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Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF- κ B

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Growing evidence links tumor progression with chronic inflammatory processes and dysregulated activity of various immune cells. In this study, we demonstrate that various types of macrophages internalize microvesicles, called exosomes, secreted by breast cancer and non-cancerous cell lines. Although both types of exosomes targeted macrophages, only cancer-derived exosomes stimulated NF- κ B activation in macrophages resulting in secretion of pro-inflammatory cytokines such as IL-6, TNF α , GCSF, and CCL2. *In vivo* mouse experiments confirmed that intravenously injected exosomes are efficiently internalized by macrophages in the lung and brain, which correlated with upregulation of inflammatory cytokines. In mice bearing xenografted human breast cancers, tumor-derived exosomes were internalized by macrophages in axillary lymph nodes thereby triggering expression of IL-6. Genetic ablation of Toll-like receptor 2 (TLR2) or MyD88, a critical signaling adaptor in the NF- κ B pathway, completely abolished the effect of tumor-derived exosomes. In contrast, inhibition of TLR4 or endosomal TLRs (TLR3/7/8/9) failed to abrogate NF- κ B activation by exosomes. We further found that palmitoylated proteins present on the surface of tumor-secreted exosomes contributed to NF- κ B activation. Thus, our results highlight a novel mechanism used by breast cancer cells to induce pro-inflammatory activity of distant macrophages through circulating exosomal vesicles secreted during cancer progression.

The dualistic role of the immune system in preventing and supporting tumor progression has been recognized in the past two decades. Immune responses against tumor antigens may inhibit tumor progression, but at the same time, inflammation generated by immune cells has been shown to enhance tumor growth and metastases¹. Along with the influence on the development and growth of primary tumors², inflammation plays a major role in preparing distant sites in the body for colonization by metastatic tumor cells³. While the presence of immune cells was originally thought to represent an attempt by the immune system to eradicate tumors, recent findings support the role of inflammation in promoting tumor growth by providing a host of molecules including growth factors, survival factors, pro-angiogenic factors, and extracellular matrix-modifying enzymes, all of which serve to adapt the microenvironment for tumor proliferation⁴.

Macrophages are among the most abundant of innate immune cell types that function in the primary tumor and at metastatic sites. As phagocytes, macrophages serve to recognize, ingest, and destroy host invaders. Equally important is their ability to secrete cytokines and chemokines to recruit other immune cells and to orchestrate an effective pathogen-eliminating response⁵. Tumor-associated macrophages (TAMs) are well known for their tumor-promoting functions such as supporting tumor angiogenesis, invasion, matrix remodeling, as well as



immune evasion^{4,6,7}. In fact, for breast cancer patients, increased density of TAMs correlates with poor prognosis⁸. Macrophages are recruited to tumor sites from circulation in response to tumor-secreted chemoattractants leading to their accumulation in hypoxic, necrotic regions of the solid tumor⁹.

Recent studies suggested that macrophages and other myeloid immune cells play a role in preparing pre-metastatic sites for tumor cell colonization. In a Lewis lung carcinoma model, a tumor-derived extracellular protein, veriscan, stimulated myeloid cells at distant sites to promote metastases¹⁰. The S1PR1-STAT3 signaling axis was shown to be activated in both tumor cells as well as myeloid cells at a pre-metastatic niche, thus promoting metastatic development¹¹. These studies highlighted the importance of intercellular communication between tumor cells and myeloid cells at a distant site to establish the pre-metastatic niche^{12–14}. In this study, we set out to characterize the modulation of macrophage activity by breast cancer cells mediated by cancer-secreted exosomes.

A large variety of different types of cells, including cancer cells, secrete significant amounts of small vesicles (40–100 nm) known as exosomes following fusion of multivesicular endosomal membranes with the cell surface¹⁵. The exosomes carry a variety of proteins, mRNAs, and small RNAs, all of which may functionally alter recipient cells that interact with exosomes. Exosomes have long been appreciated as mediators of intercellular communication, first as B cell-derived stimulators of an immune response by T-lymphocytes and more recently, as encapsulators of protein expression-modifying RNA species¹⁶. Accumulating evidence demonstrates that the contents of cancer-secreted exosomes can be transferred to other cell types in the primary tumor microenvironment and pre-metastatic niches to modulate cell function and facilitate tumor progression^{17–26}. Therefore, cancer-secreted exosomes and their molecular contents have emerged as a highly important new group of biomarkers and potential therapeutic targets for cancer.

Here, we demonstrate that breast cancer-derived exosomes are capable of inducing an inflammatory response in macrophages, which may ultimately contribute to metastatic tumor development. The cross-talk between tumor cells and macrophages required TLR2 expression on the surface of macrophages and was influenced by the presence of palmitoylated protein ligands on the surface of tumor-derived exosomes. These findings suggest an important mechanism used by cancer cells to modulate the activity of immune cells and promote metastasis.

Results

Breast cancer-derived exosomes induce NF- κ B activation in macrophages. We chose the MDA-MB-231 and MCF7 breast cancer cell lines and the MCF10A non-cancerous mammary epithelial cell line to compare the effect of exosomes from a cancerous or normal origin. Exosomes were collected from cell culture supernatants and characterized by electron microscopy (Fig. 1a), Western blot analysis (Fig. 1b), and flow cytometry (Supplementary Fig. S1a). The purity of our exosome preparations was assessed in electron micrographs showing membrane vesicles of appropriate size (50–100 nm) and a prototypical cup-like morphology (see inset for MCF10A, Fig. 1a). Expression of CD63, a characteristic protein marker of exosomes, was confirmed by Western blots of detergent solubilized exosome preparations (Fig. 1b) and by flow cytometric analysis of bead-captured exosomes (Supplementary Fig. S1a). To determine if macrophages might be targets of mammary epithelial cell-derived exosomes, a lipid-associating fluorescent dye, DiI, was used to label exosome preparations, which were later incubated with RAW264.7 macrophages. Flow cytometric analysis showed that all three types of exosomes were internalized by the vast majority (>90%) of Raw macrophages (Fig. 1c).

As a critical cell type in innate immunity, macrophages are well known for their ability to induce inflammation. Given the important link between inflammation and cancer progression, we sought to determine if cancer-derived exosomes might influence the inflammatory response in macrophages. We focused our study on NF- κ B, a critical transcription factor family involved in regulating inflammation, by making use of Raw cells stably expressing a luciferase reporter driven by a minimal murine *c-fos* promoter with two I κ B NF- κ B motifs upstream (RawkB cells). RawkB cells incubated in the presence of exosomes isolated from either the cancerous MDA-MB-231 cells (MDA-231 or 231) or MCF7 cells showed robust activation of the NF- κ B pathway as indicated by more than 60-fold induction of luciferase (Fig. 1d). Remarkably, administration of an equal amount of exosomes isolated from the non-cancerous MCF10A cells (10A) resulted in only two-fold induction of luciferase activity compared to untreated RawkB cells. The difference in exosome stimulatory capacity is not due to cell culture medium conditions as non-conditioned media processed similarly for exosome harvesting did not induce a response (Supplementary Fig. S1b). Analysis of key proteins in the NF- κ B signal transduction cascade showed that cancer exosome treatment activated the classical NF- κ B pathway. The incubation of RawkB cells with cancer exosomes induced phosphorylation of NF- κ B, IKK α /b heterodimers and I κ B α in a similar manner as in response to lipopolysaccharide (LPS) used as a positive control. In addition, levels of I κ B α in cancer exosome-treated macrophages were strongly reduced indicating NF- κ B activation (Fig. 1e). In summary, while both cancer-derived exosomes and non-cancerous exosomes are capable of associating with macrophages, only cancer-derived exosomes stimulate NF- κ B activation.

