy (EM) and a CD63 western blotting of the isolated vesicles indicated that we had successfully isolated exosomes (Fig. 1e,f). Using qRT–PCR, we found that miR-155 was contained in exosomes derived from *Wt* BMDCs but not in exosomes derived from *miR-155−/−* cells (Fig. 1g). BMDCs treated with LPS enhanced the levels of miR-155 found in the exosomal pellet, consistent with higher levels of miR-155 being produced by the activated BMDCs. In addition, we blocked exosome formation by treating donor BMDCs with GW4869, a drug that hinders exosome biogenesis by blocking neutral sphingomyelinase 2 (nSMase2) (refs 13,15). Following drug treatment, the pellet contained significantly reduced exosomes as determined by EXOCET quantification (Fig. 1h), CD63 western blotting and EM (Supplementary Fig. 1). Drug treatment also prevented the detection of miR-155 in the exosomal pellet (Fig. 1i), suggesting that miR-155 is contained within exosomes. In addition, we derived BMDCs from *Rab27a* and *Rab27b* double-knockout mice (*Rab27 DKO*), which have been previously shown to have decreased release of exosomes[23]. We found that *Rab27 DKO* BMDCs had both decreased exosome release (Fig. 1j) and a corresponding decrease in miR-155 in the exosomal pellet (Fig. 1k). Together, these data show that miR-155 can be passed between BMDCs, and that miR-155 is contained in exosomes produced by BMDCs.

Exosomal transfer of miR-155 is functionally relevant. With the knowledge that miR-155 can be transferred between BMDCs, we wanted to determine whether exosomes are sufficient for this

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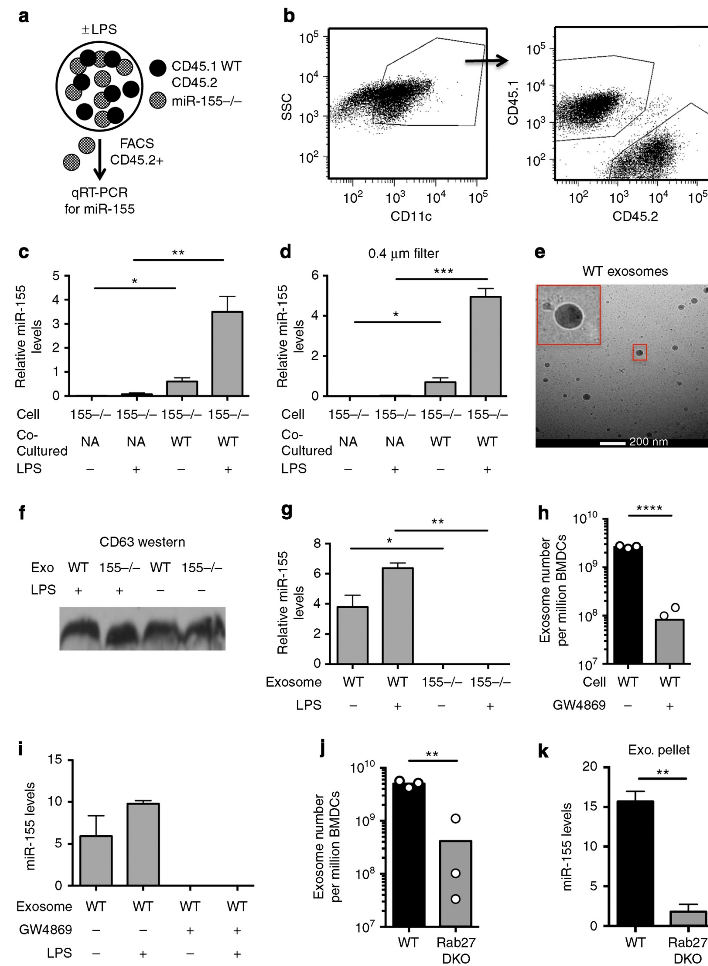

Figure 1 | miR-155 is transferred between BMDCs and is present in exosomes. (**a**) A schematic of the co-culture experiment. (**b**) Representative FACS plots where co-cultured *CD45.1*⁺ *Wt* and *CD45.2*⁺ *miR-155−/−* CD11c⁺ BMDCs were separated ($n=4$). (**c**) Relative miR-155 levels were quantified via qRT–PCR from isolated *miR-155−/−* BMDCs that had been cultured alone or with *Wt* BMDCs in the presence or the absence of LPS for 24 h ($n=4$). (**d**) Relative miR-155 levels were measured via qRT–PCR in *miR-155−/−* BMDCs either cultured alone or with *Wt* BMDCs separated by a 0.4-μm filter for 24 h with or without LPS ($n=3$). (**e**) Cryo-EM of exosomes isolated from *Wt* BMDCs. Scale bar, 200 nm. Red box is enlarged in the upper left corner. (**f**) CD63 protein levels in the exosomal pellet from *Wt* and *miR-155−/−* BMDCs treated with or without LPS. (**g**) Relative levels of miR-155 in exosomes derived from *Wt* or *miR-155−/−* BMDCs treated with or without LPS ($n=3$). (**h**) Exosome quantification of *Wt* BMDCs treated with or without GW4869 ($n=3$). Limit of detection is 2×10^7 exosomes. (**i**) Relative levels of miR-155 were measured in the exosomal pellet from *Wt* BMDCs treated with or without LPS and GW4869 as quantified by qRT–PCR ($n=2$). (**j**) Exosome quantification of *Wt* and *Rab27 DKO* BMDC-derived exosomes ($n=2$). Limit of detection is 2×10^7 exosomes. (**k**) miR-155 levels in exosome pellets from *Wt* and *Rab27 DKO* BMDC-conditioned medium ($n=2$). Data represent two independent experiments and are presented as the mean ± s.d. (error bars). $^*P<0.05$; $^{**}P<0.01$, Student's *t*-test.

transfer and whether transfer could result in knockdown of target mRNAs. To specifically investigate the impact of exosomally transferred miRNA without the effects of other factors that are released from BMDCs, we purified exosomes away from other components in the conditioned medium using differential centrifugation and washing. Next, the exosomes were re-suspended in fresh medium and administered to recipient cells. *Wt* (1×10^6) or *miR-155−/−* BMDCs produced $\sim5\times10^8$ exosomes in 24 h (Supplementary Fig. 2). Exosomes isolated from the supernatant of both *Wt* and *miR-155−/−* BMDCs treated

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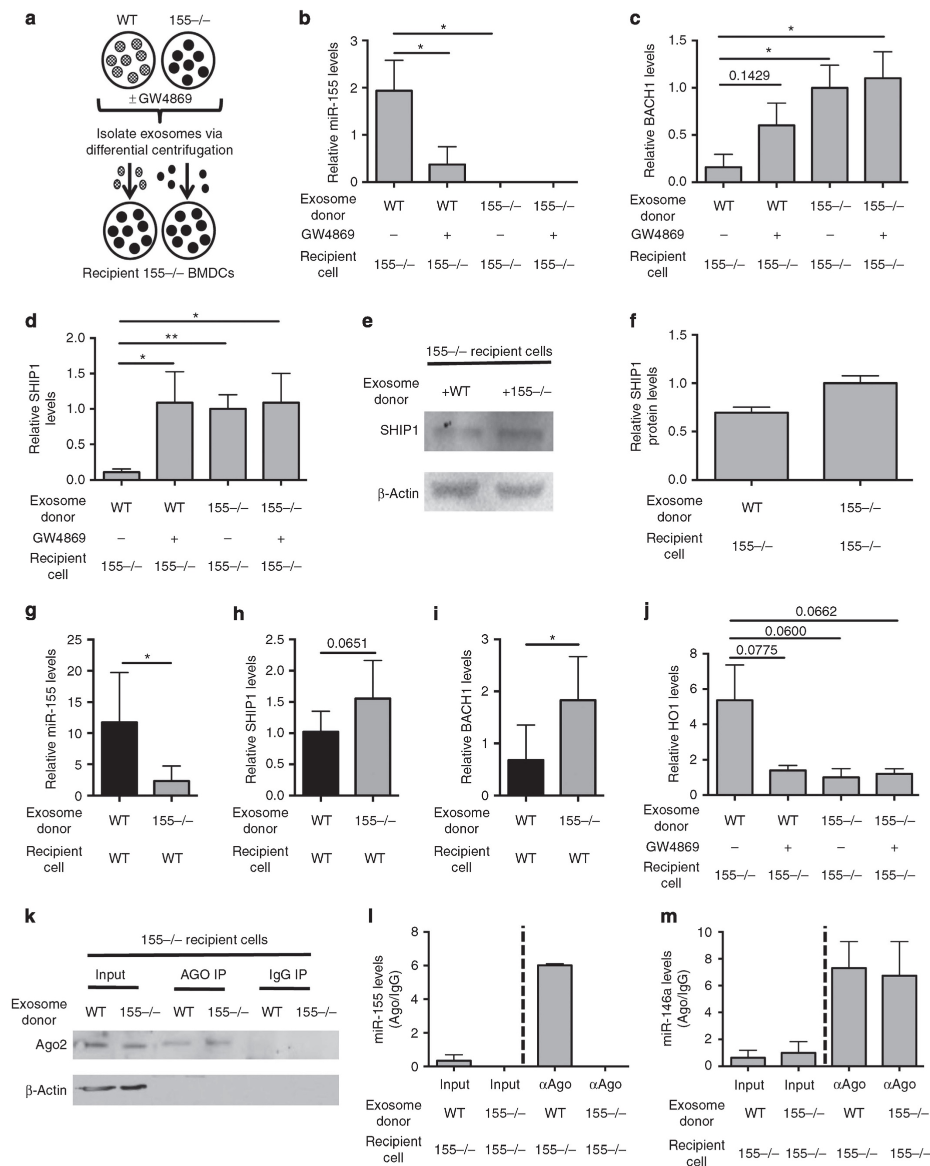
with GW4869 or dimethylsulfoxide vehicle control were transferred to *miR-155* −/− receipient BMDCs. *miR-155* −/− recipient BMDCs were incubated with donor exosomes for 24 h, to allow time for miRNA transfer and knockdown of miRNA targets (Fig. 2a). Using qRT–PCR, we detected increased miR-155 levels and decreased mRNA levels of miR-155 targets BACH1 and SHIP1 when cells were treated with *Wt* exosomes (Fig. 2b–d). These changes were prevented if the exosomes were derived from *miR-155* −/− BMDCs, or if the *Wt* donor cells were pretreated with GW4869. SHIP1 protein levels were also decreased in *miR-155* −/− BMDCs that received *Wt* exosomes (Fig. 2e,f). Exosome delivery of miR-155 brought its levels in the



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miR-155−/− recipient cells to ~20% of *Wt* miR-155 levels (Supplementary Fig. 3a,b). Furthermore, we also looked at the relative expression of a separate miRNA, miR-425, which has been previously seen to be released in exosomes[30], as a control. The levels of miR-425 increased with exosome delivery and were roughly the same in *Wt* and knockout groups (Supplementary Fig. 3c,d).

In addition to using *miR-155−/−* recipient cells, which provide a clean background to clearly detect the transferred miRNA, we also examined whether transferred miR-155 could be detected in *Wt* BMDC recipients. miR-155 levels were increased on treatment of *Wt* BMDCs with *Wt* exosomes but were not increased when treated with *miR-155−/−* exosomes (Fig. 2g). In addition, the mRNA levels of both BACH1 and SHIP1 were decreased in *Wt* BMDCs receiving exosomal miR-155 (Fig. 2h,i). These data indicate that miR-155 can be transferred between *Wt* BMDCs in exosomes, resulting in the knockdown of known miR-155 targets.

As we have found that miR-155 can knock down its targets in recipient cells, we next investigated whether the transfer of miR-155 alters downstream factors in recipient cells. We found that exosomal miR-155 increased the expression of *HO1* (Fig. 2j), an oxidative stress response gene that is well known to be repressed by *BACH1*, a gene we have shown is targeted by transferred miR-155 (ref. 29; Fig. 2c). These results indicate that transferred miR-155 not only represses its putative direct targets but can also affect factors that are downstream of these targets.

To further characterize the functional transfer of miR-155 between BMDCs, we examined whether transferred miR-155 is associated with AGO proteins that are essential for miRNA-mediated knockdown of targets. Following exosomal transfer of miR-155 into *miR-155−/−* BMDCs, an AGO immunoprecipitation (IP) was performed using a pan-AGO antibody and western blotting for AGO2 was preformed to verify pulldown was occurring. Using qRT–PCR, we found that miR-155 is associated with AGO proteins in *miR-155−/−* recipient cells (Fig. 2k,l). We did not detect miR-155-associated AGO proteins when *miR-155−/−* BMDCs were treated with *miR-155−/−* exosomes. Further, AGO2 was not detected via western blotting and miR-155 was not pulled down when an isotype control antibody was used. As an additional control, we found that another miRNA, miR-146a, was also enriched in the AGO pulldown from both groups (Fig. 2m). These data demonstrate that exosomal miR-155 is associated with AGO proteins, key components of the RNA-induced silencing complex (RISC) complex, following its uptake by recipient BMDCs.

Exosomal transfer of miR-146a is functionally relevant. miR-146a is an important anti-inflammatory miRNA involved in DC function[27] and plays an opposing role to miR-155 during inflammatory responses[25,31]. We wanted to determine whether miR-146a was also contained in BMDC-derived exosomes; hence, we isolated exosomes from *Wt* BMDCs that had been treated with or without GW4869 and/or LPS, and found that miR-146a was contained in exosomes from untreated BMDCs but was not present in the exosomal pellet from BMDCs treated with GW4869 (Fig. 3a). miR-146a is marginally increased in exosomes from BMDCs treated with LPS. In addition, reductions in miR-146a were observed in the extracellular exosomal fraction obtained from *Rab27 DKO* BMDCs compared with *Wt* controls (Fig. 3b). These data reveal that miR-146a is contained within exosomes released from BMDCs.

To test whether miR-146a could be functionally transferred between BMDCs, we isolated exosomes from *Wt* or *miR-146a−/−* BMDCs and administered them to *miR-146a−/−* BMDCs (Fig. 3c). Similar to miR-155, we observed that exosomal miR-146a was taken up by recipient BMDCs (Fig. 3d), and that miR-146a targets, IRAK1 and TRAF6, were repressed in recipient BMDCs receiving *Wt* but not *miR-146a−/−* exosomes looking at both the mRNA and protein levels (Fig. 3e–j). In addition, we calculated miR-146a copy number in *Wt* and *miR-146a−/−* exosomes where we found approximately one copy of miR-146a per exosome (Fig. 3k). miR-146a copy number was also calculated in *Wt* and *miR-146a−/−* donor BMDCs and BMDCs that received either *Wt* or *miR-146a−/−* exosomes (Fig. 3l). We observed an average of 370 copies present in recipient BMDCs following exosomes treatment. It has been suggested that 100–1,000 copies of miRNA per cell is likely to be functionally relevant[32]. As a control, we investigated the relative expression of miR-425, which was similar between the genotypes (Supplementary Fig. 3e,f). Our copy number data along with our observations of target knockdown are consistent with exosomally transferred miR-146a having functional relevance.

Recently, it has been shown in human B-cell lines that miRNAs are selectively packaged into exosomes based on 3′-non-templated nucleotide additions (NTAs)[17] where 3′-uridylation was enriched in miRNAs contained in exosomes and 3′-adenylation was enriched in miRNAs retained in cells. To address whether we observe a similar phenomenon, RNA sequencing was performed using RNA from *Wt* donor BMDCs and *miR-155* and *miR-146a DKO* BMDCs that had received *Wt* exosomes. Next, we used a previously reported approach to identify NTAs in our data set[17]. However, we did not observe an enrichment of 3′-uridylation in transferred miR-155 or miR-146a (Supplementary Fig. 4). This difference from previous findings could be due to species and cellular differences (mouse primary BMDCs versus human B-cell lines) or further processing of the transferred miRNAs in recipient cells. However, we did observe some differences at certain nucleotide positions in each respective mature miRNA sequence when comparing *Wt* donor BMDCs with *DKO* BMDCs that received *Wt* exosomes (Supplementary Tables 1 and 2). These results are consistent with the idea that alterations to the mature miRNA sequence may influence miRNA loading into exosomes versus cellular retention.

Figure 2 | Functional transfer of miR-155 via exosomes *in vitro*. (**a**) Schematic of the exosome transfer experiment. (**b**) qRT–PCR was used to measure relative miR-155 levels in *miR-155−/−* BMDCs that received either *Wt* or *miR-155−/−* exosomes derived from BMDCs treated with or without GW4869 ($n=5$). (**c,d**) mRNA levels of miR-155 targets, BACH1 and SHIP1, from the same experiment shown in **b** as measured by qRT–PCR ($n=5$). (**e**) Representative western blottings of SHIP1 and β-actin in *miR-155−/−* BMDCs given either *Wt* or *miR-155−/−* exosomes. (**f**) Protein levels of SHIP1 were quantified using ImageJ software ($n=2$). (**g**) Relative miR-155 levels in *Wt* BMDCs given either *Wt* or *miR-155−/−* exosomes as quantified by qRT–PCR ($n=6$). (**h,i**) BACH1 and SHIP1 mRNA levels were measured in the same experiment shown in **g** as quantified by qRT–PCR ($n=6$). (**j**) qRT–PCR was used to quantify HO1 mRNA levels during the experiment in **b** ($n=5$). (**k**) Western blotting for AGO2 and β-actin from *miR-155−/−* BMDCs given *Wt* or *miR-155−/−* exosomes. On the left is the input (whole-cell lysate), the middle is from the pan-AGO pulldown where one-third of input was used and the right is the IgG pulldown where one-third of the input was used. (**l**) Relative miR-155 levels were quantified via qRT–PCR in the same experiment shown in **k**. (**m**) miR-146a levels were quantified using qRT–PCR during the experiment in **k**. Levels in **l,m** are plotted as Ago:IgG. Dotted line separates input from pull-down groups. Data represent two independent experiments and are presented as the mean ± s.d. (error bars). $^{*}P<0.05$; $^{**}P<0.01$, Student's *t*-test.

