

1 **Title: Generation of novel diagnostic and therapeutic exosomes to detect and deplete pro-**
2 **tumorigenic M2-macrophages.**

3

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21 Word count for abstract: 149

22 Word count for manuscript: 2,998

23 Number of references: 50

24 Number of figures: 6

25 Number of Supplementary online-only files: 1

26

27 **Abstract**

28 Given their pro-tumorigenic function and prevalence in most malignant tumors with lower
29 survival, early detection and intervention of CD206-positive M2-macrophages may boost the
30 clinical outcome. To determine *in vivo* distribution of M2-macrophages, we adopted ^{111}In -oxine-
31 based radiolabeling of the targeted exosomes. When injected these radiolabeled targeted exosomes
32 into breast tumor-bearing mice, exosomes accumulated at the periphery of the primary tumor,
33 metastatic foci in the lungs, spleen, and liver. *Ex vivo* quantification of radioactivity also showed
34 similar distribution. Injected DiI dye-labeled exosomes into the same mice showed adherence of
35 exosomes to the CD206-positive M2-macrophages on *ex vivo* fluorescent microscopy imaging. In
36 addition, we utilized these engineered exosomes to carry the Fc portion of IgG2b with the intention
37 of augmenting antibody-dependent cell-mediated cytotoxicity. We have auspiciously
38 demonstrated that M2-macrophage targeting therapeutic exosomes deplete M2-macrophages both
39 *in vitro* and *in vivo*, and reduce tumor burden increasing survival in a metastatic breast cancer
40 model.

41

42 Exosomes have emerged as potential tools for a drug delivery system that can target
43 specific tissues or cells. Recently, the therapeutic application of exosomes has shown promising
44 results as novel therapeutic vehicles in cancer immunotherapy and suicide therapy, as well as
45 delivery of RNA-interference and drugs ¹⁻⁵. Exosomes have clear advantages over synthetic
46 nanoparticles like liposomes as a vehicle because of their improved biocompatibility, low
47 toxicity and immunogenicity, permeability, stability in biological fluids, and ability to
48 accumulate in the tumor with higher specificity ^{2, 3, 6-9}. Exosomes can be engineered to express
49 targeting peptides or antibodies on their surface for precise targeted therapeutics delivery ¹⁰⁻¹⁶.

50 Despite the exponential growth of chemotherapeutics and other targeted therapies for the
51 treatment of cancer, there have been few successes for solid tumors. Thus, instead of focusing on
52 the tumor cell alone, treatment strategies have been extended towards other cell types within the
53 tumor microenvironment (TME). Increased infiltration of tumor associated macrophages
54 (TAMs) correlates with tumor stage and poor survival ^{17, 18}. In addition to repolarization of
55 macrophages, therapeutic depletion might be an attractive approach.

56 CD206-positive M2-macrophages are shown to have a pivotal role in the dissemination
57 of breast cancer cells and prognosis ^{19, 20}. M2-macrophages participate in immune suppression,
58 epithelial to mesenchymal transition, invasion, angiogenesis, tumor progression and subsequent
59 metastasis foci formation. Investigators have utilized monoclonal antibody against CD206 or
60 multi-mannose analog diagnostic imaging compounds that target the lectin domain of CD206 as
61 imaging agents for detecting M2 macrophages in the TME or draining lymph nodes ^{21, 22}. In
62 recent year, investigators have identified a peptide sequence **CSPGAKVRC** that binds
63 specifically to CD206+ macrophages in the tumors and sentinel lymph nodes in different tumor
64 models ²². Generation of exosomes that uniquely bind to the receptor expressed by TAMs will

65 enable the design of rational therapies that specifically target TAMs, ideally leaving normal
66 macrophages unaffected.

67 Antibody-dependent cell-mediated cytotoxicity (ADCC) is a non-phagocytic mechanism
68 by which most leucocytes (effector cells) can kill antibody-coated target cells in the absence of
69 complement and without major histocompatibility complex (MHC) [29]. Targeted therapy
70 utilizing monoclonal antibodies (mAbs) has instituted immunotherapy as a robust new tool to
71 fight against cancer. As mAb therapy has revolutionized treatment of several diseases, ADCC
72 has become more applicable in a clinical context. Clinical trials have demonstrated that many
73 mAbs perform somewhat by eliciting ADCC [30]. Antibodies serve as a bridge between Fc
74 receptors (FcR) on the effector cell and the target antigen on the cell that is to be killed. There
75 has not been any report of engineered targeted exosomes inducing ADCC. In the proposed model
76 of engineered exosomes along with CD206 binding peptide, we conjugated Fc portion of the
77 mouse IgG2b that could potentially be recognized by FcR on the effector cells and stimulate the
78 ADCC events.

79

80 **Results**

81 **Determination of specificity of precision peptide *in vivo***

82 To assess *in vivo* targeting potential, rhodamine-labeled precision peptide (red) was injected
83 intravenously (IV) in metastatic syngeneic murine breast cancer (4T1) bearing Balb/C mice.
84 Three hours after injection, all animals were euthanized, and lungs, spleen and tumors were
85 collected for immune-histochemical analysis. Frozen sections from the collected tissues were
86 stained for CD206 (fluorescein, FITC) and counter stained with DAPI. The targeting peptide
87 accumulated in CD206-positive macrophages in tumors, spleen and lungs (**Figure 1a**).

88 **Generation of CD206-positive M2 macrophage-specific exosomes**

89 To confer targeting potentiality, we fused precision peptide for CD206-positive TAMs, to the
90 extra-exosomal N-terminus of murine Lamp2b, a protein found freely in exosomal membranes
91 (**Figure 1b**). A 6XHis tag in the C-terminus of the protein was added for confirming the
92 expression of the recombinant protein and luciferase was used as a reporter gene.

93 Plasmid encoding the Lamp2b construct was transfected into the HEK293 cells before exosome
94 purification (**Figure 1c**). Positively selected cells showed strong luciferase activity *in vitro*
95 following addition of luciferin substrate while non-transfected HEK293 cells did not show any
96 activity (**Figure 1d**). Induction of precision peptide in transfected cells was confirmed by
97 agarose gel electrophoresis showing single band of amplified DNA at the level of 150bp,
98 corresponding to the targeting peptide (**Figure 1e**). 6XHis tag was strongly expressed in
99 engineered exosomes compared to exosomes from non-transfected HEK293 cells and 4T1 tumor
100 cells, based on western blots with anti-6XHis tag antibody (**Figure 1f**).

