


No small matter: emerging roles for exosomal miRNAs in the immune system

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Extracellular communication is critical to the function of an organism. Exosomes, small lipid extracellular vesicles, have been recently appreciated to participate in this vital function. Within these vesicles lie critical bioactive molecules including mRNAs, proteins, and a plethora of noncoding RNAs, including microRNAs (miRNAs). Exosomal miRNAs have been shown to be produced by, trafficked between, and function in many distinct donor and recipient cell types, including cells of the immune system. For instance, loss of these critical communicators can alter the cellular response to endotoxin, and when tumor cells lose the ability to secrete these vesicles, the immune system is able to effectively suppress tumor growth. This review will highlight key findings on the known communication to and from the immune system, highlighting exosomal miRNA research in macrophages, dendritic cells, B lymphocytes, and T cells. Additionally, we will focus on three major areas of exosomal studies that involve immune responses including mucosal barriers, adipose tissue, and the tumor microenvironment. These environments are heterogeneous and dynamic, and rapidly respond to the microbiota, metabolic shifts, and immunotherapies, respectively. It is clear that exosomal miRNAs play pivotal roles in regulating cross-talk between cells in these tissues, and this represents a novel layer of cellular communication proving critical in human health and disease.

Introduction

Exosomes

Extracellular communication is critical to cellular function, and this diverse method of regulation can be mediated by many molecules including but not limited

to membrane-derived extracellular vesicles (EVs), which encompass plasma membrane budding microvesicles, exosomes, and recently described exomeres [1–3]. Seminal papers in the 1980s

Abbreviations

AIEC, adherent-invasive *Escherichia coli*; APC, antigen-presenting cell; ATM, adipose tissue macrophage; BALF, bronchoalveolar lavage fluid; BAT, brown adipose tissue; BMDCs, bone marrow-derived dendritic cells; DCs, dendritic cells; EBV, Epstein Barr virus; EC, epithelial cell; EV, extracellular vesicle; ICAM-1, intercellular adhesion molecule 1; IEC, intestinal epithelial cell; IFN- γ , interferon gamma; IL-35, interleukin-35; IL-4, interleukin 4; IS, immunological synapse; Klf4, Kruppel-like factor 4; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; MHC, major histocompatibility complex; miRNA, microRNA; MVB, multivesicular body; NDURFA4, NADH dehydrogenase 1 alpha subcomplex 4; NF κ B, Nuclear Factor kappa B; NK, natural killer; OMVs, outer membrane vesicles; PAMP, pathogen-associated molecular pattern; RFXAP, regulatory factor X-associated protein; RISC, RNA-induced silencing complex; TAMs, tumor-associated macrophages; Th1, T helper cell type 1; Th2, T helper type 2; TME, tumor microenvironment; TNF α , tumor necrosis factor α ; Treg, regulatory T cells; TXNIP, transcription factor thioredoxin-interacting protein.

simultaneously reported the discovery of exosomes in immature red blood cells (reticulocytes) from different animals [4,5], although the term exosome was not coined until 1989 [6]. After their discovery, it became apparent in the late 1990s that exosomes within the immune system were important and could be an efficacious as an anti-tumor treatment in preclinical models [7–9]. Since then, the field has greatly expanded along with the technology to understand the dynamic regulation of recipient cells by these diverse vesicles. This review will focus on exosomes, and while the definition of such vesicles has become more stringent and their protein markers more defined [10,11], we will also discuss research done in the broad area of ‘extracellular vesicles’ that has likely included exosomes in the tested extracellular material.

Exosomes are small lipid vesicles approximately 50–150 nm in size, that originate from the endosomal compartment and are released into the extracellular milieu after fusion of the multivesicular body (MVB) with the plasma membrane [12]. An in-depth review of their biogenesis can be found in van Niel *et al.* 2018 and Colombo *et al.*, 2014 [2,13]. Exosomal cargo is enriched for bioactive molecules, including but not limited to proteins, mRNAs, and noncoding RNAs [14]. It has been increasingly appreciated that miRNAs make up a large portion of the noncoding RNAs found within EVs, with some studies reporting up to 50% of the total noncoding RNA cargo being composed of miRNAs depending on the cellular origin [10,15]. Exosomal transfer of miRNAs protects against degradation of the extracellular miRNAs by free-floating RNases, making this an exciting mode of extracellular miRNA transport [10,12]. Key discoveries from recent research into exosomal miRNAs and immune cell regulation provide insight into how these noncoding RNAs can dictate responses of cells to distinct stimuli within microenvironments that can include diverse microbial pathogens.

miRNAs

While exosomes were originally described in immature red blood cells [4,5], it has been widely appreciated that almost every cell type, except for mature red blood cells, can secrete exosomes. Due to this widespread secretion, exosomes can be found in a wide variety of bodily fluids and these exosomes can have a functional effect on neighboring cells [9,13,14,16–18]. We now know a large component of the RNA species present in exosomes are noncoding RNAs including miRNAs, tRNA fragments, Y-RNAs, and long noncoding RNAs [10,15,19–21]. For over a decade, it has

been established that mast cell-derived exosomal miRNAs can regulate gene expression in recipient cells [14]. However, currently advancing inquiries into miRNAs within exosomes and their roles in modulating immune and non-immune cell functions have revealed the critical function of these vesicles in mammalian biology broadly.

miRNAs are small, ~22 nucleotide noncoding RNAs that repress or degrade target mRNAs by recruiting the RNA-induced silencing complex (RISC) and guiding it to the 6-8 nucleotide complementary seed sequences in the 3' UTR. The biogenesis and cellular role of miRNAs have been reviewed extensively in O'Connell *et al.* and Treiber *et al.* [22,23]. Briefly, miRNAs originate from Pol II-synthesized, mRNA-like primary transcripts that are then processed by the nuclear ribonuclease Drosha, exported from the nucleus, further processed by the cytoplasmic endoribonuclease Dicer, and are then loaded into the RISC complex to bind with their target mRNA. Extracellular miRNAs exist in two flavors, protected from RNases in extracellular vesicles, or bound to proteins [14,24,25]. It has been established that vesicular miRNAs can be functional in recipient cells, repressing known targets, as commonly measured by immunoblotting and qPCR [14,19]. Rescue experiments with addition of exogenous exosomes support the theory that exosomes are able to bypass the endosomal degradation pathway upon fusing with recipient cells and are loaded with enough miRNAs to execute repressive function once inside the cell [26–28], although precise mechanistic insight into exosomal fusion to the recipient cell remains unclear. The functional transfer of miRNAs and other bioactive molecules by exosomes has led to a breadth of research working to understand the dynamic cross-talk between immune cells, as well as between immune cells and other non-immune cell types such as tumor cells, adipocytes, and epithelial cells. Here, we focus on these two areas, acknowledging that this is not all-encompassing, but rather a review of these subsets of cells that are beginning to establish early paradigms in this nascent field of study.