Cancer-derived exosomes stimulate inflammatory cytokine production in macrophages. To assess which targets of NF- κ B are expressed in macrophages following cancer exosome treatment, an inflammatory cytokine array was used to identify cytokines secreted into Raw cell culture supernatants following exosome treatment. Dramatic production of GCSF, IL-6, CCL2 (MCP1), and TNF α was induced by MDA-231- and MCF7-derived exosomes but not exosomes from non-malignant MCF10A cells (Fig. 2a). The array results were confirmed by quantitative RT-PCR for Raw cells (Fig. 2b), and extended to other macrophage types, including human THP-1 macrophages (Fig. 2c and Supplementary Fig. S2a), primary bone marrow-derived macrophages (Fig. 2d), and primary Kupffer cells (Supplementary Fig. S2b). In order to definitively link cancer exosome-induced NF- κ B activation to inflammatory cytokine production, an NF- κ B pathway inhibitor, Bay 11-7082, was used to pretreat Raw cells prior to MDA-231 exosome administration. RT-PCR analysis of IL-6 and TNF α mRNA levels demonstrated almost complete inhibition of cytokine expression (Fig. 2e), indicating that cancer exosome-induced inflammatory cytokine production occurred through activation of the NF- κ B signaling pathway.

Cancer-derived exosomes stimulate tissue resident macrophages in vivo. To validate our findings *in vivo*, we administered exosomes to immunocompromised mice. Exosomes were collected from MDA-231 and MCF10A cells, fluorescently labeled using DiI, and injected intravenously through the tail vein into nude mice. Following three weeks of twice weekly injections, we examined lung and brain tissues as these sites are known to frequently house metastases in breast cancer patients. Macrophages associating with DiI⁺ exosomes could be detected in dissociated lung cells by flow cytometry following staining with anti-F4/80 (Fig. 3a). The F4/80⁺/DiI⁺ lung macrophages from mice administered MDA-231 or MCF10A fluorescent exosomes were sorted and differences in inflammatory cytokine gene expression between cell populations was queried by quantitative RT-PCR. On average, the levels of TNF α mRNA were two-fold higher in lung macrophages that internalized exosomes derived from MDA-231 cancer cells

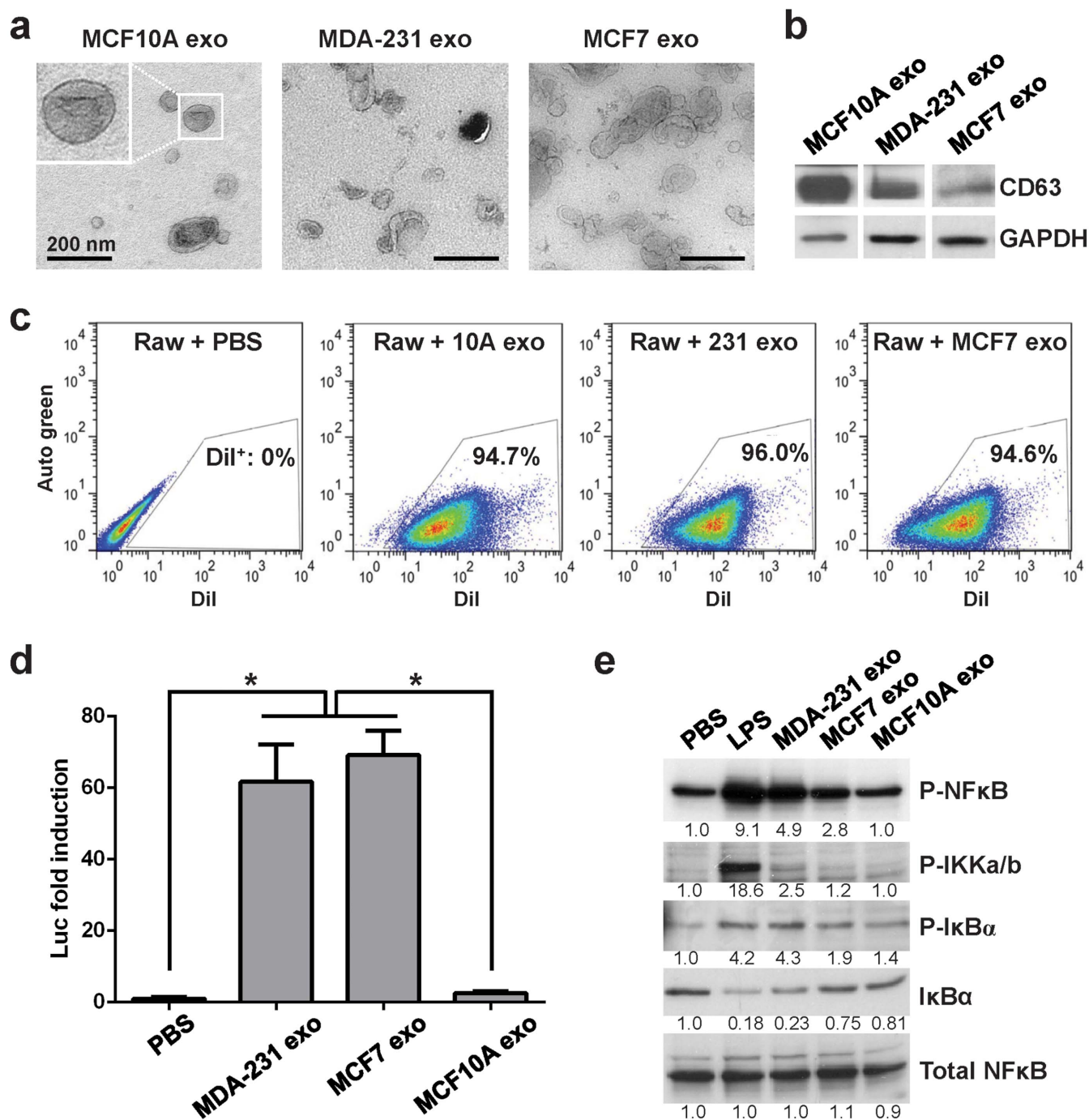


Figure 1 | Macrophages activate NF- κ B in response to breast cancer-derived exosomes. (a) Representative EM images of exosomes derived from MCF10A, MDA-MB-231 (abbreviated to MDA-231 or 231 in Figures), and MCF7 cells. Inset in MCF10A panel shows enhanced detail of cup-like morphology. Scale bar: 200 nm. (b) Western blot of indicated exosomes (exo) for CD63. Membranes/blots are cropped by molecular weights to show the proteins of interest. Samples from the three cell lines were run separately as the protein abundance was not compared to each other. The gels were run under the same experimental conditions. The original whole blot pictures are available in Supplementary Fig. S3a. (c) Raw 264.7 cells incubated with DiI-labeled exosomes derived from indicated cell types were analyzed by flow cytometry. Accumulation of DiI fluorescence, indicated along the X-axis with DiI⁺ percentage noted, reflects association of exosomes with Raw macrophages. (d) Luciferase fold induction of Raw cells fed exosomes from indicated cell types, reflecting NF- κ B activation. * $p < 0.001$. (e) Western blot of Raw cells incubated with LPS or with exosomes from cell types as specified. Treatments using exosomes were carried out for 20 min after which cells were lysed and Western blotted using indicated antibodies reflecting NF- κ B activation status. The fold difference in band intensities was quantified and indicated under the image. Membranes/blots are cropped by molecular weights to show the proteins of interest. The gels were run under the same experimental conditions. The original whole blot pictures are available in Supplementary Fig. S3b.