101 After successfully generating the engineered exosomes, we analyzed their concentration and size
102 distribution by nanoparticle tracking assay (NTA). There was no significant difference in size
103 distribution between the engineered exosomes compared to those from non-transfected HEK293
104 cells (**Figure 1g**). The mean diameter of engineered exosomes was 92.2 ± 4.6 nm and HEK293
105 cells-derived exosomes was 106.3 ± 14 nm (**Figure 1h**). Transmission electron microscopic
106 (TEM) images for engineered exosomes showed characteristic round morphology and size
107 without any deformity (**Figure 1i**).

108 **Targeting potential of CD206-positive M2-macrophage-specific exosomes**

109 To assess targeting ability of the engineered exosomes, we differentiated mouse RAW264.7
110 macrophages towards M2-macrophages by treating them with IL-4 and IL-3 *in vitro*. Then we
111 co-cultured the cells with DiI-labeled (red) engineered exosomes for 4 hours followed by
112 immunofluorescence staining for CD206-positive cells (FITC) and DAPI for nuclei. Microscopic
113 images showed that engineered exosomes were attached and internalized by the CD206-positive
114 M2 macrophages (**Figure 2a**).

115 Next, we evaluated whether the binding is mediated by CD206. We co-cultured DiI-labeled
116 engineered exosomes with CD206-negative normal mouse embryonic fibroblasts (MEF), and
117 RAW264.7 cells with or without anti-CD206 peptide treatment. After 4 hours of incubation,
118 immunofluorescence staining was done for CD206-positive cells (FITC) and DAPI for nuclei.
119 Microscopic images showed that while engineered exosomes were not bound or taken up by the
120 MEF, they were bound to the CD206-positive RAW264.7 cells (**Figure 2b**). Binding of DiI-
121 labeled engineered exosomes was attenuated by treatment with anti-CD206 peptide.

122 To confirm the targeting efficiency of the engineered exosomes *in vivo*, we injected same DiI-
123 labeled engineered exosomes in 4T1 tumor-bearing Balb/C mice. After three hours of IV
124 injection, mice were euthanized, and tumor, spleen and lungs were collected for frozen
125 sectioning. Immunofluorescence staining was done for CD206-positive cells (FITC) and DAPI
126 for nuclei (**Figure 2c**). Fluorescence microscopic images showed that DiI-labeled engineered
127 exosomes were co-localized with the CD206-positive M2 macrophages. Interestingly, the red-
128 colored engineered exosomes spared the white pulp or germinal centers of the splenic follicle
129 that accommodate T- and B-lymphocytes, implying these lymphocytes were not targeted by the
130 engineered exosomes (**Figure 2d**).

131 **Detection and quantification of *in vivo* distribution of CD206-positive M2 macrophages**
132 **targeting exosomes**

133 To investigate the validity of engineered exosomes as an imaging probe to determine the
134 distribution of M2-macrophages, we used FDA approved clinically relevant SPECT scanning
135 and labeling with ^{111}In -oxine according to our previous study ²³. We used ^{111}In -oxine-labeled
136 non-engineered control exosomes (HEK293 exo) in metastatic (4T1) mouse breast cancer
137 models, and engineered exosomes (M2-targeting exo) expressing precision peptide treated with
138 either vehicle or clodronate liposome (Clophosome®-A) 24 hours before the IV administration
139 of ^{111}In -oxine-labeled exosomes and SPECT studies. Clophosome®-A is composed of anionic
140 lipids and depletes more than 90% macrophages in spleen after a single intravenous injection ²⁴,
141 ²⁵. Clophosome®-A is not approved for human studies, and it is for experimental use only.

142 Similar to the previously-mentioned ^{131}I -labeled exosomes ²⁶, prior to IV injection into mice for
143 biodistribution, we checked the labeling efficiency of ^{111}In -oxine to the engineered exosomes
144 and serum stability of binding by thin layer paper chromatography (TLPC). While approximately
145 92% of the free ^{111}In -oxine alone moved from the spotted point in the bottom to the top half of
146 the TLPC paper (**Figure 3a**), more than 98% of ^{111}In -oxine-bound to the engineered exosomes
147 remained at the bottom, indicating that ^{111}In -oxine was bound to the engineered exosomes with
148 very little dissociation of the ^{111}In -oxine from the exosomes (**Figure 3b**). After labeling with
149 ^{111}In -oxine we also evaluated serum stability of the binding through incubating the labeled
150 exosomes with 20% FBS for 1 hour and 24 hours. TLPC showed that > 92% of ^{111}In -oxine was
151 still bound to exosomes after 1 and 24 hours (**Figure 3c**).

152 All animals underwent CT followed by SPECT scanning at 3 hours after IV administration of
153 ^{111}In -oxine-labeled exosomes. The group injected with ^{111}In -oxine-labeled HEK293 exo did not

154 show any radioactivity or localization of exosomes in tumor, lung and spleen (**Figure 3d**).
155 Significant amount of exosomes was localized in these organs of animals injected with ^{111}In -
156 oxine-labeled M2-targeting exo. Surprisingly, there was an overt accumulation of M2-targeting
157 exo in lymph nodes and bones. As Clophosome®-A treatment depleted macrophages, the treated
158 group demonstrated significantly decreased accumulation of M2-targeting exo in tumor, lung and
159 spleen compared to the untreated group. Additionally, we also created 3D surface plot of lungs
160 and tumors of above-mentioned groups using ImageJ software (**Figure 3e**). Consistent with the
161 previous findings, there was almost no radioactivity or exosome accumulation in lungs and
162 tumor of animals injected with HEK293 exo. While accumulation of M2-targeting exo in lungs
163 and tumor was conspicuously high, their localization was considerably attenuated by prior
164 Clophosome®-A injection. In the tumor, M2-targeting exo localized only at the M2-macrophage
165 prevalent rim of the tumor.

166 Activity in different organs including primary and metastatic sites (lungs) was quantified to
167 determine the percent injection dose (%ID). Estimated radioactivity demonstrated significant
168 amount of exosomes were localized in tumor, lungs and spleen of vehicle-treated animals
169 injected with ^{111}In -oxine-labeled M2-targeting exo compared to other two groups (**Figure 3f**).

170 Following the scan, animals were euthanized, and radioactivity of different organs were
171 determined as reported previously ^{27, 28}. Alike *in vivo*, *ex vivo* quantification of radioactivity also
172 showed substantially higher radioactivity in lungs, spleen and tumor of animals injected with ^{111}In -
173 oxine-labeled M2-targeting exo (**Figure 3g**).