Immune cell communication via exosomal miRNAs

Dendritic cells

Dendritic cells (DCs) are key antigen-presenting cells (APCs) of the innate immune system. Mature DCs are capable of stimulating the adaptive immune system, including effector T cells, in response to pathogens or

pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). DCs communicate with neighboring cells in a variety of ways, including via cytokine signaling, cell-to-cell contact, and secretion of vesicles [7,29]. Immature and mature bone marrow-derived DCs (BMDCs) have the capacity to secrete a large number of exosomes, although this is reduced upon maturation. This trait has created a novel field of DC vesicle research and provided a basis for understanding the basic cell biology of how exosomes are generated, transferred, and internalized [7,30–32]. The recent chapter by Kowal and Tkach [29] is a comprehensive overview of DC extracellular vesicles and their biological function, including proteins and lipids on these EVs.

Extracellular miRNA transfer via exosomes occurs readily throughout the mammalian system. We know that *in vivo*, dye-labeled BMDC exosomes can be received by splenic B cells, macrophages, plasmacytoid DCs, and conventional (CD11c-high) DCs, whereas T cells, both CD4+ and CD8+ subsets, cannot [30]. However, internalization of immature DC-derived labeled vesicles by CD4+ T cells was possible *in vitro*, highlighting the differences between *in vivo* and *in vitro* experiments within the field, as well as the impact of maturation of the vesicle-producing cell on the recipient cell [32]. This diverse repertoire of recipient cells highlights the ability of extracellular miRNA transfer into many different cell types. Interestingly, the receipt of mature DC-derived exosomes by B cells allows B-cell priming of naïve T cells [31]. Studies have also demonstrated that major histocompatibility complex class II (MHCII) and intercellular adhesion molecule 1 (ICAM-1) on the surface of mature DC exosomes are critical for stimulating T cells. This is due to lymphocyte function-associated antigen 1 (LFA-1) aiding capture of ICAM-1+ exosomes on the recipient T-cell surface [31,33,34]. Importantly, there is evidence that mature DC-derived EVs can be received by monocytes, inducing their differentiation into immature DCs that can readily release IL-12p70 [35]. Immature DC-derived EVs may also be important in skewing T cells, particularly toward T helper type 1 (Th1) vs. T helper type 2 (Th2) cells. Interestingly, this ability seems to be lost upon maturation of the vesicle-producing DCs [32]. Thus, there is a clear functional importance of DC exosomes within the immune system, suggesting that they may carry specific RNA cargos, including noncoding RNAs.

Noncoding RNAs are diverse, and many different types are found in EVs from DCs [15,19–21,29]. Upon BMDC stimulation with LPS (immune-stimulatory) or vitamin D3 (immune-suppressive), there are alterations to noncoding RNA species in EVs, including Y-RNAs

and small nucleolar RNAs (snoRNAs), as well as an enrichment in miRNAs known to be increased endogenously within DCs in response to either stimulus. This highlights how parent cell status can be reflected in the produced exosomes. This is not always the case however, notably in experiments where broad populations of RNA species found in exosomes were analyzed [21,36]. Foundational DC-derived exosomal miRNA work by Montecalvo *et al.* [30] demonstrates that miR-451 and miR-148a can be transferred from DCs to other DCs. Additionally, it is known that monocyte-derived DCs can be stimulated by activated bystander T cells to release vesicle miR-155, leading to additional activation of T cells [37].

miR-146a, an anti-inflammatory miRNA, has been found repeatedly in DC-derived exosomes [19,38] and can affect its targets IRAK-1 and Traf6 in recipient cells [19]. Additionally, our group has shown that DCs package miR-155, a pro-inflammatory miRNA, and miR-146a into exosomes and that both of these miRNAs can be received by DCs *in vitro*, modulating their target genes SHIP1, BACH1, Traf6, and IRAK-1, as measured by qPCR and immunoblotting [19,26]. Functionally, these miRNAs regulate the cellular response to endotoxin by inducing either pro- or anti-inflammatory gene programs. With the use of knockout recipient mice and bone marrow chimeras, we have been able to observe the receipt of exosomal miR-155 by B220+, CD3+, and CD11b+ cells, likely through exosome transfer. Additionally, we demonstrated that exogenous addition of WT BMDC exosomes, in contrast to exosomes from either miR-155-/- or miR-146a-/- BMDCs, restored cellular miRNA levels and, moreover, the appropriate response to endotoxin in knockout animals. Intraperitoneally injected exosomes traveled to the spleen, liver, and bone marrow, highlighting the diverse organ repertoire targeted by DC EVs [26]. Extensive research on DC exosomes highlights the myriad functions that exosomes have within the immune system itself (Fig. 1). Although much work has been done to determine how DC exosomal content affects other cells, it is still currently unknown how exosomal miRNAs are involved in differentiating monocytes into immature DCs. As this is uncovered, it will be an interesting avenue to explore and could lead to therapeutic developments for diseases that rely heavily on dendritic cell function such as cancer or inflammatory bowel disease.

Macrophages

Macrophages are a primary early defense cell of the innate immune system, acting against pathogens as

