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ORIGINAL PAPER

Multiple myeloma exosomes establish a favorable bone marrow microenvironment with enhanced angiogenesis and immunosuppression

Running title: MM exosomes enhance angiogenesis and immunosuppression

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Abstract: Multiple myeloma (MM) pathogenesis and progression largely rely on the cells and extracellular factors in the bone marrow (BM) microenvironment. Compelling studies have identified tumor exosomes as key regulators in the maintenance and education of the BM microenvironment by targeting stromal cells, immune cells and vascular cells. However, the role of MM exosomes in the modification of the BM microenvironment and MM progression remains unclear. Here, we explored the functions of MM exosomes in angiogenesis and immunosuppression *in vitro* and *in vivo*. Murine MM exosomes carrying multiple angiogenesis-related proteins enhanced angiogenesis and directly promoted endothelial cell growth. Several pathways such as signal transducer and activator of transcription 3 (STAT3), c-Jun N-terminal kinase and p53 were modulated by the exosomes in endothelial and BM stromal cells. These exosomes promoted the growth of myeloid-derived suppressor cells (MDSCs) in naive mice through activation of the STAT3 pathway and changed their subsets to similar phenotypes as those seen in MM-bearing mice. Moreover, MM exosomes upregulated inducible nitric oxide synthase and enhanced the immunosuppressive capacity of BM MDSCs *in vivo*. Our data show that MM exosomes modulate the BM microenvironment through enhancement of angiogenesis and immunosuppression, which will further facilitate MM progression.

Keywords: multiple myeloma; exosomes, myeloid-derived suppressor cells, immunosuppression; angiogenesis, STAT3

Introduction

Multiple myeloma (MM) is an incurable plasma cell neoplasm characterized by an accumulation of malignant plasma cells in the bone marrow (BM). Signals from the BM microenvironment play a pivotal role in supporting MM cell growth, spread, and survival [1]. The BM microenvironment contains hematopoietic and non-hematopoietic cells such as stromal cells (SCs), osteoblasts, osteoclasts, endothelial cells and myeloid-derived suppressor cells (MDSCs) [1-3]. During MM development, MM cells can affect BM cells through cell-cell contact or secretion of soluble factors to build up a favorable microenvironment. MM cells adhere to BMSCs in which they trigger many pathways, resulting in the secretion of cytokines such as interleukin-6 (IL-6), insulin-like growth factor-1 and vascular endothelial growth factor (VEGF), which mediate MM cell growth, proliferation, survival and drug resistance [1,3]. BM angiogenesis progressively increases during MM development from the more benign monoclonal gammopathy of undetermined significance stage to leukemic phase MM [4] and this is partly driven by pro-angiogenic cytokines including VEGF, basic fibroblast growth factor (bFGF) and metalloproteinases (MMPs) [5,6] secreted by MM cells, BMSCs and osteoclasts. Accumulation of immunosuppressive MDSCs was observed in the BM of MM patients at early stages in whom they regulate MM cell growth by inhibiting T cells [7]. De Veirman *et al.* have shown that murine MM cells directly activate MDSCs and enhance their immunosuppressive function through soluble factors such as GM-CSF [8]. All these findings emphasize the role of MM cells as modifier of the BM microenvironment

which ultimately leads to disease progression.

Exosomes are 40-100nm diameter extracellular vesicles (EVs) which mediate local and systemic cell-to-cell communication through the horizontal delivery of bioactive molecules, including lipids, mRNAs, microRNA and proteins [9]. Exosomes transfer their content by direct fusion with the membrane of recipient cells or by endocytosis [10]. The level of EVs is increased in the body fluids of cancer patients as compared to healthy donors and they are associated with progressed stages of cancer [11,12]. Exosomes derived from various cancer cells modulate immunosuppression, angiogenesis, cell differentiation, invasion and metastasis [9,13-17]. In the MM BM microenvironment, exosomes derived from BMSCs or mesenchymal stromal cells (MSCs) directly facilitate MM progression [18,19]. MM cell-derived exosomes ('MM exosomes') are involved in osteoclast differentiation and exosomes shed from hypoxic MM cells enhance angiogenesis in the BM [20,21], indicating important roles in the modification of the BM microenvironment. However, the effects of MM exosomes on other BM-derived cells are still not fully elucidated. In the present study, we investigated the effect of MM exosomes on BM endothelial cells, BMSCs, and MDSCs, *in vitro* and *in vivo* using the 5T33MM model and human samples.

Materials and methods

5T33MM model, plasma and cell culture

C57BL/KaLwRij mice were purchased from Harlan Laboratories and 5T33MM mice were generated as previously described [18]. Mice were housed and treated following conditions approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (license no. LA1230281). Human plasma was collected for routine diagnostic purposes after patients' informed consent and in accordance with the Declaration of Helsinki. All research was approved by the local ethical committee (B.U.N. 143201316382). The murine MM cell lines 5T33MMvt and 5TGM1, the murine BM endothelial cell line STR10 [22], primary BMSCs obtained from naive mice [18], as well as human MM cell lines RPMI8226, LP-1 and U266, were used and cultured as described in supplementary material and methods.

Exosome isolation

Cells were cultured without serum for 24 hours and conditioned medium was collected after centrifugation and filtered using 0.22-μm pore filter. The filtered medium was concentrated using 150KD Protein Concentrator (Thermo Scientific, Waltham, MA, USA) and filtered again with a 0.22-μm pore filter and thereafter exosomes were isolated using ExoQuick-TC exosome precipitation solution (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instruction. The concentration of exosomal proteins was determined as described previously [18].

In vivo chick chorioallantoic membrane (CAM) assay and angiogenesis array

CAM assay was performed to measure angiogenesis as described previously [23,24]. One ml conditioned medium or 200μg exosome proteins were assessed for angiogenesis-related factors using the Mouse or Human Angiogenesis Array kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

In vivo study

Naive mice were intravenously injected with 200μg 5T33MMvt exosomes 3x/week. After 3 weeks, BM and spleen cells were isolated and the percentages of MDSCs, as well as the subsets of MDSCs, were determined by flow cytometry. For short stimulation of exosomes *in vivo*, naive mice were injected with 200μg 5T33MMvt exosomes. BM and spleen cells were isolated after 24 hours and the levels of p-Stat3 in MDSCs evaluated using flow cytometry. BM $CD11b^+$ cells were also separated using MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) and their immunosuppressive capacity measured using T cell proliferation assay.

Flow cytometry

Membrane markers of MDSCs were stained with anti-Gr-1-APC (17-5931-81; eBioscience, San Diego, CA, USA) and anti-CD11b-PE-Cy7 (101216; BioLegend,

San Diego, CA, USA) or with anti-Ly6G-PE-Cy7 (127618; BioLegend) and anti-Ly6C-APC (560595; BD Biosciences, Franklin Lakes, NJ, USA). Annexin V-fluorescein isothocyanate (Annexin V-FITC, 556419; BD Biosciences) was used to determine apoptotic cells. For staining intracellular p-Stat3 in MDSCs, the whole BM was first fixed with formaldehyde and methanol, followed by staining with anti-CD11b-PE-Cy7, anti-Gr-1-APC and anti-p-Stat3-Alexa Fluor 488 (557814; BD Biosciences). For determining $CD11b^+$ subpopulations, total BM cells were stained with anti-CD11b-FITC (101206; BioLegend), anti-Ly6G-PE-Cy7 and anti-Ly6C-APC. The populations and mean fluorescence intensities were measured using a FACSCanto flow cytometer (BD Biosciences) and Flowjo software (TreeStar, Ashland, OR, USA).

Cell proliferation assay

For the $CD11b^+$ cell proliferation assay, $CD11b^+$ cells were stained with CFSE (0.1μM) and cultured with or without exosomes for 2 days. Proliferation of $CD11b^+$ cells was evaluated with CFSE dilution by flow cytometry. For T cell proliferation assay, splenocytes isolated from naive mice were stained with CFSE and cultured with RPMI1640 medium supplemented with 10% HEPES and 20μM β-mercaptoethanol for 20 min. These cells were stimulated with CD3/CD28 Dynabeads (Life Technologies) and seeded in a 96-well plate (1×10^5 cells/well) in the presence of $CD11b^+$ cells at different ratios. After a 3-day culture, cells were stained

with anti-CD3-PE-Cy7 (100219; BioLegend) and 7-AAD, and proliferation of living T cells ($CD3^{+}7\text{-}AAD^{-}$ cells) was determined by measuring CFSE dilution through flow cytometry.

Details of transmission electron microscopy (TEM), western blot, magnetic-activated cell sorting (MACS), and cell viability and apoptosis assays are provided in supplementary material and methods.

Statistical analysis

Results were analyzed with Graphpad prism 5 software. Statistical significance was determined with Mann-Whitney test or One-way ANOVA followed by Tukey's post test and $p<0.05$ was regarded as significant.