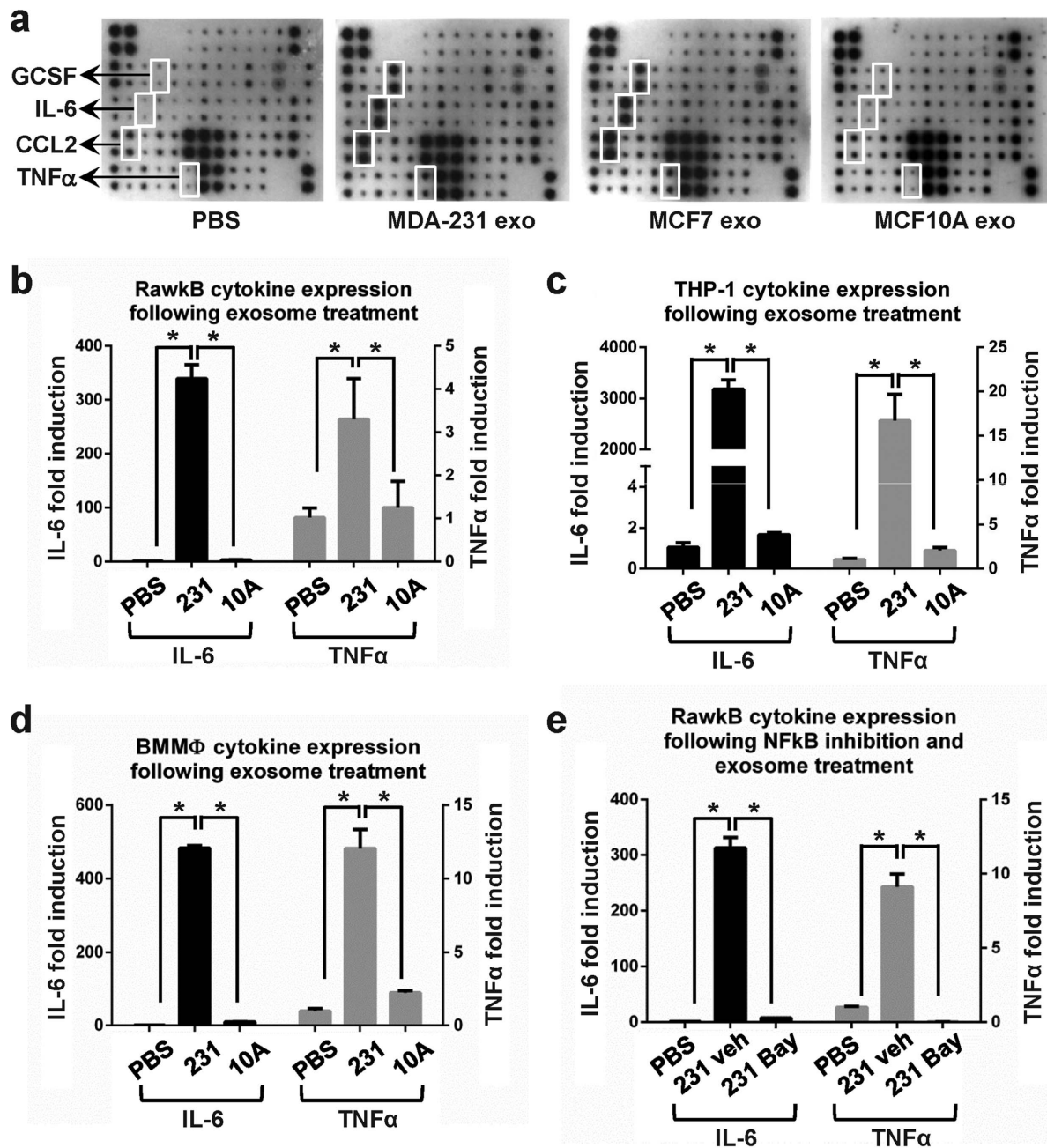


Figure 2 | Macrophages secrete inflammatory cytokines in response to breast-cancer derived exosomes *in vitro*. (a) Cytokine array identifies IL-6, CCL2, TNF α , and GCSF as the inflammatory cytokines whose expression is most highly induced following exposure to MDA-231 and MCF7 exosomes, but not MCF10A exosomes. The original full-length blots are shown. (b–d) Quantitative RT-PCR for IL-6 and TNF α in (b) RawkB cells, (c) THP-1 cells, and (d) bone marrow derived macrophages following administration of exosomes derived from MDA-231 (231) and MCF10A (10A) cells. (e) Quantitative RT-PCR for IL-6 and TNF α in RawkB cells pre-treated with 40 μ M Bay 11-7082 for 1 h followed by stimulation with 231 exosomes for 6 h. * $p < 0.001$.

compared to DiI⁻ macrophages or lung macrophages from mice receiving control MCF10A exosomes (Fig. 3b). Brain tissue sections from mice receiving fluorescent MDA-231 and MCF10A exosome injections were also analyzed by immunofluorescence. Staining with antibodies against F4/80 and IL-6 revealed populations of macrophages in the brain from MDA-231 exosome-treated mice that were exosome-associated and also expressing IL-6 (Fig. 3c).

As an alternative strategy to explore tumor exosome-induced inflammation, we examined endogenously produced exosomes from a mammary fat pad xenografted MDA-231 primary tumor. Since MDA-231 is a human breast cancer implanted into a murine host, a species-specific anti-human CD63 antibody was used to detect

tumor-derived exosomes internalized by CD68⁺ macrophages. Immunofluorescence imaging of axillary lymph node tissue sections identified populations of mouse CD68⁺ macrophages that internalized human CD63⁺ exosomes, which seemed to correlate with expression of IL-6. In contrast, CD68⁺ macrophages that did not accumulate tumor-derived exosomes were negative for IL-6 (Fig. 3d). Together, these findings demonstrate that an *in vivo* inflammatory response in tissue resident macrophages stimulated by tumor-derived exosomes could be produced similar to our *in vitro* results.