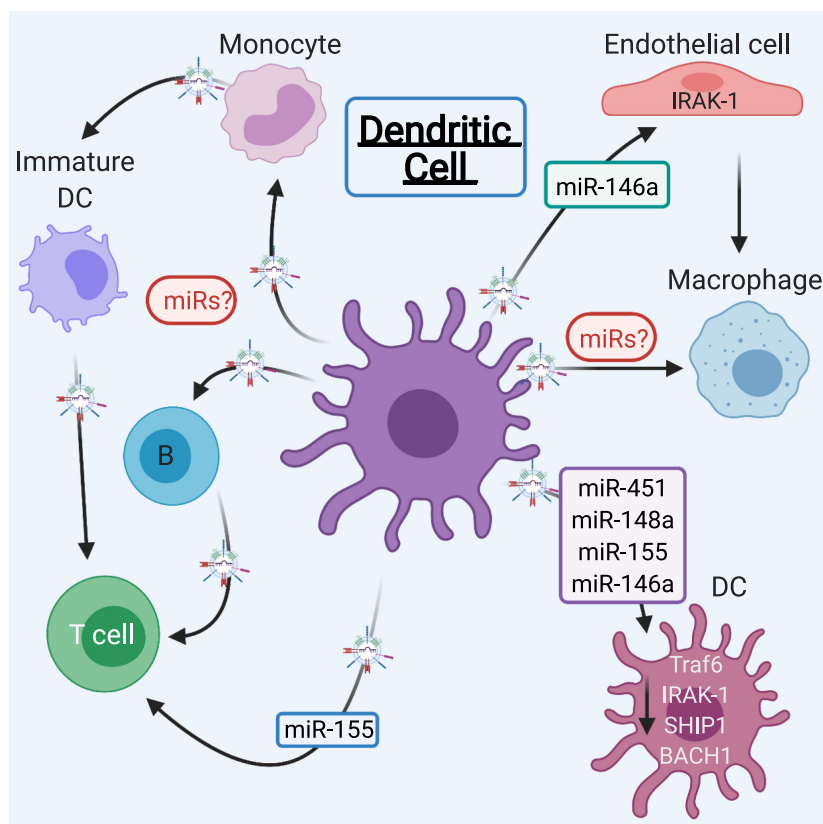


Fig. 1. Dendritic cell-derived exosomes readily modulate many different immune cells. miRNAs from DCs regulate endothelial cells, T-cell activation, and other recipient DC response to endotoxin. Exosomes from DCs also regulate differentiation of monocytes and B-cell communication with T cells.

well as promoting wound healing. They are commonly categorized as ‘classically activated/pro-inflammatory’ M1 or ‘alternatively activated/anti-inflammatory’ M2 macrophages, although increasing literature is suggesting that these cells are incredibly nuanced and complex during immune responses *in vivo* [39]. Exosomes from M2 macrophages have been increasingly appreciated for their anti-inflammatory nature and investigated as potential therapies to reduce pro-inflammatory macrophage phenotypes, common to many diseases including obesity and atherosclerosis, while also boosting other beneficial cell types within such microenvironments [40–42]. Within the intestinal microenvironment, macrophages play a critical role in response to dextran sodium sulfate (DSS)-induced colitis, a mouse model of ulcerative colitis. It has been recently suggested that M2b macrophage-derived exosomes can induce splenic T regulatory cells (Tregs) and alleviate colitis, although it is unknown if Tregs are also changed at the colonic injury site [43].

Interleukin-4 (IL-4)-treated (M2) macrophage-derived exosomes have the ability, through miR-146b, miR-99a, and miR-378a, to modulate tumor necrosis factor alpha (TNF- α) and nuclear factor kappa B (NF- κ B) pathways in recipient macrophages [40]. As is

common for most exosomal miRNA transfer studies, the authors confirmed the miRNA transfer into recipient cells by examining TNF- α and NF- κ B levels by flow cytometry after depleting the hypothesized exosomal miRs. Further, they were able to show these exosomes functionally reduce the total number of macrophages in plaques when injected into *ApoE*^{-/-} mice, in addition to increasing M2 markers, whereas naive macrophage-derived exosomes do not provide this protection. In addition to macrophage exosomes being capable of skewing macrophages within plaques of *ApoE*^{-/-} mice, it is now appreciated that mesenchymal stem cells-derived exosomes (MSCs) can also skew the macrophages in plaques to be more ‘M2’ like, through exosomal miR-let7 family members [44]. Supporting the functional relevance of M2 macrophage EVs, it was shown that miR-378a was enriched in M2 macrophage EVs, which were capable of stimulating *in vivo* bone regeneration, highlighting their anti-inflammatory and healing nature [42]. Macrophage-derived exosomal miR-21-5p can promote increased fibrogenesis in tendons after injection into mice by modulating the Smad7 signaling pathway [45]. Finally, miR-148a is important in M2 macrophage-derived exosomes and regulates the transcription factor

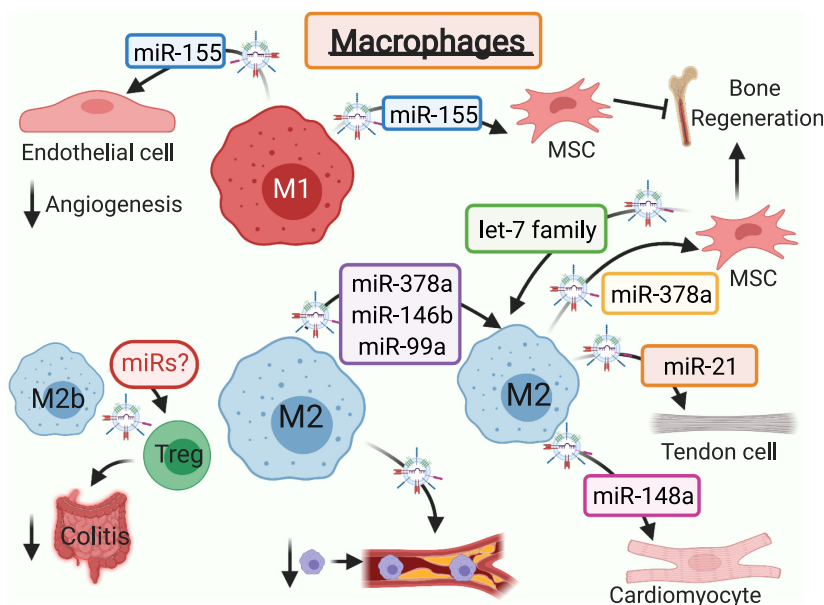


Fig. 2. Macrophage exosomes regulate diverse cell types throughout the body. M1 macrophage-derived exosomes, through miR-155, regulate endothelial cell angiogenesis and MSC regulation of bone regeneration. In contrast, M2 macrophage exosomal miRNAs can promote bone regeneration by traveling to MSCs, wound healing in cardiomyocytes, and fibrogenesis of tendon cells. M2 exosomes also can skew macrophages to an M2-like state and M2b exosomes play a role in IBD.

thioredoxin-interacting protein (TXNIP) in cardiomyocytes, allowing for a better response to myocardial injury [46].

In contrast to increasing literature highlighting the beneficial functions of anti-inflammatory EVs, pro-inflammatory EVs from M1 macrophages were found to be detrimental, halting osteoblastic differentiation and BMP signaling through miR-155 [42]. miR-155 is clearly an important exosomal miRNA, as it appears to not only function in BMP signaling, but also to be readily released by M1-like macrophages after myocardial injury, limiting wound healing and endothelial cell angiogenesis by regulating the Sirt1/AMPK α 2 and RAC1-PAK2 signaling pathways [41]. This dichotomy of function between M1 and M2 macrophage exosomes reflects their cells of origin and opens the door to therapies based on exosomal miRNAs released from M2 macrophages to stimulate wound repair and combat inflammatory diseases (Fig. 2).