174

175 **Generation of CD206-positive M2 macrophage-targeting therapeutic exosomes**

176 Following the confirmation of targeting potential of engineered exosomes for diagnostic purpose,
177 we utilize the exosomes as therapeutic carriers. We conjugated Fc portion of mouse IgG2b next
178 to the targeting precision peptide with a small linker with the purpose of inducing ADCC
179 (**Figure 4a and 4b**). Identical to the previous construct, 6XHis tag and luciferase were
180 incorporated as reporter genes.

181 Positively selected cells showed strong luciferase activity *in vitro* following addition of luciferin
182 substrate while non-transfected HEK293 cells did not show any activity (**Figure 4c**). We
183 confirmed the presence of Fc portion of mouse IgG2b on the surface of the exosomes by flow-
184 cytometry using FITC-conjugated anti-mouse IgG2b antibody, that showed ~52% of engineered
185 therapeutic exosomes express Fc portion of mouse IgG2b (**Figure 4d**).

186 We next analyzed concentration and size distribution of the engineered therapeutic exosomes by
187 NTA (**Figure 4e**). The mean diameter of engineered exosomes was significantly larger than the
188 non-engineered exosomes (**Figure 4f**). TEM images for engineered therapeutic exosomes
189 showed distinctive round morphology and size without any distortion (**Figure 4g**). Flow-
190 cytometric analysis of common exosome markers for the engineered exosomes showed ~48%
191 positive for CD9 and ~40% positive for CD63 (**Figure 4h**).

192 **Induction of cytotoxicity and depletion of M2-macrophages by engineered therapeutic
193 exosomes**

194 To ascertain the capacity of therapeutic exosomes for instigating ADCC, we treated the CFSE-
195 labeled (green) RAW264.7 macrophages with non-therapeutic CD206-positive cell-targeting
196 exosomes (LAMP-206 exo) or CD206-positive cell-targeting therapeutic exosomes (LAMP-206-
197 IgG2b exo), and without any exosome treatment (control) for 48 hours in presence of normal

198 mouse splenic mononuclear cells. Fluorescent microscopic analysis showed most of the cells
199 treated with LAMP-206-IgG2b exo were either dead or floating (**Figure 5a**). Likewise, measured
200 fluorescence intensity of the cells treated with LAMP-206-IgG2b exo was significantly lower
201 compared to the control or LAMP-206 exo treated cells (**Figure 5b**).

202 To evaluate whether the engineered therapeutic exosomes can also deplete CD206+ M2-
203 macrophages *in vivo*, we treated normal Balb/c mice with single, two and three doses of
204 therapeutic exosomes or without treatment (control). We harvested the spleens of the mice for
205 flow-cytometric analysis. Remarkably, we observed a dose-dependent decrease in M2-
206 macrophage population by therapeutic IgG2b exosome treatment compared to control (**Figure 5c**
207 **and 5d**). There was no significant difference in both CD8 and CD4 population between
208 therapeutic exosome treated and untreated group, which indicates engineered therapeutic
209 exosomes do not affect the T cell population (**Figure 5e and 5f**).

210 **Treatment with engineered therapeutic exosomes prevent tumor growth and early
211 metastasis increasing survival**

212 Furthermore, we wanted to determine *in vivo* distribution of the precision peptide after
213 therapeutic exosome treatment in mouse tumor model to see if the treatment can attenuate
214 distribution of the peptide in M2-macrophage prevalent areas. We implanted tumor cells
215 subcutaneously on the flanks of mice. After 3 weeks of tumor growth we treated one group of
216 mice with engineered therapeutic exosomes for one week (3 doses), and another group without
217 treatment. We conjugated 6-Hydrazinopyridine-3-carboxylic acid (HYNIC) with the precision
218 peptide and labeled with technetium-99m (99mTc). We injected 99mTc-labeled peptide into both
219 groups of mice and after 3 hours CT followed by SPECT images were acquired. Reconstructed
220 images and quantification displayed significant diminution of precision peptide distribution in

221 tumor and spleen of the group treated with therapeutic exosome compared to untreated group

222 **(Figure 6a and 6b).**

223 Finally, we investigated whether depletion of M2-macrophages by therapeutic exosomes can

224 prevent tumor growth and metastasis, and increase survival of tumor-bearing animals. From day

225 8, after orthotopic implantation of the tumor cells one group of mice was treated with engineered

226 therapeutic exosomes and another group without any treatment (control). Total 6 doses (3

227 doses/week) of engineered exosomes were injected intravenously for 2 weeks. Tumor growth

228 was monitored by optical imaging every week. We found slower growth of tumor (photon

229 intensities) in engineered therapeutic exosome treated mice compared to the control group

230 **(Figure 6c and 6d).** Additionally, control group presented with early metastatic foci after week 4

231 compared to the treated group, treated group did not show any metastasis even after week 6.

232 Furthermore, survival was prolonged in the group treated with therapeutic exosomes compared

233 to the control group **(Figure 6e).** These data validated the therapeutic efficacy of the engineered

234 exosomes in depleting CD206-positive M2-macrophages and subsequently averting tumor

235 growth and metastasis.

236

237 **Discussion**

238 In recent years, several pioneers have explored the possibility of using exosomes as drug

239 delivery vehicles. Owing to their defined size and natural function, exosomes appear ideal

240 candidates for theranostic nanomedicine application ²⁹. When compared to the administration of

241 free drugs or therapeutics, exosomes have certain advantages such as improved stability,

242 solubility and *in vivo* pharmacokinetics ³⁰. Exosomes can potentially increase circulation time ³¹,

243 preserve drug therapeutic activity, increase drug concentration in the target tissue or cell to
244 augment therapeutic efficacy ³², while simultaneously reducing exposure of healthy tissues to
245 reduce toxicity ³³. Since they are nanosized and carry cell surface molecules, exosomes can cross
246 various biological barriers ³⁴, that might not be possible with free drugs or targeting agents.

247 One of the concerning factors for determining *in vivo* distribution in tumor model was
248 enhanced permeability and retention (EPR) effect by which nanoparticles tend to concentrate in
249 tumor tissue much more than they do in normal tissues. Although, only a fraction (0.7% median)
250 of the total administered nanoparticle dose is usually able to reach a solid tumor, which might
251 give false positive signals of exosome distribution. Surprisingly, we did not observe any
252 retention of radioactivity for free ¹¹¹In-oxine, and non-targeted or non-cancerous exosomes
253 (HEK293 exo). This implies that our demonstration of exosome biodistribution and targeted
254 therapy is not an EPR effect, rather the exosomes were directed towards target organs by over-
255 expressed precision peptide on their surface.