Proteins on the exosome surface stimulate the macrophage inflammatory response. Scientific interest in exosomes has exploded

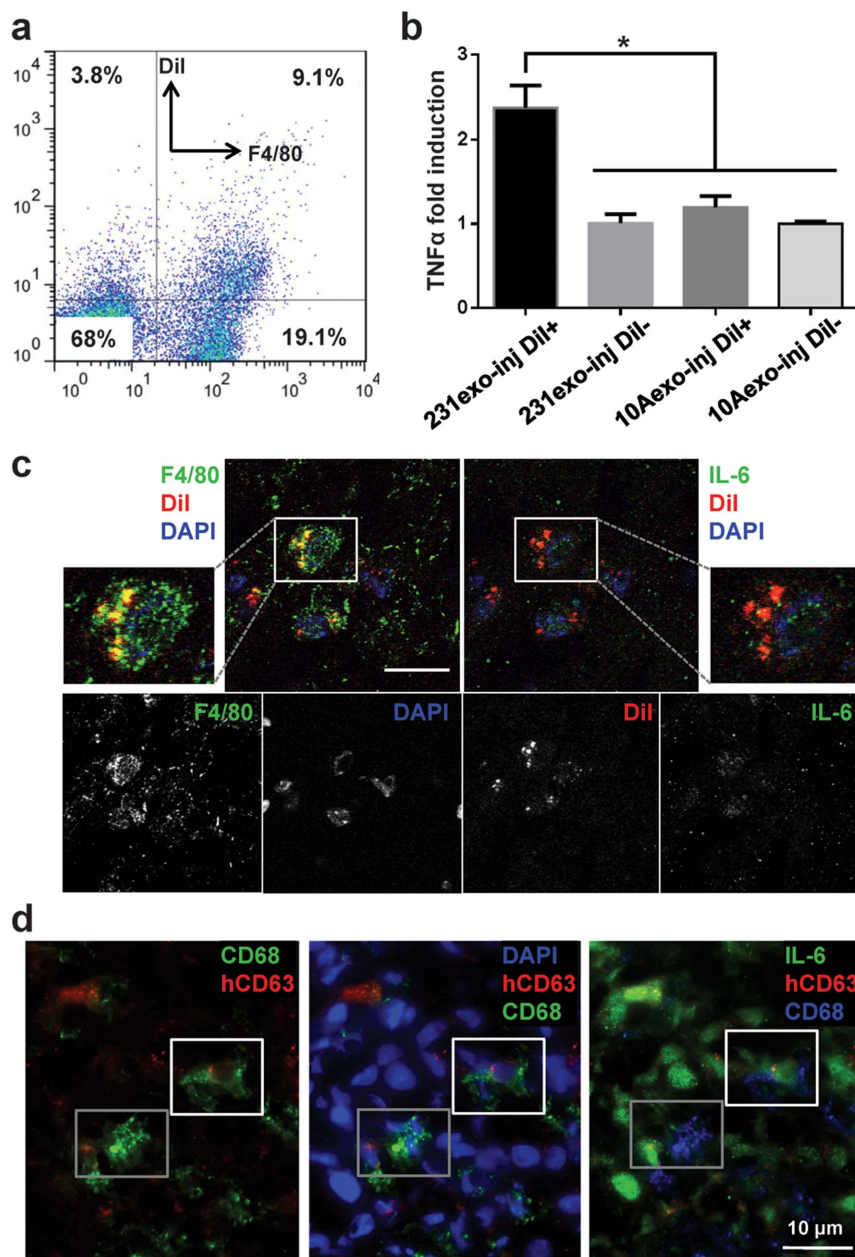


Figure 3 | Macrophages secrete inflammatory cytokines in response to breast-cancer derived exosomes *in vivo*. (a–b) DiI-labeled MDA-231 exosomes administered intravenously associate with lung macrophages and induce inflammatory cytokine production. Following tail-vein injection of DiI-labeled exosomes, lung tissue was harvested and dissociated. (a) Flow cytometric analysis following staining with anti-F4/80 antibody revealed a population of lung macrophages associating with MDA-231 exosomes (top right quadrant). (b) Quantitative RT-PCR for TNF α was carried out using RNA isolated from F4/80⁺ cells sorted on DiI^{+/+} fluorescence. * $p < 0.001$. (c) Representative immunofluorescence imaging of brain tissue from mice administered DiI-labeled MDA-231 exosomes. Left color-merged image shows F4/80⁺ macrophages in green, DAPI in blue, and DiI exosomes in red. Scale bar: 5 μ m. Insets show a higher magnification. Right color-merged image shows IL-6 in green, DAPI in blue, and DiI exosomes in red. Grayscale of each color channel is shown in bottom panels. (d) Representative immunofluorescence imaging of axillary lymph node tissue from mice with mammary fat pad xenograft MDA-231 primary tumor. White box highlights CD68⁺ macrophage with associated human CD63⁺ exosomes, which is expressing IL-6 (green, right panel). Grey box highlights CD68⁺ macrophage without associated exosomes and not expressing IL-6. Scale bar: 10 μ m.

recently following the discovery that their mRNA and miRNA contents are capable of mediating intercellular communication. We therefore sought to differentiate possible mechanisms mediated by exosomal RNA or proteins for the stimulation of macrophages by cancer-secreted exosomes. A kinetic study was carried out to determine the time course of peak macrophage stimulation by tumor exosomes. MDA-231 exosomes were most highly stimulatory 4 h after administration to macrophages (Fig. 4a). Such relatively rapid macrophage stimulation pattern likely suggests receptor-mediated

signaling rather than a miRNA-mediated effect also implicated in the effect of some tumor-derived exosomes²⁷. To test this possibility in greater detail, we sought to distinguish between proteins on the exosomal surface and proteins enclosed within exosomes. Towards this end, MDA-231 exosomes were either sonicated or left intact and subsequently treated with proteinase K to eliminate surface proteins prior to stimulation of Raw264 cells. For intact exosomes, the luciferase activity indicating NF- κ B activation was decreased by one third following proteinase K treatment. The NF- κ B-activating