T lymphocytes

T cells are a critical arm of adaptive immunity, responding to antigen presentation by APCs, including dendritic cells and B cells. Naïve T cells can become a myriad of distinct effector T-cell types following their activation. While effector T cells drive immune responses, Tregs function to repress T-cell activity and provide key negative regulatory signals in the immune system to limit autoimmunity. Akin to myeloid cells, T cells use exosomes as a method of communication for rapid cross-talk with neighboring cells, adding to the complex

regulatory mechanisms that modulate T-cell biology. Exosomes are appreciated as critical to the immunological synapse (IS) that forms between T and B cells or T cells and DCs. Within the IS of T and B cells, it has been established that exosomes are readily exchanged, potentially in a unidirectional manner [47], carrying miRNAs in this instance, that are distinct from the parent cell. Of interest, miR-355 regulates the transcription factor SOX4 in recipient Raji B cells. Building on this work, it has been shown that T-cell EVs can modulate immunoglobulin release and survival of B cells, through exosomal miR-155, miR-20, and miR-25 targeting BIM and PTEN, which was confirmed via qPCR of the targets in recipient B cells [48].

T-cell-derived exosomes are not always beneficial to the surrounding microenvironment as they can deliver cargo to β cells, resulting in deleterious effects to these critical pancreatic cells. Mechanistically, T-cell-derived exosomes carrying miR-142-3p/5p and miR-155 are capable of promoting *in vitro* β cell apoptosis in the pancreas by altering NF κ B translocation and stimulating Ccl2, Ccl7, and Cxcl10 transcription [49]. Interestingly within this same microenvironment, β cells can also release exosomal miR-29 to modulate the inflammatory state of monocytes through TNF receptor-associated factor 3 (TRAF3), highlighting the tissue-immune cell cross-talk that can be mediated by exosomes [50]. miRNAs packaged into exosomes largely function by repressing target genes in recipient cells; however, it has recently been suggested that T cells can secrete select tRNA fragments to prevent inhibition of activation, suggesting a novel ‘garbage bag’ role for EVs

in helping the cell to rid itself of certain RNA species to promote cellular maturation [15]. In addition to regulating immune cells, T-cell exosomes can alter endothelial cells through regulating integrins that control recruitment of T cells to the gut [51]. Mitochondrial or genomic DNA transfer within T-cell EVs can also trigger a response by cGAS/STING within recipient DCs [52]. This response allows for protection against infection by viruses, promoting the adaptation of recipient cells to the extracellular environment.

Recently, it has also been appreciated that exosomes from Tregs play a role in Th1 proliferation and interferon gamma (IFN- γ) secretion, mediated in part by exosomal miR-155, let-7b, and let-7d [53]. Let-7d seems to play the dominant role in regulating IFN- γ secretion and cell proliferation by targeting *Cox2* in Th1 cells, although this remains to be confirmed, as numerous potential targets were dysregulated in Th1 cells. Interleukin-35+ (IL-35+) EVs from Tregs play a role in Treg exhaustion and act *in trans* on bystander T cells, causing exhaustion and an immunosuppressive phenotype [54]. Tregs are capable of modulating effector T cells, but they can also regulate DCs. One mechanism of this regulation is through release of EVs that contain miR-150 and miR-142a, which broadly alter the

tolerogenic state of the DCs and cytokine secretion [55]. It is clear from this wealth of research that exosomal miRNAs from T cells play a critical role in cross-talk with neighboring immune cells and nonimmune cells (Fig. 3).

B cells

B lymphocytes are key antibody-producing APCs of the adaptive immune system. B cells produce exosomes containing membrane-bound MHCII, as well as many other proteins, including components of complement [9,56,57]. It has been noted that B-cell exosomes alone are capable of priming DCs by the transfer of peptide antigen-loaded MHCII, which then act in concert with CD4+ T cells and NK cells to stimulate an antigen-specific CD8 killing response [58]. Importantly, miRNAs found in exosomes are not always derived from the host genome. Epstein-Barr Virus (EBV) was the first virus known to encode its own set of miRNAs. These miRNAs have recently been shown not only to get packaged in infected B-cell exosomes, but they can function in DCs upon uptake, inhibiting CXCL11 and LMP1, as shown by 3' UTR luciferase reporter assays [59]. This transfer to noninfected cells has been

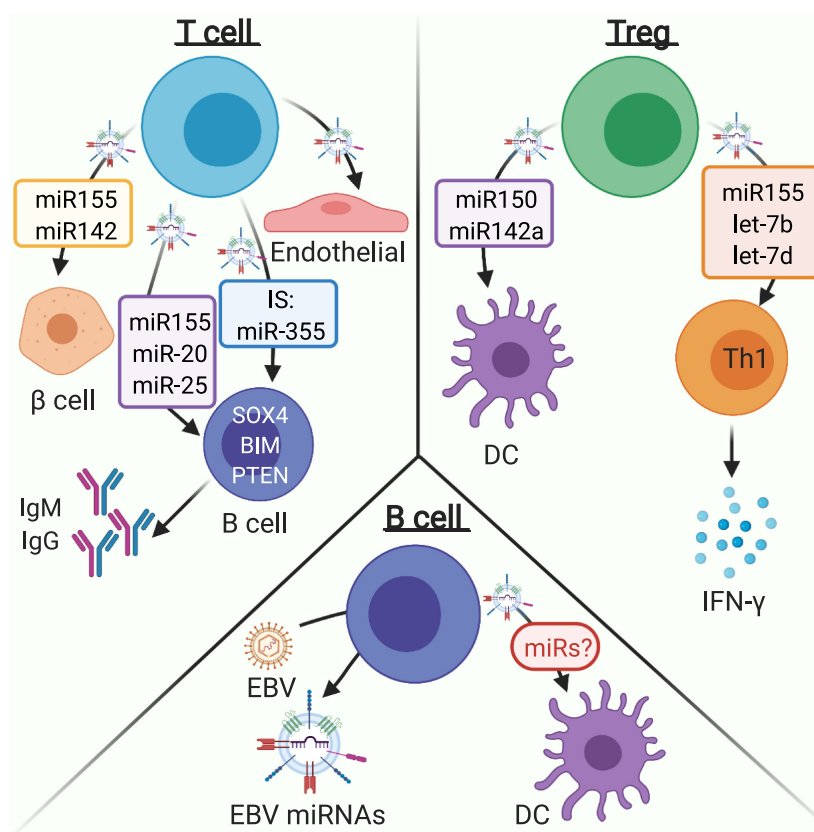


Fig. 3. Adaptive immunity functions through the use of exosomal miRNAs. T-cell exosomes regulate endothelial cells, as well as exosomal miRNAs that regulate β cell function, B-cell survival, and immunoglobulin release. In addition, Treg exosomal miRNAs regulate DCs and Th1 release of IFN- γ . B-cell exosomes can be hijacked by EBV miRNAs. Additionally, B-cell-derived exosomes communicate to DCs.

implicated in the latency potential of this possibly oncogenic virus, effectively evading the immune system. Additionally, these exosomes are capable of altering cytokine release by monocytes and macrophages following delivery of EBV miRNAs and are potential as biomarkers for EBV+ diffuse large B-cell lymphoma diagnosis [60]. Host miRNAs, such as miR-155 and miR-193b, are also affected during EBV, being upregulated in B cells and their exosomes after infection [61]. Currently, little to no attention has been paid to host miRNAs originating from B cells at steady state, and how they interact with their surrounding environment, representing an open area of investigation (Fig. 3).