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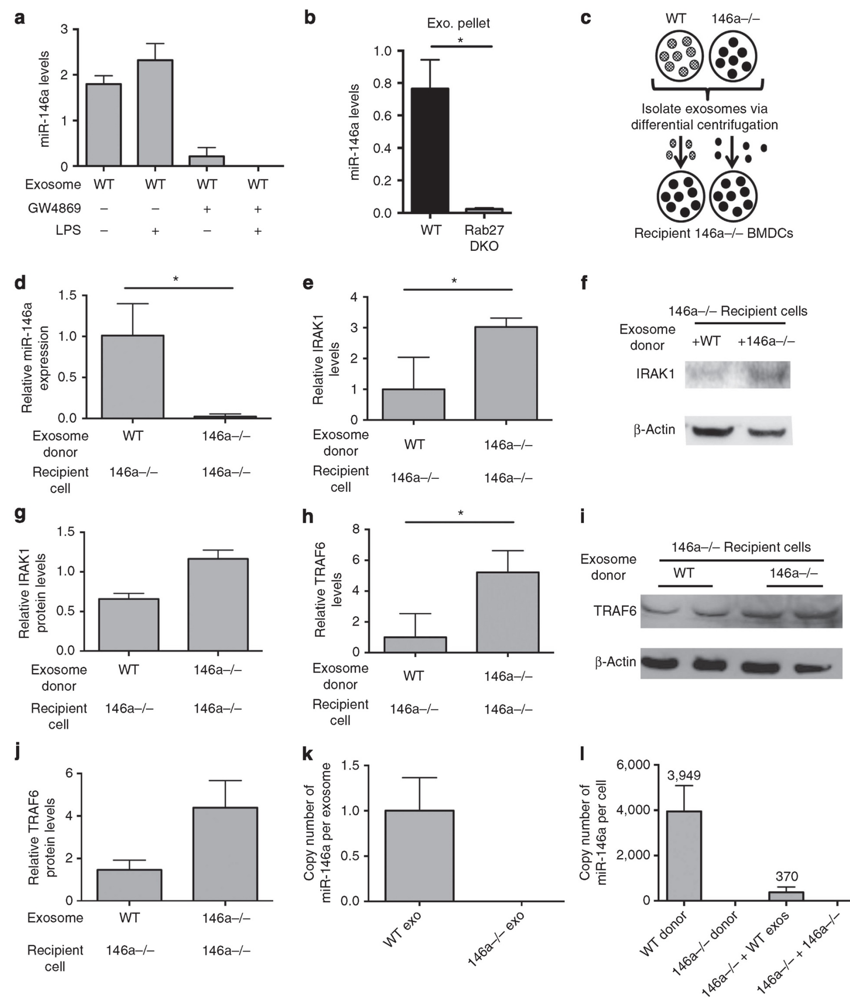

Figure 3 | Functional transfer of miR-146a via exosomes *in vitro*. (**a**) Levels of miR-146a in the exosomal pellet derived from BMDCs that were treated with or without GW4869 and LPS ($n=2$). (**b**) miR-146a levels in *Wt* and *Rab27 DKO* BMDC-derived exosomal pellets ($n=2$). (**c**) Schematic of miR-146a exosome-transfer experiment where *Wt* or *miR-146a −/−* exosomes were isolated from BMDCs and transferred to recipient *miR-146a −/−* BMDCs. RNA was isolated after 24 h and the presence of miR-146a was assayed via qRT–PCR. (**d**) Relative levels of miR-146a in *miR-146a −/−* BMDCs given exosomes derived from *Wt* or *miR-146a −/−* BMDCs ($n=4$). (**e**) mRNA levels of miR-146a target, IRAK1, were measured from the same cells as in **d** via qRT–PCR ($n=4$). (**f**) Representative western blottings of IRAK1 and β-actin from *miR-146a −/−* cells given either *Wt* or *miR-146a −/−* exosomes. (**g**) IRAK1 protein levels were quantified using ImageJ software ($n=2$). (**h**) mRNA levels of miR-146a target, TRAF6, were measured in the same cells as in **d** via qRT–PCR. (**i**) Western blottings for TRAF6 and β-actin from *miR-146a −/−* BMDCs given either *Wt* or *miR-146a −/−* exosomes ($n=2$). (**j**) Western blotting results are quantified with ImageJ software. (**k**) Copy number of miR-146a in *Wt* and *miR-146a −/−* exosomes ($n=3$). Copy number is calculated based on a standard curve where a known amount of synthetic miR-146a was spiked into *miR-146a −/−* BMDC-derived exosome pellet followed by RNA isolation and qRT–PCR. (**l**) Copy number of miR-146a was measured via qRT–PCR in *miR-146a −/−* recipient BMDCs that received either *Wt* or *miR-146a −/−* exosomes (146a −/− BMDC + *Wt* exos and 146a −/− BMDC + 146a −/− exos), as well as in *Wt* and *miR-146a −/−* donor BMDCs ($n=3$). Average copy number is displayed above. Copy number is calculated based on a standard curve where a known amount of synthetic miR-146a was spiked into *miR-146a −/−* BMDC pellet followed by RNA isolation and qRT–PCR. Data represent two independent experiments and are presented as the mean ± s.d. (error bars). *$P<0.05$, Student's *t*-test.

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Seed-dependent repression of miRNA targets. We next determined whether miR-155 and miR-146a mimic-loaded exosomes were sufficient to mediate direct target knockdown in recipient cells. *miR-155−/−* or *miR-146a−/−* BMDCs were transfected with either corresponding miRNA mimics, scrambled miRNA mimics, or seed mutant miRNA mimics for 24 h, then washed three times with PBS to remove any mimics that did not make it into the cells (Fig. 4a). We isolated exosomes from the cells after 24 h and transferred them to recipient knockout BMDCs. After 24 h, RNA was isolated from the cells and qRT–PCR was performed to assay the delivery of mimics and the knockdown of target mRNAs. We found that miRNA mimics could be successfully loaded into exosomes and delivered to recipient cells (Supplementary Figs 5 and 6). The transfer of miRNA mimics containing exosomes resulted in knockdown of respective target mRNAs in recipient BMDCs (Fig. 4b–f). However, exosomes that did not carry mimics, or that carried scrambled or seed mutant mimics, caused no change in target mRNA expression in recipient cells. These results indicate that exosomal miRNAs are responsible for direct target repression and are able to complement the target knockdown phenotype.

To further assess whether exosomal miRNA target repression was direct, we used 3′-untranslated region (UTR) luciferase reporter assays. Knockout BMDCs were transfected with 3′-UTR luciferase reporters for 6 h followed by treatment with or without *Wt* exosomes (Fig. 4g). *miR-155−/−* BMDCs were transfected with either a pmiReport empty vector control, BACH1 3′-UTR, BACH1 miR-155-binding site (bs) mutant 3′-UTR, or a miR-155-positive control (2mer). Luciferase activity in cells receiving the BACH1 3′-UTR or 2mer reporter constructs was reduced in response to miRNAs delivered by exosomes, while the exosomal miRNAs had little impact on luciferase activity in cells receiving the pmiReport empty vector or the BACH1 miR-155 bs mutant 3′-UTR reporter (Fig. 4h). In a separate experiment, *miR-146a−/−* BMDCs were transfected with either a pmiReport empty vector control, TRAF6 3′-UTR, or a TRAF6 miR-146a bs mutant 3′-UTR. The BMDCs transfected with the TRAF6 3′-UTR had decreased luciferase activity compared with the pmiReport empty vector and the TRAF6 miR-146a bs mutant 3′-UTR following exosome delivery of miRNAs (Fig. 4i). These results indicate that exosomally transferred miRNAs directly repress their targets via direct 3′-UTR interactions.

Exosomal miR-155 and miR-146a modulate the response to LPS. To determine whether the transfer of miR-155 via exosomes could affect the BMDC response to LPS, exosomes were isolated from *Wt* or *miR-155−/−* BMDCs and transferred to *miR-155−/−* BMDCs. Twenty-four hours later, cells were treated with LPS for 6 h (Fig. 5a). Consistent with a previously reported role for miR-155 in promoting interleukin (*IL*)-*6* expression[33] and previously reported roles of miR-155-regulated responses to endotoxin[34], cells that were treated with miR-155-containing exosomes produced more IL-6 on treatment with LPS for 6 h than cells having received *miR-155−/−* exosomes (Fig. 5b). These findings indicate that exosomes containing miR-155 can reprogramme recipient BMDCs in a manner that enhances their response to LPS.

miR-146a is known to induce an anti-inflammatory response to LPS[26]. Therefore, we wanted to investigate whether exosomally transferred miR-146a can programme BMDCs to respond in an anti-inflammatory manner, using a similar experimental setup as we did for miR-155 (Fig. 5c). BMDCs pre-treated with *Wt* exosomes produced more IL-10, but less IL-6 and IL-12 p40, following LPS stimulation than BMDCs that received *miR-146a−/−* exosomes (Fig. 5d–f). This gene expression profile demonstrates that miR-146a-containing exosomes reduce the pro-inflammatory response by BMDCs following LPS treatment. Without LPS treatment, there was no significant difference in *IL-10*, *IL-6* or *IL-12 p40* expression by cells receiving *Wt* versus *miR-146a−/−* exosomes (Fig. 5d–f), indicating that exosomal miR-146a specifically alters how these cells respond to LPS. Similar to miR-155, our data indicate that miR-146a is functionally transferred in exosomes and able to cause physiological changes in recipient cells. However, unlike miR-155, exosomal miR-146a acts to dampen the inflammatory response to LPS. These results are consistent with previous observations that miR-155 and miR-146a play opposing roles during inflammation[25,31].

miR-155 is transferred between immune cells *in vivo*. As we observed functional transfer of miRNAs *in vitro*, we wanted to determine whether miRNAs could be transferred between immune cells *in vivo*. We first investigated whether exosomes were present in mouse BM by isolating exosomes directly from the BM of *Wt* and *miR-155−/−* mice using differential centrifugation. We found that both genotypes had exosomes present in the BM (Fig. 6a), and that miR-155 was expressed in *Wt* BM exosomes (Fig. 6b), whereas miR-146a was present in exosomes from both genotypes (Fig. 6c). We next determined whether miR-155 was passed between immune cells *in vivo*. To investigate this, *miR-155−/−* mice were lethally irradiated and reconstituted with either an equal mix of $CD45.1^+$ *Wt* and $CD45.2^+$ *miR-155−/−* BM or just miR-155−/− BM. After allowing 3 months for reconstitution, we injected mice with LPS to stimulate production of miR-155 by BM cells (Fig. 6d). BM cells were isolated 24 h after LPS stimulation and *miR-155−/−* haematopoietic cells were sorted via FACS according to their different CD45 alleles (Fig. 6f). We also further fractionated the *miR-155−/−* BM into B-cell, myeloid cell and T-cell fractions using the surface markers B220, CD11b and CD3, respectively (Fig. 6g,h). Using qRT–PCR, we detected miR-155 expression in *miR-155−/−* B cells, T cells and myeloid cells taken from *miR-155−/−* mice that had been reconstituted with both *Wt* and *miR-155−/−* BM (Fig. 6e). As a control, no miR-155 expression was observed in cells from mice reconstituted with only *miR-155−/−* BM. These data provide evidence that miR-155 is located within exosomes within the BM and is transferred between immune cells *in vivo*.

To determine whether miRNA-containing exosomes could deliver miRNAs to various cell types *in vivo*, we intraperitoneally (i.p.) injected $\sim 10^9$ exosomes derived from *miR-155−/−* or *Wt* BMDCs into *miR-155−/−* mice. After multiple injections over a week, the spleens of these mice were harvested and $CD3^+$ T cells, $B220^+$ B cells and $CD11b^+$ myeloid cells were sorted via FACS. We found that miR-155 was delivered to all three of these cell types in the spleen (Fig. 6i–k). This indicates that exosomes are able to deliver miRNAs to various immune cell types.

Exosomal miR-155 enhances inflammatory responses *in vivo*. Our *in vitro* data suggest that exosomally delivered miR-155 can increase the BMDC response to LPS (Fig. 5b). Owing to these observations, we investigated whether we could see the same effect *in vivo*. Approximately 10^9 *Wt* or *miR-155−/−* BMDC-derived exosomes were i.p. injected into *miR-155−/−* mice, followed by administration of LPS 24 h later and collection of serum 2 h after that (Fig. 7a). The injection of *Wt* exosomes before LPS administration resulted in increased tumour necrosis factor-α (TNFα) and trending elevations in IL-6 serum concentrations compared with mice pretreated with *miR-155−/−* exosomes (Fig. 7b,c). In addition, we observed that miR-155 was delivered to the spleen, liver and BM, where we also found

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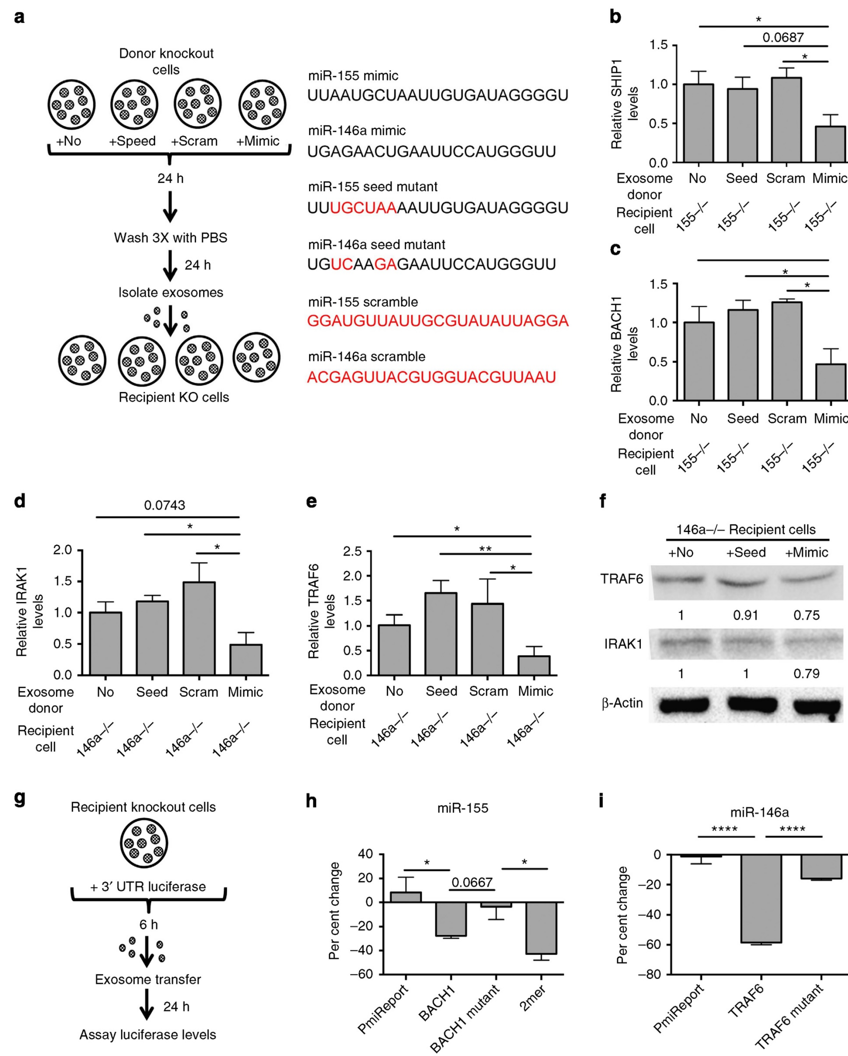

Figure 4 | Seed-dependent repression of miRNA targets by exosome-delivered miR-155 and miR-146a. (**a**) Schematic for mimic experiment. (**b**,**c**) Relative mRNA levels of the miR-155 targets SHIP1 and BACH1 were measured via qRT–PCR in recipient cells that received exosomes with no mimics (No) ($n=7$), miR-155 seed mutant mimics (Seed) ($n=4$), with scrambled mimics (Scram) ($n=3$), or with miR-155-mimics (Mimic) ($n=7$). (**d**,**e**) qRT–PCR was preformed to assay the mRNA levels of the miR-146a targets, IRAK1 and TRAF6, following treatment with exosomes containing miR-146a mimics and controls as in **b**,**c**. Results are reported normalized to exosomes with no mimics added, which is set as 1. (**f**) Protein levels of TRAF6, IRAK1 and β-actin were determined via western blotting using lysates from *miR-146a −/−* BMDCs that received exosomes containing no mimics, seed mutant mimics or *Wt* mimics. Numbers below the blot represent relative protein levels with no mimics set as 1 following normalization to β-actin. (**g**) Schematic for 3′-UTR luciferase reporter assays in **h**,**i**. (**h**) Results from 3′-UTR luciferase reporter assays where *miR-155 −/−* BMDCs were transfected with a pmiReport control vector, a BACH1 3′-UTR vector (BACH1), a BACH1 miR-155-binding site (bs) mutant vector (BACH1 mutant), or a 2mer-positive control vector. Transfected BMDCs were treated 6 h later with or without *Wt* exosomes and per cent change in luciferase activity of exosome treated BMDCs compared with no exosome treatment was calculated after 24 h ($n=4$). (**i**) Results from 3′-UTR luciferase reporter assay where *miR-146a −/−* BMDCs were transfected with a pmiReport control vector, a TRAF6 3′-UTR vector (TRAF6) or TRAF6 miR-146a bs mutant vector (TRAF6 mutant). Six hours later, the BMDCs were treated with or without *Wt* exosomes and per cent repression of luciferase activity was calculated 24 h after exosome transfer ($n=4$). Results represent two independent experiments. All data are presented as the mean ± s.d. (error bars). *$P<0.05$; **$P<0.01$, ****$P<0.0001$; Student's *t*-test.

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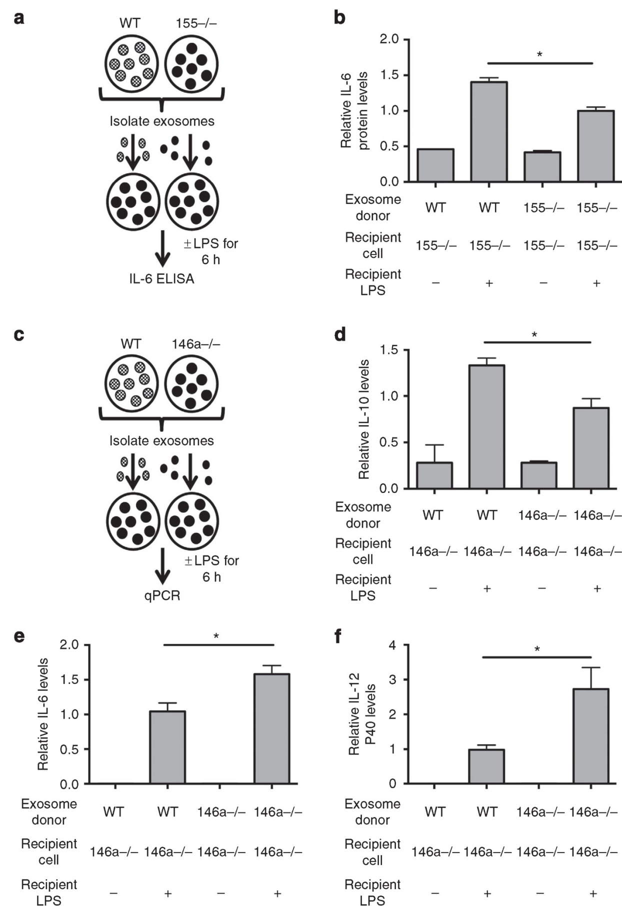

Figure 5 | Exosomal transfer of miR-155 and miR-146a programme the response to LPS *in vitro* (**a**) A schematic of the experimental design for **b**. (**b**) Exosomes were isolated from *Wt* or *miR-155 − / −* BMDCs and given to *miR-155 − / −* BMDCs for 24 h. Cells were then treated with or without LPS and media was taken after 6 h for an IL-6 enzyme-linked immunosorbent assay. Relative IL-6 protein levels are shown ($n = 4$). (**c**) Schematic for experiments in **d**–**f**. (**d**–**f**) qRT–PCR was used to quantify mRNA levels of IL-10, IL-6 and IL-12 p40 in *miR-146a − / −* BMDCs given exosomes from *Wt* or *miR-146a − / −* BMDCs for 24 h followed by stimulation with or without LPS for 6 h ($n = 4$). Data represent two independent experiments. All data are presented as the mean ± s.d. (error bars). *$P < 0.05$; Student's *t*-test.

reduced target mRNA levels consistent with miR-155 activity in these tissues (Fig. 7d–i). These data demonstrate that miR-155 can be functionally delivered to a variety of tissues and cell types via exosome injection, and that this can increase the response to LPS *in vivo*.