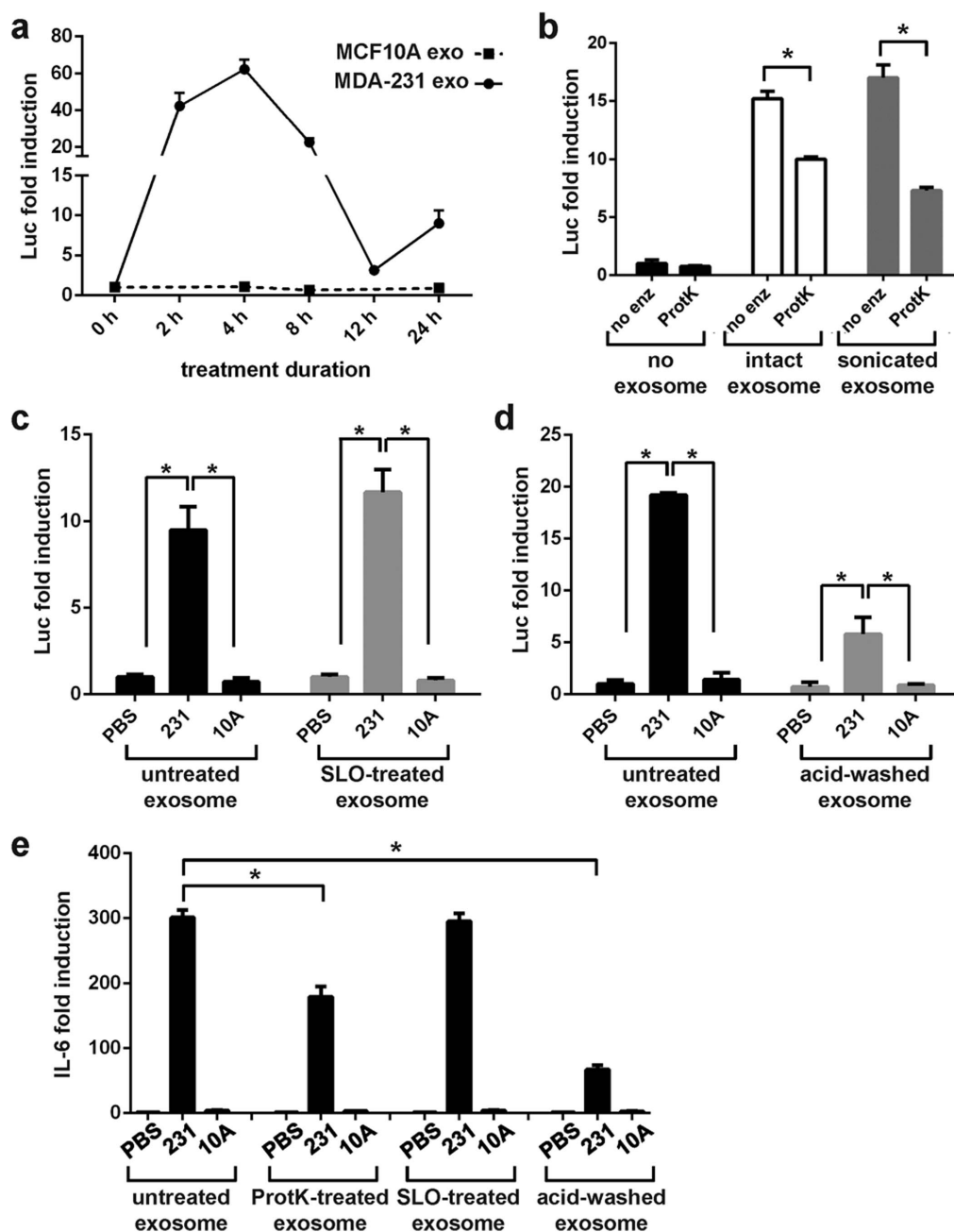


Figure 4 | Stimulation of macrophages is mediated by proteins on the surface of cancer-derived exosomes. (a) RawkB cells were fed MDA-231 (231) or MCF10A (10A) exosomes for indicated durations followed by assessment of luciferase activity. (b) 100 $\mu\text{g}/\text{mL}$ of proteinase K (ProtK) was used to treat intact or sonicated exosomes for 1 h at 37°C. Inactivation of ProtK was carried out by treatment with 5 mM PMSF. Exosome samples were used to treat RawkB cells for 4 h followed by assessment of luciferase activity. (c) Exosomes were incubated with 50 U of DTT-activated SLO on ice, washed with PBS, and used to treat RawkB cells for 4 h followed by assessment of luciferase activity. (d) Exosomes were treated with 100 mM glycine, pH 2.5, neutralized with 2 M Tris, pH 8.0, and used to treat RawkB cells for 4 h followed by assessment of luciferase activity. (e) IL-6 expression levels were determined by quantitative RT-PCR in RawkB cells treated with exosomes as indicated. * $p < 0.001$.

potential of sonicated exosomes was not significantly different from that of the intact exosomes, indicating that internal proteins do not contribute significantly to macrophage inflammation induction (Fig. 4b). To further distinguish between molecules on the exosome outer surface and those contained within, exosomes were treated with streptolysin O (SLO), a membrane-damaging extracellular toxin. MDA-231 exosomes treated with SLO to release all of the exosomal contents (and subsequently washed to remove released components) were as capable as untreated exosomes at stimulating macrophage inflammation (Fig. 4c). The content of exosomes, therefore, may be unnecessary for activating inflammatory cytokine

production by macrophages. The importance of exosomal surface-associated proteins was confirmed by acid washing exosomes to remove exosomal surface-associated proteins. Compared with untreated exosomes, the acid-washed exosomes had reduced, by about 75%, ability to stimulate macrophages (Fig. 4d). Consistent with the luciferase data, induction of IL-6 expression by MDA-231 exosomes was also partially repressed by proteinase K or acid treatment but not SLO treatment of exosomes (Fig. 4e). These findings suggest that proteins associated with the exosome surface are largely responsible for the inflammation induction observed in macrophages.



Cancer exosomes stimulate inflammatory cytokine production through TLR2 on macrophages. Central to the function of macrophages in infection and immunity is their ability to detect pathogens and to respond by initiating an inflammatory response. Initial sensing of pathogenic components is mediated by receptors on macrophages, which include the family of Toll-like receptors (TLRs). Since the response induced by cancer-derived exosomes is very similar in nature to an inflammatory response to pathogens, we investigated if exosomal stimulation might occur via similar receptors. To test whether exosomes stimulate through TLRs, bone marrow-derived macrophages were generated from mice deficient in MyD88, a common signaling adaptor for many of the different TLRs. When administered MDA-231 exosomes, MyD88^{-/-} macrophages were unable to produce the inflammatory cytokines IL-6 or TNF α (Fig. 5a–b). To determine which TLRs were specifically responsible for engaging cancer exosomes resulting in inflammatory cytokine production, we first tested macrophages derived from mice lacking both TLR2 and TLR4. Like the MyD88^{-/-} macrophages, the TLR2/4^{-/-} macrophages were also incapable of producing IL-6 and TNF α in response to cancer exosomes (Fig. 5a–b). Chloroquine treatment of C57BL/6-derived macrophages to inhibit endosomal TLRs, such as TLR3/7/8/9, failed to abrogate NF- κ B activation by exosomes (Fig. 5c). We next tested macrophages deficient in a single TLR. Although TLR4^{-/-} macrophages retained the capacity to express inflammatory cytokines in response to cancer exosomes, the inflammatory cytokine production was completely abrogated in TLR2^{-/-} macrophages (Fig. 5a–b). Using a TLR2 neutralizing antibody, we further confirmed that blockade of TLR2 in Raw macrophages completely abolished NF- κ B activation by cancer-secreted exosomes (Fig. 5d). These findings suggest that TLR2 on macrophages engages proteins on the surface of tumor exosomes, resulting in an inflammatory response by the stimulated macrophages.

Palmitoylated proteins on the cancer exosome surface bind to TLR2 contributing to NF- κ B stimulation. While TLR2 can be highly promiscuous in its binding of ligands, a common feature of pathogenic molecules that engage the receptor is the presence of a lipid modification. To test the general requirement for a thioester containing lipoprotein, we treated exosomes with 1% H₂O₂ to convert the N-terminal cysteine-thioester substructure into a TLR2-inactive sulfoxide derivative. Thoroughly washed following H₂O₂ treatment, treated exosomes could stimulate macrophages only 1/3 as effectively as untreated exosomes (Fig. 6a). To assess the type of lipid modification more specifically, hydroxylamine (HA) was used to cleave the acyl group on palmitoylated proteins. Thoroughly washed following HA treatment, exosomes lost about half of their NF- κ B-stimulatory capability as compared to vehicle-treated exosomes (Fig. 6b). The role of palmitoylation in macrophage stimulation was further tested by treating MDA-231 cells with a palmitoylation inhibiting agent, 2-bromopalmitate (2-BP), and then collecting exosomes to treat Raw264.7 cells. Exosomes derived from 2-BP-treated cells were reduced in their ability to stimulate macrophages by about 1/3 (Fig. 6c). Therefore, our results indicate that palmitoylated proteins located on the surface of cancer-secreted exosomes contribute to the exosome-induced, TLR2-dependent activation of NF- κ B.