Non-immune cell communication with immune cells via exosomal miRNAs

Intestinal epithelial cells

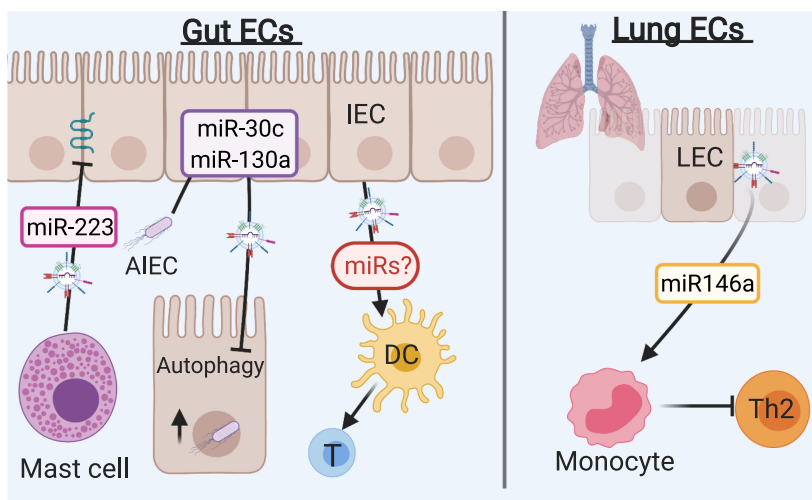
Intestinal epithelial cells (IECs) were one of the first non-immune cell types studied in an effort to understand their potential exosomal communication with immune cells. IECs uniquely monitor the lumen of the gut, endocytose and display antigens, and communicate with the underlying immune system in response to either commensal or pathogenic microbes in the gut. The important role of miRNAs within this microenvironment has been discovered using whole-body knockouts for specific miRNAs, such as miR-146a [62], but specific research on IEC-derived exosomal miRNAs has been elusive because there has been a lack of *in vivo* tools. Despite this, critical insights into the broader IEC EV biology have allowed for exploration of the roles of exosomes and hypotheses about miRNAs involved in these processes. Early on, exosomes were hypothesized to be a means of rapidly stimulating luminal immune cells by transfer of processed peptide via MHC, by bypassing the basement membrane due to their nanoscale. In the early 2000s, foundational work by Mallegol and van Niel illustrated the capability of IECs to release exosomes, especially in response to IFN- γ , and showed that IEC exosomes have a functional interaction on surrounding immune cells [63]. *In vitro* IEC-derived exosomes are found to communicate preferentially with DCs over T cells [64] and are able to deliver peptide to DCs via MHCII, lowering the threshold for these DCs to activate T cells. Injecting IEC-derived exosomes *in vivo* resulted in a specific humoral immune response [65].

To date, it remains unclear which miRNAs are found in IEC exosomes and how they may influence the intestinal microenvironment either at steady state or under disease states, such as during inflammatory

bowel disease (IBD). However, it is now known that adherent-invasive *Escherichia coli* (AIEC), which are commonly found in Crohn's patients, can lead to miR-30c and miR-130a upregulation within IECs, in contrast to commensal strains or the laboratory strain K-12. Then, these miRNAs can subsequently be packaged into IEC-derived vesicles and propagated to recipient IECs where they inhibit autophagy and lead to increased AIEC replication [66]. This was reversed with anti-miRNAs (anti-sense to miRNAs of interest) which restored autophagy machinery and intracellular AIEC to normal levels, highlighting the therapeutic potential for Crohn's patients with AIEC. In addition to understanding how IECs function during disease, it is also critical to learn how and from what immune cells they are receiving signals. One hallmark of IBD is mast cell accumulation, but the mode of deleterious effects these cells may have during disease is unknown. Recently, it has been understood that mast cells can secrete miR-223—and likely other miRNAs—in exosomes, which can modulate the intestinal barrier by downregulating key tight junction proteins, including Claudin-8, as shown by western blot [16]. Importantly, in a similar mucosal environment, lung epithelial cells are also appreciated to release exosomes containing miR-146a. This can then stimulate monocyte release of IL-10, which can suppress Th2 responses in an allergy model [67]. Interestingly, after allergic airway inflammation, EVs from hematopoietic cells are increased in bronchoalveolar lavage fluid (BALF) [68], yet the functional relevance of this remains unclear. Parallel mucosal responses could be occurring in the intestinal microenvironment but this remains to be seen.

Intestinal epithelial cells are the first line of defense against enteric pathogens and are able to discriminate between commensal and pathogenic microbes. A role for exosomes within the host-pathogen environment is slowly beginning to emerge. While bacterial and fungal infections are typically at the forefront of invasive species in the gut, the protozoan parasite *Cryptosporidium parvum* can also readily infect immunosuppressed individuals or children in developing countries. It is now appreciated that IECs respond to this infection by increasing their exosome release [69], resulting in splenocyte activation. Further, *C. parvum* RNA can be packaged into these IEC-derived exosomes [70]. While the functional role of these parasitic small RNAs is not fully understood, they are known to bind to Hmg-b1, a DNA-binding protein that can stimulate the NF κ B response through RAGE. Additional study of these IEC-derived exosomes should elucidate mechanisms of defense against *C. parvum*, potentially leading to exosome-based therapeutics. The diverse IEC-

Fig. 4. Epithelial cell-derived exosomes can cross-talk with the underlying immune system. Gut intestinal epithelial cell (EC)-derived exosomes can regulate DCs and after infection with AIEC are repurposed to allow for increased replication of AIEC within IECs. Additionally, mast cell-derived exosomal miR-223 can regulate the tight junction proteins of IECs. Within the lung microenvironment, lung epithelial cells (ECs) can regulate monocytes, inhibiting Th2 cells.



derived exosomal responses highlighted here show the complex challenges that IECs face during infection, often being hijacked by pathogens (Fig. 4). Understanding the miRNAs involved in these mucosal responses will be critical to further this field of study.