Results

MM exosomes carry pro-angiogenic proteins and promote angiogenesis *in vivo*

Exosomes were isolated from the conditioned medium of murine or human MM cells using an improved ExoQuick exosome precipitation method with extra filter and concentration steps which minimize the contamination of soluble protein and small vesicles (Figure 1A). Using this protocol, 10μg or 3μg exosomes were isolated from 10^6 RPMI822 or 5T33MMvt cells, respectively (Supplementary Figure S1A). The size and morphology of 5T33MMvt exosomes were confirmed using TEM (Figure 1B) and exosomal markers including Alix, heat shock protein 90 (Hsp90), Hsp70, flotillin-1 and Tsg101 were detected by western blot (Figure 1C). One study has demonstrated the proangiogenic function of human MM exosomes [20], therefore we next determined if murine MM exosomes have the same effect on angiogenesis using the *in vivo* CAM assay [23]. We observed many newly-formed capillaries, converging radially toward the MM exosome-loaded sponge in a "spoked-wheel" pattern (Figure 1D), indicating a strong induction of angiogenesis. As exosomes carry multiple cargos, we examined the proteins related to angiogenesis in murine or human MM cell-derived supernatant and exosomes. Several angiogenesis-related proteins were detected in 5T33MMvt (Figure 1E) or RPMI8226 exosomes (Figure 1F), showing a different pattern from those in supernatant as some proteins (eg. osteopontin in 5T33MMvt, CXCL16 and endothelin-1 in RPMI8226) are present in supernatant but absent or lower in exosomes. To exclude the possible contamination by free soluble factors, the concentrated conditioned medium was pretreated with Proteinase K to

remove free proteins and thereafter used for exosome isolation [25]. A similar pattern of angiogenesis-related proteins are observed in these exosomes (Supplementary Figure S1), suggesting limited contamination.

Multiple pathways in STR10 cells and BMSCs are modulated by MM exosomes

Since MM exosomes promoted angiogenesis, we next determined their effects on the viability of both endothelial cells (ECs) and BMSCs which play crucial roles in MM angiogenesis and progression. The BM EC line STR10 [22] and BMSCs were cultured with exosomes and we detected an increase of cell viability (Figures 2A and B). To exclude the possibility that these effects were caused by soluble contamination in the exosome suspension, 5T33MMvt exosomes suspended in PBS were re-precipitated and the supernatant was used as a negative control (Supplementary Figure S2A). Supernatant without exosomes did not increase the cell viability of STR10 and BMSC (Supplementary Figures S2B and C). Exosomes isolated from Proteinase K-treated conditioned medium (5T33MMvt exo PK) still increased the cell viability of STR10 and BMSC (Supplementary Figure S2D), confirming the positive role of exosomes. The activation of multiple apoptosis- and proliferation-related pathways in STR10 cells and BMSCs cultured with exosomes were examined to explore the underlying mechanisms of the viability increase. In STR10 cells, enhanced phosphorylation of signal transducer and activator of transcription 3 (Stat3), c-Jun N-terminal kinase (JNK) and p53 were observed after 6 or 24 hours of exposure

to exosomes, while phosphorylated Akt and p38 were reduced by exosomes at 24 hours (Figure 2C). The phosphorylation of Stat3 and JNK in BMSCs was increased by exosomes after 6, 24, or 48 hours, phosphorylated Akt was slightly reduced and the phosphorylated forms of p53 and p38 were unchanged. The level of total p53 was increased by exosomes in both cell types, especially in BMSCs. Reduced cleavage of caspase-3 at 24 hours was detected (Figures 2C and D). In addition, phosphorylation of JNK and STAT3 was detected as early as 0.5 and 3 hours respectively in both STR10 and BMSCs after incubation with exosomes (Supplementary Figures S2E and F). Moreover, enhanced cell viability of STR10 and BMSC was also induced by exosomes derived from the other murine cell line 5TGM1 (Figures 2E and F).

MM exosomes promote MDSC growth *in vivo*

MDSCs, identified as $CD11b^{+}Gr\text{-}1^{+}$ cells in mice, are classified into two major subpopulations according to the expression of the Gr-1 antigen (Ly6C/Ly6G) and nuclear morphology: granulocytic MDSCs expressing $CD11b^{+}Ly6G^{high}Ly6C^{int}$ and monocytic MDSCs expressing $CD11b^{+}Ly6G^{low}Ly6C^{+}$ [26]. We stained MACS-sorted BM $CD11b^{+}$ cells from naive and 5T33MM mice with Ly6C and Ly6G and found three distinct subpopulations of MDSCs, $Ly6G^{high}Ly6C^{int}$, $Ly6G^{low}Ly6C^{high}$, and $Ly6G^{low}Ly6C^{int}$ (Figure 3A). According to our previous study [27], these populations are granulocytic MDSCs, inflammatory or classical monocytes, and a mixture of immature myeloid cells and eosinophils, respectively. Since exosomes act as

mediators between cells, they may induce changes of MDSCs. We thus examined the effects of MM exosomes in the BM and spleen *in vivo*. Naive mice were injected with 5T33MMvt exosomes every 2 days for 3 weeks which led to an increase in spleen size (Figure 3B) and an increase of MDSCs ($CD11b^{+}Gr\text{-}1^{+}$ cells) in both BM and spleen (Figure 3C). Moreover, MDSCs from the BM of exosome-treated naive mice showed a phenotype similar to MDSCs in 5T33MM tumor-bearing mice, *i.e.* decreased granulocytic MDSCs ($Ly6G^{high}Ly6C^{int}$) and increased immature myeloid cells and eosinophils ($Ly6G^{low}Ly6C^{int}$ cells) (Figure 3D) indicating that MM exosomes play a role in these changes. In the spleen, a slight increase of $Ly6G^{low}Ly6C^{int}$ cells was observed in exosome-injected mice (Figure 3E).

MM exosomes can be taken up by MDSCs and increase CD11b in these cells

Since MM exosomes promoted the growth of MDSCs *in vivo*, we next wanted to elucidate this effect further *in vitro*. The membrane or content of exosomes were labeled with the membrane tracker DIO or cell-permeant nucleic acid stain SYTO RNASelect green fluorescent cell stain (RGFCS) respectively and they were taken up by BM cells and to a higher extent by $CD11b^{+}$ cells (Figures 4A and B), which are mainly MDSCs as nearly all of them co-express Gr-1 [27]. Moreover, a significantly higher DIO signal was observed in $CD11b^{+}$ cells *vs.* $CD11b^{-}$ cells (Supplementary Figure S3A and B), suggesting a stronger ability of MDSCs for taking up MM exosomes through membrane fusion. Following exposure to exosomes over 11 days,

BM cell survival was increased (Figures 4C and D). The pro-survival effect on $CD11b^+$ cells was detected after 7 days (Figures 4D and E) and CD11b expression on $CD11b^+$ cells was also enhanced (Figure 4F). To confirm that the CD11b increase was directly caused by exosomes, $CD11b^+$ cells were purified using magnetic-activated cell sorting (MACS) (Supplementary Figure S3C and D). 5T33MMvt exosomes increased the surface expression of CD11b about two-fold on naive and 5T33MM MDSCs (Figures 4G and H).

MM exosomes promote MDSCs viability and proliferation.

To further elucidate the direct effect of MM exosomes on MDSCs, purified $CD11b^+$ cells from naive and 5T33MM mice were cultured with 5T33MMvt exosomes. In medium with 5% serum, 100 μg of exosomes increased the viability of both naive and 5T33MM $CD11b^+$ cells by 1.5-fold after 48 hours (Figure 5A), while in serum free conditions, 12.5 μg exosomes increased the viability of these cells more than two-fold (Figure 5B). As controls, no significant increase of $CD11b^+$ cell viability was seen using the supernatant of exosomes (Supplementary Figure S4A) while we could still see increased $CD11b^+$ cell viability with exosomes isolated from Proteinase K-treated conditioned medium (Supplementary Figure S4B). As expected, 5TGM1 cell-derived exosomes also enhanced $CD11b^+$ cell viability (Figure 5C). The viability of human $CD11b^+$ cells obtained from PBMC was also elevated by exosomes derived from three human MM cell lines (Figure 5D) or MM patients' plasma

(Supplementary Figure S4C). Next, we wondered whether the enhanced viability was a result of increased proliferation or reduced apoptosis, and found that the proliferation of both naive and 5T33MM $CD11b^+$ cells was increased by 5T33MMvt exosomes (Figure 5E), while the survival of naive $CD11b^+$ cells was not affected (Figure 5F). We next studied the survival of the MDSCs subsets: exosome exposure decreased the survival of $CD11b^+Ly6G^{high}Ly6C^{int}$ cells in naive BM (Figure 5G) but increased that of $CD11b^+Ly6G^{low}Ly6C^+$ cells (Figure 5H). Different from naive $CD11b^+$ cells, exosomes increased survival of 5T33MM $CD11b^+$ cells after 4 days (Figure 5I), whereas the survival of $CD11b^+Ly6G^{high}Ly6C^{int}$ was not changed (Figure 5J). However, a significant increase of living $CD11b^+Ly6G^{low}Ly6C^+$ cells was already seen after 2 days (Figure 5K). Similar to 5T33MMvt exosomes, exosomes obtained from 5TGM1 cells also mainly promoted the survival of $CD11b^+Ly6G^{low}Ly6C^+$ cells from naive and 5T33MM mice (Supplementary Figures S4D-I).