Exosomal miR-146a reduces inflammatory responses *in vivo*. We next investigated whether exosomes containing miR-146a would have an anti-inflammatory impact following LPS administration to mice. Approximately 10^9 exosomes were isolated from *Wt* or *miR-146a − / −* BMDCs and injected i.p. into *miR-146a − / −* mice. Twenty-four hours later, the mice were given LPS and serum was collected after 2 h to assay inflammatory cytokine levels (Fig. 8a). Mice having received *Wt*, miR-146a-containing exosomes had reduced TNFα and IL-6 serum concentrations after LPS administration compared with mice having received miR-146a-deficient exosomes (Fig. 8b,c). Exosomes alone had a negligible effect on cytokine levels *in vivo*. Further, 24 h after LPS injection we isolated the spleen, liver and BM, and found that miR-146a was clearly present in these tissues from mice receiving *Wt* exosomes but was not present in tissues from mice that received *miR-146a − / −* exosomes (Fig. 8d–f). In addition, miR-146a targets involved in Toll-like receptor (TLR) signalling were repressed in tissues in mice that received *Wt* exosomes (Fig. 8g–i). Similar results were obtained when miR-

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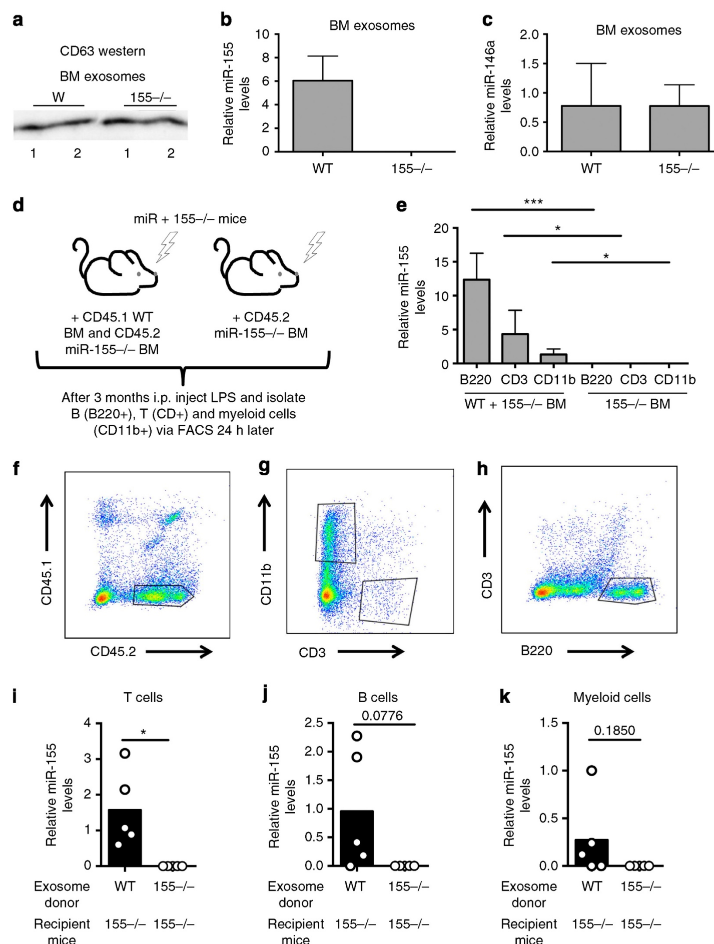

Figure 6 | Transfer of endogenous miR-155 between haematopoietic cells *in vivo*. (**a**) CD63 western blotting using exosomes isolated directly from the BM of *Wt* or *miR-155*−/− mice. 1 and 2 stand for two biological replicates. (**b**,**c**) Levels of miR-155 and miR-146a in exosomes isolated form *Wt* and *miR-155*−/− mouse BM as measured by qRT–PCR ($n=2$). (**d**) Schematic of the *in-vivo* experiment. (**e**) qRT–PCR was used to quantify levels of miR-155 in *miR-155*−/− CD45.2$^+$ BM cells that were B220$^+$, CD3$^+$, or CD11b$^+$ from *miR-155*−/− mice that were either reconstituted with *Wt* (*CD45.1*$^+$) and *miR-155*−/− BM, or *miR-155*−/− BM alone as indicated ($n=5$). (**f–h**) Representative FACS plots of the cell types in isolated BM shown in **e** ($n=5$). (**i–k**) *miR-155*−/− mice were i.p. injected multiple times over a week with either *Wt* or *miR-155*−/− exosomes. CD3$^+$ T cells, B220$^+$ B cells and CD11b$^+$ myeloid cells were sorted from mouse spleens and qRT–PCR was preformed to analyse the delivery of miR-155 to each cell type ($n=5$). All data are presented as the mean ± s.d. (error bars). *$P<0.05$; **$P<0.01$, ***$P<0.001$, Student's *t*-test.

146a-containing exosomes were administered to *Wt* recipients (Fig. 9a–i). Together, these data demonstrate that exosomal miR-146a can reduce the inflammatory response to LPS in mice.

Discussion

Although exosomes have been studied for a number of years, the biological roles of exosomal miRNAs are just beginning to be investigated[4–8]. Our data demonstrate that miRNAs 155 and 146a are released from BMDCs in exosomes, are taken up by recipient BMDCs and subsequently mediate target gene repression. In addition, we have found that the transfer of miR-155 or miR-146a can alter the ability of recipient cells to respond to inflammatory cues both *in vitro* and *in vivo*. The capacity of these transferred miRNAs to influence the response of BMDCs to a pro-inflammatory stimulus suggests that the transfer of miRNAs is an important mechanism by which immune cells are primed to respond to an imminent encounter with a microbe. As miR-155

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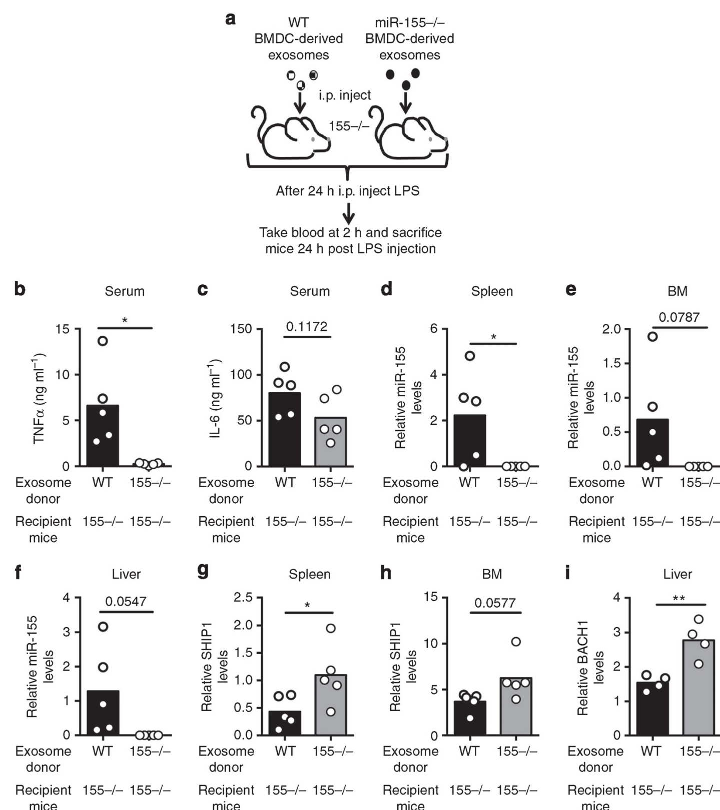

Figure 7 | miR-155-containing exosomes promote a heightened response to LPS in *miR-155 −/−* mice. (**a**) Schematic of the experimental design where *miR-155 −/−* mice were i.p. injected with either *Wt* or *miR-155 −/−* BMDC-derived exosomes and then challenged with LPS 24 h later. Blood was taken 2 h post LPS injection and the spleen, liver and BM were harvested 24 h post injection. (**b**,**c**) Serum TNFα and IL-6 concentrations were analysed via enzyme-linked immunosorbent assay 2 h after injection of LPS in *miR-155 −/−* mice that had been pretreated with either *Wt* or *miR-155 −/−* exosomes ($n = 5$). (**d**–**f**) qRT–PCR was preformed using RNA isolated from the spleen, liver and BM, to assay the relative levels of exosomally delivered miR-155 ($n = 5$). (**g**–**i**) mRNA levels of the miR-155 targets SHIP1 and BACH1 were measured in the spleen, liver and/or the BM using qRT–PCR ($n = 5$). All data are presented as the mean ± s.d. (error bars). $*P < 0.05$; $**P < 0.01$, Student's *t*-test.

and miR-146a have been shown to regulate inflammation in a variety of contexts, our findings provide novel insights into how and where they function, providing a greater understanding of how they regulate mammalian immunity. Furthermore, our study adds to the growing body of evidence that miRNA transfer within exosomes is part of the intercellular communication networks that coordinates complex immune responses[8,12,14].

Previous studies have used cell lines, miRNA overexpression and/or miRNA reporter constructs to study exosomal transfer of miRNAs[12–14]. Although these approaches have provided important evidence that miRNAs can be transferred in exosomes, we designed our approach to be as physiologically relevant as possible. miRNAs were produced at endogenous levels by primary cells and established endogenous miRNA target genes were used as readouts for miRNA activity in recipient cells. Furthermore, exosomes were purified away from other BMDC factors, such as cytokines, and miR-155- and miR-146a-deficient recipient cells were used to confidently track the delivery and specific effects of the exosomally delivered miRNA both *in vitro* and *in vivo*.

A recent report has claimed that the amounts of specific miRNAs contained within exosomes is less than one copy per exosome[35]. Our copy number analysis found there to be approximately one copy of miR-146a per exosome, consistent with exosomes having low content of individual miRNAs. However, we found that one BMDC produces ~500 exosomes after 24 h of culture, indicating that each cell is able to release at least hundreds of copies of miR-146a in exosomes to be delivered to recipient BMDCs and mediate target knockdown. Thus, it seems that the large numbers of exosomes produced per cell allows for the loading of low miRNA numbers per exosome to achieve functional relevance.

It is important to note that exosome populations produced by *Wt* cells contain both miR-155 and miR-146a, which we show

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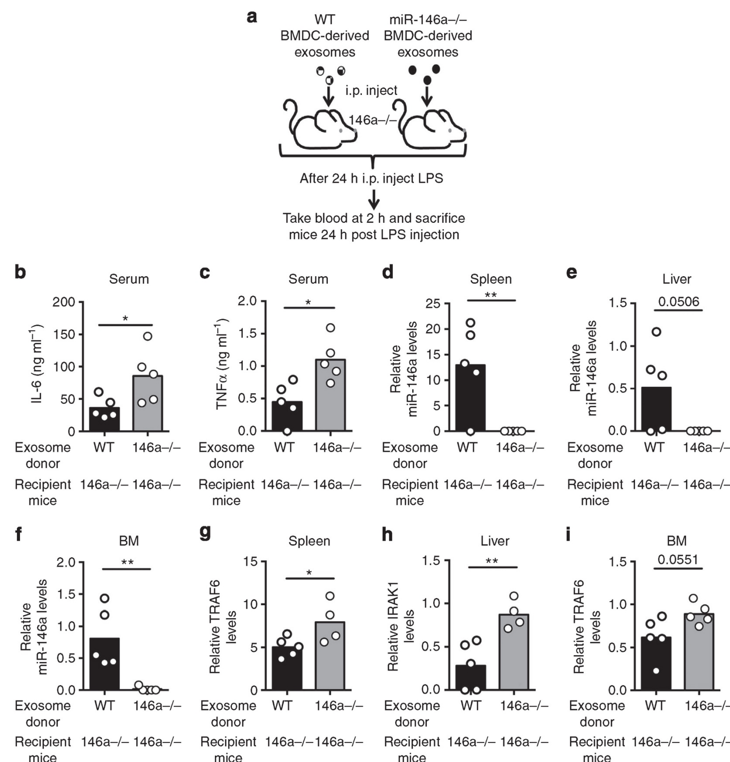

Figure 8 | miR-146a-containing exosomes reduce inflammatory responses to LPS in *miR-146a−/−* mice. (**a**) Schematic of the experimental design where *miR-146a−/−* mice were i.p. injected with either *Wt* or *miR-146a−/−* BMDC-derived exosomes and then challenged with LPS 24 h later. Blood was taken 2 h post LPS injection and the spleen, liver and BM were harvested 24 h post injection. (**b**,**c**) Serum TNFα and IL-6 were analysed via enzyme-linked immunosorbent assay 2 h after injection of LPS ($n=5$). (**d**–**f**) qRT–PCR was preformed using RNA isolated from the spleen, liver and BM, to assay the relative levels of exosomally delivered miR-146a ($n=5$). (**g**–**i**) mRNA levels of the miR-146a targets TRAF6 and IRAK1 were measured in the spleen, liver and/or the BM using qRT–PCR ($n=5$). All data are presented as the mean ± s.d. (error bars). *$P<0.05$; **$P<0.01$, Student's *t*-test.

have either pro- or anti-inflammatory effects, respectively. There are several possible reasons why exosome populations would contain both of these functionally distinct miRNAs species. First, exosomes could be transferring both pro- and anti-inflammatory miRNAs together to buffer inflammatory responses by recipient cells, to achieve the optimal magnitude of response. Second, it is plausible that miR-155 and miR-146a are located in separate exosomes that are delivered to different target cell types. A third possibility is that miR-155 and miR-146a release in exosomes is a dynamically regulated process where the ratio of miR-155 to miR-146a changes over time. For example, immune cells that have sensed a pathogen could initially release exosomes with high levels of pro-inflammatory miRNAs such as miR-155 followed by a shift to anti-inflammatory miRNAs such as miR-146a during the resolution phase of the response. These possibilities will be explored in future studies wherein the analysis of single exosomes may be required.

Exosomes are clearly complex vesicles that contain an assortment of different membrane and soluble proteins, as well as different types of RNAs, including miRNAs[3]. Thus, we cannot formally rule out that exosomes produced by *Wt* versus *miR-155−/−* or *miR-146a−/−* BMDCs may differ in some aspect other than the presence or the absence of the corresponding miRNA that has been genetically deleted, and that this may also have some influence on the inflammatory response by recipient cells. However, we have been able to address this possibility to some degree by successfully complementing the exosomal miRNA target gene phenotypes by loading miRNA mimics into miRNA knockout exosomes. Further, we were also able to demonstrate that target repression is direct through the use of seed mutant mimics that failed to repress target gene expression in recipient cells as well as 3′-UTR luciferase reporter assays where binding-site mutant 3′-UTRs were not repressed by exosomally transferred miRNAs. Collectively, these data strongly support the idea that individual miRNAs in exosomes are transferred between cells in a functionally relevant manner.

We also observed transfer of miRNAs between immune cells *in vivo*, indicating that this mechanism of cellular communication is also occurring in a physiologically relevant setting. Future work will be needed to isolate and study distinct cell types in the

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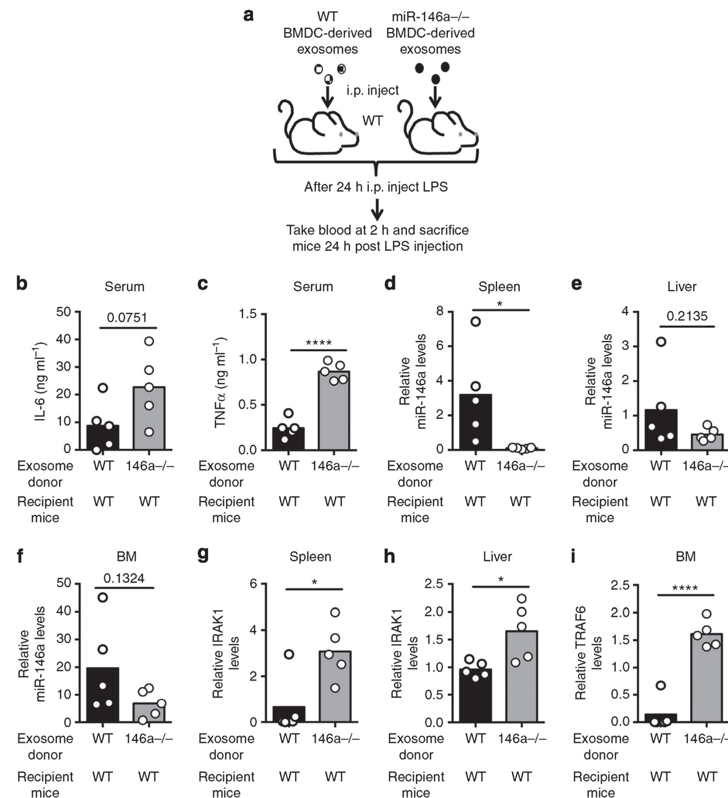

Figure 9 | miR-146a-containing exosomes reduce inflammatory response to LPS in *Wt* mice. (**a**) Schematic of the experimental design where *Wt* mice were i.p. injected with either *Wt* or *miR-146a* −/− BMDC-derived exosomes and then challenged with LPS 24 h later. Blood was taken 2 h post LPS injection and the spleen, liver and BM were harvested 24 h post injection. (**b**,**c**) Serum TNFα and IL-6 were analysed via enzyme-linked immunosorbent assay 2 h after injection of LPS ($n = 5$). (**d**–**f**) qRT–PCR using RNA isolated from the spleen, liver and BM was performed to assay the relative levels of exosomally delivered miR-146a ($n = 5$). (**g**–**i**) mRNA levels of the miR-146a targets TRAF6 and IRAK1 were measured in the spleen, liver and/or the BM using qRT–PCR ($n = 5$). Results represent two independent experiments. All data are presented as the mean ± s.d. (error bars). *$P < 0.05$; ****$P < 0.0001$, Student's *t*-test.

context of exosome production and uptake, as tissues such as the spleen, liver and BM are made up of a heterogeneous populations of cells that probably differ in their capacity to participate in these processes. Further, we predict that functional miRNA transfer via exosomes will be most relevant in defined microenvironments such as stem cell niches or within tumours, where exosome concentrations might be at their highest.

Future studies will also require reagents where the production of miRNA-containing exosomes can be specifically blocked *in vivo* to assess the relevance of this mechanism in distinct inflammatory settings. *Rab27 DKO* mice will provide one such reagent despite possible roles for Rab27 a and b in exosome-independent cellular processes. However, it is unclear whether the release of pro- or anti-inflammatory exosomal miRNAs will have a dominant impact on the inflammatory response *in vivo*, and we predict that this will probably be context dependent. However, as *Rab27 DKO* regulatory T cells have recently been shown to be functionally impaired, it is likely to be that these animals will have heightened inflammatory responses[23]. Consequently, the relevance of exosomal miRNA release by distinct immune cell types may have to be studied using cell-type-specific *Rab27 DKO* mice.

Exosomal miRNAs are currently being extensively studied as biomarkers of disease, as their serum levels are altered in a variety of pathological conditions[36–38]. Our results, in combination with others, suggest that these differences have functional consequences. As exosomes appear to be a natural way that cells transfer miRNAs, there is also growing interest in understanding the therapeutic potential of exosomes as delivery vehicles for specific miRNAs or their inhibitors[39–44]. Producing exosomes from patients' own cells may serve as an ideal vehicle for autologous therapies involving miRNA delivery, and our capacity to load miRNA mimics suggests that the miRNA content of exosomes can be manipulated. Further, we clearly demonstrate that injection of

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miR-146a- and miR-155-containing exosomes results in delivery of these miRNAs to a variety of mouse tissues, repression of target genes and an altered inflammatory response *in vivo*, where miR-155 promoted and miR-146a repressed inflammation in response to endotoxin. This suggests that exosomal miR-146a could be used as a prophylaxis or therapy to treat inflammatory diseases, such as bacterial sepsis. Conversely, exosomal miR-155 could be used as an adjuvant to improve vaccine efficacy. However, it is also clear that the full spectrum of applications whereby exosomal miRNAs can be used is potentially quite broad and will require a great deal of future work. As we refine our understanding of how miRNAs are loaded into exosomes and delivered in a functional manner to specific recipient cells, such therapeutic approaches may become feasible in the clinic.