Adipose tissue

Adipose tissue is complex, comprised of brown and white adipocytes, immune cells, and fibroblasts, and is a critical metabolic tissue within the body. Not surprisingly, it is able to communicate with many different organs and one method of doing this throughout the body is via EVs [71]. It has been suggested that a majority of serum exosomal miRNAs are derived from adipose tissue [28] and that these have the capability to regulate mRNAs in liver and muscle cells [28,72,73]. In a series of elegant *in vivo* experiments, Thomou *et al.* [28] describe a mechanism of brown adipose tissue (BAT)-derived exosomal miR-99b that travels to the liver and regulates a key metabolic gene, *Fgf21*. This distant regulation is key in understanding the importance of not only cell-to-cell communication but also organ-to-organ communication by exosomes.

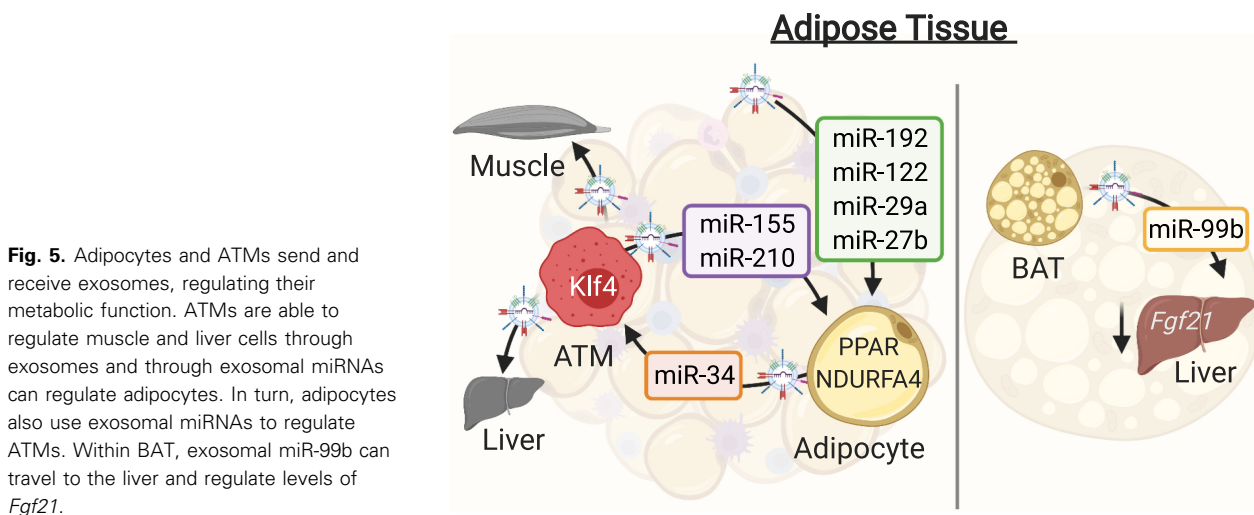
Adipose tissue can undergo obesity-induced tissue inflammation. When this occurs, there is an accumulation of pro-inflammatory M1-like macrophages, compared to the normal anti-inflammatory M2-like macrophages found in a lean dietary state. These pro-inflammatory adipose tissue macrophages (ATMs) communicate with the surrounding adipocytes as well as distant myocytes and hepatocytes through exosomes [73], and effect functional responses through the *Ppar* family of transcription factors. In particular, macrophage-derived exosomal miR-155 can regulate insulin

sensitivity through $PPAR\gamma$ in adipocytes. Conversely, exosomes from lean ATMs counteract insulin resistance in obese mice and restore normal glucose levels and insulin tolerance [73]. *Ppar* factors are readily down-regulated by exosomal miRNAs, especially during obesity, as it was also demonstrated that synthetic exosomes containing four miRNAs upregulated in obese serum exosomes (miR-192, miR-122, miR-27a-3p, and miR-27b-3p) modulate *Ppar* family expression in adipocytes [72]. Additionally, mitochondrial dysfunction occurs in obesity and it has been suggested that ATM-derived exosomal miR-210 can target NADH dehydrogenase 1 alpha subcomplex 4 (NDURFA4) in adipocytes and thereby regulates glucose levels [74].

Communication via exosomes in adipose tissue does not only emanate from ATMs, but adipocytes are also poised to communicate to ATMs via exosomes [75–77]. The *PPAR* family is clearly regulated in adipocytes by ATM exosomes and conversely adipocyte-derived exosomal miR-34a regulates Kruppel-like factor 4 (*Klf4*), a key transcription factor involved in macrophage skewing [76]. It was observed that in obese mice, adipose-derived exosomal miR-34a is elevated and that removal of this miRNA alters *Klf4* levels as well as the type of macrophages found within fat deposits. Importantly, this clear evidence of cross-talk and immune regulation within adipose tissue highlights an avenue of therapeutics that could be explored to help combat obesity (Fig. 5).

Tumor microenvironment

The dynamic relationship between immune cell EVs and cancer cells is one that has been highly researched in recent years and reviewed nicely by Wortzel *et al.*



and Kugeratski and Kalluri [17,78]. In brief, we know that EVs can drive multiple processes within the tumor microenvironment (TME), including matrix remodeling and angiogenesis, to establishing a premetastatic niche. EVs can also regulate signaling between the tumor and immune cells to provide necessary growth factors for the tumor and to exert immunosuppressive functions on the immune system [27,79–83]. Checkpoint blockade therapies have been highly efficacious for many cancers. However, this is not always the case. We now know that exosomes released from numerous different types of cancer cells have an abundance of surface PD-L1, a common immunotherapeutic target, and that this source of PD-L1 is capable of altering T-cell responses in lymph nodes, but is not targetable by immunotherapies in subcutaneous tumors [27]. In addition to understanding the basic biological functions of exosomal content within the TME, recent research has also focused on using plasma or serum vesicle miRNAs and proteins as biomarkers or predictors of many cancer types [60,84].

Tumor cells are known to secrete exosomes, whose miRNA composition is distinct from the cellular source; these exosomes can help to promote tumor growth and evade the immune system [85,86]. For instance, tumor exosomes derived from B16F0 murine melanoma cells have an effect on IFN- γ secretion and mitochondrial respiration rates in cultured T cells [87]. TLRs are also a common target of exosomal miRNAs. Lung cancer cells promote a pro-metastatic niche by releasing exosomal miR-21 and miR-29a that act as TLR7/8 agonists in peritoneal macrophages [86]; furthermore, pancreatic cancer cells down-regulate TLR4 in DCs via miR-203 and alter their ability to secrete TNF- α and IL-12 [88]. MHC II levels, regulated in

part through regulatory factor X-associated protein (RFXAP), are modulated in DCs by pancreatic cancer cell-derived exosomal miR-212-3p targeting of RFXAP [89], highlighting the pluripotent roles these exosomes can have within the TME. Further, pancreatic cancer cells can skew previously M1 macrophages to M2 macrophages; however, this can be reversed by engineering the tumor cells to express miR-155 and miR-125b-2, which are effectively released in exosomes [90]. This innate ability of pancreatic tumor cells to skew macrophages through exosomal miRNAs seems common to many cancer types and highlights a new field of therapeutic study as recently reviewed [78,91].