256 Many mechanisms have been implemented to boost the anti-tumor activities of therapeutic
257 antibodies, including extended half-life, blockade of signaling pathways, activation of apoptosis
258 and effector-cell-mediated cytotoxicity. Here we propose to target Fc gamma-receptor (Fc γ R)
259 based platform to deplete of M2 macrophages. The direct effector functions that result from
260 Fc γ R triggering are phagocytosis, ADCC, and induction of inflammation; also, Fc γ R-mediated
261 processes provide immune-regulation and immunomodulation that augment T-cell immunity and
262 fine-tune immune responses against antigens. With respect to IgG2b, part of the most potent IgG
263 subclasses can bind specifically into Fc γ RIII (KD=1.55x10⁻⁶) and IV (KD=5.9x10⁻⁸) to activate
264 Fc γ Rs ^{35, 36}. Peptibodies containing myeloid-derived suppressor cells (MDSC)-specific peptide
265 fused with Fc portion of IgG2b was able to deplete MDSCs *in vivo* and retard tumor growth of a

266 lymphoma mouse model without affecting pro-inflammatory immune cells types, such as
267 dendritic cells ³⁷. This plasticity of effector and immune-regulatory functions offers unique
268 opportunities to apply Fc γ R-based platforms and immunotherapeutic regimens for vaccine
269 delivery and drug targeting against infectious and non-infectious diseases ³⁸.

270 Investigators have used tumor cells, dendritic cells (DCs), mesenchymal stem cells (MSCs),
271 MDSCs, endothelial progenitor cells (EPCs), neural stem cells (NSCs), and other cell types to
272 generate engineered and non-engineered exosomes for both imaging and therapeutic purpose ^{8, 10,}
273 ^{15, 39-41}. We have also used tumor cells, MDSCs, EPCs, and NSCs derived exosomes in our
274 previous and ongoing studies ^{27, 41, 42}. Tumor cell-derived exosomes carry antigens and elicit
275 immunogenic reaction, therefore, these exosomes have been used in studies for tumor
276 vaccination ^{4, 5, 10}. On the other hand, both MSCs and MDSCs derived exosomes have shown to
277 be immune suppressive ⁴³⁻⁴⁵. EPC-derived exosomes may enhance neovascularization in the
278 tumors ^{46, 47}. Therefore, using these cells to generate engineered exosomes to carry CD206
279 targeting peptide may initiate unwanted effects of immune activation, immune suppression, or
280 neovascularization. Moreover, *in vitro* growth of MSCs, NSCs and EPCs may be limited due to
281 cell passage number. Ideal cell to generate engineered exosomes should have the following
282 criteria: (1) Non-immunogenic, (2) unlimited cell passage capacity without changing their
283 characteristics, (3) abundant production of exosomes both in normal and strenuous conditions,
284 (4) cells that can easily be genetically modified. HEK 293 cell is ideal for the production of
285 engineered exosomes. These cells have been extensively used by the biotechnology industry to
286 produce FDA (food and Drug Administration) approved therapeutic proteins and viruses for gene
287 therapies ^{48, 49}. Exosomes derived from these cells show no immune activation or suppression

288 following long-term injections in animal models ⁵⁰. We used HEK293 cells to generate our
289 engineered exosomes to carry precision peptide to target CD206+ M2-macrophages.

290 In conclusion, our study has demonstrated that exosomes targeting M2-macrophages could be
291 utilized effectively to diagnose, monitor and prevent tumor growth and metastasis for better
292 survival. The study provides novel insights for efficient exosome-based targeting of TME cells.

293

294 **Acknowledgements:**

295 This work was supported by NIH grants no. R01CA160216, Startup fund from Georgia Cancer
296 Center and AHA merit award 2019. We thank our former laboratory members Drs. Asm Iskander,
297 Kartik Angara, Baghelu Achyut and Adarsh Shankar. We thank Dr. Hasan Korkaya's laboratory
298 for 4T1 cells expressing luciferase gene reporter, Dr. Satyanarayana Ande's laboratory for the
299 Human embryonic kidney 293 cell line (HEK293), Dr. Nahid Mivechi's laboratory for the Mouse
300 Embryonic Fibroblast cell line (MEF), Dr. Gabor Csanyi's laboratory from the vascular biology
301 department at Augusta University for the RAW264.7 mouse macrophage cell line, and Dr. Mumtaz
302 Rojiani and Dr. Dimitrios Moskofidis for their laboratory supply support. We also thank Dr. Yutao
303 Liu's laboratory members Jingwen Cai and Hongfang Yu for the exosomes' size measurements,
304 the Histology and Electron Microscopy Core and Core Imaging Facility for Small Animals
305 (CIFSA) from Augusta University. We thank our administrative personnel Tonya Fowler, Darryl
306 Nettles, Christopher Middleton, Shelia Joyner, Denise Harper, Quar-an Green for their support.
307 We are thankful to Drs. Mark Hamrick, Hasan Korkaya, Anatolij Horuzsko, and Ahmed Chadli
308 for their valuable suggestions.

309

310 **Author contributions:**

311 MHR exosome isolation, animal treatment, *in vitro* studies data presentation. MHR, TFB collected,
312 processed tissues and performed for flow cytometer and IHC. MHR, ASA radioisotope labeling
313 and imaging analysis. RA and AA for imaging acquisition, analysis and vector design. YL and
314 ASA data interpretation and manuscript revision. MHR, TFB, ASA conceived, designed and
315 coordinated the study, data analysis, interpretation, manuscript writing, preparation and its
316 revision.

317

318 **Competing Interests:** The authors have declared that no competing interest exists.