MM exosomes activate the STAT3 pathway in MDSCs and increase their immunosuppressive capacity in vivo

STAT3 is the main transcription factor regulating the expansion of MDSCs and its activation contributes to survival and proliferation of myeloid progenitor cells [28,29]. Since the 5T33MMvt exosomes promoted MDSC proliferation, we next evaluated the activation of STAT3 in these cells. Exosomes enhanced STAT3 activation (p-stat3) in both naive and 5T33MM $CD11b^+$ cells as assessed by western

blot (Figures 6A and B) and flow cytometry (Supplementary Figure S5A), and increased the levels of pro-survival Bcl-xL and Mcl-1 (Figures 6A and B). Stattic, a selective STAT3 inhibitor, reduced the viability of naive and 5T33MM CD11b$^+$ cells even in the presence of exosomes. Although exosomes increased MDSCs cell viability, a higher concentration of stattic (400nM) reduced this viability at a similar rate in the absence or presence of exosomes (Figures 6C and D). This was most likely through the induction of apoptosis (Supplementary Figures S5B and C). Moreover, inhibition of STAT3 phosphorylation, as well as reduced Mcl-1 and Bcl-xL, by stattic was observed in CD11b$^+$ cells cultured with 5T33MMvt exosomes (Figure 6E and Supplementary Figure S5D) suggesting that the STAT3 pathway is involved, at least partially, in the exosome-induced increase of the MDSCs viability.

Activated MDSCs express high levels of both arginase 1 and iNOS, which can suppress T cell function [26]. 5T33MMvt exosomes increased the mRNA and protein level of iNOS but not arginase-1 in both naive and 5T33MM CD11b$^+$ cells (Supplementary Figures S5E-G), implicating a potential role of exosomes in T cell suppression. Intravenous injection of exosomes in naive mice increased the phosphorylation of STAT3 (Figure 6F) in MDSCs from the BM but not the spleen, which was consistent with the *in vitro* data. Furthermore, when *in vivo* activated MDSCs were sorted from the BM, they exhibited an enhancement of T cell suppression (Figure 6G).

Discussion

It is known that cancer cells secrete high levels of exosomes in contrast to their healthy counterparts [11,12]. This is also true for MM, where high levels of EVs were found in the BM and serum of MM patients and mice which were absent in healthy individuals [30-32]. We also found that healthy murine B-cells and plasma cells secrete almost no exosomes, compared to MM cells (data not shown). Furthermore, accumulating evidence demonstrates that exosomes derived from cancer cells modulate the BM microenvironment by targeting MDSCs [17,33], MSCs [34,35], ECs [20], osteoclasts [21], dendritic cells [15,36] and macrophages [36], to provide a support system for cancer progression. However, how MM exosomes modulate the BM microenvironment has not been thoroughly investigated. Here, we confirm the pro-angiogenic function of murine MM exosomes and demonstrate that these exosomes enhance the viability of BM ECs. Moreover, these exosomes increase the presence of BM MDSCs *in vivo* and change their subsets to a more tumorigenic profile. MM exosomes can intensify the activation of MDSC *in vitro* and *in vivo*, and enhance their suppressive function on T cells. Our work shows that, apart from well-identified MM cell-derived soluble factors, MM exosomes can modify the BM microenvironment by promoting BMSC growth, angiogenesis and increased immunosuppression by MDSCs (Figure 6H). This modified BM microenvironment will further facilitate MM cell growth, MM progression and induction of drug resistance.

The relationship between tumor-released exosomes and angiogenesis has been

investigated in many cancer cell types including melanoma [37], nasopharyngeal carcinoma [38], chronic myelogenous leukemia [39,40] and chronic lymphocytic leukemia [35], and the exosomes released from these cancer cells facilitate angiogenesis in the tumor microenvironment. One study has shown that human MM exosomes increase angiogenesis and that hypoxic conditions can enhance this action through upregulation of miR-135b [20]. Our results confirm a strong pro-angiogenic effect of murine MM exosomes *in vivo* and show the presence of multiple angiogenic factors in MM exosomes, such as angiogenin, bFGF [41] and VEGF [41], indicating that miR-135b is not the sole mediator in exosome stimulated angiogenesis. Additionally, our work demonstrates that exosomes directly facilitate the growth of BM endothelial cells by modulating multiple pathways, such as STAT3, JNK, Akt, p38 and p53. Although Akt activation, which is crucial for cell survival and proliferation, was slightly reduced, the pro-proliferation STAT3 pathway [42] was activated by exosomes, leading to an overall increase of cell viability. Modulation of all these pathways by exosomes implicates a complex pathway-crosstalk and suggests that exosomes, due to their different cargo, may induce multiple signaling changes instead of a single effect in target cells.

BMSCs support MM pathogenesis and progression through secretion of soluble factors and cell-cell contact [1,3,43,44]. Previous studies have shown that BMSC-derived exosomes directly facilitate MM progression and induce drug resistance [18,19,45], whereas the effects of MM exosomes on stromal cells remain

unclear. It has been demonstrated recently that exosomes secreted by cancer cells trigger MSC differentiation into pro-angiogenic and pro-invasive myofibroblasts [34], or into cancer-associated fibroblasts [35] which can favor tumor survival, proliferation and invasion [46-48]. Our previous study reported uptake of MM exosomes by BMSCs [18] and here we show that these exosomes directly promote BMSC growth and influence multiple pathways. Interestingly, p53 but not its phosphorylated form was increased by exosomes in BMSC. The exosomal factor responsible for this effect and the detailed mechanisms behind it still need to be investigated.

MDSCs accumulate in the tumor microenvironment during tumor development and have inhibitory activity on the immune response [26,49]. Accumulation of immunosuppressive MDSCs was found in the BM of newly diagnosed MM patients [7] and an increase of BM MDSCs was also detected in the 5T33MM mouse model after 1 week inoculation with MM cells [8]. Here, we found that exosomes derived from MM cells increase the proportion of BM MDSCs *in vivo* and induce changes in MDSC subpopulations which are similar to their phenotype in the BM of 5T33MM mice, suggesting the involvement of exosomes in the accumulation of MDSCs. Exosome treatment of both murine and human $CD11b^+$ cells increased proliferation and viability, indicating a direct effect of exosomes on MDSCs. In addition, MM exosomes increased CD11b, which regulates leukocyte adhesion and cell migration [26], implying multiple effects on $CD11b^+$ cells.

Two major subsets of MDSCs, $CD11b^{+}Ly6G^{high}$ and $CD11b^{+}Ly6G^{low}$ cells have been identified in the 5T33MM model [27]. MM exosomes reduced the survival of granulocytic MDSCs ($CD11b^{+}Ly6G^{high}Ly6C^{int}$ cells) from naive mice, but increased the survival of $CD11b^{+}Ly6G^{low}Ly6C^{+}$ cells, which are also increased in 5T33MM-bearing mice and in exosome-educated BM. In addition, MM exosomes promoted the survival of all 5T33MM MDSCs, with a stronger effect on $CD11b^{+}Ly6G^{low}Ly6C^{+}$ cells. Considering the higher immunosuppressive capacity of $CD11b^{+}Ly6G^{low}$ cells compared to $CD11b^{+}Ly6G^{high}$ cells [27], it is possible that MM cells increase immunosuppression in the BM through exosome-induced increase of $CD11b^{+}Ly6G^{low}$cells.

Stat3 regulates MDSC expansion by transcriptional regulation of pro-survival or pro-proliferation proteins [26]. Enhanced activation of the STAT3 pathway in MDSCs is induced by MM exosomes and inhibition of this pathway suppresses exosome-induced viability, indicating the importance of STAT3 in this situation. We have previously shown that MDSCs from 5T33MM mice are immunosuppressive and that GM-CSF secreted by MM cells is involved in their activation [8,27]. However, rather low level of GM-CSF has been detected in 5T33MMvt cell-derived exosomes [18] and a neutralizing antibody for GM-CSF did not affect MM exosome-induced MDSC viability (data not shown), suggesting a lack of its involvement. Our previous

study has shown the presence of IL-10 and IL-16 in 5T33MMvt exosomes using a cytokine array [18]. Both of these cytokines possess immunosuppressive properties [50-53]. Additionally, VEGF can also contribute to the expansion of MDSC by binding to its receptor expressed by MDSC and subsequently activating the STAT3 pathway [28,54]. Thus, these cytokines may be the mediators of exosome-induced MDSC activation.

T cell suppression induced by activated MDSCs involves two mechanisms: cell-cell contact and short-lived soluble mediators such as nitric oxide (NO) and reactive oxygen species (ROS) [26,28,29]. iNOS catalyzes the production of NO from L-arginine and activated MDSCs express high levels of iNOS [26,55]. Here, we found that MM exosomes significantly upregulate the expression of iNOS in MDSCs, especially in MM MDSCs. Moreover, these exosomes enhance the immunosuppressive function of MDSCs *in vivo*, which could lead to immune evasion of MM cells. Furthermore, our finding that activation of the STAT3 pathway in BM MDSCs was also enhanced by MM exosomes *in vivo* emphasizes the involvement of STAT3 in MDSC activation.

In summary, we demonstrate multiple functions of MM exosomes in the modification of the BM microenvironment through induction of angiogenesis and immunosuppression. Specifically, our data highlight MM exosomes as a key regulator

in the MM BM microenvironment and support the significance of new therapeutic strategies targeting exosome secretion and MDSC activation in MM.