Methods

Mice. *miR-155* − / − (Allan Bradley Lab, Sanger Institute), *miR-146a* − / − (David Baltimore Lab, California Institute of Technology), *miR-155* and *miR-146a DKO* (Ryan O'Connell, University of Utah), *Wt* (Jackson Labs) and *CD45.1 Wt* (Jackson Labs) are on a C57BL6 genetic background and housed in the animal facility at the University of Utah. *Rab27 DKO* (Rab27a ash/ash Rab27b − / −) mice (Tanya Tolmachova and Miguel C. Seabra, Imperial College London) were housed at the Imperial College London under the UK Home Office animal project license 70/7078 and the BM was sent to Utah for experiments together with the BM from the *Wt* animals of similar age, sex and background (C57BL6). 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 8–16 weeks old. For BM reconstitutions, lethal irradiation (1,000 rads) was delivered using an X-ray source. Following irradiation, mice were injected with three million BM cells via retro-orbital injection. *Escherichia coli* LPS (Sigma) was administered through i.p. injections at a sub-lethal concentration of 50 μg[29]. In other experiments, exosomes were i.p. injected 24 h before LPS injection of the same concentration.

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, β-mercaptoethanol, glutamate, sodium pyruvate, HEPES and non-essential amino acids) with 20 ng ml^{-1} granulocyte machrophage colony-stimulating factor 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} granulocyte machrophage colony-stimulating factor 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}. Cells were separated using a Transwell Permeable Support 0.4 μm Polycarbonate Membrane 24 mm insert six-well plates (Costar).

RNA sequencing. *Wt* exosomes were transferred to recipient *miR-155* and *miR-146a DKO BMDCs*. Three biological replicates from *Wt* donor and exosome-recipient *DKO* BMDCs were submitted to the University of Utah's High Throughput Genomic Core for Illumina TrueSeq Small RNA Sample Prep. NTAs were identified and frequencies of A, G, C and U additions were calculated as described previously[17] by our bioinformatics core facility. In addition, we analysed each position in the mature miRNA sequences of miR-155 and miR-146a, and calculated the percentage of observed bases at each position to determine any changes in nucleotide composition between miRNAs in donor versus exosome-recipient BMDCs. RNA sequencing data are deposited in GEO with the accession number GSE67946.

Copy-number analysis. miRNA copy number was calculated in *Wt* and *miR-146a* − / − donor cells, exosomes and *miR-146a* − / − BMDCs that received *Wt* exosomes. Total RNA was isolated (using the miRNeasy kit) from one million donor BMDCs and one million recipient BMDCs that were cultured with exosomes from one million donor BMDCs collected after 24 h or exosomes isolated from one million BMDCs after 24 h. Thirty nanograms of RNA isolated from these samples was then used for qRT–PCR analysis. To make a standard curve, 1 ng of synthetic single-stranded miR-146a (IDT custom RNA oligo—sequence: 5′-UGAGAACUG AAUUCCAUGGGUU-3′) was spiked into either one million *miR-146a* − / − BMDCs or exosomes isolated from one million *miR-146a* − / − BMDCs, and total RNA was isolated in the same manner as our experimental samples (miRNeasy). Thirty nanograms of this isolated RNA was used to preform a complementary DNA reaction to use for standard curves. Standard curves for cells and exosomes were made with these cDNA samples via serial dilutions and cp values were determined via qPCR with miR-146a primers. The BMDC standard curve was then used to determine copy number in our cellular samples and the BMDC exosome standard curve was used to determine the copy number of miR-146a in our exosome samples.

Mimic. miRNA mimics were purchased from Qiagen. Scrambled, seed mutant and miR-mimic sequences are as follows:
miR-146a scramble (5′-ACGAGUUACGUGGUACGUUAAU-3′),
miR-146a seed mutant (5′-UGUCAAGAGAAUUCCAUGGGUU-3′),
miR-146a mimic (5′-UGAGAACUGAAUUCCAUGGGUU-3′),
miR-155 scramble (5′-GGAUGUUAUUGCGUAUAUUAGGA-3′),
miR-155 seed mutant (5′-UUUGCUAAAAUUGUGAUAGGGGU-3′) and
miR-155 mimic (5′-UUAAUGCUAAUUGUGAUAGGGGU-3′). Donor cells were transfected with 30 μl of the hi-perfect transfection reagent (Qiagen) in 2 ml of serum-free media with 60 ng of each mimic. After 24 h, cells were washed three times with PBS and given fresh medium. Exosomes were isolated 24 h after washing and transferred to recipient cells for 24 h.

Luciferase reporter assay. Knockout BMDCs (2.5×10^5) were transfected with 3′-UTR luciferase reporter constructs (for *mir-155* − / − : pmiReport, Bach1, Bach1 155 mutant, 2mer[29]; (for miR-146 − / − : pmiReport, Traf6, Traf6 146a mutant[45]) using Lonza's Amaxa Mouse Dendritic Cell Nucleofector Kit, according to manufacturer's instructions. After 6 h of nucleofection, BMDCs were treated with or without *Wt* BMDC-derived exosomes and luciferase activity was measured 24 h later using a Dual Luciferase Kit (Promega). Luciferase repression of exosome-treated BMDCs compared with no exosome treatment was calculated and graphed as per cent change in luciferase activity. Renilla luciferase was used to normalize firefly luciferase values. 2 μg of each construct was transfected into BMDCs.

Exosome isolation and procedures. For *in-vitro* experiments, we isolated exosomes from approximately one million BMDCs cultured in media for 24 h and transferred them to the same number of recipient BMDCs. Differential centrifugation was performed to isolate exosomes from 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 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 tissue culture media. This protocol is based on previous exosome isolation methods[46]. We used either a Beckman ultracentrifuge with a TI75 fixed angle rotor or a Thermo Scientific Sorvall Lynx 6000 with a T26-8 × 50 rotor. GW4869 is a neutral sphingomyelinase 2 inhibitor that has been previously used to prevent exosome release[13,15]. In some experiments, we treated BMDCs with 10 μM GW4869 (Sigma-Aldrich) or vehicle for 24 h.

Exosome numbers for the miR-146a and miR-155 *in-vivo* experiments were determined using the EXOCET Exosome Quantification Assay Kit from System Biosciences, according to kit instructions. Three plates of approximately three million BMDCs each were cultured in media for 3 days. The supernatant from these plates was collected and exosomes were isolated as described above.

Western blotting and enzyme-linked immunosorbent assay. Protein was isolated with RIPA lysis buffer (RIPA buffer, phenylmethyl sulfonyl fluoride, NaF, $NaVO_4$ and protease inhibitor). Total protein levels were quantified using a Bio-Rad protein assay and equal amounts of protein were loaded and separated using 12% (TRAF6, Ago2 and CD63) or 8% (SHIP1 and IRAK1) SDS–PAGE followed by immunoblotting with appropriate antibodies. Antibodies include the following: α-TRAF6 at 1:500 dilution (EP591Y Abcam, ab33915), α-β-actin antibody at 1:1,000 dilution (mAbcam 8226, ab8226), α-Ago2/eIF2C2 antibody at 1:200 dilution (Abcam, ab32381), α-CD63 (H-193) at 1:200 dilution (Santa Cruz Biotechnology, sc-15363), α-SHIP1 (V-19) at 1:250 dilution (Santa Cruz Biotechnology, sc-1963), and α-IRAK1 D5167 at 1:500 Dilution (Cell Signaling, 4504). Western blottings were quantified using ImageJ software. The enzyme-linked immunosorbent assay used to quantify mouse IL-6 and TNFα concentrations were obtained from eBioscience and were performed using the manufacturer's suggested protocol. Images have been cropped for presentation. Full-size images are presented in Supplementary Figs 7–9.

RNA isolation and qRT-PCR. RNA isolation was performed using Qiagen's miRNeasy kit, according to manufacturer's instructions. Mature miRNA cDNA was made with a miRCURY LNA universal RT miRNA PCR kit using 10 ng of RNA from each sample (Exiqon). qPCR of mature miRNA was performed with the miRCURY LNA universal RT miRNA PCR kit SYBR green master mix (Exiqon) with LNA primers for miR-146a-5p (Exiqon), mmu-miR155-5p (Exiqon), mmu-miR-425-5p (Exiqon) and 5s rRNA (Exiqon). Custom LNA primers were also made and designed by Exiqon to detect the miR-155 and miR-146a seed mutant mimics (miR-146a design ID 410833-1) (miR-155 design ID 410829-1). 5s was used to normalize expression. cDNA from total RNA was made with qScript using 30 ng of RNA from each sample (Quanta). qPCR was performed with Promega GoTaq pPCR master mix. Primer sequences are as follows:
SHIP1-F (5′-GAGCGGGATGAATCCAGTGG-3′),
SHIP1-R (5′-GGACCTCGGTTGGCAATGGTA-3′),
BACH1-F (5′-TGAGTGAGAGTGCGGTATTTGC-3′),
BACH1-R (5′-GTCAGTCTGGCCTACGATTCT-3′),
HO1-F (5′-TGACACCTGAGGTCAAGCAC-3′),

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HO1-R (5′-TCCTCTGTCAGCATCACCTG-3′),
IRAK1-F (5′-TGTGCCGCTTCTACAAAGTG-3′),
IRAK1-R (5′-TGTGAACGAGGTCAGCTACG-3′),
TRAF6-F (5′-AAGCCTGCATCATCAAATCC-3′),
TRAF6-R (5′-CTGGCACTTCTGGAAAGGAC-3′).
L32-F (5′-AGCTCCCAAAAATAGACGCAC-3′) and
L32-R (5′-TTCATAGCAGTAGGCACAAAGG-3′). L32 levels were used to normalize mRNA expression levels.

Electron microscopy. EM samples were prepared using differential centrifugation from BMDC-conditioned media. Exosomal pellets were re-suspended in PBS and processed by the University of Utah's EM core facility for cryo-EM analysis.

Immunoprecipitations. An anti-pan Ago antibody (clone 2A8, Millipore) was was used to IP Ago proteins. α-AGO and IgG control coated beads were prepared by incubating magnetic protein G beads (Active motif) with each respective antibody in IP lysis buffer (0.5% NP40, 150 mM KCl, 1 mM NaF, 25 mM Tris, 2 mM EDTA, protease inhibitor and 0.5 mM dithiothreitol) with rotation overnight at 4 °C. One-third of the protein lysate prepared from BMDCs that had received either *Wt* or *miR-155 − / −* exosomes using IP lysis buffer was used for the IP. Bead–antibody mixes were washed three times with lysis buffer with rotation at 4 °C, re-suspended in lysis buffer and added to the lysates. Lysates were incubated with bead–antibody mix at 4 °C with rotation overnight and then washed six times with IP wash buffer (300 mM NaCl, 50 mM Tris,.01% NP40, 5 mM $MgCl_2$, 129 ml dH_2O), with the last wash done using PBS. Protein was isolated from a fraction of the sample with 1 × Laemmli diluted in lysis buffer and RNA was isolated from another fraction using miRNeasy extraction. A western blotting for AGO2 (Abcam) and qRT–PCR analysis for miR-155 and miR-146a were performed to confirm AGO pulldown and association with these miRNAs.

Flow cytometry. Fluorophore-conjugated monoclonal antibodies specific to CD45.1, CD45.2, B220, CD3, CD11b or CD11c (Biolegend) were used to stain red blood cell-depleted BM and spleen cells. These populations were sorted using a FACS Aria II in the Flow Cytometry Core Facility at the University of Utah.

Statistics. Data were analysed using Student's *t*-tests, to determine statistically significant differences between relevant samples. *P*-values were either listed or represented by the following number of asterisks: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

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

Additional information
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CHAPTER 4

RAB27-DEPENDENT EXOSOME PRODUCTION INHIBITS CHRONIC INFLAMMATION AND ENABLES ACUTE RESPONSES TO INFLAMMATORY STIMULI

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 b double knockout (Rab27DKO) mice that are deficient in exosome secretion have a chronic, low-grade inflammatory phenotype characterized by elevated inflammatory cytokines, myeloproliferation, and a metabolic syndrome. 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 proper LPS response. Further, we found that SHIP1, a direct target of miR-155 known to promote endotoxin tolerance, was 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.

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 and over time can contribute to a variety of diseases associated with aging, including obesity and type 2 diabetes (1). Noncoding RNAs are important in regulating this delicate balance as demonstrated in mice lacking miR-146a that develop an age-dependent, chronic inflammatory disease (2, 3). Chronic inflammation and the metabolic disorders associated with it are expanding at an alarming rate putting enormous stress on the U.S. medical system and overall economy (4). 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.

Proper communication between immune cells is essential for homeostasis and appropriate responses to inflammatory cues, including those that drive chronic inflammation. 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 (5–8). The transfer of cellular contents including proteins, RNAs, and other molecules by exosomes has been reported. Exosome-mediated transfer of microRNAs (miRNAs) has become of particular interest in the field (8–10) because miRNAs can be transferred between different cell types, including immune cells, to regulate cellular responses (3, 7). For instance, miRNAs can be shuttled between dendritic cells (DCs) and mediate target knockdown in recipient cells (12) as well as be transferred between T cells and DCs at the immunological synapse (13). Further, miRNA transfer via exosomes conveys resistance to hepatitis B and contributed to interferon-α antiviral responses in mice (14). There are also examples of specific miRNAs being transferred between immune cells and altering responses. For example, the exogenous delivery of

exosomal miR-155 leads to an enhanced response to inflammatory challenge while administration of exosomal miR-146a reduces response (11). Such responses have been observed both systemically and in specific tissues, including those that make up the central nervous system (15). 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 (16). 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 b 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 (17–20). 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 endogenous 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, myeloproliferation, and indicators of metabolic disease. Some of these phenotypes were cell extrinsic and could be rescued, at least in part, by WT BM cells or injections of isolated

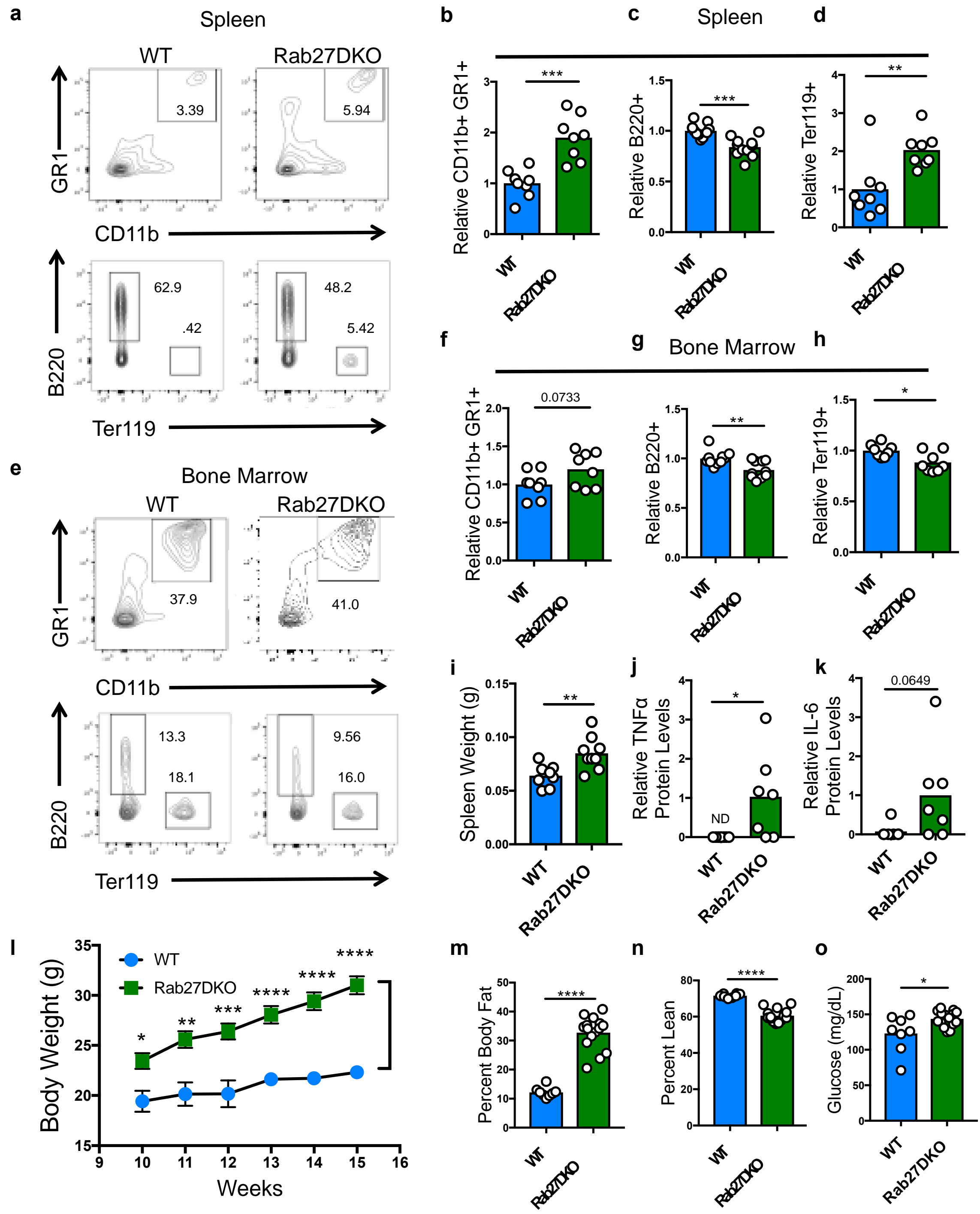
WT exosomes from BM cells. This indicates that exosome uptake is important for maintenance of hematopoietic homeostasis and prevention of aberrant chronic inflammation. Chronically inflamed Rab27DKO mice were also hyporesponsive to challenge with LPS, and thus resembled a state of endotoxin tolerance (21). This is also reminiscent of what is seen in obese patients who have defects in clearing infections despite higher resting inflammatory markers (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 proper responses to inflammatory cues. SHIP1, a miR-155 target that negatively regulates inflammatory responses (23, 24) and is involved in establishing tolerance to LPS (25), is elevated in Rab27DKO mice compared to WT mice. However, SHIP1 levels were reduced following administration of WT but not miR-155-/- exosomes in mice challenged with LPS implicating miR-155 targeting of SHIP1 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.