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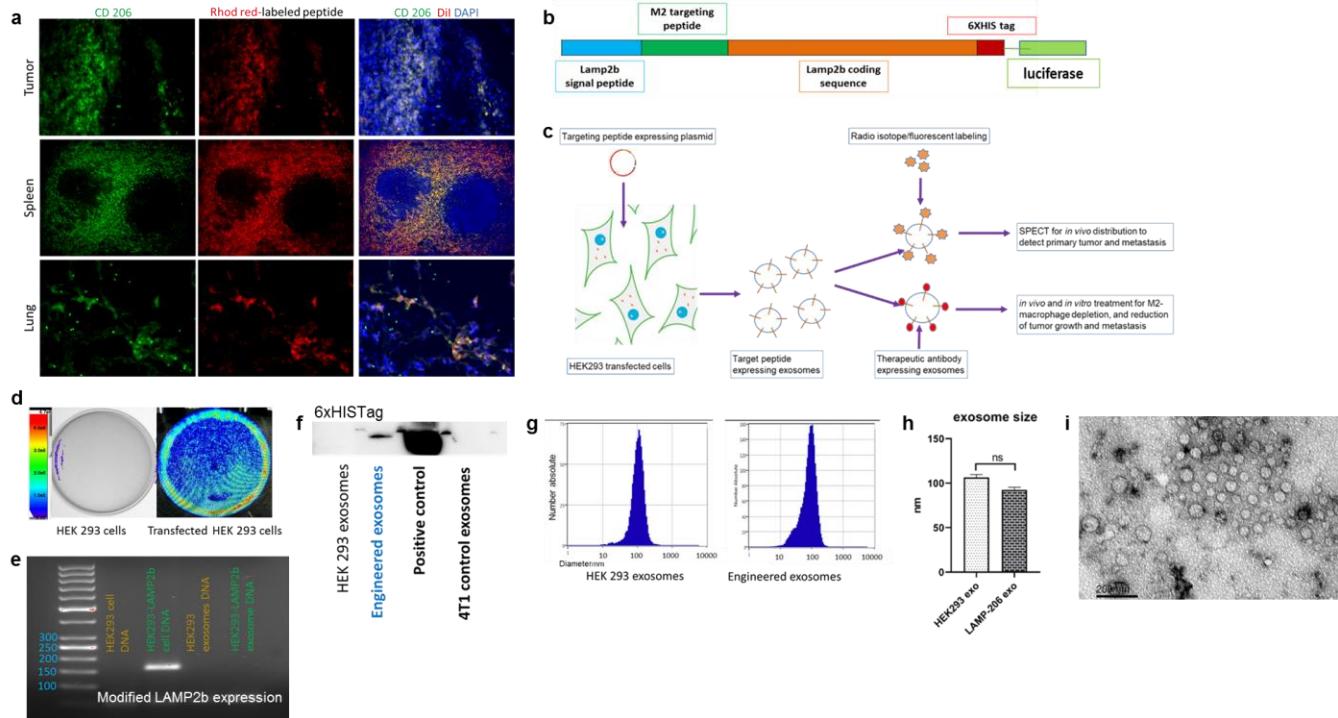
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453 **Figure and Legends:**

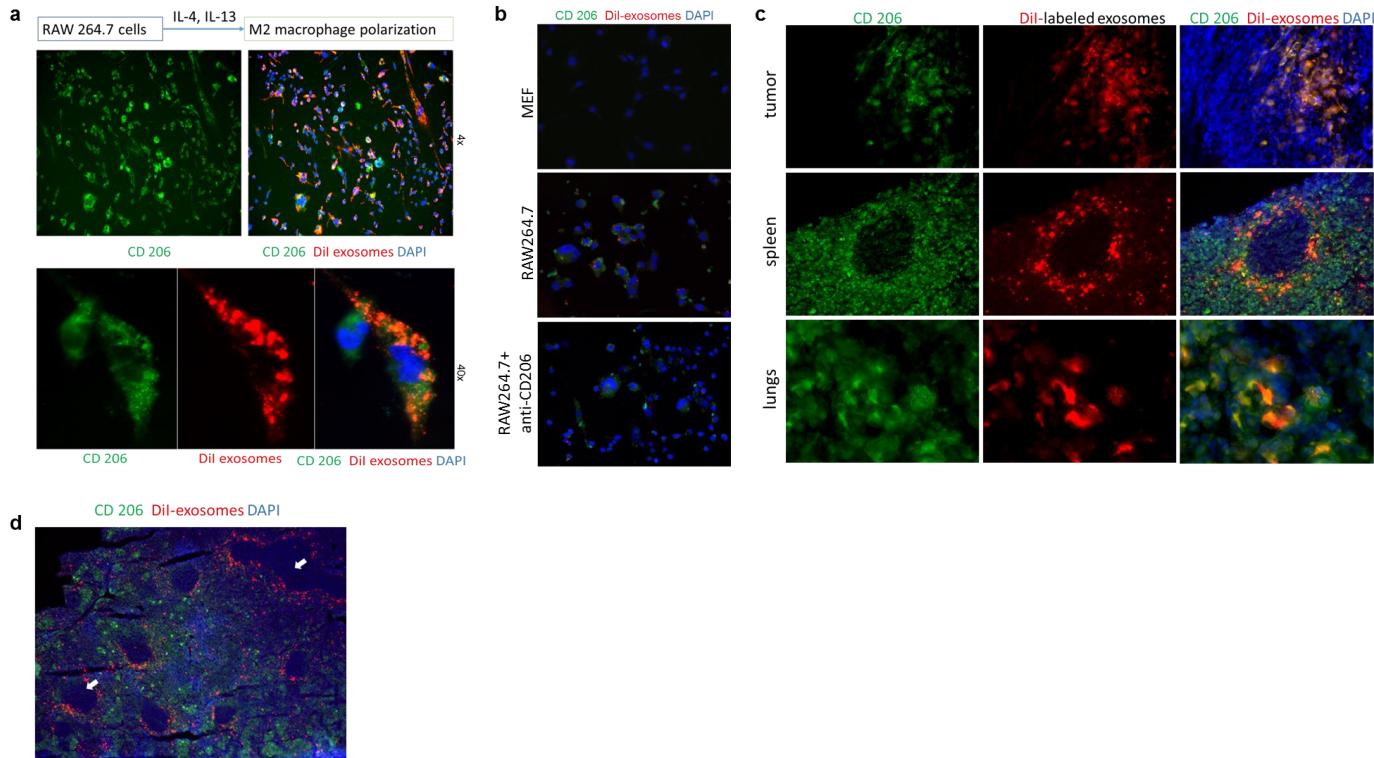
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456 **Figure 1.** Generation of engineered exosomes expressing CD206-positive M2 macrophage-specific
457 peptide along with Lamp2b. **(a)** Immunofluorescence staining of tumor, spleen and lungs sections from
458 4T1 tumor-bearing mice showing co-localization of Rhodamine red-labeled targeting peptide (injected
459 i.v.) and FITC labeled CD206-positive M2-macrophages. Nuclei were visualized by DAPI staining (blue).
460 **(b)** Schematic representation of the modified Lamp2b protein containing CD206 positive M2
461 macrophage-targeting peptide sequence following signal peptide, and a 6xHIS tag at the C terminus.
462 Luciferase was used as a reporter gene. **(c)** Schematic diagram showing generation of CD206+ M2-
463 macrophage targeting engineered exosomes for diagnostic and therapeutic purpose. **(d)** *in vitro* study
464 showing luciferase activity of transfected HEK293 cells. **(e)** Agarose gel electrophoresis showing
465 confirmation of targeting peptide sequence insert in transfected HEK293 cells. **(f)** Western blot image
466 showing anti-His tag antibody positivity in engineered exosomal protein content. **(g and h)** NTA analysis
467 showing size distribution of the engineered and non-engineered exosomes. Quantitative data are
468 expressed in mean \pm SEM **(f)** Transmission electron microscopy image for engineered exosomes, Scale
469 bar depicts 200 nm.