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Authors' contribution

Contributions: JW and EM conceived and designed the research and wrote the manuscript; JW, KDV, SF, DR and MAF performed the experiments and analyzed the data; AV and EM provided crucial suggestions and revised the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. MM exosomes promote angiogenesis. (A) Diagram illustrating the improved protocol for exosome isolation using ExoQuick solution. (B) The size and morphology of exosomes secreted by 5T33MMvt cells were observed by TEM, scale bar = 100 nm; arrows indicate typical exosomes. (C) Western blot for exosomal markers Hsp90, Hsp70, Alix, Tsg101 and flotillin-1, and the negative marker calreticulin, in 5T33MMvt cells and exosomes. (D) Sterilized gelatin sponges were loaded with PBS (Control) or 5T33MMvt exosomes (5T33MMvt exo) and implanted in chick chorioallantoic membranes (CAMs). The angiogenic response was evaluated after 4 days. Representative pictures (×50) of the CAM assays are presented in the left panel and numbers of vessels converging toward the sponge in the right panel (n=10). (E-F) Angiogenesis-related proteins in supernatant or exosomes obtained from (E) 5T33MMvt or (F) RPMI8226 cells were analyzed using a mouse or human angiogenesis array, respectively. The pixel densities of proteins in the array were quantified and normalized to reference spots. bFGF, basic fibroblast growth factor; IL-10, interleukin-10; MIP-1α, macrophage inflammatory protein-1α; MMP-9, matrix metallopeptidase 9; OPN, Osteopontin; ANG, angiogenin; ET-1, Endothelin-1; HGF, hepatocyte growth factor; TIMP-1, tissue inhibitor of metallopeptidase 1; TSP-1, thrombospondin-1. Graphs represent mean ± standard deviation. ** = $p<0.01$.

Figure 2. Multiple pathways in STR10 cells and BMSCs are regulated by MM exosomes. (A) STR10 cells or (B) BMSCs starved overnight were cultured with

5T33MMvt exosomes at the indicated final concentrations for 24 or 48 hours in serum free conditions. Relative cell viability was measured by a luminescent assay; mean ± standard deviation for at least 3 independent experiments. (C) STR10 cells or (D) BMSCs after starvation overnight were cultured with 5T33MMvt exosomes (60μg/ml) in serum free medium for different times and activation of the indicated proteins was determined by western blot. Tubulin was measured as a loading control. The pixel density of phosphorylated proteins was quantified and normalized to their total form or tubulin. (E) STR10 cells or (F) BMSCs were starved overnight and cultured with 5TGM1 exosomes at the indicated final concentrations for 24 or 48 hours in serum free conditions. Relative cell viability is shown as mean ± standard deviation for at 3 independent experiments. *p <0 .05, **p < 0.01, ***p < 0.001.

Figure 3. MM exosomes increase the growth of MDSC in vivo. (A) $CD11b^+$ cells were isolated from the BM of naive or 5T33MM mice by MACS and the indicated MDSC subpopulations were determined by flow cytometry. The percentages of the subpopulations are presented by histograms (n=3). (B-E) Naive mice (n=5) were intravenously injected with PBS (control) or 200μg 5T33MMvt exosomes 3 times a week for 3 weeks. (B) spleen sizes; (C) percentage of MDSCs ($CD11b^+Gr\text{-}1^+$ cells) in the BM and spleen by flow cytometry; (D,E) subpopulations of $CD11b^+$ cells in the BM and spleen. Graphs show mean ± standard deviation. *p < 0.05, **p < 0.01.

Figure 4. MM exosomes can be taken up by naive BM cells and affect their

survival. Total BM cells from naive mice (n=3) were cultured with (A) RGFCS- or (B) DIO-labeled 5T33MMvt exosomes (5T33MMvt exo, 100μg/ml) in medium with 5% serum for 3 or 24 hours respectively and the percentages of $RGFCS^+$ or DIO^+ cells were determined by flow cytometry after CD11b-PE-Cy7 staining. Representative profiles shown in the upper panel. (C-F) Total BM cells from naive mice (n=6) in medium with 5% serum were cultured with 5T33MMvt exosomes (50μg/ml) 3 times a week for 11 days. (C) Living cells (Annexin V^-) in the whole BM population or (E) the $CD11b^+$ population at the indicated time. (D) Representative Annexin V flow cytometry profiles of $CD11b^+$ cells after 7 days. (F) Mean fluorescence intensity of CD11b within the $CD11b^+$ population are shown. Purified (G) naive or (H) 5T33MM $CD11b^+$ cells were cultured with 5T33MMvt exosomes (5T33MMvt exo) for 2 or 4 days and the mean florescence intensity of CD11b in these cells was evaluated by flow cytometry after anti-CD11b-PE-Cy7 staining. Error bars represent mean ± standard deviation. ** = $p<0.01$, *** = $p<0.001$.

Figure 5. MM exosomes increase MDSC viability and proliferation. (A-D) Relative cell viability (luminescence assay), n=3 for each group. (A) $CD11b^+$ BM cells from naive or 5T33MM mice cultured with or without 5T33MMvt exosomes (100μg/ml) in medium with 5% serum. (B) Purified naive or 5T33MM $CD11b^+$ cells cultured with 5T33MMvt exosomes at the indicated final concentration for 48 hours in medium with 0% serum. (C) Purified naive or 5T33MM $CD11b^+$ cells cultured with 5TGM1 exosomes (100μg/ml) for 48 hours in medium with 5% serum. (D)

Purified human PBMC CD11b$^+$ cells in medium with 5% serum cultured with exosomes (100μg/ml) derived from PRMI8226, LP-1 or U266 cells for 48 hours. (E) CFSE-labeled naive or 5T33MM CD11b$^+$ cells in medium with 5% serum were cultured with 5T33MMvt exosomes (100μg/ml) for 48 hours and the percentage of proliferating cells was determined by flow cytometry (n=3). (F) Naive or (I) 5T33MM CD11b$^+$ cells in medium with 5% serum were cultured with or without 5T33MMvt exosomes (100μg/ml) for 2 or 4 days and the percentage of living (Annexin V$^-$) cells was determined by flow cytometry (n=3). After culture with exosomes, (G, H) naive or (J, K) 5T33MM CD11b$^+$ cells were stained with anti-Ly6G-PE-Cy7, anti-Ly6C-APC and Annexin V-FITC and the percentage of living (Annexin-V$^-$) cells in two subpopulations of MDSC, (G, J) Ly6G^{high}Ly6C^{int} cells and (H, K) Ly6G^{low}Ly6C$^+$ cells, was determined by flow cytometry. Histograms represent mean ± standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 6. MM exosomes activate the STAT3 pathway in MDSCs and increase their immunosuppressive capacity *in vivo*. Western blot of the indicated proteins in (A) Naive or (B) 5T33MM CD11b$^+$ cells cultured with or without 5T33MMvt exosomes (100μg/ml) in medium with 5% serum for the indicated times. β-actin was included as a loading control. (C, D) Relative cell viability of naive or 5T33MM CD11b$^+$ cells cultured for 48 hours in medium with 5% serum with or without 5T33MMvt exosomes (100μg/ml) in the absence or presence of 200nM or 400nM stattic (n=3). (E) Western blot of the indicated proteins in naive or 5T33MM CD11b$^+$

cells cultured in medium with 5% serum with or without 5T33MMvt exosomes (100μg/ml) in the absence or presence of 400nM stattic for 24 hours. β-actin was included as a loading control. (F) The levels of p-Stat3 measured by flow cytometry in BM or spleen MDSCs ($CD11b^{+}Gr\text{-}1^{+}$ cells) isolated from naive mice injected intravenously with PBS (control) or 5T33MMvt exosomes (200μg) 24 hours previously (n=4). (G) BM $CD11b^{+}$ cells were separated by MACS 24 hours after intravenous injection with PBS or 200μg exosomes in naive mice and co-cultured at the indicated ratio with CFSE-labeled splenocytes stimulated with CD3/CD28 Dynabeads. After 3 days cells were stained with anti-CD3-PE-Cy7 and 7-AAD and proliferation of living T cells ($CD3^{+}7\text{-}AAD^{-}$ cells) determined with CFSE dilution by flow cytometry. (H) Schematic showing how MM cells modify the BM microenvironment through exosome secretion. Graphs represent mean ± standard deviation (n=3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

SUPPORTING INFORMATION

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Legends for supplementary figures S1-S5.

Figure S1. MM exosomes carry angiogenesis-related proteins.

Figure S2. MM exosomes have limited contamination and promote BMSC and STR10 cell growth.

Figure S3. MM exosomes can be taken up by $CD11b^{+}$ cells.

Figure S4. Human MM and 5TGM1 exosomes increase MDSCs viability and induce the survival of $CD11b^{+}Ly6G^{low}Ly6C^{+}$ cells.

Figure S5. MM exosomes activate the STAT3 pathway and iNOS expression in MDSCs.