Results

Rab27DKO mice have a chronic, low-grade inflammatory phenotype

Upon phenotyping Rab27DKO mice, we observed that granulocyte-monocyte (GM) myeloid population, marked by CD11b+ and GR1+, were expanded in the Rab27DKO mice in both the spleen and BM compartments with a corresponding decrease in the B220+ B cell population compared to WT controls (Figure 4.1a-c, e-g). There were

Figure 4.1. 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 B220+, Ter119+, and GR1+ CD11b+ 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 B220+, Ter119+, and GR1+ CD11b+ 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 and set relative to the Rab27DKO group. (l) Weights of WT and Rab27DKO female mice were tracked from 10 to 14 weeks of age. (m-n) Body composition of the same mice tracked in L was analyzed by NMR at week 15. Percent fat and lean composition is shown. (o) Fasting glucose levels were measured in the same mice tracked in L at week 15. 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 unless otherwise noted. Data are representative of at least 3 individual experiments. *P* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$ Student's *t*-test.



a
Spleen
WT
Rab27DKO
GR1
CD11b
3.39
5.94
B220
Ter119
62.9
.42
48.2
5.42
b
c
Spleen
d
Relative CD11b+ GR1+
Relative B220+
Relative Ter119+
***
***
**
WT
Rab27DKO
e
Bone Marrow
WT
Rab27DKO
37.9
41.0
13.3
18.1
9.56
16.0
f
g
Bone Marrow
h
0.0733
**
*
i
j
k
Spleen Weight (g)
Relative TNFα Protein Levels
Relative IL-6 Protein Levels
ND
0.0649
l
Body Weight (g)
Weeks
WT
Rab27DKO
*
**
***
****
m
n
o
Percent Body Fat
Percent Lean
Glucose (mg/dL)

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 4.1a, d, e, h). Increased spleen weights were also observed in the Rab27DKO vs. WT control mice (Figure 4.1i). These data suggest that the Rab27DKO mice have a mild myeloproliferative disorder.

Beyond hematopoietic cell differences, the proinflammatory cytokines IL-6 and TNFα were elevated above baseline in the serum of Rab27DKO mice (Figure 4.1j-k). Corresponding to this chronic inflammation we noted that female but not male Rab27DKO mice gained significantly more weight than their WT counterparts and had increased body fat and a decreased lean body composition (Figure 4.1l-n). Additionally, Rab27DKO mice had higher fasting glucose levels than their WT counterparts (Figure 4.1o). These results indicate Rab27DKO mice develop a chronic, low-grade inflammatory condition.

Certain Rab27DKO chronic inflammatory phenotypes are cell extrinsic

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 4.2a-b). Rab27DKO BM that was in the presence of WT BM no longer had accumulation of Ter119+ erythroid precursor cells in the spleen as seen in the Rab27DKO mice (Figure 4.2c-d). Spleen weights were also decreased compared to the

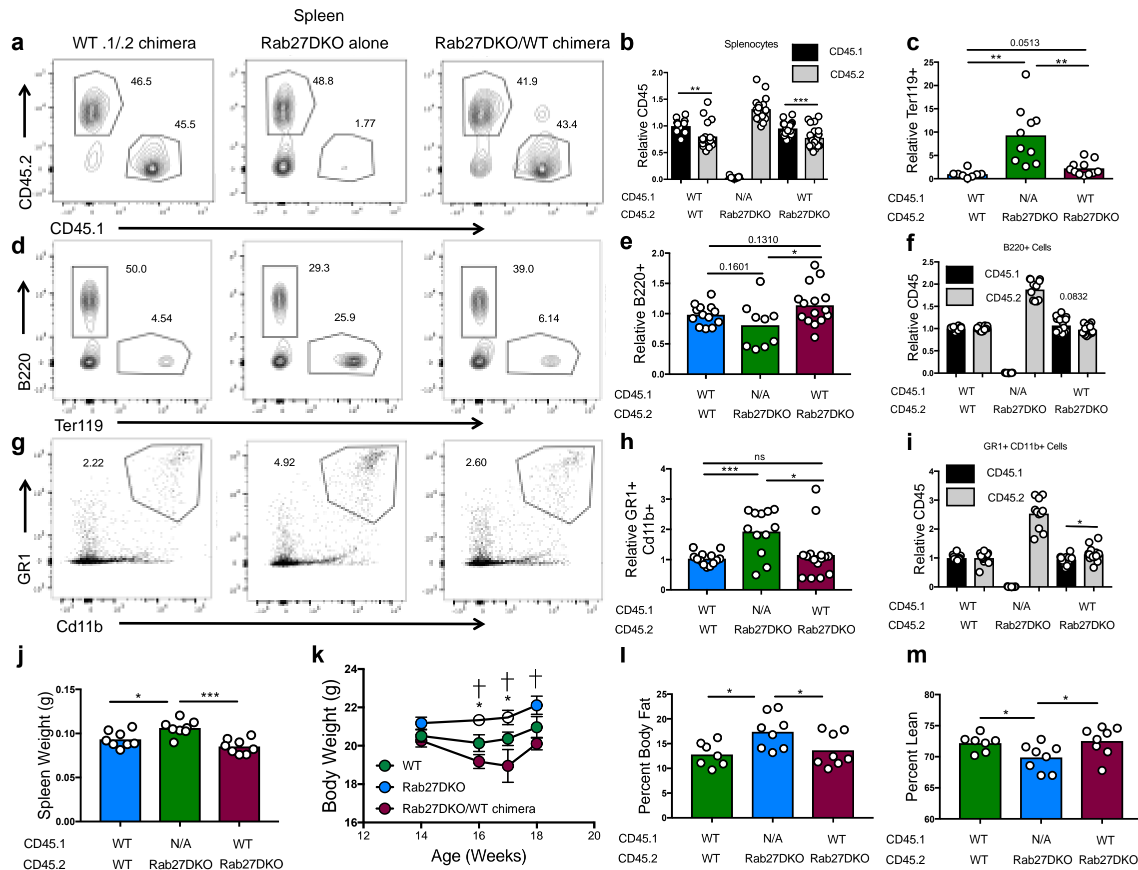


Figure 4.2. Certain Rab27DKO phenotypes are cell extrinsic. WT mice were lethally irradiated and reconstituted with either a 1:1 mix of WT (CD45.1+) and Rab27DKO (CD45.2+), Rab27DKO (CD45.2+) alone, or 1:1 mix of CD45.1+ and CD45.2+ WT bone marrow for 2 months. (a) Representative flow plots of reconstitution efficiency in the spleen using CD45 as a marker. (b) Reconstitution efficacy was quantified by CD45 markers in the spleen, genotype of CD45 marker is indicated below the graph. (c-e) Ter119+ and B220+ representative flow plots are shown for the spleen and relative levels are quantified to the right. (f) CD45 markers within the B220+ population in the spleen where black is CD45.1 and grey is CD45.2 relative levels. (g-h) GR1 CD11b+ representative flow plots and percentages with quantification of relative levels to the right. (i) CD45 markers within the GR1+ CD11b+ population. (j) Spleen weight in grams. (k) Body weight of each group was tracked over time and age of the mice is shown. Mice had been reconstituted for 6 weeks before weight measurements began. (l) Percent body fat of each treatment group at 18 weeks of age and 10 weeks post-BM reconstitution as measured by NMR. (m) Percent lean mass of each treatment group at 18 weeks of age and 10 weeks post-BM reconstitution as measured by NMR. 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. *P* values are either stated or *, $p < .05$; **, $p < 0.01$; ***, $p < .001$ Student's *t*-test. For body weight comparison * marks *P* values of < .05 between the WT and Rab27DKO reconstituted groups and T marks *P* values of < .05 between the Rab27DKO and Rab27DKO/WT reconstituted groups.

Rab27DKO BM alone group (Figure 4.2j). Additionally, there was some rescue of B220+ B and CD11b+ GR1+ GM myeloid cell populations in the spleens of mice reconstituted with the WT/Rab27DKO BM mixtures (Figure 4.2d-e, g-h). To investigate whether Rab27DKO or WT cells composed these populations, we gated on CD45.1 and CD45.2 and found that within the B220+ B cell population Rab27DKO and WT cell proportions were equivalent (Figure 4.2f). Within the GR1+ CD11b+ population there was a subtle bias towards the CD45.2+ Rab27DKO cells suggesting there could still be some cell intrinsic functions for Rab27a/b in this cellular compartment (Figure 4.2i).

Similar trends were seen in the BM with the exception of the Ter119+ population, which was not rescued with the presence of WT BM (data not shown). Further, the weight gain and increased body fat seen in the Rab27DKO mice was recapitulated by the reconstitution of Rab27DKO BM alone but not the WT/Rab27DKO chimera group (Figure 4.2k-m). Of note, this weight gain phenotype was not as drastic as that seen in the Rab27DKO mice themselves. This suggests that the weight gain in Rab27DKO mice is in part due to the hematopoietic compartment and cell extrinsic factors. 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 certain Rab27DKO phenotypes

Due to our observations that some of the Rab27DKO chronic, low-grade inflammatory phenotypes are a due to 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 the control “exosome” pellet from Rab27DKO GM-BM cells which have been previously shown to produce significantly fewer exosomes (11, 17). Injections were performed two times per week over the course of four weeks.

Ter119+ erythroid precursor cell expansion in the Rab27DKO spleens was reduced following injections of WT exosomes, but not injection of the control Rab27DKO exosome pellet (Figure 4.3a-b). The splenic GR1+ CD11b+ GM myeloid population was partially rescued by WT exosome injections (Figure 4.3d-e) while the B cell population was not (Figure 4.3a and c). Spleen weights were decreased in Rab27DKO mice that received WT exosomes (Figure 4.3f), suggesting that the extramedullary hematopoietic phenotype was rescued following administration of WT exosomes. The GR1+ CD11b+ GM population in the BM recovered after WT exosomes were injected (Figure 4.3j-k); however, the Rab27DKO Ter119+ and B220+ BM cellular populations were not altered following delivery of WT exosomes (Figure 4.3g-i). Levels of IL-6 in the Rab27DKO serum were also reduced to normal levels following injection of WT exosomes (Figure 4.3l). 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 are independent of GM-BM exosome uptake. This experiment was repeated with PBS injections as a control instead of the Rab27DKO exosomal pellet and similar results were observed (data not shown).

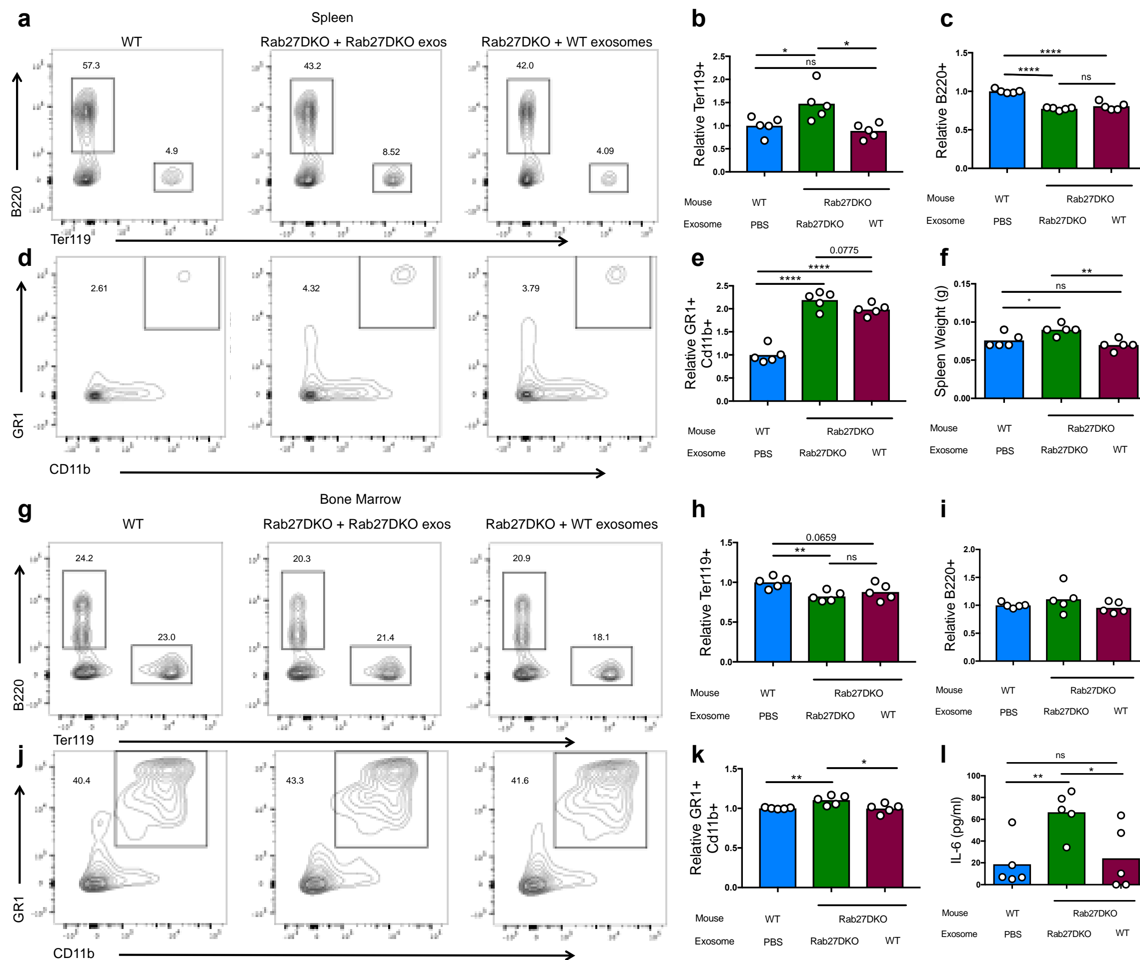


Figure 4.3. WT exosome treatment can complement certain Rab27DKO phenotypes. Rab27DKO mice were injected 2 times per week for 4 weeks with WT exosomes or the exosomal pellet from Rab27DKO GM-BMs. (a-c) Representative flow plot of Ter119+ and B220+ cells in the spleen with quantification of relative levels to the right. Mouse genotype and exosome treatment are indicated below the graphs. (d-e) GR1+ CD11b+ representative flow plots in the spleen and quantification of these percentages. (f) Spleen weights in grams of the treatment groups. (g-i) Representative flow plot of Ter119+ and B220+ cells in the bone marrow and relative levels are quantified to the right. (j-k) GR1+ CD11b+ representative flow plots in the bone marrow and quantification of these percentages. (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 1 experiment. Dots represent individual mice and the bar represents the mean. *P* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ****, $p < .0001$ Student's *t*-test.

Rab27DKO mice are refractory to treatment with LPS

Due to the increased baseline inflammation in the Rab27DKO mice, we wanted to investigate how these mice respond to a LPS challenge. To do this, we i.p. injected WT or Rab27DKO mice with a nonlethal dose of LPS and analyzed the inflammatory response by isolating serum at 2 and 6h 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 raise these levels in response to LPS to the same extent as WT mice (Figure 4.4a-c). Normally, during a LPS challenge mice will undergo a switch in hematopoietic development in the BM compartment called emergency granulopoiesis, where the GR1+ CD11b+ GM myeloid population expands and the B220+ B and Ter119+ erythroid precursor cell populations contracts by 72h 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 4.4d-h). These results indicate that Rab27DKO mice are refractory to stimulation with LPS.

Exosome injection restores the Rab27DKO response to LPS

To determine if the failure of Rab27DKO mice to respond to LPS was due to a lack of exosome uptake, we i.p. injected WT exosomes into Rab27DKO mice, or PBS, 24h before LPS injection (Figure 4.5a). Serum levels of TNFα and IL-6 were significantly increased when Rab27DKO mice were pre-treated with WT exosomes at 2- or 6-h post-LPS injection (Figure 4.5b). To analyze changes in emergency granulopoiesis, mice were injected a second time with exosomes 48h and harvested 72h post-LPS injection (Figure 4.5a). Rab27DKO mice given WT exosomes were able to expand their CD11b+ GR1+

Figure 4.4. Rab27DKO mice have a refractory response to LPS. WT or Rab27DKO mice were challenged with or without LPS. Serum was taken 2 and 6h post-LPS challenge while immune populations were examined 72h post-LPS. (a-c) Relative levels of TNFα and IL-6 in the serum at 2 and or 6h post-LPS administration with WT LPS treatment group set to 1. (d-e) Representative flow plots of myeloid (GR1+ Cd11b+), B cell (B220+) and erythroid precursor (Ter119+) populations for each condition in the bone marrow compartment. (f-h) Relative levels of B220+, CD11b GR1+, 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. *P* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$ Student's *t*-test.