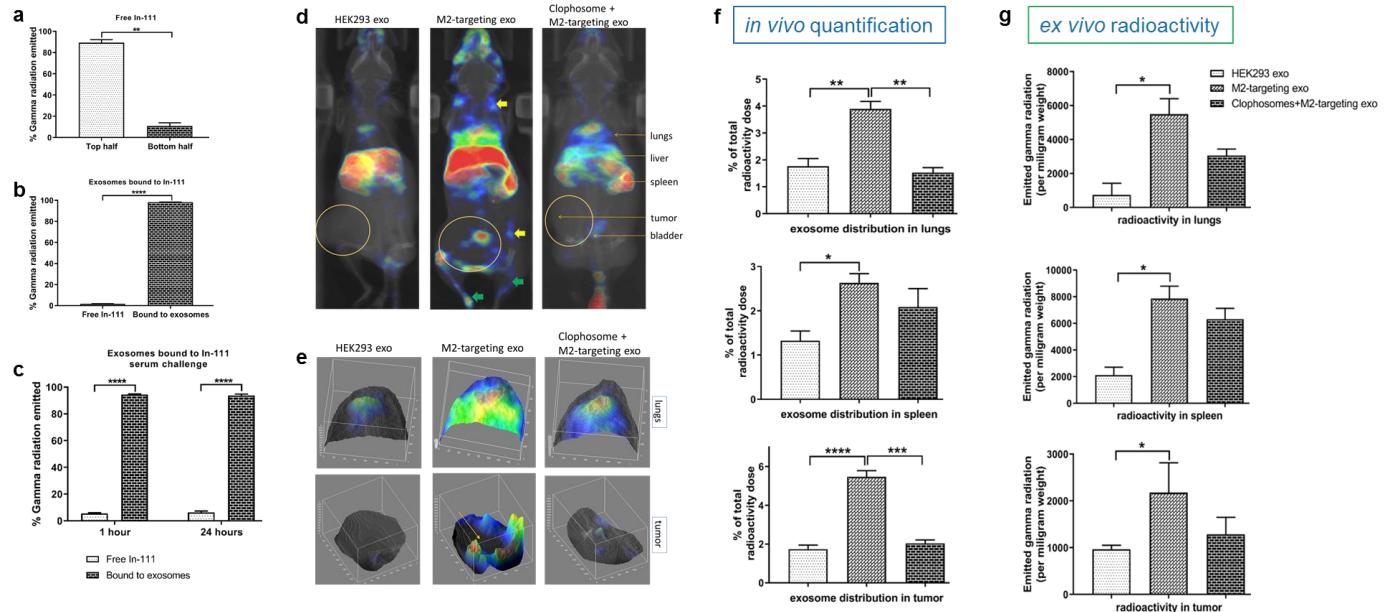
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472 **Figure 2.** Targeting efficiency and specificity of CD206-positive M2 macrophage-specific exosomes. **(a)**
473 Immunofluorescence staining showing targeting potential of DiI-labeled (red) engineered exosomes.
474 RAW264.7 mouse macrophages were differentiated to CD206-positive (FITC) cells by treating with
475 interleukin-4 and interleukin-13. Nuclei were visualized by DAPI staining (blue). **(b)**
476 Immunofluorescence staining of mouse embryonic fibroblasts (MEFs) and RAW264.7 cells treated with
477 or without anti-CD206 peptide, co-cultured with DiI-labeled (red) engineered exosomes. MEFs were
478 negative for CD206 (FITC) staining and did not take up the exosomes. Engineered exosomes bound to the
479 CD206+ RAW264.7 cells, that was prevented by anti-CD206 peptide treatment. **(c)** Immunofluorescence
480 staining of tumor, spleen and lungs sections from 4T1 tumor-bearing mice showing co-localization of
481 rhodamine red-labeled targeting exosomes (injected i.v.) and FITC labeled CD206-positive M2-
482 macrophages. Nuclei were visualized by DAPI staining (blue). **(d)** Stitched images for extended view of
483 splenic section showing engineered exosomes were not taken up by T-lymphocytes and B-lymphocytes in
484 splenic white pulp (white arrows).