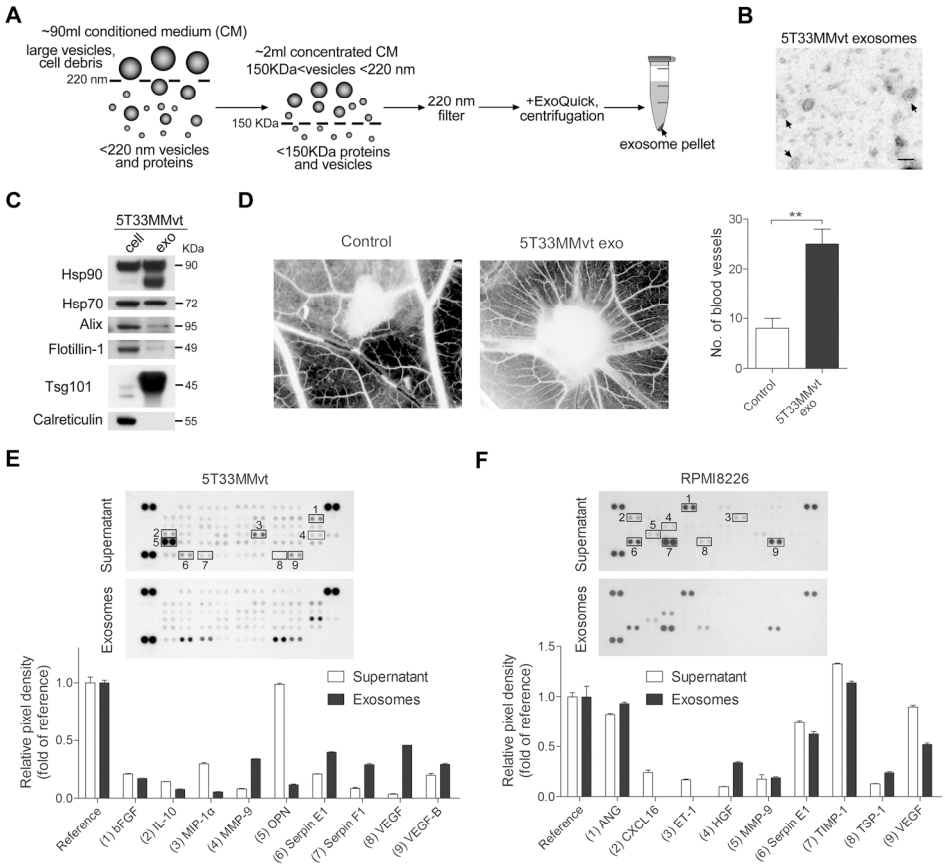

Figure 1

Figure 1
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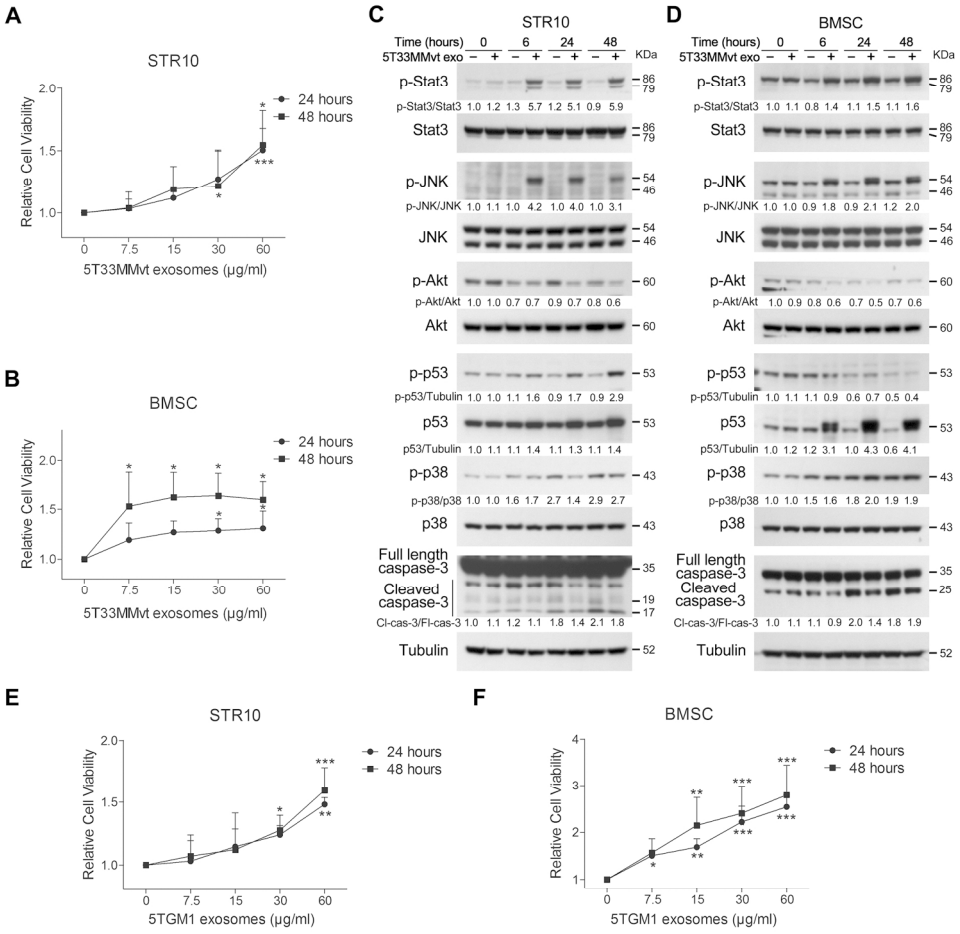

Figure 2

Figure 2
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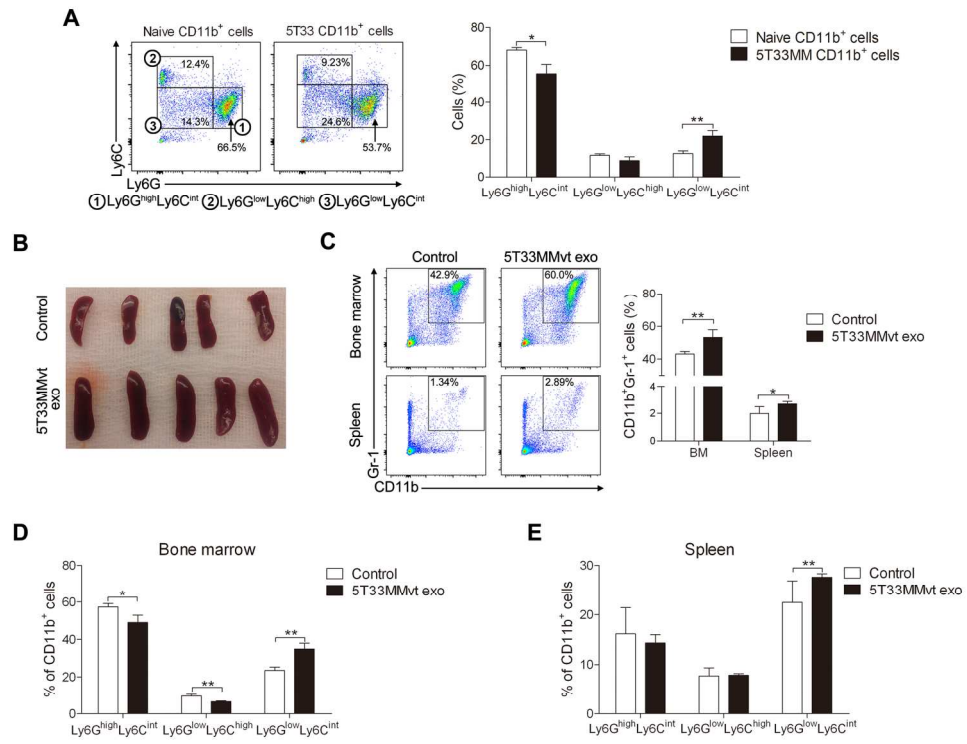

Figure 3

figure 3
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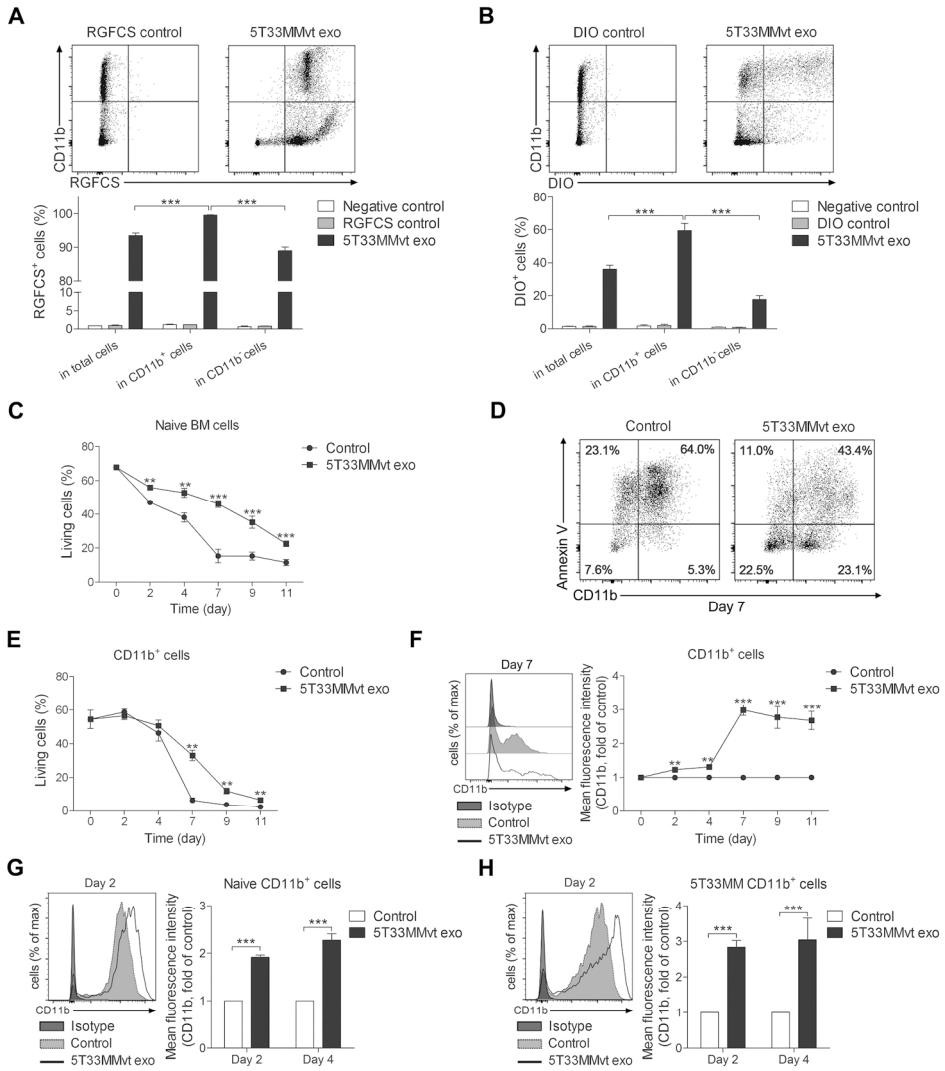