Within the TME exist myriad types of immune cell types that can vary in their tumorigenic state. Myeloid cells are critical to fight against cancer cells and utilize exosomes just as they do during homeostasis. One way this occurs is through DCs regulating cytotoxic T-cell responses within the TME and DC-derived exosomes prompting T-cell-dependent anti-tumor responses in mice [7,8]. In a recent clinical trial, DC-derived EVs were shown to induce a natural killer (NK) cell response in patients with nonsmall cell lung cancer [92]. Although specific miRNAs have not been identified, candidate identification could lead to effective cell-free anti-tumor therapies, exploiting recent advances in modulating parent cells to hyper-loading exosomes [78,93]. Another population of myeloid cells within the TME are tumor-associated macrophages (TAMs), which control tumor growth, either by promotion of tumor cell killing (M1) or tumor cell proliferation (M2). Not surprisingly, a common mode of communication by both of these cell types is exosomes. miR-16-5p from M1 macrophages can stimulate T cells to kill gastric tumor cells, through PD-L1 down-

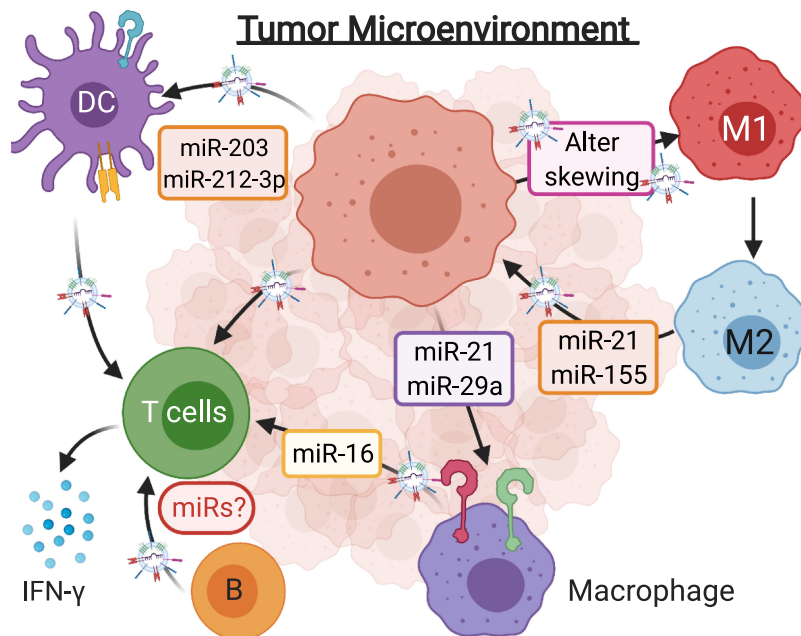


Fig. 6. The dynamic tumor microenvironment consists of exosomal cross-talk between both tumor cells and immune cells. Tumor cells can alter macrophage skewing, stimulate TLRs within macrophages, and alter T-cell responses. Macrophages can regulate the tumor cell itself as well as TME T cells and DC exosomes can regulate both the T cells and tumor cells within the TME.

regulation [94]. M2 macrophage-derived exosomal miR-21 and miR-155 can fine tune BRG1 expression on colon cancer cells, allowing for better cancer cell migration and invasion [95]. The dynamic bifunctionality of these two cell types opens the door for therapeutic intervention, ideally skewing naïve macrophages or repolarization of M2 macrophages to become M1-like and more effectively suppress tumors.

The adaptive immune system is also a powerful force within the TME. B-cell-derived EVs are capable of hydrolyzing tumor cell-produced ATP into adenosine via CD39 and CD73. These EVs can have an effect on CD8⁺ T-cell responses to chemotherapy, promoting an immunosuppressive TME [78,96,97]. The role of B-cell-derived exosomal miRNAs within the TME remains elusive. It will be informative to develop new therapies against these vesicles, as we now know they also play a role in response to cancer drug treatments, as reviewed recently [91]. The dynamic interplay between tumor cell-derived exosomes and immune cell-derived exosomes contributes to the complexity of the tumor microenvironment (Fig. 6). Recent research and development of *in vivo* models to modulate exosome release allow for better-directed inquiry, ideally leading to targetable therapeutic uses for exosomal miRNAs from both immune and cancer cells.

Future directions

Exosomes and their miRNAs are critically important to the progression of many diseases as well as the

immune system's ability to defend against such challenges (summarized in Table 1). Improvements in technologies and unification of techniques within the field have allowed for increased understanding and definition of the vesicle populations responsible for a particular phenotype [98]. In addition to the cell types reviewed here, endothelial cells also seem to be of particular importance, responding rapidly to a diverse population of immune cell exosomes and potentially guiding DC migration through their own exosomes [99], illustrating the increasing complexity of studying *in vivo* vesicle function. The continued interrogation of the miRNAs present in specific immune cell subsets, including B cells and TME DC cells, will allow for a better definition of their basic cellular function as well as their modes of action during challenge, potentially leading to therapeutic applications. In particular, understanding the natural exosomal transfer and distribution of particular miRNAs throughout an organism would also improve therapeutic targeting and thus better precision treatment of diseases. Further insight is also needed to determine how exosomes can bypass the endosomal degradation pathway once inside recipient cells. Excitingly, the new ability to modify exosomes *ex vivo* is an area of research that will continue to be informed by basic biological investigation *in vitro* and *in vivo* [36].

Over the last decade, advances in the exosome field have allowed for a better understanding and characterization of vesicles from a plethora of cell types. There has been a concerted effort to understand the differing

Table 1. Exosomal miRNAs released and received by immune cells.