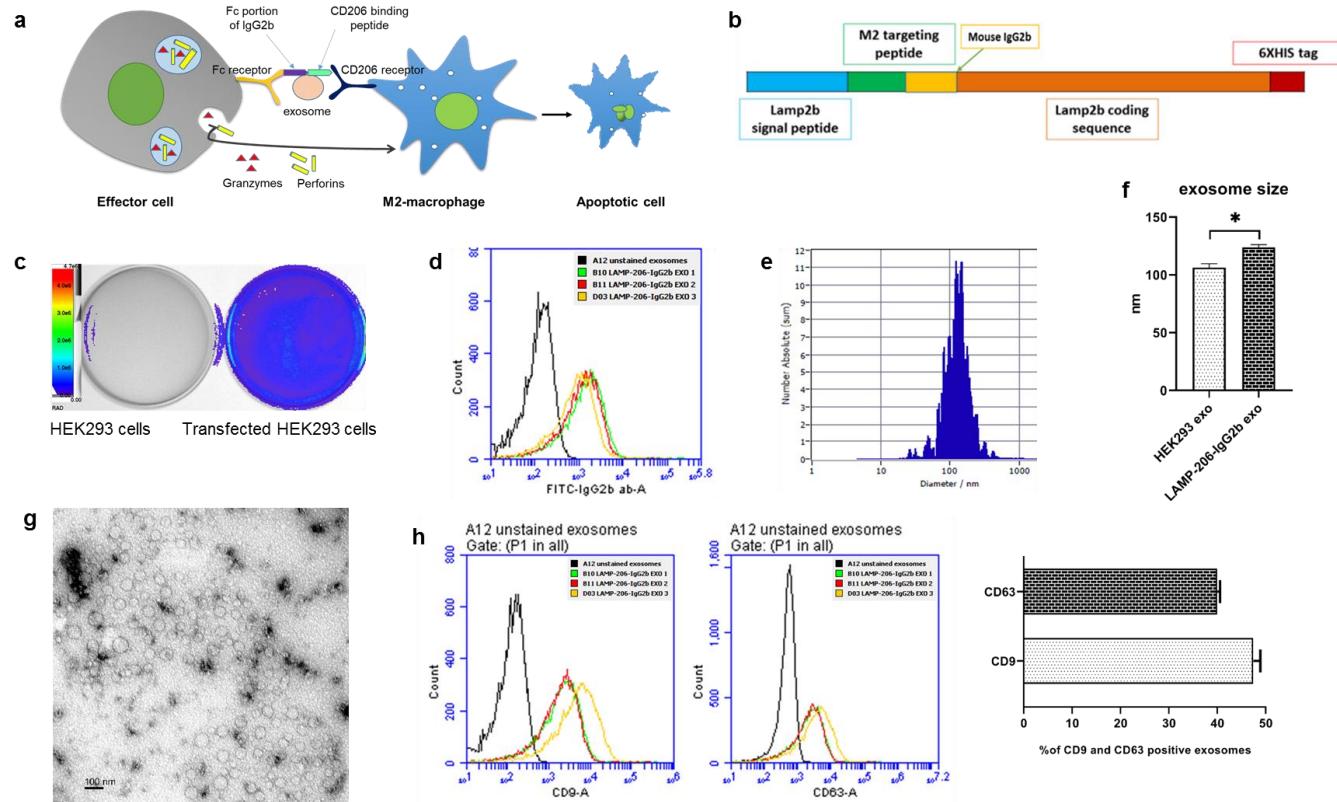
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487 **Figure 3.** Detection and quantification of biodistribution of ^{111}In -oxine-labeled exosomes targeting
488 CD206-positive M2 macrophages. **(a)** A major proportion of the free ^{111}In -oxine measured in the bottom
489 to the top half of the TLPC paper, confirming the efficacy of the eluent. **(b)** Binding of ^{111}In -oxine to
490 engineered exosomes was validated as shown by a lower percentage of ^{111}In -oxine (free, dissociated)
491 measured in the top of the paper, compared to the amount remaining in the bottom, which represented the
492 ^{111}In -oxine-labeled exosomes. **(c)** Serum stability of ^{111}In -oxine bound engineered exosomes was higher
493 compared with the small amount of free ^{111}In -oxine disengaged from the bound exosomes. **(d)** *In vivo*
494 SPECT/CT images (coronal view) after 3 hrs of intravenous injection showed significant accumulation of
495 M2-targeting exo in tumor, lung, spleen, lymph node and bones. ^{111}In -oxine-labeled non-targeting
496 exosomes (HEK293 exo) and CD206-positive M2-macrophage targeting exosomes (M2-targeting exo)
497 were injected into the 4T1 tumor-bearing mice. One group was treated with Clophosome® to deplete
498 macrophages. Yellow and green arrows denote lymph node and bone metastasis, respectively. **(e)** 3D
499 surface images showing M2-targeting exo are profoundly distributed in both lung and tumor area
500 compared to the group injected with HEK293 exo and pre-treated with Clophosome®. Yellow arrow
501 indicates the tumor center. **(f)** Quantification of *in vivo* radioactivity in lungs, spleen and tumor. **(g)** *Ex*
502 *vivo* radioactivity quantification in lungs, spleen and tumor. Quantitative data are expressed in mean \pm
503 SEM. *P<.05, **P<.01, ***P<.001, ****P<.0001. n = 3.

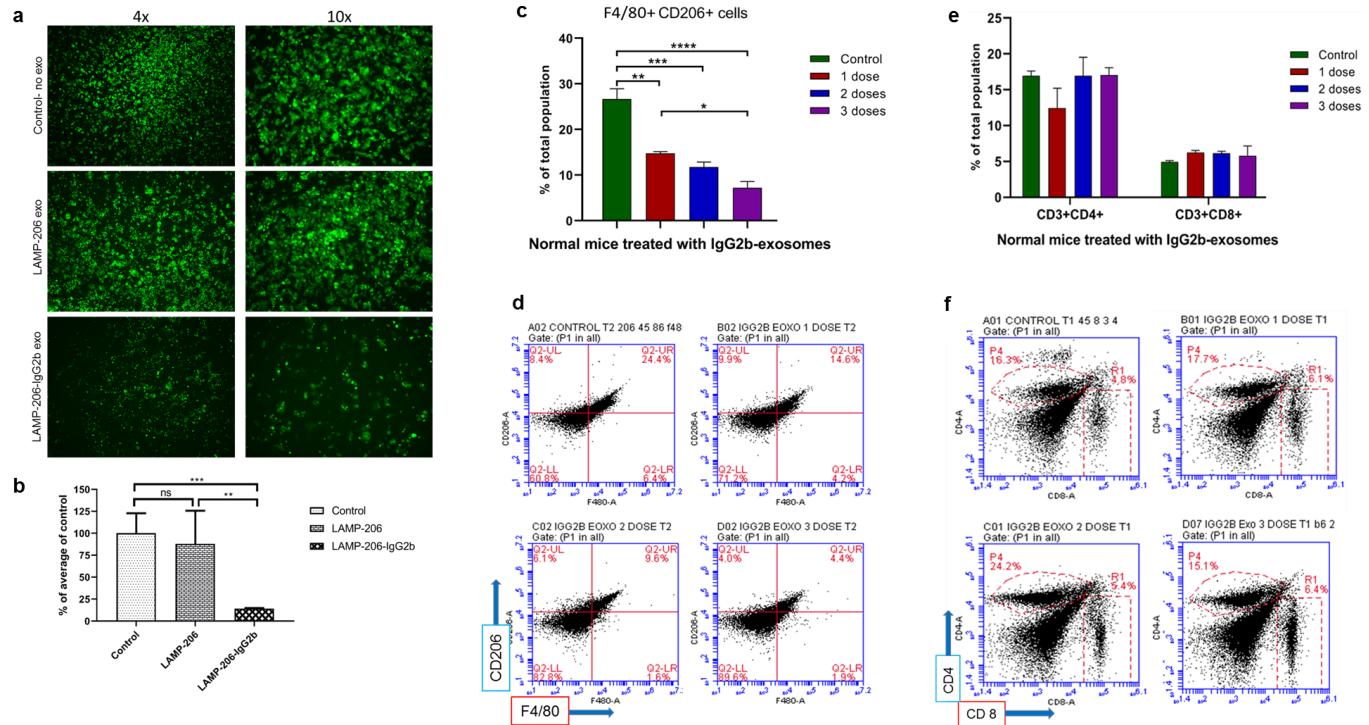
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507 **Figure 4.** Generation of CD206-positive M2 macrophage-targeting therapeutic exosomes to induce
508 antibody-dependent cell-mediated cytotoxicity. **(a)** Schematic diagram showing the proposed mechanism
509 of engineered exosome-based antibody-dependent cellular cytotoxicity. **(b)** Schematic representation of
510 the plasmid construct containing modified Lamp2b protein with CD206-targeting sequence conjugated
511 with Fc segment of mouse IgG2b. **(c)** Confirmation of luciferase activity by transfected HEK293 cells.
512 **(d)** Flow-cytometric analysis for validating the expression of Fc segment of mouse IgG2b on the surface
513 of engineered exosomes. 3 different engineered exosome samples were used for the flowcytometry. **(e**
514 **and f)** NTA analysis data showing size distribution of the engineered therapeutic exosomes. **(g)**
515 Transmission electron microscopy image for engineered therapeutic exosomes, Scale bar depicts 100 nm.
516 **(h)** Flow-cytometric analysis of exosomal markers CD9 and CD63 for the engineered therapeutic
exosomes. 3 different engineered exosome samples were used for the flowcytometry.