Figure 4

figure 4
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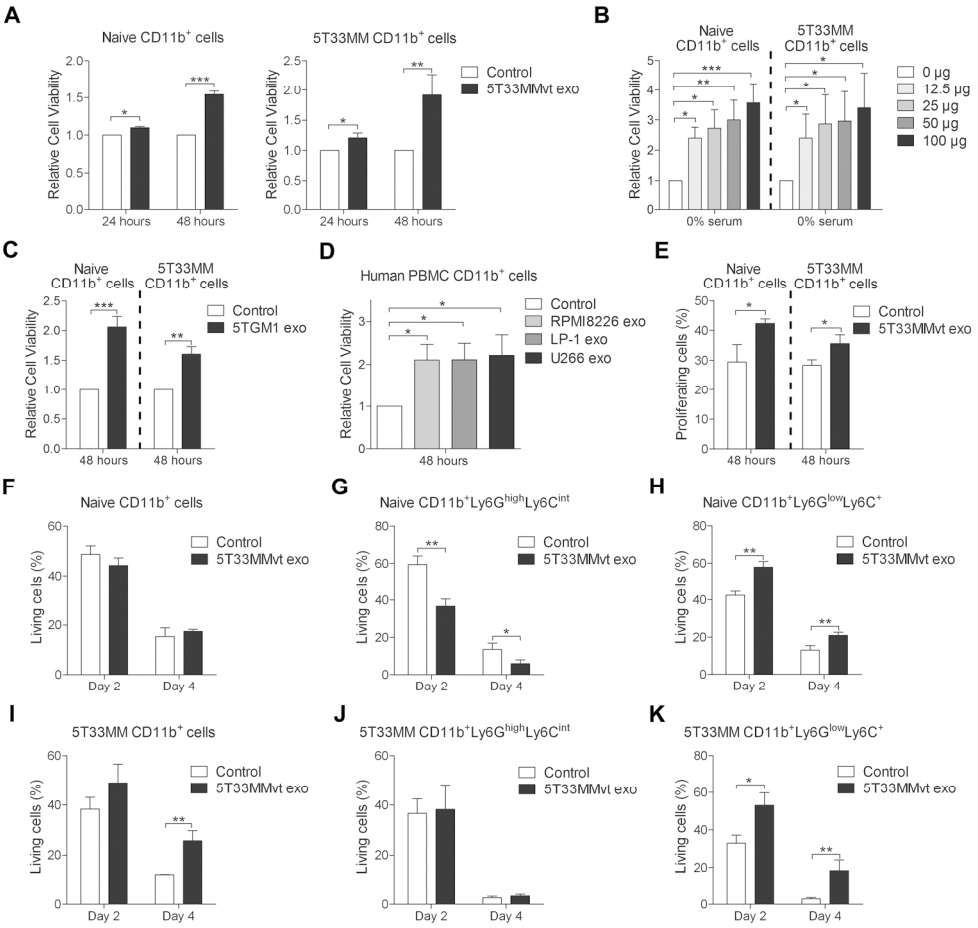

Figure 5

figure 5
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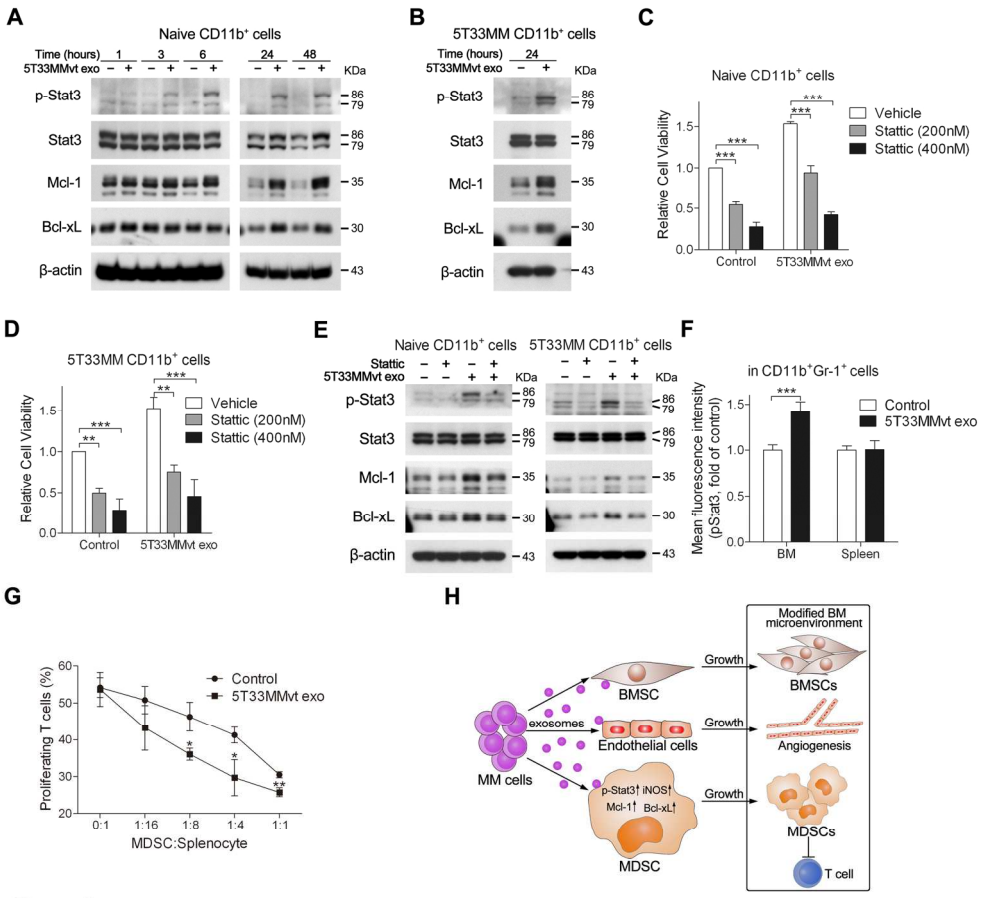

Figure 6

Figure 6
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Supplementary materials and methods

Cell culture

The murine MM cell lines 5T33MMvt and 5TGM1, the murine BM endothelial cell line STR10, as well as human MM cell lines RPMI8226, LP-1, and U266, were cultured in RPMI 1640 medium (Lonza, Visp, Switzerland), supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA), 2mM L-glutamine, and 100U/mL penicillin/streptomycin. MEM non-essential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA, USA) were also added for the 5T33MMvt and 5TGM1 cell culture. Primary BMSCs obtained from naive mice were maintained in DMEM medium (Lonza) supplemented with 10% fetal calf serum, 10% horse serum (Invitrogen), L-glutamine, and antibiotics.

Antibodies and reagents

Antibodies to flotillin-1 (3253), heat shock protein 90 (Hsp90) (4874), Hsp70 (4872), calreticulin (2891), Alix (2171), α-Tubulin (2144), Stat3 (4904), phosphorylated Stat3 (p-Stat3) (Tyr705) (9138), p-JNK (9255), JNK (9258), p-Akt (4056), Akt (9272), p-p53 (9284), p53 (2524), p-p38 (9215), p-38 (9212), caspase-3 (9665), β-actin (4967), Bcl-xL (2764), Mcl-1 (5453), horseradish peroxidase (HRP)-linked anti-mouse (7076) and anti-rabbit (7074) IgG, were bought from Cell Signaling Technology (Bioké, Leiden, The Netherlands). Anti-Tsg101 antibody (sc-7964) was bought from Santa Cruz (USA). The Lipophilic tracer DIO and SYTO RNASelect green fluorescent cell stain (RGFCS) were purchased from Life Technologies (Carlsbad, CA, USA). Stattic was bought from Selleckchem (Houston, TX, USA).

Transmission electron microscopy (TEM)

Exosomes were fixed in 2% paraformaldehyde and absorbed to a formvar-carbon coated grid. After adsorption, the grids were transferred to 1% glutaraldehyde, washed

8 times with Milli-Q water and negatively stained with 2% uranyl acetate. The grids were visualized on a TECNAI 10 transmission electron microscope (Philips, Amsterdam, The Netherlands) at 80kV and the images were captured using iTEM software (Olympus Soft Imaging Solutions, Münster, Germany).

Western blot

Exosomes or cell lysates were separated by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate and then transferred to polyvinylidene difluoride membranes. The membrane was blocked with blocking buffer, incubated with primary antibody and exposed to HRP-conjugated secondary antibody. The bands on the membrane were visualized and captured using Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) and X-ray films, respectively. The pixel densities of bands were quantified using ImageJ 1.47 software.

In vivo chick chorioallantoic membrane (CAM) assay

Fertilized white Leghorn chicken eggs were incubated at 37°C at constant humidity. On day 3, the shell was opened and 2-to-3 mL of albumen was removed to detach the CAM. On day 8, the CAMs were implanted with 1 mm3 sterilized gelatin sponges (Gelfoam Upjohn Co., Kalamazoo, MI, USA) loaded with PBS or with exosomes in PBS. On day 12, the angiogenic response was evaluated as the number of vessels converging toward the sponge at ×50 and photographed in ovo by a stereomicroscope (Olympus Italia Srl, Segrate, Milan, Italy).