Discussion

The “seed and soil” hypothesis, first set forth by Steven Paget²⁸, transformed the existing paradigm, shifting the focus of metastatic tumor development causality from intrinsic attributes of the tumor itself (the seed) to how hospitable a distant site (the soil) might be. These distant sites, or pre-metastatic niches, must evolve to become conducive to tumor cell engraftment and proliferation³. Cancer-derived adaptations of pre-metastatic niches often include one of

the enabling hallmarks of cancer: tumor-promoting inflammation²⁹. This inflammatory response is generated by immune cells following direct or indirect stimulation by cancer cells, and one mechanism could be through the action of tumor-derived exosomes. Here, we demonstrate that breast cancer-derived exosomes are capable of inducing an inflammatory response in macrophages, which may ultimately result in an enhanced rate of metastatic tumor development.

Our results are partially consistent with a previous study showing that body fluid exosomes trigger NF- κ B signaling and promote secretion of inflammatory cytokines in monocytic cells³⁰. However, unlike the reported data that exosomes of both cancerous and non-cancerous origins can induce these effects and that both TLR2 and TLR4 are required for mediating the monocytic response to exosomes, we observe that only exosomes secreted by breast cancer cell lines (MDA-MB-231 and MCF7) but not the non-cancerous MCF10A cells are capable of stimulating macrophages, and that genetic ablation of TLR2 but not TLR4 in primary bone marrow-derived macrophages abolishes exosomes’ effect. These discrepancies could be due to the different sources of exosomes and types of recipient cells used in the two studies, and may reflect a cell type- or tissue-specific manner of the exosome action. TLR2 has been implicated in the response of various types of immune cells to tumor-derived factors, including exosomes. It was recently reported that membrane-associated Hsp72 from tumor-derived exosomes triggered STAT3 activation in myeloid-derived suppressor cells in a TLR2/MyD88-dependent manner through autocrine production of IL-6, contributing to suppressed tumor immune surveillance²⁷. Cancer-secreted veriscan activates TLR2:TLR6 complexes to induce TNF α secretion by myeloid cells, therefore enhancing metastatic tumor growth¹⁰. In addition, a non-inflammatory role of TLR2 expressed in cancer cells has been documented, which contributes to the self-renewal of cancer stem cells^{31,32}. These results, along with our findings herein, provide evidence against the controversial use of TLR2 agonists in anti-tumor therapy.

TLR2 recognizes a structurally broad range of agonists, among which the lipoproteins/lipopeptides are sensed at physiological picomolar concentrations by TLR2³³. Our results show that the TLR2-dependent, macrophage-stimulating effect of cancer-derived exosomes is at least partially mediated by palmitoylated proteins on the exosome surface (Fig. 6a–c). To identify which palmitoylated protein(s) might be most important for macrophage stimulation, we performed an acyl-biotin exchange followed by a pull-down with streptavidin beads. The biotin-enriched sample was submitted for LCMS/MS analysis and a group of proteins were identified as being palmitoylated in MDA-231 cells (Fig. 6d and Table S1). Some of these proteins that are also present in palmitoylated form on MDA-231-secreted exosomes, especially those expressed or palmitoylated at lower levels in MCF10A, may mediate NF- κ B activation in macrophages through direct binding to TLR2. Towards this end, we selected CD44, NRAS, and TFR1 (transferrin receptor protein 1) for further examination as these proteins have been previously related to cancer. Although all three proteins could be detected on MDA-231 exosomes at higher levels compared to MCF10A exosomes by Western blot, knockdown of each of the genes in MDA-231 cells by RNA interference did not affect the ability of MDA-231-secreted exosomes to stimulate NF- κ B in recipient macrophages (data not shown). It is therefore possible that multiple palmitoylated proteins on the exosome surface contribute to the activation of TLR2 and thereby, NF- κ B and inflammatory cytokine expression in macrophages. In addition to exosomal proteins, cancer-derived exosomal miRNAs are reported to bind as ligands to TLRs in surrounding immune cells³⁴. As such, targeting the process of protein palmitoylation in cancer cells, interfering with exosome interaction with macrophages, and inhibiting NF- κ B activity in macrophages might be effective approaches to therapeutically block