Origin cell	miRNAs present	Recipient cell	miRNA target	Biological impact	Disease context	Reference
DC	miR-451	DCs	Unknown		Steady state	[30]
	miR-148a					
	miR-155	T cells	Unknown	Activates T cells	Steady state	[37]
	miR-146a miR-155	DCs	IRAK-1 Traf6 SHIP1 BACH1	Regulate response to endotoxin	Endotoxin exposure	[19,26]
M1 macrophage	miR-155	Endothelial cells	Sirt1/AMPKα2 RAC1-PAK2	Reduced angiogenesis, wound healing	Myocardial infarction	[41]
	miR-155	MSCs	BMP signaling	Halt osteoblastic differentiation	Bone regeneration	[42]
M2 macrophage	miR-146b	Macrophages	TNFα and NFκB signaling	Macrophage skewing	Atherosclerosis	[40]
	miR-99a					
	miR-378a	MSCs	BMP signaling	Promote bone regeneration	Bone regeneration	[42]
	miR-21-5p	Tendon cells	Smad7 signaling	Increased fibrogenesis in tendon cells	Tendon Injury	[45]
MSCs	miR-148a	Cardiomyocytes	TXNIP	Wound healing	Myocardial injury	[46]
	miR-let7 family	Macrophages	NFκB signaling, PTEN signaling	Macrophage skewing	Atherosclerosis	[44]
T cell	miR-355	Raji B cells	SOX4	Unknown	Immunological synapse	[47]
	miR-155	B cells	BIM	Modulation of Ig release and survival	Germinal center reaction	[48]
	miR-20		PTEN			
	miR-25					
Treg	miR-142-3p/5p	Pancreatic β cells	NFκB translocation	Apoptosis	Type 1 diabetes development	[49]
	miR-155	Th1 cells	Cox2 and others?	IFNγ secretion and Th1 cell proliferation	Steady state	[53]
	Let-7b Let-7d					
	miR-150	DCs	Unknown	Alter tolerogenic state, cytokine secretion	Steady state	[55]
β cell	miR-29	Monocytes	TRAF3	Cxcl10 and ICAM-1 increased	Diabetes	[50]
B cell	Unknown					
Intestinal epithelial cell	miR-30c	IECs	Unknown	Inhibit autophagy, allow for increased AIEC replication	AIEC colonization	[66]
Mast cell	miR-130a					
	miR-223	IECs	Claudin-8	Decreased tight junction proteins	IBD	[16]
Lung epithelial cell	miR-146a	Monocytes	Unknown	Modulate release of IL-10, suppress Th2 responses	Allergy model	[67]
Brown adipocyte	miR-99b	Hepatocytes	Fgf21	Altered hepatocyte metabolism	Obesity	[28]
Adipocyte	miR-34a	ATMs	Klf4	Regulation of macrophage skewing	Obesity	[76]
ATM	miR-155	Adipocytes	PPARγ	Regulation of insulin sensitivity	Obesity	[73]
	miR-210	Adipocytes	NDURFA4	Regulates glucose tolerance	Obesity	[74]
Synthetic exosomes*	miR-192	Adipocytes	PPAR family	Induces glucose intolerance, inflammation	Obesity	[72]
	miR-122					
	miR-27a-3p					
	miR-27b-3p					
Lung cancer cells	miR-21	Peritoneal Macrophages	TLR7/8 agonists	Promotes pro-meta niche	Lung cancer	[86]
Pancreatic cancer cells	miR-29a	DCs	TLR4	Impact cytokine secretion	Pancreatic cancer	[88]
	miR-203	DCs	MHCII via RFXAP	Promote tumor survival	Pancreatic cancer	[89]
	miR-212-3p	TAMs	Unknown	Macrophage skewing	Pancreatic cancer	[90]
	miR-155					

Table 1. (Continued).

Origin cell	miRNAs present	Recipient cell	miRNA target	Biological impact	Disease context	Reference
TME M1 macs	miR-125b-2 miR-16-5p	T cells	PD-L1	Promote killing gastric tumors	Gastric cancer	[94]
TME M2 macs	miR-21 miR-155	Colon cancer cells	BRG1	Promote migration and invasion	Colon cancer	[95]

*Manufactured exosomes.

RNA, protein, and lipid content of vesicles, with the creation of databases to catalog discoveries [100]. With increasingly better technology and unified techniques, the EV field will be able to solidify the role of many of these exosomal miRNAs within the immune system by building off of solid *in vitro* experiments. Until recently, few tools have existed to track the *in vivo* distribution of exosomes, but with the advent of mouse strains that have switchable fluorescent cell membranes [68] and zebrafish that have been genetically engineered to have trackable exosomes [101], investigators have exciting new tools to understand basic *in vivo* exosome cell biology. *In vitro* analysis of exosomal miRNAs has allowed for an in-depth understanding of what miRNAs can be packaged into exosomes. Potential mechanisms for sorting of RNA species, via sequence specificity and/or RNA-binding proteins, such as hnRNPA2B1 [102] and YBX1 [103], are just beginning to be explored [36]. Rarely do *in vivo* models exist to test the function of these miRNAs within the organism, due either to the lack of conditional knockouts for the miRNA of interest or inability to test prevention of exosome release. Critical to advancing the field will be mice that can block exosome release from specific cells, similar to the B-cell knockouts of Rab27A as developed by Zhang *et al.* [96], as well as tools to deplete exosomal miRNAs of interest in specific cell types, potentially by manipulating their loading into exosomes by aforementioned RNA-binding proteins [102,103]. Additionally, characterization of contents at the single vesicle level has been a goal of the field for some time. The capacity of utilizing flow cytometry, both novel semi-conductor based or imaging based for vesicle characterization, has increased in recent years with the advent of instruments that can be modified to detect these small particles and the capacity to see distinct populations of EVs [104,105], but there is still a need to be able to reliably sort purified specific populations from *in vitro* and *in vivo* sources.

Vesicles are not only released by mammalian cells but also both eukaryotic and prokaryotic organisms that may be present as commensals or disease-causing pathogens, and this is an important consideration when studying vesicle function *in vivo*. Members of the gut microbiota, including bacteria and fungi, are capable of releasing outer membrane vesicles (OMVs) similar in size to those from the host, that can have effects on the immune system [106,107]. Additionally, we know that viral RNAs, including viral miRNAs, are capable of hijacking the host system and escaping from infected immune cells in vesicles [59]. Plants are also capable of releasing vesicles, and this is particularly important when thinking about the food that we consume and the gut microenvironment, as this can trigger a cascade that alters the gut immune system [108]. Finally, parasites and pathogens can clearly communicate via vesicles, which can have functional effects on the host. *Plasmodium falciparum*, a causative parasite of human malaria, is able to communicate via vesicles transferring drug resistance plasmids and potentially regulating gametocytogenesis through vesicles [109]. Altogether, this highlights the importance of understanding vesicles, not only from mammalian cells but across kingdoms, as we strive to understand how this unique form of cellular communication contributes to human health and disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

KMB, JLR, and RMO wrote and edited the manuscript.

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