Exosome isolation from Proteinase K-treated conditioned medium or human plasma

Conditioned medium was collected from cells cultured in serum free medium and concentrated using 150KD Protein Concentrator. The concentrated conditioned medium was incubated with Proteinase K (50μg/ml, Merck Millipore, Billerica, MA, USA) at 37°C for 10 minutes [25] and thereafter incubated with ExoQuick-TC exosome precipitation solution at 4°C for 30 minutes. The exosomes were precipitated by centrifugation at 10,000 × g for 30 minutes. Exosome pellet was resuspended in PBS and incubated with ExoQuick-TC exosome precipitation solution again at 4°C overnight. Then, exosomes were isolated by centrifugation at 1500 × g for 30 minutes.

For isolated exosomes from plasma, 500µl MM patient plasma diluted with 30ml PBS was concentrated using 150KD Protein Concentrator. The concentrated plasma was incubated with Proteinase K (100µg/ml, Merck Millipore) at 37°C for 20 minutes. The exosomes were precipitated by ExoQuick solution for 2 times as described above. Informed consent was obtained from all the MM patients.

Fluorescent labeling of exosomes

Exosome suspension was stained with DIO for 30 minutes at 37° C and DIO-labeled exosomes were precipitated using ExoQuick-TC exosome precipitation and resuspended in PBS. For RGFCS labeling, the exosome suspension was directly incubated with RGFCS (10µM) at 37° C for 30 minutes. Unincorporated DIO or RGFCS in suspension were removed by Exosome Spin Columns (Life Technologies). DIO or RGFCS in PBS without exosomes were processed in the same way to get DIO control or RGFCS control solutions.

Magnetic-activated cell sorting (MACS) for CD11b$^+$ cells

The BM cells obtained from naive or 5T33MM mice, or peripheral blood mononuclear cells (PBMC) obtained from human donors after their informed consent and in accordance with the Declaration of Helsinki, were incubated with CD11b Microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 15 minutes at 4 °C, followed by separation of the CD11b$^+$ cells using LS Column (Miltenyi Biotec) and the MACS separator. The separated CD11b$^+$ cells were stained with anti-CD11b-PE-Cy7 (BioLegend, San Diego, CA, USA) and determined for purity (>90%) by flow cytometry. All research involves human samples was approved by the local ethical committee (B.U.N. 143201316382).

Cell viability and apoptosis assays

Naive or 5T33MM CD11b$^+$ cells (5×10^4) were cultured in 5% serum and treated with or without 5T33MMvt cell-derived exosomes for 24 or 48 hours. Cell viability was measured with the Cell Titer glo Luminescent Viability assay (Promega, Madison, WI, USA). CD11b$^+$ cells (5×10^5) were treated with exosomes and/or inhibitors for 2 or 4 days, and stained with Annexin V-FITC. Apoptotic cells were determined using flow cytometry.

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany), and reverse transcription of mRNA was performed using Verso cDNA kit (Thermo Scientific). The qRT-PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and specific primers using the ABI 7900TH Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were collected and analyzed by SDS 2.3 software (Applied Biosystems). The specific primer sequences for different genes were shown as follows: Inducible nitric oxide synthase (iNOS), GCTTCTGGTCGATGTCATGAG (forward), TCCACCAGGAGATGTTGAAC (reverse); for Arginase-1, GTCCCTAATGACAGCTCCTTTC (forward), CCACACTGACTCTTCCATTCTT (reverse). QuantiTect primers for GAPDH was purchased from Qiagen (QT01658692) and used as an internal control. Relative mRNA expression normalized to GAPDH was carried out using $2^{-\Delta\Delta Ct}$ method.

Coates edited

Figure S1. MM exosomes carry angiogenesis-related proteins. (A) Exosomes were isolated from RPMI8226 and 5T33MMvt cells and the protein was quantified. Quantification of exosomal protein from 10^6 cells was shown by histogram. Error bars represent mean ± standard deviation for at least 3 independent experiments. (B-C) Exosomes were isolated from (B) 5T33MMvt or (C) RPMI8226 cell conditioned medium pretreated with Proteinase K (PK) and angiogenesis-related proteins in these exosomes were analyzed using a mouse or human angiogenesis array, respectively. The pixel densities of proteins in the array were quantified and normalized to reference spots. bFGF, basic fibroblast growth factor; IL-10, interleukin-10; MIP-1α, macrophage inflammatory protein-1α; MMP-9, matrix metallopeptidase 9; OPN, Osteopontin; ANG, angiogenin; HGF, hepatocyte growth factor; TIMP-1, tissue inhibitor of metallopeptidase 1; TSP-1, thrombospondin-1.

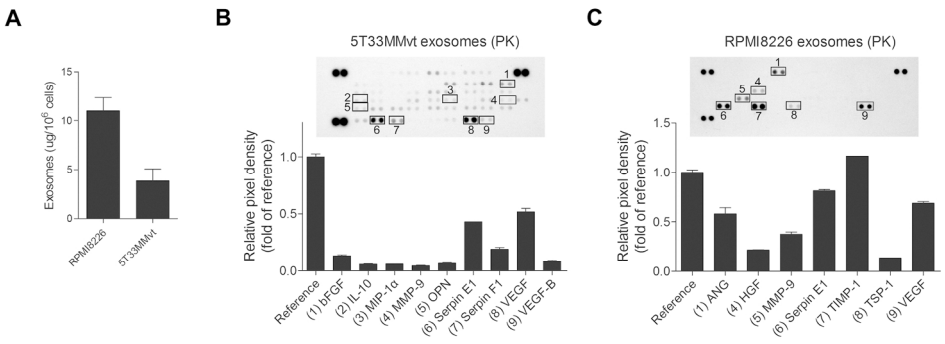
Figure S2. MM exosomes have limited contamination and promote BMSC and STR10 cell growth. (A) Diagram illustrating how to obtain a supernatant without exosomes as a negative control. (B) STR10 or (C) BMSC starved overnight were cultured in medium without serum and treated with PBS (control), ExoQuick solution diluted in PBS, supernatant without exosome, or re-precipitated 5T33MMvt exosomes for 48 hours, and the cell viability was measured by a luminescent viability assay. Graphs represent mean ± standard deviation for 3 independent experiments. (D) STR10 or BMSC were starved overnight and treated with exosomes obtained from Proteinase K-treated 5T33MMvt cell conditioned medium (5T33MMvt exo PK) for 48 hours, and the cell viability was measured by a luminescent viability assay. Graphs represent mean ± standard deviation for 4 independent experiments. (E) STR10 or (F) BMSC starved overnight were cultured with 5T33MMvt exosomes (60μg/ml) in serum free medium for indicated times, and the activation of Stat3 and JNK was determined by western blot. Tubulin was also measured as a loading control. * = $p<0.05$, *** = $p<0.001$.

Figure S3. MM exosomes can be taken up by CD11b$^+$ cells. The naive BM cells were cultured with (A) RGFCS- or (B) DIO-labeled 5T33MMvt cell-derived exosomes for 3 or 24 hour respectively. The mean fluorescence intensity of RGFCS or DIO in total BM cells, in CD11b$^+$ cells, or in CD11b$^-$ cells was determined by flow cytometry. The BM cells were isolated from (C) naive or (D) 5T33MM mice and the CD11b$^+$ cells were sorted by MACS. The percentage of CD11b$^+$Gr-1$^+$ cells or CD11b$^+$ cells before and after MACS was determined by flow cytometry. 3H2 is an anti-idiotype antibody against 5T33MM cells. Graphs represent mean ± standard deviation. *** = $p<0.001$.

Figure S4. Human MM and 5TGM1 exosomes increase MDSCs viability and induce the survival of CD11b$^+$Ly6G^{low}Ly6C$^+$ cells. (A) Naive (n=3) or 5T33MM (n=3) CD11b$^+$ cells were cultured in medium with 5% serum and treated with PBS (control), ExoQuick solution diluted in PBS, supernatant of exosome suspension, or reprecipitated 5T33MMvt exosomes for 48 hours, and the cell viability was measured by a luminescent viability assay. (B) 5T33MM (n=3) CD11b$^+$ cells were cultured in medium with 5% serum and treated with exosomes obtained from Proteinase K-treated 5T33MMvt cell conditioned medium (5T33MMvt exo PK) for 24 or 48 hours, and the cell viability was measured by a luminescent viability assay. (C) Purified human PBMC (n=3) CD11b$^+$ cells in medium with 5% serum were cultured with exosomes (100μg/ml) derived from three MM patients' plasma for 48 hours and the cell viability was measured by a luminescent viability assay. (D) Naive (n=3) or (G) 5T33MM (n=3) CD11b$^+$ cells in medium with 5% serum were cultured with or without 5TGM1 exosomes (100μg/ml) for 2 or 4 days and the percentage of living (Annexin V$^-$) cells was determined by flow cytometry. After culture with exosomes, (E, F) naive or (H, I) 5T33MM CD11b$^+$ cells were stained with anti-Ly6G-PE-Cy7, anti-Ly6C-APC, and Annexin V-FITC and the percentage of living (Annexin-V$^-$) cells