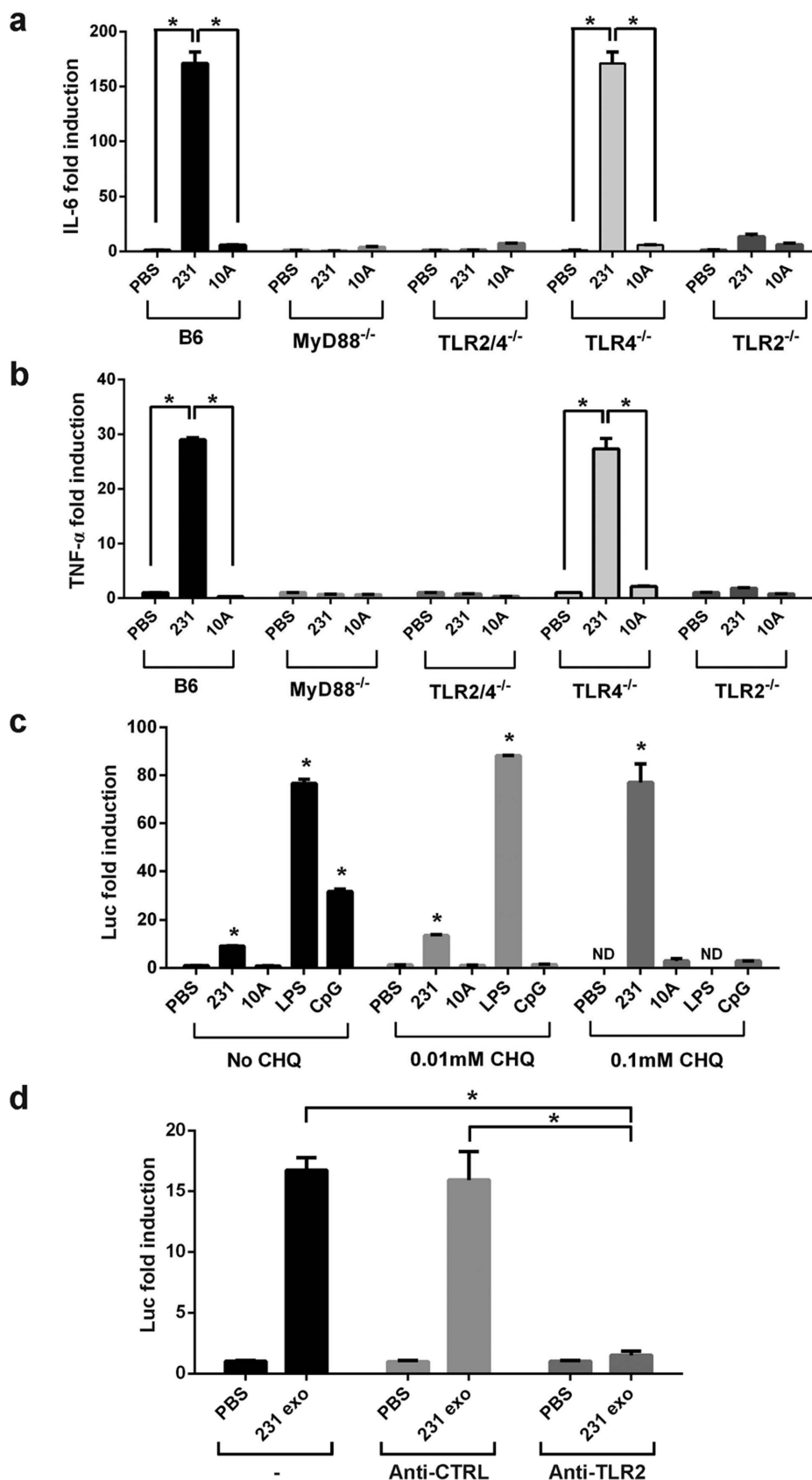


Figure 5 | Stimulation by cancer-derived exosomes occurs through TLR2 on macrophages. MDA-231 (231) or MCF10A (10A) exosomes were fed to bone marrow-derived macrophages generated from indicated mouse strains. (a) IL-6 and (b) TNF α expression levels were determined by quantitative RT-PCR. * $p < 0.001$. (c) Endosomally localized TLRs in RawkB cells were inhibited by a 2-h pretreatment with indicated concentrations of Chloroquine. Stimulation was subsequently carried out by feeding cells with exosomes or TLR ligands for 4 h followed by assessment of luciferase activity. * $p < 0.001$ compared to the PBS (control) group. (d) RawkB cells were treated with MDA-231 exosomes or PBS in the presence of a TLR2 neutralizing antibody or a control antibody at a final concentration of 30 ng/ml. Luciferase activity was assessed and compared to the PBS group. * $p < 0.001$.

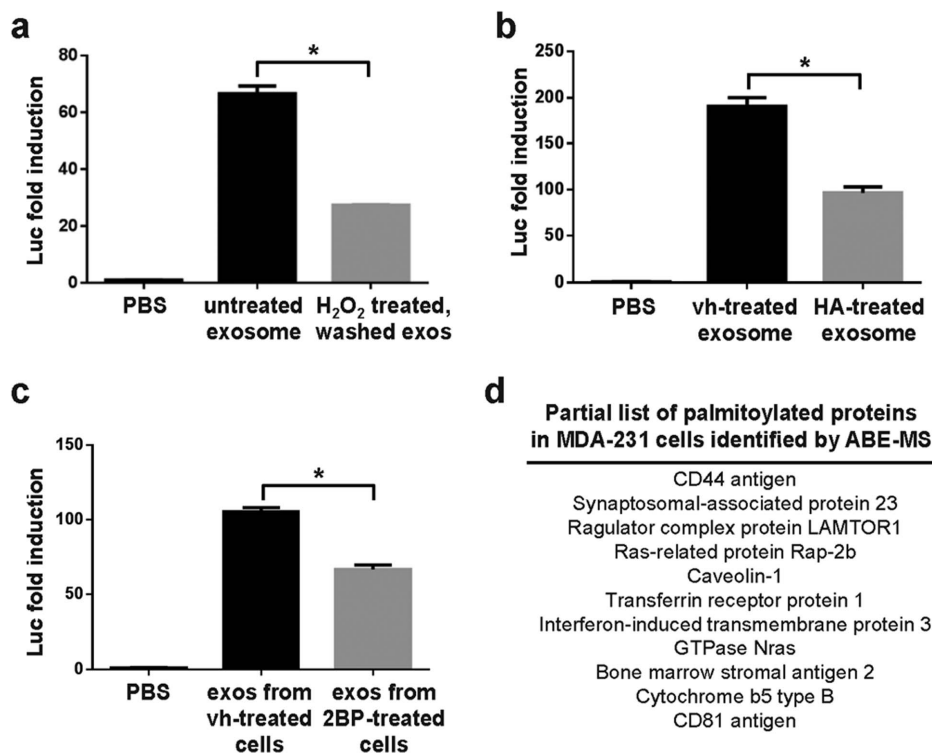


Figure 6 | Palmitoylated proteins on the surface of cancer-derived exosomes contribute to stimulation of macrophages. (a) Exosomes isolated from MDA-231 cells were treated with 1% H₂O₂ for 4 h at room temperature to oxidize lipoprotein thioesters to sulfoxide and subsequently washed with PBS to eliminate residual H₂O₂. Treated and non-treated exosomes were administered to RawkB cells for 4 h followed by assessment of luciferase activity. (b) Exosomes isolated from MDA-231 cells were treated with 1 M hydroxylamine (HA) for 1 h at room temperature to remove S-acyl groups and subsequently washed with PBS to eliminate residual HA. HA-treated and vehicle-treated exosomes were administered to RawkB cells for 4 h followed by assessment of luciferase activity. (c) Exosomes isolated from MDA-231 cells treated with 2-bromopalmitate (2BP) were administered to RawkB cells for 4 h followed by assessment of luciferase activity. (d) Identification of palmitoylated membrane proteins in MDA-231 and MCF10A cells was carried out by isolating the membrane fraction from 5×10^7 cells and performing an acyl-biotin exchange using HPDP-biotin. Streptavidin-conjugated magnetic beads were used to enrich the biotinylated (previously palmitoylated) proteins. Washed beads were reduced and alkylated, allowing for detachment of proteins from the beads, followed by trypsin digestion and HPLC-MS. *p < 0.001.

exosome-mediated activation of macrophages and the consequent pro-metastatic adaptation of tumor microenvironments.

Methods

Cells, plasmids and viruses. Human cancer cell lines, MDA-MB-231 and MCF7, and the non-cancerous cell line, MCF10A, were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in the recommended media in a humidified 5% CO₂ incubator at 37°C. RawkB cells were generously provided by Dr. Ruslan Medzhitov (Yale University)³⁵ and cultured in media identical to that recommended for traditional Raw 264.7 cells. THP-1 cells were obtained from ATCC, cultured according to supplier's instructions, and differentiated into macrophages by treatment with 5 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h¹⁰. Bone marrow-derived macrophages were generated as previously described³⁶ and used on day 6 or 7 of culture. MyD88^{-/-} and TLR2/4^{-/-} bone marrow was generously provided by Dr. Chandrashekar Pasare (University of Texas Southwestern Medical Center, Dallas, TX). TLR2^{-/-} bone marrow was generously provided by Dr. Gregory Barton (University of California, Berkeley, CA). Kupffer cells were isolated as previously described³⁷.

Exosome purification and characterization. Conditioned media was obtained from cancerous and non-cancerous mammary epithelial cell lines grown at sub-confluence for 48 h in growth media containing serum depleted of bovine exosomes (prepared by ultra-centrifugation at $100,000 \times g$ at 4°C for 16 h). Exosomes were prepared as previously described³⁸. For fluorescence labeling of exosomes, a 4 mg/ml solution of DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Sigma, St. Louis, MO) was added to the PBS wash diluted 1/20,000 and incubated light-protected at room temperature for 20 min before proceeding with the washing spin at 15°C. After resuspending isolated exosomes in PBS, a final spin was carried out to remove excess dye. In the case of PKH67 (Sigma) fluorescence labeling, PBS-washed exosomes were stained according to manufacturer's recommendations for labeling live cells. Electron microscopy (EM) analysis was carried out as previously described³⁸. For Western blot analysis, pelleted exosomes were resuspended in NP-40 lysis buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM EDTA)

containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and resolved on SDS-PAGE under non-reducing conditions. Following transfer to PVDF membranes (Biorad, Hercules, CA), blotting was conducted with anti-human CD63 antibody (Novus Biologicals, Littleton, CO) followed by goat anti-mouse HRP (Jackson ImmunoResearch, West Grove, PA) and detected by Pierce ECL Western Blotting Substrate (ThermoScientific, Rockland, IL). For on-bead flow cytometry analysis, Flash Red beads (Bangs Laboratories, Inc., Fisher, IN) were washed according to manufacturer's recommendations and rotated with isolated exosomes for 15 min at 37°C and then overnight at 4°C, followed by extensive washing. Following antibody staining of bead-adsorbed exosomes, flow cytometry data collection was carried out on a CyAn flow cytometer (Beckman Coulter, Brea, CA) and analyzed using FloJo software (Ashland, OR).