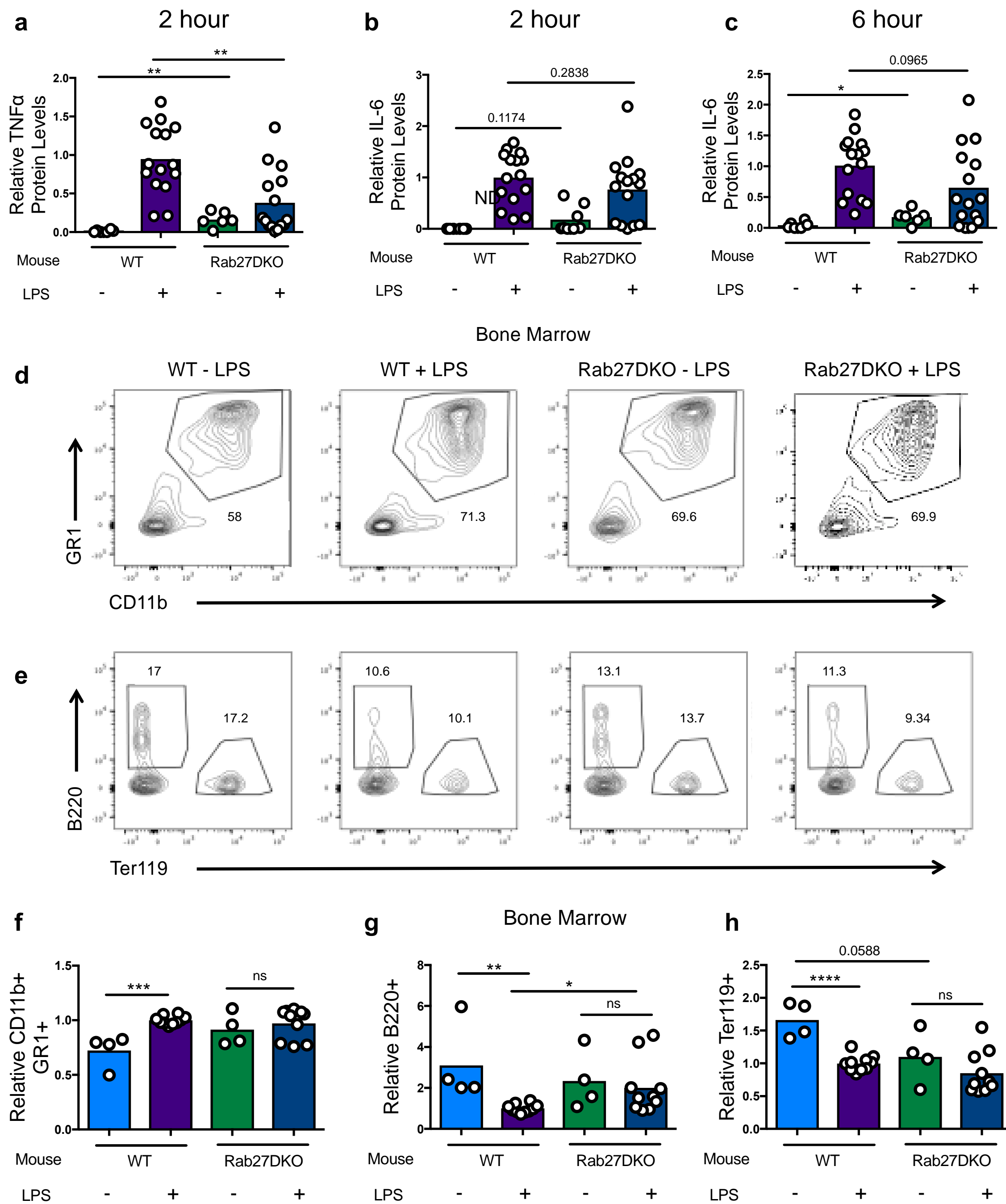
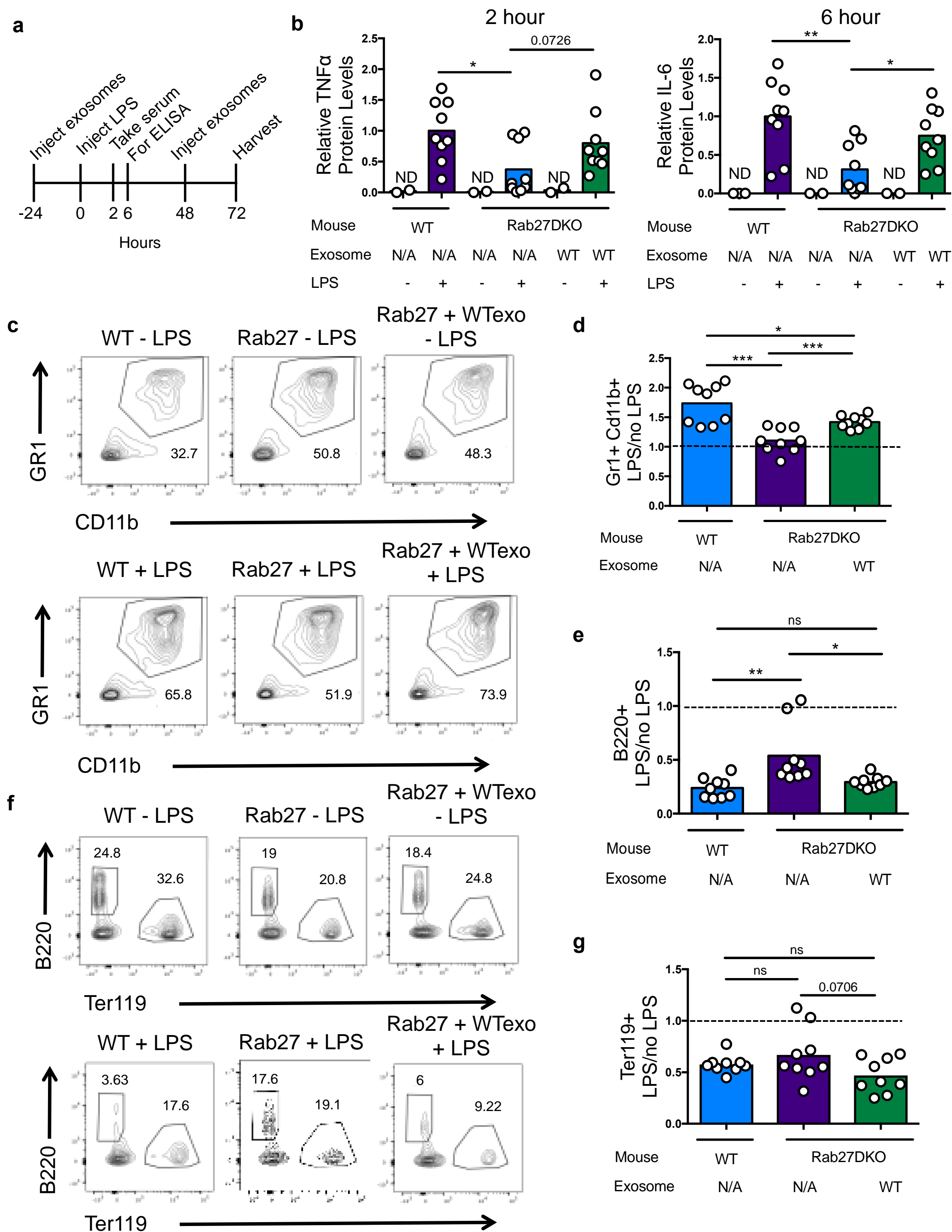

a
2 hour
Relative TNFα Protein Levels
Mouse
WT
Rab27DKO
LPS
b
2 hour
Relative IL-6 Protein Levels
0.1174
0.2838
ND
c
6 hour
0.0965
Bone Marrow
d
WT - LPS
WT + LPS
Rab27DKO - LPS
Rab27DKO + LPS
GR1
CD11b
58
71.3
69.6
69.9
e
B220
Ter119
17
17.2
10.6
10.1
13.1
13.7
11.3
9.34
f
Relative CD11b+ GR1+
g
Relative B220+
h
Relative Ter119+
0.0588
ns

Figure 4.5. 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 exosomes 24h before an LPS challenge. Serum was taken 2 or 6h post-LPS injection for ELISAs. 48h after LPS administration exosomes were injected again and 72h post-LPS immune populations were analyzed. (b) Relative levels of TNFα at 2h post-LPS and IL-6 in the serum at 6h post-LPS administration with WT treated with LPS was set to 1. (c-d) Representative flow plots of myeloid (GR1+ CD11b+) population in the bone marrow compartment with quantification to the right where the LPS treated group was set relative to the no LPS group to show the responsiveness of the population. (e-g) Representative flow plots for B cell (B220+) and erythroid precursor (Ter119+) populations in the bone marrow compartment with quantification to the right 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 separate experiments. Dots represent individual mice and the bar represents the mean. *P* values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$ Student's *t*-test.


a
Inject exosomes
Inject LPS
Take serum For ELISA
Inject exosomes
Harvest
-24
0
2 6
48
72
Hours
b
2 hour
Relative TNFα Protein Levels
0.0726
ND
6 hour
Relative IL-6 Protein Levels
Mouse
WT
Rab27DKO
Exosome
N/A
WT
LPS
c
WT - LPS
Rab27 - LPS
Rab27 + WTexo - LPS
GR1
CD11b
32.7
50.8
48.3
WT + LPS
Rab27 + LPS
Rab27 + WTexo + LPS
65.8
51.9
73.9
d
Gr1+ Cd11b+ LPS/no LPS
e
B220+ LPS/no LPS
ns
f
WT - LPS
Rab27 - LPS
Rab27 + WTexo - LPS
24.8
32.6
19
20.8
18.4
24.8
B220
Ter119
WT + LPS
Rab27 + LPS
Rab27 + WTexo + LPS
3.63
17.6
17.6
19.1
6
9.22
g
Ter119+ LPS/no LPS
0.0706

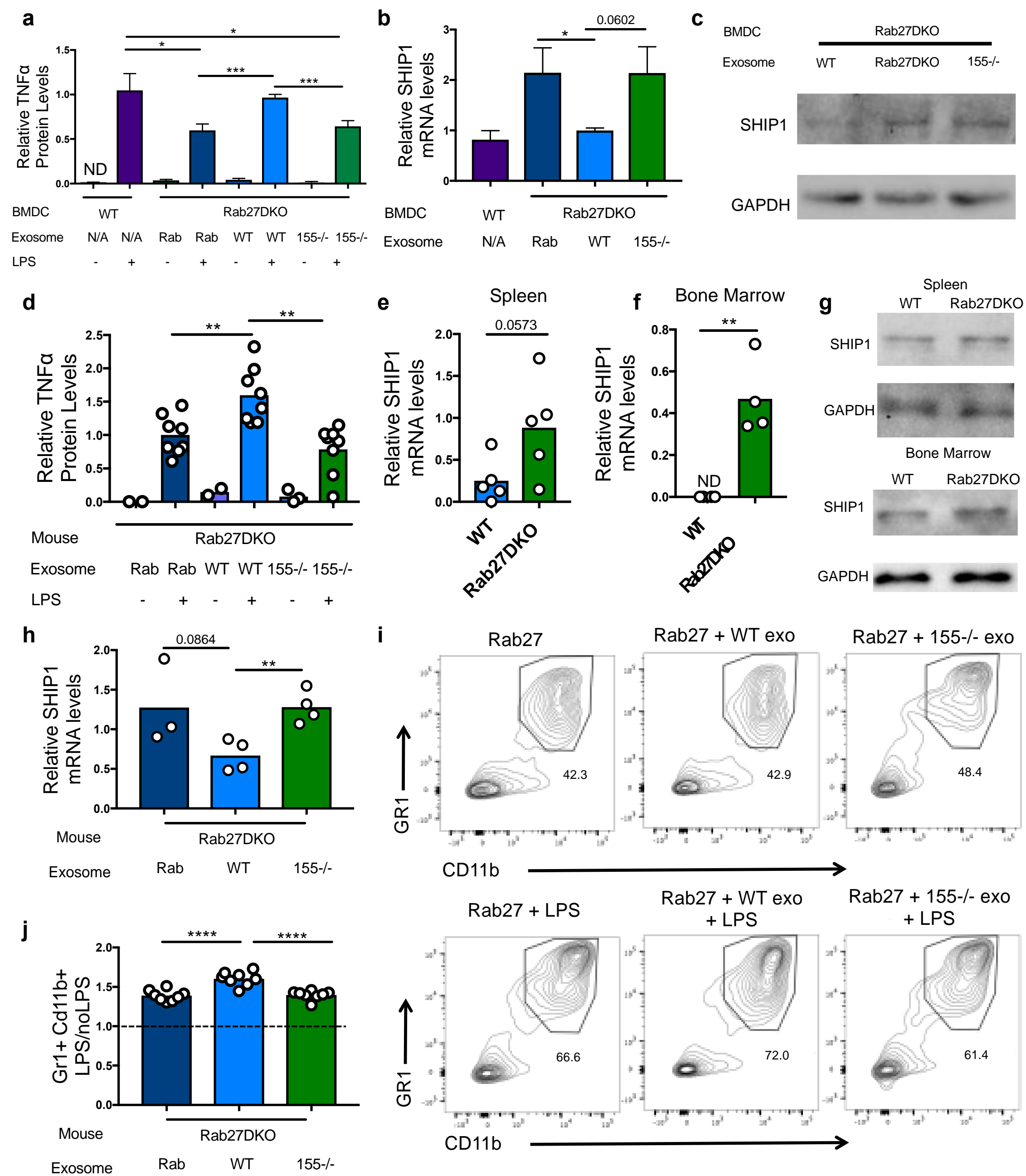
populations and reduce their B220+ B cell and Ter119+ erythroid precursor populations and in response to LPS (Figure 4.5c-g) suggesting that the uptake of WT exosomes is able to restore the ability of Rab27DKO mice to perform emergency granulopoiesis in response to LPS. It is important to note that the 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 4.5c and f). 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 in Rab27DKO BMDCs

Based on our findings that Rab27DKO mice have a refractory response to endotoxin that involves exosome uptake, we 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), we performed the following experiments. Rab27DKO, WT, or miR-155-/- GM-BM derived exosome pellets were administered to recipient Rab27DKO BMDCs, followed by LPS administration 24h later. Two hours post-LPS treatment, TNFα levels were increased in the Rab27DKO BMDCs given WT exosomes but not the Rab27DKO BMDCs that received their own exosome pellet or miR-155-/- exosomes (Figure 4.6a) 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, was affected by the administration of WT exosomes. We found that

Figure 4.6. Restoration of LPS responsiveness by Rab27DKO mice is dependent on miR-155.

(A) Rab27DKO BMDCs were treated with Rab27DKO exosomal pellet, WT exosomes, or miR-155-/- exosomes from GM-BMs 24h before LPS administration. Relative media TNFα levels 2h post-LPS administration are shown with the WT group treated with LPS was set as 1. n = 7. (B) Rab27DKO BMDCs were treated with the Rab27DKO, WT, or miR-155-/- exosomal pellet from GM-BMs 24h before LPS administration. 6h after LPS treatment RNA was harvested and SHIP1 levels were assayed with qRT-PCR with L32 as a loading control. Data are set relative to the Rab27DKO BMDC treated with WT exosomes which is set to 1. n = 5 (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) Rab27DKO mice were either i.p injected with a Rab27DKO, WT, or miR-155-/- exosome pellets 24h before an LPS challenge. Serum was taken 2 post-LPS injection for ELISAs. Values are set relative to Rab27DKO +Rab27DKO exosome LPS treatment which is set to 1. (E-F) Levels of SHIP1 mRNA in resting WT and Rab27DKO mice in the spleen and bone marrow relative to L32 loading control. (G) Westerns of SHIP1 in the spleen and bone marrow with GAPDH as a loading control. (H) Levels of SHIP1 mRNA in Rab27DKO mice BM that received Rab27DKO, WT, or miR-155-/- exosomal pellets then were treated with LPS for 72h. (I) Representative flow plots of the myeloid (GR1+ CD11b+) population for each condition in the bone marrow compartment from the same experiment set up in 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. Data is representative of at least 2 separate experiments. The error bars represent +/- S.E. Dots represent individual mice and the bar represents the mean. P values are either stated or *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$ Student's t-test.


a
Relative TNFα Protein Levels
ND
BMDC WT Rab27DKO
Exosome N/A N/A Rab Rab WT WT 155-/- 155-/-
LPS - + - + - + - +
b
Relative SHIP1 mRNA levels
0.0602
BMDC WT Rab27DKO
Exosome N/A Rab WT 155-/-
c
BMDC Rab27DKO
Exosome WT Rab27DKO 155-/-
SHIP1
GAPDH
d
Relative TNFα Protein Levels
Mouse Rab27DKO
Exosome Rab Rab WT WT 155-/- 155-/-
LPS - + - + - +
e
Spleen
Relative SHIP1 mRNA levels
0.0573
WT
Rab27DKO
f
Bone Marrow
Relative SHIP1 mRNA levels
ND
WT
Rab27DKO
g
Spleen
WT Rab27DKO
SHIP1
GAPDH
Bone Marrow
WT Rab27DKO
SHIP1
GAPDH
h
Relative SHIP1 mRNA levels
0.0864
Mouse Rab27DKO
Exosome Rab WT 155-/-
i
Rab27
Rab27 + WT exo
Rab27 + 155-/- exo
42.3
42.9
48.4
GR1
CD11b
Rab27 + LPS
Rab27 + WT exo + LPS
Rab27 + 155-/- exo + LPS
66.6
72.0
61.4
GR1
CD11b
j
Gr1+ Cd11b+ LPS/noLPS
Mouse Rab27DKO
Exosome Rab WT 155-/-

WT but not miR-155-/- exosomes were able to reduce SHIP1 levels in Rab27DKO BMDCs, corresponding to increased TNFα levels (Figure 4.6b-c), consistent with previous reports of SHIP1 negatively regulating TNFα production (27). These results indicate that exosomal miR-155 is required for exosome rescue of Rab27DKO BMDC responses to LPS through a mechanism involving repression of SHIP1.

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 responses to LPS. To study this, we injected WT, miR-155-/- or Rab27DKO GM-BM derived exosome pellets into Rab27DKO mice 24h before LPS injection and again 48h after LPS injection with the same timeline as Figure 4.5a. Rab27DKO mice treated with WT exosomes had enhanced TNFα production by 2h 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 4.6d). We also found that resting Rab27DKO mice had increased levels of SHIP1 (Figure 4.6e-g), suggesting that the Rab27DKO mice are trying to compensate for their chronic inflammatory status by upregulating a negative regulator of inflammation. This increase in SHIP1 in resting conditions could explain why the Rab27DKO mice are hyporesponsive 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 levels when LPS-treated Rab27DKO mice received WT but not miR-155-/- exosomes (Figure 4.6h). Additionally, the GM myeloid

population was increased in response to LPS administration following pretreatment with WT but not miR-155-/- exosomes (Figure 4.6i-j). These results provide evidence that miR-155 is required for exosomes to rescue LPS responsiveness by Rab27DKO mice, and that reductions in the miR-155 target SHIP1 are mediating this rescue.

Discussion

While it is clear from the literature that exosomes are important for intercellular communication between immune cells (7, 28), 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 (29, 30) and can be used as biomarkers. 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 can activate CD8+ T cells *in vivo* (31), and endogenous exosomes can mediate Treg suppression of Th1 cells via Let7d in an adoptive transfer system (32). While these studies provide evidence for the importance of endogenously produced exosomes during immunity, we designed our approach utilizing the Rab27DKO mouse model. Because these mice have defective exosome production (17, 32) we were able to study endogenous exosome production with minimal manipulation.

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 (18–20, 33). There may also be a role for the Rab27DKO proteins in recycling of membrane proteins and phagosome function (34,35).

Based on these additional functions of the Rab27 proteins in immune and cellular functions it is critical to distinguish these other functions 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.

Corresponding to increased basal inflammation and obesity, the Rab27DKO mice were hyporesponsive to LPS, consistent with previous findings that Rab27a knockout mice (ashen) are protected from LPS sepsis (36). Of clinical relevance, this effect is also observed in obese patients that have higher levels of resting proinflammatory cytokines but have defective responses to immunological challenges (22). We hypothesize that the Rab27DKO mice are less responsive to LPS because they have increased negative regulators of inflammation, such as SHIP1 (25, 27), 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). Our model for how exosome uptake is affecting the LPS response is summarized in Figure 4.7. Together our findings implicate endogenously produced miRNA containing exosomes in the regulation of an innate immune response *in vivo*.

Although our findings indicate that exosomal communication is needed to maintain hematopoietic homeostasis, it remains to be clarified which component of the exosome contributes to these physiological effects. Previous studies highlight the

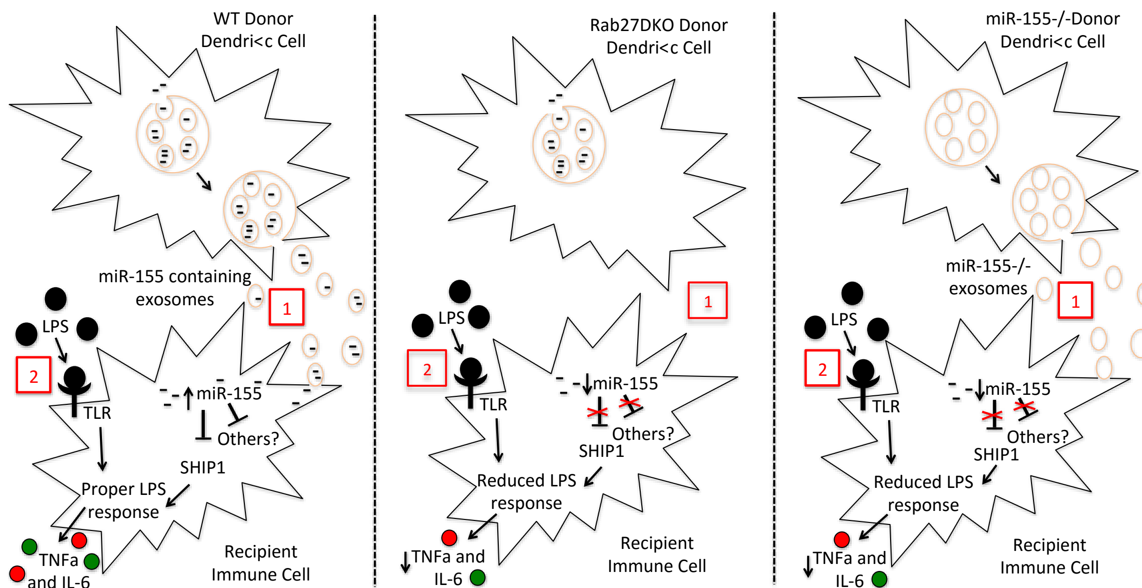


Figure 4.7. Model of the role of exosomal miR-155 in the LPS response. In a WT scenario, miR-155 can be transferred to a recipient cell leading to the knockdown of targets like SHIP1. Then the cell receives an LPS signal and can properly respond properly. However, in the Rab27DKO model, cells are defective in producing the appropriate amounts of exosomes, therefore the recipient cells do not receive miR-155 and cannot downregulate targets such as SHIP1, and thus respond improperly to LPS. In the last case, the cells are making exosomes but they do not contain miR-155 resulting in the lack of transferred miR-155, increased levels of miR-155 targets, and improper response to LPS.

importance of certain contents of exosomes for immune cell responses (7, 28). Exosomes can directly present antigen via MHC II molecules on their surface to T cells (37, 38). Activation of NK cells can be mediated by NKG2D ligands and IL-15Rα on DC exosomes (39). Further, mRNAs and miRNAs can be transferred between cells via exosomes mediate immune response (8, 12, 32). These examples and more summarized in Théry et al. and Robbins et al. (7, 28) 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 is important for proper responsiveness to LPS. However, while our experiments strongly suggest 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. One example of how the lack of miR-155 might affect exosomal communication is the recent report that deletion of miR-155 in a cell line alters exosome synthesis (40). 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 another way 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 also needed. As we begin to identify factors involved in miRNA loading, such as Ybx1 (41), 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.