in two subpopulations of MDSC, (E, H) $Ly6G^{high}Ly6C^{int}$ cells and (F, I) $Ly6G^{low}Ly6C^{+}$ cells, was determined by flow cytometry and presented by histograms. Graphs represent mean ± standard deviation. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Figure S5. MM exosomes activate the STAT3 pathway and iNOS expression in MDSCs. After culture with exosomes for 24 hours, the level of p-stat3 in naive (n=3) or 5T33MM (n=3) $CD11b^{+}$ cells was determined by flow cytometry. (B) Naive or (C) 5T33MM $CD11b^{+}$ cell were separated from the BM (n=3) and cultured with or without 5T33MMvt exosomes (5T33MMvt exo, 100μg/ml) in the absence or presence of 200nM or 400nM stattic for 48 hours. The percentage of living (Annexin V^{-}) cells in the (B) naive or (C) 5T33MM $CD11b^{+}$ population was determined by flow cytometry. (D) The pixel densities of the proteins in Figure 6E were quantified from 3 independent experiments and presented by histograms. (E) Naive (n=3) or (F) 5T33MM (n=3) $CD11b^{+}$ cellswere cultured with or without 5T33MMvt exosomes (5T33MMvt exo, 100μg/ml) for 24 hours and the mRNA expression of iNOS and Arginase-1 was detected by qRT-PCR. (G) After treatment with or without 5T33MMvt exosomes for 24 hours, the protein of iNOS in naive or 5T33MM $CD11b^{+}$ cells was detected by western blot. β-actin was included as a loading control. The pixel density of iNOS was quantified and normalized to β-actin. Graphs represent mean ± standard deviation. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$



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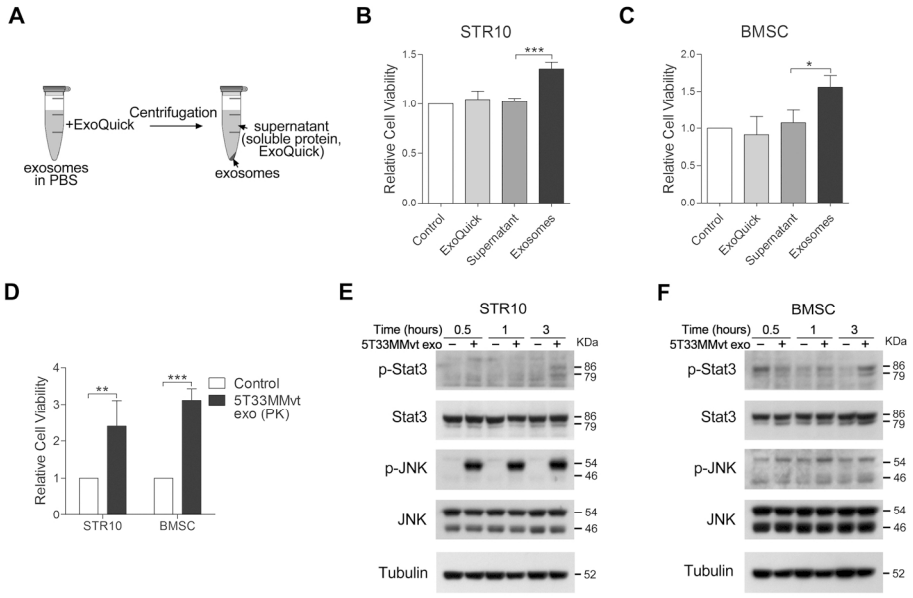

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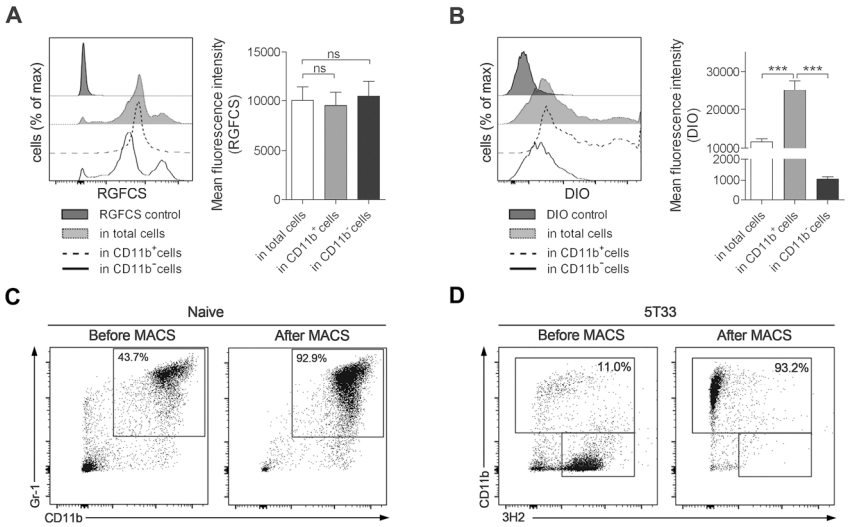

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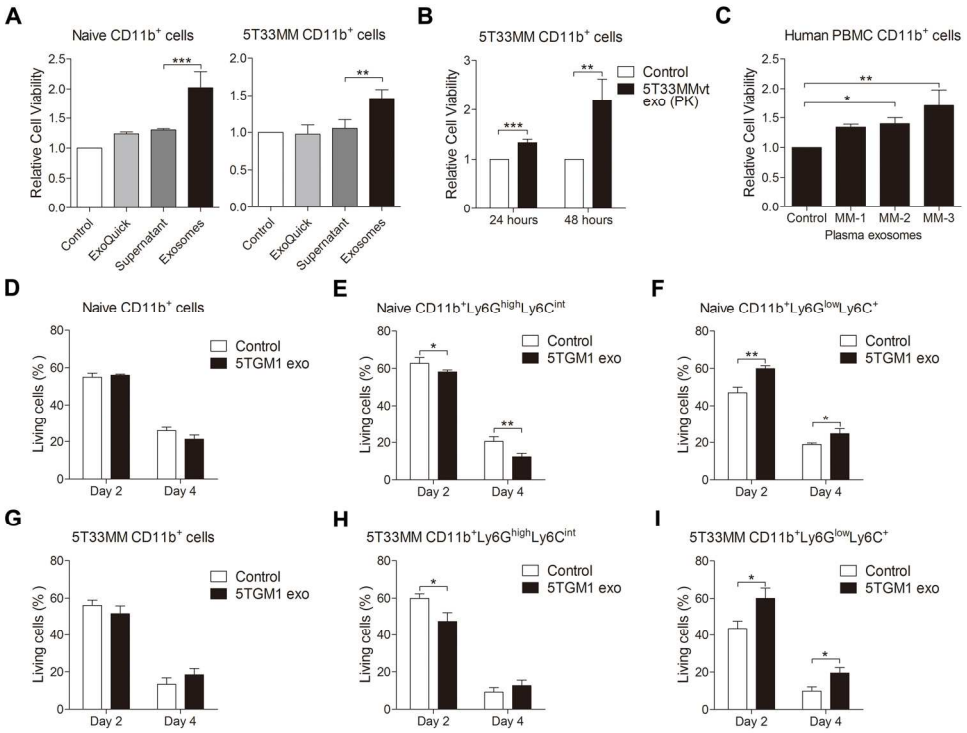

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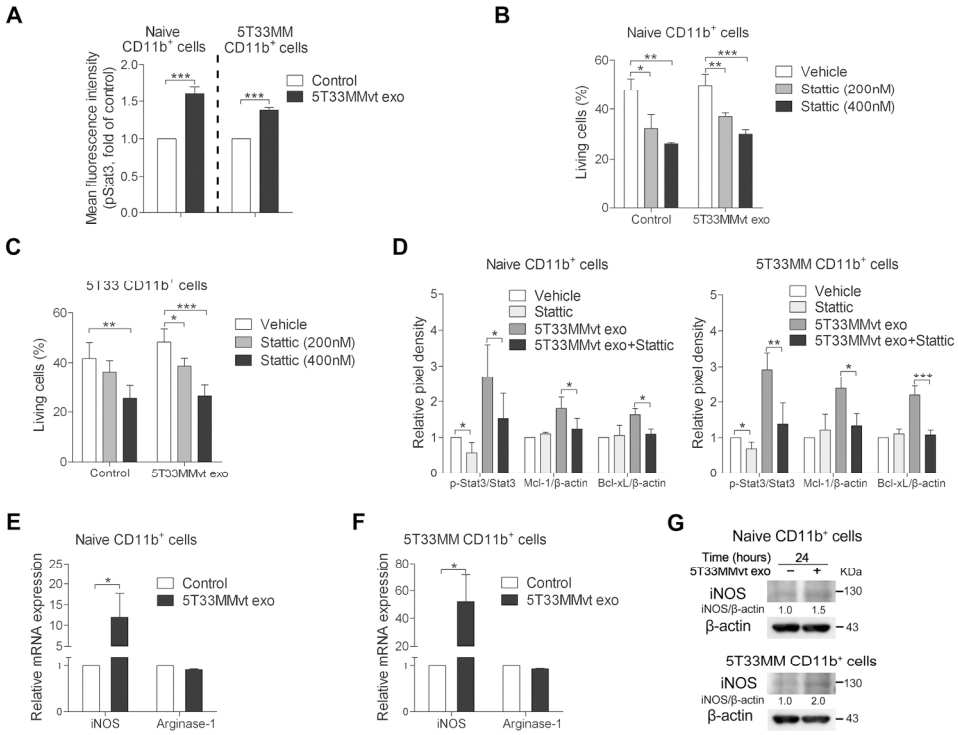

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