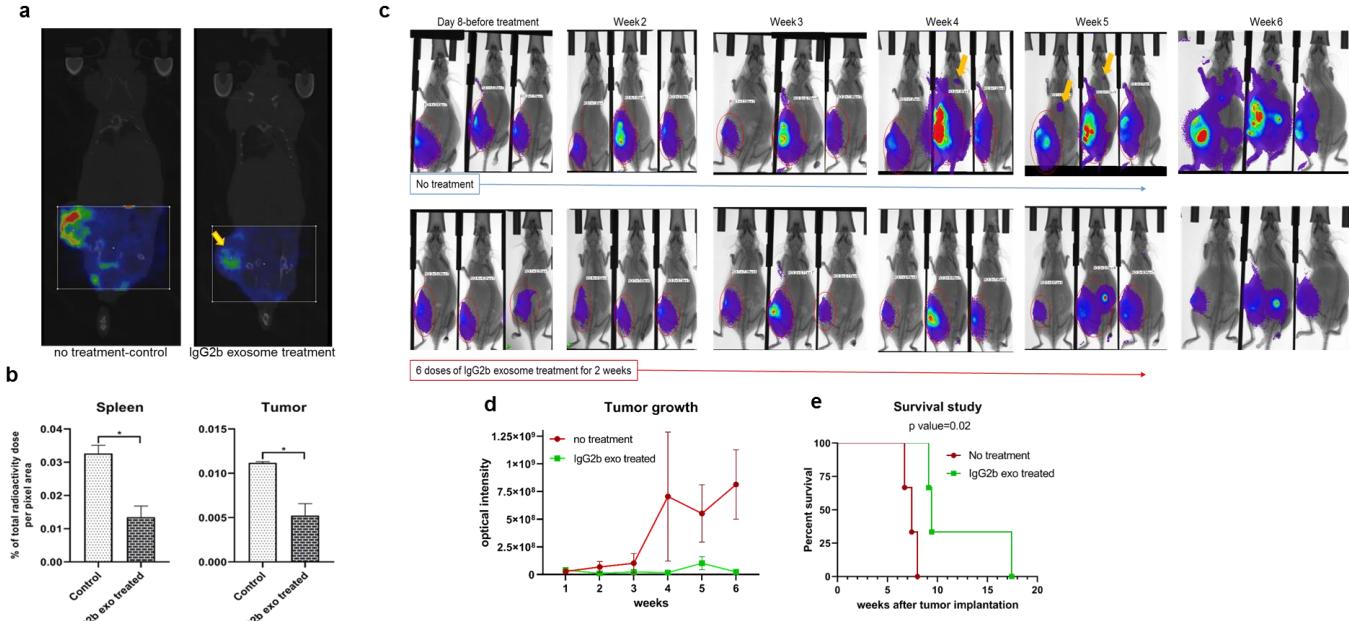
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519 **Figure 5.** Therapeutic efficiency and specificity of engineered therapeutic exosomes in depleting M2-
520 macrophages both *in vitro* and *in vivo*. **(a)** CFSE-labeled (green) RAW264.7 mouse macrophages were
521 co-cultured with non-therapeutic CD206-positive cell-targeting exosomes (LAMP-206 exo) or CD206-
522 positive cell-targeting therapeutic exosomes (LAMP-206-IgG2b exo), and without treatment (control) for
523 48 hours in presence of splenic immune cells from normal mice. Fluorescence microscopic images
524 showed decrease in cell number and increased floating dead cells in LAMP-206-IgG2b exo group
525 compared to other groups. **(b)** Measured fluorescence intensity of the above-mentioned conditions
526 showed significant decrease in LAMP-206-IgG2b exo group compared to other groups. **(c and d)** Normal
527 Balb/c mice were treated with one, two or three doses of engineered therapeutic exosomes expressing Fc
528 portion of mouse IgG2b. Flow-cytometric analysis of splenic cells showed dose-dependent decline of
529 F4/80 and CD206-positive M2-macrophage population. **(e and f)** Flow-cytometric analysis of splenic
530 cells showed no significant change in both CD4 and CD8-positive T-cell population after treating the
531 mice with different doses of therapeutic exosomes. Quantitative data are expressed in mean \pm SEM.
532 *P<.05, **P<.01, ***P<.001, ****P<.0001. n = 5.

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535 **Figure 6.** Treatment of 4T1 tumor-bearing animals with therapeutic engineered exosomes prevent tumor
536 growth and metastasis, and improve survival by depleting M2-macrophages. **(a and b)** Reconstructed and
537 co-registered *in vivo* SPECT/CT images (coronal view) and quantification of subcutaneous syngeneic
538 tumor-bearing animals (on the flank) injected with the ^{99m}Tc -labeled precision peptide after three hours.
539 Group treated with therapeutic exosomes showed lesser level of radioactivity in tumor (yellow arrow) and
540 spleen compared to untreated control group. Quantitative data are expressed in mean \pm SEM, * $P < .05$. $n =$
541 3. **(c)** Optical images of 4T1 tumor-bearing animals treated with engineered therapeutic exosomes (lower
542 panel) or without treatment (control), showing decreased tumor growth in treated animals compared to
543 control group. Metastatic foci in control group was detected (yellow arrows) as early as fourth week,
544 whereas no metastasis was detected in treated animals after 6 weeks. **(d)** Quantification of optical density
545 of the tumor area also showed decreased tumor growth in treated group compared to control group.
546 Quantitative data are expressed in mean \pm SEM. $n = 3$. **(e)** Kaplan-Meier plot showing prolonged survival
547 of the mice treated with therapeutic engineered exosomes.

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