The increased inflammation and weight gain observed in female Rab27DKO mice indicates that exosomes are key regulators of chronic inflammation, and present a valuable model to better understand the role of exosomes in this condition. Chronic inflammation and associated metabolic 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 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 from exosome treatment.

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 8–16 weeks old for all experiments.

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 resuspended 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 resuspended in PBS. This protocol is based on a previous exosome isolation methods (42). 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), where BM cells were incubated for 3 days with 20ng/ul GM-CSF and then given an additional 5ml of medium with 20ng/ul 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 3x10cm plates of 3 million GM-BMs per plate and resuspended 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.

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 pPCR master mix. L32 levels were used to normalize mRNA expression levels. Primer sequences are as follows:

SHIP1-F (5′-GAGCGGGATGAATCCAGTGG-3′),

SHIP1-R (5′-GGACCTCGGTTGGCAATGGTA-3′),

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

L32-R (5′-TTCATAGCAGTAGGCACAAAGG-3′).

BM reconstitution

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 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 sublethal concentration of 50μg. In exosome injection experiments, exosomes were i.p. injected 24h before LPS injection and again at 48h post-LPS injection. Mice were harvested at 72h post-LPS injection.

Mouse body composition and glucose testing

The composition of fat and lean tissue was determined with the NMR Bruker Minispec. Fasting glucose levels were determined by fasting the mice for 6h and then using FreeStyle Lite Test Strips and a Bayer Contour Blood Glucose Meter (Amazon).

Statistics

Data were analyzed using Student's *t*-tests with Graphpad Prism or Excel. *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.

Acknowledgments

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CHAPTER 5

IMPORTANCE OF EXOSOME RELEASE IN T-CELL ACTIVATION AND AUTOIMMUNITY DRIVEN BY T-CELLS

Abstract

T-cell activation is essential for proper adaptive immune responses. While there are many known signaling pathways involved in this activation, a novel form of intercellular communication between immune cells has been uncovered. Exosomes are small lipid packages that contain protein, mRNA and miRNAs and have been implicated in intercellular communication. However, the role of exosomes in T-cell activation has yet to be investigated. Utilizing the Rab27DKO mouse model, which has significantly decreased production of exosomes, we investigated the role of exosomes in T-cell activation. We initially observed that Rab27DKO mice have significantly increased activated T-cells in resting conditions. This increase of activated T-cells could not be rescued by the presence of WT bone marrow in a bone marrow chimera model. Additionally, the injection of BMDC derived exosomes could not rescue this phenotype. These data suggest that the aberrant activation of T-cells is a cell intrinsic phenotype that is not dependent on uptake of WT exosomes. When Rab27DKO mice were challenged with Experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, the Rab27DKO mice got significantly worsened disease phenotypes compared to their WT counterparts. This suggests that the increase of activated T-cell in the resting mice results in worsened disease outcome. It remains unclear whether lack of exosome release or some other intrinsic functions of Rab27a and b are driving this phenotype.

Introduction

During an adaptive immune response, naïve T-cells become activated in order to perform their functions. This activation is classically thought to involve key signaling

molecules such as CD3 and CD28 as well as TCR MHC interactions. Recently researchers have identified a novel form of intercellular communication where immune cells can transfer content to each other using small lipid vesicles called exosomes (1–3). The biogenesis of exosomes begins with the invagination of the limiting membrane of the endosome which forms the multivesicular body (MVB). Exosomes are release into the extracellular space when the MVB is traffic to and fuses to the plasma membrane (4–7). By knocking out Rab27a and b the MVB cannot be retained at the plasma membrane and thus the secretion of exosomes is diminished (8–11). This provides a model that can be used to investigate the role of exosomes in immune cell function and development. For example, Rab27DKO mice have been used to investigate the role of exosomes in Treg suppression of Th1 cells (12).

Using the Rab27DKO mice, we investigated whether the release or uptake of exosomes could be involved in T-cell activation. We first profiled their T-cell activation status at baseline conditions and observed that Rab27DKO mice had significantly more activated T-cells than their WT counterparts. The aberrant activation of Rab27DKO T-cells appeared to be a cell intrinsic phenotype as the presence of WT bone marrow in a bone marrow chimera did not rescue this defect. Additionally, the injection of WT exosomes into Rab27DKO mice did not rescue this defect suggesting that either exosome release or another cell intrinsic role of Rab27a or b resulted in the aberrant T-cell activation in these mice. In a model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), Rab27DKO had significantly worsened disease. This suggested that the increase of activated T-cells at resting conditions led to a worse outcome in an autoimmune disease mediated by T-cells in the Rab27DKO mice.

Results

Rab27DKO mice have increased activated T-cells

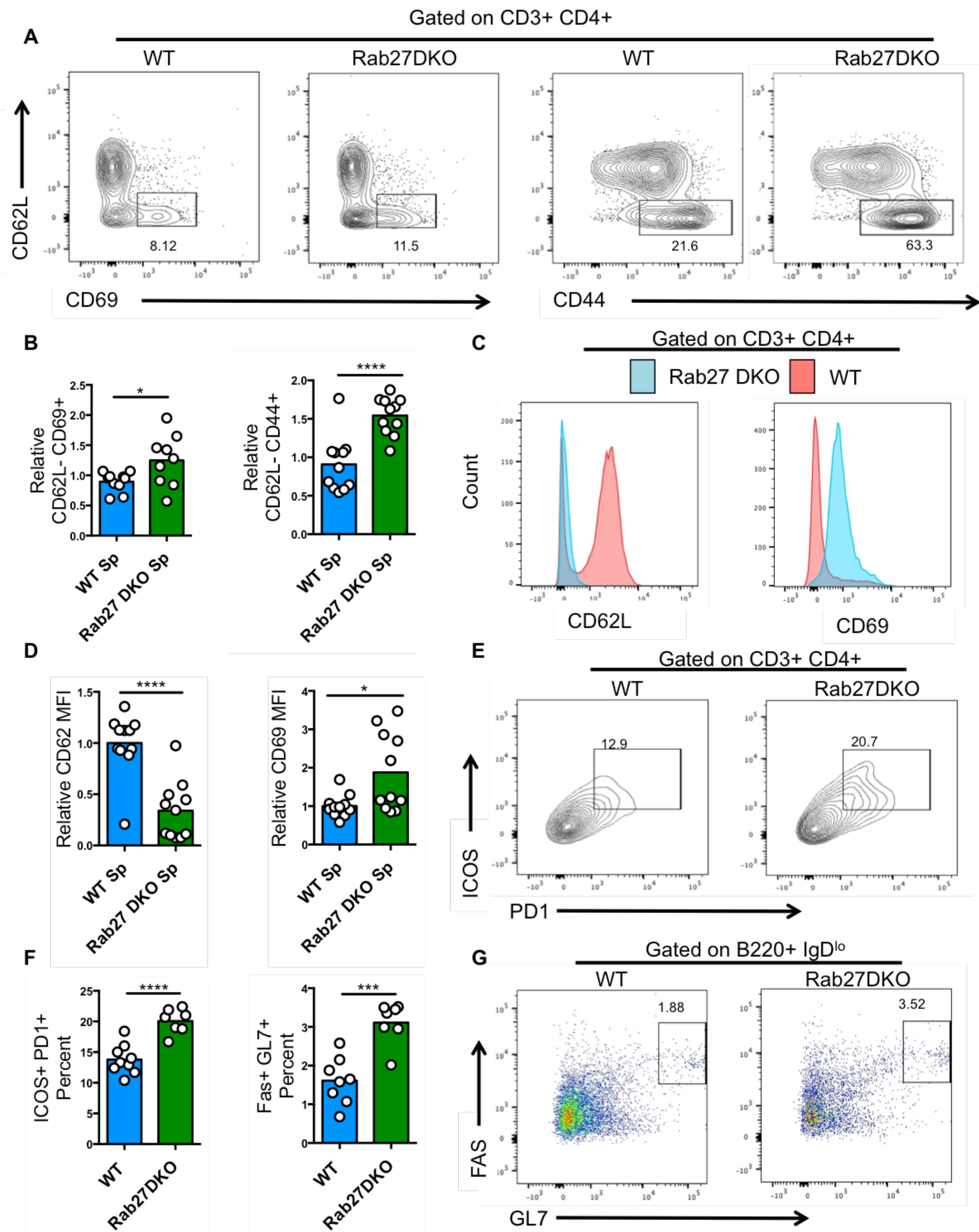
In order to investigate the role of exosome release in T-cell homeostasis we utilized the Rab27DKO mouse model, which is defective in exosome release. We analyzed T-cell populations via flow cytometry in Rab27DKO and WT mice. Overall CD4+ and CD8+ T-cell populations were not different between the groups; however, when we investigated the activation status of these cells we noted that Rab27DKO mice had significantly increased activated T-cells as indicated by an increase in CD62L- CD44+ cells, CD62L- CD69+ cells, CD69 mean fluorescence intensity (MFI) and the overall decrease in CD62L MFI (Figure 5.1a-d). Additionally, 6 month old Rab27DKO mice have increased t follicular helper cells (TFH), marked by PD1 and ICOS, and germinal center B cells (GCB) B220+ IgD low cells that are GL7+ FAS+ (Figure 5.1e-g). These results suggest that Rab27a and b are required for preventing aberrant activation of T-cells and germinal centers which may be due to the inability of these cell either to release or receive exosomes from other cells.

Activated T-cell phenotype in Rab27DKO mice is cell intrinsic

Based on our observation that T-cell activation was increased in Rab27DKO mice, we wanted to investigate whether this was due to a cell intrinsic defect, such as the inability to release exosomes, or a cell extrinsic defect, such as the inability to take up exosomes. In order to investigate this, we made bone marrow chimera mice. CD45.1 WT mice were lethally irradiated and reconstituted with either a CD45.1/CD45.2 WT chimera, a Rab27DKO (CD45.2+)/CD45.1 chimera, or Rab27DKO bone marrow alone.

After 2 months, the bone marrow and spleens from these mice were isolated and

Figure 5.1. Rab27DKO mice have increased activated T-cells, TFH cells and germinal center B cells. Spleens from 6-8-week old WT and Rab27DKO mice were analyzed for the T-cell activation status. TFH and GCB populations were analyzed in 6-month old mice. (A) CD69+ CD62L- and CD44+ CD62L- cells were analyzed in the CD3+ CD4+ T-cell population and representative flow plots are displayed. (B) Relative levels of CD62L- CD69+ and CD62L- CD44+ T-cells are displays with WT average set as 1. (C) Representative MFI plots of CD62L and CD69 within the CD3+ CD4+ population. (D) Relative MFI of CD62L and CD69 with WT average set as 1. (E) Representative flow plot of ICOS+ PD1+ (TFH) cells in the CD3+ CD4+ gate from 6-month-old mice. (F) Percentages of ICOS+ PD1+ (TFH) cells in the CD3+ CD4+ population are shown. Percentages of Fas+ GL7+ (GCB) cells in the B220+ IgDlo population are shown. (G) Representative flow plots of FAS+ GL7+ cells in the B220+ IgDlo gate are shown. Data are representative of 2 separate experiments. Dots represent individual mice and the bar represents the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$ Student's *t*-test.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Fas+ GL7+ Percent
Rab27DKO
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Gated on B220+ IgDlo
FAS
GL7
1.88
3.52

hematopoietic populations were analyzed via flow cytometry. BM reconstitution was effective as shown by CD45.1 and CD45.2 composition in the spleen (Figure 5.2d).

The presence of WT bone marrow was not able to rescue activated T-cell phenotypes, as indicated by increased CD62L- CD69+ cells, CD69 MFI and decreased CD62L MFI in mice that were reconstituted with Rab27DKO bone marrow alone as well as the mice that reconstituted with a mix of Rab27DKO and WT bone marrow (Figure 5.2a-c). Additionally, the CD62L- CD69+ cells were composed of cells derived from Rab27DKO bone marrow as shown by their CD45 markers (Figure 5.2 e-f). These data suggest that the activation of resting Rab27DKO T-cells is cell intrinsic and not due to the uptake of exosomes or from defects in the functions of other cell types such as regulatory T-cells.

<u>Exosome injection does not rescue T-cell activation phenotype in Rab27DKO mice</u>

In order to determine that exosome uptake is indeed not contributing to the activated T-cell phenotype in the Rab27DKO mice we performed BMDC derived exosome injections of resting Rab27DKO mice. Rab27DKO mice were injected 2 times a week for 4 weeks with WT exosomes while controls received PBS injections. After 4 weeks, spleens were isolated and T-cell activation status was determined via flow cytometry. Injection of BMDC derived exosomes did not alleviate T-cell activation phenotypes (Figure 5.3). These data suggest that BMDC derived exosomes cannot rescue Rab27DKO T-cell over-activation.

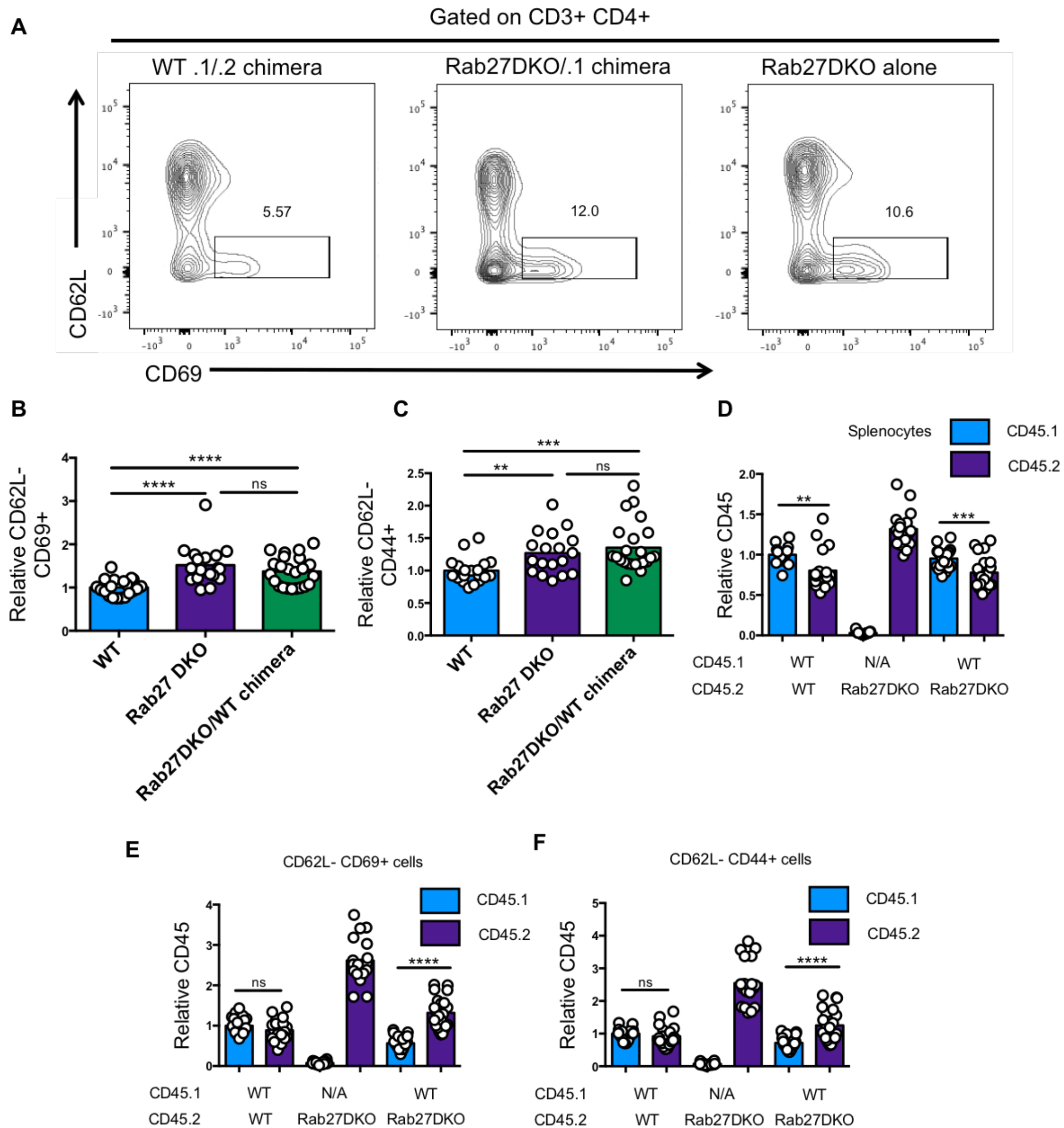

Figure 5.2. Aberrant activation of Rab27DKO CD4+ T-cells is a cell intrinsic defect. Bone marrow chimera experiments were performed to determine whether T-cell activation in Rab27DKO mice was a cell intrinsic or extrinsic defect with the following groups; WT (CD45.1)/ WT (CD45.2) bone marrow mixed, Rab27DKO (CD45.2)/ WT (CD45.1) mixed, and Rab27DKO bone marrow alone. (A) CD69+ CD62L- and CD44+ CD62L- cells were analyzed in the CD3+ CD4+ T-cell population and representative flow plots are displayed. (B-C) Relative levels of CD62L- CD69+ and CD62L- CD44+ T-cells are displays with WT average set as 1. (D) Reconstitution efficiency in the spleen as measured by CD45 composition (E) Relative CD45.1 or CD45.2 in the CD62L- CD69+ population. The CD45 marker genotype is marked below the graph. (F) Relative CD45.1 or CD45.2 in the CD62L- CD44+ population. Data is representative of 3 separate experiments. Dots represent individual mice and the bar represents the mean. **, $p < 0.01$; ***, $p < .001$; ****, $p < .0001$ Student's *t*-test.