Exosome treatment of macrophages in vitro. Isolated exosomes resuspended in PBS were quantified using the Pierce micro-BCA protein assay kit (ThermoScientific) and administered to macrophages in equal amounts. For flow cytometric analysis of macrophage association with exosomes, 5×10^5 cells were seeded in 6-well plates one day prior to exosome treatment. Following exosome treatment, cells were detached in cold PBS and analyzed by flow cytometry. For Western blot analysis of NF-κB activation, cells were similarly treated with exosomes, but then lysed in NP-40 lysis buffer containing protease inhibitor cocktail. Proteins were resolved by SDS-PAGE, transferred to PVDF, and Western blotted using the NF-κB pathway antibody sampler kit (Cell Signaling Technology, Danvers, MA). For luciferase assays, RawkB cells were seeded 5×10^4 /well in 24-well plates 16 h prior to exosome treatment. At indicated times, cells were lysed in passive lysis buffer provided with Promega (Madison, WI) luciferase assays. Lysates were combined with BrightGlo substrate and luciferase activity was measured. For the inflammatory cytokine array, conditioned media following exosome treatment of RawkB cells was concentrated and subsequently applied to the Ray Biotech (Norcross, GA) mouse Inflammatory Cytokine array C-series and processed according to manufacturer's instructions. For cytokine expression analysis, RNA from macrophages treated with exosomes was isolated as previously described³⁹ and used for quantitative PCR. Primers used for PCR are: 5'-CAATCTGGATTCAATGAGGAGAC and 5'-CTCTGGCTTG-TTCCTCACTACTC for IL-6, 5'-CAGCCTCTTCTCCTCTCCTGA and 5'-



AGATGATCTGACTGCCTGGG for TNF α , 5'-TGAGCCAACTCCATAGCGG-CCT and 5'-TCCCAGTCTTCCATCTGCTGCCA for GCSF, and 5'-AAGA-TCTCAGTGCAGAGGCTCG and 5'-TTGCTTGTCCAGGTGGTCCAT for CCL2. Reagents used for chemical disruption of exosome effects on macrophages, including proteinase K, streptolysin O, chloroquine, hydroxylamine, and 2-bromopalmitate, were all purchased from Sigma. Bay 11-7082 was purchased from Calbiochem (Billerica, MA). The mouse TLR2 neutralizing antibody (T2.5) and control IgG were purchased from InvivoGen (San Diego, CA).

Exosome treatment of mice and xenografts. All animal experiments were approved by the Institutional Animal Care and Use Committee at City of Hope, and were performed in accordance with relevant guidelines and regulations. Fluorescently-labeled exosomes were injected intravenously through the tail vein into 6-week-old female nude mice (NCI Mouse Repository, Frederick, MD) twice-weekly for three weeks. Tissues were isolated from euthanized mice and then mechanically and enzymatically dissociated to obtain a cell suspension for flow cytometry/FACS or embedded in Tissue-Tek O.C.T. (Sakura, Torrance, CA) frozen blocks for subsequent sectioning and immunofluorescence analysis. Antibodies used for staining include: F4/80 (eBioscience, San Diego, CA), anti-IL-6 (Abcam, Cambridge, MA), and anti-CD68 (AbD Serotec, Raleigh, NC). Stained sections were imaged on a Zeiss (Thornwood, NY) LSM510 confocal microscope or an IX81 inverted fluorescence microscope (Olympus; Center Valley, PA). For mammary fat pad xenografts, 2×10^5 MDA-MB-231 cells were injected into the No. 4 mammary fat pad of 6-week old female NOD/SCID/IL2R α -null (NSG) mice.

Acyl-biotin exchange (ABE) and proteomics identification of palmitoylated proteins. Membrane fractionation was carried out by mechanical disruption of cells via dounce homogenization with the tight pestle for 30 strokes. Disruption of cells was confirmed by trypan blue incorporation. Nuclei and unbroken cells were removed by slow speed centrifugation and supernatant was spun in ultracentrifuge at $200,000 \times g$ for 30 min. Pelleted membranes were resuspended in RIPA buffer with protease inhibitor cocktail (PIC) and N-ethylmaleimide (NEM; Sigma) to block free cysteines while solubilizing membranes. Following a 1-h incubation at 4°C, insoluble debris were removed by centrifugation. SDS was added to the soluble fraction for a final concentration of 4% and NEM was added for a final concentration of 10 mM. Following a 10-min incubation at 37°C, chloroform-methanol precipitation was carried out three times in succession, each time resuspending the pelleted protein in 100 μ L of 4% SDS buffer (4% SDS, 50 mM Tris, 5 mM EDTA; pH 7.4). After the last precipitation, the samples were divided in half: one subsequently diluted with 4 volumes of hydroxylamine-containing buffer (HA⁺ buffer; 0.7 M HA pH 7.4, 1 mM HPDP-biotin crosslinker, 0.1% NP-40, PIC) and the other with 4 volumes of HA⁻ buffer (50 mM Tris pH 7.4, 1 mM HPDP-biotin crosslinker, 0.1% NP-40, PIC). Following rotation for 1 h at room temperature, a second series of chloroform-methanol precipitations was performed. Each sample was then resuspended in Magnesphere pulldown buffer (0.1% SDS, 0.1% NP-40 in PBS) and rotated with streptavidin Magnesphere Paramagnetic Particles (SA-PMP; Promega) for 2 h at 4°C. Following three washes with 5 min rotation in Magnesphere pulldown buffer, four washes in buffer without rotation, and three washes in PBS, beads were submitted for LCMS/MS analysis.

For protein identification by mass spectrometry, samples were reduced, alkylated, and digested according to a published protocol⁴⁰ and then reconstituted in water with 0.1% formic acid (buffer A) for analysis. Each sample was analyzed by LCMS/MS using an Agilent 6520 Q-TOF mass spectrometer fitted with an Agilent HPLC-Chip nano-electrospray interface (G4240A), a micro-fluidic device (PN: G4240-62030 with a 360 nL trapping column and 150 mm \times 75 μ m, 3 μ m Polaris C18 particle size analytical column) and an Agilent 1200 nanoflow HPLC system. Scaffold (version Scaffold_4.2.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm⁴¹. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Statistical analyses. All results were confirmed in at least three independent experiments, and data from one representative experiment was shown. All quantitative data are presented as mean \pm standard deviation (SD). For all quantitative data, statistical analyses were performed using GraphPad software. ANOVA testing was used for comparison of quantitative data followed by Student-Newman-Keuls post-test. Values of $p < 0.05$ were considered significant.

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Author contributions

A.C., M.K., V.N., X.R. and S.E.W. contributed to project design and wrote the main manuscript text. A.C. conducted all experiments. W.Z., M.Y.F., J.C., D.V.H., A.R.C. and L.L. contributed to Figures 1 & 3–5. Z.M. and W.H. contributed to Figure S2. B.G.G. contributed to the design of Figure 6d. All authors reviewed the manuscript.

Additional information

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