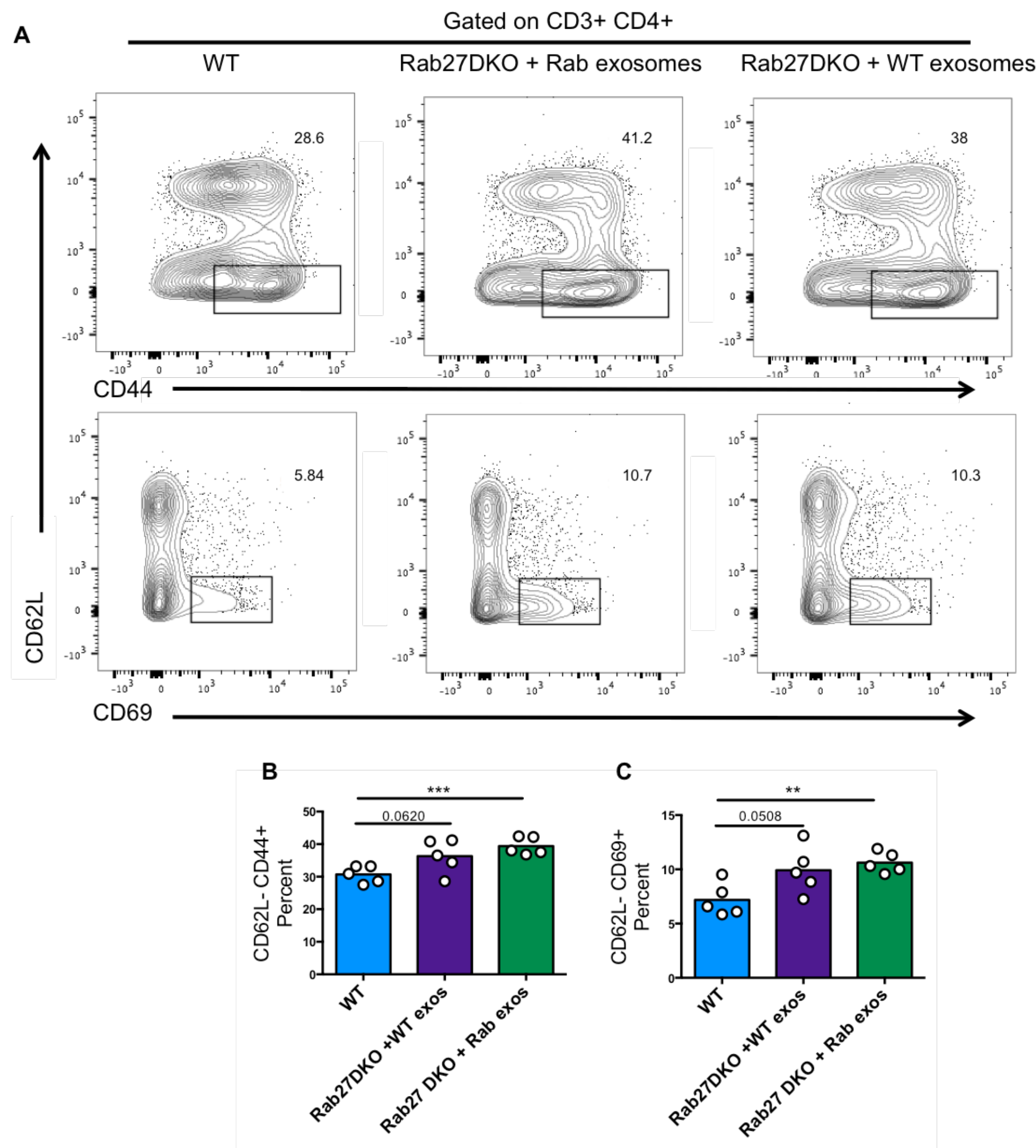

Figure 5.3. WT exosome injections cannot rescue aberrant T-cell activation in Rab27DKO mice. WT or Rab27DKO exosomes were injected into Rab27DKO 2 times a week for 4 weeks and then spleens were harvested to analyze T-cell activation status. (A) CD69+ CD62L- and CD44+ CD62L- cells were analyzed in the CD3+ CD4+ T-cell population and representative flow plots are displayed. (B-C) Relative levels of CD62L- CD69+ and CD62L- CD44+ T-cells are displays with WT average set as 1. Data are representative of 2 separate experiments. Dots represent individual mice and the bar represents the mean. **, $p < 0.01$; ***, $p < .001$; Student's *t*-test.

<u>Rab27DKO mice show increased EAE disease</u>

Due to the increase of resting activated T-cells in Rab27DKO mice we decided to challenge these mice with a disease model that is T-cell-dependent, EAE. Rab27DKO mice developed worsened EAE disease scores compared to WT mice (Figure 5.4a). Additionally, Th1 and Th17 cells were increased in the brains of the Rab27DKO mice given EAE (Figure 5.4b-d). These results suggest that the T-cells are indeed more activated in Rab27DKO mice as they are associated with increase disease.

Discussion

While much is known about the signaling events that contribute to T-cell activation, the exact mechanism of this activation remains unclear. Here, we propose a role for exosome release in this activation. While more work is needed confirm the exosome release is indeed the driving factor in our model, we do have evidence supporting this hypothesis.

We found that Rab27DKO mice have increased activated T-cells at resting conditions compared to WT mice. These results suggest that the Rab27 proteins are playing a role in repressing aberrant T-cell activation; however, it does not reveal whether this phenotype is due to exosomes or not. Our first way of addressing this was to determine if the increased activation of T-cells in the Rab27DKO mice was a cell intrinsic or extrinsic defect. We observed that the presence of WT bone marrow could not rescue this defect suggesting that something inherent to the Rab27DKO T-cells was resulting in their activation. To confirm that the uptake of exosomes was not affecting the activation of T-cells, we injected WT exosomes into Rab27DKO mice and observed that this did not rescue the activation defect. All these data suggest that either decreased exosome release is

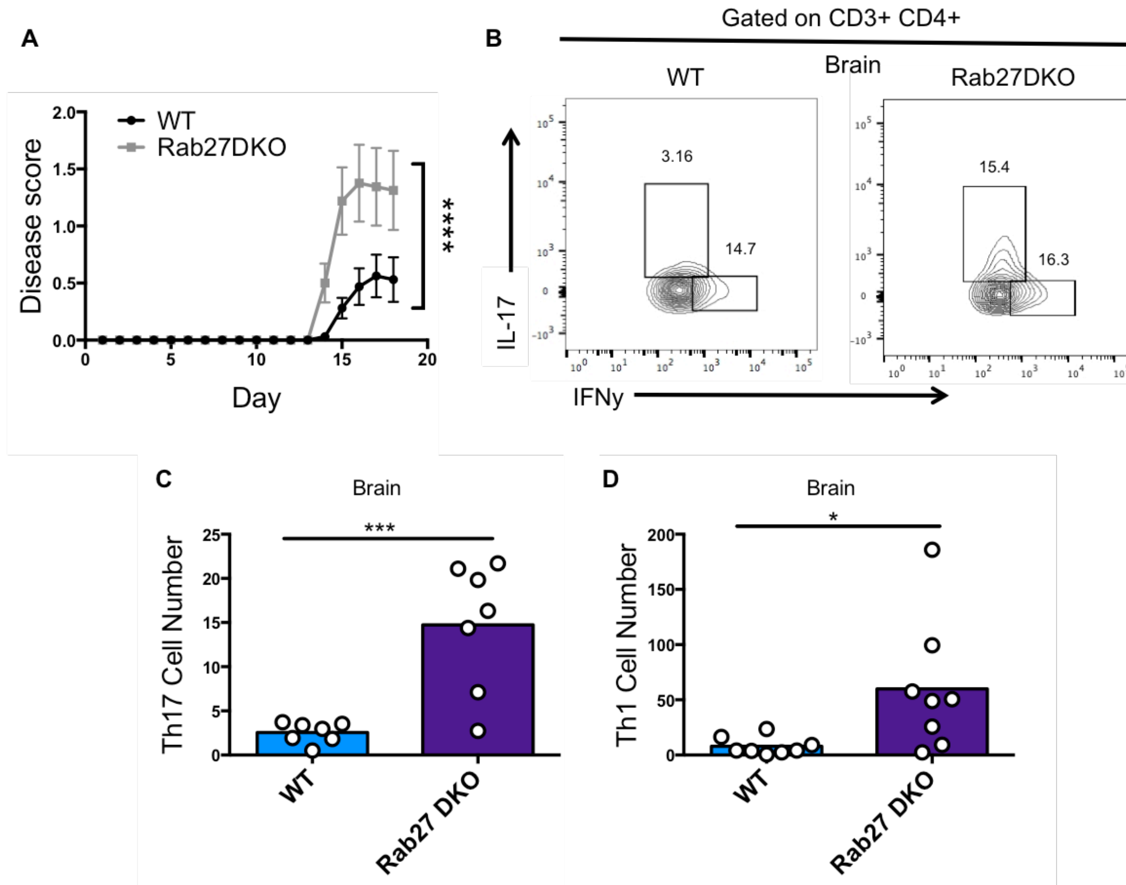

Figure 5.4. Rab27DKO mice are more susceptible to EAE. WT or Rab27DKO mice were given EAE and their disease scores were tracked over time. At day 18 mice were sacrificed and their brains were analyzed for Th1 and Th17 T-cell subsets. (A) IL-17+ or IFNy+ cells were analyzed in the CD3+ CD4+ T-cell population and representative flow plots from the brain are displayed. (B-C) Total cell numbers of Th17 cells (IL-17+) and Th1 (IFNy+) T-cells in the brain are displayed. Data are representative of 2 separate experiments. Dots represent individual mice and the bar represents the mean. *, p >.05; ***, p <.001 Student's t-test.

contributing to the T-cell activation or that Rab27 a and/or b are contributing to this activation independent of exosome release.

The implications of aberrantly activated T-cells are strong for autoimmunity. For this reason, we challenged our Rab27DKO mice with a model of MS, EAE. Rab27DKO mice have significantly worsened disease score compared to their WT counterparts. This suggests that the prevalence of activated T-cells in the Rab27DKO mice is predisposing them for worsened disease outcomes.

When interpreting our results, it is important to note the Rab27DKO mice have defects outside of exosome release. Rab27a and/or b have been shown to have defective granules release in immune cells such as cytotoxic T-cells as well as defective neutrophil chemotaxis (13–19). There may also be a role for the Rab27 proteins in the recycling of membrane proteins and phagosome function (20, 21). Based on these additional roles of the Rab27 proteins in other immune and cellular functions it is important to tease apart these other roles from their role on exosome secretion. Our approach to this was to attempt to rescue observed phenotypes with the addition of WT exosomes. If the defect is rescued, then that suggests that the lack of uptake of exosomal content is contributing to the defect. In the case of aberrant T-cell activation, the addition of WT exosome was unable to rescue the defects we observed. This suggests that the Rab27 proteins could be playing a role outside of exosomes that is having the observed effect, or that the release of exosome is the contributing factor to this phenotype.

Materials and methods

Mice

Wt (Jackson Labs) and *CD45.1 Wt* (Jackson Labs) *Rab27 DKO* (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 8–16 weeks old.

Exosome isolation and procedures

Differential centrifugation was performed to isolate exosomes from 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 resuspended in 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 resuspended in tissue culture media. We used a Thermo Scientific Sorvall Lynx 6000 with a T26-8 × 50 rotor. For the *in vivo* experiments exosomes were isolated from GM-SCF treated bone marrow where bone marrow cells were incubated for 3 days with 20ng/ul GM-CSF and then spiked with 5ml media and 20ng/ul GM-CSF for a total of 7 day of culture. For the *in vivo* experiments, one mouse receives exosomes derived from 3 10cm plates of 3 million GM-BMs, which yields approximately 10^9 exosomes as previously quantified (22). Protein concentrations are quantified and similar protein levels are injected except for Rab27DKO exosomal pellets.

Flow cytometry

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

EAE

For induction of EAE, mice were injected subcutaneously into the base of the tail with a volume of 200 μl containing 100 μg/ml MOG_{35-55} peptide (TOCRIS) emulsified in complete Freund's adjuvant (CFA). Mice were also i.p. injected with 200 ng of pertussis toxin on days 0 and 2, and clinical symptoms were scored regularly according to the following criteria: 0, no symptoms; 0.5, partially limp tail; 1, completely limp tail; 1.5, impaired righting reflex; 2, hind limb paresis; 2.5, hind-limb paralysis; 3, forelimb weakness; 4, complete paralysis; 5, death.

BM reconstitution

Mice were lethally irradiated (1,000 rads) was delivered using an X-ray source. Following irradiation, mice were injected with three million BM cells via retro-orbital injection and aged for 2 months.

Acknowledgements

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CHAPTER 6

DISCUSSION

Existing state of the exosomal miRNA communication field

Since the discovery that exosomes contain miRNAs, there has been significant progress made in understanding the roles of exosomal miRNAs during intercellular communication. Many different cell types can secrete miRNA containing exosomes and there is evidence that the miRNAs can be functionally transferred between cells (1–5). However, many of these studies do not use a miRNA knockout as a recipient of the miRNA containing exosomes, which would demonstrate that the miRNA is not endogenously upregulated but is delivered exogenously. There are additionally many examples of the functionality of transferred miRNAs in cancer and immune cells (1, 2, 5–8). For example, exosomes can transfer miRNAs between T cells and dendritic cells in a unidirectional manner to modulate gene expression in the recipient cells (1). Additionally, miRNAs are functionally transferred between mouse dendritic cells (9). In the case of cancer cells, exosomal miRNAs prime brain metastasis by inducing the loss of PTEN (10).

While these studies and many more have contributed much to what we know about the functional transfer of miRNAs via exosomes there still remain key questions that need to be addressed. One of these questions concerns the selective loading of miRNAs into exosomes during their biogenesis. Several proteins have been implicated in this loading in different cell types including hnRNPA2B1, YBX1, and SNYCRIP (11–13). However, it still remains to be determined whether these proteins play this role in many cell types or if there's a cell-specific sorting mechanism. It seems likely that there is a common mechanism across all cell types, but this remains to be elucidated.

Another major question remaining in the field concerns how recipient cells take

up the miRNA containing exosomes and how the miRNA is delivered to the cytoplasm. There is evidence of several different mechanisms of miRNA delivery by exosomes; including endocytosis, plasma membrane fusion, and phagocytosis (14–18). Integrins play an important role in the adherence of the exosome membrane to the membrane of the recipient cell (18, 19). It is unknown whether exosome uptake is receptor mediated, although there is some evidence for this (20), the specific receptors remain unclear. Tetraspanins have been implicated in receptor mediated uptake, but more work is needed to determine whether these proteins play a role in cell specific uptake of exosomes (18, 21, 22). Treatment of cells or exosomes with trypsin results in significantly reduced uptake of exosomal miRNAs, suggesting that proteins on both the cell and exosomes are needed for uptake (data not shown). Further, we have performed studies with inhibitors of cytoskeletal rearrangement and found that rearrangement is necessary for exosome uptake (data not shown).

Another outstanding question in the field is whether the transfer of miRNAs via exosomes is an important event during normal biological events. In order to properly address this question, a model where exosome secretion is defective is needed. The model that has begun to emerge to address this question is the Rab27DKO mouse model, which has been characterized as having decreased exosome production (23, 24). While there are caveats with this model, it remains the best model in the field to study the role of exosomes *in vivo*. There has been one major study investigating the role of exosome in regulatory T cell function with this model but many questions remain about the role of exosomes during normal hematopoietic development and during response to challenge

New insights into the roles of exosomal transfer of miRNAs and the roles of exosomes during intercellular communication *in vivo*

In our studies, we have observed that miRNAs miR-155 and miR-146a can be functionally transferred between immune cells and alter how those cells respond to a challenge. In the case of miR-155, we have observed that exosomes can transfer this miRNA to recipient cells resulting in the knockdown of normal miR-155 targets in a seed dependent manner and increasing the inflammatory response to endotoxin. The transfer of miR-146a in exosomes also knocks down its targets in a seed dependent manner but decreases the inflammatory response to endotoxin. These results support the idea that the transfer of miRNAs via exosomes can be used to communicate inflammatory status.

Using the Rab27DKO mouse model, we have begun to investigate the role of exosomes during *in vivo* processes. We have evidence that suggests that exosomes may be playing important roles in baseline hematopoietic homeostasis, chronic inflammation, and response to bacterial challenge. Some of these phenotypes seem to be dependent on exosome uptake as the administration of exosomes rescues some baseline hematopoietic homeostasis defects and rescues the response to endotoxin in the Rab27DKO mice. Other phenotypes seem to be cell intrinsic as the presence of WT bone marrow and the injection of exosomes does not rescue the T cell activation phenotype in Rab27DKO mice. This could mean that either the release of exosomes is needed to prevent aberrant T cell activation or that there is some other role of Rab27a and or b that is resulting in the increased activation of T cells. All of these results strongly suggest that exosomes are playing important roles *in vivo*. Additionally, in the case of endotoxin response, the

presence of miR-155 in exosomes is essential for the rescue of the defective endotoxin response in the Rab27DKO mice. This result suggests that this particular miRNA is a major factor involved in endotoxin response *in vivo*.

Future directions in the exosomal communication field

Despite all we have learned from our studies, there is still much work to be done to characterize the role of exosomes during development and immune responses as well as the role of transferred miRNAs in intercellular communication. One aspect in the field that needs to be standardized is how we determine whether a particular miRNA is functionally transferred via exosomes. In order to determine whether there is a physiological role for transferred miRNAs there are several key experiments that must be done. miRNA knockout cells should be used as recipients to assure that the increase in miRNA signal is due to exogenous delivery and not endogenous upregulation. Additionally, to determine if the delivery of the miRNA is directly targeting the 3'UTR of it's targets, luciferase assays can be performed where a WT and seed mutant version of a target 3'UTR is used. In order to address whether the differences seen between WT and miRNA knockout exosomes is due to other changes in the content of the exosomes apart for the lack of the factor of interest, mimic experiments can be performed using miRNA knockout exosomes loaded with a mimic of the miRNA of interest and transferring the mimic loaded exosomes to recipient miRNA knockout cells compared to ones that did not contain mimic. Finally, in order to determine if the transfer of the miRNA of interest is important *in vivo*, Rab27DKO mice can be used to assess whether exosome communication is important in certain scenarios and whether your favorite miRNA

contained within exosomes is sufficient to rescue defects in Rab27DKO mice. These experiments are essential when trying to determine the functionality of a particular miRNA being transferred.

In order for the field of miRNA exosome communication to move forward essential tools are required. An important new tool would be a mouse model that is unable to load miRNAs into exosomes. This model would separate the miRNA function in the exosomes from other exosome functions. While there has been some progress on elucidating what proteins might be involved in the loading of miRNA into exosomes, it remains to be seen if there is a common mechanism of loading in all cell types.

The role of exosomal communication *in vivo* is just beginning to be investigated. The Rab27DKO model provides a mouse model where the secretion of exosomes is decrease; however, this model has many caveats. The largest drawback to this model is that Rab27 a and b have other functions in the cell besides exosome release. Due to the other roles of Rab27 a and b, it cannot be assumed that phenotypes observed in the Rab27DKO mice are due to exosomal communication. One way to address this issue and specifically look at the role of exosome uptake is with exosome injection experiments. If the injection of exosomes rescues a phenotype in the Rab27DKO mice, then that supports the conclusion that exosome uptake is contributing to that phenotype.

The implications of the use of miRNAs contained within exosomes as a tool for communication are vast. Classically, cells are thought to utilize proteins as a way to signal each other of their status; however, now a new molecule, exosomally contained miRNAs, have been implicated in this signaling. Not only does the transfer of miRNAs represent a novel form of intercellular communication but also it presents a potential new

and better way to deliver therapeutics.

Implications of the exosome mediated transfer of miRNAs

With the deeper understanding of exosome and exosomal communication via miRNAs, we can begin to utilize this knowledge in potential therapeutic applications. Due to our observations that exosome delivery could rescue 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 from exosome treatment. For example, in the case where an increase in inflammatory response is desired, such as a vaccine adjuvant, miR-155 containing exosomes could be administered. In the case where a decreased response is desired, such as during sepsis, treatment with miR-146a exosomes could be used.

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